GENETIC MODIFIERS OF BETA THALASSAEMIA: PHENOTYPE-GENOTYPE RELATIONSHIP IN A SRI LANKAN POPULATION

BY

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DECLARATION

I declare that the contents of this thesis are my own work, except for that detailed below, for which I would like to thank Mr. A.A.G.S. Abeysekara for genotyping the samples for the *HBB*, *HFE* and *COL1A1* genes.
ABSTRACT

Introduction

Beta thalassaemia is a highly heterogeneous disorder in its phenotype, geographical distribution and molecular mechanism. This heterogeneity is a result of interaction of environmental and genetics factors. These genetic changes can occur in the β globin gene or its flanking regions, directly affecting the β globin gene regulation (primary modifiers), variation in the α globin chains or the level of HbF due to co-inheritance of α thalassaemia or γ globin gene variations (secondary modifiers) and genetic changes described in association with recognized complications of β thalassaemia, such as iron overload, hyperbilirubinaemia, osteoporosis, etc. (tertiary modifiers). Therefore the objectives of this study were to determine the genotypes of patients having a clinical diagnosis of β/HbE-β thalassaemia who are on regular blood transfusions and to establish the genetic diagnosis; genotype the hemochromatosis (HFE) and collagen type 1, alpha I(COL1A1) genes which are tertiary genetic modifier genes on the same population and correlate the clinical phenotype of thalassaemia with the β hemoglobin (HBB) gene mutations, and the serum ferritin levels with HFE gene mutations c.845G>A (C282Y) and c.187C>G (H63D), and the BMD (Bone Mineral Density) with the COL1A1 genotype.

Methodology

125 patients with β thalassaemia major who are on regular blood transfusions were selected for the study. One cohort was recruited (n = 93) from a thalassaemia centre in a thalassaemia endemic area Anuradhapura, and the second cohort (n = 32) was recruited from a tertiary care children’s hospital; Lady Ridgeway Children’s Hospital (LRH) in Colombo, a thalassaemia non-endemic area. Blood samples were collected into ethelene diaminetetraacetic acid (EDTA)
anticoagulant for genotyping the $HBB$, $HFE$ and $COL1A1$ genes. Blood samples were collected into tubes without anticoagulant for biochemical analysis of the levels of serum ferritin, C-reactive protein (CRP), serum glutamic-pyruvic transaminase (SGPT) and sodium citrate anticoagulant for erythrocyte sedimentation rate (ESR). Biochemical tests were carried out in a biochemistry laboratory using standard methods. The genotyping was performed for mutations in the $HBB$ gene by Amplification Refractory Mutation System - Polymerase Chain Reaction (ARMS PCR) for five common mutations previously described in Sri Lankan patients with $\beta$ and HbE/$\beta$ thalassaemia. They were; c.92+1G>A, c.92+5G>C, c.79G>A, c.126_129delCTTT and g.71609_72227del619. This was followed by sequencing the P1 region (exon 1, exon 2 and the flanking regions) of the $HBB$ gene in 3 patients who had negative results and 21 patients who had a single pathogenic allele (heterozygous) by ARMS PCR. $HFE$ gene was tested for the common two mutations; c.845G>A and c.187C>G by ARMS PCR. $COL1A1$ gene was tested for the c.104-441G>T polymorphism by Restriction Fragment Length Polymorphism (RFLP) method.

**Results**

**$HBB$ gene analysis**: Of the total study population 94 (75.2%) were homozygous or compound heterozygous for two mutations. Therefore the genetic diagnosis was established in those 94 patients. Of the 250 alleles studied, 215 (86%) alleles were genotyped by both assays and total of 8 pathogenic mutations were detected in the study population. The pathogenic mutations and their mutant allele frequencies were: c.92+5G>C 174 (69.6%), c.92+1G>A 23 (9.2%), c.126_129delCTTT 9 (3.6%), c.79G>A 2 (0.8%), c.51delC 2 (0.8%), c.27_28insG 2 (0.8%), c.46delT 2 (0.8%) and g.71609_72227del619 1 (0.4%). The ARMS PCR assay which was aimed at detecting 5 pathogenic mutations enabled the detection of 209 alleles out of 250 alleles in this
study population. 90 (72%) patients were homozygous or compound heterozygous for two mutations tested by this assay. Three mutations, c.51delC, c.27_28insG and c.46delT were detected by automated sequencing in 4 patients.

**HFE gene analysis:** No mutations were detected in the c.845G>A variant and 23 patients were heterozygous for the c.187C>G mutation. The allele frequencies of the two mutations were 0% and 9.2% respectively. The heterozygous group had a higher serum ferritin level, however there was no statistically significant association between this group and those who had the wild type allele (p value = 0.865).

**COL1A1 gene analysis:** Of the total population gene analysis was done on 46, of which 6 (4.8%) patients were heterozygous for the c.104-441G>T polymorphism. The allele frequency for the variant allele was 6.52%. Bone Mineral Density data were not available in both study cohorts to enable genotype phenotype analysis.

**Conclusions**

Total of 8 pathogenic mutations were detected in the study population either by the ARMS PCR assay or by automated sequencing. Genetic diagnosis was established in 94 patients. Of the 250 alleles studied, 215 (86%) alleles were genotyped by both assays. Genotyping of the **HBB** gene which was performed by a rapid, simple and cost effective ARMS PCR assay resulted in the confirmation of the genotypes in 72% of the study population. Therefore it can be concluded that this test can be used as a sensitive molecular diagnostic screening test for β thalassaemia in our population. The c.79G>A mutation was rare in the study group and it has been described that this mutation is unevenly distributed in the thalassaemic population in the island. All the genotypes tested by ARMS PCR and automated sequencing were compatible with the phenotypes of this
thalassaemia cohort. The results of the HFE gene analysis were comparable with the previous published data on Sri Lankan patients and larger population cohorts. The c.845G>A mutation is rare in our thalassaemic population and therefore does not have to be considered in the pathogenesis of iron overload in regularly transfused thalassaemia patients. The c.187C>G genotype may need to be considered in patients with iron overload. COL1A1 gene, c.104-441G>T variant is polymorphic in the population, however was unable to correlate its relationship with the Bone Mineral Density due to unavailability of data regarding Bone Mineral Density.
1.0 INTRODUCTION TO THALASSAEMIA

1.1 Definition of thalassaemia

The definition of *thalassaemia* is derived from ancient Greek term “the sea” (Mediterranean) in the blood (Hoffbrand 2005; Hoffman R. 2005). This condition was first described in the early nineteenth century by a Detroit physician, Thomas B. Cooley in 1925, where he described a series of infants with profound anaemia and splenomegaly in the first year of life (Hoffbrand 2005). Although initially the term thalassaemia was used to principally describe anaemic conditions seen in people in the Italian and Greek coastal regions, it is now used to refer to inherited defects in globin chain biosynthesis.

1.2 Classification of thalassaemia

Genetic disorders of human globin chain are of two main types; disorders characterized by quantitative defects in globin chain synthesis (Beta and Alpha Thalassaemias) and those with qualitative defects in globin chain synthesis; which includes haemoglobin E (HbE) thalassaemia. A third category; hereditary persistence of fetal haemoglobin is also described (Weatherall 2001).

Thalassaemias are broadly classified into α, β, δβ and εγδβ thalassaemias according to the type of globin chain which is being ineffectively synthesized (Weatherall 2001). Beta thalassaemia is characterized by a quantitative deficiency of functional β globin chain synthesis which leads to imbalanced globin chain production with an excess of α chains (Thein 2004). Similarly α thalassaemia refers to quantitative deficiency of α globin chain synthesis which leads to excess of β chains. The δβ and εγδβ thalassaemias are rare and involve in series of deletions in the β
globin gene cluster. This can remove either δ and β genes or all or greater part of the β globin gene cluster including the β gene itself (Weatherall 2001; Thein 2005).

The clinical classification of thalassaemia is based on the severity of the disease. It has a spectrum of clinical phenotypes ranging from β thalassaemia major, β thalassaemia intermedia and the β thalassaemia carrier state. At the severe end of the spectrum there are β thalassaemia major patients who present with profound transfusion dependent anaemia from early life (Hoffbrand 2005; Bain 2006). Thalassaemia intermedia is characterized by anaemia and splenomegaly and may or may not need regular blood transfusions. Thalassaemia minor is a carrier state of thalassaemia which is a transfusion independent condition.

At molecular genetics level, thalassaemia can be classified according to the presence of the number of functional β alleles; β⁰ and β⁺. β⁰ thalassaemia is a state where globin chain synthesis is absent and in β⁺ thalassaemia the globin chain synthesis occur at a reduced rate (Thein 2004; Hoffbrand 2005).

**1.3 Inheritance**

Beta thalassaemia is transmitted as an autosomal recessive disorder. In homozygotes both copies of the hemoglobin beta (**HBB**) gene situated in one locus are mutated and compound heterozygotes have two different mutations in two different loci of the **HBB** gene. Both homozygotes and compound heterozygotes inherit two mutant copies from each parent and manifest as clinically detectable disease usually form the infancy (Hoffbrand 2005).

**1.4 Prevalence of thalassaemia and the health burden**

Global epidemiology of hemoglobin disorders has been described by Angastiniotis *et al* (1998). Lahiry and his colleagues have focused on the prevalence of the disease in the Indian
subcontinent and in the Middle East (Angastiniotis et al. 1998; P. Lahiry 2008). (Fig: 1.1). According to their studies it has been estimated that there are 269 million carriers of haemoglobin disorders worldwide; out of which 80 million are carriers of β thalassaemia (Thein 2005). Globally more than 400,000 children are born with β thalassaemia annually (Angastiniotis et al. 1998; P. Lahiry 2008).

Thalassaemia has become a major health burden for some countries and occur in high frequency particularly in the Indian sub continent and many parts of South East Asia where there is high gene frequency of β thalassaemia and HbE variants. It is believed that the cause for this high occurrence in these countries is the major demographic transition following improvement in health, hygiene, diet, availability of medical services and upgrading of diagnostic procedures. Therefore neonates, babies and young adults who would have died in the early part of their lives now survive long enough to allow diagnosis and treatment (Weatherall 1998; de Silva et al. 2000; Premawardhena et al. 2004).

The prevalence of thalassaemia is also high in certain populations especially in the Mediterranean regions, Middle-East, Transcaucasia and Central Asia (P. Lahiry 2008; Cao et al. 2010). In certain countries like Cyprus, Sardinia and South East Asia the incidence is very high; most likely due to selective advantage for healthy carriers being protected against lethal effects of Plasmodium infection (Weatherall 1998; P. Lahiry 2008; Cao et al. 2010). However as a result of global migration, thalassaemia can be observed in areas where it was not endemic; for instance North Europe, North and South America, Caribbean Islands and Australia (Thein 2005; Cao et al. 2010). It is believed that thalassaemia has been introduced to these countries by the immigrants (Angastiniotis et al. 1998).
Over 700 structural haemoglobin variants have been identified to date of which HbE is the commonest structural haemoglobin variant. The gene for HbE variant is distributed at a high frequency in parts of India, Myanmar, Bangladesh, Pakistan Nepal and throughout South East Asia (Weatherall 2001). The frequency of HbE may even be as high as 60% in many regions of Thailand, Laos, and Cambodia (Vichinsky 2007). The most likely cause for this high prevalence is the resistance of HbE red cells to *Plasmodium falciparum* infection (Vichinsky 2007). HbE/β-thalassaemia affects at least a million of people globally (Vichinsky 2007). During the last two decades HbE/β-thalassaemia has become the commonest thalassaemia disorder in North America. The explanation for this high incidence is population migration as most of these immigrants have come from the areas where HbE mutation is prevalent (Vichinsky 2007).

**Fig: 1.1 Beta thalassaemia mutations in at risk populations.** Mutations listed are those which are common and represent 91-95% of affected individuals in these regions. Listed in brackets are beta thalassaemia carrier frequencies in each region. (P. Lahiry, S. A. A.-A. a. R. A. H. V. B. R. G., Robarts Research Institute and the University of Western Ontario, Canada (2008). "Understanding Beta-Thalassemia with Focus on the Indian Subcontinent")
In Sri Lanka the first thalassaemia case was identified in 1951 (De Silva et al. 1951). Increasing number of cases have been observed in the past few decades particularly in Kurunegala district due to improvement in health, hygiene and the diagnostic services (Fisher et al. 2003). According to unpublished data, there are approximately 1600 patients with thalassaemia in the country and 80 new cases of thalassaemia are being added to the list each year. Therefore thalassaemia is of considerable public health importance in Sri Lanka. According to the Ministry of Health statistics, the Sri Lankan government spends >144 million Rupees to treat these patients annually.

As described by Lahiry et al (2008), in the Indian subcontinent (South Asia) which includes Sri Lanka, India and Pakistan, there are about 45 million β thalassaemia carriers. According to their study, four common β thalassaemia mutational ‘hotspots’ have been described in the HBB gene in the Indian subcontinent (P. Lahiry 2008). They are c.92+5G>C, c.92+1G>A, g.71609_72227del619 and c.27_28insG. A study done by Fisher et al (2003) has shown that there are 24 different β globin gene mutations in the Sri Lankan population out of which three mutations; c.92+5G>C, c.92+1G>A and c.79G>A account for 84.5% of the β thalassaemia population of their study (Fisher et al. 2003).

1.5 Pathophysiology of β thalassaemia major
The pathophysiology of β thalassaemia lies upon the imbalance in the ratio of α to β globin chains (Fig: 1.2). The reduced or absent β globin synthesis, with normal α globin synthesis result in excess α globin chains; which in the absence of their partners become unstable and precipitate within the red cells forming inclusions. These inclusions, in red cell precursors interfere with red cell maturation in the bone marrow leading to red cell destruction within the bone marrow (ineffective erythropoiesis) due to oxidative damage of the cell membrane causing apoptosis.
(Cao et al. 2010). These inclusions interfere with the passage of red cells in the microcirculation leading to premature red cell destruction (before the normal life span of 120 days) especially within the splenic microvasculature (Thein 2004), (Weatherall 1998). Hence anaemia in these patients result in a combination of ineffective erythropoiesis and haemolysis (Rund et al. 2005).

Anaemia stimulates secretion of erythropoietin leading to extramedullary hematopoiesis and expansion of the bone marrow in the skull and long bones causing bony deformities particularly in the craniofacial skeleton and the long bones of the legs (Weatherall 1998; Thein 2004; Bain 2006; Cao et al. 2010). These skeletal changes include frontal bossing, prominent malar eminence, depressed nasal bridge, maxillary hypertrophy causing exposure of upper teeth and tendency to mongoloid slant of the eyes (Bain 2006; Cao et al. 2010). Due to thinning of the cortical bone these patients develop bone pain, tenderness and increased predisposition to long bone fractures (Bain 2006). Hepatomegaly and splenomegaly are marked in these patients owing to extramedullary haemopoiesis. It has been estimated that the expansion of erythroid mass in some patients with β thalassaemia can be between 10 and 30 times normal (Weatherall 1998). Splenomegaly will lead to hypersplenism causing increased peripheral destruction of the immature red blood cells (Bain 2006). Splenomegaly together with bone marrow expansion lead to increase in plasma volume accounting for about 9% to 40% of the total blood volume expansion seen in these patients with thalassaemia (Weatherall 1998).

Haemoglobin F is, the major haemoglobin in the foetus but is found in smaller proportions after birth; usually accounting for <1% of the total haemoglobin in adults. This HbF is confined to a certain subset of red cells called ‘F cells’ (Weatherall 1998; Weatherall 2001). HbF is formed by the combination of 2 α globin chains with 2 γ globin chains. The HbF percentage is determined by age, sex and inherited characteristics. In patients with β thalassaemia, the unpaired α globin
chains can combine with γ chains and form HbF resulting in raised HbF levels in these patients. Therefore the cells which can produce relatively high γ chains in the bone marrow of β thalassaemias are protected to a certain degree against the deleterious effects of excess α chains. However in β thalassaemia the combined output of β and γ globin chains is never sufficient to match the excess levels of α globin chains, hence harmful consequences of the disease will always manifest (Weatherall 1998).

Because of profound anaemia seen in β thalassaemia major, the patients are transfusion dependent. Unless a regular transfusion program is established, these patients will not survive beyond late infancy or early childhood. Once a regular transfusion program is initiated, children with β thalassaemia can lead a near normal life with normal development (Cao et al. 2010). However their quality of life will depend on other factors related to the pathophysiology of the disease, such as development of hyperbilirubinaemia and gall stones, bone complications and treatment related complications like iron overload. Development of these complications are postulated to be related to their genotype, a factor that is less understood at the present time (Cao et al. 2010).
1.6 Clinical manifestations and complications of β thalassaemia

1.6.1 Clinical manifestations at diagnosis

Infants with β thalassaemia major are not anaemic at birth. Clinical features begin to manifest after the second six months of life; at the time of developmental switch over from foetal to adult haemoglobin, which starts to occur around six months of age. These patients can present with a range of clinical features including failure to thrive, pallor, vomiting, diarrhoea, feeding problems, recurrent bouts of fever, irritability, abdominal distention due to splenomegaly and hepatomegaly (Hoffman R. 2005; Cao et al. 2010).

1.6.2 Clinical manifestations in well-transfused patients

Iron overload is one of the main complications seen in transfusion dependent thalassaemia and is associated with morbidity and mortality. Excess iron is accumulated in various organs of the body causing viceralomegaly leading to organ damage and end organ failure. Heart, liver, spleen and endocrine glands are the major organs where iron is laid down. Cardiac complications due to iron over load is still the main cause of mortality in patients with β thalassaemia (Rund et al. 2005; Cao et al. 2011). It has been estimated that 71% of deaths among patients with β thalassaemia are due to cardiac complications owing to iron overload (Cao et al. 2010). Dilated cardiomyopathy, pericarditis arrhythmias and heart failure are the main consequences of iron deposition in the myocardium. Chronic hepatitis, fibrosis and cirrhosis can result from iron deposition in the liver parenchyma (Cao et al. 2010). Excess iron is very toxic to endocrine organs. It has been reported that more than 50% of patients have hypogonadism due to iron deposition; and hypothyroidism and hypoparathyroidism have been reported in 10% and 9% of patients with β thalassaemia respectively (Cao et al. 2011). As a result of iron overload in the
pituitary gland, patients with β thalassaemia may have stunting of growth and failure of sexual maturation. Secondary diabetes mellitus is also reported in about 6.4% of patients as a consequence of iron deposition in the pancreatic tissue (Cao et al. 2011).

