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# The role of alpha thalassaemia in unexplained microcytosis at the Wits academic hospital

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# CHAPTER 1

# LITERATURE REVIEW

## LITERATURE REVIEW

### 1.0 Introduction

Epidemiological studies have shown that approximately one billion subjects are anaemic worldwide (Cook *et al.,* 1998). Cook and Colleagues reported in 1998 that the global prevalence of anaemia is about 30% with a range of 8% - 36%. Furthermore, the prevalence of anaemia depends on the socio economic circumstance as well as genetic traits, such as the common monogenetic gene disorders that are highly prevalent in the Mediterranean countries, South East Asia, Africa, Middle East and the Indian subcontinent (Higgs and Gibbons 2010).

Anaemia occurs as a result of an imbalance between production, sequestration and destruction of red cells and is confirmed with decreased levels of haemoglobin, and may be classified as moderate to severe depending on the haemoglobin concentration. The causes of anaemia are numerous and varied including substrate deficiency (iron, vitamin B12 and folate) blood loss, chronic disorders, infections (chronic bacterial infection e.g. TB, parasitic e.g. malaria and viral e.g. HIV) malnutrition, haemolysis and inherited haemoglobin disorders (haemoglobin variants and the thalassaemias (Cook *et al.,* 1998).

### 1.1 Haemoglobin

Haemoglobin (Hb) is an oxygen binding protein that transports oxygen from the lungs to the tissues. There are several subtypes of Hb in humans, a) Embryonic haemoglobins (Gower 1, Gower 2 and Portland) are produced by the yolk sac during the first eight weeks of embryonic development (Hoffbrand *et al*., 2004), b) Fetal Hb (Hb F) which is

the predominant Hb in the fetus and is produced by the liver for up to 30 weeks of fetal life and c) HbA which is the predominant haemoglobin in the human where adult levels are reached at the end of the first year of life. From this stage onwards Hb F comprises <2% and Hb A2 (subtype of HbA) 2.5 – 3.5% of total Hb, whereas the balance is made up of HbA (Clark and Thein 2004).

Haemoglobin is produced, packaged and stored in erythrocytes. Erythrocytes are produced in the bone marrow, and has a lifespan of 100-120 days. One of the major functions of haemoglobin is to carry oxygen. The amount of oxygen delivered to organs and tissues is dependant on the haemoglobin concentration

**1.2 Haemoglobin (Hb) Structure**

Haemoglobin is a tetrameric molecule, consisting of 4 globin chains, each linked to a haem molecule. The globin tetramer comprises of 2 alpha (polypeptide of 141 amino acids) and 2 non-alpha chains. The globin chains form a series of folds to create a hydrophobic recess in which resides the haem molecule (Forget 1986). The haem part of the molecule is made up of a porphyrin ring with a centrally situated iron atom which binds to an oxygen molecule. The Hb tetramer can therefore bind and transport 4 molecules of oxygen. A single globin chain complexed with a single haem is called a haemoglobin monomer. A complete haemoglobin tetramer is required for effective oxygen transport to the tissues, and a deficiency or abnormality of any component of the haemoglobin molecule could destabilize the tetramer and lead to anaemic condition

(Steinberg 2001). In iron deficiency anaemia there is inadequate incorporation of iron into the haem component, which is a rate limiting step for haem synthesis.

### 1.3 Iron deficiency and anaemia of chronic disorders (ACD)

It is important that a distinction be made between iron deficiency and anaemia of chronic disorders (ACD). Iron deficiency anaemia leads to development of microcytic hypochromic red cell indices, in contrast to ACD where the red cells are initially normochromic and normocytic but may later evolve to a microcytic hypochromic blood cell picture in more severe cases. ACD is associated with a variety of conditions but is commonly seen in chronic infections, inflammatory states (e.g. rheumatoid arthritis) and neoplastic disease. These disorders mimic an iron deficiency state in which serum iron concentrations are low and the developing red cells, may become microcytic hypochromic as a result of reticuloendothelial iron blockade. However, ACD differs from iron deficiency in that in ACD subjects, serum ferritin levels are normal or raised and the levels of transferrin are reduced whereas in iron deficiency serum ferritin levels are reduced and transferrin levels elevated (Hoffbrand *et al*., 2004) as shown in Table 1.

Table 1: Laboratory differentiation of causes of hypochromic microcytic anaemia

| | **Serum iron** | **Transferrin** | **Percentage Saturation** | **Ferritin** |
|---|---|---|---|---|
| **Iron deficiency** | ↓ | ↑ | ↓ | ↓ |
| **Anaemia of chronic disorder** | ↓ | ↓ | ↓ | N or ↑ |
| **Thalassaemia minor** | N | N | N | N or ↑ |

### 1.4 Haemolytic Anaemia

Haemolysis is defined as a state in which the red cell survival is shortened, i.e. <100 days where normal red cell survival is 100-120 days (Steinberg 2001). Haemolytic anaemia may be acquired or inherited and is classified according to the site of destruction *viz*. extravascular or intravascular. Extravascular haemolysis (EVH) takes place in the reticulo-endothelial system. Examples of EVH include hereditary spherocytosis, thalassaemia and warm antibody haemolysis. Intravascular haemolyis (IVH) takes place in the intravascular compartment. Examples of IVH include micro/macro- angiopathic haemolysis, cold antibody haemolysis. The inherited haemolytic anaemias comprise of three categories: a) Red cell membrane disorders b) Red cell enzyme disorders c) Haemoglobinopathies (Hoffbrand *et al*., 2005).

#### 1.4.1 Red Cell membrane disorders

There are 2 common red cell membrane disorders namely, hereditary spherocytosis (HS) and hereditary elliptocytosis (HE) (Hoffbrand *et al*., 2005).

HS is a common haemolytic anaemia caused by defects in the proteins involved in the interaction between the membrane skeleton and the lipid bilayer of the red cell. The loss of membrane may be caused by the disintergration of parts of the lipid layer that are not supported by the skeleton. The flexible nature of the red cell is owed to its biconcave shape. In HS the red cells undergo membrane loss and become progressively spherical and rigid as they circulate through the spleen and the rest of the reticuloendothelial system. Eventually the red cells are unable to pass through the splenic circulation where they are taken up by the macrophages. HS patients present with the classical triad of

anaemia, jaundice and splenomegaly. The peripheral blood film shows spherocytes which are round, fully haemoglonised red cells, without the central region of pallor (Hoffbrand *et al*., 2004).

Similar clinical and laboratory features are encountered in HE except for the morphology of the red cells that are characteristically elliptical in shape. However, HE is a much milder disorder than HS (Hoffbrand *et al*., 2005).

### 1.4.2 Red Cell Enzyme disorders

Several enzyme deficiencies have been described, the common ones being glucose 6 phosphate dehydrogenase (G6PD) deficiency and pyruvate kinase (PK) deficiency. The function of G6PD is to reduce NADP to NADPH (nicotinamide adenine dinucleotide phosphate) and in the process oxidise glucose-6-phosphate. It is the only source of NADPH in red cells, therefore a deficiency of reduced glutathione renders the red cell prone to oxidant injury. Furthemore, there are a wide variety of normal genetic variants of the enzyme, the most common being the type A (African) and type B (Western). The main clinical feature is episodic haemolysis in response to oxidant drugs, fava beans or infections. Rarely patients may suffer from chronic haemolysis in more severe cases. The blood film may usually show bite/ blister cells as a result of "pitting" of Heinz bodies by splenic macrophages (Beutler 1994).

In PK deficiency, there is reduced ATP formation causing a net efflux of $K^+$ and resultant water loss. The red cells become dehydrated and rigid. The blood film shows

poikilocytosis and distorted prickle cells. The severity of the anaemia is variable but is generally mild to moderate. Inheritance is autosomal recessive (Hoffbrand *et al*., 2005).

### 1.4.3 Haemoglobinopathies

Haemoglobinopathies refer to a diverse group of disorders caused by disruption of the expression of the globin gene. Haemoglobinopathies are the commonest single gene disorder and occur at high frequencies in tropical and sub-tropical regions where malaria is endemic (Flint *et al.*, 1993).

The genetic disorders of Haemoglobin can be classified into two groups.

a) Haemoglobin variants: characterized by structural alteration in one of the globin chains.
b) The Thalassaemias: characterized by partial or complete failure of synthesis of the globin chains which result in an excess of globin chains that are normally produced.

### 1.4.4 Haemoglobin Variants

The most common and important Haemoglobin variants are Haemoglobin S, Haemoglobin C (Hb C) and Haemoglobin E (Hb E). It has been suggested that these variant haemoglobins provide protection against Plasmodium Malaria (Weatherall *et al*., 1974).

**a) Sickle Cell Disorders**

Sickle cell disorders refer to a group of haemoglobin disorders in which the sickle β globin gene is inherited. The sickle β globin abnormality is caused by substitution of valine for glutamic acid at position 6 in the β chain (Hoffbrand *et al*., 2005). Sickling disorders include heterozygous states for Haemoglobin S (sickle cell trait), homozygous states (sickle cell disease), compound heterozygous states for HbS with other haemoglobin variants e.g. Hb C, D, E or co-inheritance with thalassaemic disorders. Hb S (Hb α2 βS2) is insoluble when exposed to low oxygen tension and polymerises into long fibres and tactoids each consisting of seven intertwined double strands that are cross linked (Hoffbrand *et al*., 2005). Elongation of sickle tactoids forces the red cells to sickle which is responsible for blocking different areas of the microcirculation (Weatherall 2001). The disease prevalence has reached polymorphic frequencies in sub Saharan Africa because of the protection against malaria that is afforded by the carrier state (Nagel and Fleming 1992).

**b) Haemoglobin C**

The genetic defect of Haemoglobin C is commonly found in West Africa and is caused by substitution of lysine for glutamic acid in the β- globin chain at position 6 of the β globin chain (Hoffbrand 2004). Hb C is less soluble than Hb A and precipitates to form rhomboidal crystals. In Hb C disease there is a net efflux of $K^+$ followed by water from the red cells. The ensuing dehydration and rigidity causes haemolyis and increased red cell turnover (Weatherall 2001). The homozygous state for Hb C is characterized by a mild haemolytic anemia associated with moderate splenomegaly. The haematological

changes are characterized by a moderate reduction in the Hb level, a reticulocytosis and a characteristic appearance of the peripheral blood film, which shows large numbers of target cells and intracellular crystals. This variant migrates in the same position as Hb A2 at alkaline pH electrophoresis but is separated by acid pH electrophoresis, iso-electric focusing or high performance liquid chromatography.

**c) Haemoglobin E (Hb E)**

Hb E is very common in South East Asia and is found locally in South Africa amongst the Cape Malay Coloured community (Flatz 1967). In the homozygous states there is a mild hypochromic microcytic anaemia. When Hb E is inherited with $\beta^0$ thalassaemia, there is a marked deficiency of β chain production. These patients usually present with severe anaemia and splenomegaly resembling homozygous β thalassaemia (Weatherall, 2001).

## 1.5 The Thalassaemias

### 1.5.1 Definition and classification

Thalassaemias are one of the most common single-gene disorders in the world population. They are defined as a heterogeneous group of inherited disorders of Hb synthesis, characterized by the absent or reduced synthesis of one or more of the globin chains. They can be classified in several ways: 1) Clinical classification that simply describes the degree of severity. 2) The globin chain that is synthesized at a reduced rate, which may be further classified according to the particular mutation/deletion that is responsible for the defective globin chain synthesis (Sankaran and Nathan 2010).

The human Hb molecule is a tetramer composed of two α and two non-alpha globin chains. In the α thalassaemias, there is a relative excess of β globin chains that have the ability to form tetramers (β4) called Hb H. Hb H although more stable than the β monomer, has a shortened life span due to moderate instability.

In β-thalassamias, impaired production of β-globin chains result in unpaired α-globin chains, which are unstable where they precipitate and cause membrane injury which may lead to toxicity and death of the cells, thus causing ineffective erythropoiesis (Sankaran and Nathan 2010).

### 1.5.2 Clinical classification

Thalassaemias may be divided into three broad categories:

i) Transfusion dependant – e.g. Homozygous β thalassaemia, where patients receive transfusions at regular intervals.

ii) Thalassaemia intermedia e.g. Hb H disease, here patients are anaemic but transfusion independent. However they are not free from long term complications of haemolysis

iii) Heterozygous or carrier states, which are asymptomatic and do not suffer any clinical sequalae.

### 1.5.3 Genetic classification

Classification is based on the globin gene affected with decreased/absent output. The most common types of thalassaemia encountered in adults are α and β thalassaemia followed by δβ thalassaemia and HPFH. The α globin cluster is situated on chromosome 16 and the β globin cluster on chromosome 11 (Weatherall 2001).

Microcytosis with or without red cell hypochromia is a common abnormality detected on a full blood count which prompts clinicians to investigate further in search for a cause. In the absence of iron deficiency and chronic disorder, a microcytosis may indicate a β thalassaemia trait or a deletion of one or two α globin genes (α thalassaemia trait) and is often followed by a request from clinicians for a haemoglobinopathy screen. Since a full blood count include parameters such as the mean cell volume (MCV) and red cell count (RCC), several formulae and ratios have been proposed using erythrocyte indices from a routine full blood count (Narchi and Basak 2010). These include the red cell distribution width (RDW), the RDW index (MCV X RDW/RBC), the Mentzer index (MCV/RBC), the Green and King index ($MCV^2$ X RDW/(Hb X 100), the England and Frazer index (MCV-(5 X Hb)+ RBC + 3.4), the Shine Lal index ($MCV^2$ X MCH/100) and the Srivasta index (MCH/RBC). Although these ratios are useful in distinguishing α thalassaemia trait from iron deficiency they are not absolute and do not distinguish between α and β thalassaemia.

