Detection of Haemoglobinopathies in Single Cells: An Approach For Preimplantation Genetic Diagnosis and Early Prenatal Diagnosis

By

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A Thesis submitted for the degree of Doctor of Philosophy at the University of London

1998

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To my mother

HRH El-Sharifa Fatima Deeb Mohammad Al-Badawi

Abu-Namous El-Hashemite
Acknowledgements

In the completion of this thesis, I am grateful and thankful to many people. First a huge gratitude and thanks are owed to my mother, to whom I am dedicating this thesis, who taught me how to achieve success, patience, respect and working hard to achieve proficiency. As well, My Father – HRH Professor El-Sharif Mohammed Bin King Faysal (I) El-Hashemite, and my brothers (Dr Prince Adel, Prince Adnan and Prince Ahmed) without whose support, love, helpfulness and encouragement I would not be able to complete any work.

A sincere appreciation is for my supervisor Professor Joy Delhanty, who I came to admire more and more through the days not only as a lady but as a scientist who does not spare any moment of her time without working even though she is a mother. Working with her is itself an encouragement to work harder and harder.

As well I should not forget the assistance of Dr Mary Petrou and her associates in the Perinatal Centre Evelyn and Toyin, which I really appreciate very much. I am grateful for all colleagues and friends in the MRC unit - The Galton Laboratory, particularly for Professor Sue Povey and her team, Professor Hopkinson, Nalini, Dr Julia White and John Attwood.

I would like to thank my colleagues in the National Research Centre - Cairo particularly Dr Ibtessam Hussein; Professor Amal El-Beshlawi - Cairo University, Dr Magdy Rady and the colleagues in Ain Shams University - Cairo and Professor Mohammad El-Khateeb - Jordan University, for providing samples and the good experience of collaboration with them. As well I would like to express my thanks to Asangla Ao, Pierre Ray and all in the IVF unit at Hammersmith Hospital.
Finally I would like to thank the most wonderful and lovely colleagues of lab 208: Rosemary, Margaret, Clare, Angela, Puteri, Agnes, Richa, Molly, Joyce, Patrick, Dagan, Jon, Antonis and Calum, for many things but mainly for their friendship.
Abstract

β thalassaemia is defined as a group of heterogeneous anaemias in which the β globin peptide synthesis is either absent or reduced by 30% or more. It is the world’s most widespread autosomal recessive single gene disorder and represents a major health problem. More than 200 million individuals world-wide carry the β thalassaemia gene with an estimated 75,000 thousands of affected individuals born annually. Because of this β thalassaemia has attracted a great deal of research interest both at the molecular and clinical levels, which has led to the understanding of the complex molecular pathogenesis.

Recent clinical management has improved and increased the life expectancy of affected individuals, however, the treatment required is very expensive and unsatisfactory. Therefore, the aim is to control the incidence of the disorder through the provision of carrier detection and prenatal diagnosis.

Although prenatal diagnosis has reduced the number of β thalassaemia births, potential risks to the fetus have been associated with the available procedures. Furthermore, pregnancy termination is still unacceptable for many families, and couples at risk are looking for a safer, less emotionally traumatic way to have normal offspring. An alternative is offered by preimplantation genetic diagnosis carried out on the third day after in vitro fertilisation.

With the application of nested PCR, amplification of the minute amount of DNA in a single cell has become possible and DNA analysis of single gene disorders for preimplantation diagnosis can be achieved.
To enable preimplantation diagnosis of β thalassaemia by direct detection of mutant β globin genes at the single cell level nested PCR and silver stained single stranded conformation polymorphism (SSCP) analysis were employed. However, two main problems with single cell PCR, contamination and allele specific amplification failure in addition to mosaicism can lead to misdiagnosis and the transfer of affected embryos. In this study, the reduction of allele specific amplification failure by the use of SDS/proteinsae K as a lysis buffer, and the application of fluorescence based PCR to reduce the incidence of contamination which becomes apparent when reamplification is done, are reported.

Furthermore, this study has also focused on optimising DNA amplification procedures using standard or quantitative multiplex fluorescent PCR in single cells for the detection of mosaicism and age related trisomy 21 along with specific diagnosis of single gene disorder diagnosis and developing mutation analysis techniques such as fluorescent SSCP and automated fluorescent DGGE for either early prenatal diagnosis using transcervical cells or preimplantation diagnosis.

Finally, the first step of applying any prevention program for haemoglobinopathies is the identification of mutations within the β globin gene. The molecular characterisation of β thalassaemia has been studied in samples from Jordan and Egypt to facilitate screening and prenatal programmes based on DNA methodology.
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<td>A</td>
<td>adenine residue in a DNA sequence.</td>
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<tr>
<td>bp</td>
<td>base pairs.</td>
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<tr>
<td>BSA</td>
<td>bovin serum albumin.</td>
</tr>
<tr>
<td>C</td>
<td>cytosine residue in the DNA sequence.</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis.</td>
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<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane regulator.</td>
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<td>CVS:</td>
<td>chorionic villus sample.</td>
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<tr>
<td>ddH₂O</td>
<td>deionised distilled water.</td>
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<td>DGGE</td>
<td>denaturing gradient gel electrophoresis.</td>
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<td>DNA</td>
<td>deoxyribonucleic acid.</td>
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<td>DTT</td>
<td>dithiothritol.</td>
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<tr>
<td>G</td>
<td>guanine residue in the DNA sequence.</td>
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<td>g</td>
<td>grams</td>
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<td>HA</td>
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<td>HbA</td>
<td>adult haemoglobin.</td>
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<td>haemoglobin C.</td>
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<td>HbF</td>
<td>fetal haemoglobin.</td>
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<td>HbS</td>
<td>sickle haemoglobin.</td>
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<td>IVF</td>
<td>in vitro fertilisation.</td>
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<td>l</td>
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<td>M</td>
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<td>µg</td>
<td>microgram.</td>
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<td>mg</td>
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<td>microlitre.</td>
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ml  millilitre.
μM  micromolar.
mM  millimolar
PCR polymerase chain reaction.
PGD preimplantation genetic diagnosis.
PK proteinase K.
SCA sickle cell anaemia.
SSCP single stranded conformation polymorphism.
STR: short tandem repeat.
TCC transcervical cells.
T  thymine residue in the DNA sequence.
Chapter I

INTRODUCTION
Chapter I
INTRODUCTION

I - 1: History of Thalassaemia:

The history of thalassaemia dates back to very early times even before the scientific recognition of the disorder. The modern history of \( \beta \) thalassaemia started in Prague 100 years ago. This was when Von Jaksch reported a case of an anaemic 14-month-old infant with nucleated red blood cells in the peripheral blood and with splenomegaly who died before the age of 2 years. This kind of anaemia was known at that time under the term of Von Jaksch anaemia. After this, different scientific development in the recognition of \( \beta \) thalassaemia occurred early in this century in Europe and the United States of America.

In 1925, Cooley and Lee described in the USA five children with severe anaemia associated with decreased red blood cells (RBCs) osmotic fragility, hepatosplenomegaly and changes in the bones and skull. Cooley and colleagues described another two cases in 1927 similar to those described previously by Von Jaksch and later by him which was known as Von Jaksch anaemia. The same observations were recognised and reported in Italy and Greece by Rietti in 1925, Greppi in 1928 and Micheli in 1935 (Weatherall, 1980). The conditions they described were in adult patients, who had anaemia associated with abnormal morphology and decreased number of RBCs, thus the cases were milder than those described by Cooley. The severe form of the disease was known as Cooley's anaemia and the milder form of this anaemia was known as Rietti-Greppi-Micheli anaemia.

Many cases were reported in the following years. In 1936 Whipple and Bradford described patients with the same kind of anaemia but their background origin was Mediterranean, therefore, they used the Greek term Thalassaemia to describe the condition. Since the origin of all patients described at that time in USA
or Europe were Mediterranean, the term thalassaemia which means the anaemia of
the sea was used to identify the condition.

In the USA particularly in 1944 the various observations made through Europe
and USA were linked by Valentine and Neel (1944) as they proposed the standard
Mendelian inheritance pattern of \( \beta \) thalassaemia as we know it today. Valentine and
Neel (1944) and Smith (1949) demonstrated through their genetic studies that
Cooley's anaemia represents the severe form of \( \beta \) thalassaemia major, i.e., the
homozygous condition; and the milder cases that was described by Rietti-Greppi-
Micheli represents the heterozygous condition.

Later in 1949, Chini and Valeri carried out extensive family studies and
showed that patients with \( \beta \) thalassaemia major have variable haematological
abnormalities. Therefore, they subdivided the conditions into thalassaemia
intermedia (representing the affected status of moderately severe haemolytic
anaemia) and \( \beta \) thalassaemia minor representing those with milder anaemia
(heterozygous state). In the same year, Pauling et al (1949) reported an inherited
defect in the structure of haemoglobin which resulted in sickle cell disease. The
discovery of an abnormal structure of the globin fraction of the haemoglobin either in
thalassaemia cases or in sickle cell started a world-wide initiative to study
extensively the haemoglobin pattern and structure either in normal or affected
individuals.

Many abnormal haemoglobins were discovered (Ingram & Stretlon, 1959) in
association with the clinical picture of thalassaemia. With the combination of the
clinical and genetic studies of these haemoglobin variants they reported that the
clinical manifestation of thalassaemia resulted from a heterogeneous group of defects
in the haemoglobin synthesis. This finding made it possible to classify the concept of
the disease which is based mainly on the genetic control of haemoglobin synthesis.
I - 2: Genetic Basis of Haemoglobin Synthesis:

The significant advances since 1950s in the study of the chemistry of normal and abnormal haemoglobin has led to better understanding of the human globin gene structure and chromosomal organisation (Maniatis et al, 1980).

Normal individuals inherit two β globin chain genes one from each parent; and four α globin chain genes two from each parent. The encoded genes for the globin chains are arranged in two clusters: α and β. The β globin cluster is located on the short arm of chromosome 11 distal band p14 (Gusella et al, 1979) and the α globin cluster has been localised at the tip of chromosome 16 (Buckle et al, 1988).

The earliest embryonic haemoglobin differs in the structure of fetal haemoglobin (Butler et al, 1960). The embryonic haemoglobin consists of ε (β-like) and ζ (α-like) polypeptide chains, these are: Hb Gower 1 (ζ 2, ε 2), Hb Portland (ζ 2, γ 2) and Hb Gower 2 (α 2, ε 2). At the fetal stage, i.e., by eight weeks gestation, the embryonic chains are gradually replaced by fetal haemoglobin (HbF) chains which consists of two α globin chains and two γ chains designated as G γ and A γ. Fetal haemoglobin (α 2, γ 2) is the predominant haemoglobin throughout the fetal life. From 10 weeks of gestations, the γ globin chains are gradually replaced by the adult β and δ globin polypeptides. Thus at the time of birth, fetal haemoglobin comprises approximately 60-90% of the total haemoglobin and during the first few months of postnatal life, the level of fetal haemoglobin rapidly falls to be replaced by adult haemoglobin (HbA). This switch is complete by 6 months after birth.

Weatherall (1980) showed that electrophoresis at an alkaline pH of normal haemoglobin from adult individuals showed that adult haemoglobin consists of two protein fractions: one major fraction known as HbA and one minor fraction called HbA2. In HbA2, the β globin chains are replaced by a pair of δ chains which differ from β chains by 8 amino acids (Ingram & Stretton, 1961). Within six months of birth, 98% of haemoglobin is HbA and approximately 2% are HbA2. Weatherall
showed in 1962 that the α chains of the haemoglobin tetramer have the same chemical constitutions and genetic control both in fetal or adult haemoglobin (Weatherall, 1980). In 1963 Schroeder et al worked out the amino acid sequence of the γ globin chains and found that it is similar in structure to the β chains but differing by of 17 amino acids. Thus the β like chains of the β globin gene cluster encode respectively: embryonic (ε), fetal (γ^0, γ^A), non-functional ψβ and adult (β, δ). These genes were shown to reside within about 50 Kb of chromosomal DNA on chromosome 11 in the transcriptional order from 5' to 3' which reflects the order of their expression during ontogeny (Figure 1 - 1) (Fritsch et al, 1980). These structurally related genes are normally expressed in cells of the erythroid lineage and their expression undergoes a developmental stage related switching mechanism (Weatherall, 1981).

I - 3: β globin gene cluster structure and regulation:

In 1980, the complete nucleotide sequences of the five β globin genes and their flanking regions were determined (Slighton et al, 1980; Spritz et al, 1980; Barelle et al, 1980). All five genes constitute 3 exons which are interrupted by two introns at identical locations. The first intron is located between codons 30 and 31 and it ranges in length of 122-130 bp. The second intron is longer (700-900 bp) and is located between codons 104 and 105 (Figure 1 - 2). Thus each globin gene is approximately 1600 bp in length and the five genes together occupy approximately 17% of the 50 kb segment of chromosome 11 in which they are found. The role of the remaining DNA between the genes is probably important for gene regulation (Flavell et al, 1978; Lawn et al, 1978; Kaufman et al, 1980).

The complete comparison of the human β globin gene sequences that was presented by Efstratiadis et al (1980) which demonstrated the regions of possible functional importance was of tangible benefit in studying the structural differences between normal and mutant β globin genes.
Figure - 1: The genetic control of human globin synthesis. Idea of diagram (adapted from Huehns & Shooter 1965).
Figure 1-2: The structure of the β globin gene.
**β globin gene expression:**

β globin gene expression involves 3 processes: transcription, processing and translation. The transcription and processing occur in the nucleus when genomic DNA is copied to the complementary heterogeneous nuclear RNA molecule (hnRNA). Bunn & Forget showed that there are three sets of conserved sequences at the 5’ untranslated region of the β globin locus which may be serve as regulatory signals for the nuclear RNA molecule (Bunn & Forget, 1986). These sequences are the ATAA box, which is located 30 bp upstream of the cap site; the CCAAT which is situated 80 bp upstream to the cap site and the CACCC which is 90 bp upstream to the cap site (Collins & Weissman, 1984; Efstratiadis et al, 1980). At the cap site, the initiation of transcription occurs and it continues beyond the conserved AATAAA polyadenylation signal.

The hnRNA molecule is then rapidly processed to form mRNA. The introns are removed from the precursor mRNA by splicing and the exons are joined to form mature mRNA, which is then transported from the nucleus to the cytoplasm (Green 1986; Breathnach et al, 1978). The mRNA then acts as a template in which the amino acids are sequentially joined by peptide bonds to form the polypeptide chains by a process called translation (Lewin, 1983). Once the mRNA binds to ribosome in a process called initiation, the synthesis of the protein chain continues by elongation. The translation process starts at the initiator codon (AUG) that sets the reading frame of the mRNA, and this is followed by reading the subsequent triplet nucleotide codons in a linear fashion in a 5’ to 3’ direction until the termination codon is encountered.

The important advances in the understanding of globin gene structure, function and the temporal regulation of its synthesis during development make it a good model for the investigation of β thalassaemia aetiology at the molecular level.
I - 5: β Thalassaemia:

β thalassaemia is defined as a group of heterogeneous anaemias in which β globin synthesis is either absent or reduced (Weatherall & Clegg, 1972; Bank et al, 1980). It is the world’s most widespread autosomal recessive single gene disorder and represents a major health problem. More than 200 million individuals worldwide carry the β thalassaemia gene with an estimation of 100,000 thousands affected individuals born annually (Boyo et al - WHO working group, 1982). β thalassaemia has attracted a great deal of research interest both at the molecular and clinical levels, which has led to the understanding of the complex molecular pathogenesis as well as the provision better treatment and preventive procedures.

I - 5.1: World distribution of β thalassaemia:

The prevalence of β thalassaemia in the world population is summarised in Figure 1 - 3. Although the disorder is prevalent in the Mediterranean, Middle East, Asian ancestry, Africa, South East Asia and Indian subcontinent, it has been described in every racial group. In 1982 the World Health Organisation (WHO) working group estimated the birth of 100s of thousands of affected children with β thalassaemia every year. Although the number of affected children with thalassaemia has decreased in some countries like Sardinia (Figure 1 - 4) due to the extensive work that has been done during the last 20 years toward the control of the spread of thalassaemia, the number of thalassaemics born annually in developing countries remains high. In Egypt, for example, it has been estimated that for every 1.5 million children born annually, 1000 of them are affected and 1500 are carriers of β thalassaemia. (Novelletto et al, 1990), and in India 6000 affected children with β thalassaemia major are born per year (Chakravarty et al, 1994) and 3.3% of the Indian population are known to be carriers of β thalassaemia (Modell et al, 1983). In fact the number of affected children born annually will increase massively during the
Figure 1 - 3: The world distribution of β thalassaemia mutations (Weatherall & Clegg 1996).
Figure 1 - 4: Diagram representing the decreased in number of β thalassaemia births in Sardinia following the application of prenatal diagnosis since 1975 (Cao et al 1993).
next century. Therefore it is of importance to set up global control and management programmes through the international and national agencies.

1 - 5.2: Clinical Manifestations & Pathophysiology of \( \beta \) thalassaemia:

The clinical severity of \( \beta \) thalassaemia varies in different individuals and depends on: the nature of the mutation in the \( \beta \) globin gene (Antonarakis et al, 1984, 1985); the co-inheritance of a gene for hereditary persistence of fetal haemoglobin (HPFH) (Cappellini et al, 1981); the co-inheritance of \( \alpha \) thalassaemia (Waincoat et al, 1983); the co-inheritance of the \( \beta \) globin gene cluster haplotype associated determinant that increases HbF under conditions of erythropoietic stress (Thein et al, 1987; Gilman & Huisman, 1985); and unknown factors that could be related to enzymatic deficiency.

The \( \beta \) thalassaemia is divided clinically into 3 categories based on the severity of the manifestations. These are arranged from the most severe to the least: \( \beta \) thalassaemia major which is characterised by an absent or large reduction of \( \beta \) globin chain production; \( \beta \) thalassaemia intermedia is characterised by reduced amount of the \( \beta \) globin chain synthesis; \( \beta \) thalassaemia minor which is asymptomatic but with prominent microcytic hypochromic RBCs. The clinical status however cannot be used to define the genotype of patients. Patients with \( \beta \) thalassaemia major or intermedia are either homozygous mutant or compound heterozygous.

The clinical manifestations of \( \beta \) thalassaemia major usually present by 6 months of birth when switching of \( \gamma \) chains to \( \beta \) chains usually occurs (Figure 1 - 5). Patients develop a severe haemolytic microcytic anaemia in addition to severe intramedullary and peripheral hemolysis. Untreated children have slow rates of growth and development, and in the adolescents the onset and development of
Figure 1-5: A diagram to explains in summary the pathophysiology of β thalassaemia major (Weatherall, 1996).

- Selective survival of cells containing HbF
- High $O_2$ affinity of RBCs
- Bone deformity
- Increased metabolic rate
- Wasting, Gout, Folate deficiency
- Precipitation
- Haemolysis
- Destruction of RBCs precursors
- Ineffective erythropoiesis
- Anaemia
- Transfusion
- Iron overload
- Endocrine deficiency
- Cirrhosis
- Cardiac Failure
- DEATH

N. El-Hashemite
secondary sexual characteristics are delayed. Patients have skeletal abnormalities secondary to expansion of the erythroid marrow. The hyperactive ineffective bone marrow expands in volume and enlarges the marrow cavity in the skull and other bones as they become weak and easily broken. Many patients have developed cardiomegaly which may be accompanied by signs of congestive heart failure. The abnormality in RBCs causes hypersplenism which in turn accelerate the destruction of red blood cells and shortening their life span. Hyperparathyroidism, diabetes and hypogonadism are another manifestations which result from iron overload.

I - 5. 3: Treatment

Treatment of β thalassaemia is primarily supportive but not curative. It is composed mainly of blood transfusion and iron chelation therapy using desferrioxamine, and in many cases splenectomy is considered. Regular blood transfusion is of importance to maintain haemoglobin value between 10-14 g/dl, which will allow normal growth and development. Desferrioxamine is usually given as a subcutaneous injection through a portable pump (Figure 1 – 6). The obvious benefits of iron chelating therapy are: to reduce iron overload in the body (Grady, 1976; Jacobs, 1979); stop hepatic and pancreatic damage (Barry et al, 1974; Modell et al, 1982; Weatherall et al, 1983); prevent incidence of heart disease and endocrine dysfunction; and to improve survival rate of patients.

The disadvantage of desferrioxamine is the high cost. New advance in chelating therapy is the development of the L1 oral iron chelator which appear promising in the preliminary results (Agarwal et al, 1991), however, side effects such as nausea, fatigue, arthraglia and agranulocytosis following its administration has turned its use (Cohen, 1997). Thalassaemic children who are not given any treatment die before 2 years of age. These who are adequately treated survive adult life.

Bone marrow transplantation which may provide a possible cure. Bone marrow transplantation can be provided for patients who have an HLA-matched donor, preferably a sibling to increase chances of success and rate of survival. This
Figure 1 - 6: β thalassaemia child receiving the subcutaneous injection of iron chelation therapy via a pump. Sharing the same pump between different patients may be a major source of infection [Coleey’s Anemia Foundation leaflet – USA].

Michelle receives blood every two weeks and wears her pump every day.
is usually carried out in patients receiving regular iron chelation therapy, and for those who have not developed hepatomegaly or portal fibrosis (Lucarelli, 1991; Galimberti et al, 1997). The success rate of bone marrow transplantation is still very low and deaths have occurred following transplantation either because of rejection or because of developing other pathological diseases.

The development of gene therapy could be a promising cure for genetic disorders. Extensive work has been done on developing gene therapy for the thalassaemias in the last few years. Although Grosveld et al (1987) and Higgs et al (1990) have described the possibility of development of retroviral vectors as an approach for gene therapy for haemoglobinopathies, the possibility of gene therapy will not be feasible in the near future. This is because many problems need to be solved. The major problem is with β globin gene expression. The upstream region known as locus control region (LCR) that controls the β globin gene cluster regulation (Grosveld et al, 1987) has had an enormous benefits particularly in therapeutics in which development of gene therapy and manipulation of the switching of fetal haemoglobin to adult haemoglobin production. The LCR needs to be included in order to express the β globin gene at high levels. The problem is that LCR has been found to be unstable in retrovirus. Furthermore, even when using stable vectors, viruses express β globin in in vitro cultures but not in animals as they turned out to be silent in vivo (Hughes, 1997).

Until now, the treatment required for thalassaemia is unsatisfactory and very expensive, therefore the idea of preventing the spread of β thalassaemia arose through organising genetic screening and counselling programmes and developing preventive techniques in order to provide prenatal diagnosis for couples at high risk.

I - 5.4: Molecular basis of β thalassaemia

The heterogeneity of β thalassaemia at the clinical level suggested the existence of many different β thalassaemia alleles. Sequence analysis of the β globin
gene from normal and thalassaemic individuals revealed that the β thalassaemia phenotype is usually associated with point mutations, deletion or insertion and in rare cases the third exon of the gene is deleted (Weatherall & Clegg, 1982). To date more than 150 different β globin mutations have been identified that cause β thalassaemia (Baysal & Huisman, 1995). Depending on the effect of these mutations on the globin peptide synthesis, thalassaemia mutations can be divided into two categories: β0 in which β globin peptide synthesis is completely absent, and β+ in which the β globin chains synthesis is reduced by 30% or more. Most of the mutations that have been identified are single point mutation substitutions affecting gene regulation and expression.

β thalassaemia mutations can be subdivided according to their region of location in the β globin gene and its effect on gene function:

1 - Gene deletions: A number of large deletions affecting the β globin gene cluster have been described and resulting in the majority of δβ0, γδβ0 and different forms of δβ0-HPFH. These deletions result in the removal or inactivation only of the δ and β globin genes (such as in δβ0 and γδβ0 thalassaemias) or in absence of β globin chain production. Few number of deletions affecting only the β globin gene have been described such as the 619 deletion at the 3’ end of the gene which causes β0 thalassaemia in Asian Indians (Orkin et al, 1979).

2 - Transcriptional mutations: are mainly located either 30 bp upstream from the cap site, in the ATAA box or in the proximal and distal CACACCC sequences situated 90 and 105 bp upstream from the cap site. Such mutations (Table 1 - 1) reduce the transcription of the gene and cause a mild clinical phenotype (Antonarakis et al, 1984). However, a severe form of the disease has been described in Chinese (Huang et al, 1986) which could be due to the absence of co-existence of genetic determinants that increase HbF production.
Table 1-1: Examples of types and ethnic origin of mutations causing β thalassaemia and the resulting phenotype. $\beta^0$ indicates the complete absent of β globin peptide synthesis, while $\beta^+$ indicates reduced β globin peptide synthesis (adapted from the β and δ thalassaemia repository, eighth edition, 1995).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Origin</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcriptional:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-88 C-T</td>
<td>American Black</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>-87 C-G</td>
<td>Mediterranean</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>-28 A-G</td>
<td>Chinese</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>-30 T-G</td>
<td>Chinese</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>-29 A-G</td>
<td>American Black/Chinese</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td><strong>RNA processing:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1- Splice Junction Site:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVSI nt 1 G-A</td>
<td>Mediterranean</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>IVSI nt 1 G-T</td>
<td>Asian Indian</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>IVSII nt 1 G-A</td>
<td>Mediterranean</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>IVSII 850 G-A</td>
<td>English/Scottish</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>2- Consensus Site:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVSI nt 5 G-C</td>
<td>Asian Indian</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>IVSI nt 6 T-C</td>
<td>Mediterranean</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>3- IVS Changes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVSI nt 110 G-A</td>
<td>Mediterranean</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>IVSII nt 745 C-G</td>
<td>Mediterranean</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>IVSI nt 116 T-C</td>
<td>Mediterranean</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>4- Coding Region Changes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD 27 G-T (Hb Knossos)</td>
<td>Mediterranean</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>CD 19 A-G</td>
<td>Malay</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>CD 24 T-A</td>
<td>Japanese</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td><strong>RNA Translation:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1- Nonsense:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD 15 G-A</td>
<td>Asian Indian</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>CD 39 C-T</td>
<td>Mediterranean</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>CD 127 C-T</td>
<td>English</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>2- Frameshift:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD 5 -CT</td>
<td>Mediterranean</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>CD 8 -AA</td>
<td>Mediterranean</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>CD 8/9 +G</td>
<td>Asian Indian</td>
<td>$\beta^0$</td>
</tr>
<tr>
<td>CD 41/42 -TTCT</td>
<td>Chinese</td>
<td>$\beta^0$</td>
</tr>
</tbody>
</table>

IVS = intervening sequence (intron); CD = codon; nt = nucleotide.
3 - RNA processing mutations: are divided into three different types as follow:

- Cap Site mutation A-C affects either transcription or translation (Wong et al, 1987) and produces a very mild symptoms. However its combination with a severe β thalassaemia mutation produces a seriously clinical condition.

- RNA Cleavage & Polyadenylation Mutations affect RNA cleavage efficiency in which case only a small percentage of the RNA is cleaved and the rest remains uncleaved which results in decreased β globin synthesis (Orkin et al, 1985). Such mutations of the polyadenylation signal, e.g. AATAAA-AACAAAA and AATAAA-AATGAA (Orkin et al, 1985; Jankovic et al, 1989) produces causes β⁺ thalassaemia as some of the normal transcripts are produced.

- The third type of mutations affects the RNA splicing (Table 1–1) which causes either complete absence of normal splicing that result in β⁰ thalassaemia or incomplete splicing that result in β⁺ thalassaemia (Cheng et al, 1984; Lapoumeroulie et al, 1986; Orkin et al, 1984).

4 - Chain termination mutations such as nonsense mutations, for example at CD 39 and CD 17, alter the coding signal to a termination signal, i.e. results in premature termination of translation and β⁰ thalassaemia (Trecartin et al, 1981; Chang & Kan, 1979). The frameshift mutations, which result from a deletion or insertion of few nucleotides in the coding region, produce a non-functional mRNA. Frameshift mutations cause a disrupting in the normal reading frame, e.g. the insertion of G at codons 8 and 9 results in premature termination signal at codon 22 and leads to an abnormal RNA (Kazazzian et al, 1984).

5 - Dominant β thalassaemia:
Although β thalassaemia disorders are inherited in the recessive form, a number of studies reported a rare dominant form of the β thalassaemia in heterozygote patients with dyserythropoietic anaemia associated with inclusion bodies in red cell precursors (Kazazzian et al, 1989; Thein et al, 1990). The dominant allele has been
described in single families of different ethnic groups, and found to be as a result of mutations in exon 3 of the β globin gene that cause unstable elongation of the β globin chains and produces aggregations, i.e. inclusion bodies that are resistant to intra-cellular proteolytic digestion. Unlike the recessive of β thalasaemia, the dominant form have not accomplished high gene frequencies in parts of the world, this may be because of its severity that prevented it from the positive selection pressure due to malaria.

In general, each ethnic group has their own battery of mutations (Orkin & Kazazzian, 1984), which has been defined by the number of mutations that are prevalent in a population group, for example, Mediterranean (Kazazzian et al, 1984), Chinese (Chan et al, 1987) and Asian Indian (Thein et al, 1988). The world distribution of β thalassaemia mutations is shown in Figure 1-3.

The detailed information on the nature of the mutations and their distribution on within the world populations are due to the progress in techniques of molecular analysis. The recent development of methodologies has facilitated the screening for mutations causing β thalassaemia. The most powerful is the polymerase chain reaction (PCR) (Saiki et al, 1988; Wong et al, 1987, Rosatelli et al, 1985) combined with direct and indirect methods used for the screening and detection of mutations.

1 - 6: Sickle Cell Anaemia (SCA):

1 – 6.1: Introduction:

Sickle cell disease is an autosomal recessive disorder that caused by a single base substitution in codon 6 (A-T) of the β globin gene that change glutamic acid to valine. This mutation produces sickle haemoglobin (HbS) and changes the behaviour
of haemoglobin molecules which tend to polymerise on deoxygenation. In turn, RBCs become less pliable and some are deformed into the characteristic sickle shape.

Sickle cell disease is acquired in different forms, the most common is the sickle cell anaemia (SCA) which represents the homozygous form. The next form of sickle cell disease is the double heterozygote with an abnormal haemoglobin or thalassaemia. This is common in West African populations and it gives rise to different phenotypes depending on the nature of the second mutation. The inheritance of HbS and the gene for HbC for example is usually a mild form of the disease. Sickle cell thalassaemia is frequent through West Africa and some of the Mediterranean populations. Depending on the type of the β thalassaemia mutation, a wide spectrum of clinical phenotypes can result. If the β thalassaemia mutation is mild, the result is Sβ+ which is characterised by 20-30% of HbA and a mild clinical disorder. However, in the case of β0 mutations, the result is a very severe form of the disease that is similar to SCA, and is characterised by a complete absent of HbA. The clinical picture of the sickle cell disease is heterogeneous and the frequency at birth of the above forms is dependent on the population and the frequency of the different forms of mutations.

1 - 6.2: Epidemiology of SCA:

SCA was first recognised among individuals of West African origin. The disease is quite common among Equatorial African individuals as it occurs in up to 30% of the populations, however it is less frequent in north or South African populations. The inheritance of HbS has been found to be more widespread around the Mediterranean, the eastern province of the Arabian Peninsula and throughout central India. The main factor for this distribution along with the occurrence of HbS is the gene selection by falciparum malaria, as heterozygous individuals for HbS have a relative resistance to malaria which increases the possibility for survival, breeding and passing on the gene.
1 - 6. 3: Pathophysiology & Clinical Manifestations of SCA:

The deoxygenation in sickle haemoglobin results in a hydrophobic interaction with other haemoglobin molecules triggering an aggregation into large polymers. The polymerisation of deoxygenated HbS which is considered the first event in the pathogenesis of the disorder, result in a marked decreased deformability and the sickle shape of RBCs. These rigid cells cannot negotiate the capillary beds and this results in premature destruction of RBCs (Haemolysis) and blockage of blood flow (vaso-occlusion) which are the hallmark of sickle cell disease. Figure 1 - 7 summarise the clinical results of haemolysis and vaso-occlusion in sickle cell disorder.

II: Cystic Fibrosis (CF)

II – 1: Introduction

Cystic fibrosis is considered the most common fatal genetic disorder in Caucasian children. It was recognized to be an inherited disease in 1946 by Anderson et al and found to be an autosomal recessive trait (Nadler, 1978) and affect both sexes with equal frequency. Cystic fibrosis is a general disease of the exocrine glands and is characterized by thick secretions in the airways and intestines which blocks the lumen, excessive salt secretion in the sweat, pancreatic insufficiency caused by blockage of the pancreatic duct with mucus, male infertility and sometimes liver failure.

Although the frequency of the disease varies in white populations, the incidence of affected new-borns from Northern European origin is 1 in 2500 (McGrae et al, 1990). Thus, the carrier frequency considered being 1 in 25 in this ethnic group for ΔF508 mutation.
Figure 1 - 7: Summary of the pathophysiology of SCA.

Sickle Cell Disease

Polymerisation of deoxygenated HbS

Haemolysis  Vaso-occlusion  Bone pain crisis  Others

Megaloblastic erythropoiesis
Aplastic crisis
Clinical jaundice & gallstones

Dactylitis

Acute Chest syndrome
Genitourinary problems
Leg ulcers

Splenic manifestations:
stroke  Sickle retinopathy  Impaired growth  pregnancy complications
pneumococcal septicaemia, splenic sequestration, hypersplenism
The prevalence of the disease permitted extensive studies of the cystic fibrosis phenotype at the cellular level, which led to the identification of the biochemical and physiological defects responsible for the disease. The physiological studies showed that CF is an epithelial disease, which affects the regulation of ion transport. A major defect in Cl- transport was observed in CF epithelia, which helps to explain the major findings in the CF phenotype (Quinton, 1990; Welsh, 1990). The CF epithelial tissues were classified as anion secreting (pancreatic ducts), cation absorbing (sweat gland duct) or both (airways). Such findings directed biochemical research to focus on the understanding of the mechanism and regulation of Cl- transport in epithelial cells (Kartner et al, 1992; Marino et al, 1991; Rosenfeld et al, 1992).

II - 2: Isolation of the CF gene

With the discovery of restriction fragment length polymorphisms (RFLPs) it became possible to use a limited number of DNA markers throughout the human genome and determine the approximate map location of any single gene (Botstein et al, 1980). A large number of studies were performed which excluded certain regions or chromosomes from consideration as the location for the CF gene (Tsui et al, 1985).

A genetic approach of identifying the CF gene became feasible when it was found to be linked to the gene encoding the enzyme paraoxonase (Eiberg et al, 1985) and to the DNA marker D7S15 (Tsui et al, 1985). Shortly afterwards, the gene was mapped to chromosome 7 (Knowlton et al, 1985), and detailed mapping and linkage analysis further localized the CF locus to 7q 31-32 (White et al, 1985; Wainwright et al, 1985; Zengerling et al, 1987; Duncan et al, 1988; Beaudet et al, 1986; Lathrop et al, 1988). The cloning of the CF gene along with identifying the most common mutations was reported in 1989 (Rommens et al, 1989; Riordan et al, 1989; Estivill et al, 1989).
II - 3: Structure of the CF gene

Zeilenski and colleagues (1991) identified the genomic DNA sequence of the CF gene and it was found to be of approximately 250 kb in length (Figure 1 - 8). The 6.5 kb mRNA transcript observed in most tissues by Northern blot analysis consists of a relatively short 5' untranslated region of approximately 60-100 bp, 4.44 kb of open reading frame, a 1.6 kb 3' untranslated region and a polyadenylic acid tail (Riordan, 1989). The CF gene consists of a total of 24 exons ranging in size from 38-724 bp and intervening sequences which range from 1.1 to 40 kb (Zielenski et al, 1991).

The product encoded by the CF gene was termed the cystic fibrosis transmembrane conductance regulatory (CFTR) protein. The protein structure predicts a repeated motif consisting of a transmembrane domain with six membrane spanning regions (Riordan et al, 1989). The motifs are separated by a highly charged hydrophilic domain which is designated as R (regulatory) which contains several potential protein kinase A and protein kinase C phosphorylation sites (Figure 1 - 9).

Based on the predicted domain structure of the polypeptide, it has been found that the CF proteins are members of the class of proteins which includes the multidrug resistance P-glycoprotein and several bacterial amino transport proteins. Such proteins are characterized as pumping proteins into or out of the cells which when altered or absent could lead to the pathophysiology of cystic fibrosis.

II - 4: Expression of the CFTR

The expression of the CFTR gene is consistent with on the pathology of the disease in the epithelial cells. Using Northern blot analysis of total RNA, it has been found that CFTR is detected at high levels in the pancreas and nasal polyps; less in the lungs, liver, testis and sweat glands (Riordan et al, 1989). It has been found that other organs such as stomach, intestine and kidney express the CFTR transcripts at levels comparable to or greater than the level of expression in the lung (Gregory et al, 1990; Riordan et al, 1989).
Figure 1 - 8: Schematic diagram of the steps taken for identifying the CF gene and the structure of the CF gene which consists of 24 exons and its position on chromosome 7.
Figure 1-9: The structure of the cystic fibrosis transmembrane conductance regulator embedded in the lipid layer of the cell membrane.
Although the expression of the CFTR is generally consistent with the pathology of the disease, other studies showed that the level of expression is quite variable and does not always correlate with the pathology (Trezise & Buchwald, 1991; Sarkadi et al, 1992; Trapnell et al, 1991). For example, the level of CFTR as assayed by RNA and protein appears to be low in the lungs in comparison with the kidney which have a relatively high level of expression.

The characterization of the CFTR gene and its mutations helped in the understanding of the CFTR biology and in identification the factors that influence its expression and function.