Regular transfusions help to maintain normal haemoglobin levels and prevent bony deformities, organomegaly, etc. However without adequate iron chelation therapy, gradual accumulation of excess iron in organs / tissue will result in above mentioned complications. Children who are adequately chelated too could have problems in growth, sexual maturation and osteoporosis due to extreme sensitivity of endocrine glands to even mild iron overload (Hoffbrand 2005).

1.6.3 Clinical manifestations and complications in untreated or undertreated patients

Children who do not receive treatments usually die in late infancy or early childhood as a result of severe anaemia. Untreated patients will show typical features described in the pathophysiology of β thalassaemia major. Anaemia is worsened by progressive splenomegaly and folate deficiency, due to increased folate requirement following excessive hematopoiesis and haemolysis. Hypersplenism can aggravate the anaemia, and cause thrombocytopenia and bleeding tendency. Bleeding manifestations may be partly attributed to liver damage as well. Because of bone marrow expansion bony deformities arise especially in the skull bone, giving rise to classical “mongoloid facies” of β thalassaemia (Hoffbrand 2005). Additionally dental complications with poorly formed teeth and malocclusion may also develop. Because of skull deformities sinuses and middle ear drainage is inadequate, leading to chronic sinus infection and deafness. These patients are hypermetabolic and develop intermittent fever and failure to thrive. Occasionally they develop hyperuricaemia and secondary gout owing to increased turnover of red cell precursors (Hoffbrand 2005).
Osteoporosis is another complication seen in patients with β thalassaemia, and occurs due to bone marrow expansion, hypogonadism, diabetes mellitus, hypothyroidism, hypoparathyroidism, cardiac dysfunction and presence of genetic predisposing factors (Rund et al. 2005).

Genetic variations are known to modify or aggravate the level of iron overload and bone defects in patients with β thalassaemia major and the relevant genetic aspects will be dealt in detail in the later parts of this dissertation.

1.7 Laboratory investigations

The initial investigations in a patient with suspected thalassaemia at presentation consist of a full blood count (FBC) and blood picture. Characteristic features in a FBC are low haemoglobin level, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and haematocrit (Hct) levels with increased red cell distribution width (RDW). The blood picture will reveal variations in shape and size of red cells (anisocytosis and poikilocytosis) with predominantly hypochromic microcytic cells, and nucleated red blood cells (Bain 2006; Cao et al. 2010). Presence of hypochromic, often wrinkled and folded cells (leptocells) containing irregular inclusion bodies of precipitated α globin chains is a characteristic feature in the blood film (Hoffman R. 2005). These inclusions are prominent in splenectomized patients. Basophilic stippling, Pappenheimer bodies and target cells are also present. The low reticulocyte count present in these patients indicates the severity of intramedullary erythroblast destruction (Hoffman R. 2005). Leukocytosis with increased neutrophil count is usually noted (Bain 2006). In patients with hyperslenism due to massive splenomegaly, leucopenia with thrombocytopenia can also be seen.
Bone marrow examination, although not routinely performed, will show gross erythroid hyperplasia with reversed myeloid/erythroid ratio from the normal 3 or 4 to 0.1 or less (Cao et al. 2010). Dyserythropoiesis with nuclear lobulation and fragmentation, basophilic stippling, defective haemoglobinization and α chain precipitates are also visible in the bone marrow aspirate (Bain 2006). As a result of precipitated α chains in erythroid precursors, accelerated apoptosis is noted in the polychromatophilic and orthochromic stages (Mathias et al. 2000; Rund et al. 2005). Bone marrow iron stores are increased and actively phagocytic macrophages and pseudo-Gaucher cells are also present.

Diagnosis of β thalassaemia major is depended on the finding of the relative levels of mainly adult haemoglobin (HbA) and foetal haemoglobin (HbF) using Haemoglobin electrophoresis, isoelectric focusing or High Performance Liquid Chromatography (HPLC) methods. The findings will be varied according to the functional haemoglobin allele which is present in the patient; β0 and β+ alleles. In homozygous or compound heterozygous for β0 allele (β0β0), HbA is absent, the predominant type is HbF (approx. 95-98%) and HbA2 can be variable. When there is homozygous state for β+ allele (β+β+) or compound heterozygous state for β0 and β+ alleles (β0β+) HbA is also present in variable amounts; can be up to 35% of total haemoglobin (Bain 2006; Cao et al. 2010).

High serum ferritin and saturated iron binding capacity are signs of iron overload and seen in most patients with transfusion dependent thalassaemia. This change is reflected as increased iron in both reticuloendothelial and parenchymal cells in the liver and increased bone marrow iron stores. Bilirubin level is usually elevated and haptoglobins are absent. Serum aspartate aminotransferase (AST) levels are frequently high at the time of diagnosis due to hemolysis. Alanine aminotransferase (ALT) levels are usually normal prior to transfusion therapy and
subsequently ALT levels may elevate due to iron induced hepatic damage or viral hepatitis (Hoffbrand 2005; Hoffman R. 2005).

1.8 Molecular genetics in thalassaemia

1.8.1 Molecular basis of globin chain synthesis

Among genetic disorders thalassaemias were the first disorders to be analyzed at the molecular level, and thus enabled detailed analysis and understanding of the correlation between the different phenotypes with the genotypes (Weatherall et al. 1996).

Adult human haemoglobin molecule consists of four sub units, each having one polypeptide chain and one heme group. All the types of haemoglobin bear the same prosthetic heme group iron protophophyrin IX which is associated with two pairs of unlike globin chains; two α and two β chains. Each α chain consists of 141 amino acid residues whereas each β chain has 146 residues. Both α and β globin chains are almost similar in length but differ in their sequences (Marengo-Rowe 2006). Each of the globin chain is encoded by distinct genes; two genes exist for α globin chains hemoglobin alpha 1 (HBA1) and hemoglobin alpha 2 (HBA2) and one gene; HBB for β globin chains.

Beta globin chains are encoded by HBB gene which is located on the short arm of chromosome 11(11p15.4) which spans 70 Kb in length. HBB gene complex has five functional genes; 5’Epsilon(ε), G gamma(Gγ), A gamma(Aγ), Delta(δ) and Beta(β) 3’ arranged according to their developmental expression (P. Lahiry 2008). (Fig: 1.3). These genes become active in sequential order from 5’ to 3’ direction in specific time periods of development. During the embryonic period up to six weeks of gestation ε gene is expressed, following which transcription of two γ genes take over in the fetal liver. During the prenatal period δ and β genes are expressed (Thein
At time of birth, \(\gamma\) globin synthesis decreases and \(\beta\) globin synthesis increases and around 6 months of age HbF levels declines to \(<5\%\) of the total haemoglobin. This level gradually decreases to \(<1\%\); the adult level by the age of 2 years (Thein 2004). By this stage the mutations affecting the \(\beta\) gene become clinically apparent. Due to persistence of small amount of \(\gamma\) expression in adult life, all adults have small amount of fetal haemoglobin in their blood (Thein 2004).

1.8.2 Molecular basis of β thalassaemia and phenotypic heterogeneity

Phenotypic heterogeneity of β thalassaemia is known to be dependent on both genetic and environmental factors. The primary genetic changes can occur in the β globin gene or its flanking regions, directly affecting β globin gene regulation thus, these genetic defects can be called as primary modifiers. Variation in the α globin chains or the level of HbF due to co-inheritance of α thalassaemia or γ globin gene variations are called secondary modifiers. Genetic changes described in association with recognized complications of β thalassaemia, such as iron overload, hyperbilirubinemia, osteoporosis; are called tertiary modifiers (Weatherall 2001; Thein 2004; Thein 2005). (Fig: 1.4).

1.8.2.1 Primary Modifiers - Introduction

Primary modifiers are the numerous β globin mutations in the *HBB* gene. More than 200 mutations have been identified in the *HBB* gene to date which are known to cause different clinical phenotypes of β thalassaemia (Weatherall 2001). Globin Gene Server Web Site ([http://www.globin.cse.psu.edu](http://www.globin.cse.psu.edu)) provides a complete update of all the mutations identified in the *HBB* gene to date. Except for a few deletions the majority of the mutations are point mutations within the gene or its flanking sequences. These mutations interfere in gene expression at the levels of transcription, translation and post translation (Thein 2004; Cao et al. 2010).

1.8.2.2 Genomic structure of the *HBB* gene

The genomic region of β globin gene mainly comprises of transcribing regions and conserved sequences. Transcribing region consists of three exons and two intervening sequences which code for 146 amino acids. ([Fig: 1.3](#)). Conserved sequences are found in the 5’ promoter region, at the exon-intron junctions, and in the 3’ untranslated region (3’-UTR) (P. Lahiry 2008). Beta globin gene promoter region has three elements; a TATA box, a CCAAT box and duplicated CACCC motifs. Out of these three promoter sequences CACCC motifs are erythroid cell specific promoters and are very important in the normal adult β globin gene expression (Thein 2004). The most important transcription factor Erythroid Kruppel-like factor (EKLF) binds to the proximal CACCC box. Upstream to the promoter region there are two other binding motifs for the erythroid transcription factor GATA-1. In the 5’UTR region there is a 50 nucleotide region where transcription starts and the initiator codon; ATG is the hallmark of it. There are two prominent conserved sequences in the 5’ UTR region in both α and β genes; CTTCTG and CACCATG (Thein 2004). The 3’-UTR extremity consists of 132 nucleotides and has termination codon (TAA) and poly A tail. In between the termination codon and poly A tail there
is a conserved sequence; AATAAA and two enhancers are found in intron 2 and 3’end, 600-900 bp downstream of the poly A site. Mutations in all above described regions have been implicated with the β thalassaemia phenotype (Thein 2004).

1.8.2.3 Mutations/ Polymorphisms

1.8.2.3 (a) $\beta^0/\beta^+/\beta^{++}$ mutations

Different classes of β thalassaemia mutations are responsible for the phenotypic heterogeneity of this disorder (Thein 2005). $\beta^0$ thalassaemia in which there is a complete absence of β globin production is mainly responsible for the thalassaemia major phenotype. $\beta^+$ alleles are able to produce variable amounts of β globin chains, and are extremely heterogeneous. $\beta^{++}$ alleles are the ones which allow production of significant proportion of β globin chains resulting in a mild phenotype (Thein 2004).

Table 1.1 summarizes the different allele types in β thalassaemia and their relationship to the disease severity.

**Table 1.1: Disease severity vs Allele types in β thalassaemia**

<table>
<thead>
<tr>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Disease severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta^0$</td>
<td>$\beta^0$</td>
<td>Severe (thalassaemia major)</td>
</tr>
<tr>
<td>$\beta^0$</td>
<td>Severe $\beta^+$</td>
<td>Severe (thalassaemia major)</td>
</tr>
<tr>
<td>$\beta^0$</td>
<td>Mild $\beta^+$</td>
<td>Moderate (thalassaemia intermedia)</td>
</tr>
<tr>
<td>$\beta^0$</td>
<td>$\beta^{++}$</td>
<td>Moderate (thalassaemia intermedia)</td>
</tr>
<tr>
<td>$\beta^+$</td>
<td>$\beta^+$</td>
<td>Mild/moderate (thalassaemia intermedia)</td>
</tr>
<tr>
<td>$\beta^+$</td>
<td>$\beta^{++}$</td>
<td>Mild</td>
</tr>
<tr>
<td>$\beta^{++}$</td>
<td>$\beta^{++}$</td>
<td>Mild</td>
</tr>
<tr>
<td>$\beta^0$ or $\beta^+$</td>
<td>Normal</td>
<td>Carrier/trait/thalassaemia minor</td>
</tr>
<tr>
<td>3rd exon mutation</td>
<td>Normal</td>
<td>Dominant type of β thalassaemia</td>
</tr>
</tbody>
</table>
Approximately half of β thalassaemia causing mutations trigger premature termination of β chain extensions due to nonsense and frameshift mutations which introduce premature termination codons resulting in β0 alleles. Most mutations of this nature cause termination of transcription within exon 1 and 2 (Thein 2004). These mutations are associated with minimal steady-state levels of mutant β mRNA in erythroid cells. This is due to nonsense mediated mRNA decay (NMD); a process called accelerated decay of mutant mRNA in erythroid precursors (Thein 2004; Thein 2005). In heterozygous state for these mutations β chains are not produced from the mutant allele and only half the normal β globin protein is present, resulting in a less severe phenotype; the patients are typically asymptomatic (Thein 2005).

Mutations in the splice junction in which normal splicing is completely eliminated will result β0 thalassaemia phenotype. These mutations can affect mRNA processing and involve in either of the invariant dinucleotides (GT at 5’ and AG at 3’) at the splice junctions. Additionally mutations in the initiation codon (ATG) can also result in β0 alleles (Thein 2005).

However Cao and his colleagues (2010) have described few β0 alleles which demonstrate a mild thalassaemia phenotype due to linkage with G- gamma gene (-158 G-γ) which result in production of high HbF levels during haematologic stress. Mutations in cd 6-A and cd 8-AA are two such examples (Cao et al. 2010).

Mutations involving the promoter region, (either the CACCC or TATA box), polyadenylation site, 5’ or 3’ UTR regions and mutations within the consensus sequences at the splice junctions will result in β+ thalassaemia allele (Cao et al. 2010).

β+ thalassaemia mutations can be further sub-classified according to the extent of the reduction of β chain output, as severe, mild, and silent. (Fig: 1.5). Phenotype of mild β thalassaemia (β++
is classified as clearly definable changes in the red cells in heterozygotes and of intermediate severity in homozygotes (Weatherall 2001). β⁺⁺ allele is responsible to produce moderate amount of β globin chains. Mutations in the proximal CACCC box, TATA box, 5’ UTR, or exon 1 causing alternative splicing or in the consensus splicing sequences, 3’ UTR and poly-A site will result in mild β thalassaemia phenotype (Weatherall 2001; Cao et al. 2010).

A cryptic splice site that contains the sequence; GT GGT GAG G in exon 1 creates an alternative splicing site at codon 19, 26 and 27 and is associated with mild or silent phenotype giving rise to Hb Malay, Hb E and Hb Knossos respectively (Thein 2004; Cao et al. 2010).

‘Silent’ β thalassaemia is caused by minimal defect in β globin chain synthesis, thus mild imbalance of the α/β globin chain ratio. These mutations are rare except for the -101 C>T mutation which is frequently observed in the Mediterranean region. These mutations are usually observed in compound heterozygous state with a severe β thalassaemia allele resulting in thalassaemia intermedia phenotype. In the homozygous state it produces β thalassaemia trait phenotype. Several mutations in the distal CACCC box, in the 5’UTR, polyadenylation signal and some splicing defects are included in this category (Weatherall 2001; Thein 2004; Cao et al. 2010). (Fig: 1.5)

HBB gene deletions cause complete absence of β globin chains. Therefore the phenotype should be severe β thalassaemia. However these mutations are associated with high levels of HbF and HbA2 and the phenotype seen is not as severe as expected. The increase in HbF is variable and moderate in heterozygotes. But in homozygotes for these deletions the increase in the level of HbF is adequate to compensate the complete absence of β globin chains. These deletions can be varied in sizes. Gross deletions affecting the β globin gene are very rare, except for g.71609_72227del619, which removes a region from -125 to +78 in the 5’-β promoter, which includes the CACCC, CCAAT and TATA elements (Thein 2004). This mutation is very common in Sind and Panjab population of India and Pakistan (Cao et al. 2010).

1.8.2.3 (b) Dominantly inherited β thalassaemia

Some forms of β thalassaemia are dominantly inherited where an inherited single β thalassaemia allele creates clinically detectable disease (Faustino et al. 1998; Thein 2004). These
heterozygotes have thalassaemia intermedia phenotype in spite of normal α globin genotype (Faustino et al. 1998). These patients present with moderate anaemia, splenomegaly and thalassaemic blood picture with increased levels of HbA2. The remarkable feature of this phenotype is the large inclusion bodies observed in red cell precursors, similar to those seen in patients with β thalassaemia major (Thein 2004). Therefore the original term for this phenotype was labeled as ‘inclusion body β thalassaemia’.

This clinical entity was first recognized in an Irish family. Since then more than 30 dominantly inherited β globin alleles have been identified to date (Thein 2004). The molecular mechanisms for these mutations include missense mutations, minor deletions leading to the loss of intact codons, frameshift mutations, and nonsense mutations leading to truncated β variants (Weatherall 2001; Thein 2004). (Fig: 1.6). In general, the stop codons and frameshift mutations terminate in exon 1 or 2 are recessively inherited whereas those that are terminating in exon 3 or beyond are considered as dominantly inherited. Few dominantly inherited mutations have been reported in the exon 1 and 2 as well (Faustino et al. 1998; Weatherall 2001; Thein 2004).
Fig: 1.6 Dominant β thalassaemia. The lengths (in amino acids) of the abnormal products are indicated, with the normal product shown at the top. Nonsense (NS) or Frameshift (FS) mutations in exon 1 and 2 of the β globin gene are recessively inherited and those that are terminating in exon 3 are dominantly inherited. (Weatherall, D. J. (2001). "Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias." Nat Rev Genet 2(4): 245-255.)