### 1.6 β Thalassaemia

The β thalassaemias are a markedly heterogenous group of autosomal recessive disorders resulting from reduced ($\beta^{+}$) or absent ($\beta^{0}$) production of the β-globin chains (Cao *et al*., 2002). The shortage of β chains results in an excess of unpaired α-chains, which precipitate and damage membrane of red cell precursors, causing destruction of the precursors. Three different clinical and haematological conditions are described, the β-thalassaemia carrier state, thalassaemia intermedia and thalassaemia major.

The β-thalassaemias have a high frequency in the Mediterranean area, the Middle East, the Far East and East Asia. A relatively high incidence is also observed in people of African origin (Cao *et al*., 2002). The β thalassaemia carrier state is clinically asymptomatic. Haematologically it is characterized by microcytosis, a reduced mean cell volume (MCV), a reduced haemoglobin content per red cell (MCH), and an increased percentage of haemoglobin A2 (greater than 3,5%) (Cao *et al*., 2002).

In thalassaemia intermedia, the patients in this group can sustain a haemoglobin level without the need for transfusion therapy and present later in life.

Thalassaemia major is characterized by severe anemia and extramedullary haemopoiesis with resultant spleen and liver enlargement, characteristic skeletal abnormalities results from expansion of the bone marrow cavity to compensate for the premature destruction of red blood cell precursors. Hb sub-fraction shows absent or reduced haemoglobin A (0 – 30%) variable haemoglobin A2 (2-5%) and the balance is made up with >70% of Hb F. The management of thalassaemia major entails regular blood transfusion and iron chelation therapy to eliminate the iron overload. Desferrioxamine poses a challenge for

adherence as the mode of administration is continuous subcutaneous infusion (Nathan 2010). In addition, treatment should begin at an early age as these patients are transfusion dependant.

### 1.7 Hereditary persistence of fetal haemoglobin (HPFH)

This is a group of conditions in which there is a persistence of foetal haemoglobin production in adult life with no haematological abnormalities. Homozygotes are clinically asymptomatic and have a mild thalassaemic like picture. Hb F constitutes 100% of total Hb in homozygotes, and 20-30% in heterozygotes (Weatherall 2001).

### 1.8 α Thalassaemia

Alpha thalassaemias may be divided into three main categories, the inherited α thalassaemias, α thalassaemia associated with mental retardation and acquired haemoglobin H disease (Alli 2005).

#### 1.8.1 Inherited α thalassaemia

Under normal conditions there are four α-globin genes. Deletions or mutations affecting one or more α-globin genes, results in decreased or absent production of the α-globin chain, giving rise to α thalassaemia. The clinical severity of the disease depends on the number of α-globin genes involved. Individuals with the loss of either one or two α globin genes (either on the same chromosome or one on each of the two chromosomes) are usually asymptomatic. Those with the loss of function of three genes (HbH disease, β4) is characterized by hypochromia, microcytosis and haemolytic anaemia usually that is

transfusion dependant but frequently associated with hepatosplenomegaly and mild jaundice (Laosombat 2009).

Complete deletion of four alpha genes (Hb Barts - hydrops fetalis), is the most severe form of α thalassaemia and is incompatible with life since the affected fetus is unable to produce any alpha globin chains to make HbF or HbA. The clinical picture is characterized by severe anaemia, marked hepatosplenomegaly and cardiac failure, and the affected fetus is either stillborn or dies soon after birth (Galanello and Cao 2011).

### 1.8.2 α thalassaemia mental retardation syndrome

Several cases of mental retardation associated with the phenotype of HbH has been described (Gibbons *et al*., 1991). Unusual features were noted about this form of α thalassaemia. Firstly the patients were Caucasian in origin, and secondly one parent showed evidence of a mild form of α thalassaemia whereas the other parent was completely normal. As this condition was unlike inherited HbH disease it was suggested that these patients might have acquired a *de novo* mutation, which had occurred in the germ cells of one of the parents and which was responsible for both α thalassaemia and mental retardation (Galanello and Cao 2011). Two groups of this syndrome exist:

**a)** ATR-X syndrome, a rare condition that is inherited in X-linked fashion presenting with severe mental retardation and mild α thalassaemia trait (Higgs and Weatherall 2009).

**b)** ATR-16 syndrome, is characterized by large chromosomal rearrangements that remove many genes from the short arm of chromosome 16 including the α globin cluster (Higgs and Gibbons 2010). This condition is associated with less severe physical and intellectual disabilities.

In both (a) and (b) α thalassaemia does not cause clinically significant haemolysis, but presents with a mild hypochromic microcytic morphology, with occasional morphological detection of "golf balls" on the reticulocyte smear that serve as a diagnostic tool.

### 1.8.3 Acquired Haemoglobin H (HbH) disease

Hb H disease has been reported in a number of haematological malignancies which include myelodysplastic syndrome, myeloproliferative disorders, Fanconi's anaemia and erythroleukaemia (Alli 2005). Acquired H disease being most commonly encountered in myelodysplastic syndromes, is referred to a α thalassaemia-myelodysplastic syndrome (ATMDS). The subjects are much older, with a strong male predominance. Most patients present with a severe anaemia that is transfusion dependant. The peripheral blood morphology shows dimorphic red cells (normal and hypochromic red cells) with an abundance of Hb H inclusions on the reticulocyte smear and a fast moving Hb H band on Hb electrophoresis (Alli 2005).

The inherited variety ([--/-α]) represents by far the commonest form of Hb H disease where affected individuals suffer from lifelong but usually fairly well compensated

haemolysis. Alpha thalassaemia trait ([--/αα], [-α/αα] or [-α/-α]) poses no significant clinical problem and is usually identified serendipitously through detection of microcytic red cell indices (sometimes with a mild anaemia), during family studies, or population screening. Hb H disease affects individuals throughout Southeast Asia, Mediterranean islands and some areas of the Middle East (Kan *et al*., 1977; Embury *et al*., 1979), but is rare among individuals of African descent owing to the rarity of the $\alpha^0$ mutation (Higgs *et al*., 1979; Felice *et al*., 1984).

Although Hb H disease and the β thalassaemias (homozygous as well as heterozygous) is readily detected by Hb electrophoresis, α thalassaemia trait is not. Occasional Hb H inclusions (seen on incubated reticulocyte preparations) may be seen in some cases with 2 α gene deletions, but the majority of α thalassaemia trait patients remain undetected.

A previous study, on alpha thalassaemia, in the South African population described the profile of RFLP'S (restriction fragment length polymorphisms) associated the α–and β-globin gene clusters, and used these to estimate the frequency of α thalassaemia in the South African population (Ramsay 1986). In addition Krause *et al.,* in 1994 carried out a population based survey and characterised the α and the β- globin gene clusters in the South African Asian population (Krause 1994). However, neither of the two studies addressed the question of unexplained microcytosis and α thalassaemia.

Chong *et al*., (2000), described seven common deletions $\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{MED}$, $-\alpha^{20.5}$, $--^{SEA,\ --Thai,}$ and $--^{FIL}$. Although the gold standard used for the identification of α globin gene deletions has been done using the Southern blot analysis (Southern 1975), there are disadvantages using this technique, as it is time consuming and there are difficulties in setting up the technique.

Cage Johnson's study on alpha thalassaemia in American black subjects found the frequency of α thalassaemia-2 (single gene deletion) to be 0.07 (Johnson *et al*., 1982). Therefore 13% of the population are carriers of α thalassaemia-2 trait, and 0.5% are expected to be homozygous for this mutation. Dozy *et al.*, (1979), reported a frequency of α thalassaemia-2 to be 27,5% using the method of restriction endonuclease mapping. This finding agrees with that of Pierce *et al.*, (1977), who detected a high incidence of α thalassaemia in black recruits by globin chain synthesis studies.

Double heterozygosity with a non-deletion mutant, particularly Hb Constant Spring and Hb G Philadelphia, is a fairly common variant of the disease (Clegg and Weatherall 1974; Reider 1976). Deletions or mutations have also been reported in North America (Bergeron *et a*l., 2005) and Brazil (Borges *et al*., 2001), however the clinical phenotype remains asymptomatic for the one or two α gene deletion types.

With recent advances in molecular techniques particularly polymerase chain reaction (PCR), it is now possible to detect the seven most common α thalassaemia deletions in a

single tube, single tube multiplex PCR reaction (Chong *et al*., 2000); (Tan *et al*., 2001). This method has been shown to amplify across junction fragments for the following deletions: $-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{MED}$, $-\alpha^{20.5}$, $--^{SEA,\ --Thai,}$ and $--^{FIL}$. Although the distribution of haemoglobinopathies and α thalassaemia overlaps the geographic distribution of malaria, it must be noted that such disorders are increasingly encountered in previously non-endemic areas as a result of population movement (Higgs and Weatherall 2009). Since it has been reported in several studies that there is a high incidence of α thalassaemia in blacks, it may be reasonable to suggest that the major cause of the unexplained microcytosis that is presently observed in our laboratory setting may be due to α thalassaemia trait. Patients in this subgroup are clinically asymptomatic and require no specific treatment. However, these patients are often subjected to fairly extensive investigations and iron supplementation is administered although presenting with normal iron levels. This may result in complications associated with iron overload. It is therefore vital to investigate the prevalence of α thalassaemia trait in the South African population with unexplained microcytosis.

# CHAPTER 2

# AIM

## 2.1 Aim

The aim of this study:

- To investigate a possible α thalassaemia trait in South African subjects with unexplained microcytosis using a multiplex PCR assay.

# CHAPTER 3

# STUDY DESIGN

### 3.1 Study Design

Currently the diagnosis of haemoglobinopathy is carried out in several steps, ranging from basic haematological testing to DNA analysis. The main screening tool relies on the detection of haematological indices which are determined by an automated full blood count analyser, haemoglobin chemical and structural determination by HPLC and cellulose and citrate agar Hb electrophoresis. Moreover, while the common abnormal haemoglobin molecule can be characterized using these methods, definitive identification can be only achieved by DNA analysis or gene sequencing.

There are a number of techniques that are available to amplify DNA, such as beta Q replicase, transcription- mediated amplification, strand displacement amplification and the polymerase chain reaction (PCR).The latter method is the most successful and most widely used technique to achieve DNA amplification (Ross 1996).

The polymerase chain reaction (PCR) is a three step process, referred to as a cycle, that is repeated a specified number of times. One PCR cycle consists of the following steps, denaturation, annealing and extension. This process takes place in a thermal cycler, an instrument that automatically controls and alternates the temperatures for programmed periods of time for the appropriate number of PCR cycles usually between 30 to 40 cycles. Almost all the methods for DNA analysis of the haemoglobinopathies used today are based on PCR.

The PCR based techniques used in haemoglobin diagnostics include allele-specific oligonucleotide (ASO), hybridization or dot-blot analysis, reverse dot-blot analysis, allele-specific priming or refractory mutation system (ARMS), restriction enzyme analysis, amplification created restriction analysis, mutagenitically separated PCR and GAP PCR. GAP PCR uses specific primers to flank the deletion breakpoints and detects deletions associated with α thalassaemia. GAP PCR combined with the methodology of multiplex PCR enables the amplification of two or more products in parallel in a single reaction tube.

Although Southern blotting is probably one of the few non-PCR based molecular techniques that still has a significant role to play in the molecular diagnosis of haemoglobinapathies (Southern 1975), PCR methods have proven to be simple sensitive and specific for the diagnosis of α thalassaemia. Yet it is considered difficult to achieve reliable reproducible results with a single tube multiplex PCR assay. Single tube multiplex assays are less laborious, less expensive and more rapid. Therefore the single-tube multiplex assay developed by Tan *et al.,* (2001), was successfully used to diagnose the seven most widely occurring α globin gene deletions. This method has been shown to reliably amplify across junction fragments for the following deletions: $-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{MED}$, $-\alpha^{20.5}$, $--^{SEA,}$ $--^{Thai,}$ and $--^{FIL}$. The sequence homology between the α1 and α2 genes and the high GC concentration of the α globin gene locus had previously made PCR amplification of this region difficult with problems of reliability and reproducibility. However, these have now been overcome by the use of a multiplex PCR kit that contains HotStarTaq polymerase, an innovative PCR additive that facilitates amplification of

difficult regions by modifying the melting behaviour of DNA and a buffer that contains the synthetic factor MP, which allows efficient primer annealing and extension irrespective of primer sequence (Qiagen Multiplex PCR Handbook, 2008). Amplification of the α2 gene, totally or partially removed by all the above 7 deletions, is used to indicate heterozygosity, and primers for the LIS1 gene acts as a control (internal control) for amplification within each individual PCR reaction. As shown in Figure 1, a PCR gel, highlighting a control set.

Therefore, the most logical method to determine the cause of unexplained microcytosis seen in these patients was to first study the haematological indices, iron studies, Hb electrophoresis, HPLC and then to screen for the seven common deletions by performing a multiplex PCR.