II - 5: Mutation analysis of the CF

The identification of 3 bp deletion in a partial cDNA from a CF patient (Kerem et al, 1989) which has been found to remove a phenylalanine residue at the position 508 of the translation product, was a primarily confirmation for the identity of the gene and its involvement in CF. Different types of mutations have been found in the CFTR, with the most common type being missense, followed by frameshift and nonsense mutations (Table 1 - 2) (Kerem et al, 1989; Zielenski et al, 1991).

The ΔF508 mutation, which is considered the most common mutation, was found in 68% of the CF chromosomes worldwide and more than 70% of CF chromosomes in northern European populations with highest frequency reported in Copenhagen and Stockholm. The frequency of the ΔF508, however, was found to be lower in other ethnic groups (Figure 1 - 10). For example, among southern European only 1/2 of the CF chromosomes bear ΔF508 (Gasparini et al, 1991) and among the Jewish population it is seen in approximately 1/4 of the CF chromosomes (Ng et al, 1991). Based on this variation in the frequency of ΔF508 among the Euro-Asia Caucasian populations, haplotype analysis to study the geographic origin of ΔF508 has been conducted (Serre et al, 1990).
Table 1-2: Different mutations causing cystic fibrosis at different position within the gene, the most common types are missense, nonsense and frameshift mutations.

<table>
<thead>
<tr>
<th>Name of Mutation</th>
<th>Amino Acid Change</th>
<th>Nucleotide Change</th>
<th>Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>241delAT</td>
<td>Frameshift</td>
<td>-AT at nt 241</td>
<td>2</td>
</tr>
<tr>
<td>D44G</td>
<td>Asp-Gly at CD 44</td>
<td>A-G at nt 263</td>
<td>2</td>
</tr>
<tr>
<td>G85E</td>
<td>Gly-Glu at CD 85</td>
<td>G-A at nt 386</td>
<td>3</td>
</tr>
<tr>
<td>444delA</td>
<td>Frameshift</td>
<td>-A at nt 444</td>
<td>4</td>
</tr>
<tr>
<td>R117H</td>
<td>Arg-His at CD 177</td>
<td>G-A at nt 482</td>
<td>4</td>
</tr>
<tr>
<td>621+1G-T</td>
<td>Splice mutation</td>
<td>G-T at nt 621 +1</td>
<td>intron 4</td>
</tr>
<tr>
<td>G178R</td>
<td>Gly-Arg at CD178</td>
<td>G-A at nt 664</td>
<td>5</td>
</tr>
<tr>
<td>711 +1G-T</td>
<td>Splice mutation</td>
<td>G-T at nt 711 +1</td>
<td>intron 5</td>
</tr>
<tr>
<td>R297Q</td>
<td>Arg-Gln at CD 297</td>
<td>G-A at nt 1022</td>
<td>7</td>
</tr>
<tr>
<td>1078delT</td>
<td>Frameshift</td>
<td>-T at nt 1078</td>
<td>7</td>
</tr>
<tr>
<td>1342 -1G-C</td>
<td>Splice mutation</td>
<td>G-C at nt 1342-1</td>
<td>intron 8</td>
</tr>
<tr>
<td>A455E</td>
<td>Ala-Glu at CD 455</td>
<td>C-A at nt 1496</td>
<td>9</td>
</tr>
<tr>
<td>G480C</td>
<td>Gly-Cys at CD 480</td>
<td>G-T at nt 1570</td>
<td>10</td>
</tr>
<tr>
<td>Q493X</td>
<td>Gln-Stop at CD 493</td>
<td>C-T at nt 1609</td>
<td>10</td>
</tr>
<tr>
<td>ΔF508</td>
<td>Deletion of Phe at CD 508</td>
<td>3 bp del between nt 1652 and 1655</td>
<td>10</td>
</tr>
<tr>
<td>1717-1G-A</td>
<td>Splice mutation</td>
<td>G-A at nt 1717 -1</td>
<td>intron 10</td>
</tr>
<tr>
<td>G524X</td>
<td>Gly-Stop at CD 542</td>
<td>G-T at nt 1756</td>
<td>11</td>
</tr>
<tr>
<td>1784delG</td>
<td>Frameshift</td>
<td>-G at nt 1748</td>
<td>11</td>
</tr>
<tr>
<td>Y563N</td>
<td>Tyr-Asn at CD 563</td>
<td>T-A at nt 1819</td>
<td>12</td>
</tr>
<tr>
<td>1898+1G-A</td>
<td>Splice mutation</td>
<td>G-A at nt 1898 +1</td>
<td>intron 12</td>
</tr>
<tr>
<td>G628R</td>
<td>Gly-Arg at CD 628</td>
<td>G-A at nt 2014</td>
<td>13</td>
</tr>
<tr>
<td>2043delG</td>
<td>Frameshift</td>
<td>-G at nt 2043</td>
<td>13</td>
</tr>
<tr>
<td>Y913C</td>
<td>Tyr-Cys at CD 913</td>
<td>A-G at nt 2870</td>
<td>15</td>
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<tr>
<td>R1158X</td>
<td>Arg-Stop at CD 1158</td>
<td>C-T at nt 3604</td>
<td>19</td>
</tr>
<tr>
<td>3662delA</td>
<td>Frameshift</td>
<td>-A at nt 3662</td>
<td>19</td>
</tr>
<tr>
<td>S1225X</td>
<td>Ser-Stop at CD 1255</td>
<td>C-A at nt 3896</td>
<td>20</td>
</tr>
<tr>
<td>N1303H</td>
<td>Asn-His at CD 1303</td>
<td>A-C at nt 4039</td>
<td>21</td>
</tr>
<tr>
<td>E1371X</td>
<td>Glu-Stop at CD 1371</td>
<td>G-T at nt 4243</td>
<td>22</td>
</tr>
<tr>
<td>4374 +1G-A</td>
<td>Splice mutation</td>
<td>G-A at nt 4374 +1</td>
<td>intron 23</td>
</tr>
</tbody>
</table>

N. El-Hashemite 52
Figure 1-10: The frequency of ΔF508 mutation causing cystic fibrosis in Europe
Up to date, more than 140 different mutations causing CF have been identified. From these analyses, it has been found that only a small number of the mutations are common in each ethnic group and the rest are described only in individual families. A variety of types of mutation have been found in the CFTR gene, and many more remain to be discovered. The indication of uneven distribution of mutations among the various presumptive functional domains of the protein, simply reflects the localization of the more frequent mutations. This helped to define the biochemical activities of the mutant alleles which confirmed that the CFTR is intrinsically a chloride channel protein (Cutting et al., 1990; Kerem et al., 1990; Rich et al., 1990; Drumm et al., 1990; Anderson et al., 1991; Rommens et al., 1991; Bear et al., 1992).

II - 6: Genotype-Phenotype correlation in CF

The clinical presentation of CF is heterogeneous and the severity of the disease varies at different ages. The most remarkable clinical finding is the pancreatic function status among affected individuals suggesting that the degree and the rate of the progression of the pancreatic disease is predetermined by the patient's genotype (Corey et al., 1989).

Analysis has demonstrated a correlation between pancreatic function and mutations in the CFTR gene (Kerem et al., 1990; Kristidis et al., 1992). Therefore, CF mutant alleles were classified into two classes (Kerem et al., 1989): one that contributes to pancreatic insufficiency (PI) and one that confers pancreatic sufficiency (PS). The PI results from the acquisition of two severe alleles whereas the PS phenotype is conferred by mild CFTR mutations. In fact, since most CF patients have the PI phenotype, this indicates that most of the CF mutations are severe ones, and very few one are considered as mild ones, i.e., each genotype is associated with either PI or PS but not both.
II - 7: Clinical manifestations of CF & Treatment

The significant physiological defect in CF is related to the sodium and chloride transport. The dysfunction of the exocrine glands produce viscid secretions of low water content that results in pancreatic insufficiency and respiratory failure. The pancreatic insufficiency results in consequent malabsorption while respiratory failure results as the mucus that fills airways provides a rich environment for organisms such as pseudomonas species which cause recurring bronchial infection that leads to progressive lung damage.

Along with respiratory problems CF patients usually present with abnormal bowel function, failure to gain weight, and distended abdomen. Enlarged liver is also seen in CF patients due to excessive fat deposition. Hypoproteinaemia and generalized oedema have been described. The low levels of pancreatic enzymes also cause intestinal problems such as intestinal obstruction. The improved life expectancy of CF patients has increased the incidence of diabetes mellitus. The early signs of puberty may be delayed which is associated with delayed skeletal maturity, which could result from the chronic pulmonary problems. The majority of males are sterile because of the abnormal development of the vas deferens and epididymis. Episodes of arthritis such as swelling joints, pain, tenderness and limitation of movements also have been reported in CF patients.

The clinical management of CF patients mainly depends on relieving and treating the symptoms. Currently, lung transplantation offers the means of optimal survival for CF patients, however, it remains an expensive procedure and may not be successful in all patients due to organ rejection.

II - 8: Prenatal Diagnosis of CF

Cystic fibrosis remains a serious disorder and is considered life shortening, despite the improvement of the clinical management that have occurred in the last few
years. The median survival age has been indicated to be 26 years in USA and 19 years in UK (Boat 1989; British Pediatrics Association 1988). Prior to the identification or establishment of genetic linkage of the CFTR gene locus, attempts were made to provide neonatal screening of CF based on theoretical pathophysiology. Hosli and colleagues (Hosli et al, 1981) suggested that the induction of alkaline phosphatase in the cultured amniotic fluid cells by Tamm-Horsfall protein could be used to diagnose an affected fetus with CF. Because the biochemical basis of this test was not clear or explained, other laboratories could not repeat the test.

Another test proposed by Nadler et al (Nadler et al, 1980a; 1980b) based on the titration of trypsin-like protease against the artificial substrate - 4 methyllumbelliferylguanidinobenzoate (MUGB) - that showed initial promise in prenatal detection of affected fetuses through measuring the esterolytic component of amniotic fluid albumin (Tummler et al, 1982), but sooner has been declined because it was not reproducible in other centers.

Just before the discovery of the CFTR gene, a system, which has a physiological basis, was introduced for prenatal diagnosis of CF (Brock et al, 1984). The method involving an assay of microvillar enzymes in second trimester amniotic fluid supernatant, in which the activity of two peptidase g-glutamyltranspeptidase (GGTP) and amniopeptidase M (AMP) were significantly depressed in the presence of an affected fetus (Carbans et al, 1983; Baker et al, 1983). This system is still occasionally used particularly when the parental genotype is uncharacterized.

The identification of the CF gene and the ability to directly detect the mutation alleles have opened an era for a preventive programs for carrier testing, genetic counseling and prenatal or preimplantation diagnosis (Lemna et al, 1990; Rommens et al, 1990, Halianos et al, 1989; Handyside et al, 1992). Antenatal diagnosis of CF through direct detection of mutant alleles can be invariably detected after PCR amplification either by heteroduplex analysis, allele specific oligonucleotides (ASO), restriction enzyme digestion or allele refractory mutation system (ARMS) (Kerem et al, 1989; Halley et al, 1989; Taylor, 1989; Mathew, 1989; Bellabio et al, 1990; Newton et al, 1989; Rommens et al, 1990; Ng et al, 1991;
Preimplantation genetic diagnosis was performed successfully for cystic fibrosis by amplifying across the ΔF508 locus (Handyside et al, 1992). In UK, a total of 18 PGD cycles for the ΔF508 mutation for 12 couples have been performed and resulted in 5 singleton births of homozygous normal genotype (Ao et al, 1996).

III: PRENATAL DIAGNOSIS:

III - 1: Introduction

As the management of thalassaemia major is expensive and life long, therefore, the aim of controlling the disease became a major public health goal for many countries particularly those with high incidence of β thalassaemia. The preventive programme included screening the population to identify carriers, genetic counselling and the offer of prenatal diagnosis (Figure 1 - 11). The aim of prenatal diagnosis is to give couples at high risk an informed choice (Petrou et al, 1990).

1II - 1.1: Screening

A successful prevention programme of β thalassaemia major by antenatal diagnosis depends on the success of screening programmes. The strategy of heterozygous screening and identification can be performed either by clinical haematology tests or at the molecular genetic level. The country's geographical location, the population history as well as its interaction with other societies and populations and the mutations which are prevalent in the neighbouring countries have to be considered. However, haematology tests are more sensitive for carrier screening, and should be combined with molecular tests.
Figure 1-11: Diagram to show a preventive programme strategy. Adapted from Petrou et al 1990.
The clinical haematology tests involve measuring the Mean Corpuscular Haemoglobin (MCH), Haemoglobin values and Mean Cell Volume (MCV). The values of haemoglobin, MCV and MCH are reduced in heterozygous individuals. Also the osmotic fragility of RBCs is reduced.

The advantage of combining both tests in national screening programmes is that some samples could show negative results for the most common mutations prevalent at this region, and this does not mean that this particular person is normal, because many mutations are still uncharacterised in certain populations. If haematology tests showed that an individual is heterozygous for β thalassaemia, sequencing analysis must be carried out to identify the mutation causing the disease, which will help in mutation detection and fetal diagnosis. Figure 1 - 12 illustrate a flow chart strategy in population screening for thalassaemia.

**III – 1.2: Counselling**

Once carriers are identified, genetic counselling is the next step of prevention programme. In fact, genetic counselling is considered the cornerstone of a preventive procedure and is an important service. Through genetic counselling, couples at high risk will know how the occurrence and the risk of recurrence of β thalassaemia within their families. This helps couples at high risk to understand the implications of thalassaemia and to consider the reproductive options. The main objectives of genetic counselling are: to provide education to parents about their condition, to prevent the spread of affected individuals with genetic diseases by encouraging couples at risk to attend prenatal diagnosis and to explain to them that through this they have the opportunity of confirming the normality of their future offspring. Thus the most important adjunct of prenatal diagnosis is genetic counselling.
Figure - 12: Diagram illustrates flow chart for laboratory strategy in population screening for thalassaemia (Cao et al 1980).

Indices

MCH > 27 pg = Not thalassaemia

Sickledex +ve

electrophoresis

-ve = not HbS trait

Positive

HbS trait

Normal

Microcytosis, MCH < 27 pg

Hb Lepore
HbH disease
HbE trait
Hb CS

electrophoresis

positive

HbA2 estimation

Low or normal

HbF estimation

If MCH > 25 pg

α-thalassaemia trait

Normal child or unknown diagnosis

If MCH < 25 pg

Globin biosynthesis study

unbalanced

Raised > 3.5%

Classical β thalassaemia trait

δβ thalassaemia trait
HPFH

Raised > 2%

α1 thalassaemia trait
“normal A2” β thalassaemia trait
III - 2: Prenatal diagnosis

Prenatal diagnosis of β thalassaemia has played a major role in decreasing the frequency of β thalassaemia major in some countries such as Sardinia (Cao et al, 1990), Cyprus (Angastiniotis & Hadjiminas, 1981) and Greece (Loukopoulos et al, 1990). Such programmes have benefited couples at high risk by allowing them to have healthy families and the countries have successfully reducing the annual rate of affected children to around zero (Old, 1992). Since the middle of 1970s prenatal diagnosis of β thalassaemia became possible as a third trimester procedure with the advent of fetal blood sampling. Developing second trimester (amniocentesis) and then first trimester (chorionic villus sampling) procedures as soon as fetal DNA analysis became possible followed this.

III – 2.1: Fetal Blood Sampling

In 1974, prenatal diagnosis of β thalassaemia was first achieved by fetal blood sampling through the analysis of fetal blood by of globin chain synthesis (Fairweather et al, 1978). The method was initiated after the development of safe techniques for fetal blood sampling at 18 to 20 weeks of gestation.

Valenti in 1973 collected fetal blood from a patient who was scheduled for abortion (Valenti, 1973). In 1974, Hobbins et al (1974a) developed a special cannula to obtain fetal blood under endoscopic control from continuing pregnancies. In the same year the method was first used in prenatal diagnosis for β thalassaemia (Hobbins et al, 1974b).

Rodeck and Campbell also used the fetoscopy (Rodeck & Campbell, 1979), as to collect fetal blood from the umbilical cord in its area of insertion into the placenta. Although this approach allowed the retrieval of fetal blood which was not contaminated with either maternal blood or amniotic fluid, a high rate of
complications and substantial 5-10% risk to the fetus was reported (Alter, 1985) and limited the use of fetoscopy as a technique for fetal blood sampling.

Following the advances in high resolution ultrasound, Daffos et al introduced in 1983 an approach of umbilical cord blood sampling under direct ultrasound guidance (Figure 1 - 13) (Daffos et al, 1983). Fetal blood is then withdrawn into a syringe containing a small amount of anticoagulant. Once an appropriate volume of fetal blood is obtained, the needle is withdrawn and fetal heart rate, uterine activity and umbilical cord punctured site are monitored for fetal well being and bleeding respectively. The volume of fetal blood that can be obtained increases with the gestational age. In most cases, blood will be obtained from the umbilical vein because puncturing the umbilical artery has been associated with fetal bradycardia (Weiner, 1987). Furthermore, the more advanced the gestational age the more success in obtaining pure fetal blood is achieved because the umbilical vessels increase in size. The duration of the procedure does not exceed 10 minutes (Daffos et al, 1985; Boulot et al, 1990) and it is mainly dependent on the placenta location and the operator expertise.

The first application of fetal blood sampling was the prenatal diagnosis of haemoglobinopathies. The diagnosis is based globin chain biosynthesis studies. The process involves incubation of fetal blood with radioactive Leucine to label the globin chains. Lysing RBCs and precipitation of globin follows this. The precipitated globin are then separated into carboxymethyl cellulose chromatography (Clegg et al, 1965; Alter et al, 1976) to assess the amounts of β chains present (Alter, 1983). Manca et al, (1986) introduced the analysis of fetal blood for diagnosing β thalassaemia by isoelectric focusing of haemoglobin tetrarmeres and thereafter avoiding radioactivity and the need for biosynthesis.
Figure 1 - 13: Diagram showing precutaneous fetal umbilical blood sampling (cordocentesis).
Prenatal diagnosis of haemoglobinopathies using fetal blood sampling was performed in more than 20 centres worldwide and more than 13000 cases have been reported to the World Health Organisation (Alter, 1990). Although this method was reasonably successful, it was associated with many problems. It relies on obtaining fetal blood, which is not possible before 18 weeks of pregnancy ([Alter, 1990); this makes the decision of elective abortion difficult for the mother because of the late stage of pregnancy and 2% risk of fetal loss. All of this turned the attention towards developing earlier prenatal diagnosis using amniotic fluid DNA. In addition, the advent of prenatal molecular genetic diagnosis techniques particularly the polymerase chain reaction (PCR) (Saiki et al, 1988) has decreased the use of fetal blood sampling which has been replaced by amniocentesis as a midtrimester test and later by chorionic villus sampling in the first trimester.

11 – 2.2: Amniocentesis

Amniocentesis was first introduced in early 1880s in Germany as a surgical technique by Lambl to treat polyhydramnios and later in 1937 Aureau had used amniocentesis procedure for pregnancy termination through introducing hypertonic saline into the amniotic sac (James, 1956). In 1956 several investigators reported the determination of fetal gender from amniotic fluid cells (Fuchs et al, 1956; James et al, 1956; Makowski et al, 1956). This was followed by a formal introduction of prenatal diagnosis of genetic disorders by Steele & Berg in 1966 by performing chromosomal analysis of cultured amniotic fluid cells (Steele & Berg, 1966). Jacobson and Barter (1967) reported the first antenatal diagnosis of chromosomal abnormalities, and Valenti et al reported the diagnosis of Down syndrome which was confirmed after elective abortion (Vlenti et al, 1967), and Nadler reported the diagnosis of galactosemia in utero from amniotic fluid cells (Nadler, 1968). Nadler & Grebie (1970) published a summary of their initial experience with prenatal genetic diagnosis in 142 patients using amniocentesis. They revealed the low risk of the procedure for the mother and the fetus as well as the accuracy of diagnosis. This
report helped to establish fetal genetic diagnosis by amniocentesis as a part of modern obstetric care (Nadler & Grebie, 1970).

The procedure of amniocentesis is traditionally performed at about 15-16 weeks of gestation, this is because the uterus can be approached abdominally and it contains a sufficient amount of amniotic fluid, which ranges between 200 to 250 ml, to permit the aspiration of 20 ml from the fluid with safety (Emery, 1970). Transvaginal amniocentesis has been associated with difficulty, infection and spontaneous abortion following the procedure therefore the abdominal approach has been recommended (Scrimegeour, 1973).

Amniocentesis is performed by the abdominal approach (Figure 1 - 14) under ultrasonographic monitoring to visualise the needle continuously. About 20 ml of the amniotic fluid is then aspirated and subjected to genetic analysis. Several investigators (Golbus et al, 1979; Romero et al, 1985; Tabor et al, 1986) have reported that the efficiency of amniocentesis is dependent on 3 factors: the experience of the operator, the gestational age and the use of ultrasonography.

Although midtrimester amniocentesis is a highly efficient and accurate procedure for prenatal diagnosis, it has been associated with potential risks that could affect the mother and the fetus. The fetal potential risks are: spontaneous abortion which could be as a result of infection of the umbilical cord injury, premature labour, injuries due to withdrawal of amniotic fluid, or gangrene of an arm, peripheral nerve injury or ocular trauma (the last 3 are not considered frequent) and chorioamnionitis (UK working party on amniocentesis 1978; NICHD national registry for amniocentesis study group 1976). In addition to the fetal risk, maternal ones have been reported such as amnionitis which occurs in 1:1000 women undergoing amniocentesis (Murken et al, 1979). Vaginal leakage of amniotic fluid has been reported in 2-3% of women which could lead to pregnancy loss and uterine contractions (NICHD, 1979).
Figure - 14: The amniocentesis procedure.
Although amniotic cells have been used since the 1960s for prenatal diagnosis of chromosomal abnormalities, they have not been used for diagnosing β thalassaemia or other single gene disorders until more recently. In fact it was the advances in techniques of that molecular biology made it possible to use amniotic cells DNA for the molecular diagnosis of haemoglobinopathies. A sufficient amount of DNA can be prepared directly from amniotic fluid cells (Old, 1986) and β thalassaemia can be diagnosed by methods using PCR.

**III – 2.3: Chorionic villus sampling (CVS):**

Chorionic villus sampling for genetic analysis was first suggested by Mohr in 1968 and was developed gradually throughout the 1970s. The procedure of sampling was done using the transcervical approach, either blindly (Rhine, 1979) or guided by endoscopic vision. The early attempts at CVS were discontinued because the endoscopic method was associated with difficulties and discomfort in addition to major complications of uterine infection, low success rate in sampling and membrane perforation.

Following the introduction of real time ultrasound, Kazy et al reported early in 1980s CVS using ultrasound guidance of a thin biopsy forceps and sampling was done through a transcervical approach (Kazy et al, 1982). Following the report of Old et al (1982) on genetic diagnosis of haemoglobinopathies and Brambati & Simoni (1983) of diagnosing chromosomal abnormalities from chorion tissues, the procedure has attracted attention as a method for first trimester prenatal diagnosis. The procedure has then developed and the efficiency and safety was demonstrated through clinical trials.

The working group of the WHO has evaluated the status of prenatal genetic diagnosis and the potential for increasing the acceptability of fetal diagnosis by CVS (WHO working group 1984). The studies presented in this evaluation showed that first trimester prenatal diagnosis by CVS have fewer psychological and behavioural
effects on the mother than amniocentesis, this is because with amniocentesis the waiting period of the genetic test is relatively long and the elective abortion is difficult and painful. Furthermore, selective abortion during the first trimester seems less of a problem from the medical, legal or religious viewpoint (Cao et al, 1987; McCormack et al, 1990, El-Hashemie, 1997). Following this evaluation by the WHO working group, CVS has become an integral part of fetal medicine.

Chorionic tissue can be obtained by two approaches either transabdominally by needling or transcervically through the cervical canal by a catheter or biopsy forceps (Figure 1 - 15) Smidt-Jensen et al, 1986; Brambati et al, 1990). The general timing of sampling is between 10-13 weeks of gestation, because during this time the placenta is clearly identified and the success rate of sampling is practically almost 100%. In fact, the main clinical and practical advantage of CVS as an early stage antenatal diagnosis comes in the case of elective abortion as it is easier to introduce medical termination rather than surgical.

However, CVS is associated with many complications which vary in frequency depending on whether the procedure has been performed transabdominally or transcervically for some complications and for others depend on the timing of sampling and the experience of the operator (Hogge et al, 1986; WHO consultation on first trimester prenatal diagnosis 1986). Vaginal spotting and bleeding (Roads et al, 1989) is the most common complication of the transcervical approach as it has been reported in 32% of the patients. On the other hand, haemorrhagic complication is less frequently observed after the transabdominal approach. Intrauterine haematoma is a complication which is related to the amount of chorionic tissue collected (Jahoda et al, 1989). Infection is a common complication particularly with transcervical CVS that is of consequent morbidity to the mother and the fetus (Blakemore et al, 1985). The vagina is a field heavily colonised with microorganisms and during the passage of the cannula through the cervical canal infection occurs. Chorioamnionitis is another type of infection that occurred in up to 0.5% of cases (Hogge et al, 1986). Infection is a serious complication as the clinical history of patients who experienced spontaneous abortion after transcervical CVS
Figure - 15: Transvaginal sampling of chorionic tissue.
showed that infection was the main cause of fetal loss in up to 14% of cases. On the other hand, infection complications following transabdominal CVS have only been occasionally reported.

The most frequent risk of CVS is fetal loss which ranges between 2.2-5.4% (Smidt-Jensen et al, 1991). Factors that dramatically affect the rate of fetal loss after CVS are the experience of the operator and the gestational age as well as the multiple attempts at sampling during a single session (Brabati et al 1989). Some other complications such as the disruption of chorioamniotic or trophoblastic vessels could lead to structural defects (Christiaens et al, 1989; Planteydt et al, 1986), protrusion or rupturing of the chorion membrane could result from improper movement of the catheter. The most serious is increased risk of limb abnormality (Boyd et al, 1990; Firth et al, 1991).

Although amniocentesis is potentially safer than CVS, the later has a major advantage over amniocentesis, which is that pregnancy termination if indicated occurs in the first trimester and thereby avoids the psychological and social burdens of second trimester abortion.

**III - 2.4: Non-invasive prenatal diagnosis**

III – 2.4.1: Introduction

Many women who are under the age of 40 years and at risk of having children with chromosomal anomalies (such as age related aneuploidy) do not prefer to consider attending prenatal genetic diagnosis and check up. This could be because of the risks and complications associated with sampling procedures as amniocentesis or CVS still considered invasive techniques. Having a non-invasive first trimester method to obtain fetal cells that could identify genetically abnormal fetuses without harming the pregnancy or the mother will encourage the participation in prenatal
diagnosis either for age related aneuploidy, chromosomal abnormalities (such as translocations) or diagnosis of specific genetic diseases.

The non-invasive recovery of fetal cells in the maternal blood has dominated the attempt to revolutionise prenatal medicine. In 1975 Sheroder demonstrated the entry of fetal erythrocytes and platelets into the maternal blood as he was clinically studying maternal - fetal blood group antigen incompatibility (Sheroder, 1975). In 1969 Walknowska and associates obtained blood from 30 pregnant women at 14 weeks of gestation and cultured peripheral blood lymphocytes (Walknowska et al, 1969). While they were screening maternal metaphases, they found an occasional 46, XY karyotype in 21 samples in 19 of which the women gave birth to male infants. They demonstrated as well that the frequency of male karyotypes ranged between 0.2-1.5%. In fact, although this proportion is low it is considered relatively high considering the volume of fetal blood at this early stage of gestation.

The migration of placental-derived trophoblasts through the uterine veins to the maternal peripheral blood has been reported in maternal blood samples obtained from both the uterine vein at hysterectomy (Douglas et al, 1959) and in the pulmonary veins of a pregnant women who died from eclampsia (Trotter et al, 1956). It has been demonstrated that the presence of trophoblasts in the circulating blood of pregnant women from 18 weeks of gestation until term is a normal process in pregnancy (Douglas et al, 1959). All these findings gave an incentive to studying or developing an approach for early non-invasive antenatal genetic diagnosis without risk to the fetus or the mother.

Although a variety of fetal cells of different types such as nucleated RBCs, lymphocytes and trophoblasts have been detected in the peripheral blood of pregnant women, only a very small number of fetal cells are present in the maternal blood. In addition, many issues still need to be understood and clarified regarding the transfer of fetal cells to the maternal blood: the type of cells that cross the placenta and when during pregnancy they enter the maternal circulation and, in addition, what is the life span of these cells in the maternal blood.
In 1971, Shettlers mentioned the presence of trophoblastic cells derived from the chorionic villi in the endocervical canal (Shettlers, 1971). When examined by Y chromosome fluorescent dye, he proved that these cells are of fetal origin as are derived from a male fetus. Many studies have been followed by several investigators, which revealed the detection of fetal cells (trophoblasts) in the endocervical canal (Warren et al, 1972; Varner et al, 1977; Rhine et al, 1977, 1979; Griffith-Jones et al, 1992; Adinolfì et al, 1993). The presence of trophoblasts in the endocervical canal can be caused either by the invasive nature of trophoblasts or necrosis and degeneration of the decidua capsularis that exposed the chorionic villi to the uterine cavity (Rodeck et al, 1995).

Following the advances in molecular genetic diagnostic techniques, which enables direct diagnosis from a few cells by the application of fluorescent in situ hybridisation (FISH) and PCR, the interest to developing a non-invasive first trimester prenatal diagnosis using transcervical cells (TCC) has increased and opened a new route in the recent years for the possibility of its routine clinical application (Adinolfì 1995, 1996).

III – 2.4.2: Collection of TCC samples

TCC samples can be collected between 6 to 15 weeks of gestation by several techniques (Rodeck et al, 1995; Adinolfì, 1996) (Figure 1 - 16). Sampling method can be done either by: cotton swabs; cytobrush; aspiration of the cervical mucus with a catheter attached to a syringe filled with 3-5 ml of saline; aspiration with a tube for endometrial biopsy (Pipelle De Cornier); lavage of the endocervical canal with 5 ml of sterile saline; or by uterine lavage with up to 20 ml of saline. Although by using transcervical and intrauterine lavage more fetal cells can be obtained, such techniques seem to be more invasive than using mucus aspiration and cytobrush sampling (Rodeck et al, 1995). Furthermore, cell collection by cotton swabs has produced false positive or negative results (Morris & Williamson, 1992) and shown to be unreliable (Adinolfì et al, 1995). The published data presented by Adinolfì et al
Figure 1 - 16: Transcervical cells sampling procedures (Rodeck et al 1995).

- Aspiration
- Cytobrush
- Endocervical lavage
- Intrauterine lavage
(1995a, 1995b) and Briggs et al (1995) suggested that fetal cells could be detected in 50-70% of collected TCC samples following aspiration of the endocervical mucus.

The majority of sampling has been collected by mucus aspiration and prior to termination of pregnancy and only few studies revealed the collection during ongoing pregnancies and before to invasive prenatal diagnosis procedures (Adinolfi et al, 1995; Massari et al, 1995). Once collected, samples are examined to record the degree of cellularity. The presence of maternally derived cells is variable (Bulmer et al, 1995) and is mainly depend on the method of collection used. TCC clumps can be collected on the basis of morphological characteristics of trophoblasts, or by using a combination of antibodies that recognise fetal cells (His et al, 1987). Collected clumps are then washed and separated into small aliquots for the use of various analysis either by fluorescent in situ hybridisation (FISH) or PCR (Figure 1–17).

Different studies have revealed that the methods used for sampling and the molecular technique employed for the detection of fetal cells, are the main two factors affecting the detection of fetal cells in TCC samples obtained between 6 to 15 weeks of gestation (Adinolfi et al, 1995; Chang et al, 1997; Rodeck et al, 1995; Kingdom et al, 1995). FISH analysis and PCR techniques have been used widely to confirm the incidence of trophoblastic cells in TCC samples retrieved from mothers with male fetuses (Kingdom et al, 1995; Adinolfi et al, 1993, 1995a, 1995b; Briggs et al, 1995; Massari et al, 1995; Chang et al, 1996; Daryani et al, 1996). The results presented in these studies show reasonable hope about the future application of TCC for prenatal diagnosis of inherited disorders. The main concern remained with TCC sampling is the contamination incidence with maternal cells. However, the combination of using fetal cell-specific antibody with cell morphology and sensitive molecular techniques will overcome the problem of contamination not only with maternal cells but as well with spermatozoa.
Because CVS before 9 weeks of gestation has been associated with limb defects (Firth, 1997), TCC sampling seems to be promising as a non-invasive first trimester technique, once the presence of fetal genetic materials in TCC collected by aspiration is determinant and the safety of this method in on-going pregnancy evaluated.

IV: Preimplantation genetic diagnosis (PGD)

IV – 1: Introduction:

The end step of prenatal diagnosis is pregnancy termination if it is indicated. There are several side effects and concerns in prenatal diagnosis. Couples at high risk usually face emotional and psychological side effects in order to have a normal child. Additionally, repeated abortions have medical complications such as reduced fertility. Furthermore, pregnancy termination is not acceptable for many populations or ethnic groups because it still raises ethical, moral, legal and religious objections.

β thalassaemia remains a prominent public health problem. Economical and psychological factors make it difficult for many patients to benefit from the available treatment. In many countries, medical care management as well as complete programs of genetic screening, counselling and prenatal diagnoses are not feasible yet for cultural and financial reasons. Furthermore, there is as yet no cure for β thalassaemia as bone marrow transplantation has succeeded on very few patients worldwide. Therefore, prenatal diagnosis represents a valuable method for reducing the level of β thalassaemia births. However, the major concern with prenatal diagnosis is the end step, which is pregnancy termination. Ethical, moral, legal, religious and psychosocial objections to pregnancy termination have produced negative reactions and have limited the application of some prenatal programs. All of this make couples at high risk seek a safer, non-invasive, painless and less emotionally traumatic method which
is ethically more acceptable in order to have healthy children. An alternative to prenatal diagnosis is offered by preimplantation diagnosis (Handyside, 1993; Delhanty, 1994).

1V - 2: An approach for Preimplantation Genetic Diagnosis:

The advances in in vitro fertilization (IVF), micromanipulation and techniques of molecular biology have made possible diagnosis of genetic disorders from single cells prior to implantation. Preimplantation genetic diagnosis (PGD) has many advantages over prenatal diagnosis. PGD is ethically and religiously more acceptable because it is performed prior to implantation and at the 8 cell stage. PGD helps couples at high risk to overcome the dilemma associated with terminating a pregnancy after prenatal diagnosis and it gives couples at high risk the opportunity to start a pregnancy knowing that the fetus is unaffected with a particular genetic disease. Couples with subfertility as a result of gonadal mosaicism or chromosomal translocation that require IVF treatment or those who are infertile and have the risk of transmitting genetic disorders would greatly benefit from PGD in order to avoid the termination of a wanted pregnancy.

The clinical application of PGD is entirely based on the methods of superovulation and IVF. This allows the generation of several embryos in one cycle, which are then accessible for genetic analysis and diagnosis. PGD involves four main steps: IVF, biopsy of either polar bodies for preconception diagnosis or one to two cells from cleavage stage embryos; genetic analysis and diagnosis, and transfer of selected healthy embryos. The selection of embryos usually depends on the morphology and the stage of development.

1V - 3: In vitro fertilization (IVF)

The standard procedure of IVF is based on superovulation treatment by follicle stimulating hormone (FSH) to increase follicular recruitment, human chorionic gonadotropin (hCG) to complete oocyte maturation and gonadotropin releasing hormone agonist (GnRH), at the beginning of the menstrual cycle. The mature
Oocytes are collected through the vaginal route and placed in culture medium for insemination. The oocytes are checked under the microscope after the first 24 hours. The presence of two pronuclei indicates the occurrence of normal fertilization (Figure 1-18). The embryo then starts dividing. The zygote (one cell) divides to form 2-4 cells by the second day, 8-10 cells on the third and 12-16 cells (morula stage) on day four which represents the end of the cleavage stage. On day 5, the embryo enters the blastocyst stage with separation of the inner cell mass from which the embryoproper will be derived and implantation occurs in vivo.

1V - 4: Embryo Biopsy

The advances in techniques of micromanipulation have heralded a new era in the treatment of fertility and in preimplantation diagnosis. Successful implantation of biopsied embryos and their development into live offspring was reported in mice (Monk et al, 1988) and marmosets (Summers et al, 1988). Simultaneously, pioneering work was carried out at the Hammersmith hospital – London, to establish genetic diagnosis in human embryos obtained by IVF and prior to implantation (Hardy et al, 1990). Prior to any clinical attempt, Hardy et al (1990) assessed the proportion of embryo development to the blastocyst stage following biopsy, including the total number of cells in the trophectoderm and inner cell mass, as well as the uptake of glucose, pyruvate and energy substrate from the day of biopsy till day 7 after fertilization. Following the removal of one or two cells from cleavage stage embryos, the proportion of embryo development to the blastocyst stage was found to be high (Hardy et al 1989 a & b). Thus it has been found that development of human embryos to the blastocyst stage following removal of one to two cells from embryos on day 3 is not affected adversely by the biopsy (Hardy et al, 1989b, 1990). Handyside et al first reported pregnancies following the transfer of biopsied human embryos (Handyside et al, 1990). Recent studies have been done to analyze the pregnancy outcome following biopsy of cleavage stage embryos, and revealed that such pregnancies have no significant differences in biochemical and ultrasound measurements when compared to controls (Soussis et al, 1996 a & b).
Figure 1 - 18: Steps of in vitro fertilisation and development of embryos: cleavage stage from day 1 to 4. Blastocyst stage starts on day 5.
Preimplantation diagnosis can be performed on biopsied cells obtained from either cleavage stage embryos (Handyside et al, 1989), blastocyst embryos (Dokras et al, 1990) or prior to conception by separating the first polar body from the oocyte (Monk & Holding, 1990).