In dominant β thalassaemia, mutations in exon 3 and beyond are able to produce substantial amounts of abnormal cytoplasmic m-RNA leading to production of highly unstable β chain variants. These highly unstable products are precipitated in the cytoplasm of the red cell precursors causing intra-erythroblastic inclusions. Some less unstable products can combine with α globin chains to produce haemoglobin tetramers which ultimately precipitate in mature red cells (Weatherall 2001). Therefore, in dominant β thalassaemia the inclusion bodies comprise of both α and β chains in contrast to homozygous β thalassaemia which consist of only precipitated α globin chains (Ho et al. 1997; Thein 2004).

1.8.2.3 (c) HbE/β thalassaemia

This variant results in substitution of glutamic acid for lysine at the 26th codon of β globin chain creating an alternate splice site within that exon (Dora Bachir 2004; Vichinsky 2007). The
mutated HbE β chain is synthesized at a reduced rate compared to normal adult β chain which form HbA. As a consequence, heterozygotes, compound heterozygotes and homozygotes show β thalassaemia phenotype (Vichinsky 2007).

Haemoglobin E is a heterogeneous disease. Clinical severity is highly variable ranging from β thalassaemia minor through thalassaemia intermedia to thalassaemia major phenotypes (Dora Bachir 2004; Vichinsky 2007). Patients with the same mutation within the same family show significant difference in their clinical severity. Heterozygous HbE state is a complete asymptomatic condition with no clinical significance. Homozygous haemoglobin E disease (EE genotype) is defined by the presence of two HbE alleles. Individuals with this genotype are usually asymptomatic. There is no anaemia, the spleen is not generally enlarged and hemolysis is rarely observed in these patients (Dora Bachir 2004).

Co-inheritance of HbE along with β thalassaemia mutations β⁰ or β⁺ (compound heterozygous) bear a resemblance to homozygous β thalassaemia, ranging from severe anaemia with transfusion dependency, through thalassaemia intermedia to β thalassaemia minor (Thein 2004). The most severely affected HbE/β syndrome patients have HbE/β⁰ allele composition. They are transfusion dependent and have hepatosplenomegaly, growth retardation and bone marrow expansion causing facial deformities (Vichinsky 2007). Less severely affected individuals do not require regular transfusions but facial deformities and splenomegaly may be present and their allele composition is HbE/ β⁺. To differentiate the two HbE /β⁰ and HbE/ β⁺ genotypes, DNA analysis is required and is increasingly being used nowadays (Vichinsky 2007).

The exact phenotype of β thalassaemia cannot be determined by analysis of the HBB gene alone, other confounding factors have to be analysed as well. These confounding factors are the other
influencing genes; the secondary and tertiary modifier genes described above and environmental factors. The final clinical outcome is a result of interaction of all these factors. The following sections of this dissertation will be focusing on these secondary and tertiary modifier genes.

1.8.2.4 Secondary Modifiers

The imbalance in the ratio of α and β globin chains, that contribute to the pathophysiology of β thalassaemia can be genetically modulated at two loci; α globin and γ globin genes (Camaschella et al. 1997; Weatherall 1998); (Thein 2005).

1.8.2.4 (a) α globin genes

Co-inheritance of α thalassaemia along with β thalassaemia is not an uncommon phenomenon. Inheritance of a single α gene deletion along with β0 allele does not have a significant effect on the phenotype. On the other hand individuals with two α genes deletions and homozygous β+ allele may show mild form of thalassaemia intermedia. Inheritance of three α gene deletions; HbH disease along with β globin gene mutations may result in moderately severe anaemia which might confound the correct diagnosis of Hb H disease (Chui 2005; Thein 2005).

A study done by Ho et al. (1998) has confirmed that co-inheritance of triplicated α globin genes can increase the clinical severity of the β thalassaemia phenotype (Ho et al. 1998; Thein 2004). Another study done by Camaschella and his colleagues have suggested that interaction with triplicated α genes should be considered in patients with heterozygous β thalassaemia who have a severe clinical phenotype than expected (Camaschella et al. 1997).

1.8.2.4 (b) γ globin genes

The globin chain imbalance can be minimized by increased production of γ globin chains which combine with excess α globin chains and form fetal haemoglobin (Hb F; α2γ2) (Agarwal et al.
In all thalassaemias the inherent capacity to produce HbF is high because of selective survival of erythroid precursors that synthesize γ chains (Thein 2005).

Genetic polymorphism involving C > T substitution at -158 position in the Gγ globin gene (Xmn1-Gγ polymorphism) is a common sequence variation in all populations. Clinical studies have shown that in stressful haemopoietic situations like homozygous β thalassaemia and sickle cell disease, the presence of the Xmn1-Gγ polymorphism increases the HbF level (Thein et al. 1987). As this polymorphism is widely distributed in the population it is one of the important genetic determinants of the diverse phenotypes of β thalassaemia, especially in the β^0 allele of the intermediate variety (Weatherall 2001).

Ho and his colleagues have demonstrated in their study that the presence of the in-cis Xmn1- Gγ site is a modulating factor for phenotypic severity in β thalassaemia. It was further found when the Xmn1-Gγ polymorphism was present with the β^0 c.92+1G>A mutation in Asian Indians, it results in increased HbF response leading to a milder phenotype than expected in homozygotes (Ho et al. 1998). This is due to the mutations in the promoter region in the HBB gene that cause increased HbF output thereby reducing the globin chain imbalance (Thein 2005). Family studies have shown that the inherent capacity to produce HbF is partly due to the genetic determinants located outside the globin gene cluster, that trans acting. Although these loci have been mapped to chromosomes 6q23, Xp22 and 8q, the molecular basis of these different entities has not been characterized as yet (Thein 2005).

### 1.8.2.5 Tertiary Modifiers

Genetic loci unlinked to globin chain production have important phenotypic effects, particularly associated with complications of the disease (Weatherall 2001). These tertiary modifiers include
many different genetic polymorphisms, some of which have co-selected with thalassaemia. The importance of these genetic associations are increasingly being recognized in populations where β thalassaemia is widespread (Weatherall 2001).

Iron overload is the major complication observed in patients with β thalassaemia owing to blood transfusions and increased intestinal absorption. Co-existence of hereditary haemochromatosis along with β thalassaemia can aggravate and accentuate this problem.

### 1.9 Hereditary Haemochromatosis and β thalassaemia

Iron overloading diseases can be classified as either primary (hereditary) or secondary (inborn or acquired) (Oliveira et al. 2006). Sideroblastic anaemia, congenital dyserythropoietic anaemia, myelodysplastic syndromes are examples of other haematological diseases with secondary iron overload. These diseases have inappropriately elevated iron absorption from the gut because of increased demand of haemoglobin synthesis. Therefore these patients develop iron overload in the absence of blood transfusions and transfusions can further aggravate the problem (Swinkels et al. 2006). Primary cause of iron overload is due to inherited abnormalities in proteins associated with iron transport and regulation, leading to inappropriate iron absorption from the gut (Hash 2001).

Hereditary haemochromatosis is defined as an inherited disorder characterized by inappropriately high absorption of dietary iron which leads to abnormal accumulation of iron in parenchymal organs (Hash 2001). It is a genetically heterogeneous disease and has five major forms, caused by sequence variations in different genes. The classic form is due to the mutation in hemochromatosis (*HFE*) gene (type 1). Juvenile form, although less common, is the most severe type and usually results from mutations in the hemochromatosis type 2 (juvenile) (*HJV*) gene or
more rarely in hepcidin antimicrobial peptide (HAMP) gene (Lok et al. 2009). An autosomal dominant form (type 4) of haemochromatosis has also been documented and is due to mutations in the solute carrier family 40 (iron-regulated transporter), member 1 (SLC40A1) gene which encodes ferroportin.

**1.10 HFE associated haemochromatosis and β thalassaemia**

**1.10.1 Introduction**

HFE associated hereditary haemochromatosis has an autosomal recessive inheritance and is thought to be the most common inherited disorder in White populations. This disorder is commonly found in populations of Northern European origin and rarely occurs in Africans or Asians (Hash 2001; Lok et al. 2009). It is estimated that approximately 1 in 200 whites in United States are homozygous for the variant allele and is the most commonest inherited disease in the United States (Hash 2001). One in seven Australians of Anglo-Celtic descent are heterozygous for the c.845G>A mutation (Rossi et al. 2004). The reason for the difficulty in recognizing hereditary haemachromatosis in Africans and Asians is the presence of haemoglobin disorders like thalassaemia that result in secondary iron overload (Lok et al. 2009).

Hepcidin deficiency is the key factor allowing excessive iron absorption and development of iron overload in hereditary haemochromatosis (Origa et al. 2007). Haemochromatosis is frequently observed in patients with β thalassaemia due to increased absorption of iron from the gastrointestinal tract and frequent blood transfusions. Studies have shown that interaction of hereditary haemochromatosis with thalassaemia may have a synergistic effect, resulting in increased iron intake and storage. Rees et al (1997) have demonstrated the interaction between common mutation, c.845G>A in the HFE gene and β thalassaemia intermedia phenotype (Rees et al.
Another study done by Oliveira et al (2006) revealed the higher frequency of inheritance of the c.845G>A mutation in the HFE gene among β thalassaemia trait individuals may contribute to the worsening of their clinical picture (Oliveira et al. 2006). According to Melis et al (2002) β thalassaemia carriers who are homozygous for the variant allele c.187C>G have higher serum ferritin levels than carriers with the wild type allele (Melis et al. 2002). Piperno et al (2000) have demonstrated that β thalassaemia trait aggravates the clinical picture of c.845G>A homozygotes, favoring higher rates of iron accumulation and development of severe iron-related complications (Piperno et al. 2000).

1.10.2 Pathophysiology of iron overload in β thalassaemia

Pathophysiology of iron overload in patients with β thalassaemia major has been explained at length in the medical literature. According to Origa et al (2007) and Papanikolaou et al (2005) iron overload is mainly attributed to blood transfusions and partly due to increased gastrointestinal absorption (Papanikolaou et al. 2005; Origa et al. 2007; Cao et al. 2010). In patients with β thalassaemia major due to regular transfusions, it is estimated, that approximately 6-8 grams of iron is accumulated in the body per year (Origa et al. 2007). Unlike in patients with β thalassaemia major, in patients with β thalassaemia intermedia, the main cause for the iron overload is the increased intestinal absorption secondary to chronic anaemia and ineffective erythropoiesis (Origa et al. 2007).

Hepcidin; a small peptide produced in the liver is the key iron regulatory hormone in iron homeostasis. It inhibits iron absorption from the intestine and iron release from hepatic stores and reticuloendothelial cells, thus reduces the levels of elemental iron in the circulation (Papanikolaou et al. 2005; Cao et al. 2010). The expression of hepcidin is increased in iron
overloading conditions and in inflammation whereas the level declines with anaemia and hypoxia (Origa et al. 2007; Cao et al. 2010). It is found that the patients with thalassaemia major and intermedia have inappropriately low levels of hepcidin in their circulation hence increasing the intestinal absorption of iron (Papanikolaou et al. 2005; Rund et al. 2005).

1.10.3 Iron overload and evaluation of iron overload

The degree of iron overload can be evaluated by multiple parameters. Most commonly used methods for assessing the iron overload include measurement of serum ferritin and liver iron concentration.

Estimation of liver iron concentration is the “gold standard” method for assessing body iron stores as it can predict the total body iron stores accurately (Beutler et al. 2003; Musallam et al. 2011). This can be measured either invasively or non-invasively. In the invasive method liver iron concentration is measured by liver biopsy; but this can be inaccurate due to liver fibrosis, cirrhosis or uneven distribution of iron. Superconducting quantum interface device (SQUID) or Magnetic resonance imaging (MRI) can be used to assess the liver iron concentration in a non-invasive way (Beutler et al. 2003).

Plasma ferritin concentration is often used for the indirect estimation of body iron stores. Ferritin is the main storage form of iron and is synthesized in the liver and secreted into the plasma in small amounts (Brittenham et al. 1993). Under normal circumstances, plasma ferritin concentration is related to the magnitude of body iron stores because, the amount of plasma ferritin synthesized and secreted is proportional to the amount of ferritin produced in the internal iron storage pathway (Brittenham et al. 1993). When there are no complicating factors, plasma
Ferritin level decreases with depletion of storage iron and increases with accumulation of storage iron.

Ferritin is an acute phase protein in which the levels may be non-specifically elevated in systemic effects of inflammation. Therefore in conditions such as an acute infection and chronic inflammatory disorders ferritin is expected to be elevated. Both acute and chronic liver damage, destruction of other ferritin rich tissues can increase the serum ferritin level due to release of tissue ferritin from damaged parenchymal cells and also due to inflammatory process itself. Hepatic dysfunction, malignancies, hemolysis and ineffective erythropoiesis are some other conditions where ferritin levels are expected to be high. Serum ferritin levels can be depleted in ascorbate deficiency independent to that of decrease in iron stores (Brittenham et al. 1993; Beutler et al. 2003). Biochemical analysis of C-reactive protein (CRP), serum glutamic-pyruvic transaminase (SGPT) and the erythrocyte sedimentation rate (ESR) is helpful to exclude co-existing infections, liver parenchymal disease and inflammation respectively.

The precise diagnosis of hyperferritinemia is very difficult and needs a detailed medical history, biochemical analysis of blood and sometimes genetic analysis as well (Estevao et al. 2011). Estimation of iron overload in thalassaemia is relied upon serum ferritin measurements regardless of evidence showing that it does not always correlate with body iron concentrations (Origa et al. 2007).
1.11 HFE gene

1.11.1 Introduction

The HFE gene, originally called human histocompatibility leucocyte-H (HLA-H), is a non-peptide binding major histocompatibility complex class I homologue. HFE gene encodes a β2-microglobulin associated protein and it binds with β2 microglobulin and modulates iron transport by binding with the transferrin receptor-1 (TfR1) on the cell surface in a pH-dependent manner (Drakesmith et al. 2002; Kaur et al. 2003). Binding with TfR1 occurs in competition with transferrin and in vitro; this reduces cellular iron by reducing iron uptake. In vivo HFE gene is strongly expressed by liver macrophages and intestinal crypt cells, which behaves as if they are relatively iron-deficient, in hereditary haemachromatosis (Drakesmith et al. 2002).

According to Drakesmith et al (2002), the wild type HFE protein raises cellular iron by inhibiting iron efflux from the monocyte/macrophage cell line THP-1. This study has concluded that the HFE gene has two mutually exclusive functions, binding to TfR1 in competition with transferring and inhibition of iron release (Drakesmith et al. 2002).

1.11.2 Genomic structure

HFE gene was first identified in 1996 by Feder et al (1996) and the genomic region was mapped to chromosome 6; HLA-H region (Feder et al. 1996). According to Totarto et al (1998), the locus was further defined and mapped to 6p22 which spans approximately 9.5 Kb in length (Totaro et al. 1998). The precise location of the HFE gene, is 6p21.3 and at least nine alternative spliced variants have been described for this gene (Roy et al. 2001). The open reading frame has six exons and an additional non-coding exon is located within the 3’ UTR. The sizes of the seven introns range from 160 bp (intron 4) to 3 Kb (intron 1). Human HFE gene promoter has
structural features in common with several housekeeping genes. It does not have classical TATA and CAAT boxes and has a region relatively high in GC content (64%) from base 1 to -78 (Totaro et al. 1998).

1.11.3 Mutations/ Polymorphisms

Two common missence mutations have been identified in the HFE gene in hereditary haemochromatosis patients. They are c.845G>A (C282Y>G>A) and c.187C>G (H63D). Considerable ethnic variation is observed in the distribution of HFE gene mutations. World wide literature has shown that the c.845G>A mutation is mainly restricted to North European populations and has low prevalence in African, Australian and Asian populations (Merryweather-Clarke et al. 1997; Karimi et al. 2004; Elmrghnì et al. 2011) (Table 1.2). In the north European population the allele frequency can be even as high as 10% (Rees et al. 1997). The distribution of c.845G>A mutation overlaps with populations where haemochromatsis has been reported (Merryweather-Clarke et al. 1997; Rochette et al. 1999). Hereditary haemochromatosis is rare in India and so far HFE mutations have not been explored comprehensively (Kaur et al. 2003). The allele frequency of the c.187C>G mutation is greatly variable worldwide, highest being reported in Spain (Melis et al. 2002).