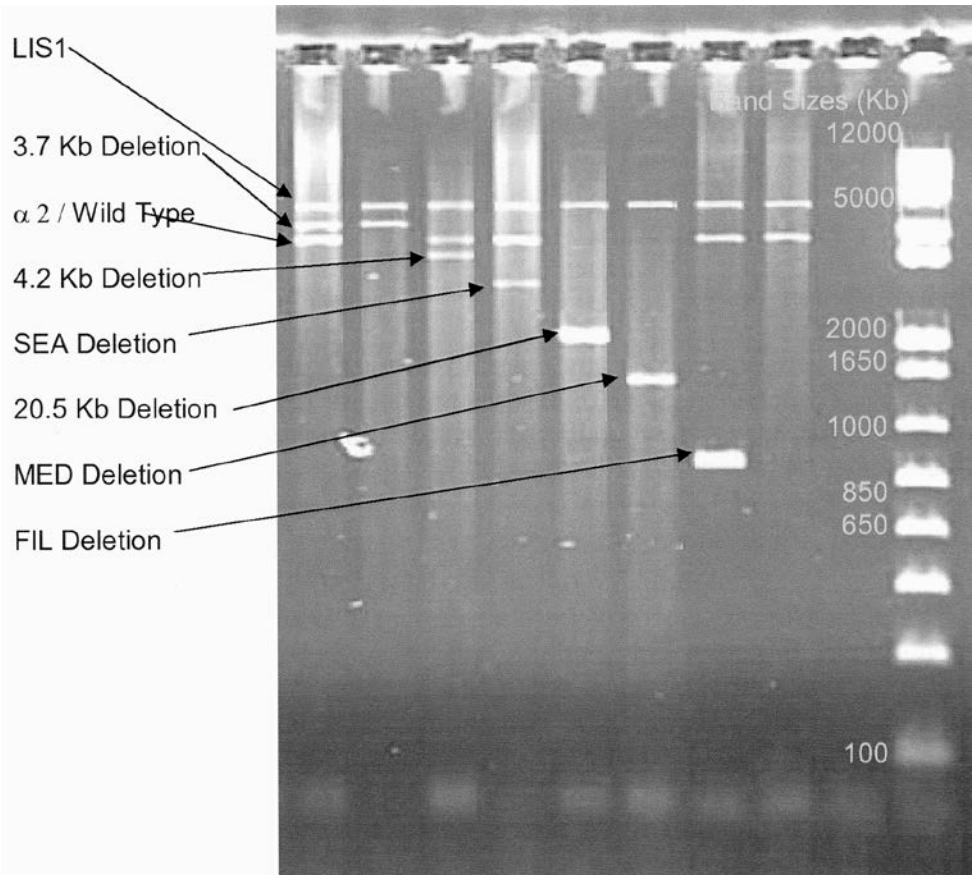



Figure 1: Representation of band patterns for single tube multiplex PCR of the common α thalassaemia deletions. 1.0 X TBE, 1.5 % agarose.

3.7/ 4.2 = $-\alpha^{3.7}/-\alpha^{4.2}$

SEA HET = $--^{SEA}/\alpha\alpha$

MED/3.7 = $--^{MED}/-\alpha^{3.7}$

20.5/3.7 = $-\alpha^{20.5}/-\alpha^{3.7}$

NORM = αα/αα

MED HET = $--^{MED}/\alpha\alpha$

3.7 HET = $-\alpha^{3.7}/\alpha\alpha$

# CHAPTER 4

# MATERIALS AND METHODS

## 4.1 MATERIALS

Table 2: List of reagents/ kits used for all analysis and reagents for PCR testing

| Reagents | Supplier |
|---|---|
| Agarose Gel Grade L | Bioline |
| Bovine serum albumin | Promega |
| Ethidium bromide | Merck |
| Fuji film | Kodak |
| High Pure PCR template extraction Kit | Roche Diagnostics |
| HPLC | Biorad |
| HYDRAGEL 7 ACID (E) Hemoglobin (E) | Separation Scientific |
| HYDRAGEL 15 ACID (E) Hemoglobin (E) | Separation Scientific |
| Iron Kit | Roche Diagnostics |
| New Methylene Blue stain | DMP |
| Oligonucleotides as shown in Table 2 | Whitehead Supplier |
| Qiagen Multiplex PCR kit | Southern Cross Technology |
| Wrights stain kit | Siemens |
| GeneRuler 100 bp plus DNA ladder | Inqaba Biosciences |
| | |
| | |
| | |

### 4.2 Sample collection

Patient samples were selected from routine specimens arriving at the Wits academic complex laboratories *viz*, Chris Hani Baragwanath Hospital (CHBH), Charlotte Maxeke Johannesburg (CMJH) and Helen Joseph Hospitals for full blood count analysis. One hundred samples were collected but only 86 fulfilled all of the criteria for unexplained microcytosis. These samples were collected during a period of 36 months. The initial referral of a suspected haemoglobinopathy was received from the physician to CMJH haematology laboratory. After routine screening tests for a haemoglobinopathy, there was a proportion of samples where the microcytosis still remained unexplained. As the routine laboratory investigations yet do not offer tests for α thalassaemia trait, these samples were collected and included in this study. The criteria for inclusion in the study were patients that had a microcytosis (mean corpuscular volume (MCV) <81 fl), with or without hypochromia (mean cell haemoglobin (MCH) < 27 pg), normal/ borderline iron levels (≥10 µmol/l) and low/ normal Hb A2 levels (≤2.5%).

Individual red cell parameters were analysed for sensitivity and specificity. The optimal sensitivity and specificity for analysis in selected parameters were determined using the Youden index. The cut-off values for the MCV, MCH, Green King index and Mentzer index were 81, 27, 65 and 12 respectively.

Control group*:* Adult Control subjects (staff members >18 years) were recruited from Chris Hani Baragwanath Hospitals and Charlotte Maxeke Johannesburg Hospital. Subjects with normal full blood counts and iron profiles were enlisted as the control group.

## 4.3 Method

**Test procedures**:

Venous blood (5 ml EDTA and 5 ml clotted tube) were collected from control subjects and patients for full blood counts, White cell count (WBC), Red cell count (RBC), Haemoglobin (HB), Haematocrit (HCT), Mean cell volume (MCV), Mean cell Haemoglobin (MCH), Mean cell haemoglobin concentration (MCHC), Red cell distribution width (RDW), and Platelet count (PLT), differential counts, (neutrophils, lymphocytes, monocytes, eosinophils and basophils), automated Reticulocytes (RET). Full blood count was obtained from the EDTA blood samples using the Sysmex XE 5000 analyser. Serum iron studies (serum iron, serum ferritin, transferrin concentrations and percentage saturation), separation of the Hb subtypes using High performance liquid chromatography (HPLC) and Haemoglobin electrophoresis with HbA, HbA2 and Hb F quantification was performed using an EDTA blood sample. Genomic DNA was extracted from an EDTA blood sample and multiplex PCR was performed using standard laboratory procedures.

The FBC with differential counts was performed at CHBH, Department of Haematology, HPLC, Hb electrophoresis performed at CMJH Department of Haematology, and iron studies performed at CHBH, Department of Chemical Pathology as per standard laboratory protocols.

**Morphology**:

Blood smears for differential counting and red cell morphology evaluation was prepared and stained using either the Sysmex Automated SP 1000 I (Slide maker and stainer) or manual methods for preparation and staining using the semi automated method utilizing the Hematek slide stainer. Romanowsky stains were used for staining of blood smears. The property of the Romanowsky stain is its ability to make subtle distinctions in the staining of granules differentially depending on the two components namely the azure B and eosin Y. This also enhances the staining of the nucleoli, polychromatic red cells and allows the differentiation between normal granulation of a neutrophil and toxic granulation in a neutrophil (Lewis 2001). The blood film was examined in conjunction with the full blood count and iron studies. The blood count, including Hb and red cell indices, provided valuable information useful in the diagnosis of both α and β thalassaemia, also the interactions with the Hb structural variants. The film examination showed characteristic red cell morphological changes such as microcytosis, hypochromia, as depicted in Figure 2a, Normal peripheral blood film, Figure 2b, Hypochromic microcytic blood film. Other features that can be seen are target cells, sickle cells and rhomboidal shaped cells as seen in Hb C and sickle cells in HbS.

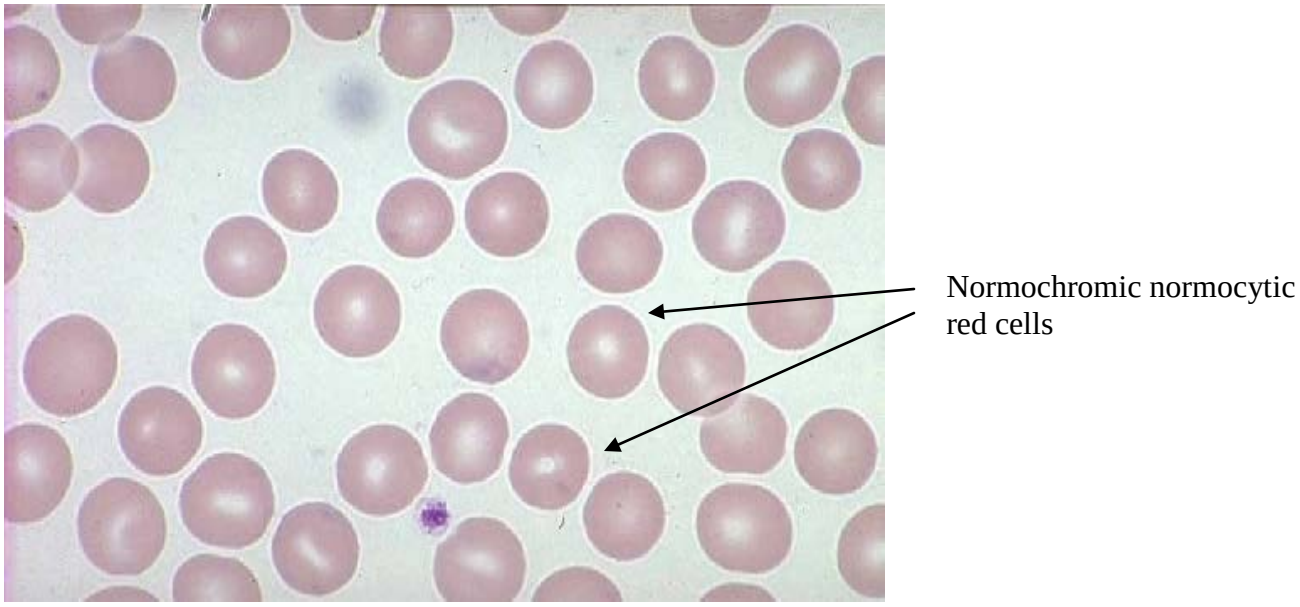



Figure 2a: Normal peripheral blood smear X 100 objective.

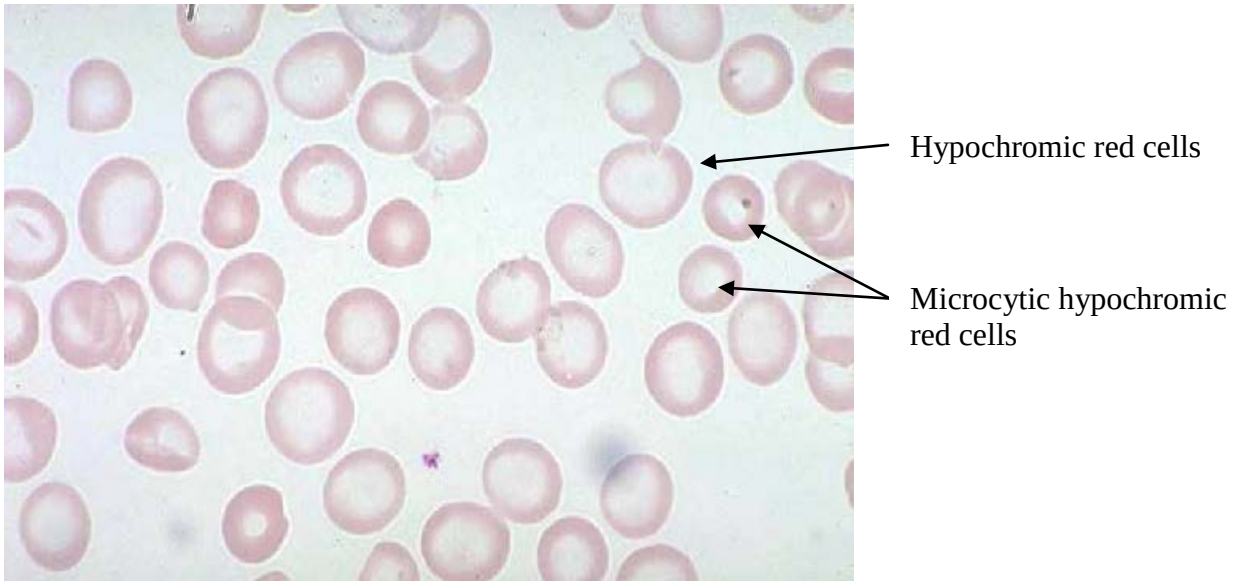



Figure 2b: Hypochromic microcytic blood picture X 100 objective.