**IV – 4.1: Polar Body analysis:**

Biopsy of first polar body opens the possibility for preconception diagnosis of inherited diseases. The aim is to select oocytes which contain an unaffected allele for fertilization and subsequent embryo transfer.

The standard method of IVF is used for polar body analysis. Oocytes are retrieved and cultured for 3 hours. Oocytes that extrude their first polar bodies are selected for micromanipulation. Genotyping of oocytes is based on removing the first polar body (Figure 1 - 19) and subjecting its DNA material to genetic analysis (Verlinsky et al, 1990). Following the first polar body biopsy, genetic analysis is performed either by PCR for single gene disorders or by FISH for chromosomal aneuploidy.

For single gene disorders, if the polar body is homozygous (i.e. each chromatid carries the same allele) for the defective gene, then the oocyte is considered to be normal. If the polar body is heterozygous, the oocyte will also be heterozygous and the embryo might inherit the affected allele at the completion of the second meiotic division. If the polar body is homozygous normal then the oocyte will contain the affected allele. Oocytes presumed to be normal can be fertilized and generated embryos transferred. The remaining embryos including those whose DNA failed to amplify, or those where corresponding polar bodies analysis show they are heterozygous or normal, will be diagnosed at the cleavage stage and unaffected embryos will be transferred either in the same cycle or will be frozen (Verlinsky et al, 1990).

Polar body analysis has been clinically applied in two centers in USA for the diagnosis of cystic fibrosis (Strom et al 1998), β thalassaemia (Kuliev et al 1998) and
Figure 1 - 19: A schematic diagram showing polar body biopsy.

1. Holding pipette
2. Acid Tyrode’s pipette
3. Biopsy of 1st polar body
4. Genetic Analysis
5. Fertilisation of normal oocytes
6. 2nd polar body
7. 2nd polar body biopsy
8. Transfer of healthy embryos
age related aneuploidy in women undergoing routine IVF treatment (Verlinsky et al, 1992, 1996; Munne et al, 1995). However, there are many problems associated with preconception diagnosis. First is the problem of crossing over in heterozygous females. In the absence of crossover, the first polar body will be homozygous for the allele which is not in the oocyte. If crossing over occurs, the genotype of the oocyte and the polar body will be heterozygous; this is difficult to detect. Therefore, the test is not efficient for loci distal to the centromere as the crossover rate will be high. The second problem with polar body biopsy is that almost half of diagnosed oocytes will fail to fertilize. Polar body analysis can not be used for gender determination in the case of X-linked disorders. An additional weakness of this method is that the genotype of the oocyte is inferred rather than determined directly, which could explain the 76% success rate following the analysis of first and second polar bodies. Although the only advantages of this method is that it is non-invasive for the embryo as well as the absence of mosaicism at this stage, it has many disadvantages that make it limited in practice particularly following misdiagnoses of CF and β thalassaemia.

IV – 4.2: Blastocyst Biopsy

On day 5 postinsemination, embryos enter the blastocyst stage. The embryo will differentiate to form the inner cell mass which later will form the fetus and the trophectoderm cells that will form the placenta. Embryo biopsy can be performed at the blastocyst stage and it seems to be a potential approach for PGD. Gardner and Edwards first reported it for PGD in rabbits in 1968 (Gardner & Edwards, 1968). In 1990, Dokras et al (1990) first undertook biopsy at the blastocyst stage from human embryos and were able to obtain as many as 10-30 cells from the trophectoderm at this stage. This is the main advantage of embryo biopsy at the blastocyst stage because the number of cells has increased up to more than 100, which makes more material available for genetic analysis. In addition, the trophectoderm cells that may be biopsied will not affect the inner cell mass of the embryo. However, at the present time, the problems of mosaicism at the cleavage stage and the subsequent divergence of inner cell mass and trophectoderm and the fact that the proportion of embryos that may reach the blastocyst stage in vitro is very low (Hardy et al, 1993) make it
impractical. Even if the development of IVF embryos to the blastocyst stage is improved, the viability of blastocyst biopsy for PGD will depend on the pregnancy success rate following the transfer of blastocyst embryos, which currently seems to be low in comparison to cleavage stage transfer (Dawson et al, 1988; Bolton et al, 1991).

**IV – 4.3: Cleavage stage Biopsy:**

Preimplantation genetic diagnosis was first achieved by blastomere biopsy from cleavage stage embryos (Handyside et al, 1989; 1990). The embryo biopsy is performed on day 3 post insemination, when the embryo reaches 6-10 cells (Figure 18). One or two cells may be biopsied (Figure 1 - 20) from each generated embryo using a fine glass pulled micropipette by drilling a hole through the zona pellucida with acid Tyrodes solution (pH 2.4). The removal of one or two blastomeres from 8 cell stage embryos has been found not to affect the development of biopsied embryos to the blastocyst stage (Hardy et al, 1990). This is because embryos at this stage are able to regulate their metabolism and development and cells are not yet irreversibly committed to specific fates. Furthermore, biopsy at this stage was found not impair the implantation rate as a high success rate in achieving pregnancy followed blastomere biopsy which appeared to be the same as that for ordinary IVF. However, biopsy at an earlier stage, e.g. the 4 cell stage, appeared to affect the growth and development of embryos. This may be due to ratio alteration of the inner cell mass to trophectoderm (Tarin et al, 1995) as the embryonic genome is normally activated by this stage and functions such as cavitation can then be initiated (Braude et al, 1988; Tesarik, 1998).

Cleavage stage biopsy is the main procedure used in almost all PGD centers worldwide because of its many advantages over other procedures mentioned above. With the application of blastomere biopsy, it is possible to diagnose the sex of embryos, which is not possible in the case of polar body biopsy. In addition, PGD can be performed at the cleavage stage for all cases including autosomal recessive or dominant disorders and chromosomal abnormalities (e.g. translocations) providing an accurate diagnosis of the conceptus. Furthermore, when PCR has failed to identify the genotype of the polar body,
Figure 1 - 20: Cleavage stage embryo biopsy.

Acid Tyrode’s pipette with short & polished tapering end, diameter is around 10 μm or less to allow fine and proper control of acid flow.

Sampling pipette should be polished at the edges to prevent lysing of blastomeres. Diameter should be between 35-40 μm, as larger pipettes may give the possibility for sucking the whole embryo out of the zona, while smaller one may lyse blastomere during aspiration.

Aspiration of single blastomere.
biopsy of blastomere is required, thus drilling a hole in the zona pellucida twice may affect the chances of implantation; this can be avoided by direct blastomere biopsy.

1V - 5: Genetic Analysis of Embryos

The biopsied cell is subjected to genetic analysis either by fluorescent in situ hybridization (FISH) for gender determination (Griffin et al, 1993) or for the detection of chromosomal anomalies (Conn et al, 1996, 1998) or by PCR for single gene disorders (Handyside et al, 1990) (Figure 1 - 21).

PGD has been successfully attempted for the diagnosis of X-linked diseases, single gene disorders or chromosomal abnormalities. Over 100 babies were born following PGD with no report of congenital malformations (ESHRE Special Interest Group, Edinburgh 1997). Table 1 – 3 summarize the world data, till the end of December 1996, for PGD following cleavage stage embryo biopsy.

IV – 5.1: Single cell analysis by PCR:

The first application of preimplantation diagnosis was carried out to avoid X-linked disease. The procedure was to amplify a specific sequence of the Y chromosome to sex the embryo (Handyside et al, 1990). Two rounds of PCR amplification (nested PCR) are necessary to amplify sufficient amount of DNA from single cells (Figure 1 – 22) for conventional gel electrophoresis (Mullis et al, 1987; Li et al, 1988; Cui et al, 1989; Coutelle et al, 1989). The use of nested primers reduces the incidence of non-specific amplification and the carry-over contamination. Amplified products are then subjected to mutation detection and analysis following gel electrophoresis by heteroduplex analysis (Lesko et al, 1991; Liu et al, 1992) for small deletion or insertions; single strand confirmation polymorphism analysis for point mutations (El-Hashemite et al, 1996; Ao et al, 1998) or restriction enzyme digestion (Ray et al 1996).
Figure 1-21: Genetic analysis of biopsied blastomeres from IVF embryos.

PCR Amplification of the target gene for single gene disorders, followed by gel electrophoresis for mutation detection

FISH analysis for gender determination or chromosomal abnormalities
Table 1 – 3: The world data of PGD cycles following cleavage stage embryo biopsy [Harper 1996].

<table>
<thead>
<tr>
<th>Analysis</th>
<th>No of cycles</th>
<th>Embryo transfers</th>
<th>Pregnancies</th>
<th>Deliveries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sexing</td>
<td>223</td>
<td>184</td>
<td>48</td>
<td>31</td>
</tr>
<tr>
<td>Single gene defects</td>
<td>173</td>
<td>139</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>Translocations</td>
<td>32</td>
<td>27</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Age related aneuploidy</td>
<td>127</td>
<td>100</td>
<td>34</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>565</td>
<td>450</td>
<td>114</td>
<td>71</td>
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Figure 1 – 22: Diagrammatic presentation of nested PCR procedure to amplify genomic DNA from single cells. A: In the External amplification reaction, the outer primers anneal to the genomic target sequence encompassing the mutation. At the end of the external amplification, an aliquot of the amplified products is used as a template and then subjected to Internal amplification (B) in which the inner (nested) primers anneal internally to the target sequence, to allow the amplification of a smaller fragment. The amplified products are then subjected to gel electrophoresis to allow mutation detection and diagnosis.
Several single gene defects have been diagnosed prior to implantation, and healthy babies were born. This includes cystic fibrosis (Handyside et al, 1992), Duchenne muscular dystrophy (Liu et al, 1995), Tay Sachs disease (Gibbons et al, 1995), Myotonic dystrophy (Sermon et al, 1997), and familial adenomatous polyposis coli (Ao et al, 1998).

IV – 5.2: Single cell analysis by FISH:

Fluorescent in situ hybridization (FISH) has been used to identify the sex or the chromosomal status of preimplantation embryos. The in situ hybridization (ISH) technique was first applied to detect one or both sex chromosomes from interphase nuclei (Jones et al, 1987; West et al, 1987, 1988). The advent of dual fluorescent ISH using DNA probes specific for both sex chromosomes allowed the simultaneous detection of the X and Y chromosomes in different colors (Griffin et al, 1992), and live female births were achieved (Griffin et al, 1994). The direct identification of the nucleus and its embryonic origin, the avoidance of sperm contamination and detection of the accurate numbers of chromosomes which helps to avoid mosaicism and aneuploidy (Griffin et al, 1994), made FISH analysis for preimplantation gender determination the method of choice. Recently, the development of multicolor FISH made it possible to detect numerical and structural chromosomal abnormalities from embryos (Conn et al 1996).

The procedure of FISH analysis involves spreading of biopsied blastomeres onto glass slides using spreading solution that contains HCl and Tween 20. The spreading solution will lyse the cell membrane and dissolve the cytoplasm, leaving the nuclei clear. Fluorescent probes are then added to the slide and following denaturation allowed to hybridize to their specific sequence. The nuclei are then visualized under fluorescent microscope and counting the number of different colored signals makes diagnosis possible. The probes that are used for FISH analysis are of two types: either DNA probes that will bind to a repetitive sequence as in the case of sexing, or locus specific YAC or cosmid probes which bind specifically to the...
corresponding chromosomes. The latter is used for the diagnosis of chromosomal abnormalities and may require indirect labeling.

1V - 6: Potential for misdiagnosis in PGD

Despite widespread use there are many difficulties associated with cleavage stage embryo biopsy for preimplantation diagnosis; these are: the availability of limited material, i.e. one or two cells, for analysis; cell lysis upon biopsy because human embryonic cells are so fragile; and limited time for analysis. Analysis must be carried out within 24 hours to avoid having to freeze the embryo. Therefore, methods used for analysis must be sensitive, reliable, fast and give accurate results. Speed is an issue in PGD, as ideally all steps should be performed within one working day so that selected embryos can be transferred by the end of the third day post insemination to make the chances of implantation optimal.

As PGD becomes legally, ethically and religiously more acceptable than prenatal diagnosis as a preventive procedure and comes to be used in many countries worldwide, it is important to address the problems of clinical application to minimize the incidence of misdiagnosis. There are three main problems that arise with PGD: mosaicism, allele specific amplification failure (allele drop out) and contamination. The last two are mainly associated with single gene disorder diagnosis. However, mosaicism can generally affect diagnosis in human embryos.

1V - 6.1: Mosaicism:

Cytogenetic analysis of embryos generated from IVF has indicated the important problem of chromosomal mosaicism. A high proportion of numerically abnormal karyotypes has been demonstrated when analyzing IVF embryos at early stages of development (Michaeli et al, 1990; Bongso et al, 1991; Zenzes et al, 1992). The development of multicolor FISH analysis has facilitated the analysis of almost all

Data from multicolor FISH analysis presented by Delhanty et al (1997) shows that the incidence of chromosomal abnormalities (mosaicism) in embryos is the same whether these embryos are from routine IVF patients or from fertile women attending a PGD program. The incidence of chromosomal abnormalities in IVF embryos falls into four categories (Harper & Delhanty, 1996): normal embryos with all cells being uniformly diploid; abnormal with cells being uniformly abnormal; mosaic embryos in which most of the cells are diploid with a few nuclei that could be haploid aneuploid or polyploid; , and chaotic embryos in which there is a random variation in the chromosomal constitution from one cell to another. It has been revealed that the incidence of chaotic embryos in repeated IVF cycles is related to particular patients (Delhanty et al, 1997). Although these data could explain the generally poor success rate of the IVF procedure because it is likely that most cleavage stage embryos have mosaicism, normal infants are generated following IVF or PGD. Thus despite the high frequency of mosaicism, IVF embryos may have a possible mechanism that allows normal development.

The underlying mechanism leading to such high levels of mosaicism is still unknown, however, the frequent presence of chromosomal abnormalities in embryos has direct implications for the accuracy and efficiency of PGD. The presence of mosaicism will not affect the accuracy of sexing embryos for X-linked diseases (Delhanty et al, 1996) but may have a greater impact on the diagnosis of single gene disorders particularly the dominant ones and compound heterozygotes as well as aneuploidy. In the case of dominant disorders and compound heterozygotes for recessive diseases, the biopsy of a mosaic cell (e.g. haploid cell) can lead to misdiagnosis and the transfer of affected embryos, and the same could apply in the case of trisomies. This problem can be minimized by analyzing two cells from each
embryo and by using a sensitive mutation detection method such as SSCP analysis (El-Hashemite et al, 1997b).

**1V – 6.2: Allele Drop out**

Allele specific amplification failure (allele drop out) is a particular problem when amplifying genomic DNA from single cells. It is a phenomenon in which one of the alleles carried by the cell fails to amplify (Delhanty, 1994) or amplifies preferentially (Findlay et al, 1996). This affects the detection of variants and can lead to misdiagnosis. Allele drop out (ADO) will not lead to the transfer of affected embryo if the genotype of both parents is the same in the case of recessive disorders; but it will decrease the number of available embryos to be transferred. This is because if the normal allele failed to amplify in heterozygous embryo, the embryo will be diagnosed as homozygous affected (Figure 1 – 23 a). On the other hand, if the mutant allele failed to amplify in a heterozygous embryo, the embryo will be diagnosed as normal and will be considered for transfer. The later is not a problem in the case of recessive disorders, as heterozygotes are phenotypically normal and such embryos will be transferred.

ADO is considered a serious problem in recessive disorders when both parents are carrying different mutations leading to compound heterozygous offspring or when diagnosing a dominant disorder, which it could lead to misdiagnosis and the transfer of affected embryos. If diagnosing a dominant disorder in which heterozygotes are phenotypically affected, failing to amplify the mutant allele in heterozygous embryos might lead to its transfer. In the case of compound heterozygous, if one of the mutant alleles failed to amplify, the embryo will be diagnosed as heterozygous for the other mutation and might be transferred (Figure 1 – 23b). Depending on the method used for mutation detection and diagnosis, misdiagnosis and transfer of affected embryos as a result of ADO can be minimize. For example the application of single stranded conformation polymorphism (SSCP) analysis allows the identification of each allele and the assessment of ADO (El-Hashemite et al, 1997b; Ao et al, 1997).
Figure – 23: Diagram explains the incidence of ADO and the possible misdiagnosis in preimplantation embryos when using insensitive methods for mutation detection. A) in case of recessive disorders and when both parents carrying the same mutation causing the disease. 1: represent when the mutant allele fails to amplify; and 2 when the normal allele fails to amplify. M = mother, F = Father

A:

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<th>M</th>
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<tr>
<td>F</td>
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<td></td>
<td>Normal allele</td>
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<td></td>
<td>Mutant allele</td>
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B: In case of compound heterozygous, if the mutant allele failed to amplify (Lane 1) in heterozygous embryo carrying either maternal or fetal mutation, the embryo will be diagnosed as normal, but if the normal allele failed to amplify (Lane 2) the embryo will be diagnosed as affected and will be excluded from transfer. If the embryo is compound heterozygous and one of the two mutant alleles failed to amplify (Lane CH) this will lead to misdiagnosis. F = father, M = mother, CH = compound heterozygous.
The main source of misdiagnosis in single gene disorders is contamination with extraneous DNA or cellular materials, which could lead to false negatives and false positives. There are five main sources of contamination, these are: from an additional sperm that remained attached to the embryo, cross-contamination between samples, airborne cellular materials or carry-over contamination from previous PCR products that enter when handling the sample or from reamplifying the DNA as in the case of nested PCR. Previous research works showed that contamination that results from carry-over, such as extraneous DNA, reagents, pipetting devices, laboratory surfaces or even the skin of researchers is the major concern (Gibbs & Chamberlain, 1989; Kwok & Higuchi, 1989; Kitchin et al, 1990).

The PCR is a remarkably sensitive technique that has wide applications in many fields such as in medical diagnostics. The PCR methodology allows the detection of only few molecules of a target DNA sequence. False positives or mistyping may occur when the majority of molecules to be detected arise from exogenous source rather than from the sample itself. Obviously the fewer molecules one is trying to detect the more one should guard against this possibility. The use of PCR for sensitive detection is complicated by the fact that the product of the amplification serves as the substrate for the generation of more products. A single PCR cycle produces a very large numbers of amplifiable molecules that can potentially contaminate subsequent amplifications of the same target sequence. Such problem of contamination constitutes a major drawback to the widespread of the technology for sensitive applications such as PGD. There are many methods that have been described for either avoiding false results with PCR or removal of contaminating DNA from PCR reagents. These include Ultraviolet (UV) irradiation of PCR mixtures (Ou et al, 1991), the use of specific nucleases (Zhu et al, 1991) or by using ultrafiltration method for elimination of genomic DNA from PCR reagents (Wages et al, 1994).
Determining the source of such contamination can be difficult and sometimes time consuming, therefore physical precautions can be taken and is indeed of practical value to reduce the carryover and contamination to a minimum.

Unlike FISH procedures, in PGD of single gene disorders and following to routine IVF procedure, the problem of contamination can arise from tubing an additional sperm that remained attached to the embryo during the process of biopsy. Such contamination does affect dramatically the diagnostic results. For example, if the embryo is a homozygous affected with that particular disease, and the blastomere tubed has been contaminated by a sperm that carry the normal gene, the resulted diagnosis will be diagnosing the embryo as a heterozygote and may be considered for transfer, and the same applied for compound heterozygous (Figure 1 – 24). This is of a major problem and main concern when diagnosing recessive disorders. The same can be applied when diagnosing dominant disorders as contamination of normal embryos with sperm carrying the mutant gene can lead to diagnosing the embryo as heterozygous and reducing the number of available embryos for transfer. The problem of sperm contamination when performing PGD of single gene disorders can be avoided by using intracytoplasmic sperm injection (ICSI) as a method of fertilisation.

The problem of contamination with a sperm can be avoided by fertilizing embryos subjected to PCR for PGD of single gene disorders by intracytoplasmic sperm injection (ICSI). Sterile environment for embryo biopsy or isolating single cells, using dedicated micropipettes, filtered tips and specific hood and room to set up and run the initial PCR, can overcome and reduce the problems of contamination which result from handling the sample. However, nested PCR cannot be avoided, because it is the only procedure available and used for amplification of the only two copies of a particular gene from single cell.
Figure 1 – 24: Incidence of sperm contamination in PGD of single gene disorders.

Blastomere from normal embryo

\[
\begin{array}{c}
\text{N} \\
\text{Sperm carrying mutant allele}
\end{array}
\]

PCR amplification

Gel electrophoresis

Embryo sample Normal control

Blastomere from homozygous affected embryo attached to it a sperm carrying normal allele

\[
\begin{array}{c}
\text{N} \\
\text{Sperm carrying normal allele}
\end{array}
\]

PCR amplification

Gel electrophoresis

Embryo sample Affected control

N. El-Hashemite
V: Mutation Analysis

The advances in the techniques of molecular biology have clarified the DNA defects, which cause a variety of single gene disorders. The advent and development of restriction endonuclease digestion, Southern blot, DNA hybridization, DNA sequence analysis and the polymerase chain reaction has made it possible to routinely screen for and detect the rapidly growing number of mutations causing diseases. The progress in identifying and cloning numerous disease genes has resulted in an increasing need for the development of techniques which have 100% detection rate, have no false positives or negatives, are straightforward, simple, and do not require complex equipment or harmful reagents. The methods must also be fast and cheap. Thus methods used for mutation screening and detection must be economical and effective. There are many direct and indirect methods in use for detecting mutant genes, and each has its advantages and applications.

V - 1: Restriction enzyme digestion

Restriction endonuclease enzymes are part of the bacterial defense mechanism. These endonucleases recognize a sequence of 4 or more nucleotides and cleave double stranded DNA at a specific recognition sites. The presence of restriction sites in human DNA made it possible to cleave the double stranded DNA into fragments (Malcom 1981). The restriction enzymes are used for direct detection of either gene deletions or single base substitutions by adding the appropriate restriction enzyme that recognize a specific restriction site which was created or abolished by the mutation. The extracted DNA with the enzyme is then incubated at 37 °C for at least 2 hours (time required for digestion mainly depends on the enzyme), then digested products are visualized on 4% agarose gel by ethidium bromide staining following electrophoresis.

Most of the DNA mutations causing monogenic disorders are single base substitutions, and few of these mutations (for example, few of the more than 100
different β globin mutations have a natural restriction site (Kazazian, 1989). In addition enzymes required for detecting such mutations are very expensive. This makes restriction enzyme digestion for screening and diagnosing β thalassaemia at the molecular level impractical particularly for laboratories where β thalassaemia mutations are heterogeneous.

V - 2: Southern Blot

The Southern blot technique is well established and widely used either for research purposes or diagnosis of large deletions causing disease. The Southern blot method has been used widely for the prenatal diagnosis of haemoglobinopathies (Old, 1986; Boehm, 1989), and it is based on restriction enzyme digestion, electrophoresis for 24 hours and then blotting into a nylon filter. The DNA fragments are then hybridized with radioactive probes, washed to remove background and autoradiographed.

Although this method is has been extensively used for diagnosis, its importance and applications has decreased particularly after the development of in vitro DNA amplification by PCR. However, many laboratories are still using it for the diagnosis of α thalassaemia, which results from large DNA deletion.

V - 3: Polymerase Chain Reaction (PCR)

The advent of PCR (Saiki et al, 1985; Mullis & Faloona, 1987) has resulted in the development of many simple and fast techniques for mutation analysis, which have had a great impact on human genetics. The main idea of PCR is that it allows enzymatic in vitro amplification of a short specific DNA sequence through repeated cycles of heat denaturation of double stranded DNA. This is followed by annealing of two synthesized oligonucleotide primers which flank the DNA fragment to their complementary sequences, and finally an extension of the annealed primers with DNA
polymerase (Figure 1-25). After 30 cycles of amplification, a sufficient amount of DNA (approximately 10 million copies of the target fragment) can be produced which greatly simplifies subsequent manipulation of the DNA sample. The specificity of PCR amplification results from the precise selection of the two primers that flanking the DNA segment to be amplified. These are typically between 20 to 25 nucleotides in length and are designed to hybridize to opposite strands of the target sequence, so the synthesis of DNA by Taq polymerase proceeds across the targeted region, i.e. between the primers, to effectively doubling the amount of that DNA segment. A new advantage of PCR amplification of genomic DNA is that primer sequences not complementary to the template DNA can be added to the 5' end of the primers. Such sequences become incorporated into the double stranded DNA generated by PCR and provide a means of introducing an artificial restriction sites (Scharf et al, 1986) which could be useful to detect variants by restriction enzyme digestion.

V – 4: PCR Mutation detection techniques:

Since the invention of the PCR, many methods of mutation analysis and detection have been developed. This has been fundamental for identifying and characterizing the DNA alterations such as base substitutions, deletions and insertions causing genetic disorders in order to facilitate screening and prenatal diagnosis. Each of these techniques has its advantages and disadvantages, with varying sensitivity, accuracy, cost and time effect, and reliability. Although, almost all-available techniques have been applied to prenatal screening, not every method is suitable for preimplantation genetic diagnosis of single gene disorders. However, techniques developed for preimplantation diagnosis can be easily applied and adapted for prenatal diagnosis.
Figure 1 - 25: A diagram explains the cycles of PCR amplification of a target DNA sequence.
V – 4.1: Dot Blot Hybridization

Wallace et al (1981) to detect single base substitutions and frameshifts in genomic DNA first used allele specific oligonucleotides (ASOs) by hybridizing a specific labeled oligonucleotide to its complementary sequence. Sickle cell anaemia and the most common Mediterranean mutations causing β thalassaemia were the first disorders to be diagnosed by this approach (Orkin et al, 1983; Conner et al, 1983).

The technique is based on testing a specific genomic DNA sequence, which was first amplified by PCR. PCR products are then denatured and dot blotted onto a nylon filter. Under controlled conditions a labeled oligonucleotide will hybridize only to its matched complementary sequence of fixed DNA. The filter is then washed (Wood et al, 1985) to remove mismatched probes from the filter leaving only perfectly matched ones (Figure 1 - 26).

Although this method has proved to be accurate, it is expensive and requires a long time to identify mutations. In addition, this approach is perfect for laboratories diagnosing a few mutations, like in Sardinia were there is only one common β thalassaemia mutation and very few rare ones. However, in laboratories were β thalassaemia mutations are heterogeneous, different methods are used which allow the screening of more than one mutation at the same time.

V – 4.2: Denaturing Gradient Gel Electrophoresis (DGGE)

The main application of this technique is the direct detection of mutations causing disease and polymorphic changes in specific sequences. DGGE allows the resolution of DNA fragments differing by as little as a single base substitution, by electrophoresis of double stranded DNA fragments through a linearly increasing denaturing gradient, formamide and urea are mainly used (Myers et al, 1985). As the DNA proceeds through the gradient gel, it reaches a position where the concentration of the denaturing agent equals the melting temperature of its lowest melting domain.
causing denaturation and retardant. The melting temperature is determined by the DNA sequence and is altered by the presence of a mismatch in the sequence. DNA fragments can then be visualized by either ethidium bromide staining or silver staining. Heterozygous samples will show heteroduplex and homoduplex formation (Figure 1 - 27).

Because DGGE can not resolve DNA fragments differing by a base variant which is located within the highest melting domain, due to loss of sequence dependent migration upon a complete strand dissociation. This is overcome by adding a GC rich sequence (GC clamp) to the primers where high temperature will present complete denaturation (Myers et al, 1985). The introduction of GC clamp primers has increased the percentage of mutations that can be detected by DGGE up to approximately 100% (Myers et al, 1985).

Since its introduction, DGGE has been widely used for screening for β thalassaemia mutations rather than for diagnosis, because of the time it takes and the running expense.

V - 4.3: Allele specific priming

Allele specific priming for the detection of point mutations, small deletions or insertions can perform in vitro amplification of mutant DNA samples. This method has been developed by Newton et al in 1989 for the analysis of α-1 antitrypsin alleles and called Amplification Refractory Mutation System (ARMS). This method has been then adapted for mutation detection of β thalassaemia and is based on amplifying a specific sequence of the DNA fragment in the presence of four primers, in which one of them (ARMS primer) is designed to amplify when it only forms a perfect match with the DNA sequence (either mutant or normal). This will allow the ARMS normal primer to hybridize only to the normal sequence and the mutant primer to the mutant sequence. The second pair of primers is control one to amplify a region, which will be as a control band to avoid misdiagnosis in the case of failure of amplification.
Figure 1 - 26: Diagram explains mutation detection by Dot Bolt Hybridisation. The target DNA sequence is hybridised onto strips containing ASOs for the normal sequence and the mutant one. DNA will hybridise to its complementary sequence. In the case of heterozygous sample, the DNA will hybridise to its normal and mutant complementary, and if the sample is affected it will only hybridise to the mutant sequence while normal samples will hybridise only to the normal.

Hom = homozygous; Het = heterozygous; Nor = normal.
Figure 1-27: Diagram explains the theory of DGGE analysis. The amplification of the same DNA fragment using GC clamp primers will introduce high melting domain temperature which allows the resolution of single base substitutions by DGGE. During electrophoresis, double stranded DNA differing by a single base in their lowest melting domain will result in branching of DNA molecule at different position along the denaturing gradient.
Following PCR cycles, the amplified product is then visualized on 3% agarose gel stained by ethidium bromide after electrophoresis which will give only the normal and in the case of normal, two bands in the case of heterozygous which indicates the amplification of both normal and mutant alleles and only the mutant band in the case of homozygous affected (Figure 1 - 28).

V - 4.4: Heteroduplex analysis

During PCR amplification and throughout the cycles of denaturing and reannealing of single stranded DNA molecules, homoduplex molecules are formed. The presence of a single mismatch within the DNA sequence will result in the formation of heteroduplex molecules.

The mixing of the wild type and the mutant DNA strands during the PCR amplification forms Heteroduplexes. The method of analysis is based on denaturing PCR products for 5 minutes at 95 °C to maximize heteroduplex formation, electrophoresis of samples on 6% polyacrylamide gel and then visualize DNA fragments by either ethidium bromide staining or silver staining. In the case of a heterozygous sample four different fragments will be generated: the normal homoduplex, the mutant homoduplex and two Heteroduplexes. In the case of homozygotes, only homoduplexes will be generated. The normal homoduplex will migrate with a different mobility on the gel matrix from the mutant one, leaving the fragments in a different position (Figure 1 -29).

The stability and the sensitivity of heteroduplex formation depend mainly on the type of mutation and the viability of electrophoretic conditions. Frameshift mutations, which result from more than 3 base pair deletions or insertions will create very, stable Heteroduplexes. However, the sensitivity and stability of heteroduplexes formation decreases in the case of single base substitutions. Therefore, variation in the electrophoresis conditions such as temperature and the ionic strength of the buffer, increases the sensitivity of mutation detection by heteroduplex formation, because
Figure 1 - 28: Schematic representation of the principle of ARMS technique. C = common primer; M mutant primer; N normal primer; Het = heterozygous; Hom = homozygous; Nor = normal.

Gel electrophoresis followed by ethidium bromide staining

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heteroduplex and homoduplex mobility during electrophoresis is small (White et al., 1992; Keen et al., 1991). Furthermore, the stability of heteroduplex formation depends on the neighboring base in the case of point mutations. Heteroduplex analysis is very sensitive in the case of frameshift mutations, and this explains its wide application for detection of ΔF508 causing cystic fibrosis (White et al., 1992).

V – 4.5: Single Stranded Conformation Polymorphism Analysis (SSCP)

PCR-SSCP analysis has become a widely used method for mutation screening and detection because of its simplicity and versatility. In this method, the region of interest in genomic DNA is amplified enzymatically by PCR using two normal primers. The amplified products are denatured to form single strands and then subjected to nondenaturing polyacrylamide gel electrophoresis. The principle of this method is that under nondenaturing conditions, each single stranded molecule of the DNA will adopt a specific conformation at a given temperature (Figure 1 - 30). Any changes in the DNA sequence will result in a change of the conformation (Orita et al., 1989). The single stranded DNA carrying mutation can be identified by virtue of altered mobility during nondenaturing polyacrylamide gel electrophoresis. Because of the lack of a theoretical background to predict conformation and the mobility of the single stranded DNA in gel electrophoresis, optimal conditions for separation of conformers have to be determined. There are many factors that affect the sensitivity of mutation detection by SSCP. The main parameters are those of the polyacrylamide concentration, temperature of the running gel and the concentration of the running buffer (Glavac et al., 1993; Hayashi et al., 1993). The temperature of the running gel dramatically affects the sensitivity of single stranded migration pattern and mutation detection. This is because the stability of single stranded conformations is altered by temperature. Thus at a certain temperature, most point mutations, small deletions and insertions in fragments up to 500 bp can be simultaneously detected by gel mobility shift without the use of restriction enzymes, probes or blotting hybridization. Furthermore, the most common and rare mutations within a region of the gene can be detected by using a single set of normal primers avoiding the need to synthesis.
Figure 1 - 29: Heteroduplex analysis.

2 alleles

denaturing during PCR

Het  Nor  Hom

-  +
Figure 1 - SSCP analysis

Double stranded DNA

Denaturing at 95°C in the presence of formamide
specific primers for each mutation. This method has proven to be sensitive, fast, reliable, cost effective, accurate and economical for mutation screening and diagnosis.

Following gel electrophoresis, the DNA bands can be easily visualized by either autoradiograph following radioactively labeling of DNA fragments during PCR amplification (Orita et al, 1989) or by silver staining method for non-radioisotopic PCR amplification (Ainsworth et al, 1990). The sensitivity of SSCP in a single run is generally about 80% (Glavac & Dean, 1993; Hayashi & Yandell, 1993). This percentage is variable and is mainly dependent on the target sequence, experimental conditions and length of the fragment analyzed.

**V – 4.6: Fluorescent PCR**

Fluorescent PCR is a new modification of the PCR procedure. The concept of fluorescent PCR is the same as the conventional one except for using fluorescently labeled primers and an automated DNA sequencer machine. The sensitivity of fluorescent PCR is around 1000 times more than of conventional agarose or polyacrylamide gel analysis (Hattori et al, 1992), which allows the detection of signals far below the threshold.

Fluorescently amplified products can be analyzed on an automated DNA sequencer, which detects molecules passing through a fixed laser beam. Once it reaches the laser beam, the laser will excite the fluorescent dye by penetrating the gel perpendicular to band migration. The emitted light is detected by photodiodes and signals are automatically collected and sent to the computer for storage (Figure 1 – 31).

Fluorescently amplified products can be analyzed by different approaches, either by fragment analysis, quantitatively, ARMS or even by SSCP. The detection system of Fluorescent PCR allows the accurate sizing of fragments to single base pairs (Ziegle et al, 1992). This has a major advantage for the fragment analysis of
mutations resulted from either deletions or insertions such as the detection of the 3 bp deletion ΔF508 causing CF (Findlay et al, 1996). In addition, quantitative analysis can be conducted on fluorescent PCR samples, which allows the identification of the quantity of the initial target sequence (Ferre, 1992). This has been used to recognize the copy numbers of specific chromosomes following the amplification of small tandem repeats (STR) markers unique for each chromosome pair (Mansfield, 1993; Sherlock et al, 1997). Mutations resulted from single base substitution can be analyzed either by the ARMS technique (Monk et al, 1993) or by SSCP. The sensitivity of fluorescent PCR detection methodology made it possible to detect the amplified products of target sequences from single cells following 30-35 PCR cycles (Findlay et al, 1996; Sherlock et al, 1997b). This has opened a new era in PGD, which will negate the need for nested PCR to amplify target sequences from single cells.
Figure - 32: Diagram explains the method of signal detection of fluorescently amplified DNA using automated DNA sequencer.
Objectives of project:

1 - To enable preimplantation diagnosis of β thalassaemia by direct detection of mutant β globin genes at the single cell level.

2 - To optimize DNA amplification procedures from single cells and develop mutation analysis techniques as an application for either early prenatal diagnosis using transcervical cells or preimplantation genetic diagnosis of monogenic disorders.

3 - To help characterize the spectrum of β thalassaemia mutations in Egypt and Jordan where β thalassaemia is a major health problem, to facilitate the application of a preventive programs.
Chapter 2

MATERIALS & METHODS
Chapter 2

Materials & Methods

2.1 Materials:

2 - 1.1: Chemicals

All the chemicals for general use were supplied by British Drug House (BDH) - Poole, Dorset. Sigma chemical company supplied proteinase K, dithiothretol, bovine serum albumin, ethidium bromide, standard grade agarose, ultra pure low melting point agarose, Ficoll paque solution and phosphate buffered saline (PBS) tablets. Acrylamide was supplied as a 40% solution and 6% solution by Severn Biotech Ltd. MDE nondenaturing polyacrylamide solution was supplied by Flowgen. Ultrapure Sequagel XR acrylamide solution and its buffer were supplied by National Diagnostic. Fluorescent Thermosequenase sequencing kit was supplied by and Solid Phase sequencing kit by Amersham-Pharmacia Biotech. TEMED and ammonium persulphate were supplied by Bio-rad. Restriction endonucleases, Hank's, trypsin and versene solutions were obtained from Gibco BRL. All primers were obtained from Oswel or Amersham-Pharmacia Biotech.

2 - 1.2: Patients & DNA samples

When establishing the technique, DNA samples were obtained from normal individuals and from patients known to be carriers to the following mutations: IVSI

Patients’ samples were obtained from Dr F Chehab – University of California San Francisco; Dr J Old - Oxford University; Dr I Hussein - National Research Institute - Cairo, and Dr. Mary Petrou at the Perinatal Centre at University College London Hospital.