<table>
<thead>
<tr>
<th>Region</th>
<th>N</th>
<th>H63D allele frequency</th>
<th>C282Y frequency</th>
<th>References</th>
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<td><strong>Europe</strong></td>
<td></td>
<td></td>
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<tr>
<td>North</td>
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<td></td>
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</tr>
<tr>
<td>North Ireland</td>
<td>404</td>
<td>14.10%</td>
<td>9.9%</td>
<td>Murphy, 1998</td>
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<tr>
<td>Denmark</td>
<td>200</td>
<td>12.75%</td>
<td>6.75%</td>
<td>Steffen, 1998</td>
</tr>
<tr>
<td>Norway</td>
<td>94</td>
<td>11.20%</td>
<td>6.4%</td>
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<td>3.84%</td>
<td>Cordoso, 1998</td>
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<td>20.40%</td>
<td>3.09%</td>
<td>Sanchez, 1998</td>
</tr>
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<td>Turkey</td>
<td>70</td>
<td>13.57%</td>
<td>0%</td>
<td>Clarke, 1997</td>
</tr>
<tr>
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</tr>
<tr>
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<td>118</td>
<td>8.5%</td>
<td>0%</td>
<td>Clarke, 1997</td>
</tr>
<tr>
<td>North</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>171</td>
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<td>9.0%</td>
<td>Aguiler, 1997</td>
</tr>
<tr>
<td>Benghazi-Libya</td>
<td>100</td>
<td>17%</td>
<td>0%</td>
<td>Current study</td>
</tr>
<tr>
<td>South</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>39</td>
<td>1.3%</td>
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</tr>
<tr>
<td>Senegal</td>
<td>130</td>
<td>0%</td>
<td>0%</td>
<td>Clarke, 1997</td>
</tr>
<tr>
<td>Kenya</td>
<td>78</td>
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</tr>
<tr>
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<td>1.9%</td>
<td>0%</td>
<td>Clarke, 1997</td>
</tr>
<tr>
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<td>0.7%</td>
<td>0%</td>
<td>Clarke, 1997</td>
</tr>
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<tr>
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<td>Clarke, 1997</td>
</tr>
<tr>
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<td>90</td>
<td>2.8%</td>
<td>0%</td>
<td>Clarke, 1997</td>
</tr>
<tr>
<td>Taiwan</td>
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<td>0%</td>
<td>Clarke, 1997</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia (aboriginals)</td>
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<td>0%</td>
<td>0%</td>
<td>Clarke, 1997</td>
</tr>
<tr>
<td>Australia (aboriginalts)</td>
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<td>0.6%</td>
<td>0%</td>
<td>Clarke, 1997</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>54</td>
<td>6.5%</td>
<td>0%</td>
<td>Clarke, 1997</td>
</tr>
<tr>
<td>Jamaica</td>
<td>90</td>
<td>2.2%</td>
<td>1.1%</td>
<td>Clarke, 1997</td>
</tr>
<tr>
<td>United States</td>
<td>5171</td>
<td>13.5%</td>
<td>5.4%</td>
<td>Karen, 2001</td>
</tr>
</tbody>
</table>

According to Feder et al (1996), c.845G>A mutation is the commonest one out of the two mutations and has been detected in 83% of the study population in their study. The mutational change is, G > A transition at nucleotide 845 of the open reading frame that results in a Cysteine to Tyrosine substitution at amino acid 282(Feder et al. 1996). The c.845G>A mutant protein is primarily an intracellular protein which alters the conserved cysteine residues in the wild type.
HFE protein thereby completely disturbing HFE-β2 microglobulin interaction (Feder et al. 1998).

The second missense variant; the c.187C>G caused by C > G transversion at 187 nucleotide of the open reading frame. This mutation occurs in exon 2, which results in a histidine to aspartic acid substitution at position 63 (Feder et al. 1996; Takeuchi et al. 1997). However the implication of this second mutation was controversial in early days as it was detected in a lower frequency in patients with hereditary haemochromatosis than the c.845G>A mutation and occurs at a high frequency in the general population, and therefore considered as a genetic polymorphism (Takeuchi et al. 1997). Unlike the c.845G>A mutation, c.187C>G mutation in vitro alters the HFE gene’s ability to reduce the affinity between TfR and Fe bound-transferrin (Melis et al. 2002).

However the significance of the c.187C>G mutation as a disease causing variant has been established by clinical and molecular genetic studies. According to Feder et al (1996) there is an excess of c.187C>G alleles among hereditary haemochromatosis patients. Compound heterozygous state for the c.845G>A; c.187C>G mutations was found in these patients; providing convincing evidence for the c.187C>G variant as being a strong candidate for hereditary haemochromatosis (Feder et al. 1996). Beutler et al (1997) concluded that the c.845G>A mutation was the main but not the only mutation in the HLA-H region that gives rise to hereditary haemochromatosis. The compound heterozygous state of the c.187C>G mutation along with the c.845G>A mutation is also significant as a causative variant in hereditary haemochromatosis (Beutler 1997). In addition, in an analysis done by Fairbanks et al (1998) it was confirmed that the c.187C>G variant is an independent risk factor for hereditary haemochromatosis (Fairbanks et al. 1998).
A large population study done by Burt et al (1998) has shown that serum iron indices, particularly transferrin saturation was higher in subjects heterozygous for c.187C>G allele than in those with wild type allele (Burt et al. 1998). Therefore the causative role of the c.187C>G mutation in iron overload and hereditary haemochromatosis has been established even though the penetrance of this allele has not yet been defined.

The homozygosity for the c.187C>G allele can be considered as a genetic variant which increases iron overload when associated with other genetic conditions such as β thalassaemia (Melis et al. 2002). The association between hereditary haemochromatosis and β thalassaemia has been dealt in a previous chapter of this dissertation.

1.12 Bone disease and β-thalassaemia

Progressive osteopenia and osteoporosis is another common complication seen in young adult patients with β thalassaemia. The average reported frequency of osteoporosis in patients with thalassaemia major is 90% (range 52-96%) (Origa et al. 2005). This complication can be seen in both well and inadequately transfused patients and reasons for this being, anaemia causing bone marrow expansion, hypogonadism, diabetes mellitus, hypothyroidism, hypoparathyroidism, cardiac dysfunction and presence of genetic predisposing factors (Cao et al. 2010).

Bone disease is defined by reduced bone mineral density (BMD) which is measured by dual-energy X-ray absorptiometry (DXA) (Thein 2004). Osteoporosis is characterized by low bone mass and deterioration of micro architecture of bone tissue, leading to enhanced bone fragility and higher tendency to fracture development (Origa et al. 2005). Osteoporosis is defined by BMD and is present when the BMD is <2.5 SD of the mean value in young adults (T score < -2.5), and osteopenia is present when T score is between -1 and -2.5 (Origa et al. 2005).
According to the World Health Organization, the diagnosis of postmenopausal osteoporosis is based on the presence of a BMD T-score 2.5 SD or greater below the mean for young women. The International Society of Clinical Densitometry (ISCD) uses the same BMD criteria for the definition of osteoporosis in males, premenopausal women and children (Voskaridou et al. 2004).

Bone disease can manifest as; diffuse bone pain especially in the lower back, vertebral fractures, cord compression, spontaneous fractures and femoral head necrosis (Thein 2004).

Bone mass is under strong genetic control which includes multiple loci (Grant et al. 1996; Perrotta et al. 2000; Mann et al. 2001; Jin et al. 2009). During the last few years many studies have recognized the importance of genetic factors in the regulation of Bone Mineral Density (BMD). It has been estimated that 50-85% of BMD is under genetic regulation (Origa et al. 2005). Several candidate genes including oestrogen receptor gene, vitamin D receptor gene (VDR), collagen, type I, alpha 1 (COL1A1) and collagen, type I, alpha 2 (COL1A2) genes, and transforming growth factor β1 gene (TGFβ1) have been implicated in this aspect (Thein 2004). A polymorphism in intron 8, Bsm1, in the VDR gene is associated with progressive osteoporosis and osteopenia in patients with β thalassaemia (Dresner Pollack et al. 2000). Besides Ferrara et al (2002) have shown a significant association between BMD and osteopenia by another polymorphism in the exon 2 of the VDR gene; Fok 1 (Ferrara et al. 2002). Polymorphisms in the ESR1 gene; XbaI and PvuII are also associated with osteoporosis and osteopenia (Gennari et al. 2005). Of these multiple genes, COL1A1 gene will be discussed in detail in the next few sections of this dissertation.
1.13 COL1A1 gene

1.13.1 Introduction

Type 1 collagen is the major protein of the bone matrix and is the most abundant type of collagen in the human body. This protein helps to strength and support many tissues of the body including bone, cartilage, tendon, skin and sclera. It is encoded by two genes; COL1A1 and COL1A2. COL1A1 gene encodes the α1 (I) protein chain of type I collagen (Mann et al. 2001). Collagen synthesis begins as pro-collagen molecules and each rope like pro-collagen molecule is made up of three chains; two pro α1 (I) chains which are encoded by the COL1A1 gene and one pro α2 (I) chain encoded by the COL1A2 gene (van Dijk et al. 2011). After pro collagens are processed; the mature collagen is formed as long, thin fibrils. Within these fibrils collagen molecules are cross-linked to one another and formation of these cross-links produces strong type I collagen (van Dijk et al. 2011).

1.13.2 Genomic structure

COL1A1 gene is mapped to chromosome 17 at an exact locus of 17q21.31-q22 (Origa et al. 2005). This gene consists of 51 exons and >17kb in length. (Fig: 1.7).
Fig: 1.7 The COL1A1 gene locus with all coding and non-coding regions to scale. DNA sequenced region in human includes 1223bp of the promoter (striped box), 263bp of the 5’ and 3’ mRNA untranslated regions(white boxes), the 4392bp of coding sequence for all 51 exons (black boxes), with non coding region in between (*). (Stover, D. A. and B. C. Verrelli (2011). "Comparative vertebrate evolutionary analyses of type I collagen: potential of COL1a1 gene structure and intron variation for common bone-related diseases." Mol Biol Evol 28(1): 533-542)

DNA sequence region of the promoter region of COL1A1 gene includes 1,223 bp; 263 bp of the 5’ and 3’ mRNA untranslated regions, and 4,392 bp of coding sequence of all 51 exons. There is a 496 bp gap in the collected sequence of intron 25 for the presence of an Alu element.

Over 400 human disease-associated mutations have been identified within the COL1A1 gene and majority of them are linked to osteoporosis. Most of these disease-associated mutations involve in the protein coding region, specifically within the triple helix domain. This triple helix domain is composed of repeating amino acid sequences with glycine in every third position separated by proline residues. This repetition is essential as it facilitates the triple helix domain to wind into its compact structure in type I collagen (Stover et al. 2011). Disease associated mutations resulting in substitution of the glycine residues with other amino acids cause severe phenotype and those causing alteration of the length of the triple-helix domain causes less severe phenotype (Stover et al. 2011).
The non-coding region of the \textit{COL1A1} gene is functionally important when disease causing mutations are considered. The c.104-441G>T polymorphism in the first intron gives rise to bone disease (Stover \textit{et al}. 2011).

1.13.3 Mutations/ Polymorphisms

Most studies have concluded that the c.104-441G>T polymorphism in the \textit{COL1A1} gene is significantly associated with reduced bone mass and osteoporotic fractures (Grant \textit{et al}. 1996; MacDonald \textit{et al}. 2001; Mann \textit{et al}. 2001; Mann \textit{et al}. 2003; Jin \textit{et al}. 2009). This polymorphism was first described by Grant \textit{et al} (1996) and it is a G>T substitution (rs 1800012) in the regulatory region of the \textit{COL1A1} gene at a recognition site for the transcription factor Sp1 (Grant \textit{et al}. 1996; Jin \textit{et al}. 2009). Functional studies have shown that this polymorphism alters binding of Sp1 to its recognition site in DNA and is associated with disturbances in \textit{COL1A1} gene transcription and collagen protein production, hence resulting in alteration of biochemical properties of bone (Mann \textit{et al}. 2003). The c.104-441G>T polymorphism lies within the first intron of the \textit{COL1A1} gene at the position of +1245 (Gen Bank accession no.J03559) and is considered as a functional variant. Normally in the bone matrix when collagen is produced, there is 2:1 ratio of collagen type I α1 chains to α2 chains (Jin \textit{et al}. 2009). When this polymorphism is present it modulates Sp 1 binding site and \textit{COL1A1} gene regulation, resulting in excess production of collagen α1 (I) chains relative to α2 (I) chains thereby altering the structural integrity of bone leading to brittle bones (Mann \textit{et al}. 2001; Mann \textit{et al}. 2003).

According to Grant \textit{et al} (1996), at the c.104-441G>T polymorphic site, the G/T heterozygotes (Ss) had significantly lower BMD than the G/G homozygotes (SS) in a study of two populations of British women. The BMD was further reduced in T/T homozygotes (ss).
The *COL1A1* c.104-441G>T polymorphism has been recognized as an independent risk factor for genetic susceptibility to osteoporosis in patients with β thalassaemia major. As described by Perrotta *et al* (2000) they have concluded that subjects who were either homozygous or heterozygous for the variant allele G>T showed a lower BMD than the subjects with the wild type allele (Perrotta *et al*. 2000). Therefore studying this polymorphism may help to identify thalassaemia patients who are at high risk for the development of osteoporosis and pathological fractures. A study by Wonke *et al* (1998) found that approximately 30% of the thalassaemia major patients in the population to be heterozygotes (GT) and 4% to be homozygotes (GG) for this variant allele. This polymorphism was commoner in females (female to male ratio 2:1); in contrast to female patients the male patients had severe osteoporosis of the spine and hip (Wonke *et al*. 1998; Voskaridou *et al*. 2004).

This variant has a prevalence of >20% in some populations, but is absent in others. Therefore this mutation may contribute to ethnic and geographic variation in bone strength and fracture risk (Stover *et al*. 2011).

### 1.14 Other Tertiary modifiers

Ineffective hematopoiesis coupled with haemolysis leads to chronic hyperbilirubinaemia and cholelithiasis in β thalassaemia. Studies show that the level of bilirubin and the incidence of gall stones are related to a polymorphic variant in the TATA box of the promoter region of the uridine diphosphate-glucoronyltransferase IA (*UGT1A*) gene which codes the enzyme UDP-glucuronosyltransferase-1 enzyme which is important in bilirubin metabolism by the liver (Galanello *et al*. 2001). The wild type allele has six TA repeats in the promoter region, whereas the variant allele has seven TA repeats. The variant allele causes reduced expression of the *UGT1A* gene giving rise to hyperbilirubinaemia (Bosma *et al*. 1995).
Cardiac complications are a common cause of death in patients with β thalassaemia. A polymorphism in the APOE ε4 gene; Cystein -Argnine interchange is a genetic risk factor for the development of LVH in patients with β thalassaemia (Economou-Petersen et al. 1998). Other genetic defects associated with complications in β thalassaemia include genetic thrombophilia variants, Methylenetetrahydrofolate Reductase (MTHFR) 677C>T, Factor V 1691G>A (Leiden), and Prothrombin 20210G>A (Eldor et al. 2002) and hepatitis and liver cirrhosis associated with specific human leucocyte antigen alleles.

Thalassaemia as described above is a heterogeneous disorder with genetic variants playing a significant role in its pathogenesis. The role of the modifier genes in the Sri Lankan thalassaemic population has not been studied to a great extent in previous studies, especially in correlation the genetic variants with biochemical parameters. Due to financial and time constraints faced, only the primary modifier HBB gene and the two tertiary modifiers; HFE and COL1A1 genes have been selected for the investigations described in this dissertation.
1.15 Objectives

1. To determine the genotypes of patients with β thalassaemia who are on regular blood transfusions and establish the correct genetic diagnosis.

2. To determine the genotypes of the *HFE* and *COLIA1* genetic modifiers in patients with β thalassaemia who were transfusion dependent.

3. To correlate the clinical phenotype of thalassaemia with *HBB* gene mutations; serum ferritin level with *HFE* gene mutations; and BMD (Bone Mineral Density) with the *COLIA1* c.104-441G>T polymorphism.
2.0 METHODS

2.1 Ethical consideration

This study was conducted according to the Declaration of Helsinki (2008). Ethical clearance was obtained from the Ethics Review Committee (ERC) of the Faculty of Medicine, University of Colombo, Sri Lanka and Hospital Ethics Committee of the Lady Ridgeway Hospital for Children (LRH), Colombo. The study was done in collaboration with all the Paediatricians who were informed about the study before hand, hence a network of Paediatricians were involved into this study. The study has social value because it contributes to the advancement of generalizable knowledge in the field of β thalassaemia in Sri Lanka. Knowledge of the genotypes of the different modifier genes including HFE gene and COL1A1 gene in β thalassaemia patient population would enhance the knowledge in the field and facilitate in the clinical management of these patients. The study was designed appropriately to ensure scientific validity. The study was open to all clinically diagnosed patients with β thalassaemia major, thus there was fare participant selection.

The patients and parents were interviewed privately while the child was warded for the monthly blood transfusion at his or her respective ward. This was done to ensure privacy and to discuss the study privately with the investigators / participant without the presence of others. Physical examination was done in a way to ensure the privacy of the participant; this was done by conducting the examination in the privacy of an examination room with the presence of a chaperone.

Written informed consent was obtained after providing the necessary information and allowing adequate time for the parent/guardian to make the decision in private. Consent form was designed in all three languages; Sinhala, Tamil and English in order to prevent racial discrimination. Some Moors were able to give their consent in Tamil while others were able to
give in Sinhala. Since all subjects were minors, consent was obtained from parents/guardians.
The parents were given an information sheet which included all the details about the study and a
consent form to read and sign before consenting for their children to participate in the study. So
the subjects and their parents/guardians were able to read and understand the information and
make a voluntary decision to participate in the study. If the participants had any questions about
the study they were free to ask the investigator and were answered before consenting. Any
parent/guardian was free to withdraw his/her consent at any time, with no penalty or effect on
medical care or loss of benefits received by the child. All the participants were given contact
details of the investigators and the Ethics Review Committee (ERC) in case if they needed to
clarify any doubts about the study.
The data collection booklet was designed to ensure confidentiality of information gathered.
Personal data was collected to keep a track of the participants for future follow up. Soon after
collecting the personal information, the identification page was removed and filed separately.
The only identification number in the rest of the booklet was a coded subject study number
which cannot be linked to an individual without the page containing the personal information
which was kept by the principal investigator under lock and key. The electronic database
containing the data had only the subject study number thus ensuring confidentiality. The
database and the computer containing the database were password protected and will be kept
under the care of the supervisors at the end of the study. These measures were taken to ensure
that the main risk of this study, i.e. loss of confidentiality, to be minimized. No personal
information by which subjects can be identified would be released or published. The data will
not be used in a way that the subjects can be identified in any public presentation or publication.
The other risk to the subjects participating in this study was the risk of pain, bruising, and
infection at the venepuncture site. These were minimized by performing venepuncture under aseptic conditions by a trained phlebotomist.

In contrast to these risks, the subjects were benefited by participating in this study in the following ways: Firstly since they were tested for β thalassaemia mutations, their genetic diagnosis was found; as genetic testing is not freely available in Sri Lanka this was very valuable opportunity to these patients. Secondly three genetic disease modifiers were analysed; knowledge about genetic mutations affecting the phenotype would enable the Paediatricians caring for them to understand their clinical manifestations and predict disease outcomes better and make appropriate management decisions. A written summary of the results, in lay language will be posted to all subjects. In addition, subjects and parents/guardians will be provided genetic counseling about the genetic test results.