**Reticulocyte Count**:

The automated reticulocyte count was obtained from the Sysmex XE 5000 automated Full blood count analyser. In addition a manual Reticulocyte preparation was performed using a supra vital stain (1% Brilliant Cresyl Blue) and examined for HbH inclusion bodies. Equal volumes of blood and stain was incubated at 37 º C for 2 hours. A wedge smear slide was prepared and examined under a 100 X objective (Bain and Bates 2001).

The presence of Hb H inclusions after incubation is an additive screening test for α thalassaemia. The brilliant cresyl blue dye both precipitates and stains β chain tetramers-β4 which are present in a small proportion of α thalassaemia. The precipitated Hb is visible under light microscopy by its "golf ball appearance" as shown in Figure 3.

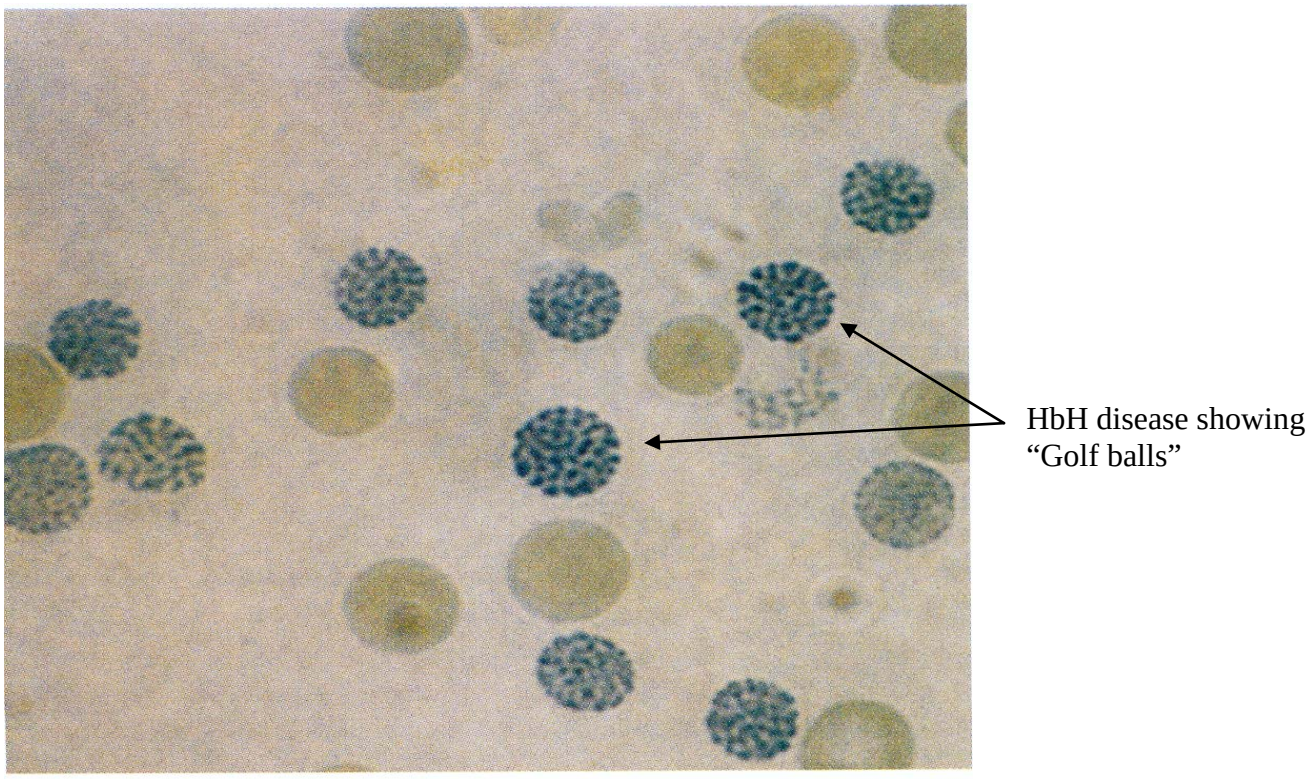



Figure 3: Reticulocyte preparation showing HbH inclusions in a patient presented at CHBH hospital with HbH disease (Golf Balls)

**Iron studies**:

Clotted blood samples were centrifuged at 300 g for 10 minutes and the serum stored at –20°C for iron studies. The iron studies (serum iron, serum ferritin, transferrin and percentage saturation) was performed on the Roche analyser using a combination of methods (NHLS, CHBH, Department of Chemical Pathology). The method for iron estimation is based on the ferrozine method without deproteinization, whereas estimation of transferrin and ferritin is based on an immunological agglutination principle with enhancement of the reaction by latex for ferritin. The percentage saturation was calculated using the serum iron and transferrin levels (Higgins 2006).

**High Performance liquid chromatography (HPLC)**:

Hb subtypes were studied by HPLC using the Bio-Rad D10 machine. In the liquid buffer, the sample was injected and pumped under high pressure through the column where the protein was separated. Protein separation was based on charge and polarity differences leading to exit from the column at different times. These are detected spectrophotometrically and recorded as peaks. Separation of the Hb subtypes (HbA, HbA2, HbF, abnormal Hb) is based on the principle of ion exchange, which separates different proteins based on charge (CMJH, Department of Haematology). The blood samples were brought to room temperature before performing the assay. No sample preparation was required if the EDTA tube was > 2 ml of blood. Samples < 2 ml, were pre-diluted. With 1,5 ml of wash/diluent into a labeled 1,5 ml vial, followed by 5 µl of the well mixed whole blood sample. The sample was capped and mixed well. A representation of the print out result of HPLC as seen in Figure 4.

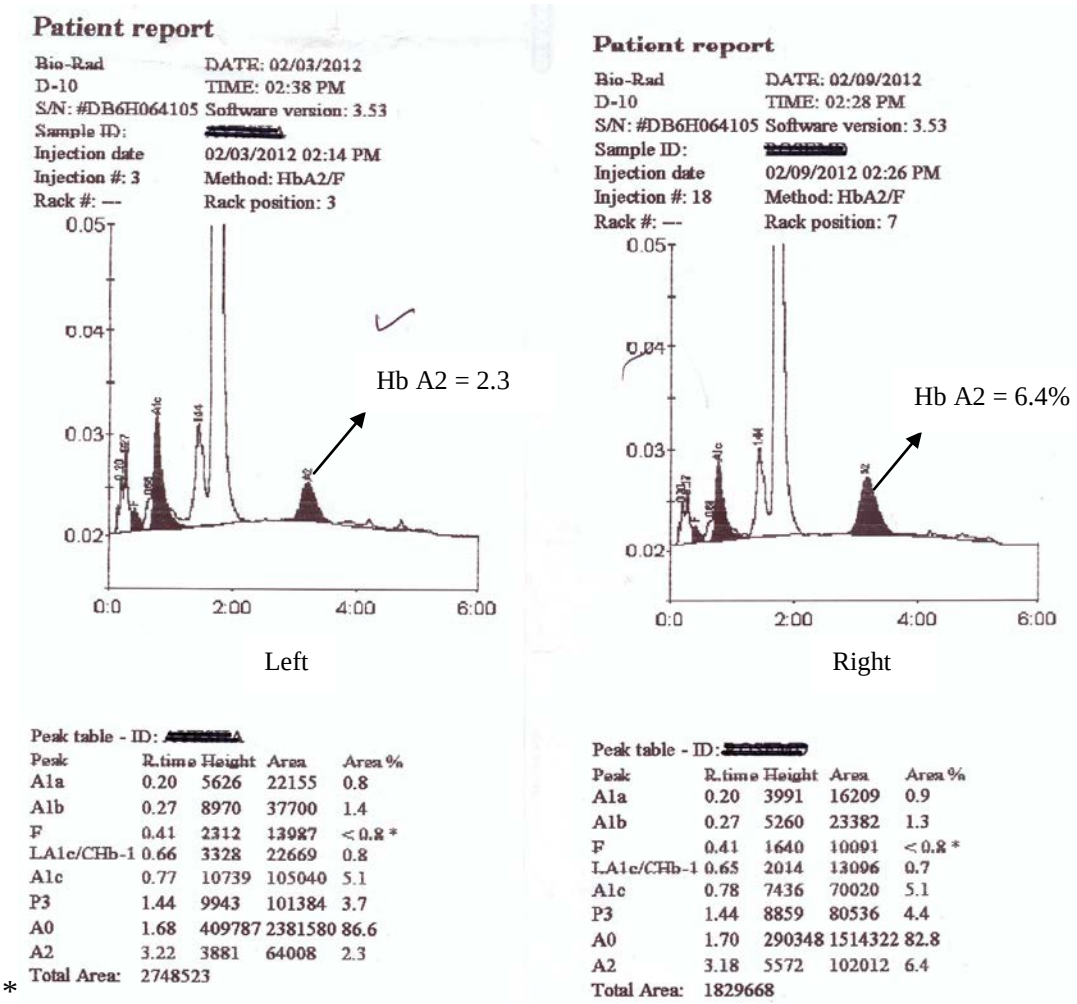



Figure 4: HPLC representation of a sample analysed at the CMJH NHLS Haematology laboratology, showing a normal HbA2 (left) and an abnormal HbA2 result (right).

**Hb electrophoresis** (**Sebia- Norcross, G.A)**

The Hydragel 7 Hemoglobin (E) and Hydragel 15 Hemoglobin (E) kits are designed for the separation of the normal haemoglobins (A, A2 and F) and the detection of the major haemoglobin variants S or D and C and E, by electrophoresis on alkaline agarose gels (pH 8,5). These kits are used in conjunction with the semi-automated HYDRASYS system. The electrophoregrams were visually evaluated. Furthermore, electrophoresis on acidic gels follows to confirm the identification of haemoglobin variants, in particular to differentiate hemoglobin S from D and E from C.

Fresh anticoagulated (EDTA) blood samples were used for analysis. The HYDRASYS is a semi-automated multi-parameter instrument. The automated steps include processing of HYDRAGEL agarose gels in the following sequence: sample application, electrophoretic migration, drying, staining destaining and final drying. Known commercial controls (Haemoglobins A, F, S and C) was included into each analysis processed (alkaline and acid) as shown in Figure 5.

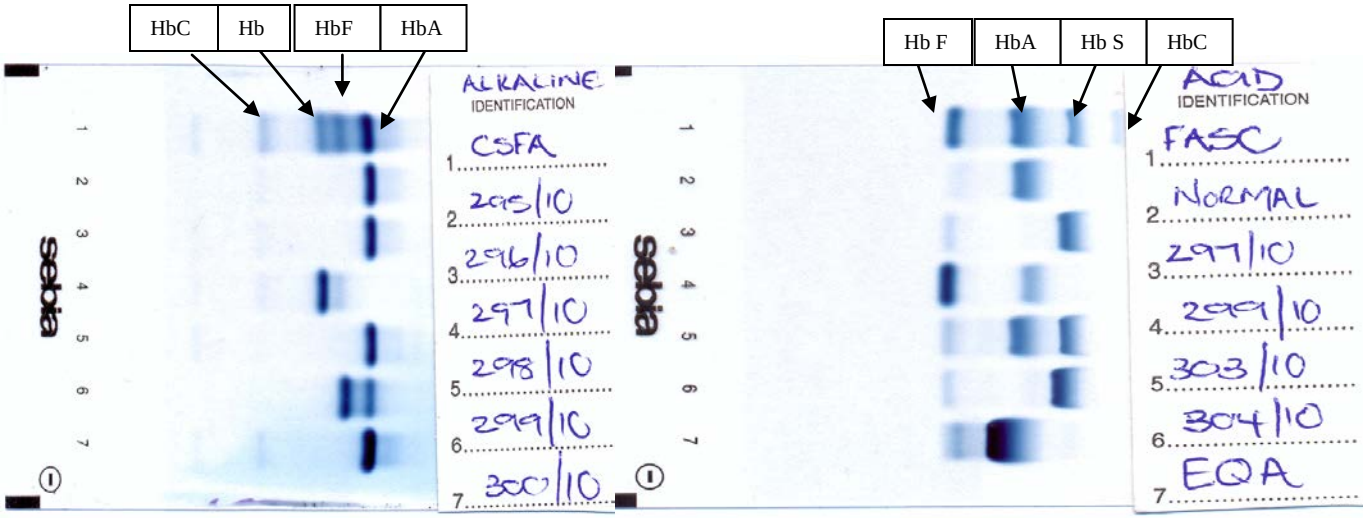



Figure 5: Electrophoregram of an Alkaline (left) and Acid electrophoresis (right)

| KEY | Alkaline gel | Acid gel |
|---|---|---|
| **Commercial control** | Lane 1 | Lane 1 |
| **Normal** | Lane 2 | Lane 2 |
| **Sickle cell disease** | Lane 4 | Lane 3 & 5 |
| **High HbF syndrome** | Lane 6 | Lane 4 |

**Extraction of Genomic DNA:**

**Principle**: DNA was extracted from 5ml EDTA anti-coagulated blood using a High Pure PCR Template preparation Kit (Roche, Switzerland). The blood cells were lysed during a short incubation with the lysing solution. Proteinase K in the presence of a chaotrophic salt (guanidine-HCL) which inactivated all nucleases. Cellular nucleic acid (NA) bound selectively to special glass fibres pre-packed in a high pure purification filter tube. Bound NA was purified in a series of rapid wash and spin steps to remove contaminating cellular components. The DNA was obtained using a low salt elution solution that released the NA from the glass fibre.