Another group of DNA samples were obtained from the Perinatal Centre - UCLH from patients undergoing prenatal diagnosis. Samples were in family sets containing parental DNA and CVS DNA from either ongoing or terminated pregnancies that were previously diagnosed at the Perinatal Unit mainly using ARMS.

2.1.3: Solutions and buffers

All solutions were prepared using distilled and deionised water and were stored at room temperature. Proteinase K, KOH / DTT and neutralising buffer were stored at -20 C. The component of solutions and buffers used are presented in appendix 2.1.
2 - 2: Methods

2 - 2.1: Single cell Isolation

The analysis was performed on various types of single cells (buccal, fibroblasts and lymphocytes) isolated from different individuals some known to be carriers of β thalassaemia mutations, the ΔF508 mutation causing cystic fibrosis, SCA patients, and from single embryonic blastomeres.

A total of 294 single cells was isolated from β thalassaemia patients and lysed using 17 μM SDS and 125 μg/ml Proteinase K (lysis buffer). Single cells were obtained from different individuals of known genotype with the following mutations causing β thalassaemia within intron 1: IVSI-1 G-A, IVSI-1 G-T, IVSI-5 G-C, IVSI-6 T-C and IVSI-110 G-A. These mutations were chosen to start with because they are very common in the at risk populations in the UK. Furthermore, there are couples known to be carriers of these mutations who, having experienced pregnancy termination after prenatal diagnosis, are interested in preimplantation diagnosis.

Single lymphocytes were also isolated from the blood of normal individuals and blastomeres were obtained from IVF embryos donated for research at Hammersmith Hospital - London.

2-2.1.1: Buccal cell isolation:

To prepare single buccal cells, a mouth wash was obtained from normal individuals and thalassaemic patients in double distilled water. Following
centrifugation at 1500 xg for 5 minutes, the supernatant was discarded and cells were then washed and diluted in PBS, isolated using a pulled glass micropipette, and transferred to 0.5 ml eppendorf tubes containing the lysis buffer.

2-2.1.2: Single lymphocytes:
Single lymphocytes were isolated in the same way as above following separation using the Ficoll paque method (Sigma). Three mls of blood was collected in EDTA and diluted 1:1 in PBS. An aliquot of 3 ml of diluted blood was gently pipetted onto a layer of Ficoll-paque (Figure 2 - 1). Following 30 minutes of centrifugation at 400 xg, separated lymphocytes were aspirated, transferred into a sterile tube and washed twice with 10 ml PBS buffer supplemented with 10 mg/ml bovine serum albumin and centrifuged for 10 minutes at 250 xg. Lymphocytes were then diluted by PBS and isolated.

2-2.1.3: Single fibroblasts:
Single fibroblasts were isolated from chorionic villus cultures after first washing with Hanks' saline, displacing cells from the substrate with trypsin-versene solution, and washing with medium to inactivate the trypsin (appendix 2.2). Following centrifugation, the pellet was washed twice and diluted with PBS and cells isolated.

2-2.1.4: Single blastomere isolation:
Cleavage stage embryos were disaggregated under a binocular microscope. The zona pellucida was removed by acid Tyrode's (pH 2.4) solution by leaving

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Figure 2 - 1: The separation of lymphocytes from blood using the Ficoll-paque method.

Blood / Centrifugation for 30 minutes at 400 xg

Ficoll-paque

Plasma
Lymphocytes
Ficoll-paque
Red Blood Cel
embryos in a drop of acid for approximately 30 seconds. Embryos were then returned to HEPES buffered handling medium. Single blastomeres were gently separated by pipetting using a fine glass pulled pipette. Single blastomeres were washed by PBS, isolated and placed in 0.5 ml thin walled eppendorf tubes containing 2 μl of PBS and 2 μl of lysis buffer.

2-2.1.5: Isolation of single cells:

Single cells were isolated using a mouth-tube with a fine glass pulled pipette under an inverted or dissecting microscope. Cells (buccal, fibroblasts or lymphocytes) were diluted by PBS and then rediluted to contain 3 - 4 cells maximum into a microdrops of PBS (Figure 2 – 2). Single cells were isolated and washed 3 times in microdrops of PBS, then transferred into eppendorf tubes containing lysis buffer.

2 - 2.2: Lysis Protocol

2 - 2.2.1: SDS/Proteinase K (PK)

All cells were lysed by SDS/PK unless otherwise indicated. Three different protocols were used when lysing cells by SDS/PK:

An aliquot of 1 μl of 17 μM SDS and 2 μl of 125 μg/ml of PK was added to each of the eppendorf tubes and kept on ice. Isolated cells were pipetted into each tube and then all tubes were incubated at 37 °C for 1 hour. Tubes containing cells were then incubated at 95 °C for 20 - 30 minutes to inactivate the enzyme PK after
Figure 2-2: Isolating Single Cells

1. Place glass plate on Petri dish
2. Add PBS drops for washing
3. Add cells in PBS drop
4. Add cells in PBS
5. Add cells in PBS
6. Add cells in PBS
7. Add cells in PBS
8. Add cells in PBS
9. Add cells in PBS
10. Add cells in PBS
50 μl of mineral oil was added to each tube. This was followed by a centrifugation of all tubes which were then kept at room temperature for setting up the PCR or stored at -20 °C for up to 3 months to be used later.

To make the protocol more suitable for preimplantation diagnosis the length and temperature of incubation was modified. Proteinase K was activated at 37°C prior to aliquoting. Aliquots of SDS/PK were added to each tube as described above and tubes were kept at room temperature into which isolated single cells were then pipetted. Cells were then incubated at 95°C for 20 - 30 minutes on a PCR block to inactivate the enzyme.

During the time of investigation it was found that the more chemicals added to the single cell genomic DNA the less efficient is the amplification. Therefore, SDS was excluded from the lysis buffer and only PK was used to act as a lysis buffer. As described previously, PK was activated at 37 °C prior to aliquoting and 2 μl of it was added to each tube into which cells were pipetted and this was followed by incubation at 95 °C for 20 minutes to inactivate the enzyme. The quality and concentration of the proteinase K has been found critical for the preparation of genomic DNA from single cells. For the preparation of single cells, PK concentration should be 125 μg/ml, and for clump of cells 400 μg/ml. If the concentration of PK has increased to more than 125 μg/ml for single cells it has been found to decrease the efficiency of amplification may be due to the damage that PK could cause for genomic DNA.
2 - 2.2.2: KOH/DTT (Alkaline buffer)

To compare the actual effect of different lysis buffers on the efficiency of amplifying both alleles in single cells, KOH/DTT was used to lyse 40 single cells. Five µl of 200 mM KOH and 50 mM DTT was added to each tube. Following transfer of the isolated single cells to each tube, lysing was completed by heating the samples for 10 minutes at 65 °C after adding 50 µl of mineral oil to each tube.

2 - 3: Polymerase Chain Reaction (PCR)

2 - 3.1: Oligonucleotides:

Oligonucleotide primers were purchased as purified solutions. The annealing temperature of the primers was calculated by the formula Tm = 4(G+C) 2(A+T). Appendix 2.3 shows the sequences of primers used and their annealing temperatures.

2 - 3.2: DNA amplification from samples with known mutations

When establishing the technique, DNA samples with known mutations at 100 ng/µl concentration were amplified using different primers depending on the region investigated, in a final reaction volume of 25 µl for a total of 30 cycles. All the above mentioned mutations were detected using primers B30; Ext I β and B1.1, however, mutations in exon II and IVSII nt 1 were detected using exon II primers, and intron II mutations using B1-2 primers.
2 – 3.3: Contamination Control

In order to minimise contamination with extraneous DNA during the experimental work, a specific hood for setting up and running the initial PCR was used. In addition to that, filtered pipette tips and dedicated Gilson micropipettes for PCR were used. Blanks where no DNA was added were used as negative controls to assess the incidence and source of contamination. Two types of blanks were used: the first type contains only PCR mix, and the second type contains small volumes of the medium that contained single cells during the isolation process.

2-3.4: Nested PCR

2-3.4.1: β globin gene

In vitro amplification of single cell genomic DNA was performed by nested PCR using Taq DNA polymerase. Two sets of specific oligonucleotide primers Ext I β and Int I β; B30 & Ext I β (appendix 2.3) were designed and used to allow amplification of the β globin DNA sequence where the most common mutations within the different ethnic groups of the UK population are located (Figures 2 – 3).

The mixture for the outer amplification contained: 7.5 p moles/μl of each outer primer, 0.2 mM nucleotides (dNTPs), 10X Super Taq buffer and 1U Super Taq polymerase (HT Biotechnology). Reaction volumes were made up to 50 μl and overlayed by paraffin oil (BDH). Five μl of the outer amplification products were aliquoted and PCR reagents were added as described above except that 1 p moles/μl
of each inner primer was used. Amplification was performed for 30 cycles in each run as follows: with 30 seconds denaturing at 94 °C, 45 seconds primer annealing at 60 °C and extension for 45 seconds at 72°C.

2.3.4.2: Comparison between different lysis buffers

To investigate the effect of the lysis buffer on the efficiency of amplifying both alleles in heterozygous cells, a total of 177 single fibroblasts and lymphocytes was amplified following lysis by one of two methods (Table 2 - 1). Cells were obtained from patients and CVS cultures known to be carriers for the ΔF508 mutation causing CF and a base substitution mutation in the β globin gene causing haemoglobin C (HbC). For all cells the amplification was performed by nested PCR using Taq polymerase (HT Biotechnology).

Cells lysed by KOH/DTT

In vitro amplification of exon 10 of the cystic fibrosis transmembrane regulatory (CFTR) gene from single cells that were lysed by KOH/DTT was performed using the two sets of primers which will generate 154 bp in case of the normal and 151 bp in case of the mutant with ΔF508 (appendix 2.3). The outer amplification mix contained: neutralising buffer (900 mM Tris HCl - pH 8.3, 300 mM KCl, 200 mM HCl); 0.2 mM nucleotides (dNTPs), 1 U Taq polymerase, 0.8 μM of each of the outer primers and PCR buffer (1 in 10 dilution of 25 mM MgCl₂, 1 mg/ml gelatine, 100 mM Tris HCl - pH 8.3). Amplification was performed for 25 cycles as follows: denaturation at 96 °C for 45 seconds, annealing at 40 °C for 45
Figure 2-3: Sequence of Ext I β, Int I β and B30 primers used to amplify β globin gene from single cells.

**ExtIβ**

5' - GCA ACC TCA AAC AGA CAC CA -3'

5' - CAA AGA ACC TCT GGG TCC AA -3'

**IntI**

5' - CTG AGG AGA AGT CTG CCG TT -3'

5' - GGT AGA CCA CCA GCA GCC TA -3'

**B30**

5' - ACC TCA CCC TGT GGA GCC AC -3'

5' - CAA AGA ACC TCT GGG TCC AA -3'
Table 2-1: Single somatic cells of different types used to analyze the difference between the effect of KOH/DTT and SDS/PK lysis buffers on the efficiency of DNA amplification.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Lysis buffer</th>
<th>No cells</th>
<th>Cell type</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF</td>
<td>KOH/DTT</td>
<td>18</td>
<td>lymphocytes</td>
<td>ΔF508</td>
</tr>
<tr>
<td></td>
<td>SDS/PK</td>
<td>57</td>
<td>fibroblasts</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>58</td>
<td>lymphocytes</td>
<td></td>
</tr>
<tr>
<td>HbC</td>
<td>KOH/DTT</td>
<td>22</td>
<td>fibroblasts</td>
<td>CD6 G-A</td>
</tr>
<tr>
<td></td>
<td>SDS/PK</td>
<td>22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
seconds, and extension for 1.5 minutes at 72 °C. When the outer amplification was completed, 5 μl of the amplified product was added to a second PCR mixture containing: 0.8 μM of each of the inner primers, 0.2 μM nucleotides (dNTPs), 5 μl of 10X PCR buffer II and 1 U Taq polymerase in a final volume of 50 μl. The same amplification cycles were performed as described above except that the annealing temperature for the inner primers was 50 °C.

The same nested PCR protocol was followed for amplifying exon I of the β globin gene using B30 and Ext I β primers described previously. The amplification was performed in each run for 25 cycles with 30 seconds of denaturing at 94 °C, annealing and extension for 45 seconds at 60 °C and 72 °C respectively.

**Cells lysed by SDS/PK**

The mixture of the outer amplification contained: 7.5 p moles/μl of each of the β globin primers described above or 0.8 μ moles of the CF outer primers; 0.2 mM nucleotides (dNTPs); 10X Super Taq PCR buffer (HT Biotechnology) and 1U of Taq polymerase, in a final volume reaction of 50 μl. Five μl of the outer amplified products were aliquoted and subjected to further amplification using the same mixture as for the outer amplification with only a change to the inner set of primers (5 p moles/μl of β globin and 0.8 μ moles of the CF inner primers). PCR cycling conditions were the same as described above for KOH/DTT.
2 - 3.5: Fluorescence based PCR

In fluorescent PCR usually the forward primers are labelled with fluorescent molecule which allows the PCR product to be detected by laser analysis on an automated DNA sequencer. The type of the fluorescent dye is mainly dependent on the type of the laser automated machine used for DNA analysis. In all fluorescent PCR studies described here forward primers were labelled with Cy5' at 5' end and analyses were conducted on the ALF Express automated DNA sequencer (Pharmacia Biotech).

2 - 3.5.1: β globin samples

DNA samples from β thalassaemia patients with known mutations (IVSI nt 110, IVSI nt 1 G-A, IVSI nt 1 G-T, IVSI nt 6, IVSI nt 25, IVSI nt 5, FS8/9 +G, CD 39, HbS) were obtained to act as positive controls when establishing the technique.

A total of 159 single cells (38 cells HbS/N, 50 cells IVSI nt 110/N, 20 cells IVSI 110, 36 cells IVSI nt 6/N, and 15 cells IVSI nt 6/IVSI nt 110) were isolated from buccal cells and CVS cultures as described above. Seven blastomeres were obtained from IVF embryos donated for research at Hammersmith Hospital - London. All cells were tubed in 2 μl of PBS and lysed with 125 μg/ml of PK as described previously, making the final volume of 4 μl.

Exon and intron I of the β globin gene were amplified using Taq polymerase and one set of normal primers (B30). The forward primer was fluorescently labelled at the 5' end with Cy5™. The PCR mixture contained: 1 μl of 100 ng/μl genomic
DNA or 4 μl of lysed cells, 7.5 p moles/μl of each primers, 0.2 mM nucleotides mixture (dNTPs), 10X Super Taq buffer and 1 U of Super Taq polymerase. Reaction volumes were made up to 25 μl and overlayed by 50 μl of paraffin oil. Amplification was performed for 19 cycles from 100 ng/μl DNA samples and 30 cycles from single cells as follows: denaturing at 94 °C for 30 seconds, annealing at 60 °C for 45 seconds and extension at 72 °C for 45 seconds.

2 - 3.5.2: CF ΔF508 samples

A total of 96 single buccal cells, fibroblasts and lymphocytes was obtained from normal, heterozygous and homozygous individuals for the ΔF508 mutation causing CF (Table 2 - 2), and 17 single blastomeres obtained from embryos known to be heterozygous or homozygous for the mutation following a PGD cycle (Hammersmith Hospital - London) were lysed by 2 μl of 125 μg/ml PK and analysed.

The 154 bp fragment of exon 10 of the CFTR gene where the ΔF508 mutation is located was amplified using the inner set of primers described in appendix 2.3. The forward primer was labelled at the 5' end by Cy5. The amplification was performed for 30 cycles as follows: 45 seconds denaturing at 94 °C, 45 seconds annealing at 50 °C and extension at 72 °C for 1.5 minutes.
Table 2-2: A total of 113 cells of different types isolated for the analysis of the CFTR gene by fluorescence based PCR.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Number</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal cells</td>
<td>12</td>
<td>Normal</td>
</tr>
<tr>
<td>Lymphoblasts</td>
<td>12</td>
<td>ΔF508</td>
</tr>
<tr>
<td>Lymphoblasts</td>
<td>70</td>
<td>ΔF508/N</td>
</tr>
<tr>
<td>Blastomeres</td>
<td>12</td>
<td>ΔF508/N</td>
</tr>
<tr>
<td>Blastomeres</td>
<td>5</td>
<td>ΔF508</td>
</tr>
</tbody>
</table>
2 - 3.5.3: Quantitative Multiplex PCR

Quantitative multiplex PCR methods have been recently used for the detection of chromosomal aneuploidy for chromosomes 21, 13, X and 18 by using highly polymorphic small tandem repeat (STR) markers (Mansfield 1993; Pertl et al 1996). The aims of this study were to optimise single cell PCR conditions for PGD of single gene disorders particularly in the case of mosaic embryos and to find out if it was possible to detect age related chromosome 21 aneuploidy in patients undergoing PGD for monogenic disorders.

The small tandem repeat D21S11 marker was used along with the CF primers to allow simultaneous amplification of the STR and exon 10 of the CFTR gene. Quantitative Fluorescent PCR (QF-PCR) was first carried out on genomic DNA samples obtained from normal and patients heterozygous for AF508. The PCR mix contained: 7 pM of the D21S11 and 8.5 pM of the CFQ (see Appendix 2 - 3) primers; 0.2 mM nucleotides (dNTPs), 10X Super Taq PCR buffer and 1U of Taq polymerase. Amplification was performed for 20 cycles as follows: denaturing at 94 °C for 30 seconds, annealing at 60 °C for 50 seconds and extension for 1 minutes at 72 °C. The expected fragment size of the CF is 100 bp for normal allele and 97 bp for the mutant, while the size of the resulting amplified STR is variable.

A total of 117 single cells of different types (Table 2 - 3) was amplified as described above but for 30 cycles. In all 53 single blastomeres from 10 IVF embryos donated for research (Hammersmith Hospital) were analysed to see if it was possible to assess the incidence of age related chromosome 21 aneuploidy in women receiving...
Table 2-3: Single cells used to analyse exon 10 of the CF gene and the D21S11 STR marker by quantitative multiplex fluorescent PCR.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Number</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocyte</td>
<td>6</td>
<td>Normal</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>8</td>
<td>ΔF508/N</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>50</td>
<td>Trisomy 21; normal for CF</td>
</tr>
<tr>
<td>Blastomeres</td>
<td>53</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>117</td>
<td></td>
</tr>
</tbody>
</table>
routine IVF. Six unfertilised oocytes were analysed to see the pattern in haploid cells. All cells were lysed by 2 µl of 125 µg/ml PK as described previously.

To test the efficiency of the procedure for PGD of single gene disorders, 7 single blastomeres from 3 different embryos that had undergone preimplantation diagnosed for CF at Hammersmith Hospital were tested blindly.

2 - 4: Mutation analysis

2 - 4.1: Agarose gel electrophoresis

On completion of the PCR cycles either from genomic DNA samples or single cells following nested PCR, 5 µl of each reaction mix including negative controls were aliquoted and mixed with 3 µl of agarose gel loading buffer on a nescofilm™ then loaded on 2% agarose gel in 1X TBE buffer. Gel electrophoresis was conducted at 100 V for 30 - 40 minutes, and then gels were stained by 2 µl/ml ethidium bromide. For the visualisation of the presence of amplified DNA products and the presence or absence of any contamination, gels were viewed under UV transillumination light.

2 - 4.2: Silver stained SSCP analysis

For SSCP analysis, 1.5 µl of amplified DNA was denatured at 95 °C for 10 minutes in a PCR block in the presence of 2 µl of 95% deionised formamide. Denatured samples were placed immediately on ice until the time of loading. Loading
wells were formed with Nescofilm™ using an applicator template mould (Pharmacia Biotech). Two µl of the denatured samples were loaded into the wells, and DNA samples were taken from the Nescofilm by a 12 well sample comb applicator (Pharmacia Biotech). The comb applicator was then placed in the electrophoresis chamber which has a pre-prepared homogenous 20% nondenaturing polyacrylamide gel (Phast gels - Pharmacia Biotech) that had been pre-run for 9 Vh with solid native buffer strips (Pharmacia Biotech). SSCP analysis was performed using automated gel electrophoresis (Phastsystem™ - Pharmacia Biotech). Several different conditions were tested for performing SSCP analysis in order to determine the most efficient for mutation detection. The Phastsystem conditions were varied by altering the temperature, duration of the pre-run and the total running time of separation. The pre-run conditions were set up as follows (as described by manufacturer): 400 V, 10 mA, 2 W at temperature 15 °C or 18 °C; while the electrophoresis conditions were: 400 V, 5 mA, 2 W for 2 Vh then followed by 400 V, 10 mA, 2 W for 350-400 Vh at the same temperature. DNA bands were detected by silver staining which was carried out automatically through 16 washes by the Phastsystem developer chamber as described by manufacturer. All solutions required for silver staining (Appendix 2.4) were prepared fresh at the time of use with distilled deionised water.

2 - 4.3: Fluorescent SSCP

Nondenaturing polyacrylamide gel was prepared in a final concentration of 0.5X of the original concentration as follows: 25 ml of MDE acrylamide gel
(Flowgen) mixed with 69 ml of Nanopure water, 6 ml of 10X TBE buffer (pH 7.4),
400 µl of 10% Ammonium persulfate and 40 µl of TEMED.

PCR products from 100 ng/µl DNA samples were diluted 1:10 and 2 µl was
aliquoted for analysis, alternatively, 2 µl of amplified products from single cells were
aliquoted. Aliquotted PCR amplified fluorescent DNA fragment of the β globin gene
were mixed with 5 µl of loading buffer containing formamide and Dextran blue
(Pharmacia) and subjected to heat denaturation at 95 °C for 10 minutes, and then
were put on ice until the time of loading. The ALF Express gel thermoplates were
rinsed with MilliQ water, after drying, the plates were rinsed with isopropanol then
set up on the machine after pouring the gel. Five µl of the denatured samples were
then loaded into the 0.35 mm nondenaturing polyacrylamide gel set on the ALF
Express with a gel temperature controlling system - The Multitemp (Pharmacia
Biotech). Gel electrophoresis was conducted using the ALF Express short plates at a
constant power of 25 W, 1500 V, 60 mA for a maximum of 200 minutes and the
running gel temperature 15 °C. Electrophoresis was carried out in an off-vertical gel
cassette thermoplate. Temperature control of the gel was achieved by circulating
water from the integral water circulator through one of the plates which forms the
cassette. During electrophoresis, the fluorescently labelled fragments in each lane
migrate down through the gel. The fixed laser beam, which passes through the glass
spacers that are located between the plates and is directed into the gel perpendicular
to the direction of band migration, excites the fluorescently labelled DNA bands and
the light emitted is detected by photodetectors located behind the gel; there is one for each lane. The photodetector signals are collected, analysed and sent to the computer for processing. Single stranded DNA bands were detected as peaks, and the image data was analysed by the ALF fragment manager programme (Allele links).

2 - 4.4: Heteroduplex analysis (HA)

During amplification of heterozygous DNA samples, heteroduplexes will be formed. On polyacrylamide gel electrophoresis, heteroduplexes migrate slower than homoduplexes. In the heterozygous cells heteroduplex formation shows that both alleles have amplified and the presence of the homoduplex band alone indicates allele drop out. SSCP and heteroduplex analysis were performed using automated gel electrophoresis (Phastsystem™). 1.5 μl of the amplified products were denatured in the presence of 2 μl of 95% deionised formamide at 95 °C for 10 minutes to produce single stranded DNA. Samples were then loaded on 20% nondenaturing polyacrylamide gels (Phast gels - Pharmacia Biotech) which had been pre-run as described previously for 49 Vh. Denaturation of samples for SSCP is an imperfect process leaving a significant proportion of the DNA in a double stranded state. Consequently SSCP and heteroduplex analysis can be performed simultaneously. The combination of SSCP-HA allows the identification of the allele that failed to amplify.

An increased pre-run allows the visualisation of heteroduplexes but may also cause single strand patterns to be less clearly resolved. Heteroduplex analysis - SSCP
was conducted when the running gel temperature was 15 °C for β globin mutations and 10 °C for the CF mutation under the same electrophoresis conditions described above with a total separation time of 145 Vh.

2 – 4.5: Quantitative Analysis

A 6% of acrylamide gel (Severn Biotech Ltd) was prepared and poured on 0.35mm ALF gel large thermoplate and 2 μl of the amplified products of single cells was loaded in the gel after denaturation in the presence of 5 μl of the loading buffer containing formamide at 95 °C for 5 minutes. Gel electrophoresis was run on an automated DNA sequencer, the ALF Express, under the same conditions described above but when the gel temperature was ambient. DNA bands were analysed quantitatively by the ALF Fragment Manager Programme.

2 – 4.6: Automated DGGE

An automated fluorescent DGGE method has been developed to overcome the limitations of the conventional method. Because of the sensitivity of the automated DNA sequencer in detecting variations, DNA samples were generally amplified using non-GC clamp primers (as used before for SSCP analysis).

DNA samples from normal, β thalassaemia (IVSI nt 110, IVSI nt 6, IVSI nt 1 G-A, IVSI nt 5, CD5 -CT, CD30, CD8 -AA) and CF (ΔF508) patients were amplified using the sets of normal non-GC clamped primers (B30 for β globin gene and CF inner for CF) which are fluorescently labelled at the 5' end with Cy5 as
described above (appendix 2.3). DNA samples were amplified as previously described for 19 cycles. 2 μl of the diluted (1:10) amplified DNA was then subjected to DGGE analysis on an automated DNA sequencer the ALF Express. To create a gradient gel of 45% - 70%, the short plate of the ALF was sealed with 10% agarose gel to allow loading the gel in the vertical position. Seventy per cent denaturing acrylamide was prepared by adding 5.95 ml of 100% denaturing solution with 2.55 ml of 0% denaturing solution; and the 45% contains 3.825 ml of 100% denaturing solution and 4.675 of 0% denaturing solution. The 100% denaturing solution contains 21.67 ml of 40% acrylamide; 40 ml of 100% deionised formamide; 72.04g of 7 M urea and 5 ml of 20X TAE buffer, making the final concentration of acrylamide up to 6.5%. The 0% denaturing solution contains the same chemicals excluding only the urea and formamide as denaturant materials. Solutions were prepared with 20X TAE buffer pH 9, to improve the quality of mutation detection. The gel gradient was made from 45% to 70% using a gradient maker. Electrophoresis was performed when the running temperature was 50 °C and on constant power 30 W for a maximum time of 180 minutes, and peaks were analysed by the ALF Fragment Manager. To test the efficiency of automated DGGE using non-GC clamped primers for diagnosis, five CVS samples were tested blindly for patients known to be carriers for CD5 -CT, IVSI nt 110, IVSI nt 6, CD 30, IVSI nt 5.

To compare the efficiency of mutation detection of IVSI nt 110 when using GC clamp and non GC clamp primers, 5 DNA samples from heterozygous and
homozygous individuals were amplified using GC clamp primers designed for conventional DDGE (appendix 2 - 3).

2 - 4.7: Sequencing Analysis

2 - 4.7.1: Egyptian Samples

Exon I, intron I and exon II of the β globin gene were amplified from genomic DNA of 16 samples of unrelated patients using Taq polymerase and one set of primers (B15-16) for 30 cycles as described previously. Screening for β thalassaemia mutations was carried out by silver stained SSCP analysis using automated gel electrophoresis under non-denaturing conditions and at different temperatures to detect abnormal conformations of mutant alleles (Phastsystem - Pharmacia). One DNA sample in which the SSCP pattern was suggestive of the FS 8/9 +G mutation was amplified with mutant ARMS primer for FS 8/9 +G (appendix 2.3) in a separate amplification reaction and products were visualised on 6% agarose gel (3% standard agarose, 3% ultra pure low melting point agarose) after electrophoresis in 1X TAE buffer at 150 V for 50 minutes with ethidium bromide staining. Samples that could not be identified by SSCP analysis were then subjected to direct sequencing using the autolode Solid Phase sequencing kit (Pharmacia). The PCR for sequencing was performed, as described in the protocol of the autolode Solid Phase sequencing kit, using one standard primer and the forward one was biotin labelled at the 5' end; amplification was for 30 cycles.
The sequence analysis was done by different steps as described by the manufacturer. Eighty \( \mu l \) of the Binding/Washing buffer (2M NaCl, 10mM Tris-HCl, pH 7.5, 1mM EDTA) was added to each well of the 10 well plate. 40 \( \mu l \) of the PCR product was added to each of the 10 wells plate as the sequencing combs were placed in the plate wells, mixed gently and incubated at 65 °C for 30 minutes. The teeth of the comb which contains immobilized streptavidin will capture the PCR products. Following incubation, the combs were washed by dipping them in Binding/Washing buffer. The non-biotinylated strands of the PCR products were removed by alkaline denaturation. 100 \( \mu l \) of freshly prepared 0.1 M NaOH solution was added to each well of a new plate. The combs were placed in the wells and incubated at room temperature for 5 minutes. The combs were then washed in 3 different solutions: first with 0.1 M NaOH followed by TE buffer (appendix 2.1) and last in sterile water. The immobilised single stranded products that remained bound to the sequencing combs was then used as a template for dideoxy sequencing reaction with a Cy5™ labelled primer.

A new 10 well plate was used for sequencing reaction and to each well 12 \( \mu l \) of annealing buffer, 4 \( \mu l \) of Cy5 labelled B30 primer (5 pmol) and 104 \( \mu l \) sterile water was added. The combs were then placed in the plate and incubated at 65 °C for 10 minutes. Then the plate was left at room temperature to cool for 20 minutes. The sequencing reaction was then prepared for each of the four nucleotides as follows: 30 \( \mu l \) of nucleotide mix, 20 \( \mu l \) of annealing buffer, 10 \( \mu l \) of extension buffer 120 \( \mu l \) of
sterile water, 10 μl of T7 DNA polymerase and 10 μl of DMSO. Nineteen μl of the master reaction was added to its corresponding well in a 40 well plate. The wells were pre-warmed at 37 °C for 2 minutes and the combs were then added to the plate, mixed and then incubated for 5 minutes at 37 °C. The plate was then placed on ice until the time of loading.

A 0.5 mm thickness of 6% ultra pure Sequagel XR (National Diagnostic) polyacrylamide gel was prepared by adding 40 ml of acrylamide solution, 10 ml of sequagel buffer and 200 μl of 20% ammonium persulfate on the large plate and placed on the ALF Express machine. The water temperature was set at 55 °C and the gel wells were washed with 1X TBE buffer, then 12 μl of stop solution was added to each well to release the labelled sequencing fragments from the combs. The combs were placed in the wells gently and left for 10 minutes and then removed as sequencing was performed. DNA sequencing was performed on the ALF Express under the following conditions: 1500 V, 35 W, 65 mA, sample interval 2 seconds for a maximum running time of 600 minutes, and analysed by the ALF Sequencing Programme. During electrophoresis, the raw data was displayed as a chromatogram in real time on the computer screen. Four different colours are used to display each of the four bases, in which one peak represents each nucleotide in the sequence (Figure 2-4).
Figure 2-4: Sequencing analysis on automated fluorescence system.

Gel electrophoresis  Computer Screen

N. El-Hashemite
Figure 2-5: Sequence of primers used to amplify the whole β globin gene.

5' - CGA TCT TCA ATA TGC TTA CCA AG -3'

5' - GGG CCT ATG ATA GGG TAA T -3'

Exon

Introduction
2 - 4.7.2: Jordanian Samples

Eighteen DNA samples from β thalassaemia individuals were sequenced using the Thermosequenase kit (Amersham-Pharmacia Biotech). The whole β globin gene was amplified using two sets of primers (Figure 2 - 5). The PCR cycling conditions were as follows: one cycle of denaturing at 94 °C for 5 minutes; 35 cycles of denaturing at 94 °C for 1 minute, annealing and extension at 56 °C and 72 °C respectively for 2 minutes each; one cycle of denaturing at 94 °C for 1 minute, annealing at 56 °C for 2 minutes and final extension at 72 °C for 8 minutes. Cycle sequencing was performed on the samples as following: 3 μl of the PCR amplified products were mixed with 4 μl of sequencing primer (5 pmol) labelled at 5' with Cy5 and mixed with 18 μl of steril distilled water. An aliquote of 5 μl of the master mix was added to each tube containing 2 μl nucleotide. 20 μl of mineral oil was added to each tube and tubes were then subjected to cycle sequencing for 18 cycles of denaturing at 94 °C for 30 seconds and annealing at 60 °C for 30 seconds. After the cycles of sequencing finished, the mineral oil was removed and 5 μl of loading buffer was added to each tube. Samples were then denatured at 95 °C for 5 minutes on a PCR block and then kept on ice while loading. Samples were loaded on 0.35 mm 6% ultrapure sequagel (National Diagnostic) polyacrylamide gel, and sequencing analysis was performed on the ALF Express under the same conditions as described above.
2 - 5: Efficiency of SSCP for first trimester Prenatal Diagnosis

2 – 5.1: Blind Studies

2 – 5.1.1: silver stained SSCP

Different blind studies were done to test the efficiency of silver stained SSCP analysis in detecting β globin mutations and the accuracy of diagnosis. The first blind study was done on 16 different DNA samples representing 7 different mutations obtained from normal, carrier and β thalassaemia trait individuals. Samples were divided into three different sets which represented the combination of heterozygous parents and their resulting offspring (Table 2 - 4). DNA samples were amplified using Ext I β primers as described previously and the PCR products of each set were analysed by SSCP along with positive controls to confirm the identification of the abnormal migration pattern of the amplified samples.

The second study was done to confirm the efficiency and accuracy of silver stained SSCP prior to application for clinical use either for first trimester prenatal diagnosis or preimplantation genetic diagnosis. This study set out to identify the genotype of CVS samples from ongoing and terminated pregnancies which had been previously diagnosed by ARMS in the Perinatal Centre - UCLH. Each set contained the parental DNA samples which were known to be heterozygous as well as the CVS DNA.
Table 2-4: DNA sets used in the first blind study representing heterozygous parents and possible resulting offspring.

<table>
<thead>
<tr>
<th>Set Number</th>
<th>Heterozygous possible parent</th>
<th>Possible resulting offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F: IVSI nt 6</td>
<td>Homozygous normal</td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 6</td>
<td>Homozygous affected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygous</td>
</tr>
<tr>
<td>1</td>
<td>F: IVSI nt 110</td>
<td>Homozygous normal</td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 110</td>
<td>Homozygous affected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygous</td>
</tr>
<tr>
<td>2</td>
<td>F: IVSI nt 6</td>
<td>Heterozygous (X2)</td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 110</td>
<td>Compound heterozygous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homozygous normal</td>
</tr>
<tr>
<td>3</td>
<td>F: IVSI nt 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 110</td>
<td></td>
</tr>
</tbody>
</table>
5.1.2: Fluorescent PCR-SSCP:

Eleven CVS DNA samples were tested blindly by fluorescence based PCR-SSCP analysis for the following mutations IVSI nt 110, IVSI nt 6, IVSI nt 1 G-A, IVSI nt 1 G-T, IVSI nt 5, CD 39 and HbS. The blind study was set up to identify the genotype of CVS samples which had been previously identified in the Perinatal Centre - UCLH. Each CVS sample was tested along with the parental DNA and positive controls.

5.2: Transcervical cell samples

Transcervical cell clumps were collected during ongoing pregnancies by aspiration of the cervical mucus from seven couples attending first trimester prenatal diagnosis for haemoglobinopathies and prior to the CVS procedure at UCLH. Couples were known to be carriers for HbS or β thalassaemia mutations (Table 2 - 5). Clumps of cells were collected and separated depending on the morphological characteristics of trophoblasts using an inverted microscope, after being suspended in PBS. Separated clumps were then washed in PBS and transferred to 0.5 ml eppendorf tubes. Genomic DNA was extracted by the additional of 2 µl of 17 µM SDS and 400 µg/ml PK which was incubated at 37 °C for one hour, 50 µl of paraffin oil was added to each tube, which was then incubated at 95 °C to inactivate the PK for 30 minutes. The β globin gene fragment from extracted DNA was amplified by nested PCR as described previously using primers B30 and BI.1 and the amplified
products were tested blindly along with parental DNA samples by silver stained SSCP.