The samples and data will be stored for future studies in the field of β thalassaemia. Appropriate consent was obtained for this purpose and any future studies need to be subjected to ethical clearance prior to commencement.

### 2.2. Recruitment of subjects and study protocol

This study is a descriptive study and all the patients were recruited prospectively. Almost all the patients with β thalassaemia major who were referred to ward 2, 3, 4 and 9, Lady Ridgway Hospital, Colombo for their monthly blood transfusions were recruited to the study. This cohort consisted of 32 patients with β thalassaemia major phenotype. Prior to this recruitment ethical clearance was obtained from the Hospital Ethics Committee; Lady Ridgway Hospital, Colombo. Patients were also recruited from the Thalassaemia centre, Teaching Hospital, Anuradhapura and this cohort consisted of 83 patients who were transfusion dependent. The ethical clearance
obtained from the ERC, Faculty of Medicine, University of Colombo was considered as adequate for patient recruitments by the hospital administration.

2.2.1. Study Population

Study population consisted of 125 clinically diagnosed patients with β thalassaemia major. Since this study was a descriptive one and most studies of this nature have been done with smaller samples we believed that this sample size would be adequate to describe the phenotypes observed in these patients adequately.

2.2.2 Inclusion Criteria

Subjects who met the following criteria were recruited into the study

- Patients with a clinical diagnosis of β thalassaemia who were on regular blood transfusions.
- E/β thalassaemia patients with thalassaemia major phenotype who were on regular blood transfusions.
- Ability to provide written informed consent or in case of a minor, the availability of a parent/guardian who can provide written informed consent.

2.2.3 Exclusions criteria

Subjects who met the following criteria were not recruited into the study.

- Patients who were unable to give consent or in case of minors where a suitable parent/guardian was not available to give consent.
Patients with a clinical diagnosis of β thalassaemia who were not on regular blood transfusions.

2.2.4 Clinical evaluation and data collection

At the time of recruitment, subjects and parents/guardians were interviewed by the principle investigator to gather demographic data and clinical data (Please see the data collection booklet). Detailed clinical history including the age at which the diagnosis was made and how the diagnosis was made, past medical and surgical history relevant to β thalassaemia, drug history with a view of all the medications currently the child was on and the chelating history with transfusion regimen were documented. Family history was recorded which included a three generation pedigree; consanguinity and presence of any other family member with thalassaemia were also recorded. History of ingestion of alcohol base products or alternative medicinal products were inquired to exclude any possibility of occurrence of liver damage owing to them. Extensive clinical examination was carried out including anthropometric measurements, general examination, and systemic examination including cardiovascular and respiratory system and examination of the abdomen. According to the Tanner staging protocol, genitalia of males and females, breast examination and pubic hair distribution were assessed.

In addition to the clinical data, details of other biochemical investigations were gathered by examining medical records of the subjects. Pre transfusion haemoglobin levels were recorded in the preceding 12 months period prior to recruitment date. Mean pre transfusion haemoglobin level was calculated. Detailed transfusion history was recorded from the previous medical records and the annual blood consumption in the previous year; ml/kg was calculated in each
patient. If ECG, 2D Echo and USS abdomen findings were available those were also documented.

2.3 Biological sample collection

At the time of recruitment, a sample of 5ml venous blood was obtained from each subject for genotyping. This was done at the same time when the cannula was inserted to give the blood transfusion after admission. Additionally 2ml of blood was taken to a tube without anticoagulant to perform serum ferritin, Serum Glutamic Pyruvic Transaminase (SGPT), and C- Reactive Protein (CRP) levels and another 2ml of EDTA blood was taken for the Erythrocyte Sedimentation Rate (ESR). These samples were immediately sent to a biochemical and haematology laboratories to perform the above tests. Genotyping blood samples were stored in the refrigerator at -80°C. DNA extraction was performed using commercial kits. Genotyping was done for HBB, HFE and COL1A1 genes using published methods. (Takeuchi et al. 1997; Mann et al. 2001; Old et al. 2001). The left over samples after genotyping were stored in a -80°C freezer under the supervision of the supervisors of this study indefinitely for future research into genetics of β thalassaemia. Appropriate consent was obtained for this purpose.

2.4 Bio chemical Testing

All the investigations including Serum Ferritin, CRP, ESR and SGPT were done in one biochemical laboratory in order to minimize laboratory errors. Serum ferritin was done by a solid-phase, two site chemiluminescent enzyme immunometric assay. This test has been standardized in terms of the WHO second international standard for ferritin. Paediatric and adolescent reference ranges were taken according to the published laboratory standards (refer laboratory
reference ranges Table 2.1 and Table 2.2). **Table 2.1 Adult reference range for Serum Ferritin (ng/ml)**

<table>
<thead>
<tr>
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<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>28</td>
<td>397</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>159</td>
</tr>
</tbody>
</table>

**Table 2.2 Paediatric and Adolescent reference range (ng/ml)**

<table>
<thead>
<tr>
<th>Age</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1 day-6 weeks</strong></td>
<td>Up to 400</td>
<td>Up to 400</td>
</tr>
<tr>
<td><strong>7 weeks – 1 year</strong></td>
<td>10-95</td>
<td>10-95</td>
</tr>
<tr>
<td><strong>1 year – 9 years</strong></td>
<td>10-60</td>
<td>10-60</td>
</tr>
<tr>
<td><strong>10 years – 18 years</strong></td>
<td>10-300</td>
<td>10-70</td>
</tr>
</tbody>
</table>

The reference range for SGPT and CRP were 0-40U/L and 0-5.0mg/l respectively. ESR was done using standard Westergren method and the reference ranges were as follows in table 2.3 and table 2.4 according to the age and sex (Lewis S.M. 2006).
Table 2.3 The mean ESR values for age distribution according to Westergren method


<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Mean value (/mm for the 1&lt;sup&gt;st&lt;/sup&gt; Hr)</th>
</tr>
</thead>
<tbody>
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<td>Westergren</td>
<td></td>
</tr>
<tr>
<td>4-7</td>
<td>13</td>
</tr>
<tr>
<td>8-14</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Table 2.4 The mean ESR value for sex distribution according to Westergren method *(Lewis S.M., B. B. J., Bates I., Ed. (2006). *Dacie and Lewis Practicle Haematology, Elsivier*)

<table>
<thead>
<tr>
<th>Men (Age in years)</th>
<th>ESR in mm for the 1&lt;sup&gt;st&lt;/sup&gt; hrs</th>
<th>Women (Age in years)</th>
<th>ESR in mm for the 1&lt;sup&gt;st&lt;/sup&gt; hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-50</td>
<td>10 or &lt;</td>
<td>17-50</td>
<td>12 or &lt;</td>
</tr>
<tr>
<td>51-60</td>
<td>12 or &lt;</td>
<td>51-60</td>
<td>19 or &lt;</td>
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<tr>
<td>61-70</td>
<td>14 or &lt;</td>
<td>61-70</td>
<td>20 or &lt;</td>
</tr>
<tr>
<td>&gt;70</td>
<td>30 or &lt;</td>
<td>&gt;70</td>
<td>35 or &lt;</td>
</tr>
</tbody>
</table>
2.5 Molecular genetic testing

2.5.1 DNA extraction

5ml of venous blood was obtained from all subjects. Blood was collected into EDTA containing tubes and stored at -80°C prior to DNA extraction. DNA extraction was done using Promega Wizard® Genomic DNA purification kit according to the manufacturer’s protocol. The Promega Wizard® Genomic DNA purification procedure was carried out as follows: 300 μl of whole blood was added to 900 μl of cell lysis solution. The mixture was incubated for 10 minutes at room temperature to lyse the red blood cells. Following incubation, the mixture was centrifuged at 14,000 rpm for 20 seconds. The supernatant was discarded without disturbing the white pellet formed. This was followed by vigorous vortexing for approximately 10-15 seconds until the white blood cells were resuspended. Next 300 μl of nuclei lysis solution was added to the microcentrifuge tubes containing the resuspended cells. This solution was pipetted 5-6 times to lyse the white blood cells. The extraction process next involved addition of 1.5 μl of RNase solution to the nuclear lysate and incubate at 37°C for 15 minutes and cooling back to room temperature. This was followed by addition of 100 μl of protein precipitation solution and vortexing vigorously for 10-20 seconds. Next the DNA extraction process continued by centrifugation at 14,000 rpm for 3 minutes and the supernatant was transferred to 1.5μl microcentrifuge tubes containing 300 μl of isopropanol at room temperature. This mixture was then gently mixed until thread-like strands of DNA formed a visible mass. This was again centrifuged at 14,000 rpm for 1 minute and the supernatant was discarded. Next 300 μl 70% ethanol was added to the DNA at room temperature and the tubes were gently inverted to wash the DNA pellet and the sides of the microcentrifuge tubes. Then the ethanol was carefully aspirated and the tubes were inverted on clean absorbent paper and the pellets were air-dried for
10-15 minutes. Lastly, 100 μl of DNA rehydration solution was added to the microcentrifuge tubes containing DNA and the DNA was rehydrated by incubating the solution overnight at 4°C. The eluted DNA samples were labeled and stored at -20°C. These samples were used for the PCR experiments, which is described next. The left over blood samples were stored in a -80°C freezer under the supervision of the supervisors of this study indefinitely for future research into genetics of β thalassaemia.

2.5.2 HBB Gene

HBB gene was first screened by the ARMS (Amplification Refractory Mutation System) PCR method (Newton et al. 1989) for five common prevailing mutations in the Sri Lankan population. According to Fisher et al (2003); c.92+5G>C, c.92+1G>A and c.79G>A are the three most common mutations in our population (Fisher et al. 2003). Another two common mutations which have been described in the Sri Lankan β thalassaemia population were also included in the study, those were c.126_129delCTTT and g.71609_72227del619. Standard protocols published elsewhere were used (Newton et al. 1989; Old et al. 2001).

2.5.2.1 ARMS-PCR (Amplification Refractory Mutation System - Polymerase Chain Reaction)

PCR method of amplification refractory mutation system (ARMS) was carried out according to the method described by Newton et al (1989) (Newton et al. 1989). The target DNA was amplified using the primers complementary to the five common β - chain mutations described earlier. (Table 2.5 and Table 2.6). Genotyping was performed by wild type primers in patients who were detected to have common mutations selected to the study. Another set of primers; CNT_R and CNT_F were used to amplify the 861 bp fragment of the distal part of the β - globin
gene, to serve as an internal control for the PCR and to detect the g.71609_72227del619 mutation.

2.5.2.2 Conditions for PCR

PCR for the β - chain mutations was carried out in a 25 µl reaction volume containing 100 ng genomic DNA, 50 pmol primers, 0.1 mM dNTPS, 5 µl of reaction buffer ( 50 mM Tris HCl, 250 mM KCl), 1.5 mM MgCl₂ and 1.5 Unit Taq. The amplification on the thermal cycler was carried out which consisted of an initial denaturation at 94°C for 5 minutes followed by 30 cycles; each cycle consisted of denaturation at 94°C for 1 minute, primer annealing at 65°C for 1 minute and DNA extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes.

Table 2.5 ARMS-PCR primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.92+1G&gt;A</td>
<td>5' TTAAACCTGTCTTGTAAACCTTGATACCGAT 3'</td>
</tr>
<tr>
<td>c.92+5G&gt;C</td>
<td>5' CTCCTTAAACCTGTCTTGTAAACCTTGTTAG 3'</td>
</tr>
<tr>
<td>c.79G&gt;A</td>
<td>5' TAACCTTGATACCAACCTGCCAGGGCGTT 3'</td>
</tr>
<tr>
<td>c.126_129delCTTT</td>
<td>5' GAGTGGACAGATCCCCAACAGGACTCAACCT 3'</td>
</tr>
<tr>
<td>Common_F</td>
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</tr>
<tr>
<td>CNT_R</td>
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<tr>
<td>CNT_F</td>
<td>5' CAATGTATCAGCTCTTTGCACC 3'</td>
</tr>
</tbody>
</table>
Table 2.6 Primers used for genotype the respective mutations.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
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<td>c.92+1G&gt;A Wild</td>
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</tr>
<tr>
<td>c.92+5G&gt;C Wild</td>
<td>5' CTCCTAAACCTGTCTTGTAACCTTTGTTAC 3'</td>
</tr>
<tr>
<td>c.79G&gt;A Wild</td>
<td>5' TAACCTTGATAACCTGGCAGGCGTC 3'</td>
</tr>
<tr>
<td>c.126_129delCTTT Wild</td>
<td>5' GAGTGGACAGATCCCCAAAGGACTCAAAGA 3'</td>
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<tr>
<td>Common_F</td>
<td>5' ACCTCACCTGTGGAGGCAC 3'</td>
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<td>HBB-COMM-2</td>
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</tr>
<tr>
<td>CNT_R</td>
<td>5' GAGTCAAAGGCTGAGGAGTCAGGA 3'</td>
</tr>
<tr>
<td>CNT_F</td>
<td>5' CAATGTATCATGCTCTTTGACC 3'</td>
</tr>
</tbody>
</table>

2.5.2.3 Agarose gel electrophoresis

The presence of the PCR amplified products were confirmed by electrophoresing a fraction of the reaction mix on an agarose gel. Amplified product (8µl) was subjected to electrophoresis on a 3.0% agarose gel; 0.5 µg/ml of ethidium bromide were also added to each gel. 60mA current and 100V of voltage was applied to move the negatively charged nucleic acid molecules. Subsequently the patterns created by different size DNA fragments were visualized by examining the agarose gel on a UV transilluminator.

2.4.2.4 Automated Sequencing

The samples which gave negative results and heterozygous for one mutation from the ARMS PCR were amplified using three overlap M13 sequence tag primers: They were exon 1, exon 2 and the flanking region (P1), exon 2 and the flanking region (P2) and exon 3 and the flanking region (P3) using PCR primes described elsewhere (Huisman 1997; Weatherall 2001). (Fig 2.1)
PCR conditions were 25 µl reaction volume containing 100 ng genomic DNA, 25 pmol primers, 0.2 mM dNTPS, 5 µl of reaction buffer (50 mM Tris HCl, 250 mM KCl), 1.5 mM MgCl₂ and 1 Unit Taq. The amplification of P1 region on the thermal cycler was carried out consisting of an initial denaturation at 95°C for 5 minutes followed by 35 cycles; each cycle consisted of denaturation at 95°C for 30 seconds, primer annealing at 58°C for 30 seconds and DNA extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes.

The amplification of P2 and P3 regions on the thermal cycler was carried out consisting of an initial denaturation at 95°C for 5 minutes followed by 35 cycles; each cycle consisted of denaturation at 95°C for 30 seconds, primer annealing at 60°C for 30 seconds and DNA extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes.

**Fig: 2.1 Relative locations of the PCR primers complimentary to the HBB gene.**

The PCR products were purified using ExonucleaseI (ExoI) and Shrimp Alkaline Phosphatase (SAP) (Fermentas) according to the manufactures instructions.

The DNA sequencing was performed with the M13 primers using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing fragments were purified using Centriflex® gel filtration cartridge Edge Biosystem (USA).
Filtered sequencing fragments were analyzed using an ABI 3130 Genetic Analyzer. This system works on the basis of the Sanger dideoxy chain termination principle. It contains four ddNTPs with different fluorescence labels and AmpliTaq® DNA polymerase. All the patients were tested for mutations in the *HBB* gene as follows: At the end of the reaction PCR products of varying sizes all terminating with a fluorescent-labelled dideoxy nucleotide are generated. The sequence is then read out automatically by capillary electrophoresis of cycle sequencing products through an automated sequencing machine. DNA sequencing analysis was done using Chromas Lite 2.01 software (Technelysium Pty Ltd 2008).

The published human *HBB* gene sequence (GenBank ID NG_00007.3) was used for the comparison of the sequences generated from the participants and to confirm the presence of any mutations in the *HBB* gene other than the common mutations detected by the ARMS PCR method.

### 2.5.3 *HFE* Gene

#### 2.5.3.1 ARMS - PCR

A rapid and a simple method was used to detect the c.845G>A (rs.1800562) and c.187C>G (rs.1799945) mutations in the *HFE* gene. It was based on an allele specific PCR (AS-PCR) method which was a multiplex PCR. Takeuchi *et al* (1997) in his population based study included four different oligonucleotide primers; two non allele specific primers and two allele specific primers (Takeuchi *et al*. 1997). (Table 2.7)
Table 2.7 Primers used to detect c.845G>A mutation in the ARMS PCR

<table>
<thead>
<tr>
<th>Two non-allele specific primers</th>
<th>Two allele specific primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1: AAGCAGCCAATGGATGCAAG</td>
<td>Rw: CCTGGGTGCTCCACCTGGC</td>
</tr>
<tr>
<td>R1: CCACTGATGACTCCAATGACTA</td>
<td>Fm: GGGAAGAGCAGAGATACGTA</td>
</tr>
</tbody>
</table>

We also used the same set of primers for the c.845G>A mutation. For the c.187C>G mutation, an in house method was used where we designed the primes at the Genetics laboratory of the Human Genetics Unit, Faculty of Medicine, University of Colombo. The primers were as follows.

63Fw -AGCTGT TCGTGTCTATGATC
63F4- AGCTGTTCGTGTTCTATGATG
63R3- CTGTGGTTGTGATTTTCCATAA

2.5.3.2 Conditions for PCR

PCR reaction mixtures consisted of 3 μL of the sample DNA as template, 200 μmol/L of each dNTPs, 200 nmol/L Rw, 1000 nmol/L Fm, 1000 nmol/L F1, 1000 nmol/L R1 and 0.7 U Amplitaq Gold for the hot start PCR on 20 μL total volume. Conditions for the amplification were initial denaturation at 95°C for 11 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 64°C for 30 seconds and extension at 72°C for 30 seconds with a final extension at 72°C for 10 minutes(Takeuchi et al. 1997).
2.5.3.3 Agarose gel electrophoresis

Presence of PCR amplified products were confirmed by electrophoresing a fraction of the reaction mix on an agarose gel. 60mA current was applied to move the negatively charged nucleic acid molecules. Subsequently the patterns created by different sized DNA fragments were visualized by examining the agarose gel on a UV transilluminator.