To 200 µl of blood sample, 200 µl of binding buffer and 40 µl of Proteinase K was added. Mixed immediately and incubated at 72 º C for 10 minutes. 100 µl of isopropanol was added and mixed well. The sample was pipetted into the upper reservoir of a combined filter tube – collection tube assembly. The sample was centrifuged for 1 min at 8000 rpm in a microfuge. After centrifugation the flow through and collection tube was discarded. The filter tube was attached to a new collection tube and 500 µl of inhibitor removal buffer was added to the upper reservoir. Centrifuged for 1 minute at 8000 rpm. The flow through and collection tube was discarded. The filter tube and the new collection tube was combined. 500 µl wash buffer was added to the upper reservoir. Centrifuged for 1 minute at 8000 rpm. Discarded the flow through and collection tube. This step was repeated twice. The flow through tube was discarded. The filter tube on the same collection tube was centrifuged to remove wash buffer for 1 second at 8000 rpm. The collection tube was discarded. Inserted the filter tube in a sterile 1.5 ml

microfuge tube. Added 100 µl of pre-warmed elution buffer (56°C) to the filter tube. Centrifuged for 1 minute at 8000 rpm. The microfuge tube contained the eluted DNA.

The concentration of the DNA was measured using the Nanodrop-1000, (Applied Biosciences) a full spectrum spectrophotometer that measures 1 µl sample with high accuracy and reproducibility. It utilizes sample retention technology, whereby the sample is pipetted onto the end of a fibre optic cable. A second fiber optic cable is then brought into contact with the sample liquid causing the liquid to bridge the gap between the fibre optic ends. A pulsed xenon flash lamp provided the light source and a spectrophotometer using a linear CCD array was used to analyse the light after passing through the sample. The purity of the DNA sample was assessed from the 260/280 nm ratio. A ratio of ~1,8 was generally accepted as pure for DNA.

Table 3: Oligonucleotide primers for single tube multiplex PCR of common α thalassaemia deletions and PCR fragment sizes.

| | *PRIMER SEQUENCE (5'-3')* | PCR FRAGMENT SIZE (bp) |
|---|---|---|
| LISI-F | *GTCGTCACTGGCAGCGTAGATC* | 2503 |
| LISI-R | *GATTCCAGGTTGTAGACGGACTG* | |
| A 2/3.7-F | *CCCCTCGCCAAGTCCACCC* | 2022/2029 |
| A 2-R | *AGACCAGGAAGGGCCGGTG* | |
| 20.5-F | *GCCCAACATCCGGAGTACATG* | 1007 |
| 3.7/20.5-R | *AAAGCACTCTAGGGTCCAGCG* | |
| 4.2-F | *GGTTTACCCATGTGGTGCCTC* | 1628 |
| 4.2-R | *CCCGTTGGATCTTCTCATTTCCC* | |
| SEA-F | *CGATCTGGGCTCTGTGTTCTC* | 1349 |
| SEA-R | *AGCCCACGTTGTGTTCATGGC* | |
| THAI –F | *GACCATTCCTCAGCGTGGGTG* | 1153 |
| THAI –R | *CAAGTGGGCTGAGCCCTTGAG* | |
| FIL-F | *TGCAAATATGTTTCTCTCATTCTGTG* | 1166 |
| FIL-R | *ATAACCTTTATCTGCCACATGTAGC* | |
| MED-F | *TACCCTTTGCAAGCACACGTAC* | 807 |
| MED-R | *TCAATCTCCGACAGCTCCGAC* | |

**4.4 Preparation of Stock Reagents**

**Oligonucleotides Primers**

All primers were diluted in sterile water to give a working stock of 100µM.

Preparation of primer multiplex mix, diluted all 14 primers according to the Table 4 below.

Table 4: Diluted primers for PCR reaction

| No | Primer Name | Stock µM | Primer Volume µl | Water Volume µl | Final Concentration µM |
|---|---|---|---|---|---|
| 1 | LISF | 100 | 75 | 0 | 100 |
| 2 | LISR | 100 | 75 | 0 | 100 |
| 3 | 4.2F | 100 | 37,5 | 37,5 | 50 |
| 4 | 4.2R | 100 | 37,5 | 37,5 | 50 |
| 5 | All others | 100 | 13 | 52 | 20 |

**Bovine serum albumin (BSA)**

Ten mg/ml stock of BSA was diluted into 1,6 mg/ml concentration. Both the stock and diluted BSA is stored at -20°C.

**Qiagen Multiplex PCR Kit**

A kit contains PCR buffer, sterile water and Q solution. These reagents were stored at -20°C. The multiplex mix was kept on ice for the duration of the PCR preparation.

**GeneRuler 100 bp Plus DNA ladder**

The loading dye is supplied with the DNA molecular marker.

**4.5 Quality Control**

Previously identified samples (obtained from Kings College London) of all deletions were tested with each PCR analysis. A negative sample was also processed with each run and a blank as a control for any reagent contamination. A set of seven positive controls

was obtained from Singapore, compliments from Dr Tan (Department of Paediatrics, National University of Singapore, National University Hospital).

### 4.6 Method Multiplex of Alpha Thalassaemia PCR

On initial set up, each genotype was optimized individually. Subsequently all genotypes was optimized in a multiplex system using the seven sets of primers.

Table 5: PCR Master mix for single tube multiplex PCR of common α thalassaemia deletions.

| Reagent | 1 tube volume μl | Final Concentration μM |
|---|---|---|
| 2 x Qiagen Master mix */** | 25 | 1 x (1,5 mM $MgCl_2$) |
| Muliplex primer mix (As shown in table 4 | 3,5 | 10 |
| BSA | 2,0 | 20 |
| Q Solution | 7,5 | |
| Sterile water | 7,0 | |
| For Blank add purified water/ Patient or control DNA (100 ng/μl) | 5,0 | Not added to master mix |
| Total reaction volume | 50,0 | |

*(Contains :HotStarTaq DNA polymerase, Multiplex PCR Buffer, dNTP mix)

**Also contains 1 mM $MgCl_2$.

The volumes referred to, are for a single PCR reaction. For quality assurance, a positive and negative control, together with a blank control were included in every set of PCR. The conditions of the cycler were programmed as follows. The PCR reaction was initiated at 95 °C for 10 minutes, thereafter 35 cycles consisting of a denaturation step of 45seconds at 97 °C, an annealing step of 90 seconds at 61°C and an elongation step at 72°C for 90 seconds and a final elongation of at 72°C for 5 minutes. A 1.5% agarose gel in TBE buffer was prepared stained with ethidium bromide. Twenty three µl of each PCR reaction together with 5 µl of loading dye (supplied with the DNA marker) were loaded in the wells. Ten µl of 1 kb DNA marker was loaded in a designated well. The gel was placed in an electrophoretic tank containing TBE buffer and was subjected to 100 volts for 90 minutes. The bands were visualized under ultra violet trans-illumination and photographed. The bands were identified using the 1 kb DNA marker and the genotypes identified using the known controls (Chong *et al*., 2000). (as shown in Figures 6, 7, 8, 9 and 10).

**4.7 Statistical Analysis**

Statistical analysis was carried out using the Statististical Analysis System (SAS) software version 9.1 programme. The Wilcoxin two-sample test was used to analyse the haematological and biochemical parameters between the patient and control subjects. The Fisher's Exact test was used to calculate the frequency of the genotypes and the percentage differences in the population groups. In addition the Youden index was used as a measure of the receiver operating characteristic curve (ROC). This measures the effectiveness of a parameter and also allows the selection of an optimal cut-off point.

# CHAPTER 5

# RESULTS

Demographic data of patient and control subjects are detailed in Table 6. All patient and control subjects were adults, with a mean age of 34.5 ± 14.5 and 36.4 ± 12.7 years respectively. The gender split showed a male: female ratios of 0.45 and 0.46 in the patient and control group. Race groups were determined as per hospital classification. In the patient group, Blacks, Indians and Whites comprised 51.1%, 40.7% and 8.1% respectively. There were two Coloured subjects who were incorporated in the Black group for analysis. A similar distribution was noted in the control group where Blacks, Indians and Whites comprised of 54.6%, 28.8% and 16.4% respectively in the control group.

Haematological parameters as detailed in Table 7 were determined on all patient and control subjects. All these parameters *viz*, Hb, MCV, MCH, RDW, HbA2 and as well the Mentzer and Green King indices were significantly lower ($p < 0.05$) in the patient group as compared to the control group.

In this study, 146 subjects were recruited for the control group of which 49 had microcytosis. In the latter group, 26 subjects (17.8%) were confirmed to have iron deficiency and were thus excluded from the control group. The remainder 23 subjects (15.7%) tested positive for PCR and were also excluded from the control group, but were incorporated in the patient group, since they fulfilled the criteria for patient selection using the positive predictive value of the MCV.

Although 61 patients had full iron study profiles, the total iron level was significantly different ($p=0.441$) between the patient and control subjects, these results were within the

normal limits (>10µmol/l). However, the transferrin and ferritin levels were not significantly different (see Table 8).

Table 6: Patients and Controls subject profile of age, sex and race.

| SUBJECT PROFILE | | | |
|---|---|---|---|
| | **Patient (n=86)** | **Control (n=97)** | **Fisher's Exact test** |
| **Age** | 34.5 (±14.5) | 36.4 (±12.7) | 0.1576* |
| **Male** | 34 | 44 | 0.4566 |
| **Female** | 52 | 53 | 0.4566 |
| **Blacks** | 44 | 53 | 0.1079 |
| **Indians** | 35 | 28 | 0.1079 |
| **Whites** | 7 | 16 | 0.1079 |

* P value

Table 7: Haematological results of patient and control group subjects.

| HAEMATOLOGICAL DATA | | | |
|---|---|---|---|
| **Parameter** | **Patients (n=86)** | **Controls (n=97)** | **P value** |
| **Hb (g/l)** | 12.9 ± 2.35 | 14.3 ± 1.28 | <0.0001 |
| **MCV (fl)** | 73.6 ± 7.06 | 87.8 ± 4.53 | <0.0001 |
| **MCH (pg)** | 23.8 ± 3.08 | 30.3 ± 1.88 | <0.0001 |
| **RDW (%)** | 16.1 ± 4.13 | 13.3 ± 0.89 | <0.0001 |
| **HbA2 (%)** | 2.47 ± 0.63 | 2.80 ± 0.67 | 0.0027 |
| **Mentzer Index** | 13.8 ± 3.26 | 18.7 ± 2.43 | <0.0001 |
| **Green King Index** | 70.8 ± 27.44 | 72.4 ± 11.1 | 0.0001 |

Table 8: Biochemical data (iron profile) results of patients and control subjects.

| BIOCHEMICAL DATA | | | |
|---|---|---|---|
| **Parameters** | **Patients (n=61)** | **Control (n=97)** | **P value** |
| **Total Iron (µmol/l)** | 13.96 ± 6.6 | 15.75 ± 5.2 | 0.0441 |
| **Transferrin (g/l)** | 2.87 ± 0.63 | 2.90 ± 0.46 | 0.9758 |
| **Ferritin (µg/l)** | 118.9 ± 182.8 | 98.6 ± 89.0 | 0.8126 |

Multiplex PCR for detecting the seven common deletional α thalassaemia variants was performed in all patient and control subjects (see Figures 6-10). From a total of 86 in the patient group, 64 (78%) tested positive for α thalassaemia PCR. The respective incidences amongst the Black, Indian and White population groups was 80%, 85.2% and 37.5% respectively. There were four indeterminate results which were excluded from analysis. This leaves 18 subjects (22%) of the patient cohort that remain unexplained (see Table 9a). Table 9b), shows the breakdown of control subjects in the respective population groups, and PCR results on control subjects yielded a negative result.

On analysing different ethnic groups, the rate of PCR positivity in Black and Indian patients were similar viz., 76% and 80% respectively. The White patient group showed a positivity rate of only 37.5%. However, here the numbers were too small for meaningful analysis (Table 9a).

The breakdown of the various α thalassaemia genotypes encountered in the study group is detailed in Table 10. The commonest α thalassaemia genotype was $-\alpha^{3.7}$ which accounted for 92.1% wheares the $-\alpha^{4.2}$ (Figure 9) genotype accounted for 4.68% of the total number of positive subjects. In addition two subjects had a double hetrozygous phenotype *viz*., $-\alpha^{3.7}/\alpha^{4.2}$ and $-\alpha^{3.7}/ --^{SEA}$.

The subject with the $-\alpha^{3.7}/ --^{SEA}$ mutation is of Malaysian descent who had HbH disease (Figure 10), and was incorporated into the Indian group for analysis.