2-6: Clinical cases

Prenatal diagnosis was carried out in six clinical cases by silver stained SSCP for β thalassaemia and one case for CF ΔF508 using fluorescent DNA fragment analysis. Preimplantation genetic diagnosis was performed in one case where both parents are known to be heterozygous for the IVSI nt 6 (maternal) and IVSI nt 110 (paternal) mutations causing β thalassaemia, using fluorescent SSCP analysis.
<table>
<thead>
<tr>
<th>Case No</th>
<th>Parental Genotype</th>
<th>Possible fetal genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: IVSI nt 110/N</td>
<td>Normal, IVSI nt 110, IVSI nt 110/N</td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 110/N</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F: CD 30 /N</td>
<td>CD 30 /N, IVSI nt 5 /N, Normal, CD 30/ IVSI nt 5</td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 5 /N</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F: IVSI nt 5/N</td>
<td>IVSI nt 5 /N, Normal, HbS/N, sβ thal</td>
</tr>
<tr>
<td></td>
<td>M: HbS/N</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>F: IVSI nt 6/N</td>
<td>IVSI nt 6/N, IVSI nt 110/N, Normal, IVSI nt 6/IVSI nt 110</td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 110/N</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F: IVSI nt 110/N</td>
<td>Normal, IVSI nt 110, IVSI nt 110/N</td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 110/N</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F: -88</td>
<td>sβ thal, -88/N</td>
</tr>
<tr>
<td></td>
<td>M: HbS/N</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>F: HbS/N</td>
<td>Normal, HbS/N, IVSI nt 2/N, sβ thal</td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 2/N</td>
<td></td>
</tr>
</tbody>
</table>
**Appendix 2.1: General Solutions & buffers.**

<table>
<thead>
<tr>
<th>Category</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – Electrophoresis buffers:</td>
<td></td>
</tr>
<tr>
<td>TAE</td>
<td>40 mM Tris-acetate, pH 8; 40 mM Sodium-acetate; 1 mM EDTA. Made up as 20X concentrate.</td>
</tr>
<tr>
<td>TBE</td>
<td>90 mM Tris-HCl, pH 8; 90 mM boric acid; 2 mM EDTA. Made up as a 10X concentrate</td>
</tr>
<tr>
<td>2 – Lysis buffer:</td>
<td></td>
</tr>
<tr>
<td>SDS/proteinase K for single cells</td>
<td>17 µM SDS; 125 µg/ml proteinase K</td>
</tr>
<tr>
<td>for clump of cells:</td>
<td>17 µM SDS; 400 µg/ml proteinase K.</td>
</tr>
<tr>
<td>KOH/DTT (Alkaline buffer)</td>
<td>200 mM KOH; 50 mM dithiothritol (DTT)</td>
</tr>
<tr>
<td>Neutralising buffer</td>
<td>900 mM Tris-HCl, pH 8.3; 300 mM KCl; 200 mM HCl.</td>
</tr>
<tr>
<td>3 – PCR buffers:</td>
<td></td>
</tr>
<tr>
<td>Super Taq PCR buffer</td>
<td>500 mM Tris-HCl, pH 9; 500 mM KCl; 70 mM MgCl₂; 160 mM (NH₄)₂SO₄</td>
</tr>
<tr>
<td>10X PCR buffer [K+] free *</td>
<td>25 mM MgCl₂; gelatine 1 mg/ml; 100 mM Tris-HCl, pH 8.3</td>
</tr>
<tr>
<td>10X PCR buffer II *</td>
<td>500 mM KCl; 100 mM Tris-HCl, pH 8.3; 25 mM MgCl₂</td>
</tr>
<tr>
<td>4 – Acid Tyrode's solution</td>
<td>2.0 g NaCl; 0.05g HCl; 0.06 g CaCl₂.2H₂O; 0.025g MgCl₂.6H₂O; 0.25g glucose; 1.0g PVP. Made in a final volume of 250 ml and to a pH 2.3 with 5N HCl</td>
</tr>
<tr>
<td>5 – TE buffer</td>
<td>10 mM Tris-HCl; 1 mM EDTA, pH 8</td>
</tr>
<tr>
<td>6 – Binding/Washing solution</td>
<td>2 mM NaCl; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA</td>
</tr>
</tbody>
</table>
Appendix 2.2: Cell Culture Media:

**Dulbecco's MEM medium**

74 ml sterile deionized distilled water; 9 ml Dulbecco's MEM; 10 ml fetal calf serum; 7.5 ml 5% w/v sodium bicarbonate; 1 ml GPS (Glutamine, penicillin, streptomycin); 1-1.5 ml NaOH in a final volume of 100 ml

**HEPES buffered Eagle's MEM medium**

72 ml steril deionised distilled water; 8 ml 10X MEM Eagles; 1 ml 10 mM HEPES buffer; 20 ml 10% serum; pH 7.2 with sodium bicarbonate, 1 ml GPS.
Appendix 2.3: Oligonucleotide primer sequences and annealing temperatures.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequence</th>
<th>Annealing temp °C</th>
</tr>
</thead>
</table>
| Ext 1β         | F: 5'-GCA ACC TCA AAC AGA CAC CA-3'  
R: 5'-CAA AGA ACC TCT GGG TCC AA-3' | 60 |
| Int 1β         | F: 5'-CTG AGG AGA AGT CTG CCG TT-3'  
R: 5'-GGT AGA CCA CCA GCA GCC TA-3' | 60 |
| B30            | F: 5'-ACC TCA CCC TGT GGA GCC AC-3'  
R: 5'-CAA AGA ACC TCT GGG TCC AA-3' | 60 |
| II10GC-clamp   | F: 5'-AAG GAG ACC AAT AGA AAC TG-3'  
R: 5'-GGT AGA CCA CCA GCA GCC TA-3' | 65 |
| B15-16         | F: 5'-CAA TGT ATC ATG CCT CTT TGC ACC-3'  
R: 5'-GAG TCA AGG CTG AGA GAT GCA GCC GGA-3' | 62 |
| B1-2           | F: 5'-AGT GCT GCA AGA AGA ACA ACT ACC-3'  
R: 5'-CCT TGC ACT ATG GGC AGT GAG CTC-3' | 62 |
| FS 8/9 ARMS    | F: 5'-ACC TCA CCC TGT GGA GCC AC-3'  
R: Mutant: 5'-CCT TGC CCC ACA GGA CAG TAA CGG CAG ACC-3' | 65 |
| Whole gene     | F: 5'-CGA TCT TCA ATA TGC TTA CCA AG-3'  
R: 5'-GGG CCT ATG ATA GGG TAA T-3' | 55 |
| CF Outer       | F: 5'-GCA TTC ACT TCT AAT GAT GAT-3'  
R: 5'-CTC TGC ACT ATG GGC AGT GAG CTC-3' | 40 |
| CF Inner       | F: 5'-TGG GAG AAC TGG AGC CTT-3'  
R: 5'-GGT TTG ATG ACG CTG TTC TAT-3' | 55 |
| D21S11         | F: 5'-TAT GTG AGT CAA TTC CCC AAG TGA-3'  
R: 5'-GGT GTA TTA GTG AAT GTT GTC CAG-3' | 60 |
| B1.1           | F: 5'-ACC TCA CCC TGT GGA GCC AC-3'  
R: 5'-GGT AGA CCA CCA GCA GCC TA-3' | 60 |
| CFQ            | F: 5'-GTT TTC CTG CAT TAT GCC TGG CAC-3'  
R: 5'-GTT GGC ATG CCT TGA TGA CGC TTC-3' | 60 |
| Exon II        | F: 5'-GGC CAA TCT ACT CCC AGG AG-3'  
R: 5'-ACA TCA AGG GTC CCA TAG AC-3' | 62 |

F: Forward primer; R: Reverse primer
Appendix 2.4: Silver staining procedure for native homogenous 20% polyacrylamide gels (Phast Gels).

<table>
<thead>
<tr>
<th>Step No</th>
<th>Solution</th>
<th>Time</th>
<th>Temp</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20% w/v trichloroacetic acid</td>
<td>5</td>
<td>20</td>
<td>Fixing solution</td>
</tr>
<tr>
<td>2</td>
<td>50% w/v ethanol; 10% Acetic acid</td>
<td>2</td>
<td>50</td>
<td>Wash solution</td>
</tr>
<tr>
<td>3</td>
<td>10% w/v ethanol; 5% Acetic acid</td>
<td>2</td>
<td>50</td>
<td>Wash solution</td>
</tr>
<tr>
<td>4</td>
<td>10% w/v ethanol; 5% Acetic acid</td>
<td>4</td>
<td>50</td>
<td>Wash solution</td>
</tr>
<tr>
<td>5</td>
<td>5% w/v gluteraldehyde</td>
<td>6</td>
<td>50</td>
<td>Protein sensitisation</td>
</tr>
<tr>
<td>6</td>
<td>10% w/v ethanol; 5% Acetic acid</td>
<td>3</td>
<td>50</td>
<td>Wash solution</td>
</tr>
<tr>
<td>7</td>
<td>10% w/v ethanol; 5% Acetic acid</td>
<td>5</td>
<td>50</td>
<td>Wash solution</td>
</tr>
<tr>
<td>8</td>
<td>Dionised water</td>
<td>2</td>
<td>50</td>
<td>Wash solution</td>
</tr>
<tr>
<td>9</td>
<td>Dionised water</td>
<td>2</td>
<td>50</td>
<td>Wash solution</td>
</tr>
<tr>
<td>10</td>
<td>0.4% w/v silver nitrate</td>
<td>10</td>
<td>40</td>
<td>Silver staining</td>
</tr>
<tr>
<td>11</td>
<td>Dionised water</td>
<td>0.5</td>
<td>30</td>
<td>Wash solution</td>
</tr>
<tr>
<td>12</td>
<td>Dionised water</td>
<td>0.5</td>
<td>30</td>
<td>Wash solution</td>
</tr>
<tr>
<td>13</td>
<td>2.5% w/v sodium carbonate; 0.02% formaldehyde</td>
<td>1</td>
<td>30</td>
<td>Developing solution</td>
</tr>
<tr>
<td>14</td>
<td>2.5% w/v sodium carbonate; 0.02% formaldehyde</td>
<td>10</td>
<td>30</td>
<td>Developing solution</td>
</tr>
<tr>
<td>15</td>
<td>3.7% Tris-HCl, 2.5% sodium thiosulphate</td>
<td>2</td>
<td>30</td>
<td>Reducing background</td>
</tr>
<tr>
<td>16</td>
<td>5% w/v glycerol</td>
<td>5</td>
<td>50</td>
<td>Stop solution</td>
</tr>
</tbody>
</table>
Chapter 3

RESULTS
Chapter 3

RESULTS

3-1: Mutation analysis of \(\beta\) thalassaemia mutations using single strand conformation polymorphism (SSCP)

3-1.1: Silver stained SSCP

When establishing the technique, a total of 22 different mutations (Table 3-1) were subjected to silver stained SSCP analysis. All the \(\beta\) globin mutations investigated at this stage were detected from DNA samples. The principle of SSCP is that under nondenaturing conditions, each single stranded DNA molecule will adopt a specific conformation at a given temperature. Thus, the single stranded DNA molecule carrying a mutation can be identified by virtue of its altered mobility during electrophoresis.

The presence of a mutation within the amplified sequence of the gene can be detected by the appearance of a novel conformation for that amplicon. Figures 1 and 2 show detection of PCR amplified DNA from individuals with different mutations in the \(\beta\) globin gene. Theoretically, in the case of a heterozygous individual, DNA molecules show the appearance of four bands; these represent the normal allele and the mutant (see introduction Figure I - 30). The homozygous affected will show only the conformation of the mutant allele. This shows the advantage of SSCP over many
Table 1: The most common β thalassaemia mutations detected by SSCP analysis and the optimal temperature for mutation detection by SSCP.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Ethnic Origin</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbS ¹</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>HbC ¹</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>HbE ¹</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>CD 5 -CT ¹</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>CD 8 -AA ¹&amp;²</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>FS 8/9 +G ¹&amp;²</td>
<td>Asian Indian</td>
<td>18</td>
</tr>
<tr>
<td>CD 15 ²</td>
<td>Asian Indian</td>
<td>15</td>
</tr>
<tr>
<td>CD 16 ²</td>
<td>Asian Indian</td>
<td>15</td>
</tr>
<tr>
<td>CD 30 ²</td>
<td>Turkish</td>
<td>15</td>
</tr>
<tr>
<td>- 88 T-A ¹</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>CD 39 ³</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>FS 41/42 -TCTT ³</td>
<td>Asian Indian</td>
<td>18</td>
</tr>
<tr>
<td>IVSI nt 1 G-A ²</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>IVSI nt 1 G-T ²</td>
<td>Asian Indian</td>
<td>15</td>
</tr>
<tr>
<td>IVSI nt 5 ²</td>
<td>Asian Indian</td>
<td>15</td>
</tr>
<tr>
<td>IVSI nt 6 ²</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>IVSI nt 25 ²</td>
<td>Kuwaiti</td>
<td>15</td>
</tr>
<tr>
<td>IVSI nt 2 ²</td>
<td>African</td>
<td>15</td>
</tr>
<tr>
<td>IVSI nt 110 ²</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>IVSII nt 1 ³</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>IVSII nt 745 ⁴</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
<tr>
<td>IVSII nt 848 ⁴</td>
<td>Mediterranean</td>
<td>15</td>
</tr>
</tbody>
</table>

1 = mutations detected when using B30 primers; 2 = mutations detected when using Ext I β primers; 3 = mutations detected when using exon II primers; 4 = mutations detected when using B15-16 primers.
Figure 3-1: Detection of a mutation in exon II of the β globin gene using silver stained SSCP from template DNA. Lane 1 = normal; Lane 2 = C39 /N. The arrow indicates the novel conformer of the mutant allele in exon II. Samples were run at 15 °C for 250 Volt hours (Vh).
Figure 3 - 2: Detection of a frameshift mutation in exon 1 of the β globin gene at codon 8/9 (+G) by SSCP. The temperature of the running gel was 18 C. Lane 1 = homozygous for CD 8/9 +G; Lane 2 = Normal control; Lane 3 = heterozygous for CD 8/9 +G. Arrow indicates the novel conformer of the mutant allele.
of the other existing techniques for mutation detection since it allows the visualisation of each allele. However, the behavior of the heterozygous single stranded DNA molecules on the nondenaturing polyacrylamide gel sometimes gives different conformations and numbers of bands, which might depend on the type of the mutation and the presence of polymorphisms. Thus, detection of mutations by SSCP relies on the presence of a different conformation in mutant samples from the normal, for example Figure 3 – 3. Where two mutations are close to each other, for example HbS and HbC in which a mutation affects the adjacent nucleotide at codon 6 of the β globin gene, the DNA from an individual with sickle cell anaemia will give a different conformation from that of HbC (Figure 3 - 4).

The spectrum of β thalassaemia mutations in the UK is heterogeneous due to the different ethnic groups of the immigrants. The main ethnic groups at risk of β thalassaemia in the UK are Asian Indian, Pakistanis, Mediterranean and African, with each population having their own battery of mutations. The mutational panel investigated initially included 19 mutations common to all these ethnic groups which covers around 90% of mutations. By using one set of primers, the most common and rare mutations occurring within exon I and intron I were detected by SSCP analysis. However, the optimal conditions for detection varied between different mutations. The most important factor is temperature, but increasing the running time was also found to improve separation of single strands. As well, an increased pre-run allowed visualisation of heteroduplexes, however, single strand patterns of β globin variants were then less resolved. The optimal conditions for the detection of β globin
Figure 3 – 3: SSCP analysis of the IVSI nt 110 mutation causing β thalassaemia. The heterozygous sample (Lane 3) shows the normal allele and the mutant in comparison with the normal (Lane 2), while the homozygous affected samples shows only the mutant allele (Lane 1). The arrow indicates the conformation of the mutant allele.
Figure 3 - 4: The presence of different changes in adjacent nucleotides in codon 6 of the β globin gene causing sickle cell anaemia and HbC, give different conformations when analysed by SSCP. Lane 1 = heterozygous for HbC; Lane 2 = normal control; Lane 3 = heterozygous for HbS. Arrow indicates the mutant allele conformer of each mutation.
variants using automated silver stained SSCP were found to be when the running temperature was 15°C or 18 °C (Table 3 - 1), the pre-run was 9 Volt hours (Vh) and the total running time 350 - 400 Vh.

3 - 2: Efficiency of nested PCR on single cells

3 - 2.2: Efficiency of nested PCR on single somatic cells

To enable preimplantation diagnosis of β thalassaemia by direct detection of mutant β globin genes at the single cell level, nested PCR followed by silver stained SSCP analysis was applied for the analysis of 304 single cells of different types.

Successful amplification of a fragment encompassing exon I and intron I of the β globin gene from single somatic cells was achieved by nested PCR (Table 3 – 2). The amplified products were clearly visualised by UV transillumination following electrophoresis on a 2% agarose gel stained with ethidium bromide. A total of 282 somatic cells of different types amplified successfully (96%). Only twelve buccal cells (4%) did not amplify which could be due to DNA degeneration after cell death before setting up the initial PCR, or failure to transfer the isolated cell to the tube.

3 - 2.3: Contamination

In all, 86 negative control tubes were used to assess the incidence of contamination. Only four tubes (4.6%) from 3 different reactions demonstrated any
Table 3 - 2: Amplification of 233 bp fragment from intron I of the β globin gene by nested PCR and detection of heterozygous β thalassaemia mutations at IVS-I nt 1, nt 5, nt 6, and nt 110 by SSCP analysis.

<table>
<thead>
<tr>
<th>Single cells</th>
<th>Mutation</th>
<th>No</th>
<th>No amp</th>
<th>Genetic diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal</td>
<td>Normal</td>
<td>33</td>
<td>31</td>
<td>n/n</td>
</tr>
<tr>
<td>Buccal</td>
<td>IVSI 6/IVSI 110</td>
<td>53</td>
<td>53</td>
<td>C.het</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>IVSI 110/N</td>
<td>21</td>
<td>21</td>
<td>m/n</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>IVSI 1/IVSI 5</td>
<td>15</td>
<td>15</td>
<td>C.het</td>
</tr>
<tr>
<td>Buccal</td>
<td>IVSI 110/N</td>
<td>79</td>
<td>78</td>
<td>m/n</td>
</tr>
<tr>
<td>Buccal</td>
<td>IVSI 1/N</td>
<td>35</td>
<td>30</td>
<td>m/n</td>
</tr>
<tr>
<td>Buccal</td>
<td>IVSI 110</td>
<td>58</td>
<td>54</td>
<td>m/m</td>
</tr>
<tr>
<td>Blastomere</td>
<td>Normal</td>
<td>10</td>
<td>10</td>
<td>n/n</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>304</td>
<td>292</td>
<td></td>
</tr>
</tbody>
</table>

No = number of cells; amp = amplified; n = normal; C.het = compound heterozygous; m = mutant
DNA amplification. This amplification was not specific and would not be confused with products derived from either normal or mutant β-globin samples.

3 - 2.4: Silver stained SSCP

Following the amplification of exon and intron I of the β-globin gene from single cells, the PCR products were subjected to SSCP analysis for mutation detection and analysis. All β-globin mutations investigated at the single cell level which were obtained from individuals known to be normal, heterozygous and compound heterozygous for the mutations within intron I at nucleotides 1 G-A, 1 G-T, 5, 6, 110, were detected by SSCP (Figure 3 - 5).

3 - 2.5: Sensitivity of nested PCR for PGD

To assess the efficiency of nested PCR and amplification of the β-globin gene from single blastomeres prior to clinical application, DNA from 10 nucleated single blastomeres from morphologically normal embryos was subjected to amplification by nested PCR using primers for exon and intron I. Genomic DNA from all ten amplified without any contamination and gave the expected normal pattern when analysed by SSCP (Figure 3 - 6).

3 - 2.6: Allele specific amplification failure

The occurrence of allele specific amplification failure (allele drop out – ADO) following PCR amplification from heterozygous single cells affects the detection of variants and can lead to misdiagnosis. Therefore extensive preliminary work was
Figure 3 - 5: Detection of compound heterozygotes IVSI nt 6/ IVSI nt 110 from single cells (Lanes 1 & 2) using silver stained SSCP analysis. Lane 3 = IVSI 6/N; Lane 4 = IVSI 110/N; Lane 5 = Normal.
Figure 3-6: SSCP analysis of exon I and intron I of the β globin gene amplified from single blastomeres by nested PCR (Lane = 1) comparison with normal DNA (Lane = 2). Electrophoresis analysis was at 15 °C for 200 Vh.

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done on different types of single cells to assess the incidence of allele drop out when amplifying exon and intron I of the β globin gene. Interestingly, allele drop out was not observed in any of the 200 PCR amplifications (Figure 3 - 7) performed on single cells that were heterozygous or compound heterozygous for IVSI nt 110, IVSI nt 6, IVSI nt 1 G-T, IVSI nt 5, IVSI nt 1 G-A (Table 3 - 2).

3 - 3: Comparison between SDS/PK and KOH/DTT lysis buffers

To investigate the role of lysis buffer on the efficiency of amplifying both alleles in heterozygous cells, a total of 117 single cells from patients heterozygous for CF ΔF508 and HbC was used to assess the affect of the DNA preparation method by using two different lysis buffers (SDS/PK and KOH/DTT) on the incidence of allele drop out.

The efficiency of amplification of exon 10 of the CFTR gene and exon I of the β globin gene by nested PCR following both lysis methods was high (Table 3 - 3). DNA from all cells was amplified successfully without any evidence of contamination (a total of 28 blanks in total).

The amplified products from 108 single cells were subjected to heteroduplex analysis to assess the incidence of allele dropout. Three of the 17 (17.6%) cells heterozygous for the CF mutation ΔF508 that were lysed with the alkaline buffer (KOH/DTT) did not show heteroduplexes (Table 3 - 3), indicating the presence of
Figure 3 - 7: The amplification of both alleles in heterozygous cells obtained from a patient known to be a carrier for the mutation IVSI nt 110 (Lanes 2 to 4) in comparison to Normal (lane = 1). The arrow indicates the conformation of mutant allele.
Table 3-3: The incidence of allele drop out when amplifying exon 10 of the CFTR gene, and exon I of the β globin gene, from a total of 117 single heterozygous cells.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Lysis</th>
<th>No</th>
<th>Cell type</th>
<th>Mutation</th>
<th>No</th>
<th>No with</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>KOH/DTT</td>
<td>18</td>
<td>Lymphocyte</td>
<td>ΔF508</td>
<td>17</td>
<td>3</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>SDS/PK</td>
<td>115</td>
<td>Fibroblast</td>
<td></td>
<td>110</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>HbC</td>
<td>KOH/DTT</td>
<td>22</td>
<td>Fibroblast</td>
<td>CD6 G-A</td>
<td>21</td>
<td>1</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>SDS/PK</td>
<td>22</td>
<td>Fibroblast</td>
<td></td>
<td>20</td>
<td>none</td>
<td></td>
</tr>
</tbody>
</table>

amp = amplified; ADO = allele drop out; PK = proteinase K; No = number of cells.
allele drop out (Figure 3 - 8), whereas all 115 CF cells that were lysed by SDS/PK showed heteroduplexes without any example of ADO (Figure 3 - 9). Similarly, ADO was not observed in any of the 22 HbC samples that were lysed by SDS/PK (Figure 3 - 10) in comparison with 4.7% (1 in 22) (Table 3 - 3) in those lysed with KOH/DTT (Figure 3 - 11).

3 - 4: Efficiency of silver stained SSCP for diagnosis

The efficiency of the silver stained SSCP technique in mutation detection and the accuracy of diagnosis prior to its clinical application either for prenatal or preimplantation genetic diagnoses was tested in two different blind studies. In the first blind study, DNA samples were chosen to represent the possible heterozygous parents and the possible resulting offspring (See Materials & Methods Table 2 - 4). Samples were run along with normal and positive controls for each mutation and all samples representing the possible resulting offspring in this study were identified correctly (Figure 3 - 12).

The second blind study was aimed at identifying the genotype of CVS DNAs, which had been obtained for first trimester prenatal diagnosis. The CVS samples had been obtained from either ongoing or terminated pregnancies that had been diagnosed previously in the Perinatal Centre - UCLH using the ARMS techniques. A total of twenty two different CVS samples of unrelated families were studied and diagnosed using silver stained SSCP (Table 3 - 4). CVS DNA from each family was run along with parental DNA samples as heterozygotes positive controls and if
Figure 3 - 8: Heteroduplex analysis of single cells heterozygous for CF ΔF508. All cells that were lysed with SDS/PK showed heteroduplexes, indicating the amplification of both alleles.
Figure 3-9: Heteroduplex analysis of heterozygous single lymphocytes for ΔF508. Three of the cells that were lysed with KOH/DTT showed homoduplexes only indicating the presence of allele drop out, e.g., Lane = 3.
Figure 3 - 10: Assessment of allele drop out in single cells heterozygous for haemoglobin C by heteroduplex analysis. All cells that were lysed with SDS/PK showed heteroduplexes.
Figure 3 - 11: Assessment of the incidence of allele drop out in single heterozygous cells for HbC. One of the cells that was lysed with KOH/DTT (Lane 4) presented with homoduplex only indicating the presence of allele drop out.
Figure 3 - 12: SSCP analysis of DNA samples in the first blind study. Lane 1 = normal; Lane 2 = control homozygous for IVSI nt 110; Lane 3 = sample identified as heterozygous for IVSI nt 110; Lane 4 = IVSI nt 110/N positive control. The arrow indicates the novel conformer of the mutant allele.
<table>
<thead>
<tr>
<th>Case No</th>
<th>Parental genotype</th>
<th>CVS genotype identified by ARMS</th>
<th>CVS genotype identified by SSCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: IVSI 110/N</td>
<td>* Normal</td>
<td>* Normal</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 110/N</td>
<td>* IVSI 110/N</td>
<td>* IVSI 110/N</td>
</tr>
<tr>
<td>2</td>
<td>F: IVSI 5/N</td>
<td>IVSI 5/N</td>
<td>IVSI 5/N</td>
</tr>
<tr>
<td></td>
<td>M: CD 30/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F: IVSI 110/N</td>
<td>*IVSI16/IVSI110</td>
<td>* IVSI16/IVSI110</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 110/N</td>
<td>* IVSI 110/N</td>
<td>* IVSI 110/N</td>
</tr>
<tr>
<td>4</td>
<td>F: FS 8-9/N</td>
<td>FS 8-9/N</td>
<td>FS 8-9/N</td>
</tr>
<tr>
<td></td>
<td>M: FS 8-9/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F: HbS/N</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>M: HbS/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F: HbS/N</td>
<td>HbS</td>
<td>HbS</td>
</tr>
<tr>
<td></td>
<td>M: HbS/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>F: IVSI 5/N</td>
<td>IVSI 5/IVSI 5</td>
<td>IVSI 5/IVSI 5</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 5/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>F: IVSI 110/N</td>
<td>IVSI 110/IVSI</td>
<td>IVSI 110/IVSI 110</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 110/N</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F: IVSI 110/N</td>
<td>IVSI 6/N</td>
<td>IVSI 6/N</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 6/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>F: HbS/N</td>
<td>HbS</td>
<td>HbS</td>
</tr>
<tr>
<td></td>
<td>M: HbS/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>F: IVSI 110/N</td>
<td>IVSI 1/IVSI 110</td>
<td>IVSI 1/IVSI 110</td>
</tr>
<tr>
<td></td>
<td>M: IVSI nt 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>F: Not available</td>
<td>Normal</td>
<td>HbS/N</td>
</tr>
<tr>
<td></td>
<td>M: HbS/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>F: IVSI 110/N</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 110/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>F: IVSI 6/N</td>
<td>IVSI 6/N</td>
<td>IVSI 6/N</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 6/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>F: FS 8-9/N</td>
<td>FS 8-9/N</td>
<td>FS 8-9/N</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 1/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>F: IVSI 110/N</td>
<td>IVSI 110/N</td>
<td>IVSI 110/N</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 110/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>F: IVSI 110/N</td>
<td>IVSI 110/N</td>
<td>IVSI 110/N</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 110/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>F: FS 8-9/N</td>
<td>IVSI 5/N</td>
<td>IVSI 5/N</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 5/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>F: IVSI 110/N</td>
<td>IVSI 110/IVSI 110</td>
<td>IVSI 110/IVSI 110</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 110/N</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>F: FS 41-42/N</td>
<td>FS 41-42/N</td>
<td>FS 41-42/N</td>
</tr>
<tr>
<td></td>
<td>M: FS 41-42/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>F: IVSI 5/N</td>
<td>IVSI 5/N</td>
<td>IVSI 5/N</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 5/N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>F: IVSI 110/N</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>M: IVSI 110/N</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* different CVS DNA samples for same patients
available normal and affected DNA samples from the same family. Twenty one samples that were run along with the parental samples and positive controls were identified correctly. Figure 3 - 13 shows example of the identification of the CVS genotype using SSCP analysis.

One CVS sample (No 12) from a sickle cell anaemia case showed the heterozygous pattern (Figure 3 - 14) when analysed by SSCP while ARMS analysis showed that the fetus was normal. In this case, only the mother's DNA sample was available as a positive control for HbS, but the paternal was not. The identification of this CVS genotype as heterozygous could be explained by the presence of a polymorphism, possibly at codon 2, that has been inherited from the father, particularly since the primers cover that region. Polymorphisms are silent changes in the DNA sequence and their presence can affect the conformation of single strand DNA molecules (Figure 3 – 15) and this could explains the slight variation in the conformation of certain mutations (such as HbS) from different samples. One of the main aims of SSCP analysis is to detect the most common and rare mutations within a segment of 300 bp by using one set of primers. The codon 2 polymorphism is considered the most common one in exon I of the β globin gene, and it cannot be avoided when designing the primers because many families are known to be carriers for mutations in exon I and intron I. Thus by using one primer such as Ext I both parental mutations and compound heterozygous can be detected. In order to avoid the problem of polymorphism in diagnosis when applying SSCP analysis, both parental DNA samples along with normal and affected DNA samples if available
Figure 3 - 13: SSCP analysis of parental and CVS DNA of case 8 where both parents are known to be carriers for IVSI nt 110. Lane 1 = heterozygous sample control; Lane 2 = normal control; Lane 3 = homozygous affected control; and Lane 4 = CVS DNA sample identified as homozygous affected for IVSI 110. Arrow indicates novel conformer of mutation.
Figure 3-14: Case 12 in which the fetal DNA was identified as heterozygous for HbS while ARMS analysis showed that the fetus was normal. The extra band present in Lane 1 (CVS sample) in comparison with the Normal DNA (Lane 2) and the maternal sample (Lane = 3) could be the result of a polymorphism.
Figure 3-15: To show how the presence of a polymorphism in a DNA sequence may affect the conformation of the single stranded DNA when analysed by SSCP. Lane = 2 Normal DNA; Lane = 1 Normal DNA heterozygous for codon 2 polymorphism in the β globin gene.
from the same family’s children must be analysed along with CVS samples to assess whether a polymorphism that could affect the accuracy of a diagnosis is present.

3 - 4: Efficiency of nested PCR-SSCP analysis on transcervical cell samples

Isolated transcervical cell (TCC) clumps that were retrieved from patients known to be carriers for HbS and β thalassaemia attending for first trimester CVS prenatal diagnosis, were subjected to nested PCR to amplify exon I and intron I of the β globin gene. The PCR amplified products were then analysed along with parental DNA by silver stained SSCP to determine the fetal genotype and to assess the success of retrieving clumps of fetal origin for future application of non-invasive first trimester prenatal diagnosis using TCC sampling.

In case one, where both parents were carrying IVSI nt 110 (Table 3 - 5) three clumps were available for analysis. Two of the clumps gave the normal pattern (Figure 3 - 16) and one clump showed the heterozygous pattern for IVSI nt 110 mutation. Since the CVS pattern was normal, the result indicates that the first two clumps were of fetal origin, while the third clump consisted of maternal cells.

In the second case each parent was known to be a carrier for a different mutation (IVSI nt 5 and CD30) causing β thalassaemia (Table 3 - 5). All four clumps gave the pattern of heterozygous for IVSI nt 5 mutation (Figure 3 - 17) which was the maternal genotype. This indicates two possibilities: either the fetus was carrier for the maternal mutation or there was a maternal cell contamination of a normal sample.
Table 3 - 5: Results of the tested TCC clumps and the actual CVS diagnosis.

<table>
<thead>
<tr>
<th>Case No</th>
<th>Mutation</th>
<th>CVS</th>
<th>Clumps</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>1  2  3  4  5  6  7  8  9  10 11 12</td>
</tr>
<tr>
<td>1</td>
<td>IVSI 110/N</td>
<td>IVSI 110/N</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>IVSI 5/N</td>
<td>CD 30/N</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>Hbs/N</td>
<td>IVSI 5/N</td>
<td>IVSI 5/N</td>
</tr>
<tr>
<td>4</td>
<td>IVSI 110/N</td>
<td>IVSI 6/N</td>
<td>IVSI 6/N</td>
</tr>
<tr>
<td>5</td>
<td>IVSI 110/N</td>
<td>IVSI 110/N</td>
<td>IVSI 110/N</td>
</tr>
<tr>
<td>6</td>
<td>Hbs/N</td>
<td>-88</td>
<td>-88/N</td>
</tr>
</tbody>
</table>

AS = heterozygous for Hbs
Figure 3 - 16: Detection of the β globin genotype from the TCC clumps in case 1 by SSCP analysis. Lane 1 = TCC showing the heterozygous conformation for IVSI nt 110; Lane 2 & 3 = TCC clump showing normal conformation in comparison to normal DNA control (Lane 5); Lane 4 = maternal DNA control (IVSI nt 110/N).
Figure 3 - 17: SSCP analysis of exon and intron I of the β globin gene of TCC clumps from the second case showing the pattern of IVSI 5/N (Lanes 2-4) in comparison with paternal sample CD30/N (Lane 1), maternal sample IVSI nt 5/N (Lane 6) and normal control (Lane 5).
The actual CVS diagnosis showed that the fetus was normal (Table 3 - 5) which makes the second possibility more favourable, i.e., the presence of maternal cells is the explanation. The parents involved in the third case were known to be carriers for HbS (maternal) and IVSI nt 5 (paternal). Three clumps were analysed and gave the pattern of HbSβ thalassaemia while the fetus was a carrier for the paternal mutation (IVSI nt 5), according to the CVS result, therefore there was maternal tissue in the clumps.

Three clumps were isolated from case four, and gave the conformation of the paternal mutation (Figure 3 - 18), which indicates that the fetus was heterozygous for the IVSI nt 6 mutation and all clumps were of fetal origin. In the fifth case both parents were known to be carriers for IVSI nt 110 causing β thalassaemia, the three clumps showed the conformation of heterozygous for the mutation. Although CVS DNA results confirmed the result of the fetus being carrier for IVSI nt 110, it was difficult to determine the origin of such cells.

In the sixth case, the father was known to be a homozygous affected for a mild mutation at position -88 C-T causing β thalassaemia, and the mother was heterozygous for the sickle cell anaemia. DNA from seven out of eight clumps amplified. Five out of the amplified seven clumps showed the pattern of the heterozygote for -88 mutation and two clumps gave the conformation of HbS heterozygote (Figure 3 - 19). Such results indicate that the fetus was a carrier for the paternal mutation (-88 C-T) and the other two clumps were maternal cells.
Figure 3 - 18: SSCP analysis of amplified DNA for intron I of the b globin gene of TCC clumps from case 4 showing the conformation of heterozygous IVSI nt 6 (Lanes 4 - 5) in comparison to the paternal DNA control (Lane 3). Lane 1 = normal and Lane 2 = compound heterozygous (IVSI 6/IVSI 110) control sample.
Figure 3 - 19: SSCP analysis of amplified DNA for the β globin gene of five TCC clumps from case 6 showing the conformation of heterozygous -88 (Lanes 1,3,5,6) compared to the -88/N control (Lane 7). Two clumps (Lanes 2 & 4) showed the conformation of HbS compared with maternal control (Lane 9). Lane 8 = Paternal DNA sample homozygous for -88.
The couple of case seven was heterozygous for IVSI nt 2 and HbS. Four clumps were separated and one to three cells were isolated from each clump and tubed individually hoping to avoid the problem of maternal contamination. A total of 12 tubes were amplified and three gave the conformation of the maternal mutation (heterozygous IVSI nt 2) while the rest showed the pattern of the HbS carrier (paternal genotype). The CVS analysis showed that the fetus was a carrier for the IVSI nt 2 mutation which confirms that the first 3 cells could be either maternal or fetal cells. However, this does not explain the presence of paternal mutation in 9 clumps. There are two possibilities that could explain the presence of paternal cells particularly since TCC sampling remain an experimental procedure and women are not asked whether they have had a sexual intercourse prior to sampling. Either these cells were epithelial cells that come as a component of the ejaculation fluid, or white blood cells that are secreted in the semen as a result of infection of the genitalia. In either case, such cells will be collected from the accumulated seminal fluid during the transcervical aspiration of the mucus.

3 - 5: Fluorescence based PCR amplification from single cells

Nested PCR has many advantages over one run of 50 cycles of PCR particularly for amplifying a gene from single cells, by creating sufficient PCR product for analysis by conventional methods. However, there are two main problems with nested PCR, these are the longer time required for PCR cycling as 55 - 60 cycles are required, i.e., around 6 hours for amplification. In addition to the longer time of amplification, contamination with extraneous DNA sequences
increases even in a sterile environment. Such contamination may give false positives, which can lead to misdiagnosis. The problem of contamination with extraneous DNA in single cell PCR becomes apparent when more than 40 PCR cycles are performed or when reamplification is done. For this reason, the efficiency and reliability of a simplified and optimal PCR procedure for the diagnosis of β globin gene and CFTR gene variants from single cells was evaluated.

3 - 5.1: Diagnosis of haemoglobinopathies using Fluorescent PCR – SSCP analysis

The aim of this study was to assess the possibility of applying fluorescent PCR - SSCP analysis to detect mutant β globin genes from single cells. Initially, nine different mutations (IVSI nt 110, IVSI nt 1 G-A; IVSI nt 1 G-T; IVSI nt 6; IVSI nt 5; CD 8/9 +G; CD 39 and HbS) were investigated by analysis of DNA samples. When the temperature of the running gel was 15 °C (Figure 3 - 20), all 9 mutations could be detected and distinguished from the normal.