2.5.4 COL1A1 Gene

2.5.4.1 Polymerase Chain Reaction (PCR)

Primer pairs described elsewhere (Mann et al. 2001) were used to amplify the coding regions of COL1A1. Polymerase chain reactions (PCR) were carried out in 25 µl reaction volume containing 100ng genomic DNA, 25pmol primers, 0.2mM dNTPs, 5 µl of reaction buffer (50mM Tris HCl, 250mM KCl) 1.5mM MgCl2 and 1 Unit Taq. The first round of nested-PCR was carried out with the following primer set: 5’- TAACTTCTGGACTATTTGCGGACTTTTTGG- 3’ (p1) and 5’ - GGGCGAGGGAGGAGGGAA- 3’ (p3), which amplifies a 283-bp region surrounding the polymorphic site in the first intron of COL1A1. The thermal cycling protocol was: 95°C for 2 minutes, 58°C for 1 minute (one cycle), 95°C for 50 seconds, 58°C for 1 minute (13 cycles); 95°C for 50 seconds, 58°C for 1 minute, 72°C for 3 minutes (one cycle). Undiluted products from this reaction were used as template in a second-round PCR using a forward primer of the same sequence as in the first round and the following reverse primer: 5’ - GTCCAGCCCTCTCATCTGGCC - 3’ (p2), which introduced a restriction site for the enzyme Mscl in products derived from the c.104-441G>T allele.

For the second round of PCR, the following thermal cycling protocol was used: 94°C for 3 minutes, 62°C for 10 seconds, ramping at 1°C per 10 seconds to 72°C, 72°C for 15 seconds (one
cycle); 94°C for 50 seconds, 62°C for 10 seconds, ramping at 1°C per 10 seconds to 72°C, 72°C for 15 seconds, (26 cycles); 94°C for 50 seconds, 62°C for 10 seconds, ramping at 1°C per 10 seconds to 72°C, 72°C for 5 minutes (one cycle).

2.5.4.2 Restriction Fragment Length Polymorphism (RFLP)

Amplified product (8µl) was electrophoresed on a 1.5% Agarose gel to confirm the presence of PCR products. After electrophoresis (60mA, 100V), the DNA was visualized under UV illumination using a transilluminator, followed by digestion of 10 µl of the PCR product with 1 U of the MscI enzyme Promega (Madison, WI USA) and 1.5 µl of its 10× reaction buffer in a 15-µl reaction volume. The mixture was incubated at 37 °C overnight. The digested products were electrophoresed on 4% standard agarose at 60mA, 100V for 50 min. The fragments were visualized by ethidium bromide under UV transilluminator.
2.6 Software tools and electronic database information

Data that were collected were entered into an electronic database which is maintained by the Human Genetics Unit, Faculty of Medicine, University of Colombo in order to organize the data. Blood and DNA of patients were stored in the Unit using subject study numbers.

The software and electronic databases used in these investigations are listed below;

- GenBank at the National Centre for Biotechnology Information (NCBI), USA was searched to obtain DNA sequences and gene mapping information. This is a free database that can be accessed online at URL: http://www.ncbi.nlm.nih.gov.

- Primer3 on the WWW for general users and for biologist programmers (Rozen and Skaletsky, 2000) was used to design oligonucleotide primers for PCR. This is a free program that can be accessed online at URL:http://www.broad.mit.edu/genome_software/other/primer3.html


2.7 Statistical methods

Phenotypic data which were entered directly into an electronic database were manually verified for errors. In the phenotypic analysis, summary values are presented as the mean [standard deviation (SD)] when data were normally distributed, and as the median (inter quartile range) when not. Student’s t-test was used to test for differences between groups where the data were normally distributed. Summary values for categorical data are presented as percentage distributions.
3.0 RESULTS

3.1 β Thalassaemia Phenotypic Data

In this chapter the analyses of the phenotypic data of the 125 patients with β thalassaemia is presented.

3.1.1 Demographic characteristics

The study population consisted of 60 (48%) male patients and 65 (52%) female patients. The age distribution of these patients varied between 0.09 years to 23.06 years with a mean age of 8.86 years (SD± 4.7) and majority of them were around 8.09 years. The mean age at which the diagnosis was made was 1 year and most of them were diagnosed by the age of 0.06 years.

The diagnostic methods that have been used to confirmed β thalassaemia are shown in the Figure 3.1. Among 125 patients with β thalassaemia major; the diagnosis was confirmed in 41 (32.8%) by HPLC; in 46 (36.8%) by Hb electrophoresis. Genetic diagnosis was available in 3 (2.4%) patients. In 30 (24%) patients; the thalassaemia diagnosis confirmation investigation results were not available at the time of data collection and 5 (4%) patients had only a clinical diagnosis of β thalassaemia major, and had been transfusion dependent from infancy.
Figure 3.1: Modes of diagnosis of β thalassaemia patients in the study group.

The age of onset of blood transfusion ranged from 0.02 years to 2.01 years and in the majority; a regular transfusion regimen was initiated by the time they were 0.06 years old.

3.1.2 Family History

Analysis of the three generational pedigrees showed that third degree parental consanguinity was observed in 33 (26.4%) patients, a majority of 84 (67.2%) did not have a history of consanguinity. Family history of consanguinity was not been ascertained in 8 (6.4%) patients. Only 24 (19.2%) patients with β thalassaemia major had a positive family history of having another relative with thalassaemia major. Five (4%) patients had a history of miscarriage of another sibling in their families.
3.1.3 Clinical History

Data on the haemoglobin levels were not available in three patients. The mean pre transfusion haemoglobin level was 8.82g/dl (SD±0.85) and it ranged from 5.9g/dl to 10.65g/dl in this cohort. The majority of patients have received monthly blood transfusions with a pre transfusion haemoglobin level of 8.7g/dl.

Twenty five (20%) patients of the study group were on calcium supplements. They were either on kalzana or cal-lactate; 1 alpha treatment, alendronate therapy, kalzana+ alendronate combined treatment and 1 alpha+ cal lactate combination. Only 1 (0.8%) case was reported to have a history of bony fracture in this cohort.

Seventeen (13.6%) had undergone splenectomy and in 1 (0.8%) patient cholecystectomy had been performed. There were 7 (5.6%) patients with diabetes mellitus, 5 (4%) patients with hypothyroidism and 1 (0.8%) with hypoparathyroidism. Two patients who had diabetes mellitus had hypothyroidism as well.
3.2. Results of the study: Genetic, Biochemical and ESR

The investigation results carried out on the study population are given in Table 3.1.

Table 3.1 Results of Biochemical investigations, ESR and genotyping of HBB, HFE and COLIA1 genes.

<table>
<thead>
<tr>
<th>Thal Number</th>
<th>Serum Ferritin (ng/ml)</th>
<th>CRP (mg/l)</th>
<th>ESR (mm/1st hr)</th>
<th>SGPT (U/L)</th>
<th>HBB mutations</th>
<th>Type of allele; β'/β o</th>
<th>c.845G&gt;A</th>
<th>c.187C&gt;G</th>
<th>c.104-441G&gt;T</th>
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</thead>
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<td>46.40</td>
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<td>CG</td>
<td>GG</td>
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<td>CC</td>
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<td>CC</td>
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<td>GG</td>
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**c.92+5G>C**

**HOMO**

**β₀/β₀**

**GG**

**CC**

**β₀/β₀**

**GG**

**CC**

**β₀/β₀**

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**β₀/β₀**

**GG**

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**β₀/β₀**

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**β₀/β₀**

**GG**

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**β₀/β₀**

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**β₀/β₀**

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3.2.1 Results of the \textit{HBB} gene mutation analysis

The mutational analysis of the \textit{HBB} gene was carried out by ARMS PCR and automated sequencing. The figure 3.2 summarizes the sequential order in which the \textit{HBB} gene was tested. (Fig: 3.2)

\begin{center}
\begin{tikzpicture}
  \node (total) {Total of 125 patients with $\beta$ thalassaemia};
  \node (arms) [below of=total] {ARMS PCR}
  \node (homo_compound) [below of=arms] {Homozygous / Compound Heterozygous (n=90)}
  \node (heterozygous) [right of=arms] {Heterozygous for one mutation (n=29)}
  \node (no_mutation) [right of=arms] {No Mutation (n=6)}
  \node (automated) [below of=heterozygous] {Automated Sequencing of the P1 region}
  \node (comp_hetero) [below of=automated] {Compound Heterozygous n=2}
  \node (homo_mutation) [right of=automated] {Homozygous Mutation n=2}
  \node (no_mutation_region) [right of=automated] {No Mutation in the P1 region n=20}
  \draw [->] (total) -- (arms);
  \draw [->] (arms) -- (homo_compound);
  \draw [->] (arms) -- (heterozygous);
  \draw [->] (arms) -- (no_mutation);
  \draw [->] (heterozygous) -- (automated) node [midway, above] {n=21};
  \draw [->] (no_mutation) -- (automated) node [midway, above] {n=3};
  \draw [->] (comp_hetero) -- (automated);
  \draw [->] (homo_mutation) -- (automated);
  \draw [->] (no_mutation_region) -- (automated);
\end{tikzpicture}
\end{center}

\textbf{Fig: 3.2} \textit{HBB} gene analysis and results
3.2.1.1 ARMS PCR

Of the 125 patients studied 90 (72%) were homozygotes or compound heterozygotes for two mutations tested by the ARMS PCR assay. Twenty nine (23.2%) cases had only one mutation (heterozygous for any mutation). No mutations were detected in 6 (4.8%) cases. (Fig: 3.2). The agarose gel images of the results by the ARMS PCR are shown in figures 3.3, 3.4, and 3.5. The commonest mutation was c.92+5G>C and the second commonest was c.92+1G>A. Two hundred and nine (83.6%) alleles were detected in total out of 250 alleles in this study population. Two patients (1.6%) were compound heterozygous for c.92+5G>C; c.79G>A mutations. Genotype distribution and their frequencies are shown in Table 3.2. Previous molecular genetic test results of the HBB gene were available in 3 patients of the study group. The study findings were similar to their previous molecular genetic test results.

Fig: 3.3 Gel image showing ARMS PCR for the detection of HBB gene mutations; Primer mix B contained c.92+5G>C mutation specific primer. Primer mix C contained c.92+1G>A, c.126_129delCTTT mutation specific primers. Each mixture (B and C) contained control primers to detect NG_000007.3:g.71609_72227del619 and to act as an internal quality control. Primer mix B: 100bp ladder, B1-Thal No1 –ve, B2-Thal No21 +ve, B3-Thal No 66 +ve, B4 +ve control, B5- -ve control, B6-Thal 120 +ve, B7-Thal 125+ve. Primer mix C: 100 bp ladder, C1,C2,C3 –ve for Thal 21, Thal 66, Thal 100 respectively. C4+ve control, C6 –ve control, C6,C7 –ve for Thal 125, Thal 133 respectively.
Fig: 3.4 Gel image of g.71609_72227del619 mutation, only control primers were used (861bp). 100bp ladder, L1- Thal 134 (–ve) , L2- Thal 106 (–ve), L3- Thal 52 (+ve), L4- (-ve) control

Fig: 3.5 Gel image showing genotyping for (+ve) c.92+5G>C samples. L1, L2- Homozygous for c.92+5G>C, L3-L7- Heterozygous for c.92+5G>C, L8- (+ve) control, L9- (-ve) control
Table 3.2 Pathogenic mutations detected in 119 patients with β thalassaemia major by ARMS PCR method.

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<th>Genotype/Mutation</th>
<th>N (%)</th>
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<tr>
<td><strong>Homozygosity</strong></td>
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<tr>
<td>c.92+5G&gt;C</td>
<td>69 (55.2%)</td>
</tr>
<tr>
<td>c.92+1G&gt;A</td>
<td>7 (5.6%)</td>
</tr>
<tr>
<td>CD41/42 (-TCTT)</td>
<td>1 (0.8%)</td>
</tr>
<tr>
<td><strong>Compound Heterozygosity</strong></td>
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</tr>
<tr>
<td>c.92+5G&gt;C; c.126_129delCTTT</td>
<td>5 (4%)</td>
</tr>
<tr>
<td>c.92+5G&gt;C; IVS1-1 G&gt;A</td>
<td>5 (4%)</td>
</tr>
<tr>
<td>c.92+5G&gt;C; g.71609_72227del619</td>
<td>1 (0.8%)</td>
</tr>
<tr>
<td>c.92+5G&gt;C; c.79G&gt;A</td>
<td>2 (1.6%)</td>
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<tr>
<td><strong>Single pathogenic allele</strong></td>
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<tr>
<td>c.92+5G&gt;C</td>
<td>23 (19.2%)</td>
</tr>
<tr>
<td>c.92+1G&gt;A</td>
<td>4 (3.2%)</td>
</tr>
<tr>
<td>c.126_129delCTTT</td>
<td>2 (1.6%)</td>
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</table>

The allele frequencies of the pathogenic mutations detected by this method are tabulated in Table 3.3.

Table 3.3 The allele frequencies of pathogenic mutations detected in the *HBB* gene by ARMS PCR method

<table>
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<th>Mutation</th>
<th>N (%)</th>
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<tr>
<td>c.92+5G&gt;C</td>
<td>69.6%</td>
</tr>
<tr>
<td>c.92+1G&gt;A</td>
<td>9.2%</td>
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<tr>
<td>c.126_129delCTTT</td>
<td>3.6%</td>
</tr>
<tr>
<td>g.71609_72227del619</td>
<td>0.4%</td>
</tr>
<tr>
<td>c.79G&gt;A</td>
<td>0.8%</td>
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</table>
3.2.1.2 Automated sequencing

DNA sequencing of P1 region of the *HBB* gene was done in 24 patients. They consisted of 21 patients out of 29 heterozygotes for any mutation and 3 out of 6 patients who were negative for any common mutation by ARMS PCR. (Fig: 3.2)

Of the 24 patients sequenced, 3 pathogenic mutations were found in four patients. The findings are summarized in Table 3.4. Detected mutations were c.51delC, c.26_27insG and c.46delT (Fig 3.6, 3.7 and 3.8). Additional mutations were not detected by sequencing the P1 region in 19 of the heterozygous group. In four patients both ARMS PCR and automated sequencing of the P1 region failed to detect any mutations. Automated sequencing of the P1 region was not done in 11 patients of the study group due to technical difficulties.

Table 3.4 Pathogenic mutations detected by automated sequencing

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<th>Genotype/Mutation</th>
<th>N (%)</th>
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<tr>
<td>c.51delC</td>
<td>1 (0.8%)</td>
</tr>
<tr>
<td>c.27_28insG</td>
<td>1 (0.8%)</td>
</tr>
<tr>
<td><strong>Compound Heterozygosity</strong></td>
<td></td>
</tr>
<tr>
<td>c.92+5G&gt;C ; C46delT</td>
<td>2 (1.6%)</td>
</tr>
</tbody>
</table>


Fig: 3.6 Chromatogram view showing homozygous c.51delC mutation (Thal 012).

Fig: 3.7 Chromatogram view showing Homozygous c.27_28insG mutation (Thal 033).
3.2.2 Results of the HFE gene mutation analysis

Of the 125 cases none had the c.845G>A mutation. The c.187C>G mutation was detected in the heterozygous state in 23 (18.4%) cases. Of the study population 102 (81.6%) were homozygous for the wild type allele. The agarose gel image results are shown in Fig: 3.9 and Fig: 3.10. The c.187C>G allele frequency was 9.2% and genotype distribution was in Hardy-Weinberg equilibrium at this locus (Santiago Rodriguez 2009). (Table 3.5)
Fig: 3.9 Gel image showing the c.845G>A mutation: Lane1-L6 – patient samples for homozygous wild type variant (GG), Lane 7-Standard (–ve) control.

Fig: 3.10 Gel Image showing the c.187C>G mutation: 100bp ladder, Lane1- heterozygous for H63D mutation (CG). Lane2- CC, Lane3- CG, Lane4- CC, Lane5 CC, Lane6- (+ve ) control, Lane7- (-ve) control.
Table 3.5 Prevalence of the c.187C>G and the c.845G>A mutations in the study group (N/N – wild type)

<table>
<thead>
<tr>
<th>c.187C&gt;G genotype</th>
<th>Number of patients</th>
<th>% of the population</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.187C&gt;G/N</td>
<td>23</td>
<td>18.4%</td>
</tr>
<tr>
<td>c.187C&gt;G/ c.187C&gt;G</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>c.845G&gt;A/ c.187C&gt;G</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>N/N</td>
<td>102</td>
<td>81.6%</td>
</tr>
</tbody>
</table>

Serum ferritin levels were compared between patients with the wild type allele for c. 187C>G (CC) and the variant allele for c. 187C>G (CG) by using independent ‘student t test’ to find an association between serum ferritin levels and the genotypes. Four serum ferritin reports were not available for the analysis. Serum ferritin levels were high in both groups and relatively higher in those with the variant allele compared to those with the wild type. This difference was not statistically significant (p value= 0.865). Table 3.6 illustrates the mean serum ferritin levels with the genotyping data.

Table 3.6 Mean serum ferritin levels and genotyping data of the c.187C>G mutation. CC- homozygous for the wild type, CG- heterozygous for the variant allele.