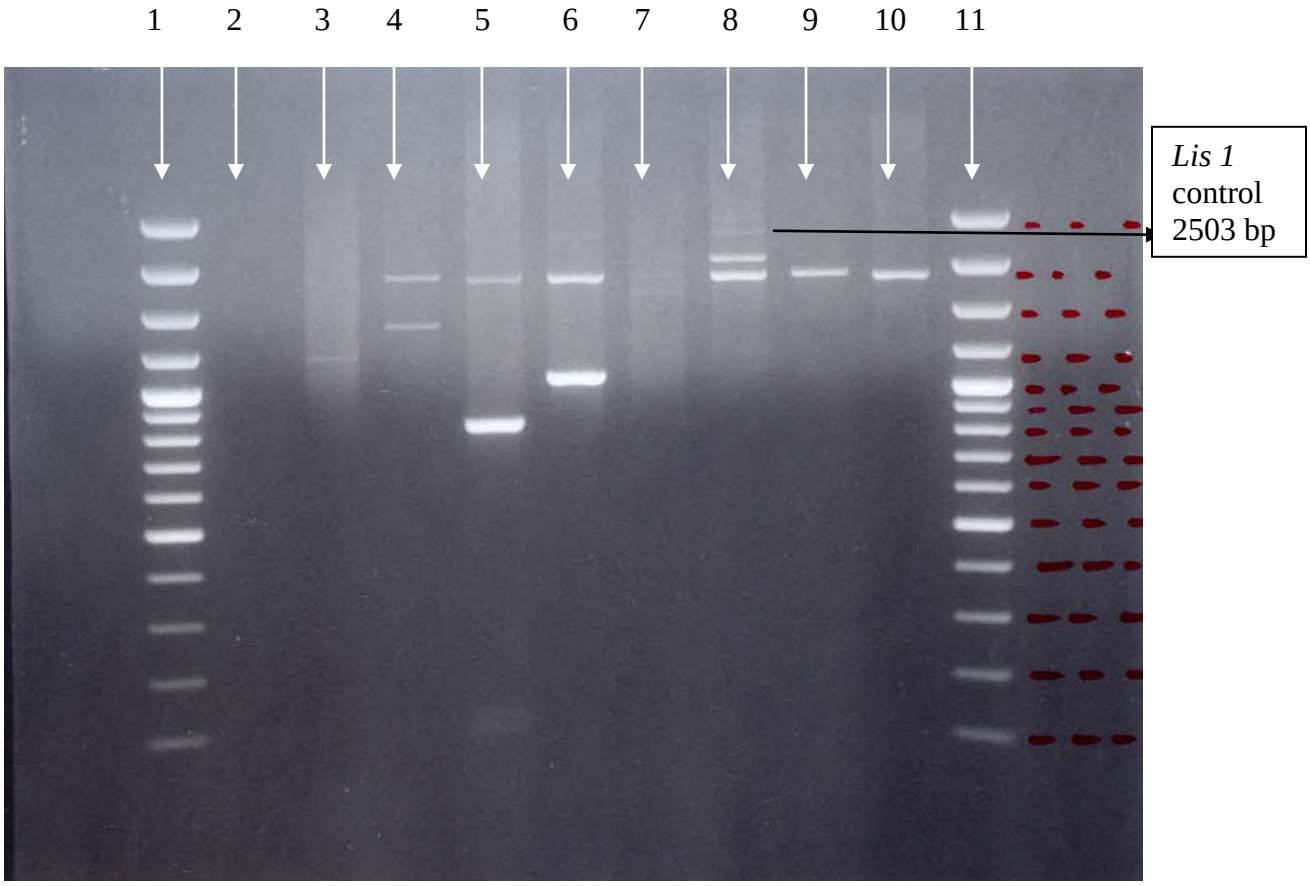



Figure 6: PCR gel with known positive controls showing six common α thalassaemia deletions including αα/-- $^{FIL}$ deletion (lane 3).

| | | | |
|---|---|---|---|
| Lane 1. | GeneRuler 100 bp Plus DNA ladder | Lane 7. | αα/-α $^{4.2}$ deletion (1628 bp) |
| Lane 2. | Blank | Lane 8. | αα/-α $^{3.7}$ deletion (2022 bp) |
| Lane 3. | αα/-- $^{FIL}$ deletion (1166 bp) | Lane 9. | αα/αα normal (Control) |
| Lane 4. | αα/-- $^{SEA}$ deletion (1349 bp) | Lane 10. | αα/αα normal (Control) |
| Lane 5. | αα/-- $^{MED}$ deletion (807 bp) | Lane 11. | GeneRuler 100 bp Plus DNA ladder |
| Lane 6. | αα/-- $^{20.5}$ deletion (1007 bp) | | |

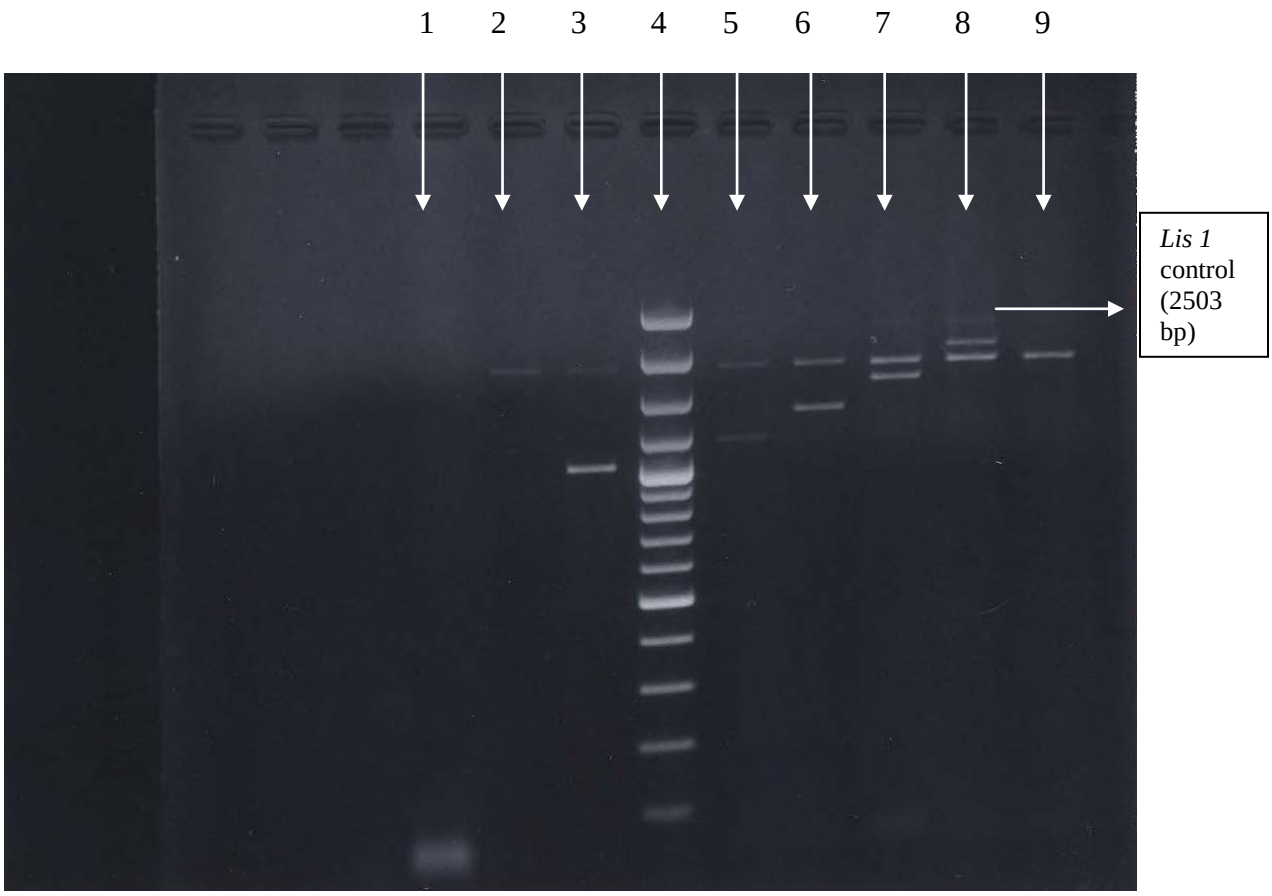



Figure 7: PCR gel with known positive controls showing six common α thalassaemia deletions including αα/--$^{THAI}$ deletion (lane 5).

Lane 1. Blank

Lane 2. αα/αα normal

Lane 3. αα/-- $^{20.5}$ deletion (1007 bp)

Lane 4. GeneRuler 100 bp Plus DNA ladder

Lane 5. αα/--$^{THAI}$ deletion (1153 bp)

Lane 6. αα/-- $^{SEA}$ deletion (1349 bp)

Lane 7. αα/-α $^{4.2}$ deletion (1628 bp)

Lane 8. αα/-α $^{3.7}$ deletion (Control)

Lane 9. αα/αα normal (Control)

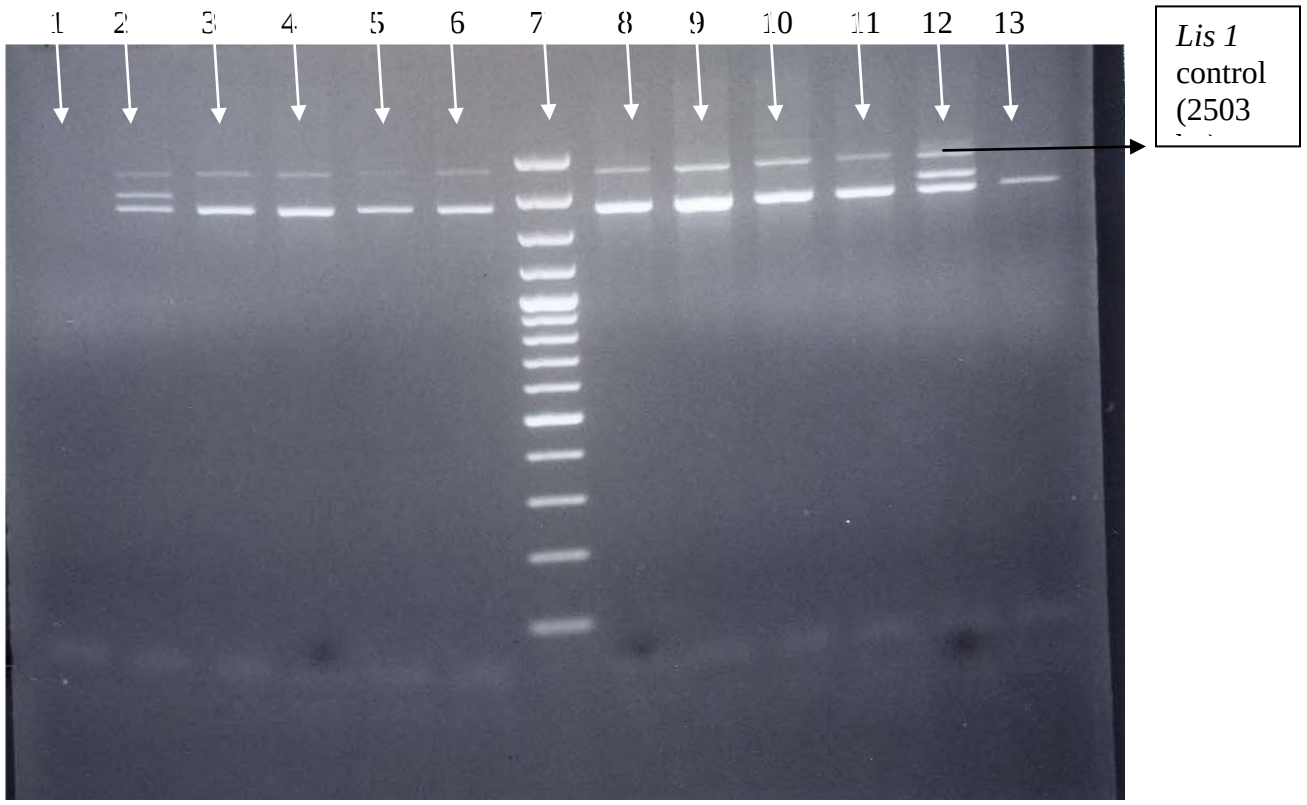



Figure 8 : PCR gel indicating negative PCR analysis of the Control group and -α $^{3.7}$ deletion.

| | | | |
|---|---|---|---|
| Lane 1. | Blank | Lane 7. | GeneRuler 100 bp Plus DNA ladder |
| Lane 2. | αα/-α $^{3.7}$ deletion (Control) | Lane 8. | αα /αα negative |
| Lane 3. | αα /αα negative (Control) | Lane 9. | αα /αα negative |
| Lane 4. | αα /αα negative | Lane 10. | αα /αα negative |
| Lane 5. | αα /αα negative | Lane 11. | αα /αα negative |
| Lane 6. | αα /αα negative | Lane 12. | αα/-α $^{3.7}$ deletion |
| Lane 13. | αα /αα negative | | |