To determine if preimplantation genetic diagnosis could be reliably and accurately performed on single cells, genomic DNA from a total of 159 single cells (Table 3 – 6) was fluorescently amplified with primers B30 (Appendix 2.3). Three fibroblasts did not amplify giving a 98% success rate. The failure of amplification could be due to either the failure to transfer the cell to the tube, particularly as thin walled tubes had not been used at this stage of the research work to allow one to see the cell going into the tube; or DNA degradation prior setting up the PCR. All β globin mutations (HbS; IVSI nt 110; IVSI nt 6; IVSI nt 6/IVSI nt 110) tested were detected at the single cell level (Figure 3 - 21).
Figure 3 - 20: Detection of β globin mutations in exon I and intron I from fluorescently amplified products by SSCP. The analysis was conducted on the ALF Express when the running gel temperature was 15 °C.
Table 3 - 6: A total of 159 single cells of different types analysed for β globin mutation by Fluorescent PCR-SSCP analysis.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No of cells</th>
<th>Genotype</th>
<th>No amp</th>
<th>ADO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast</td>
<td>38</td>
<td>HbS/N</td>
<td>38</td>
<td>none</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>50</td>
<td>IVSI 110/N</td>
<td>50</td>
<td>none</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>20</td>
<td>IVSI 110</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>Buccal</td>
<td>36</td>
<td>IVSI 6/N</td>
<td>36</td>
<td>none</td>
</tr>
<tr>
<td>Buccal</td>
<td>15</td>
<td>IVSI 6/IVSI 110</td>
<td>15</td>
<td>none</td>
</tr>
<tr>
<td>Blastomere</td>
<td>7</td>
<td>Normal</td>
<td>7</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3-21: β globin mutations were detected at the single cell level following 30 cycles of fluorescent PCR amplification, and analysed by SSCP on the ALF Express. Lane 1: heterozygous cell for IVSI nt 6; Lane 2: Normal cell. Arrow indicates the conformation of the mutation.
As expected from the previous results with PK lysis buffer, allele specific amplification failure was not observed in any of the 139 heterozygous and compound heterozygous single cells when amplifying the β globin gene (Table 3 - 6).

To confirm the efficiency of amplification from single blastomeres prior to clinical application of fluorescent PCR - SSCP to either prenatal or preimplantation diagnosis, genomic DNA from seven blastomeres that were obtained from morphologically normal embryos was amplified fluorescently. DNA from all seven blastomeres amplified and gave the expected normal pattern of the β globin gene when analysed by SSCP (Figure 3 - 22).

The amplification of single cells using fluorescent PCR was completed in 3 hours, representing a 50% reduction in time, in comparison with the time required to complete amplification using the conventional nested PCR protocol. In addition, the entire procedure of electrophoresis from loading the gel to diagnosis took a maximum of 3 hours in which time a total of 40 samples can be analysed simultaneously.

In all, 17 negative control tubes were used to assess the incidence of contamination when amplifying the β globin gene from single cells using fluorescent primers. No amplification signals were detected in any of the negative control tubes indicating no contamination, this is in comparison to 4.6% contamination when using nested PCR to amplify the same gene under the same conditions.
Figure 3 - 22: Fluorescent amplification of intron I of the β globin gene from single blastomeres (Lanes 1-2) compared to normal DNA (Lane 3).
3 - 5.2: Efficiency of Fluorescent PCR - SSCP analysis in diagnosis

To determine the reliability of fluorescent PCR and the accuracy of SSCP in detecting β globin variants for diagnosis, eleven CVS cases were diagnosed blindly. All CVS genotypes were identified correctly as compared with ARMS results obtained previously by the Perinatal Centre (Figure 3 - 23).

3 - 6: Optimising single cell PCR and mutation detection of CF ΔF508

A total of 113 single cells of different types from individuals of known genotype were fluorescently amplified (Table 3 - 7). The reliability of fluorescent PCR in amplifying exon 10 of the CFTR gene was 97.3% (110/113). Twenty eight negative control tubes were used to assess the incidence of contamination and none of the blank tubes showed any example of contamination with extraneous DNA. Internal size markers of different sizes mixed with the amplified products prior to electrophoresis allow sizing of the fragment to single base pairs which yield accurate mutation detection. All analysed samples including blastomeres gave the expected pattern of 151 bp for the homozygous alleles bearing the 3bp deletion and 154 bp for the normal allele. The combination of the two alleles will appear in heterozygous status (Figure 3 - 24).

Using proteinase K as a lysis buffer, allele drop out was not observed in any of the heterozygous cells of either somatic or embryonic types (Table 3 - 7). The analysis of exon 10 of the CF gene from single cells can be performed within 4 hours including 3 hours for PCR amplification and 45 minutes for mutation detection and
Figure 3 - 23: Identification of IVSI nt 6 and IVSI nt 110 mutations within intron I from CVS DNA amplified fluorescently and analysed by SSCP analysis on the ALF Express, when the running gel temperature was 15 C. Lane 1 = maternal DNA sample heterozygous for IVSI nt 6; Lane 2 = paternal DNA sample heterozygous for IVSI nt 110; Lane 3 = compound heterozygous control DNA; Lane 4 = CVS DNA sample identified as heterozygous for IVSI nt 6; Lane 5 = Normal control.
Table 3-7: Different types of cells known to be carriers for ΔF508 mutation causing CF were analyzed by fluorescent PCR.

<table>
<thead>
<tr>
<th>No cells</th>
<th>Type</th>
<th>No amp</th>
<th>Genotype</th>
<th>ADO</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>buccal</td>
<td>11</td>
<td>normal</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>lymphocyte</td>
<td>12</td>
<td>ΔF508</td>
<td>-</td>
</tr>
<tr>
<td>70</td>
<td>lymphocyte</td>
<td>70</td>
<td>ΔF508/N</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>blastomere</td>
<td>9</td>
<td>ΔF508/N</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>blastomere</td>
<td>8</td>
<td>ΔF508</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3 - 24: Fragment analysis of CF ΔF508 mutation in single cells. Normal cells showed the 154 bp fragment (Lane 1); homozygous affected cells with 3bp deletion showed 151 bp fragment (Lanes 8 & 9) while heterozygous cells gave the pattern of both alleles (Lanes 2-7). Total gel electrophoresis time was a maximum of 90 minutes; CF peaks were detected at 40 minutes of total running time.
fragment analysis using the Fragment Manager of the ALF Express automated DNA sequencer.

3 - 7: Determination of CF ΔF508 and chromosome 21 aneuploidy using quantitative multiplex fluorescent PCR

Quantitative multiplex fluorescent PCR was applied at the single cell level to assess the feasibility of simultaneous detection of age related chromosome 21 aneuploidy causing Down's syndrome and the cystic fibrosis ΔF508 mutation in human embryos prior to implantation. Additionally, this study has aimed to investigate the possibility of applying multiplex PCR in increasing the accuracy of preimplantation diagnosis of monogenic disorders particularly with the existing problem of mosaicism.

Quantitative analysis of the fluorescent intensity of chromosome 21 STR marker D21S11 amplified products was expected to fall in three categories. Trisomic samples are expected to show 3 peaks of similar intensity 1:1:1 or two peaks with a ratio of 2:1, the first representing the presence of 3 different STR alleles and the second represents the presence of two identical STR copies when the third is a different allele. The third category is when one peak for the STR allele is present. This can be seen in either monosomic cells or rarely in normal or trisomic individuals who are homozygous for the STR allele (Figure 3 – 25).
Figure 3 – 25: Diagram to explain detection of trisomies using specific STR polymorphic markers by quantitative analysis. A: Most normal diploid samples will show two STR peaks with intensity ratio of 1:1 (1), i.e. diallelic. Highly polymorphic STR markers will show one peak in few homozygous individuals (2).

B: In trisomic samples, the presence of 3 STR peaks with a ratio 1:1:1 (1) represent trisomic triallelic; the presence of 2 STR peaks with a ratio of 1:2 represents trisomic diallelic (2), and very rarely and depending on how highly polymorphic is the STR marker some trisomic samples will show one STR peak (3), i.e., monoallelic.

A:

**Chromosome 21**

Maternal

Paternal

**Normal Diploid**

Gel electrophoresis

```
1 2
```

```
1 2
```

Primer peak

N. El-Hashemite

Results 201
Chromosome 21

Maternal

Extra copy of chromosome 21

Paternal

Trisomic Samples

Gel electrophoresis

Primer peak
Quantitative multiplex fluorescence PCR was employed to amplify 100 bp of exon 10 of the CFTR gene and the D21S11 STR polymorphic marker in chromosome 21. The multiplex PCR conditions for the concentration and annealing temperature of primers used was optimised. All amplified products were analysed quantitatively on the ALF Express fragment manager program to assess their fluorescent intensities and calculations. The total running time of amplification and analysis was 5.5 hours.

Fifty single fibroblasts isolated from a CVS culture that was obtained from a fetus diagnosed with trisomy 21 were analysed using primers for CFΔF508 and D21S11. All cells amplified without any incidence of contamination. Ten blanks were used to assess the incidence of contamination and were negative when analysed. The normal 100 bp peak of CFΔF508 was detected in all the 50 cells indicating that the fetus was normal for the CF. When analysing the STR alleles for D21S11 marker (Table 3–8), the fluorescent intensity of the peaks of 45 cells gave the ratio of 1:2 (Figure 3–26) and five cells gave the normal STR ratio of 1:1, these five cells could be maternal cells.

The amplified products from six unfertilised oocytes obtained from patients attending routine IVF and were known to be normal for CFΔF508 were analysed. The normal peak of CF was seen and a single peak for the STR D21S11 was detected indicating the presence of one copy of chromosome 21 (Figures 3–26 and 27). Eight lymphocytes that were obtained from chromosomally normal patients heterozygous for the CF mutation ΔF508 showed two peaks for the CFTR gene
Table 3 - 8: Single somatic cells tested with STR marker D21S11 for the detection of trisomy 21 by quantitative multiplex fluorescent PCR.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Total No</th>
<th>No amp</th>
<th>Mean Peak Ratio *</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte</td>
<td>8</td>
<td>8</td>
<td>1.06</td>
<td>1.0 - 1.2</td>
</tr>
<tr>
<td>Fibroblast (CVS)</td>
<td>50</td>
<td>45</td>
<td>1.97</td>
<td>1.87 - 1.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>1.1</td>
<td>1.03 - 1.22</td>
</tr>
</tbody>
</table>

* Mean Peak Ratio was calculated by dividing the larger area by the smaller area.

amp = number of cells amplified
Figure 3 - 26: Quantitative multiplex fluorescent analysis of single cells. Lane 3 is a single fibroblast isolated from CVS culture previously diagnosed with trisomy 21 by cytogenetics analysis showing the STR D21 S11 marker with a peak ratio of 1:2, and a normal CF peak for dF508. Lane 1 shows a single STR D21S11 peak from a single oocyte representing one copy of chromosome 21 and the normal CF peak for dF508 while Lane 2 is a normal single buccal cell showing a normal peak of CF dF508 and two STR peaks with a ratio of 1:1.
Figure 3 - 27: The amplification of exon 10 of the CFTR and STR D21S11 from an unfertilised oocyte showing a single peak for chromosome 21.
which represent the normal and the mutant allele; and two STR peaks with an intensity ratio of 1:1 (Figure 3 - 28).

Ten embryos (grade II.5 and III) that were donated for research from different patients undergoing IVF treatment were investigated for the number of chromosome 21 copies and the status of the cystic fibrosis mutation ΔF508. The age of the two patients was 32 and 37 years. Six embryos were from one patient who was aged 37 and the other four were from the other patient. The ten embryos were disaggregated into single cells and each amplified fluorescently. A total of 38 negative blanks was used to assess the incidence of contamination, and no signals were detected from any of the negative control tubes. As expected the blastomeres showed only the normal peak for the CF, however, the STR D21S11 peaks varied between different embryos and within the same embryo (Table 3 – 9). The amplified products of blastomeres from three different embryos (embryos 1, 3 and 8) showed two STR peaks with an intensity ratio of 1:1 (Figure 3 - 29) indicating the presence of two copies of chromosome 21. Three cells were available from embryo 2, DNA from two of the lysed cells did not amplify. The genomic DNA from the third cell of embryo 2 amplified and the STR peak analysis showed the peak ratio of 2:1 giving the possibility that 3 copies of the chromosome are present in the cell.

Genomic DNA of fourteen blastomeres was amplified from embryo 4 in which the STR peak ratio of 11 cells was 1:2 (Figure 3 – 30). The amplified product of from 3 cells of the same embryo showed a normal peak value of the STR marker D21S11 with a 1:1 ratio. Mosaicism could explain the presence of two different
Figure 3 - 28: Quantitative multiplex PCR from two single cells DNA obtained from a patient heterozygous for the ΔF508 showing two peaks for exon 10 of the CF gene that represent the normal and mutant alleles of the mutation and two STR peaks for D21S11 marker of chromosome 21 with a peak ratio of 1:1.
Table 3-9: Testing human embryonic single blastomeres from 10 embryo from two different IVF patients for chromosome 21 aneuploidy with quantitative multiplex fluorescent PCR using polymorphic STR marker D21S11.

<table>
<thead>
<tr>
<th>Embryo No</th>
<th>No of Cells</th>
<th>No amp.</th>
<th>Mean Peak Ratio*</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1.03</td>
<td>1.0 - 1.15</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1.6</td>
<td>1.79</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1.1</td>
<td>1.07 - 1.2</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>11</td>
<td>2.0</td>
<td>1.6 - 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1.1</td>
<td>1.0 - 1.2</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>3</td>
<td>1.9</td>
<td>1.8 - 2.1</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>4</td>
<td>1.2</td>
<td>1.05 - 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.8</td>
<td>1.87</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>6</td>
<td>1.0</td>
<td>1.05 - 1.12</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>1.9</td>
<td>1.77 - 1.99</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>2</td>
<td>1.06</td>
<td>1.0 - 1.15</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>4</td>
<td>1.1</td>
<td>1.03 - 1.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.7</td>
<td>1.76 - 2.1</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>2</td>
<td>1.95</td>
<td>1.9 - 1.97</td>
</tr>
</tbody>
</table>

* Mean Peak Ratio was calculated by dividing the larger area by the smaller area.

amp = number of cells amplified

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Figure 3 - 29: Detection of exon 10 of the CF gene and the D21S11 STR marker in single blastomeres from embryo 1. This cell shows the normal peak for CF and two STR peaks with a ratio of 1:1.
Figure 3 - 30: Quantitative analysis of the D21S11 STR marker in single blastomeres from embryo 4. The two cells show two STR peaks (diallelic) with a ratio of 1:2.
peak ratios of the STR marker within the same embryo. The STR peak ratio in disaggregated blastomeres from embryos 5 and 10 indicated that these embryos have 3 copies of chromosome 21 in each cell. All blastomeres from embryos 6, 7 and 9 showed the normal peak ratio with the exception of one cell from embryo 6, three cells from embryo 7 and two cells from embryo 9 which showed the peak ratio of 1:2, these cells could be mosaic.

Although this study was aimed at assessing the possibility of simultaneous detection of age related chromosome aneuploidy in patients requesting PGD of single gene disorders, the problem of preferential amplification may affect the accuracy of quantitation of the above results. In addition, only one STR marker for chromosome 21 was used to assess the copy numbers of chromosome 21 in single cells. In fact, using more than one marker was avoided in this study in order to minimise the competition between different regions to be amplified with regards to the nucleotides and Taq polymerase in the PCR mix. Although these could be considered as weak points for clinical application of this method for the detection of aneuploidy, the procedure of multiplex PCR using a polymorphic STR marker can be of great advantage for improving the efficiency of PGD of monogenic disorders. For example, it is useful in identifying haploid and diploid cells, and assessing the incidence of allele specific amplification failure. Furthermore, because the D21S11 STR marker has a variable size of amplified product between different individuals, it can be used for identifying the source of contamination during diagnosis, if present.
3 - 7.1: Reliability of quantitative multiplex fluorescent PCR in preimplantation diagnosis

A blind study was done in collaboration with the Hammersmith Hospital, aimed to assess the efficiency and reliability of applying quantitative multiplex PCR in preimplantation diagnosis of single gene disorders. The study was designed to analyse blindly the spare embryos from a PGD cycle that had been previously diagnosed prior to implantation for the cystic fibrosis ΔF508 mutation using heteroduplex analysis. The method used was quantitative multiplex fluorescent PCR to determine the cystic fibrosis status and the copy number of chromosome 21. Three spare embryos were available and they ranged from grade II to grade III.

Genomic DNA of the seven individual blastomeres was amplified without any contamination (5 blanks in total). Five cells were available from embryo 5 (Grade II). Four out of five gave the pattern of heterozygous for CF ΔF508 and two D21S11 STR peaks were seen with a peak ratio of 1:1 (Figure 3 - 31). However, one cell showed only the presence of the affected allele of the CF with a DNA fragment of 97 bp and a single peak for the STR marker. The results indicate that the embryo was heterozygous for the ΔF508 mutation and the fifth cell was haploid.

One cell was available from each of embryos 2 and 3 (grade III) and fragment analysis of the CF gene revealed that the embryos were homozygous affected. When analysing the STR alleles, embryo 2 showed that the presence of two peaks of STR alleles with a ratio of 2:1 giving the possibility of the presence of 3 copies of chromosome 21; while the STR mean peak value of the cell embryo 3 was of quite
Figure 3 - 31: Multiplex fluorescent PCR analysis from embryos following a PGD cycle for ΔF508. One cell gave the pattern of homozygous affected for CF and showed one STR allele (Lane 2) compared to the positive DNA control of homozygous patient for ΔF508 with normal D21S11 STR peak ratio (Lane 1).
high value, 2.5, which may be due to the presence of several copies of the chromosome 21 in this cell.

The CF status was diagnosed correctly by fluorescent PCR in these embryos confirming the accuracy of the results. Interestingly, it was found that the blastomere that was available from embryo 3 was multinucleated which explains the high STR mean peak value. Additionally, during the diagnostic procedure at Hammersmith Hospital, two cells were biopsied from embryo 5 in which one showed the heteroduplex and the other showed only the mutant homoduplex, and no contamination was observed. It was not clear whether there was allele drop out or the cell was haploid, therefore embryo 5 was not transferred because of a conflict in the results from the two biopsied cells. The diagnostic result of embryo 5 confirms the results of fluorescent PCR which revealed the probable presence of mosaicism for haploid.

3 - 8: Automated fluorescent DGGE

Conventional DGGE techniques have been employed for a wide spectrum of research and diagnostic applications. Although DGGE has proved to be highly sensitive and accurate in the detection of a wide spectrum of mutations and polymorphisms, it still has many disadvantages. The conventional method is expensive as it requires the synthesis of relatively long GC-clamp primers and time consuming preliminary work before analysis.
The first ever automated fluorescent non GC-clamped DGGE procedure has been developed in which a DNA fragment is amplified fluorescently and mutation analysis is conducted on an automated DNA sequencer - the ALF Express, to improve the sensitivity of mutation detection by DGGE and to overcome the problems of costly GC-clamped oligonucleotides and the length of time for analysis.

A representative group of the most common β globin mutations and the CF ΔF508 was investigated. Interestingly all mutations were detected in an approximate time of 180 minutes when using the short thermoplate of the ALF Express without the need for GC clamped primers (Figure 3 - 32). Frameshift mutations seem to give better allele separation than single base substitution when the pH of the running buffer was 7.4 and the temperature of the running gel was ambient. However, with single base substitutions it has been found that increasing the pH of the buffer up to 9 and the running gel temperature to 50 °C, gave a better resolution for the separation of alleles and the quality of mutation detection was improved. This could be because DGGE is based on heteroduplex analysis. Initially, the optimal conditions either of the duration of electrophoresis, gradient ranges, temperature of the running gel and the pH of the running buffer were optimised during experimental work.

Different DNA samples from patients known to be carriers for IVSI nt 110 were investigated to compare the efficiency of single base substitution detection by automated DGGE when using GC-clamped primers and non GC-clamped ones.. The GC-clamped automated DGGE analysis of IVSI nt 110 was clearer and of better
Figure 3 - 32: Detection of β globin mutations from genomic DNA obtained from normal and heterozygous individuals using non GC-clamped automated DGGE. Lane 1 = HbS/N; Lane 2 = normal; Lane 3 = CD5-CT; Lane 4 = IVSI nt/N.
resolution than those analysed with non GC clamped primers. However, in both situations the mutation can still be detected because of the sensitivity of the automated DNA sequencer.

After optimising of the electrophoretic conditions, four CVS samples were tested blindly to determine the accuracy and reliability of the developed automated DGGE for diagnosis. All CVS samples produced an altered migration pattern (Figure 3 - 33) in the region containing the mutation. There were no difficulties in either the detection of frameshift or single base substitutions. However, optimising the electrophoretic conditions were critical for the detection of single base substitutions.

3 - 9: Molecular characterisation of β thalassaemia mutations in Egypt and Jordan

3 - 9.1: Egyptian samples

Sixteen DNA samples with 21 β thalassaemia mutations that remained unidentified in a study of 54 patients reported by Rady et al in 1996 were examined. SSCP analysis of amplified DNA demonstrated several variant conformation patterns in the PCR product at 15 °C (Figure 3 - 34) in the samples, suggesting that different mutations were involved. One sample gave the conformation of the Asian Indian mutation FS 8/9 +G (Figure 3 - 35) which was confirmed by ARMS (Figure 3 - 36).
Figure 3 - 33: Non GC-clamped automated DGGE analysis from CVS DNA diagnosed blindy. Lane 1 = Maternal DNA sample heterozygous for VSI nt 5; Lane 3 = Paternal sample heterozygous for CD30; Lane 5 = DNA sample from the affected child for CD30/IVSI nt 5; Lane 7 = CVS DNA diagnosed as heterozygous for IVSI nt 5; Lanes 9&10 are normal DNA controls.
Figure 3 - 34: Screening for β thalassaemia mutations in the Egyptian population using silver stained SSCP analysis. Lanes 1-2 showed the variant pattern compared with the normal in lane 3, indicating the presence of mutations causing the disease within the region covered by the primers.
Figure 3 - SSCP analysis showing the presence of the Asian Indian frameshift mutation at codon 8/9 (+G) in one sample in lane 3 in comparison with the positive control in lane 2. Lanes 1 and 4 show the normal controls.
Figure 3 - 36: ARMS analysis of one sample (lane 1) confirmed the presence of FS 8/9 (+G) compared to positive heterozygous control in Lane 2 and normal control in Lane 3.
Direct sequencing of enzymatically amplified DNA detected a framshift at CD 29-G in samples from four unrelated patients (Figure 3 - 37), this mutation has been described previously in the Japanese population. In addition, a novel framshift at codon 28-C was seen in one allele from a different patient (Figure 3 - 38). The framshifts at codons 29 and 28 were recognised by restriction enzymes EcoRII and StuI, respectively (Figure – 39). Interestingly, 15 samples revealed two different single base substitutions in exon I (Figure 3 - 40). A silent mutation (polymorphism) at CD 17 (G-A) was seen in all samples and a missense mutation at CD 22 (A-C) was seen in 15 alleles, these have not previously been described (Table 3 - 10).

3 - 9.2: Jordanian samples

A total of 178 alleles from 89 different samples of unrelated β thalassaemia Jordanian patients was examined. Samples were first screened for the most common Mediterranean mutations using the ARMS method. Mutations in 36 alleles remained unidentified by ARMS. Sequencing analysis showed the prevalence of Mediterranean (Figure 3 - 41) mutations (Table 3 - 11). Interestingly, the Asian Indian IVSI nt 5 (G-C) was found in 3 chromosomes (Figure 3 - 42); this mutation has been identified previously in North Jordan (Sadiq & Huisman 1994) and neighbouring countries of the Middle East such as Iraq and Iran. A single base substitution at position -30 (T-A), which has previously been described in Turkey and Egypt, was seen in 17 alleles. Unexpectedly, a few DNA samples were found to be homozygous for one mild mutation and heterozygous for another. For example,
Figure 3 - 37: Sequencing analysis showing the presence of a Japanese frameshift mutation at codon 29 (-G) in four alleles of unrelated Egyptian patients.
Figure 3 - 38: The novel frameshift mutation at codon 28 (-C) as seen in one allele of one patient in the Egyptian DNA samples.

Normal Sequence

Mutant Sequence

GCC CTG GGC AGG

GCC TG GGC AGG
Figure 3 – 39: Restriction enzyme digestion of DNA samples from Egyptian patients with the frameshift mutations at codons 29 (-G) and 28 (-C) that were recognised by EcoR II and Stu I, respectively.

CD28:
Mutant: GAG GCC TG GGC AGG

CD29:
Mutant: GAG GCC CTG GC AGG
Figure 3-40: Direct sequencing analysis from Egyptian samples showing a silent single base substitution in codon 17, and a missense mutation that cause the disease at codon 22.

- **Codon 17**: Normal Sequence
  - TGG GGC AAG GTG AAC GTG GAT GAA
- **Codon 22**: Mutant Sequence
  - TGG GGC AAA GTG AAC GTG GAT GCA
Table 3 - 10: Molecular characterization of β thalassaemia mutations and their frequencies in Egypt. The genotype of 87 chromosomes were identified by Rady et al (1997). Twenty one chromosomes were analyzed by SSCP and direct sequencing.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No of alleles</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>-87 C-G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD 5 -CT</td>
<td>2</td>
<td>1.85</td>
</tr>
<tr>
<td>CD 6 - A</td>
<td>1</td>
<td>0.925</td>
</tr>
<tr>
<td>IVSI nt 1 G-A</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>IVSI nt 6 T-C</td>
<td>20</td>
<td>18.5</td>
</tr>
<tr>
<td>IVSI nt 110 G-A</td>
<td>34</td>
<td>31.5</td>
</tr>
<tr>
<td>CD 39 C-T</td>
<td>1</td>
<td>0.925</td>
</tr>
<tr>
<td>IVSII nt 1 G-A</td>
<td>3</td>
<td>2.8</td>
</tr>
<tr>
<td>IVSII nt 745 C-G</td>
<td>8</td>
<td>7.4</td>
</tr>
<tr>
<td>IVSII nt 848</td>
<td>4</td>
<td>3.7</td>
</tr>
<tr>
<td>CD 22 A-C *</td>
<td>15</td>
<td>13.88</td>
</tr>
<tr>
<td>CD 29 -G *</td>
<td>4</td>
<td>3.7</td>
</tr>
<tr>
<td>CD 28 -C *</td>
<td>1</td>
<td>0.925</td>
</tr>
<tr>
<td>FS 8/9 +G *</td>
<td>1</td>
<td>0.925</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>108</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

* Mutations identified in this study
Figure 3-41: Sequencing analysis of the β globin gene from Jordanian patients showing the prevalence of Mediterranean mutations. A: patient is homozygous for IVSI nt 1 G-A; B: heterozygous sample for Hb Knossos changing the G at codon 27 to T.
<table>
<thead>
<tr>
<th>Mutation</th>
<th>Classification</th>
<th>No of alleles</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSI nt 110 G-A</td>
<td>β+</td>
<td>51</td>
<td>28.6</td>
</tr>
<tr>
<td>IVSI nt 1 G-A *</td>
<td>β0</td>
<td>33</td>
<td>18.5</td>
</tr>
<tr>
<td>IVSI nt 6 T-C *</td>
<td>β+</td>
<td>15</td>
<td>8.4</td>
</tr>
<tr>
<td>IVSII nt 1 G-A</td>
<td>β0</td>
<td>10</td>
<td>5.6</td>
</tr>
<tr>
<td>IVSII nt 745 C-G</td>
<td>β+</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>CD39 C-T</td>
<td>β0</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>CD37 G-A</td>
<td>β</td>
<td>13</td>
<td>7.3</td>
</tr>
<tr>
<td>-87 C-G</td>
<td>β+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CD8 -AA *</td>
<td>β0</td>
<td>4</td>
<td>2.2</td>
</tr>
<tr>
<td>CD5 -CT</td>
<td>β0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CD6 -A</td>
<td>β0</td>
<td>15</td>
<td>8.4</td>
</tr>
<tr>
<td>CD27 G-T *</td>
<td>β</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>-30 T-A *</td>
<td>β+</td>
<td>17</td>
<td>9.5</td>
</tr>
<tr>
<td>IVSI nt 5 G-C *</td>
<td>β0</td>
<td>3</td>
<td>1.7</td>
</tr>
<tr>
<td>IVSI nt 23 *</td>
<td></td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>+20 *</td>
<td>β+</td>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td>CD1 -G *</td>
<td>β0</td>
<td>3</td>
<td>1.7</td>
</tr>
<tr>
<td>CD26 G-T *</td>
<td>β+</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Mutations identified by Direct Sequencing Analysis
Figure 3-42: Direct sequencing analysis showing the Asian Indian single base substitution in IVSI nt 5 (G-C) in a homozygous Jordanian patient.
one of the samples was heterozygous for IVSI nt 6 and homozygous for a base substitution in the Cap site at +20 (C-T), a combination which has not been described previously. The analysis also revealed the high frequency of the common polymorphism at codon 2, that was present in 11 alleles out of the 36 that were sequenced. A single base substitution was identified at codon 26 (G-T) in one chromosome. This mutation has been previously identified in the Thai population and was detected in one allele in the Egyptian samples. Interestingly, a mutation at IVSI nt 23, which has not been previously reported, was detected in one allele.

3 - 10: Clinical diagnosis

3 – 10.1 Prenatal diagnosis:

Parallel with standard prenatal diagnosis by ARMS carried out at the Perinatal Centre; samples were also analysed using silver stained SSCP in β thalassaemia cases and fragment analysis of fluorescent PCR for the CF case.

- Case One:

Both parents were known to be carriers for the Asian Indian mutation IVSI nt 5 and for the ΔF508 mutation causing cystic fibrosis. The family had two children, one known to be a carrier for CF and affected with β thalassaemia who died following bone marrow transplantation, and another living affected child with CF who is a carrier for β thalassaemia.
Fluorescent multiplex PCR was performed on the CVS DNA sample to amplify Exon 10 of the CFTR gene and the STR D21S11 in one reaction, and intron I of the β-globin gene in another reaction. Silver stained SSCP analysis was employed to test for β-thalassaemia while fragment quantitative analysis on the ALF Express was used to diagnose CF and age-related chromosome 21 aneuploidy. The fragment analysis showed that the fetus was heterozygous for the CF AF508 mutation and that there were two peaks for the STR with a ratio of 1:1 (Figure 3 - 43). The cytogenetics analysis of CVS confirmed the result of the presence of two copies of chromosome 21. Silver stained SSCP analysis showed that the fetus was a carrier for the IVSI nt 5, confirming the same results obtained by ARMS at the Perinatal Centre - UCLH.

- **Case Two:**

  Both parents of the second case were carriers of sickle cell anaemia. The amplified DNA of the CVS and parental DNA were analysed by silver stained SSCP along with a normal control. The CVS sample gave the pattern of homozygous affected for the sickle cell mutation (Figure 3 - 44). The same result was seen by ARMS at the Perinatal Centre – UCLH.

- **Case Three:**

  The mother was known to be a carrier for HbS and the father for a mutation at the Cap site +1. The fetal DNA gave the pattern of heterozygous for Cap site +1 mutation (Figure 3 - 45) when analysed by SSCP.
Figure 3 - 43: Quantitative multiplex fluorescent PCR analysis of CF ΔF508 and D21S11 in prenatal diagnosis case one showing that the fetus is heterozygous for CF mutation with 1:1 peak ratio of STR marker showing the presence of two copies of chromosome 21.
Figure 3 - 44: Silver stained SSCP analysis of fetal DNA from case 2 gave the pattern of homozygous normal for HbS (lane 5) compared to normal control in lane 1. Lanes 2 & 3 are control parental samples and Lanes 4 is a homozygous affected with HbS control.
Figure 3 - 45: Silver stained SSCP analysis of prenatal diagnosis of case 3. CVS sample (Lane 4) showing the conformation of the paternal mutation +1 (Lane 2). Lanes 1 & 3 are maternal (HbS/N) and normal positive controls, respectively.
The main technique for mutation screening and prenatal diagnosis used in the National Research Institute - Cairo is reverse dot blot hybridisation. Because SSCP is a fast, sensitive and economical technique, four amniotic fluid DNA and parental samples were sent from the National Research Institute to be diagnosed by SSCP to test the efficiency of the technique in diagnosis as a step towards using SSCP for prenatal diagnosis in Cairo.

In cases 4, 5 and 6 both parents of each case were known to be carriers for the single base substitution mutations IVSI nt 6, IVSII nt 848 and frameshift at codon 5 - CT, respectively. Parental and amniotic DNA samples were amplified and subjected to silver stained SSCP analysis. The fetuses of cases 4 and 5 were found to be homozygous affected for IVSI nt 6 and CD 5 -CT respectively (Figures 3 - 46). The fetus of case 6 was found to be heterozygous for IVSII nt 848 (Figure 3 - 47).

3 - 10.2: Preimplantation genetic diagnosis of β thalassaemia:

One couple who are at risk of β thalassaemia have attended the PGD programme. The couple were Italian and known to be carriers for the common Mediterranean mutations IVSI nt 6 (mother) and IVSI nt 110 (father). The couple had experienced pregnancy termination following prenatal diagnosis that was performed by fetal blood sampling in Bologna – Italy. The patients have had difficulties in conceiving normally and are in need of IVF.
Figure 3 - 46: Silver stained SSCP analysis of fetal DNA in case 5 showed the pattern of homozygous affected for CD 5 –CT causing β thalassaemia (Lane 5). Lane 1 = normal control; Lanes 2& 3 are parental heterozygous DNA control for CD 5 –CT and Lane 4 is a homozygous affected positive control. Arrow indicates the conformation of the mutation.
Figure 3-47: Prenatal analysis of case 6 by silver stained SSCP. Fetal DNA gave the pattern of heterozygous for IVSII nt 848 (Lane 4) compared to parental DNA samples (Lanes 1 & 3) and normal control (Lane 2). Arrow indicates the novel conformer of IVSII nt 848.
Prior to the case, a preliminary analysis was performed on 150 buccal cells isolated from both parents. A total of 143 cells was fluorescently amplified and gave the correct conformation when analysed by SSCP on the ALF Express. No contamination was observed from 24 negative control blanks.

Seven oocytes were collected and fertilised by intracytoplasmic sperm injection (ICSI). Five embryo fertilised and four were available for blastomere biopsy. Five biopsied blastomeres were lysed by PK and were subjected to fluorescent PCR to amplify intron I of the β globin gene, followed by fluorescence SSCP on the ALF Express. Four blanks containing 2 µl of culturing medium for every biopsied embryo and one PCR negative blank were used to assess the incidence of contamination. Amplified samples were analysed along with parental heterozygous samples and those from normal, affected compound heterozygous for IVSI nt 6/IVSI nt 110 and homozygous affected for IVSI nt 6 and IVSI nt 110 DNA controls. The homozygous DNA samples were used to assess the presence of haploid cells or ADO. Table 3 - 12 summarises the results of analysis.

Unfortunately and completely unexpectedly, the PCR and the embryo medium negative blank controls showed contamination with the normal allele (Figure 3 – 48). Only the blank from embryo 4 showed the heterozygous for IVSI 110 bands, which could be due to inclusion of part of the lysed cell, as this cell was not seen going into the thin walled tube. The amplified products from three embryos showed the normal conformation for intron I of the β globin gene and one cell from embryo 4 showed the normal peak and the second cell showed the heterozygous peak for IVSI nt 110.
Table 3-12: PGD cycle for the diagnosis of β thalassaemia indicating the number of each embryo biopsied, blastomere condition following biopsy, tubing of blastomeres and mutation analysis of each blastomere and blanks for each embryo.

<table>
<thead>
<tr>
<th>Embryo No</th>
<th>Tube No</th>
<th>Cell conditions</th>
<th>Tubing comments</th>
<th>Mutation analysis results</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>Lysed, nucleus seen</td>
<td>Nucleus transferred</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Blank</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Lysed and stuck to bottom of dish</td>
<td>Was not seen going into tube</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>Blank</td>
<td></td>
<td></td>
<td>Contaminated with normal allele</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>Intact with nucleus</td>
<td>Cell transferred</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>Lysed, nucleus intact</td>
<td>Nucleus transferred, was not seen going into tube</td>
<td>IVSI nt 110/N</td>
</tr>
<tr>
<td>7</td>
<td>Blank</td>
<td></td>
<td></td>
<td>Contaminated with IVSI nt 110/N allele</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>Intact with nucleus</td>
<td>Cell transferred</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>Blank</td>
<td></td>
<td></td>
<td>Contaminated with normal allele</td>
</tr>
</tbody>
</table>

B = blank, each blank consist of approximately 2 μl of media taken from the same drop as the single cell.
Figure 3 - 48: Fluorescent SSCP analysis of the amplified intron I of the β globin gene from biopsied blastomeres and the medium blanks from the PGD case.

Embryo 3:

Normal DNA control

Blastomere

Medium blank from embryo 3
Embryos 4 & 1; PCR blank

Embryo 4
Cell 1

Embryo 4
Cell 2

Embryo 4
Medium blank

Embryo 1
Cell

Embryo 1
Medium blank

PCR blank
Embryo 2 & Positive DNA controls

Embryo 2
Cell

IVSI 110/N
DNA control

IVSI 6/IVSI 110
DNA control

IVSI 6/N
DNA control

N. El-Hashemite
The presence of the normal peak in three embryos does not mean that the embryos can be reliably diagnosed as normal, as this peak could be resulted from the contaminating source rather than the cell amplified. On the other hand, in embryo 4 one cell showed the heterozygous peak while the other one showed the normal peak. The normal peak could either result from failure to amplify the mutant allele or the DNA of the cell failed to amplify completely and the contamination caused the presence of such normal conformation.

The patients were informed about the results, but they chose to take the risk and transfer two embryos (numbers 3 and 1) that showed the normal peak, knowing the risk of contamination and the possibility of the embryos being affected.