<table>
<thead>
<tr>
<th>C&gt;G mutation</th>
<th>Number of patients</th>
<th>Mean ferritin level (ng/ml)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>98</td>
<td>4571</td>
<td>265</td>
</tr>
<tr>
<td>CG</td>
<td>23</td>
<td>4987</td>
<td>541</td>
</tr>
</tbody>
</table>
3.2.3 Results of the COL1A1 gene mutation analysis

In the COL1A1 gene the c.104-441G>T polymorphism was analyzed in 46 subjects. (Fig: 3.11). Genotype distribution was in Hardy-Weinberg equilibrium at this locus. Out of which, 6 (4.8%) were heterozygous for the variant allele (GT) and the allele frequency was 6.52%. The prevalence details are given in table 3.7.

Fig: 3.11 Gel image showing COL1A1 gene c.104-441G>T polymorphism: L1- Thal 001- GG, L2 Thal 008- GG, L3 Thal 009- GT, L4 Thal 010- GG, L5 Thal 011- GG, L6 Thal 012 GG, L7 (+ve) control , L8 (-ve) control.

Table 3.7 Genotyping results of the COL1A1 gene c.104-441G>T polymorphism. GG- homozygous for the wild type allele, GT- heterozygous for the variant allele

<table>
<thead>
<tr>
<th>Mutation</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>40 (32%)</td>
</tr>
<tr>
<td>GT</td>
<td>6 (4.8%)</td>
</tr>
</tbody>
</table>
3.3 Iron Chelation and Serum Iron Studies

The minimum age at which the iron chelation was initiated in the study population was 0.03 years and the maximum age was 5.05 years. By the time the patients were 1.06 years old majority of them were on some form of chelation therapy.

The Bar graph (Fig: 3.12) summarizes the modes of iron chelation and the percentage of patients under each category. At the time of recruitment 65 (52%) patients were on deferoxamine therapy either on intravenously or subcutaneously. Most of them were using subcutaneous pumps at home and took intravenous treatment while in hospital for monthly transfusions. Deferasirox (Asundra) was also popular as an iron chelator among this study population. There were about 43 (34.4%) patients who were on this therapy. Of the study population 14 patients were on combined therapy for the iron chelation; 3 (2.4%) patients were on both deferoxamine and deferasirox, 5 (4%) patients were on deferoxamine + deferiprone combined therapy, 6 (4.8%) patients were on monthly deferoxamine therapy with daily oral deferasirox. Two (1.6%) patients were not on any form of iron chelation; due to recently been diagnosed as β thalassaemia.
The mean serum ferritin of the study group was 4650ng/ml (SEM=237) with a skewed distribution from the mean. Ferritin reports of 4 patients were not available to the analysis. The serum ferritin levels varied between 157ng/ml to 12470ng/ml.

The mean serum ferritin level of male patients was 4654ng/ml (SEM= 346) and female patients was 4646ng/ml (SEM= 326). Although the mean serum ferritin level was higher in males, there was no statistically significant difference of the mean serum ferritin levels between the two groups ($p$ value = 0.987).

**Fig: 3.12 Modes of iron chelation and the percentages of patients.**
The frequency of transfusion was grouped into four classes. The mean serum ferritin of each group was calculated. Results were tabulated in Table 3.8.

Table 3.8 The frequencies of transfusion and the mean serum ferritin levels of each group of patients. (Group 1 = in 5 or more weekly intervals; Group 2 = in 4 – 4.9 weekly intervals; Group 3 = in 3 – 3.9 weekly intervals; Group 4 = in 2 – 2.9 weekly intervals). SEM=Standard error of mean

<table>
<thead>
<tr>
<th>Frequency of transfusions</th>
<th>Mean serum ferritin (ng/ml)</th>
<th>Number of patients</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5700</td>
<td>5</td>
<td>1502</td>
</tr>
<tr>
<td>2</td>
<td>4527</td>
<td>36</td>
<td>454</td>
</tr>
<tr>
<td>3</td>
<td>4611</td>
<td>78</td>
<td>289</td>
</tr>
<tr>
<td>4</td>
<td>5725</td>
<td>2</td>
<td>625</td>
</tr>
</tbody>
</table>

The highest serum ferritin levels were observed in Group 4 patients who received blood transfusions in 2 to 2.9 weekly intervals and the mean serum ferritin level was 5725ng/ml (SEM=625). The majority of patients; 78 (62.4%) received blood transfusions in 3 to 3.9 weekly intervals and the mean serum ferritin level was 4611ng/ml (SEM=289). Thirty six (28.8%) patients had blood transfusions in 4 to 4.9 weekly intervals and their mean serum ferritin was 4527ng/ml (SEM= 454). Mean ferritin level was 5700ng/ml in 5 patients who had received blood transfusions in 5 weekly intervals. However there is no statistically significant difference between the frequency of transfusion and mean serum ferritin measurements between the groups (p value=0.748).
The blood consumption in the previous year (ml/kg) was grouped into five classes and the calculated mean serum ferritin level of each group was compared between the groups. The results are summarized in Table 3.9.

**Table 3.9 Blood consumption in the previous year in ml/kg and the mean serum ferritin level of each group.**

<table>
<thead>
<tr>
<th>Blood Consumption(ml/kg/year)</th>
<th>Mean Ferritin level(ng/ml)</th>
<th>Number of patients</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;199</td>
<td>4345</td>
<td>2</td>
<td>3575</td>
</tr>
<tr>
<td>200-249</td>
<td>4027</td>
<td>22</td>
<td>576</td>
</tr>
<tr>
<td>250-299</td>
<td>4206</td>
<td>53</td>
<td>336</td>
</tr>
<tr>
<td>300-349</td>
<td>5392</td>
<td>31</td>
<td>383</td>
</tr>
<tr>
<td>&gt;350</td>
<td>6185</td>
<td>11</td>
<td>1015</td>
</tr>
</tbody>
</table>

Majority of patients; 53 (42.4%) had consumed 250-299 ml/kg/ of blood per year and their mean serum ferritin level was 4206ng/ml. Thirty one (24.8%) patients had received 300-349ml/kg/ of blood per year and the mean ferritin level of them was 5392ng/ml. The mean ferritin levels were 6185ng/ml and 3939ng/ml of patients who had consumed >350ml/kg and 200-249ml/kg of blood in the previous year respectively. Two (1.6%) patients who had received <199ml/kg/year of blood in the past year had mean ferritin level of 4345ng/ml. Association between mean serum ferritin and the blood consumption in the previous year between groups was statistically not significant and the $p$ value was 0.090.
4.0 DISCUSSION

4.1 HBB gene

4.1.1 ARMS PCR test results

Beta thalassaemia in Sri Lanka has been extensively studied in the context of its distribution, phenotypic variability and molecular mechanisms previously by others (de Silva et al. 2000; Fisher et al. 2003; Premawardhena et al. 2004; Premawardhena et al. 2005). In these studies, all molecular genetic testing has been carried out in genetic testing laboratories outside the country. To our knowledge this is the first large scale study of thalassaemia patients carried out in a molecular genetics laboratory in Sri Lanka using the ARMS PCR method and automated sequencing. The ARMS-PCR provides an easy, rapid and cost effective assay for direct detection of HBB gene mutations. This method is also capable of testing a large number of samples. Therefore the test was set up in order to identify the most common four mutations in the HBB gene described in previous studies in the Sri Lankan thalassaemia population. Mutations c.92+1G>A, c.92+5G>C, c.79G>A, c.126_129delCTTT and g.71609_72227del619 were detected. The initial four listed are the most commonly found mutations in Sri Lanka, while the fifth was used as a control primer.

The ARMS PCR was able to detect all 5 pathogenic mutations in the study population. Of the study group in 119 cases (95.2%) a HBB gene mutation was detected, of which 90 (72%) were homozygous and 29 (23.2%) were heterozygous for a mutation. No mutations were detected in 6 (4.8%) patients. ARMS PCR assay was able to genotype 209 (83.6%) alleles out of 250 alleles; out of which 180 (72%) were either homozygous or compound heterozygous. Therefore genetic diagnosis was established in 90 patients by ARMS PCR assay. Because this assay was able to
genotype a significant proportion of the study population it can be concluded that this can be used as a sensitive molecular diagnostic screening test for β thalassaemia in our population.

The commonest mutation detected by this assay was the c.92+5G>C mutation accounting for an allele frequency of 69.6%. The next two common mutations were c.92+1G>A and c.126_129delCTTT with allele frequencies of 9.2% and 3.6% respectively. The c.92+5G>C and c.92+1G>A alleles accounted for 78.8% of the alleles tested and the c.79G>A allele was rare, accounted for only 0.8% (n=2). This is in contrast to the finding of Fisher et al (2003) who described a much higher allele frequency of the c.79G>A in the Sri Lankan thalassaemia population (Fisher et al. 2003).

In this cohort the two cases with c.79G>A allele were compound heterozygous with c.92+5G>C mutation; thereby enabling the identification of two patients with HbE/β thalassaemia in the study population. This c.79G>A mutation causes a β+ functional allele which has a mild reducing effect on β globin chain synthesis (Weatherall 2001; Patrinos 2004). It has been described that most severely affected HbE/β syndrome patients have β+β0 genotype and are transfusion dependant and have hepatospleenomegaly, growth retardation, and bone marrow expansion causing facial deformities (Vichinsky 2007). The functional allele composition of those two patients was β0β+. Both these patients were transfusion dependant and had a thalassaemia major phenotype. Therefore the allele composition was compatible with the clinical phenotype of those two patients.

The prevalence of the HbE/β thalassaemia patients in the study population was 2 (1.6%), this is in contrast to the observation made in the previous large scale study (de Silva et al. 2000); of 703 both regularly transfused and intermittently transfused β thalassaemia patients recruited from
clinics in nine hospitals across the country except the Northern province; here the prevalence of 
HbE/β thalassaemia had been relatively high, 40%. The majority of the study population had 
originated from the Kurunegala district, a highly thalassaemia endemic area where the 
prevalence of HbE/β thalassaemia had been 30.9% whereas in Anuradhapura and Colombo 
districts it had been 23% and 1% respectively. The authors of that study had concluded that the 
common β thalassaemia causing mutations are unevenly spread in the population and this uneven 
prevalence could account for the low detection rate of patients with HbE/β thalassaemia in this 
study group.

**4.1.2 Automated sequencing test results**

After automated sequencing of the P1 region, genetic diagnosis was established in 4 patients. 
The three mutations detected by automated sequencing were c.27_28insG, c.51delC, and 
c.46delT. These mutations have been described in the Sri Lankan population before (Fisher et al. 
2003). The next few sections of this dissertation will discuss the phenotype genotype correlation 
of these mutations.

c.27_28insG mutation results in an insertion of G nucleotide between codons 8/9 (+G); and 
thereby change in the amino acid sequence AAG TCT(Lys;Ser) > AAG G TCT. This mutation is 
responsible for a β⁰ allele, which completely abolishes β globin chain synthesis (Patrinos 2004). 
Therefore a patient who is homozygous for this mutation has β⁰/β⁰ functional allele composition 
with the β thalassaemia major clinical picture, and this therefore correlates with transfusion 
dependent phenotype of the study patient.

Mutation c.51delC also referred to as CD16 (-C) gives rise to a β⁰ allele. This is a result of a 
deletion in the C nucleotide at Codon 16 (-C); GGC(Gly)>GG- causing a frameshift mutation. It
causes termination of protein translation at codon 18 (TGA) (Patrinos 2004). As a result of this the patient who is homozygous for this mutation has $\beta^0/\beta^0$ allele composition hence $\beta$ thalassaemia major phenotype. The patient with homozygous c.51delC mutation in this cohort had the same phenotype; thalassaemia major.

c.46delT mutation is also depicted as CD15 (-T). There is a deletion of the T nucleotide at codon 15; TGG(Trp)-$\rightarrow$-GG causing a frameshift mutation, hence resulting $\beta^0$ functional allele (Patrinos 2004). There were two patients who were compound heterozygous for this allele with c.92+5G>C mutation in this cohort. The c.92+5G>C is a major contributor for $\beta^0$ functional allele. So the two patients with c.92+5G>C; c.46delT mutations had the same functional allele composition; $\beta^0/\beta^0$ which corresponded with the thalassaemia major phenotype.

After automated sequencing two silent mutations were detected; and both were phenotypically insignificant. Those mutations were, c.9T>C (H3H) and c.33C>A (A11A) and in both, the nucleotide change had taken place at the third position of the codon which does not affect coding of the amino acid sequence of the globin gene.

After sequencing the P1 region of the $HBB$ gene in 9 patients the c.315+16G>C (IVSII+16G>C) variant was found in the IVSII region. Most of the existing literature describes this mutation as a polymorphisms in the $HBB$ gene (He et al. 2006). In contrast in a recent study by Gururaj et al (2012) this has been reported as a pathogenic variant in the Indian Karnataka population (Kulkarni et al. 2012). They have compared their data with a control population, hence due to the absence of the c.315+16G>C variant in the control population, this had been considered as a mutation by the authors. It was unable to conclude this variant as a polymorphism or a mutation in this study population as it was not possible to sequence the entire $HBB$ gene to exclude the possibility of another mutation and a control population was not investigated.
This entire thalassaemia population in this cohort belonged to a single phenotype; β thalassaemia major. Considering the functional allele composition of the five common mutations that was selected for the ARMS PCR were $\beta^0$ except the c.79G>A mutation which was $\beta^+$. Therefore all the 90 patients detected by ARMS PCR had $\beta^0/\beta^0$ allele composition except the two patients who were compound heterozygous for c.92+5G>C; c.79G>A mutations whose allele composition was $\beta^0/\beta^+$. $\beta^0/\beta^0$ patients belong to thalassaemia major phenotype whereas $\beta^0/\beta^+$ composition can be either thalassaemia major or intermedia phenotype. Hence the clinical classification of patients in this cohort was compatible with their allele composition.

The three mutations which were detected by automated sequencing; c.51delC, c.27_28insG and c.46delT give rise to $\beta^0$ alleles. Therefore after automated sequencing, the allele composition of those 4 patients was confirmed as $\beta^0/\beta^0$, again corresponding to their clinical phenotype.

Following both ARMS PCR and automated sequencing of the P1 region a genetic defect was not detected in the $HBB$ gene in 20 (16%) patients of the study population. The reasons for this could be, the presence of other $HBB$ gene mutations which are reported to be present in Sri Lankan β thalassaemia population or the presence of α gene deletions which have not been tested for in this study.

4.2 $HFE$ gene

To identify the mutations; c.845G>A and c.187C>G in the $HFE$ gene ARMS PCR was used. These two mutations are the commonest mutations giving rise to hereditary haemochromatosis.

4.2.1 c.845G>A mutation (C282Y)

None of the patients in the study had this mutation (allele frequency 0%). The data of this study were consistent with the described incidence of the c.845G>A mutation in the Asian population.
(Elmrghni et al. 2011). It can be assumed that this is due to the global prevalence pattern of the c.845G>A HFE gene mutation; being rare in the Asians, was not detected in the study population. (refer Table 1.2).

A study on Sri Lankan patients with β thalassaemia by Rochette et al (1999) has described a single patient who had been compound heterozygous for c.845G>A; c.187C>G mutations. Following haplotype analysis it has been shown that these two mutations; c.845G>A and c.187C>G have arisen independently in our island rather than by population admixture. Authors have described three new haplotypes associated with the c.187C>G mutations and one new haplotype associated with the c.845G>A mutation which were different from the European populations (Rochette et al. 1999). According to their analysis after assessing 130 chromosomes (260 alleles) the allele frequency for c.845G>A mutation was 0.008. This study results support the fact that this mutation is rare in the Sri Lankan thalassaemia population.

Previous studies further confirm our findings; a detailed analysis done by Merryweather-Clarke et al (1997) on the frequency of the c.845G>A mutation discovered findings similar to this study. Mutation analysis of 109 referral samples from Sri Lanka sent for the diagnosis of haemoglobinopathy had found that the c.845G>A mutation was absent (Merryweather-Clarke et al. 1997).

4.2.2 c.187C>G mutation (H63D)

Of the 125 patients with β thalassaemia; only 23 patients were heterozygous for the variant allele (CG). The total allele frequency for the variant allele was 9.2%. The reported global allele frequency of the c.187C>G mutation in the general population is variable (refer Table 1.2).
The allele frequency of the c.187C>G mutation in Sri Lankan patients with β thalassaemia has been described before. According to Rochette et al (1999) the allele frequency for this variant was 10.8% (Rochette et al. 1999). A study done by Merryweather-Clarke et al (1997) has obtained similar results for this variant; the allele frequency being 9.2% (Merryweather-Clarke et al. 1997). The data from this study supported the same findings.

The three studies done on this variant allele to date in Sri Lanka has been summarized in table 4.2.

**Table 4.2 Available previous research studies of the HFE gene mutations in Sri Lanka**

<table>
<thead>
<tr>
<th>Study Author</th>
<th>Number of alleles studied</th>
<th>c.845G&gt;A allele frequency</th>
<th>c.187C&gt;G allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merryweather-Clarke et al (1997)</td>
<td>260</td>
<td>0%</td>
<td>9.2%</td>
</tr>
<tr>
<td>Rochette et al (1999)</td>
<td>218</td>
<td>0.08%</td>
<td>10.8%</td>
</tr>
<tr>
<td>Current Study</td>
<td>250</td>
<td>0%</td>
<td>9.2%</td>
</tr>
</tbody>
</table>

**4.2.3 Co-relation between serum ferritin and c.187C>G genotype**

Co-relation between the mean serum ferritin and CC/CG genotypes were calculated using independent Student’s ‘t’ test. Even though the mean serum ferritin level was higher in the heterozygous CG group as compared to the CC wild type group, the results were not statistically significant (p value is 0.865).
Correlation between the c.187C>G mutation and serum ferritin levels has been widely studied by Melis et al (2002) (Melis et al. 2002). These authors have reported that the serum ferritin levels were higher in β thalassaemia carriers who were homozygous for the c.187C>G mutation than in carriers who were heterozygous for this variant. They have concluded that the c.187C>G mutation may have a modulating effect on iron absorption. But they were unable to find a statistically significant association in serum ferritin values between those homozygous for the wild type allele and those heterozygous for the variant allele. Another study done by Piperno et al (2000) has demonstrated that the β thalassaemia trait aggravates the clinical picture of c.845G>A homozygotes favoring higher rates of iron accumulation and the development of the severe iron related complications. However in both studies the iron parameter; the serum ferritin levels did not correlate with the heterozygous c.845G>A and c.187C>G variants (Piperno et al. 2000). After analyzing 168 Brazilian patients with β thalassaemia trait Oliveria et al (2006) found that coinheriance of the c.845G>A mutation along with β thalassaemia may contribute to worsening the clinical picture (Oliveira et al. 2006).