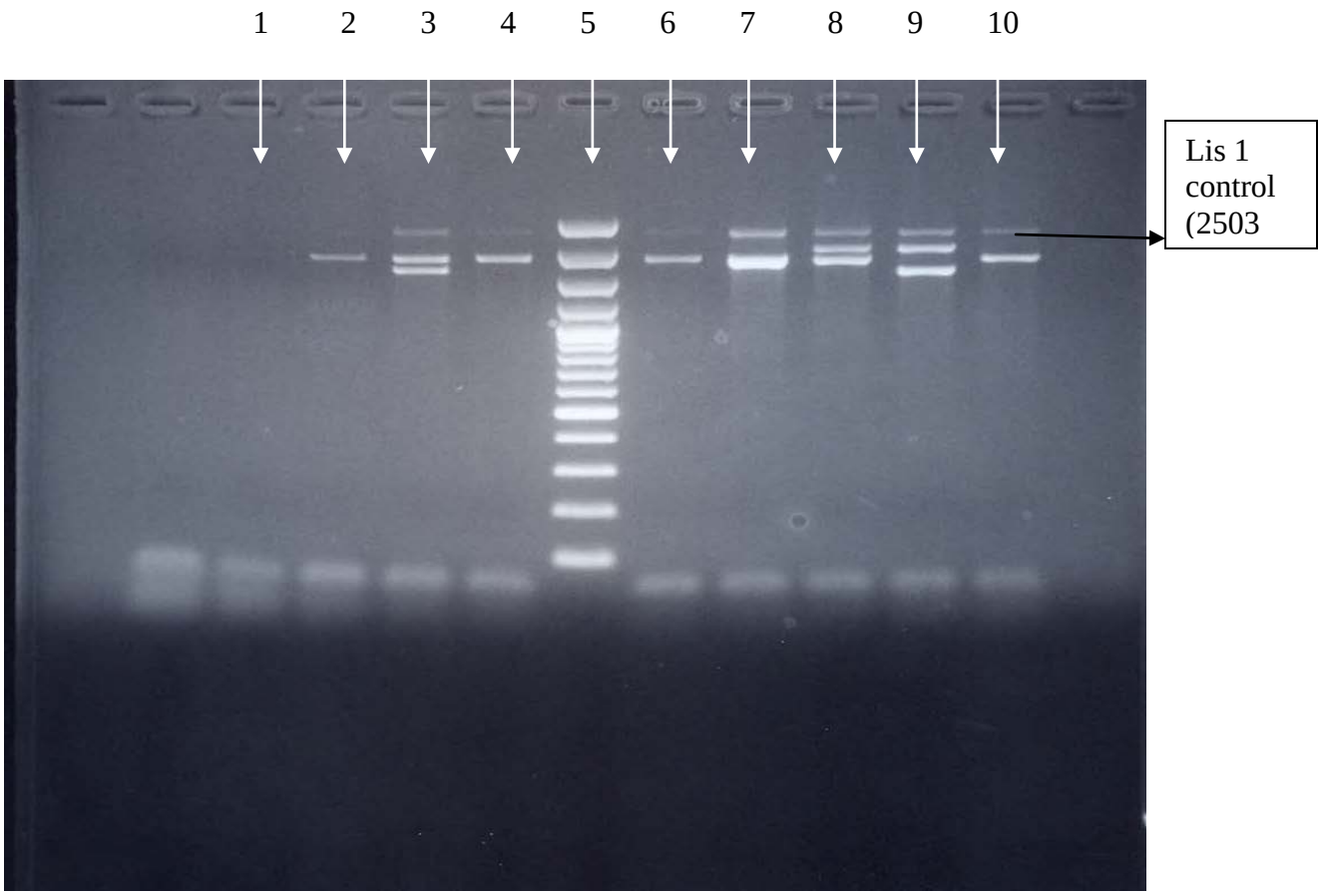



Figure 9: PCR gel showing negative analysis from the Control group and -α $^{3.7}$ and –α $^{4.2}$ deletions from the Patient group.

| | | | |
|---|---|---|---|
| Lane 1. | Blank | Lane 6. | αα/αα negative |
| Lane 2. | αα/αα negative (Control) | Lane 7. | αα/αα negative |
| Lane 3. | αα/-α $^{4.2}$ deletion (Control) | Lane 8. | αα/-α $^{3.7}$ deletion (Control) |
| Lane 4. | αα/αα negative | Lane 9. | -α$^{3.7}$/-α $^{4.2}$ deletion |
| Lane 5. | GeneRuler 100 bp Plus DNA ladder | Lane 10. | αα/αα negative |

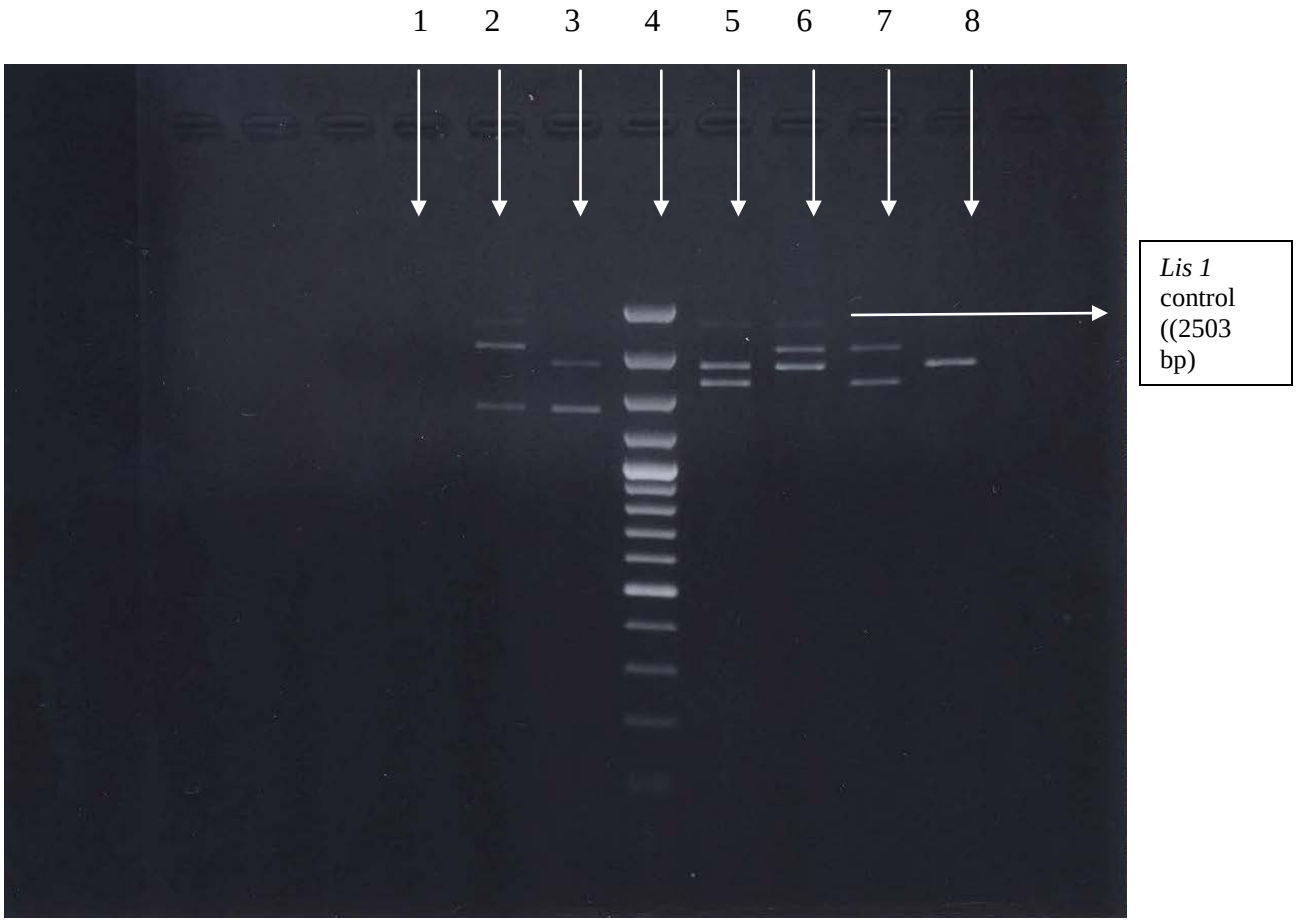



Figure 10: PCR gel showing -α $^{3.7}$, -α $^{4.2}$ and -α $^{SEA}$ deletion from the patient group.

| | | | |
|---|---|---|---|
| Lane 1. | Blank | Lane 5. | αα/-α $^{4.2}$ deletion |
| Lane 2. | -α $^{3.7}$/ --$^{SEA}$ deletion | Lane 6. | αα/-α $^{3.7}$ deletion |
| Lane 3. | --$^{SEA}$ (Control) | Lane 7. | -α$^{3.7}$/-α $^{4.2}$ deletion (Control) |
| Lane 4. | GeneRuler 100 bp plus DNA ladder | Lane 8. | αα/αα negative (Control) |

Table 9a: Breakdown of positive, negative and indeterminate results in each population group.

| Population group | Total patient group | Positive PCR | Negative PCR | Indeterminate PCR |
|---|---|---|---|---|
| **Black** | 42 | 32 | 8 | 2 |
| **Indian** | 36 | 29 | 5 | 2 |
| **White** | 8 | 3 | 5 | 0 |
| **Total number** | 86 | 64 | 18 | 4 |

Table 9b: Negative PCR results in each population group.

| Population group | Total control group | Negative PCR |
|---|---|---|
| **Black** | 53 | 53 |
| **Indian** | 28 | 28 |
| **White** | 16 | 16 |
| **Total number** | 97 | 97 |

Table 10: Alpha globin gene deletion types in the various population groups

| **Population Group** | **Total positives** | **$-\alpha^{3.7}$** | **$-\alpha^{4.2}$** | **$-\alpha^{3.7}/-\alpha^{4.2}$** | **$-\alpha^{3.7}/-\alpha^{SEA}$** |
|---|---|---|---|---|---|
| **Black** | 32 | 30 | 1 | 1 | |
| **Indian** | 29 | 26 | 2 | | 1 |
| **White** | 3 | 3 | | | |
| **Total** | 64 | 59 | 3 | 1 | 1 |

The results of the MCV and MCH were analysed for sensitivity and specificity using the Youden index. The cut off values for the MCV, MCH, Green King index and Mentzer index was 81, 27, 65 and 12 respectively. In addition, ROC curve analysis was performed for these indices (see Figures 11, 12 and 13). Here the MCV was demonstrated to be the most sensitive tool for suspecting α thalassaemia. Using these cut-off values the positive and negative preddctive rates were determined (see Table 11).

Table 11: Positive and negative predictive values for α thalassaemia using MCV, MCH Green king index and Mentzer index.

| | **MCV (fl)** | **MCH (pg)** | **Green King index** | **Mentzer index** |
|---|---|---|---|---|
| **Youden index** | 64.4 | 55.3 | 30 | 42 |
| **Optimal cut-off value** | 81 | 27 | 65 | 12 |
| **Positive predictive value** | 79.6 | 67.1 | 51.5 | 21.8 |
| **Negative predictive value** | 86.0 | 86.0 | 77.3 | 95.6 |

Figure 11: ROC analysis for MCV as a predictor of α thalassaemia trait.

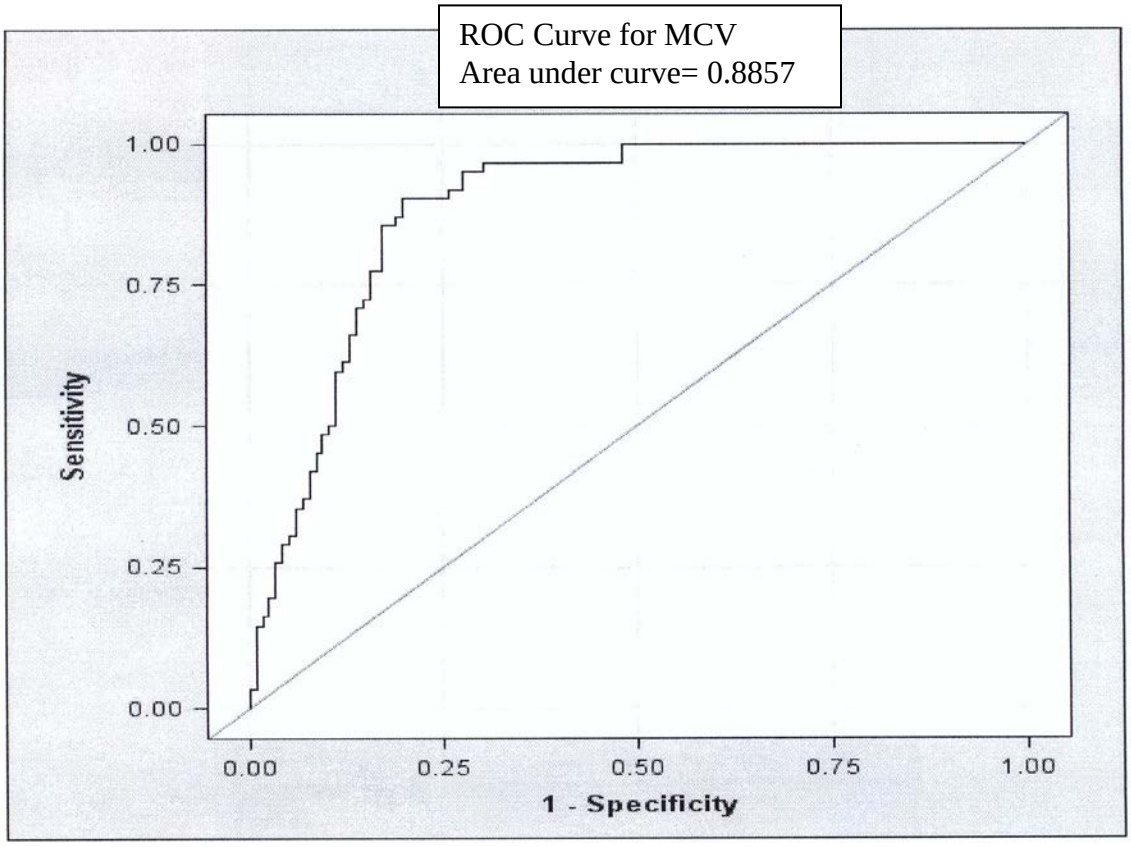



Figure 11: ROC analysis for MCV as a predictor of α thalassaemia trait.