To investigate the source of contamination, all PCR solution aliquots used during the diagnostic case were used to amplify a range of DNA samples. All 6 DNA samples (IVSI nt 6/N; IVSI nt 110/N; IVSI 6/IVSI 110; IVSI 6/IVSI 6; IVSI 110; IVSI 110; Normal) amplified and gave the expected pattern when analysed by SSCP, without any incidence of contamination in the 3 PCR negative blanks used. The only difference was the oil used during the diagnosis and when investigating the contamination source. This gives the possibility that the oil used to overly the PCR mix during the diagnosis was contaminated. If quantitative multiplex PCR using informative STR marker was applied during the case, this would allow to know the source of contamination in the mineral oil. However, multiplex PCR is avoided in SSCP because it could interfere and affect the single stranded DNA molecule conformation.
The non-transferred embryos (numbers 2 and 4) were examined to confirm diagnosis. Embryo 2 was at 3 cell stage and was tubed as a whole, however, embryo 4 was five cells stage and individual blastomeres were tubed following disaggregation with acid Tyrode’s solution. All cells amplified without any contamination from either the 2 medium blanks or PCR negative control. Embryo 2 showed the normal peak and embryo 4 showed the heterozygous conformation for IVSI nt 110 mutation (Figure 3 – 49).
Figure 3 - 49: Non-transferred embryos from the PGD case were analysed to confirm the accuracy of mutation analysis during diagnosis. Lanes 4 – 6 are amplified intron I of the β globin gene of 3 single blastomeres from embryo 4 showing the conformation of heterozygous for IVSI nt 110 in comparison to positive DNA IVSI 110/N control (Lane 3). Lane 2 represents amplified genomic DNA from embryo 2 showing the normal conformation in comparison to normal positive control (Lane 1). Lanes 7 & 8 are medium blank from embryo 4 and PCR negative control, respectively. SSCP analysis was conducted on the ALF express when the running gel temperature was 15C.
Chapter 4

Discussion
Chapter 4
Discussion

VI - 1: Preimplantation Genetic Diagnosis (PGD) of single gene disorders

Since its development, preimplantation genetic diagnosis has become a method of choice for preventing genetic disorders as an alternative to prenatal diagnosis. Over 100 unaffected babies have been born following preimplantation diagnosis at the cleavage stage using either FISH for gender determination or PCR for single gene disorders (Harper et al 1996, ESHRE Special working Group, 1997). Multicolour FISH has been developed and used for embryo sexing and detection of chromosomal abnormalities. However, FISH is a reliable technique at the chromosomal level but not applicable to detect single gene disorders caused by base substitutions, deletions or insertions. Therefore PCR remains the only technique to diagnose monogenic disorders prior to implantation.

With the application of nested PCR, amplification of the minute amount of DNA in a single cell has become possible and has been widely used for several applications (Li et al 1988; Boehnke et al 1989; Zimmerman et al 1993). The PCR technique has been successfully applied for PGD programmes. The first clinical application of PGD used a nested PCR protocol to amplify a specific repeat of the Y chromosome to sex embryos (Handyside et al 1990). Nested PCR allows the
amplification of the targeted sequence in two steps: the outer amplification and then
the inner amplification, which allows a subsequent reamplification, and provides the
sensitivity and the specificity required to detect and analyse the gene in single cells.
Many single gene disorders have been diagnosed in preimplantation embryos and
successful pregnancies have been achieved (Handyside et al 1992; Holding et al 1993;

PGD of single gene disorders relies on the success and efficiency of PCR
amplification of the two copies of the targeted gene from single cells. The precision
of the diagnosis from single cells is dependent on two factors: first the efficiency of
amplification and second on the method used for genotype analysis. There are many
factors that could affect the efficiency of PCR amplification these are: experimental
design, quality of reagents, amplification conditions, single cell transfer and the lysis
buffer used for preparing single cell genomic DNA. Selecting cells with normal nuclei
is also crucial for highly efficient amplification and mutation detection.

Following amplification of the target sequence, diagnoses have been made either
by heteroduplex analysis for the ΔF508 mutation causing CF (Ao et al 1996), SSCP
for APC (Ao et al 1998), restriction enzymes digestion for β thalassaemia (Van de
Velde et al 1997; Kuliev et al 1998) and ARMS for CF (Ke-Hui et al 1995). Each of
these methods has its advantages and disadvantages when clinically applied for PGD.
The major aim of PGD is to offer couples at high risk a chance to have only healthy children without going through pregnancy termination. However, misdiagnoses, which have led to the transfer of affected embryos, have been reported in 3 centres (Hardy & Handyside 1992; Verlinsky & Kuliev 1993; Grifo et al 1994). The first misdiagnosis occurred when using the PCR method for amplifying a specific target of the Y chromosome. This was most likely as a result of amplification failure. The second misdiagnosis occurred following the diagnosis of the cystic fibrosis ΔF508 following the amplification of exon 10 of the CFTR gene from the single cell genome. ADO or mosaicism possibly combined with contamination could be the cause of misdiagnosis. Amplification of genomic DNA from single cells has variable reliability and efficiency rates and remains a procedure with difficulties and problems (Navidi et al 1991). Variation in amplification failure from 2 to 36% has been reported (Handyside et al 1990; Handyside et al 1992; Chong et al 1993; Wu et al 1993; Avner et al 1994). Achieving high efficiency of PCR amplification and combining it with the highly sensitive and reliable detection methods remains the aim in PGD. Whatever the reasons behind each misdiagnosis, several issues must be addressed and evaluated such as the efficiency of DNA amplification from single cells, reliability of mutation detection methods used, accuracy of diagnosis and the possibility of misdiagnosis due to ADO, mosaicism or contamination. Therefore, extensive preliminary work at the single cell level is needed to ensure the sensitivity and reliability of experimental conditions prior clinical application.
VI - 2: An approach to PGD of haemoglobinopathies

To assess the feasibility of preimplantation genetic diagnosis for the haemoglobinopathies, comprehensive preliminary work was done in this study on different types of single cells.

Four other groups have recently reported preliminary work towards PGD of βthalassaemia. Ray and colleagues (Ray et al 1996) used the strategy of artificially introducing a restriction site in the PCR primers followed by a specific digest of the amplification product to detect the mutant allele. Cohen & Levinson (1988) were the first to show that by using an appropriate mismatch primer in the PCR reaction, an artificial restriction site can be created in which differential cleavage between mutant and wild type amplification product occurs. This technique however, is not straightforward particularly when no such creation of restriction site during PCR amplification occurs. Furthermore, there are many limitations upon the use of restriction enzyme digestion either for the diagnosis of β thalassaemia or as an application for preimplantation diagnosis. There are over 180 mutations causing β thalassaemia and only a few mutations create a natural restriction site, which makes it generally unsuitable as a detection method. Moreover, the digestion of the product requires additional time for cutting the product before gel electrophoresis the length of which mainly depends on the enzyme. Most importantly, if the enzyme fails to...
cut the sequence it will lead to misdiagnosis because the analysis relies on the presence or absence of a product in the gel.

The main problem that arises with restriction enzyme digestion is in the case of compound heterozygotes where it is quite impossible to perform an accurate diagnosis in one step. Van de Velde et al (1997) has reported the application of restriction enzyme digestion for two common β thalassaemia mutations: IVSI nt 6 and IVSI nt 110. The IVSI nt 6 creates a natural restriction site (SfaNI) while for the IVSI nt 110 mutation an artificial site (AvalII) was created through the reverse inner primer. This protocol involves many steps for genotyping and the use of different enzymes, which may have special conditions for digestion. The problems with this protocol will be increased in the case of allele drop out and preferential or weak amplification.

Recently, Kuliev et al (1998) has reported the clinical application of restriction enzyme digestion for the detection of IVSI nt 110 and IVSII nt 745 mutations causing thalassaemia in 12 clinical cycles following first and second polar bodies analysis. The polar body analysis is based on the biopsy of the first polar bodies from retrieved oocytes during maturation, followed by DNA amplification and mutation analysis. If the results revealed that the polar body is homozygous affected for the investigated disease, then the oocyte is presumed to be carrying the normal allele. Following the fertilisation of such oocytes, second polar bodies are biopsied to
confirm the analysis prior to embryo transfer. Although polar body analysis has been efficient in the diagnosis of age related aneuploidy (Verlinsky et al 1996; Munne et al 1995) the existing problem of allele drop out could be a source of misdiagnosis in single gene disorders, particularly if the first polar body was heterozygous due to crossing over and the normal allele failed to amplify. Figure 4 – 1 explains the possibility of transferring an affected embryo if allele drop out occurred in the first polar body. In order to avoid the possibility of misdiagnosis due to allele drop out in the first polar bodies, it has been said that both polar bodies must be analysed (Verlinsky et al 1997). However, Figure 4-1 shows that in some cases this will not avoid misdiagnosis. In addition, Kuliev et al applied multiplex nested PCR to allow the detection of polymorphic linked markers to the β globin gene along with β thalassaemia mutations (Kuliev et al 1998). This technique allowed them to detect ADO in five first polar bodies and prevented the transfer of affected embryos resulting from their corresponding oocytes. Although 2 pregnancies have been achieved, the application of restriction enzyme digestion for the detection of β thalassaemia may be unreliable, in this case not due to ADO, but due to preferential amplification or enzyme digestion failure.

Palmer et al (1996) performed single cell analysis of β globin mutations by conventional DGGE followed by ethidium bromide staining. The protocol involved the amplification of the region of interest of the gene using 40-60X GC-clamped primers to prevent a complete denaturation of the fragment and to improve mutation
Figure 4.1: Diagram illustrates the possibility of misdiagnosis following first polar body analysis if allele drop out occurred following crossing over between non-homologous chromatids.

- Oocyte
- 1st polar body biopsy
- Nested PCR amplification
- Gel electrophoresis
- Normal allele failed to amplify
- Heterozygous polar body results control

Result: Oocyte is presumed normal

- Fertilization
- Sperm carrying affected allele
- Transfer of affected embryo
detection on denaturing gradient polyacrylamide gel. Although with conventional DGGE reliability of mutation detection is close to 100% (Meyers et al. 1985) it has many restriction which make it unsuitable for single cell analysis. These limitations are: the method requires GC-clamp primers, which mainly are efficient for short sequences, and in turn each mutation will have a different melting temperature. In addition to its cost, GC-clamping may reduce the efficiency of amplifying the target gene from single cells, as it is more efficient in higher concentrations of template DNA, this could explain the reason for failure of more than 50% of amplifications from single cells or 5 pg of DNA in the study by Palmer et al. The longer time of gel electrophoresis - at least 5 hours – also makes it unsuitable for preimplantation diagnosis.

An alternative approach has been used in this study that offers significant improvements compared with restriction digestion or DGGE to enable PGD of β thalassaemia by direct detection of mutant β globin genes at the single cell level.

To detect β globin alleles following nested PCR, silver stained SSCP analysis was first used. It proved to be a rapid, sensitive, straightforward and effective technique not previously applied to the detection of β thalassaemia mutations. The principle of this method is that under non-denaturing conditions, each single stranded molecule of DNA will adopt a specific conformation at a given temperature. Most changes in the DNA sequence result in a change of the conformation (Orita et al.
The single stranded DNA carrying the mutation can be identified by virtue of altered mobility during non-denaturing polyacrylamide gel electrophoresis.

The temperature of the running gel dramatically affects the single stranded DNA migration pattern and sensitivity of mutation detection. The β globin mutation analysis using silver stained SSCP was conducted when the temperature of the gel during separation was 15 °C and 18 °C. Although temperature is the most important factor influencing the sensitivity of SSCP analysis, other factors could affect mutation detection (Hayashi & Yandell 1993; Glavac & Dean 1993). For the purpose of our work, we kept all other factors constant and assessed mutations in fragments of DNA shorter than 500 bp.

The results obtained in this study show the efficiency of silver stained SSCP analysis in the detection of mutations in intron I of the β globin gene at the single cell level. The direct identification of each allele that SSCP analysis allows, simplifies the diagnosis of heterozygotes and compound heterozygotes. Additionally, using one set of normal primers, SSCP analysis allows a range of mutations to be detected without the need for a change in experimental conditions or approach (Delhanty et al 1995, El-Hashemite et al 1997b).

SSCP analysis has been found to have clear advantages over other methodologies especially in assessing the level of allele drop out (ADO) or haploid
cells for preimplantation diagnosis. The direct visualisation of each allele in SSCP analysis allows immediate detection of ADO in the case of compound heterozygotes and aids to the deduction of its extent. Figure 4 - 1 demonstrates the advantage of SSCP analysis for mutation analysis with regard to allele specific amplification failure or haploid cells. In brief, if both parents are known to be carriers of the same mutation, the single strand conformation of their DNA fragment will show the normal allele and the mutant one. The resulted offspring will be either normal, heterozygous for the mutation or homozygous affected. The homozygous affected will show the conformation of the mutant allele only, while the normal will gave the normal allele only. In the case of allele drop out in the heterozygous cell, if the mutant allele has failed to amplify the sample will give the conformation of the normal allele only, and may lead to the transfer of heterozygous embryos as a normal. This is not a problem in the case of autosomal recessive disorders as heterozygous individuals are phenotypically normal, but is a serious problem in the case of dominant diseases. On the other hand, if the normal allele failed to amplify, the embryo will be diagnosed as homozygous affected and will not be selected, which in turn reduces the number of available embryos to be transferred. If the parents are known to be carriers of different mutations, each parental DNA will produce a different conformation when analysed by SSCP. The resulting offspring will be heterozygous for either of the parental mutations, normal or compound heterozygous. If allele drop out has occurred in a heterozygous sample, the DNA fragment will show the conformation of the mutant allele which will appear as a homozygous affected with that particular
Figure 4.2: Diagram to show the results of SSCP analysis of heterozygous cells in the case of allele drop out and haploid cells.

A: when both parents are known to be carriers of the same mutation. P = parent; N = normal; H = homozygous affected; 1 = if mutant normal failed to amplify in heterozygous cells; 2 = if mutant allele failed to amplify.

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<tr>
<td><img src="image1" alt="Mutant allele" /></td>
<td><img src="image2" alt="Normal allele" /></td>
<td><img src="image3" alt="Allele failed to amplify" /></td>
<td><img src="image4" alt="Mutant allele" /></td>
<td><img src="image5" alt="Normal allele" /></td>
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B: If each of the parents is known to be a carrier of a different mutation within the same region. F = father; M = mother; N = normal; CH = compound heterozygous; 1 & 2 = if the normal allele failed to amplify in heterozygous cells; 3 = if the mutant allele failed to amplify in heterozygous cells; 4 = if either of the mutant alleles in compound heterozygous cells failed to amplify. The same as in 1 - 4 applies for haploid cells.

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Mutant allele

Normal allele

Allele failed to amplify
mutation, an unexpected situation, and it will be quite clear that the normal allele has failed to amplify in this heterozygous sample. If one of the mutant alleles failed to amplify in the compound heterozygous cell, it will also show an odd conformation with one band only, which will indicate the presence of ADO in this affected sample, and will not lead to the transfer of an affected embryo.

A 15% haploidy rate has been reported in human blastomeres from embryos at the 8-10 cell stage (Harper et al 1995), and is a potential problem for the diagnosis of dominant disorders. However, the absence of a chromosome carrying a disease allele will not lead to an affected embryo being diagnosed as normal in the case of β thalassaemia or other recessive disorders if SSCP analysis was employed for mutation analysis (Figure 4-1).

There are currently several different methods of analysing amplified DNA that are in use for carrier detection and prenatal diagnosis of β thalassaemia. Most of them require high concentrations of DNA or an extended time in order to perform diagnosis. The most common and widely used techniques in routine prenatal diagnosis or carrier screening are reverse dot blot hybridisation and ARMS. Reverse dot blot hybridisation is a useful technique to screen for β thalassaemia mutations in populations who have one or two common mutation(s) and just a few rare ones that may be described in individuals families such as in Sardinia. Because it requires a battery of probes and primers, which are expensive, reverse dot blot hybridisation is not suitable for laboratories that encounter a large heterogeneous spectrum of β globin
mutations. In addition, for the purpose of preimplantation diagnosis, reverse dot blot hybridisation relies on the hybridisation of a probe to its sequence, which makes it unsuitable for preimplantation diagnosis because it requires a high concentration of DNA and more importantly if for any technical reason the hybridisation failed it can lead to misdiagnosis particularly in the case of compound heterozygous samples. The same is true for the ARMS technique; it is quite difficult to identify rare or uncharacterised mutations if ARMS primers are not available. Furthermore, ARMS analysis relies on the intensity of the products, and if preferential amplification is present, the sample may be incorrectly diagnosed. This makes the basic ARMS technique unsuitable for single cell diagnosis.

For preimplantation diagnosis SSCP analysis has been found to be preferable because it minimises the risk of errors that may occur in indirect systems such as those requiring restriction digestion or the ARMS procedure.

Speed is an issue in PGD, all steps for PGD from embryo biopsy to diagnosis should ideally be performed within one working day to allow the transfer of selected embryos to the mother by the end of the third day post insemination optimising the chances of implantation. In this respect silver staining for SSCP performed by automated gel electrophoresis - the Phastsystem, is preferable to radioactive detection methods. This approach to β globin mutation detection uses a single set of nested primers to detect common and rare mutations within intron I avoiding the need to synthesise specific primers for each mutation. At an optimum temperature, most
single base substitutions, small deletions or insertions in fragments up to 500 bp can be simultaneously detected by gel mobility shift without the use of restriction enzymes, probes or blotting hybridisation. Because silver staining is so sensitive, mutations in single cells, which showed only weak amplification, can still be detected. The cost of materials needed to analyse each sample is low. Thus, this method could be applied also for prenatal diagnosis in laboratories where β thalassaemia mutations are heterogeneous and financial resources are limited.

VI – 3: Possibility of misdiagnosis

There are three main problems with single cell PCR. The first is contamination with extraneous DNA, which could lead to false results; mosaicism could affect the accuracy of diagnosis of single gene disorders and allele specific amplification failure. ADO is considered the most serious which can cause problems in detecting variants and can lead to misdiagnosis (Delhanty 1994).

VI – 3.1: Contamination

Contamination is a serious problem in single cell PCR as it gives rise both to false negative and false positives and can lead to misdiagnosis. Contamination with extraneous DNA comes about either when handling the samples or during amplification. When more than 40 PCR cycles are performed or when reamplification is done, the incidence of contamination increases. In addition,
contamination with sperm or cumulus cells that remain attached to the zona following IVF, may occur during biopsy.

Contamination with sperm can be avoided by using intracytoplasmic sperm injection (ICSI) for fertilisation instead of ordinary IVF. It is important that care is taken to remove all cumulus cells prior to biopsy. Using filtered tips, dedicated micropipettes and a specific hood and room to set up and run the single cell initial PCR can reduce the chances of contamination arising when handling samples. However the problem of contamination with extraneous DNA that could arise with reamplification of products can lead to misdiagnosis particularly when normal and heterozygous embryos are considered for transfer. But if only normal embryos are to be transferred (as in the case of dominant disorders) then contamination with normal DNA will not lead to the transfer of affected embryos providing that amplification failure has not occurred. This is can be explained by the four possibilities: If normal DNA contaminates a homozygous affected genotype, the result will be heterozygous and the embryos will not be transferred. If a normal DNA contaminates a normal embryo it will not alter DNA diagnosis and the same when it contaminates a heterozygous embryo. If affected DNA contaminates a homozygous affected embryo or heterozygous embryo, the DNA diagnosis will not be altered, but when affected DNA contaminates a normal embryo, DNA misdiagnosis will occur and a normal embryo will be wasted and not transferred. It is clear that contamination is remarkably problematic during clinical cases, as it is difficult to know whether the
normal allele in normal samples has resulted from the amplification of the target sequence or if it arose from the contamination source. The inclusion of an STR marker may help to overcome this problem.

VI – 3.2: Allele specific amplification failure (Allele drop out- ADO)

The incidence of allele drop out was first reported by Ray et al (1994, 1995) when blastomeres from embryos following preimplantation genetic diagnosis of CF ΔF508 were investigated to confirm the accuracy of diagnosis. The failure to amplify either normal or mutant alleles in heterozygous cells was not specific and occurred randomly. The significant incidence of ADO during preimplantation diagnosis of cystic fibrosis has affected the expected proportions of the Mendelian ratio 1:2:1 (Ao et al 1996).

In the case of CF, the frequency of ADO ranges from 0-27% in heterozygous cells (Gitlin et al 1996; Ray et al 1996). The phenomenon of ADO has been reported solely during the amplification of specific genes from a single or a few cells. It is not known whether ADO is a result of inefficient amplification by PCR in which the different degrees of folding or the GC richness of the DNA molecule may affect the accessibility for annealing of the oligonucleotide primers and the extension of the target sequence by Taq polymerase, or if it arises from degradation of one copy of the gene prior to amplification or unsuitable DNA preparation methods.
Fortunately, in this study the problem of ADO when amplifying the β globin gene from single cells has rarely been encountered. This is in contrast to the approach used by Ray et al (1996) which resulted in 14% ADO in heterozygotes, Van de Valde et al (1997) with 8% allele specific amplification failure in heterozygous cells and that of Palmer et al (1996) who reported failure of 80% of heterozygous samples starting from 5 pg of DNA template. One reason for the difference could be the initial lysis buffer used to prepare genomic DNA from single cells.

The first step of the PCR protocol is the preparation of genomic DNA from single cells. Many protocols were used; Handyside et al (1990) prepared the DNA for analysis by lysing single cells using liquid nitrogen (LN2). The protocol involves tubing single cells in tubes containing sterile water, freeze-thawed in LN2 twice and then boiling at 100 ºC for approximately 10 minutes. Although by using this protocol successful diagnosis was achieved, the efficiency of amplification was quite low and decreased the number of available embryos for transfer (Gitlin et al 1996). In addition, a potential problem of selective failure to amplify one of the two copies of the targeted gene was seen (Strom et al 1994) when amplifying genomic DNA of heterozygous cells lysed by LN2. This problem of allele specific amplification failure has been reported in high frequency when using LN2 (Gitlin et al 1996) and the reason behind it is still unclear. This turned attention toward using a lysis buffer to prepare single cell genomic DNA for amplification. Li et al (1991) introduced the alkaline lysis buffer, which consists of KOH and dithiothritol (DTT). They reported an increased amplification efficiency of DNA from single sperm from 75% to 95%.
when switching from an SDS/PK/DTT and denaturing protocol. Several studies have compared different lysis protocols for the efficiency of DNA amplification and it has been found that when KOH/DTT has been used to lyse cells the efficiency of amplification has increased for more than 90% and the incidence of ADO decreased (Gitlin et al 1996; Sermon et al 1995; Ray et al 1996).

Ray et al (1996) have suggested that ADO could be the result of broken sequence of the target DNA during lysis of single cells. Furthermore, they showed that increasing the denaturing temperature during the first cycles of amplification reduced the incidence of ADO from 21% to 5%, but did not consider the role of the lysis buffer. In fact, the reduced percentage of ADO in their experiments could be the result of more efficient lysis of the cells caused by an increase in denaturing temperature.

Sermon et al (1995) and Gitlin et al (1996) have compared different lysis methods (liquid nitrogen, H2O and KOH/DTT) on the efficiency of amplification from single cells. The alkaline buffer was found to be preferable because the efficiency of amplification was high (Sermon et al 1995) and it gave the least percentage of ADO when amplifying heterozygous cells (Gitlin et al 1996). Accordingly, the problem of ADO appears to be related not only to the PCR running conditions, but also more likely to the method of preparing DNA from single cells.
To investigate the role of the lysis buffer in the efficiency of the amplifying both alleles in heterozygous cells, a total of 177 cells of different types were tested. Cells were obtained from patients and CVS cultures known to be carriers for the ΔF508 mutation causing CF and carriers for a base substitution CD 6 G-A causing Haemoglobin C (HbC).

In this study, there is a strong correlation between the incidence of allele drop out and the lysis protocol used to prepare the genomic DNA from single cells. Two different lysis buffers were used in this study KOH/DTT and SDS/PK. None of the cells lysed by SDS/PK showed any example of allele specific amplification failure whereas ADO was observed in 4.7% and 17.6% of the HbC and CF cells lysed with KOH/DTT, respectively.

There are several reasons which could explain why efficient amplification of both alleles occurred after lysis by SDS/PK without change in the PCR conditions. SDS is a strong detergent, which disrupts membrane lipids. The enzyme PK digests proteins without interfering with the DNA template prior amplification. In contrast, the contents of the alkaline buffer solution may interfere with the template, as KOH (a strong alkali) denatures DNA and leaves it in a single stranded state. Moreover, DTT breaks the disulphide bonds (S-S bond) but does not digest them. The denatured DNA and the presence of proteins could lie behind the failure of allele specific amplification, which is not the case with SDS/PK. Further studies are
needed to understand how different lysis buffers and chemicals could affect the genomic DNA in single cells.

This study clearly demonstrates that allele specific amplification failure is related to the DNA preparation protocol used. Therefore, using SDS/PK as a lysis buffer for single cells is more reliable and efficient than KOH/DTT and should be applied for clinical use in preimplantation diagnosis of single gene disorders.

VI - 4: Optimizing Single Cell PCR amplification

The inefficiency of PCR amplification has decreased the accuracy of PGD particularly that of heterozygous and compound heterozygous genotypes. This could be due to either the possibility of complete amplification failure of one of the two alleles carried by the cell, i.e., ADO; or by the failure of one allele to reach the threshold of amplification, i.e., preferential amplification. The inaccuracy of diagnosis that has been reported could be in part due to complete ADO or preferential amplification that may be incorrectly interpreted as ADO because of the insensitive methods used so far for mutation detection and analysis which is based on the ethidium bromide staining of either agarose or acrylamide gels.

By reviewing the techniques that have been used for molecular genetic diagnosis, several methods have been investigated to establish an optimal system for single cell analysis. However, combining the main criteria for diagnosis of monogenic disorders from single cells which is: efficiency of amplification, elimination of
contamination and sensitivity, accuracy and reliability of method applied for
diagnosis has partially been successful.

The new modification of the PCR protocol which used a fluorescent labelled
primer that allows the visualisation of amplified products on automated DNA
sequencer (Hattori et al 1992; Ziegle et al 1992; Kimpton et al 1993) has improved
the efficiency and sensitivity of the PCR methods and enabled many diagnostic
applications to be considered. Fluorescent PCR is highly sensitive in amplifying the
locus of interest from single cells. Following 30-35 cycles of PCR amplification, the
target sequence from single cells can be easily visualised after polyacrylamide gel
electrophoresis as electrophorectogram peaks on the computer screen of an
automated laser DNA sequencer - The ALF Express, with ALF Fragment Manager
software. Thus the sensitivity of Flu PCR eliminates the need for nested PCR and
reduces the time required for amplification by 50%.

There are many advantages of Flu PCR protocols which offer an exceptional
resolution for amplifying the two copies of a gene from single cells: it allows the
visualisation of signals from weakly amplified products even if below the normal
threshold, which conventional gel analysis does not allow and so the result may be
seen as amplification failure. In the conventional methods for the detection of
variants, if one allele amplifies better than the other (preferential amplification)
complete ADO will be reported mistakenly. However, the sensitivity of fluorescent-
based PCR techniques allows the detection of both alleles even when the signal of one allele is significantly weaker than the other. Furthermore, using Fluorescence PCR can avoid the problem of contamination with extraneous DNA that arises from reamplification of DNA using nested PCR. Fluorescently amplified products can be analyzed by different approaches, either by fragment analysis of mutations resulting from either deletions or insertions, quantitatively for the identification of the quantity of the initial target sequence, ARMS or even by SSCP for mutations due to single base substitution.

VI - 4.1: Fluorescent PCR-SSCP analysis

Although silver stained SSCP analysis for detecting β globin variants at the single cell level demonstrated the advantages of using SSCP over other existing methodologies particularly in PGD and is sensitive enough to detect even weakly amplified products from single cells (El-Hashemite et al 1997), it requires nested PCR to enable the DNA to be visualised. In this study the efficiency and reliability of a simplified and optimal PCR procedure for the diagnosis of β globin variants from single cells by employing fluorescent based PCR has been evaluated. β globin alleles were then detected by SSCP analysis, which was, performed on an automated DNA sequencer the ALF Express (Pharmacia Biotech).

A reduction in the time taken to complete the diagnosis is advantageous because it assures the transfer of selected embryos on day 3 post insemination,
optimising the chances of implantation. In this respect, Flu PCR-SSCP is preferable to nested PCR and silver stained SSCP. The data of this study demonstrated that by using this simplified protocol, PGD of single gene disorders could be completed in a maximum of 5 hours with improved efficiency.

The temperature of the running gel dramatically affects the single stranded DNA migration pattern and the sensitivity of mutation detection. The β globin mutation analysis using silver stained SSCP was conducted when the temperature of the gel during separation was 15 °C (El-Hashemite et al 1997). In this study SSCP analysis was performed using Flu PCR products on an automated DNA sequencer - the ALF Express. The ALF Express has many advantages over other automated machines, first the ALF gel plates are equipped with water jacketed electrophoresis apparatus. This allows strict control of the running gel temperature by using the Multitemp (Pharmacia Biotech). Secondly, a total of 40 samples can be simultaneously analysed allowing comparison of different samples on the basis of conformation. Thirdly, the DNA molecules can be visualised as peaks or can be read as bands.

This approach to β globin mutation detection uses a single set of primers to detect the common and rare mutations within exon and intron I avoiding the need to synthesis specific primers for each mutation. At an optimum temperature, most single base substitutions, and small deletions or insertions in fragments up to 500 bp can be simultaneously detected by gel mobility shift. The direct entry of data into the
computer provides objective interpretation of the results and reduces the possibilities of errors.

The combination of Flu PCR-SSCP analysis using the ALF Express offers exceptional resolution and speed which is essential for preimplantation diagnosis. This protocol is simple, sensitive, fast and reliable to detect alterations in the β globin sequence at the single cell level. Eleven CVS DNAs were tested blindly and all gave the correct results, which confirms the accuracy and efficiency of fluorescent SSCP analysis in diagnosis.

VI - 4.2: Fluorescent PCR and mutation detection of CF
ΔF508

There are several protocols that have been reported PCR amplification of exon 10 of the CFTR gene from single cells were the most predominant mutation (ΔF508) causing the disease is located (Ray et al 1996; Findlay et al 1996; Ke-Hui et al 1995). Ray et al and Ke-Hui et al have used the conventional method of nested PCR (Table 4 - 1), while Findlay et al applied the fluorescent PCR method.

Ray et al used the alkaline buffer (KOH/DTT) to lyse the cells then nested PCR for a total of 55 cycles. They reported that by increasing the denaturation temperature up to 96°C in the first 10 cycles of the outer amplification, the incidence of ADO has decreased down to 5% and the efficiency of amplification was improved by using KOH/DTT lysis buffer. In fact, one of the reasons for improving the efficiency of amplification and reduction in the incidence of ADO following an
increase in the denaturing temperature for outer amplification may be due to increasing the chance of the outer primer annealing as its annealing temperature is very low – 40 °C. The amplified product from each cell was mixed with normal and homozygous affected amplified DNA, subjected to heat denaturing then polyacrylamide gel electrophoresis followed by ethidium bromide staining to visualise heteroduplexes for analysis.

Ke-Hui et al (1995) have reported the application of the ARMS technique for the detection of mutant ΔF508 from single cells. Single cells were lysed using PCR reaction buffer followed by an outer amplification using one set of normal primers. Two aliquots from each PCR product of the outer amplification were put into different tubes and each tube was subjected to inner amplification in which one tube contained the mutant primer and the other has the normal one. The amplified products were then visualised on ethidium bromide stained agarose gel and diagnosis made upon the presence or absence of product. Although they have reported 100% amplification from cells, they did not report on the presence or absence of either contamination or allele drop out.

Findlay et al (1995) reported the application of fluorescent PCR to amplify exon 10 of the CFTR gene from either somatic or embryonic cells following lysis with the alkaline buffer. Although amplification was achieved in 157 cells out of 163 (96% efficiency) allele drop out occurred in 7 cells (4.3%), which means that correct diagnosis has been achieved in only 150 cells, and contamination was seen in 4.9%.
Table 4.1: Comparison between different PCR protocols reported by Ke-Hui et al (1995); Ray et al (1996) Findlay et al (1996) and the protocol developed in this study, to amplify exon 10 of the CF gene from single cells.

<table>
<thead>
<tr>
<th>Lysis buffer</th>
<th>Ray et al</th>
<th>Ke-Hui et al</th>
<th>Findlay et al</th>
<th>El-Hashemite (This Study)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 mM KOH/ 50 mM DTT; followed by neutralising buffer (900 mM Tris-HCl, pH 8.3; 300 mM KCl; 200 mM HCl)</td>
<td>10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂ /L; pH 8.0</td>
<td>200 mM KOH/ 50 mM DTT; followed by neutralising buffer (900 mM Tris-HCl, pH 8.3; 300 mM KCl; 200 mM HCl)</td>
<td>125 µg/ml proteinase K</td>
</tr>
<tr>
<td>PCR amplification</td>
<td>Nested PCR: Outer amplification: 10X PCR buffer [K+] free (25 mM MgCl₂; gelatine 1 mg/ml; 100 mM Tris HCl, pH 8.3; 10 mM dNTPs; Taq polymerase; Primers. Inner amplification: 10X PCR buffer (500 mM KCl; 100 mM Tris HCl, pH 8.3); 25 mM MgCl₂; 10 mM dNTPs; Taq Polymerase; Primers. 55 cycles for amplification</td>
<td>Nested PCR: 10X buffer (500 mM KCl, 100 mM HCl/ L; pH 8.3); MgCl₂ 10 mM dNTPs; Taq Polymerase; Primers (normal primers for outer amplification; ARMS primers for inner amplification). 60 cycles for amplification</td>
<td>Fluorescent PCR: 10X PCR buffer [K+] free (25 mM MgCl₂; gelatine 1 mg/ml; 100 mM Tris HCl, pH 8.3; 10 mM dNTPs; Taq polymerase; Primers. 45 cycles for amplification</td>
<td>Fluorescent PCR: 10X Super Taq Buffer; 2 mM dNTPs, Primers; Taq polymerase. 30 cycles for amplification</td>
</tr>
<tr>
<td>Mutation detection</td>
<td>ARMS method. Agarose gel electrophoresis, followed by ethidium bromide staining.</td>
<td>Fragment analysis</td>
<td>Fragment analysis</td>
<td></td>
</tr>
<tr>
<td>Contamination</td>
<td>14.2%</td>
<td>Not reported</td>
<td>4.9%</td>
<td>0%</td>
</tr>
<tr>
<td>ADO</td>
<td>5%</td>
<td>Not reported</td>
<td>4.3%</td>
<td>0%</td>
</tr>
<tr>
<td>Time of analysis</td>
<td>~8 / 9 hours</td>
<td>~8 / 9 hours</td>
<td>5 / 6 hours</td>
<td>~ 5 hours</td>
</tr>
</tbody>
</table>
In this study, to optimise PCR conditions for the detection of ΔF508 mutation at the single cell level, fluorescent primers flanking the region of the deletion were used to amplify 154 bp of exon 10 from single cells that were lysed by proteinase K. Following 30 cycles of PCR, the amplified products were subjected for simultaneous fragment analysis on the ALF Express automated sequencer. The efficiency of amplification was 97% and allele specific amplification failure was not observed in the heterozygous cells; however, a partially preferential amplification was seen in two cells.

When comparing the methods of Ray et al, Findlay et al and Ke-Hui et al with the one developed in this study, it can be seen that this protocol is more efficient, accurate, simpler and faster for single cell analysis (Table 4 - 1) because of the following:

1 - Ke-Hui et al reported using allele specific priming techniques for mutation detection. In fact, ARMS technique relies on the annealing of either the mutant or normal primer to the sequence, therefore for the purpose of preimplantation diagnosis it cannot be considered as a method of choice for many reasons. First this technique relies on the presence or absence of either normal or mutant product, therefore, if DNA in either tube failed to amplify there will be a misdiagnosis. Secondly, in the case of haploid cells or if complete allele drop out occurred this will lead to misdiagnosis. Thirdly, if one of the two alleles in the cell is preferentially amplified, the weak amplification might be considered as background but not as an amplified product, as the intensity of the DNA band is critical in ARMS analysis.

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2 - Ke-Hui et al and Ray et al have applied protocols that have many steps of analysis which itself could be a major source of errors.

3 - With regards to allele drop out; Ke-Hui et al have not mentioned the incidence of ADO in their experimental report, but Ray et al and Findlay et al have reported it. Although the percentage of ADO has been reduced to 5% when increasing the denaturation temperature (Ray et al 1996) and to 4.3% when using the fluorescent PCR (Findlay et al 1996) because preferential amplification was detected; ADO remained a problem that caused incorrect diagnosis in 7 cells. In this study, the incidence of ADO was not observed and the CFTR status has been efficiently diagnosed negating the need to change the PCR conditions and protocol. The three groups used many chemicals in the PCR mix while in this study only the basic reagents required for amplification were used. It is not known whether the large number of chemicals used in the PCR mix by Ray et al and Findlay et al such as alkaline buffer and neutralising buffer has an impact on the efficiency of amplifying both alleles of the gene in a single cell.

4 - The incidence of contamination was not reported by Ke-Hui et al, but has been reported as 14.2% by Ray et al and 4.9% by Findlay et al, this compared to 0% in the fluorescent protocol of this study. The reamplification of DNA by nested PCR could explain the presence of contamination in the case of Ray et al, however the large number of PCR cycles in one run performed by Findlay et al could be the cause of contamination even though they used fluorescent PCR.
The results of this study demonstrates that our protocol for PCR amplification and mutation detection meets all the criteria of efficiency, sensitivity, and speed required for single cell analysis.

VI - 4.3: Quantitative Fluorescent Multiplex PCR (QF-MPCR)

Although pregnancy rate following preimplantation genetic diagnosis is comparable to that of routine IVF, around 22%, it is variable and low considering that the women are usually fertile. The reasons behind implantation failure are still unknown and raise many questions. Could the low implantation rate be as a result of embryo biopsy, the age of women requesting preimplantation diagnosis or it is related to chromosomal abnormalities of embryos?

It has been reported that 16-53% of IVF embryos that appear morphologically normal have chromosomal abnormality, usually mosaicism. The actual cause of mosaicism is still unknown, and to what degree it could affect the embryonic development and implantation is still under investigation. Could the actual hormonal treatment of IVF and the culture media and the environment during fertilisation affect mitosis and lead to chromosomal aneuploidy or other abnormalities? Women attending for prenatal diagnosis or those who are over the age of 35 years of age are usually screened for chromosomal abnormalities in their pregnancies. However, in PGD programmes, where many women requesting PGD are over 35 years of age, transferred embryos are usually normal or healthy for the genetic disorder concerned but are not investigated for age related trisomy, e.g. of chromosome 21. The risk of
chromosomal abnormalities increases with advancing maternal age. The most common aneuploidy is trisomy 21, which occurs in 1:600 new-borns (Connor and Ferguson-Smith 1993). Down's syndrome which results from trisomy 21 usually arises because of meiotic nondisjunction of chromosome 21 in the mother (Jacobs et al 1995). Therefore, other techniques should be developed to investigate both the incidences of mosaicism and trisomy 21 especially in older patients requesting preimplantation diagnosis of single gene disorders such as cystic fibrosis.

This can be achieved by two different ways: either performing FISH analysis along with PCR, however this is not possible because of the limited number of cells available from each embryo or by performing multiple PCR reactions, but the DNA of a single diploid cell is limited.

The development of a whole genome amplification procedure (Zhang et al 1992) makes it possible to investigate several loci. By using primer extension preamplification (PEP) from single cells which supplies sufficient amount of template for extended PCR analysis, several nested PCR amplification can be carried out separately and in different reactions to amplify the gene of interest (e.g., CF) and polymorphic markers related to the chromosomes (e.g., 13, 18, 21). However, this requires at least 14 hours of PCR amplification followed by electrophoresis. Following such a protocol PGD will be performed over two days and selected embryos will be transferred by the end of day four. This may decrease the number of available embryos to be selected, as some embryos will be arrested in development.
An alternative to whole genome amplification is fluorescent PCR technology, which allows the examination of multiple loci by combining several unrelated primers specific for loci throughout the genome in multiplex PCR. The amplified products can then be analysed quantitatively by automated polyacrylamide gel electrophoresis. Quantitative PCR was described firstly by Lubin et al (1991) to detect X chromosome aneuploidy, and in 1993 Mansfield has modified the procedure to detect trisomy 21, 13 and 18. The quantitative analysis is based on the amplification of highly polymorphic DNA sequences unique and specific for each chromosome pair which are known as small tandem repeats (STR) of variable length. The quantitative PCR depends on the amount of specific DNA produced proportionally to the quality of initial target sequence (Ferre 1992) which makes the normal and trisomic samples distinguishable.

This work aimed to investigate the extension of quantitative fluorescent multiplex PCR for the determination of the embryonic CF status for AF508 and the copy numbers of chromosome 21 at the single cell level. The main clinical value was to investigate the possibility of detecting the incidence of chromosomal aneuploidy in women undergoing PGD who are over the age of 35 years, and to assess the possibility of applying multiplex PCR to improve the accuracy of single gene disorder diagnosis prior to embryo implantation, particularly when mosaicism is present.
Fluorescent quantitative analysis of normal DNA samples will show two peaks with an intensity ratio of 1:1 which corresponds to the presence of two alleles at each STR locus; while trisomic cells fall in two major groups. The first group present with three alleleic patterns which will show 3 peaks of similar intensity 1:1:1, while the second group will show two peaks with an intensity ratio of 2:1 which indicates the presence of two identical alleles and the product of the third.

In some cases, Down's syndrome results from translocation of the long arm of the chromosome to the acrocentric chromosome 13, 14, 18, 15 and 22 (Patterson 1987; Hassold and Jacobs 1984). The development of homologous gene quantitative PCR by Lee et al (1997) makes it possible to detect an extra piece of the long arm of chromosome 21 in translocation cases of Down's syndrome.

Although the data presented in this study demonstrates the advantages of this approach for specific diagnosis of the CF ΔF508 and chromosome 21 status, it is important to emphasise that the results could be of potential source of error when using this method for wider clinical applications. In this study only one STR marker was used for the detection of the number of copies of chromosome 21. However, for the purpose of diagnosis of trisomy 21 more than one marker should be used to avoid misdiagnosis when a single STR peak appears from the very few homozygous individuals or from signal overlapping. Moreover, the analysis of aneuploidy needs another STR marker of the same chromosome to improve the accuracy of diagnosis because of the errors of quantitative analysis that could arise as a result of preferential amplification of one allele within a single cell. However, the application
of multiplex PCR during PGD is particularly useful to assure accuracy of diagnosis in the presence of haploid cells in mosaic embryos. Furthermore, using an STR marker of variable size such as D21S11, which is informative in the family could be used to detect the source of any contamination.

The possibility of examining several loci in one PCR reaction clearly improves the reliability and accuracy of monogenic disorders at the single cell level and widens the spectrum of molecular analysis either for early prenatal diagnosis using transcervical cells to distinguish between maternal and fetal cells or in preimplantation diagnosis. However, the problem of allele specific amplification failure and preferential amplification which is associated with single cell PCR makes the application of QF-MPCR for the diagnosis of chromosomal abnormalities unreliable.

Findlay et al (1997) have reported fluorescent multiplex PCR for the diagnosis of sexing, cystic fibrosis ΔF508, trisomy 18 and 21. Their results revealed that multiplex diagnosis of several loci could be made in 80% of cases. The same was observed by Sherlock et al (1998) as diagnosis can be at the single cell level in 75% of cases. The competition for Taq polymerase and nucleotides between different loci in the same reaction could be the reason behind the relatively efficiency of multiplex PCR in diagnosis of single cells. The mechanisms that could interfere with the amplification of the genomic DNA from single cells might be the tangling of super coiled genomic DNA, or blockage or degradation of particular target sequences. The actual cause of ADO and preferential amplification remains unclear as well as its
incidence whether it can be generalised to other loci still needs to be assessed. However, although other studies have assessed the diagnostic value, reliability and accuracy of chromosome specific STR markers in single cells, the results presented in this study demonstrate that the procedure itself by using one STR marker can be a useful tool to assess the incidence of haploid mosaicism in embryos during diagnosis of monogenic disorders from single cells, particularly when the reliability of using one marker has been shown to be 100% in comparison to 75% when using two markers in the same chromosome (Findlay et al 1997). In addition, using one STR marker, which is linked to the mutation causing disease, can be beneficial to assess the incidence of ADO during clinical diagnosis.

VI – 4.4: Fluorescent Automated DGGE

DNA fragments differing by changes as simple as a single base substitution, deletion or insertion can be separated physically by electrophoresis of double stranded DNA through acrylamide gel containing linear gradient of denaturants, i.e., DGGE procedure. The principle of this method is that as the gradient denaturant concentration is raised the double stranded DNA goes through a distinct pattern of melting behaviour. The fragments reaches a certain point where its lowest melting domain that is based on the DNA sequence melts to create a branched molecules that effectively does not move any further through the gel matrix. When DNA fragments differ in the sequence that change their lowest melting domain, consequent retardation of their mobility will occur at different positions along the gel during their electrophoresis separation on denaturing gradient gel (Myers et al 1987). DNA
fragments differing by single base change within the highest melting temperature domains can not be resolved by DGGE because of the loss of sequence-dependent migration upon complete strand dissociation. Myers et al (1985) have overcome this problem by introducing into the fragment to be analysed a G-C rich domain (Clamp) through PCR amplification primers. The GC clamps, which will serve as the high melting temperature domain for DGGE analysis can be introduced by one of the two PCR primers, used to amplify the target DNA sequence (Sheffield et al 1989).

The combination of PCR - DGGE analysis is extremely powerful for the detection of heterozygous nucleotide variants. During PCR, denaturation and reannealing of single stranded molecules the formation of homoduplexes occurs. The presence of single mismatch in the DNA sequence will result in the formation of heteroduplexes that will separate from homoduplexes on the gel, thus heterozygous sample will show a homoduplex and a heteroduplex representing the normal and mutant alleles. During electrophoresis, double stranded DNA molecules will migrate at a linear rate that depends on its molecular weight. The DNA fragment will reach a position in the gel, where the denaturing concentration and temperature of the running gel equal the temperature of the melting domain temperature, were it suddenly becomes branched with a double stranded portion and single stranded portion, i.e., homoduplex and heteroduplex, respectively.

Although DGGE is a highly accurate method for screening and diagnosis, it is expensive, requires a long running time which ranges between 6 to 24 hours, and its
efficiency in mutation detection of single base variants is mainly dependent on the presence of GC clamp primers.

The original DGGE protocol has been subjected to many efforts to improve some of its features and efficiency (Meyrs et al. 1985; Attree et al. 1989; Satoh et al. 1993; Abrams et al. 1990; Takahashi et al. 1991), either by introducing GC clamps which will detect the majority of base variants within a 500 bp fragment encompassed within a single melting domain; or by enzyme digestion of PCR amplified long DNA fragments prior to electrophoresis; or amplifying DNA fragments by two GC clamp primers. Hoving et al. (1991) represented another modification of DGGE by using a constant concentrations of denaturing gel to allow increased resolutions of mutant fragments through constant migration with different electrophoretic mobility on the gel. The constant denaturing gel electrophoresis has been useful for screening of the p53 gene but it does not seem to represent a valid alternative to DGGE.

Although the conventional DGGE method is efficient and accurate technique for mutation detection, it is not convenient for single cell analysis. The main idea of using the GC rich domain is to improve the efficiency and sensitivity of mutation detection. Combining the sensitivity of mutation detection with the speed of analysis will make DGGE a suitable technique for diagnosis of monogenic disorders from single cells. The first ever-automated DGGE analysis for mutation detection and subsequently clinical diagnosis through an improvement of the original DGGE method by using the combination of fluorescent based PCR - DGGE analysis was

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developed. Non-GC clamp primers were used to amplify a fragment of 397 bp of the β globin gene. Amplified products were subjected then to DGGE analysis which was performed on an automated DNA sequencer - the ALF Express. The sensitivity of the mutation detection of the ALF express, the possibility to create a denaturing gel gradient with the temperature control of the ALF gel plates, and the speed of analysis, all of these were combined to detect β globin gene variants without using any GC clamps. Nineteen PCR cycles were needed to amplify a DNA fragment of interest, and the total running time of gel electrophoresis was for a maximum of 200 minutes using the short plates, this in fact allowed same day genetic determination of β globin status in a sample of either genomic DNA of CVS or from single cells.

The primary aim of this study was to establish an assay based on DGGE which offers simultaneous mutation scanning from single cells obviating the need for GC clamp primers and with shorter electrophoresis time that will be suitable for PGD. The availability of one or two single cells from each embryo makes it critical to choose a very sensitive method for mutation analysis that requires a very simple means of PCR amplification. Palmer et al (1996) employed analysis by the conventional DGGE using GC clamp primers. They reported failure of co-amplification in 80% of heterozygous samples starting from 5 pg of DNA template.

The sequence of bases on a strand determines its degree of stacking and stability. Changes in the base sequence as small as a single base substitution significantly alters the stacking of the DNA fragment and thus change the melting
temperature of that fragment. In practice, for more efficient and accurate mutation
detection by DGGE, usually primers are designed to amplify not more than 200 bp.
This is a problem particularly when two different mutations are present in a fragment
of more than 200 bp and representing different melting temperature, this will require
two different sets of GC clamp primers to amplify the two regions were the
mutations are located. This could be practical in the case of prenatal diagnosis where
at least 20 μl of DNA template is available for analysis and two different PCR
reactions can be made. Thus the experimental conditions are generally different for
each mutation and different denaturing gradients and running time will be required.
Such variation in conditions does not constitute a problem where the time schedule is
not crucial and funds are available. However, in a diagnostic setting where a single
DNA sample has to be analysed and where same day results are required, the
conventional DGGE assay is not satisfactory. In addition, the conventional DGGE
protocol requires a long time for analysis and the DNA bands can then be visualised
by either ethidium bromide staining or silver staining.

To establish one common protocol for PCR amplification of genomic DNA
from single, a target sequence of the β globin gene was amplified using one set of
normal primers, in which the forward primer was labelled with Cy5 at the 5' end.
Only 19 cycles of PCR were needed to amplify the region of interest from template
DNA and 30 cycles from single cells. Amplified products were then subjected for
automated DGGE analysis using the ALF express (an automated DNA sequencer -
Pharmacia). One step PCR-DGGE assay was applied to scan for mutations within

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exon I and intron I of the β globin gene with a minimum experimental manipulations including one PCR run and subsequent short time of DGGE analysis.

There are several potential reasons for the relatively high frequency of mutation detection in this technique:

1- a large fragment (up to 500 bp) can be analysed in this study;

2- the DNA fragments can be visualised as peaks, and the quality of the Microsoft programme can magnify the peaks to assess the real ones from that of artefact, as well the sensitivity of the machine allows the detection and visualisation of strands that did not separate enough from each other through measuring the peak area and distance, that is hard to distinguish by the conventional method.

3 - The maximum running time of the analysis is 200 minutes.

4 - GC clamp primers have been shown to give an excellent resolution for mutation detection, however, normal primers, i.e., non-GC clamp primers can be used to detect the mutations.

5 - Because DGGE depends on the detection of DNA duplexes, it was found that the pH of the running buffer does affect the quality of DNA fragment separation. The more alkali the buffer the better the separation of the DNA molecules, as well we found that the all mutation investigated have been analysed on a gel gradient 45%-70% and when the temperature of the running gel was up to 50 C has gave better separation and quicker analysis.

6 - because the protocol relies on the presence of a melting domain with a melting temperature higher than that of the domain where the variant is located within the
fragment to be analysed only a subset of all single base substitutions investigated have been detected. Furthermore we found that the presence of polymorphisms within the DNA fragment may affect the quality of the separation.

The Fluorescent based PCR-DGGE assay described in this study is technically simple, requires a minimum time for analysis and gives reliable results. In addition, all data of the entire analysis is presented on the computer screen with a straightforward interpretation. This has advantages in clinical diagnosis particularly as the ability to determine within 6 hours the embryo status with high fidelity may provide the basis for a rapid and secure diagnostic approach.

**VI - 4.5: Strength, Weakness and Future requirements for mutation detection at the single cell level**

There are many methods available for mutation detection and analysis, each has its advantages and disadvantages. Not all methods are suitable for determination of gene variants for diagnostic purposes. Some methods require hazardous chemicals and radioactive materials which make it less suitable for routine screening and diagnosis; some are relatively labour intensive, very expensive, and require longer time for analysis.

With the features of current methods used for either screening or diagnosis of single gene disorders, it is very difficult to prove that a particular method is the ideal one particularly for single cell analysis. A method is considered ideal when it has the following criteria: sensitivity, fast, accurate, reliable and cheap. In fact, any method
developed for preimplantation diagnosis of single gene disorders can be ideal for prenatal diagnosis screening, however, the opposite is not always correct.

By comparing different methods, it has been found that combined SSCP heteroduplex analysis is the most useful method for screening and mutation detection because it is cheap, fast, straightforward, efficient and accurate and requires a single step for analysis. SSCP heteroduplex analysis is simple and relatively highly sensitive, and has become one of the most common techniques and most frequently used for the detection of point mutations, small deletions or insertions. Hayashi (1991) has estimated that the sensitivity of the method is highly dependent on many factors but most importantly is the length of the PCR product to be analysed, the shorter the fragment the higher the efficacy of the method in mutation detection. He demonstrated that when the DNA fragment is between 100-300 bp the frequency of mutations detection is higher than 92% and it decreases as the fragments get larger than 500 bp or shorter than 100 bp.

Using one set of primers the most common and the rare mutations can be analysed by SSCP which makes it suitable for screening where mutations are heterogeneous. Furthermore, it is a simple method for screening, and sometimes diagnosis can be performed by conformational analysis without the need for molecular characterisation of the mutation causing the disease. Additionally, it allows the visualisation of each allele which makes it the most suitable technique for PGD. However, if amplification of multiple loci by multiplex PCR is to be considered for clinical application to PGD, SSCP will not be the ideal method for mutation detection.
and quantitative analysis. This is because other products could interfere with the conformation of single stranded DNA molecule.

Conventional DGGE offers a valid technical approach for the identification of mutant genes particularly that of single base substitutions. DGGE has many advantages: its sensitivity of detection is almost 100%, it makes the detection of heterozygous samples easier through the detection of heteroduplexes and homoduplexes and it is a non radioactive technique. The disadvantages of DGGE include: it is laborious and time consuming, expensive particularly for the synthesis of relatively long primers and has a limit to the size of the DNA fragment that can be analysed (around 500 bp) efficiently. The development of automated fluorescent DGGE can overcome or reduce the disadvantages of the conventional method.

The availability of 1 or 2 cells from each embryo for analysis and the short time for diagnosis are considered limitations of preimplantation diagnosis of single gene disorders. Such limitations make automation of genotyping techniques a necessity. Although fluorescent PCR requires an expensive automated DNA sequencer for mutation detection; the speed and accuracy of analysis particularly when different methods can be combined, will outweigh its only disadvantage which the cost of such machines.

ARMS or dot blot hybridisation are not considered to be sensitive enough techniques for PGD, because if the mutant primer fails to hybridise, it could lead to misdiagnosis. The application of fluorescent ARMS technique may be an ideal
method for the detection of single base substitutions from single cells particularly when combined with multiplex PCR for polymorphic markers linked to the mutation. This can allow the assessment of ADO, if present, haploid cells and the identification of the source of contamination. Fluorescent PCR seems to be the way forward for the PGD of monogenic disorders.

**VI - 5: Non-invasive Prenatal diagnosis using TCC**

Several attempts have been made to develop non- or minimally invasive first trimester prenatal diagnosis tests based on either the detection of fetal cells in the maternal peripheral blood or in endocervical samples collected at early stages of gestation (Adinolfi 1995). Both approaches are still in the experimental stage because the frequency of fetal cells that can be collected is variable in either approaches, particularly in the case of TCCs, it depend on the methods employed for the retrieval (Rodeck et al 1995) and testing (Adinolfi 1996).

Fetal cells were successfully retrieved from the TCC samples obtained in the seven cases where both parents were known to be carriers for haemoglobin disorders. Two major problems remain with using clumps of trophoblastic cells for non-invasive prenatal diagnosis: one is that the possibility of contamination with maternal cells. The second problem is to distinguish between maternal and fetal cells particular if the fetus is a carrier for the maternal mutation.

Three possibilities may overcome these problems: First is testing single cells isolated from available clumps either by monoclonal antibodies after digestion with
collagenase (Rodeck et al 1995). Another option is to use fluorescent multiplex PCR amplification specifically to diagnose the fetal status for haemoglobinopathies and to use polymorphic STR markers to distinguish between the fetal and maternal cells by fingerprinting.

Although the results from the seven cases show only partial success, non-invasive prenatal diagnosis using TCC sampling could possibly be applied clinically once improvements in cells sampling, isolation and accurate identification of fetal cells have been achieved or when combining different techniques for mutation detection, fingerprinting and chromosomal analysis can be achieved.

VI - 6: Prevention of haemoglobinopathies in Arab countries

β thalassaemia causes significant morbidity and mortality in affected individuals, burdening not only the patients and their families but also national health services. Worldwide, there are over 180 different types of mutations causing the disease through which there are few common mutations and some rare ones in any population. Currently, the high frequency of β thalassaemia can be reduced by offering genetic counselling and prenatal diagnosis for couples at high risk (WHO working group 1983).

The haemoglobinopathies particularly β thalassaemia are a major health problem in the Middle East. In order to develop an easy, cheap and accurate DNA based method for national thalassaemia control programmes as enunciated by the WHO, it is necessary first to identify the spectrum of mutations of the β globin gene
in this region. \( \beta \) thalassaemia affects the people of Mediterranean, African and Asian ancestry. In the past few years there had been intensive studies on the \( \beta \) thalassaemia genes from most of the different ethnic groups of the Mediterranean, but the molecular basis of this disease in Egypt and Jordan remains to be investigated.

There has been revolution in the sequencing methods used over the last 15 years (Sanger et al. 1977; Baringa 1991; Bain 1991; Streyoska et al. 1993) particularly with the advent of PCR. The most important development is sequencing by automated fluorescent laser DNA sequencers which proved to be efficient at sequencing up to 1000bp in a short time with high efficiency (Grothnes et al. 1993; Early et al. 1994). This technique has been applied to screen for \( \beta \) globin mutations in Egyptian and Jordanian patients.

VI - 6.1: Molecular characterization of \( \beta \) thalassaemia in Egypt

\( \beta \) thalassaemia is relatively common in Egypt. It has been estimated that out of 1.5 million live births per year, over 1000 children with \( \beta \) thalassaemia major are born (Noveletto et al. 1990). In contrast to other Mediterranean countries, few studies on the molecular basis of \( \beta \) thalassaemia have been done in Egypt. In the course of screening for \( \beta \) thalassaemia mutations [-87 C-G; CD5 -CT, CD 6 -A, IVSI nt 1 G-A, IVSI nt 6, IVSI nt 110, CD 39, IVSII nt 1, IVSII nt 745, IVSII nt 848] were detected by reverse dot blot hybridisation in 87 alleles out of 108 (Rady et al. 1997). Twenty one allele in 16 DNA samples were negative for the mutations investigated. These
samples were subjected to SSCP analysis to screen for mutations followed by direct sequencing analysis.

The few studies that have been done on the molecular basis of β thalassaemia in Egypt have screened and identified the most common Mediterranean mutations (Novelette et al. 1990; Hussein et al. 1993; Rady et al. 1997). However, the entire spectrum of β thalassaemia mutations has not been studied adequately in Egyptians, and many more mutations are expected to be identified.

In this study screening and direct sequencing analysis was performed on 16 DNA samples from unrelated patients with previously undetected mutations in 21 alleles. Upon screening the 21 chromosomes by SSCP, a frameshift 8/9 (+G) was detected in one allele. This Asian Indian mutation was reported previously in 13 chromosomes in Turks (Basak et al. 1992). Therefore, it is not surprising that this mutation is present in Egyptian as well as the Turkish populations, particularly as historical evidence has revealed that these populations have interacted in the past.

When variant DNA samples were sequenced, a frameshift at CD 29 (-G) was observed in four different alleles of unrelated patients. The identification of a Japanese mutation in Egypt is unexpected. Thus, it is most likely that the mutation arose independently in these two populations, particularly since frameshifts are among the most common lesions affecting the β globin gene (Huisman 1992). Furthermore, the identification of another frameshift at codon 28 (-C) in one allele was not surprising.
A change at codon 22 (A-C) of the β globin gene has been found in 15 of 21 chromosomes. Although this mutation was thought to cause β thalassaemia (El-Hashemite et al 1997) it is in fact a polymorphism causing Hb G-Coushatta. Whether the phenotype of thalassaemia in those patients may be due to the co-existence of genetic determinants that decrease the HbF, additional investigations are required to confirm the actual causing of the β thalassaemia phenotype among the patients. Similarly a silent nucleotide change at codon 17 (G-A) seen in all samples investigated, is probably a polymorphism in that population. Further studies are needed to confirm the frequency of this polymorphism among Egyptians, and its presence within neighbouring countries. Such studies will help in tracing the geographic origin of this mutation.

These results suggest that the disease-causing mutations identified in this study may account for up to 20% of the β thalassaemia alleles in Egyptians. The results also confirm that a heterogeneous group of mutations is causing β thalassaemia in Egypt. Unlike in other Mediterranean populations, β thalassaemia in Egypt is caused not by one or two predominant mutations, but by over ten. Four mutations (IVSI nt 110, IVSI nt 6 and IVSI nt 1) constitute about 77% of the mutations causing β thalassaemia in Egyptians. In addition, this demonstrates that SSCP analysis is a reliable, sensitive and cost-effective method of mutation screening and detection. Using the set of primers described, all the common β thalassaemia mutations within a
500 bp fragment of the gene can be detected without the use of restriction enzyme digestion, probes or blot hybridisation. Since most mutations causing β thalassaemia in Egyptians have been identified, the challenge for the future is to organise a comprehensive national screening programme in Egypt.

VI - 6.2: Molecular characterization of β thalassaemia in Jordan

β thalassaemia is a common genetic disorder in Jordan with high prevalence. There has not been any kind of estimation for the carrier status in that country. The characterisation of mutations and their frequencies in this population is an important prerequisite to the implementation of DNA based methods for screening and prenatal diagnosis programmes.

A total of 89 DNA samples from unrelated Jordanian patients who have been diagnosed clinically with β thalassaemia major and intermedia were analysed. Because of its geographic location between Mediterranean countries and the historical relations with neighbouring countries such as Israel, Iraq, Egypt, Lebanon, Arabia and Syria, the samples were first screened for the most common Mediterranean mutations using ARMS which were known to be prevalent in the neighbouring countries. Sequence analysis was carried out on 32 samples whose mutations were not elucidated.

In general, the spectrum of the β globin variants observed in this study were similar to those found in North of Jordan (Sadiq and Huisman 1994) and the
neighbouring countries with slight difference in the frequencies. This study shows the presence of 17 different mutations causing thalassaemia in the Jordanians. The mutational profile reflects the delicate balance between population exchange in the region as well as local consanguinity marriage. Although the precise rate of consanguinity in Jordan is not known, its prevalence can be seen from the high frequency of homozygous patients despite the heterogeneity of mutations.

The identification of IVSI nt 5 G-C mutation is interesting because it has been previously found in North Jordan (Sadiq and Huisman 1994), United Arab Emirates (Quaife et al 1994), Iraq (M. Petrou - personal communication), Gaza Area - Palestine (Filon et al 1995) and Asian Indian (Kazazian et al 1984) populations. A single base substitution at codon 26 which has been identified previously in the Thai population was seen in one allele which has two possible explanations: either this mutation was introduced to Jordan by the gene flow from south east Asia, or it arose independently in these two population. Two novel mutations have been identified at the Cap site at position +20 C-T and the other at IVSI nt 23 A-C. The cap site mutation was seen in one patient who was homozygous for it and carrying another mutation in intron I. Generally, this mutation with its position known to cause either mild β+ thalassaemia or β thalassaemia trait phenotype. The base change in intron I at nt 23 has been found in two different alleles, and it is quite difficult to postulate whether this mutation is causing the disease or it is a polymorphism.
The molecular characterisation of β thalassaemia mutations in Jordan reflects that the spectrum of mutations for any population is essential for formulating a strategy of prenatal diagnosis although the ethnic origin may reflect the range of possible mutations. The data presented in this study represent a true incidence of the different β thalassaemia mutations in the Jordanian population because they are derived from the study of unbiased samples which were obtained from patients attending the haematology department for transfusion.

Recently, there is growing population awareness on the possibility of prevention of the thalassaemia. Based on the data presented in this study, a simple approach to national screening and the early prenatal diagnosis can be a feasible option and reality in Jordan.

VI - 6.3: Comparison between β thalassaemia mutations in the Middle East

Several interesting observations emerge from the analysis of the β globin genes in the previous studies regarding the distribution and frequency of the molecular defects in these countries.

In general, the IVSI nt 110 mutation is the predominant defect in the Middle East. The IVSI nt 1 G-A has been found to be with high frequency in Egypt, Jordan and Gaza Area but less frequent in Israel. The nonsense mutations CD 39 has been found at almost the same frequency in Jordan, Gaza and Israel but rarely described in
in low frequency while it is predominant in Egypt and Israel.

Although the spectrum of β thalassaemia mutations in Jordan and Egypt are mostly Mediterranean, the frequency of mutations is variable in these countries compared to the neighbouring countries. Generally, this mutational profile reflects a population exchange in the region as well as local isolation, which explains the presence of new polymorphisms and mutations.

**VI - 6.4: Anthropological study on the spectrum of β thalassaemia in the Middle East**

Haemoglobinopathies are major genetic and public health problem in Arab countries, where they have been selected by malaria, migrations and a highly endogenous social system. The Middle East is a melting pot of populations from Arabs, African, Mediterranean, Asian and European origins. The combination of historical, geographical, epidemiological and molecular data makes it possible to explain the distribution and frequencies of haemoglobinopathies in such countries.

A remarkably detailed picture of the distribution of β thalassaemia worldwide has been provided while attempting to find the genetic mutations underlying β thalassaemia. As a result of these studies a large amount of data on genotypes has been accumulated. On the basis of the data published in the literature, it can be postulated that β thalassaemia mutations may have become established at similar
times throughout the world. The question herein is can selection by malaria be responsible for the high frequencies of β thalassaemia worldwide? Just to think about the fact that similar observations have been made for β thalassaemia in different parts of the world makes it likely that a single factor, malaria, is responsible for the high frequency of β thalassaemia alleles. The idea that certain haemoglobinopathies provide protection against malaria is over 40 years old (Haldane 1949). This suggestion however, only relies on the association between the present distribution of β thalassaemia and the assumption about the past distribution of malaria in the world.

On the other hand, it has been reported that in areas avoid of malaria new mutations appeared to be identical to those characterised in the malaria belt. Such mutations occurred on haplotypes that indicate their independent local origin (Kazzazian et al 1986; Chehab 1986). Hence other factors such as genetic drift, migration and population size and the social system may be responsible for the presence of β thalassaemia mutations in areas devoid of malaria. Worldwide, we can see that migrations have distributed mutations at high frequencies. This can be seen through the high frequency of CD 39 and IVSI nt 110 mutations in the β globin gene through the Mediterranean countries, which reflect a high rate of gene flow in this region (Aulehla et al 1990; Anthanassiably et al 1987; Ottolenghi et al 1986; Rund et al 1991). These mutations, however, have different haplotype distributions
suggesting the presence of other factors as well. Furthermore, admixture of different populations may promote haplotype diversity.

By combining the molecular characterisation of \( \beta \) thalassaemia mutations in the above studies with other data published in literatures about Middle Eastern countries, all these factors could explain the high frequencies of \( \beta \) thalassaemia mutations. Up to date, few molecular studies were undertaken in United Arab Emirates, Saudi Arabia, Egypt, Jordan, Iran and Israel in order to determine the genotypes of mutations and their frequencies in these populations. \( \beta \) thalassaemia is a common disorder in the populations of the Middle East, and its distribution is uneven among the different ethnic groups. Unlike other populations, \( \beta \) thalassaemia in these countries is not associated with one or two predominant mutations but with many different ones. Therefore, the elucidation of the molecular defect of \( \beta \) thalassaemia in the Middle East should be informative on both scientific (molecular) as well as anthropological levels.

The Middle East is a melting pot of populations, and it has attracted migrations from all over the world. This is because of its geographic location as it connects Asia, with Africa and Europe, and because of its holiness for the main three religions: Judaism, Christianity and Islam. Hence, there is a great diversity of mutations. One might postulate a continuous admixture in time, space or in both. Historical evidence suggests that admixture and expansion of modern humans are likely to have occurred in the Middle East, as it began with the spread of traders between Indian and South
East Asia. This could explain the presence of two Asian Indian mutations in Egypt (FS 8/9 +G) and the IVSI nt 5 in Jordan. Interestingly the IVSI nt 5 have been seen as the major mutation causing thalassaemia in Eastern Arabia as in United Arab Emirates, Kuwait and Iraq as well as in Iran; and the FS 8/9 mutation was seen in Turkey, Egypt, United Arab Emirates and Iran.

The knowledge of the different distribution of β globin variants according to the geographic location and historical background of populations is indeed of great importance in order to determine and offer comprehensive prenatal diagnosis and prevention programmes. Such knowledge is crucial and useful particularly in Western countries with multicultural ethnic groups who are dealing with patients from this region. For example, in UK where almost all β thalassaemia mutations are brought over by immigrants from different part of the world. The careful study of the geographic origin of patients’ families will very much help in choosing the appropriate set of primers and mutation to begin screening.

**VI - 7: Clinical application of SSCP analysis for preimplantation diagnosis**

One treatment cycle was performed for the diagnosis of β thalassaemia prior implantation. This cycle was the first PGD for single gene disorder and performed by Fluorescent PCR to amplify exon and intron I of the β globin gene. Unfortunately, contamination was seen in all medium blanks and in the PCR negative control. The PCR negative control and three of the medium blanks showed
contamination with the normal allele. This contamination might came either from the assisted conception unit, particularly that during tubing the biopsied blastomeres, one of the embryologist was preparing sperm samples for IVF in the same hood, or it could be due to amplified normal alleles present in the room where PCR amplification was carried out. Although mutation analysis of the biopsied blastomeres showed that three embryos were normal, it was difficult to judge whether this normal peak was from the amplified product of a normal allele carried by the cell or from the contaminating source. One of the biopsied cells from embryo 4 showed the conformation of normal while the second one showed the conformation of heterozygous for IVSI nt 110, and the medium blank from this embryo showed the conformation of IVSI nt 110/N. The presence of two different results particularly the conformation of normal within one embryo could be either due to allele drop out of the mutant allele in heterozygous embryo, or that DNA failed to amplify and the normal conformation is the result for contamination in the PCR blank. The presence of IVSI nt 110/N conformation in the medium blank of embryo 4 could be either as a result of a lysed fragmented cell from the embryo that was present in the medium, particularly since the cells were tubed directly from the medium drop where the biopsy was performed and were not washed due to extensive cell lysis.

To investigate the source of contamination, all PCR reagents used during the clinical case were used to amplify genomic DNA from 6 samples. Samples were amplified without any source of contamination. The only difference was the actual
mineral oil used to cover PCR mix. Thus maybe the oil was the source of contamination.

With all the precautions taken during preliminary work and in actual clinical cases, contamination remains a major problem when diagnosing monogenic disorders from single cells and does affect the accuracy of diagnosis dramatically.

VI - 8: Moral and ethical issues on genetic preventive procedures

Despite the international resolution of the World Health Organisation (WHO) to prevent the spread of genetic disorders, the number of babies born with genetic anomalies remains high and represents a global health problem. The treatment of genetic disorders, if available, is still unsatisfactory and expensive.

Advances in modern developmental biology and genetic diagnostic techniques raise as many question as they solve. How are different societies to deal with many legal, moral, ethical and religious issues that arise from genetic screening, counselling and preventive procedures? In many countries the use of available preventive procedures for couples at high risk of transmitting genetic disorders to their offspring is restricted.

Public health authorities are now increasingly concerned by the high rate of births with genetic disorders particularly among minorities, which may have an adverse impact on their budget plans and control strategies. Among those minorities are Muslims many of whom do not attend genetic counselling and genetic programmes because of poor understanding of genetic concepts which makes them
believe that they should not interfere with natural events, or because of their cultural and religious beliefs. Unfortunately, such people do not distinguish between what it is permissible according to the religion of Islam and their traditional and cultural beliefs.

Learning about how patients' cultural values and religion beliefs affect their perceptions of health and illness will allow health professionals to improve the quality of assessment and interventions and provide culturally appropriate care. Such an approach is needed when dealing with Muslim couples at high risk of transmitting a genetic disorder to their children. Roberts and colleagues (1996) study in Birmingham - UK, about the representation of ethnic minorities at genetic clinics showed that family doctors refer Pakistani families with genetic disorders less frequently to genetic clinics than families from other ethnic groups. This could be because physicians believe that Pakistani families do not wish to attend prevention programmes because of their religious beliefs.

Although prenatal diagnosis has led to a reduction in the number of births with genetic disorders in some countries, termination of affected pregnancy remains a contentious issue. The Muslim juriconsults (Sunni and She'at) agreed that if genetic tests proved definitely that a fetus is affected by a serious disease which will make him disabled after birth, then abortion is permissible and lawful. Pregnancy termination, however, should be carried out before the time of breathing the soul, i.e., before 120 days of gestation (El-Hashemite 1995).
The development of preimplantation diagnosis of genetic disorders has offered couples at high risk an alternative to prenatal diagnosis so that selective abortion can be avoided. Preimplantation diagnosis is based on in vitro fertilisation (IVF) procedures and genetic diagnosis of embryos on day 3-post insemination. Only healthy embryos are then transferred to the mother's uterus to initiate pregnancy. The use of preimplantation genetic diagnosis in human clinical genetics would, unlike CVS and amniocentesis, avoid abortion of affected cases, which are ethically difficult to justify. As it is impossible to foresee whether the conceptus will be one embryo or monozygotic twins at this stage of embryonic development, the separation of one blastomere for diagnostic purposes can not be considered as a sacrifice of an individual but as a diagnostic procedure.

In vitro fertilisation is permissible in Islam if the sperm and oocytes are from the husband and wife. The Muslim juricounsults (Sunni & She'at) have agreed that preimplantation diagnosis of genetic disorders is permissible in Islam because it is based on IVF and using such technique will not conflict with God's desire and might [El-Hashemite 1995]. Furthermore, this technique is not considering an alteration or modification for God's creation as it is a kind of treatment. Preimplantation diagnosis is preferable to prenatal diagnosis because it is done when embryos are only at the eight-cell stage and breathing the soul has not yet occurred at this stage.
VII: Future development of PGD of single gene disorders

The future of PGD of single gene disorders can be seen in the development of many mutation detection techniques to improve the efficiency and accuracy of diagnosis. The application of fluorescent multiplex PCR for specific diagnosis of X-linked diseases such as Duchene Muscular Dystrophy. The application of fluorescent ARMS technique and detection of amelogenin for sexing can achieve this.

Direct sequencing from the amplified genomic DNA of single cells could be beneficial, if the problem of ADO did not exist. The application of whole genome amplification followed by fluorescent PCR could be of importance particularly were large deletions are involved such as the 619 bp deletion in the β globin gene or for α thalassaemia.

A main development could be the combination of FISH analysis for the detection of chromosomal aneuploidy and the PCR technique for the diagnosis of single gene disorders. Four cells are required from each embryo in order to achieve this and it would be necessary to consider biopsy at the morula or blastocyst stage. However, the high incidence of mosaicism in the trophectoderm of blastocysts and the low rate of embryo survival in culture to reach the blastocyst stage along with the low pregnancy rate achieved following blastocyst transfer makes it difficult to consider.
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