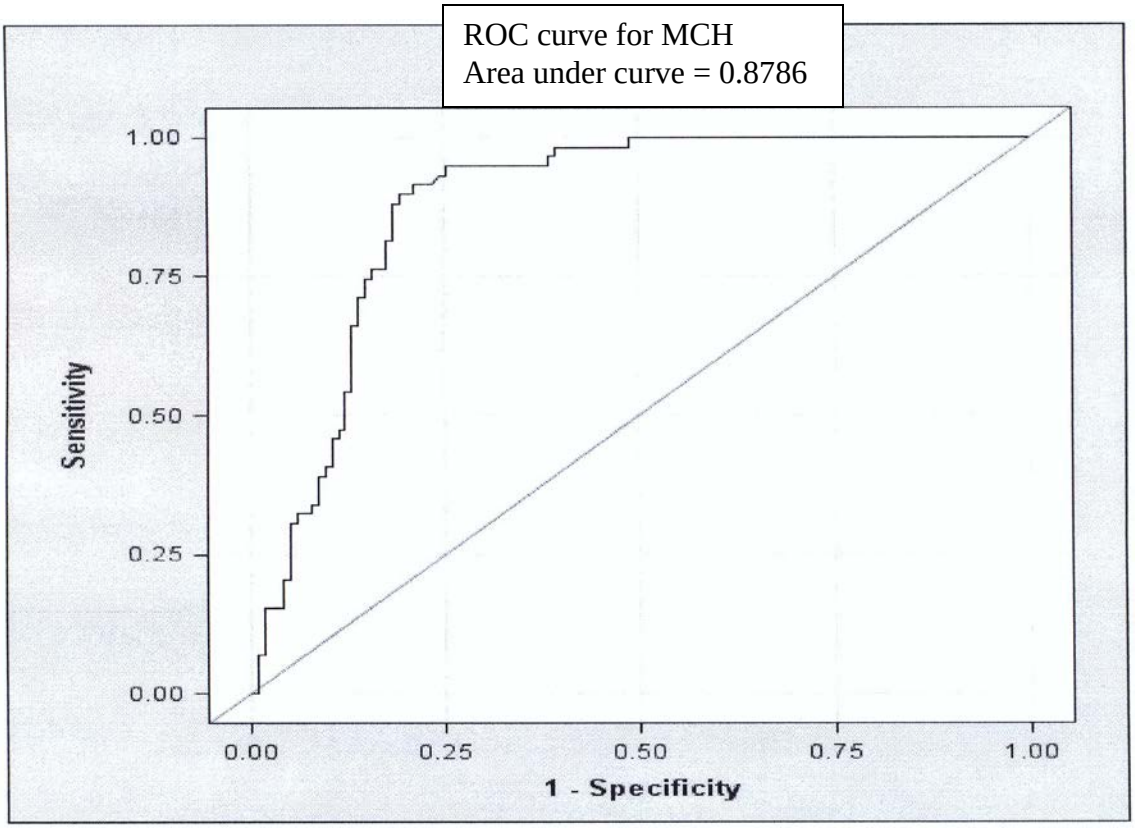



Figure 12: ROC curve analysis for MCH as a predictor of α thalassaemia

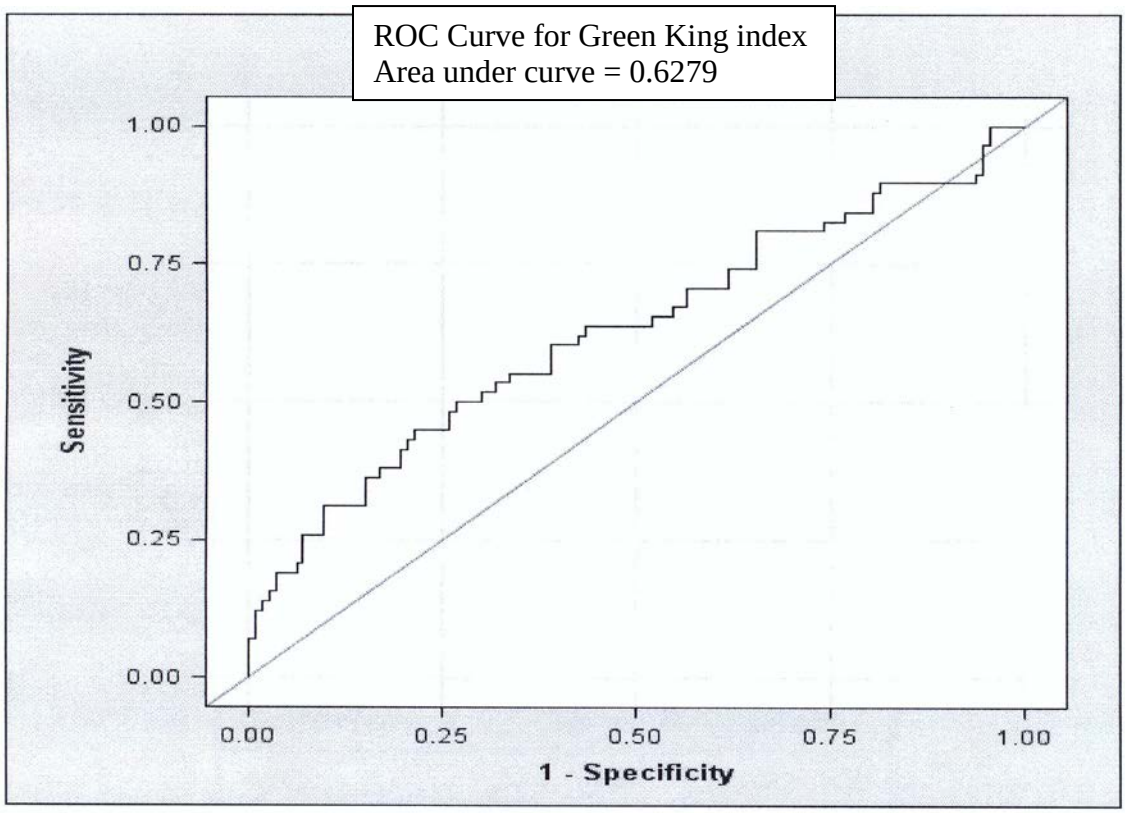



Figure 13: ROC curve analysis for Green king index as a predictor of α thalassaemia trait.

# CHAPTER 6

# DISCUSSION

Differentiating between the causes of microcytosis and hypochromia in iron deficiency and haemoglobinopathy states is obviously important and poses a challenge to clinicians. Not only does an accurate diagnosis allow for adequate management and appropriate counselling (in the case of haemoglobinopathies) but it also has therapeutic implications particularly with respect to unnecessary iron therapy. Mach-Pascual *et al.*, (1996), showed that iron deficiency remains the leading cause of microcytosis, followed by deletional α thalassaemia which is the most frequent haemoglobinopathy globally.

As stated above, microcytic hypochromic anaemia is commonly encountered in the clinical setting, and α thalassaemia has to be considered in the differential diagnosis.
Several studies have been performed worldwide. More than 40 different alpha thalassaemia deletional variants have been described (Cao *et al.*, 2002). The α-thalassaemia phenotypes range from an asymptomatic clinical state to a chronic haemolytic state. The former is due to one or two alpha gene deletions and is detected serendipitously or in the course of family screening, whereas the latter is caused by three gene deletions, also known as HbH disease. Haemolytic anaemia in HbH disease is usually compensated and does not necessitate blood transfusion (Wasi 1983; Galanello 1992). However, during periods of bone marrow stress such as infection, the Hb often drops to a level that demands blood transfusion. A minority may be severely affected that renders them to be transfusion dependant. Regardless of transfusion dependency, these patients have chronic anaemia (and consequent hypoxia). Hypoxia is a potent stimulus for increased iron absorption, which can lead to iron overload in these patients. Such cases need iron chelation therapy to prevent organ damage and dysfunction.

In instances where all four α genes are deleted, anaemia develops after eight weeks of foetal life when production of embryonic Hb ceases. The anaemia is progressive and ultimately leads to cardiac failure and hydrops foetalis.

In the unadjusted control population of 120 subjects, 23 tested positive for alpha thalassaemia. The extrapolated incidence of α thalassaemia in the normal population is calculated at 19.1%. Two additional haemoglobin abnormalities were detected in the control group: a) Two cases of β thalassaemia trait and, b) One case of HPFH who had a HbF level of 30% and a pancellular distribution of HbF.

From a total of 86 in the patient group, 64 (78%) tested positive for α thalassaemia PCR. Four results were indeterminate. This leaves 18 subjects (22%) of the patient cohort that remain unexplained. Possible reasons for a negative PCR result include:

a. Presence of α thalassaemia deletions not tested for such as the αα/-- $^{SA.}$ mutation. This mutation has been described by Vadenplas *et al.,* (1987), in a South African family. As mentioned above, 7 deletional variants were tested for, since they account for the majority of the α thalassaemia mutations. More than 40 deletional variants have been described in the literature.
b. Non-deletional α globin mutations, which are rare in α thalassaemia. Sequencing of α globin genes would be necessary to detect non-deletional alpha thalassaemia mutations as these are not detected by GAP PCR.

It is worthy of mention that of the 18 PCR negative subjects, only 4 did not have an iron profile, precluding the possibility of a deranged iron profile as a major player in causing the microcytosis.

Analysis of all positive PCR results revealed the $-\alpha^{3.7}$ genotype to be the commonest, namely 92.1%. The percentage of the $-\alpha^{3.7}$ genotype was similar amongst the Black and Indian groups viz 80% and 85.2% respectively (refer Table 9). Other alpha genotypes were uncommon, with $-\alpha^{4.2}$ and $--^{SEA}$ constituting 4.68% and 1.56% (single case) respectively. The subject with the $--^{SEA}$ deletion has HbH disease, with HbH inclusions present in over 50% of the red cells. She was of Malaysian origin, which was not surprising as this deletion is found in high frequencies in Southeast Asia (Kattamis *et al*., 1996). No double deletions in cis were encountered in the Black population group, a phenomenon which explains the rarity of HbH disease amongst Blacks.

This pattern of alpha genotype distribution has been mirrored in other studies. Sankar *et al.,* (2006), reported the incidence of $-\alpha^{3.7}$ as the commonest genotype (35.7%) in microcytic hypochromic subjects of Indian descent. Here, other causes of microcytosis such as iron deficiency and β thalassaemia trait were not excluded from the cohort.

A Canadian group, Bergeron *et al.,* (2005), yielded a PCR positive rate of only 24.5% in the unexplained microcytosis cohort. In this study however, 29% of the population were Asian immigrants, with the remainder being of Canadian descent. Of the positive PCR results, $-\alpha^{3.7}$ , $-\alpha^{4.2}$ ,$--^{SEA}$ and $--^{MED}$ comprised 81.25% , 6.25%, 10.4% and 2%

respectively. In both these studies, the $-\alpha^{3.7}$ was to be the commonest mutation causing α thalassaemia as was the finding in our study (59%).

In the subgroup of SS subjects 5 out of the 7 (71.5%) tested positive for α thalassaemia (Steinberg, *et al*., 1983) PCR, which is comparable to the non sickle population viz 74.6%. The beneficial effect of α thalassaemia on sickling disorders has been reported in the literature (Steinberg *et al*., 1983). They have shown that sickle cell disorders in conjunction with homozygous or heterozygous α thalassaemias is associated with higher Hb levels, less haemolysis, fewer episodes of leg ulceration and acute chest syndromes. The intracellular concentration of HbS is decreased in α thalassaemia. The decrease in HbS content leads to an increase in the delay time to sickling, resulting in fewer episodes of sickle crisis (Vasavda *et al*., 2008). In our study, one would expect a higher prevalence of α thalassaemia given the clinical advantage conferred by α thalassaemia. However, the number of SS patients is too small for statistical analysis and a larger series would be required to draw any conclusion to this effect.

In resource poor settings, PCR analysis may prove prohibitive for routine testing. In this vein, the optimal sensitivity and specificity for analysis in selected parameters and formulae were determined using the Youden index. As shown in Table 11 and Figures 11 and 12, the MCV offers the highest positive and negative predictive values followed by the MCH. The Green king and Mentzer indices were shown to be of minimal value in respect of predicting α thalassaemia trait. However, upon utilising the MCV, a significant percentage would still be undetected, a scenario that is far from ideal during prenatal/ premarital screening. It appears therefore, that the best method to detect the α thalassaemia deletions would be to perform PCR testing.

# CHAPTER 7

# CONCLUSION

## CONCLUSION

Unexplained microcytosis is a frequently encountered phenomenon in clinical practice. In the present study 78% of the unexplained microcytosis was shown to be associated with deletions within the alpha globin cluster, underpinning the importance of molecular testing for α thalassaemia in this setting.

This is the first study in South Africa that answers the question of unexplained microcytosis, and further studies are warranted to explore the possibility of other deletional defects within the α globin cluster.

# CHAPTER 8

# REFERENCES