All the above studies have been done on patients with β thalassaemia trait whereas the current study was on patients with β thalassaemia major. The main difference in the results was that there were no homozygotes for the variant allele in the current study.

Table 4.3 summarizes existing literature done on HFE gene mutations in β thalassaemia major and intermedia patients (Rees et al. 1997; Cappellini et al. 1998; Longo et al. 1999; Kaur et al. 2003).
Table 4.3 *HFE* gene mutation analysis: summary of previous studies done on β thalassaemia major and intermedia patients and the present study.

<table>
<thead>
<tr>
<th>Number</th>
<th>Study Design</th>
<th>Authors</th>
<th>Type of thalassaemia</th>
<th>Country</th>
<th>N</th>
<th>c.845G&gt;A allele frequency</th>
<th>c.187C&gt;G allele frequency</th>
<th>Correlation with serum ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Case/control</td>
<td>Longo et al (1999)</td>
<td>Major</td>
<td>Italy</td>
<td>71</td>
<td>1.4%</td>
<td>12.7%</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>Case/control</td>
<td>Kaur et al (2003)</td>
<td>Major</td>
<td>India</td>
<td>75</td>
<td>4%</td>
<td>12.6%</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Cases only</td>
<td>Rees et al (1997)</td>
<td>Intermedia</td>
<td>Mix ethnic group</td>
<td>81</td>
<td>0.6%</td>
<td>0%</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>Cases only</td>
<td>Cappellini et al (1998)</td>
<td>Intermedia</td>
<td>Italy</td>
<td>37</td>
<td>0%</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Descriptive</td>
<td>Current study</td>
<td>Major</td>
<td>Sri Lanka</td>
<td>125</td>
<td>0%</td>
<td>9.2%</td>
<td>No</td>
</tr>
</tbody>
</table>

In both studies done on thalassaemia major; (study number 1 and 2) authors were unable to detect statistically significant results between serum ferritin level and the *HFE* gene genotypes (Longo *et al.* 1999; Kaur *et al.* 2003).

Both studies done on patients with thalassaemia intermedia have reported very low allele frequencies for both variants. Therefore authors could not correlate the results with iron parameters (Rees *et al.* 1997).

Irrespective of whether the studies were done on patients with β thalassaemia major, intermedia or minor; none of the studies could demonstrate a statistically significant difference in serum ferritin values with the heterozygous variants. Therefore the results of this study were compatible
with the previous published data where it has been shown that only the homozygous state is associated with high serum ferritin levels.

4.3 COLIA1 gene

The c.104-441G>T polymorphism in COLIA1 gene was analyzed in 46 patients out of 125 study population. There were only 6 patients who were heterozygous (GT) for this variant allele and the allele frequency was 6.52%. Therefore this allele is polymorphic in the study population. The data on Bone Mineral Density of these patients was not available at the present time. So could not correlate the genotyping data with the Bone Mineral Density and this will be analyzed in a future study.
CONCLUSION

This study was designed to analyze the \textit{HBB} gene in a group of patients with transfusion dependent β or HbE/β thalassaemia and to analyze two tertiary modifier genes; \textit{HFE} and \textit{COL1A1}, genes which have been shown to modify the phenotype of the associated complications of thalassaemia which are, iron overload and bone complications respectively.

ARMS PCR assay was able to genotype a significant proportion of the study population, therefore it can be concluded that this can be used as a sensitive molecular diagnostic screening test for β thalassaemia in our population. All the genotypes tested by ARMS PCR and automated sequencing were compatible with the phenotypes of this thalassaemia cohort. In Sri Lanka geographical variation of the prevalence of the c.79G>A mutation may exist, with low prevalence in the Anuradhapura and the Colombo districts. This needs further large scale study.

The c.315+16G>C variant too needs further studies in both thalassaemic and non thalassaemic populations to determine the role of its pathogenic significance in transfusion dependent β thalassaemia.

The c.845G>A mutation is rare in our thalassaemic population and therefore does not have to be considered as a contributor of iron overload in regularly transfused patients with β thalassaemia. The c.187C>G variant of the \textit{HFE} gene may need to be considered for testing in patients with β thalassaemia; based on the strength of evidence of the association of the c.187C>G mutation and iron overload.

The variant c.104-441G>T in the \textit{COL1A1} gene is polymorphic in the study population and needs further study including correlation of the genotyping data with the Bone Mineral Density.
References


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APPENDIX 1: List of Abbreviations

DNA    Deoxyribonucleic acid
IVS    Intervening sequence
UTR    Untranslated region
HbA    Adult haemoglobin
HbF    Foetal haemoglobin
MHC    Major Histocompatibility Complex
ARMS   Amplification Refractory Mutation System
PCR    Polymerase Chain Reaction
BMD    Bone Mineral Density
ESR    Erythrocyte Sedimentation Rate
CRP    C-reactive protein
SGPT   Serum Slutamic-Pyruvic Transaminase
HPLC   High Performance Liquid Chromatography
EDTA   Ethelene Diaminetetra Acetic acid
HH     Hereditary Haemochromatosis
HbE    Haemoglobin E
FBC    Full Blood Count
ALT    Alanine aminotransferase
TfR1  Transferrin receptor-1

AS-PCR  Allele specific PCR

RFLP  Restriction Fragment Length Polymorphism
APPENDIX 2: List of Suppliers

Applied Biosystems  7 Kingsland Grange, Woolston, Warrington, Cheshire, WA1 7SR, UK.

Promega UK Ltd.  Delta House, Chilworth Research Centre, Southampton SO16 7NS, UK.

Integrated DNA Technology  (IDT) – USA.
APPENDIX 3: Documents used for subject recruitment

This appendix contains all the documents which were used for subject recruitment.

- Information sheet for study participants used for the recruitment (English, Sinhala and Tamil translations)
- Consent form used for the recruitment (English, Sinhala and Tamil translations).
- Data collection booklet which was designed to collect personal and clinical data of patients with β thalassaemia major.
INFORMATION SHEET

Genetic modifiers of Beta Thalassaemia: phenotype-genotype relationship in a Sri Lankan population

(Version 1.0, 18-08-11)

This study is conducted by me, Dr Padmapani Padeniya, a MSc student in Clinical Genetics attached to the Human Genetics Unit, Faculty of Medicine, University of Colombo. I would like to invite you to take part in the research study titled “Genetic modifiers of Beta Thalassaemia: Phenotype-genotype relationship in a Sri Lankan Sample” conducted by myself under the supervision of Prof. Vajira Dissanayake, Prof. Rohan Jayasekara and Dr. Hemali Goonasekara at the Human Genetics Unit, Colombo Medical Faculty.

1. Purpose of the study

The purpose of this research is to assess the genetics modifiers of Beta thalassaemia and to correlate clinical phenotypes with these genetic modifiers.

2. Voluntary participation

Your participation in this study is voluntary. You are free to not participate at all or to withdraw from the study at any time despite consenting to take part earlier. There will be no loss of medical care or any other available treatment for your illness or condition to which you are otherwise entitled. If you decide not to participate you may withdraw from the study at any time by informing us.
3. **Duration, procedures of the study and participant’s responsibilities**

Patients referring to Lady Ridgeway Hospital for monthly blood transfusion will be recruited. If you allow your child to participate in this study, we would like to interview you, have access to your child’s medical records, and examine the child. We also need your permission to publish the data collected in a scientific journal. We will not mention your child’s name or any other identifiable information about him/her when we publish the results. Furthermore your permission is required to draw 5ml of blood to assess the genetic diagnosis of beta thalassaemia and genotype all the modifier genes. Your consent is essential to undergo a “DEXA SCAN” to assess the bone mineral density. This is done by placing the scan instrument on the body of the child. Details of the other biochemical investigations would be obtained from the previous records.

4. **Potential benefits**

The genetic diagnosis would be known to the participants in this study, as genetic testing is not freely available in Sri Lanka this would be beneficial to these patients. Knowledge about the different genetic mutations modifying their phenotype would enable the Paediatricians caring for them to understand their clinical phenotype better and care for them better. In addition subjects and parents/guardians will be provided genetic counseling about each subject’s test results freely. This study has social value because it would contribute to generalizable knowledge in the field. So all the participants will contribute to increase the generalizable knowledge in β thalassaemia in Sri Lanka.
5. **Risks, hazards and discomforts**

Blood will be drawn for the assessment of the molecular diagnosis of beta thalassaemia and to identify the possible genetic modifiers of the disease. Approximately 5 ml of blood will be taken. The risk to subjects participating in this study is the risk of pain, bruising, and infection at the venepuncture site. These would be minimized by performing venepuncture under aseptic conditions by a trained phlebotomist.

6. **Reimbursements**

There will be no reimbursement for participating in the study, but you will be given a written summary of the test results, in lay language.

7. **Confidentiality**

Confidentiality of all records is guaranteed and no information by which you can be identified will be released or published. The data collection booklet is designed to ensure confidentiality of information gathered. The electronic database containing the data will have only the subject study number and the database and the computer containing the database would be password protected. These data will never be used in such a way that you could be identified in any way in any public presentation or publication without your express permission.
8. Termination of study participation

You may withdraw your consent to participate in this study at any time, with no penalty or effect on medical care or loss of benefits. Please notify us as soon as you decide to withdraw your consent. However, it will not be possible for you to withdraw once the results are sent for publication or once the results are published.

9. Clarification

If you have questions about any of the tests / procedures or information please feel free to ask any of the persons listed below.

Dr Padmapani Padeniya
MSc Student
Human Genetics Unit
Faculty of Medicine
Colombo
0112689545

Prof Vajira Dissanayake
Senior Lecturer
Human Genetics Unit
Faculty of Medicine
Colombo
0112689545
තනනීල්ක ආර්ථික

විශේෂයෙන්, පීටතාපුව කෙනැරෙයි විශේෂයෙන් ප්‍රකාශයක් ලබාදීමෙන්තමිකයින් උපකාරිතාව නිර්මාණය විසින් ප්‍රකාශයක් ලබාදෙයින් ප්‍රකාශයක් ලබාදීමෙන්තමිකයින් උපකාරිතාව නිර්මාණය විසින් ප්‍රකාශයක් ලබාදෙයින්.

මෙම ප්‍රකාශයක් ලබාදෙයින් විශේෂයෙන් ප්‍රකාශයක් ලබාදෙයින් විශේෂයෙන් ප්‍රකාශයක් ලබාදෙයින්.

1. ප්‍රකාශයක් ලබාදෙයින්
2. ප්‍රකාශයක් ලබාදෙයින්
3. ප්‍රකාශයක් ලබාදෙයින්
4. මින් ඼ද නිර්ණයක

5. ප්රකාලික උදාහරණක

6. අලධියම් කාලයක

7. අලුතුරු කාලයක

8. ප්‍රකාලික මාර්ගයක

9. ආකාලයක

011-2689545
தகவல் புனிதம்

1. தகவல் புனிதமாலம் புந்தகபொருள் பாதுகாப்புகள்

2. தகவல் புனிதமாலம் பொருள் பாதுகாப்பு உரையாட்டுகள்

முன்னிலை மாணவை (MSc முன்னிலை) அறிவியல் நுட்பங்கள் மற்றும் புரோச்சொலி அலைகள் பிறகு, புரோச்சொலிப் பல்கலைக்கழகங்களில் (MSc – புரோச்சொலி புேரூபந்தருளின்) அறிவியல் பிறந்திருந்து போதும் உள்ள.

1. முன்னிலை பிறந்திருத்தம்

2. பேருந்து செய்யும் பொருள் பாதுகாப்பு
3. அமைப்பங்கள், வேளுற்றுக்குரிய பாதுகாப்புச் சாதனங்கள் உயர்வாக்ககள்

புகழ்பெறும் வேளுற்றுக்குரிய பாதுகாப்புச் சாதனங்கள் உயர்வாக்க பாதுகாப்புகள் உருவகமடைந்து வலசு விளைவு பெறும் உயர்வாக்க பாதுகாப்புச் சாதனங்கள் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும் உயர்வாக்க பாதுகாப்புச் சாதனங்களின் உருவாக்கம் பெற்று உயர்வாக்க பாதுகாப்புச் சாதனங்கள் உற்பத்தியாகவும் உருவாக்குவதற்கு விளையாடும்
4. சாத்திய குறுக்கு செய்யக்கூடாது

நின்னும் மறுமலை விளைப்படுட்புரிந்து நிவாரிகள் வழங்கப்படுவது சிறுவர்களுக்கு முன்பு விளையாடப்பட்டு விளையாடும் பொழுது. சிறுவர்களுக்கு நின்னும் மறுமலை விளையாடும் பொழுது விளையாடும் வல்லர்களை நிவாரிகளாக விளையாடும்.

5. மூக்காம், கிளைப்பலம் முடியாத அழகாமையானது

பெருந்தாய ஆளால் இருந்து காதல் குறைய கால் அடுத்து விளையாடப்பட்டுக் காணப்படுகிறது, அச்சுறுத்துக்கு விளையாடும் காலாலை குறைய விளையாடும் காலாலை விளையாடும் பாதிக்கும்.
6. செயலிப்பு

தினகாலத்தில் மக்களின் விளக்கங்களைத் தெரிந்து பார்த்து தலைமை வகிக்கப்படும் முயற்சிகளை மன்னர் விளக்கம் பின்வருமாறு நோக்கியுள்ளது. தொடர்ந்து தினகாலத்தில் முன் முன் மேற்குத்து மற்றும் பிற்குறுத்து

7. விளக்கம் விளக்கம்

அரசுபுரவு ஆணத்தில் பிறிக்கப்படும் விளக்கமாக வெளியாகவும், அரசுத்துவ குழுவில் விளக்கம் பிறிக்கப்படும் முறைக்காணியும்.

குடிநோய்வாளர் விளக்கம் தமது நான்கு புதுக்கு விளக்கம்.

சுருக்கமாக குடிநோய்வாளர் நோக்கியுள்ள முறையில் குடிநோய்வாளர் நலமான நோக்கியுள்ள விளக்கம் விளக்கம் பிறிக்கப்படும் முறைக்காணியும்.

அரசுபுரவமாக எந்த ஆரம்பப்புரோஷ்டெஞ்சுப்பாட்டு போக்குமாறு அதைத் தக்கங்கொண்டு அரசுபுரவம் விளக்கினை செய்யும் முறையில் விளக்கம் விளக்கமாக விளக்கம் விளக்கம்.
8. முப்பதருவிள் விளையாட்டு தொடர்பு

இன்னும் உள்ளது சுருக்கிய வேளையுஸ்த்ஸ், முடிவு ரோஜர் விளையாட்டு நிரலிடத்துடன் வேளையுஸ்த்ஸ் நிரலிடத்துடன் வேளையுஸ்த்ஸ் நிரலிடத்துடன் வேளையுஸ்த்ஸ் நிரலிடத்துடன் வேளையுஸ்த்ஸ்.

9. மதிப்பிட்டுதலால் விளையாட்டுதலும்

இன்னும் உள்ளது சுருக்கிய வேளையுஸ்த்ஸ், முடிவு ரோஜர் விளையாட்டு நிரலிடத்துடன் வேளையுஸ்த்ஸ் நிரலிடத்துடன் வேளையுஸ்த்ஸ் நிரலிடத்துடன் வேளையுஸ்த்ஸ் நிரலிடத்துடன் வேளையுஸ்த்ஸ் நிரலிடத்துடன் வேளையுஸ்த்ஸ்.

Dr. பூம்பாலி பாங்காரினம்

Msc பாலாமை

மாதிக் பொப்பாடுபலான மாதிக்

மாதிக் பொப்பாடுபலான மாதிக்

0777-334750
CONSENT FORM

Genetic modifiers of Beta Thalassaemia: phenotype- genotype relationship in a Sri Lankan population

(Version 1.0, 18-08-11)

To be completed by the participant/guardian

The participant/ guardian should complete the whole of this sheet himself/herself.

1. Have you read the information sheet? (Please keep a copy for yourself) YES/NO

2. Have you had an opportunity to discuss this study and ask any questions? YES/NO

3. Have you had satisfactory answers to all your questions? YES/NO

4. Have you received enough information about the study? YES/NO

5. Who explained the study to you? .................................................................

6. Do you understand that you are free to withdraw from the study at any time, without having to give a reason and without affecting your future medical care? YES/NO

7. Sections of your medical notes, including those held by the investigators relating to your participation in this study may be examined by other research assistants. All personal details will be treated as “STRICTLY CONFIDENTIAL”. Do you give your permission for these individuals to have access to your records? YES/NO
8. Do you agree to have leftover blood samples be stored for future research into β thalassaemia under the supervision of the supervisor?  

YES/NO

9. Have you had sufficient time to come to your decision?  

YES/NO

10. Do you agree for the samples to be sent abroad?  

YES/NO

11. Do you agree to take part in this study?  

YES/NO

Participant’s/Parent’s/ Guardian’s signature……………………………………

Date……………………

Name (BLOCK CAPITALS)………………………………………………………………

……………………………………………………………………………………………………

To be completed by the investigator

I have explained the study to the above volunteer and he/ she has indicated her willingness to take part.

Signature of investigator………………………….

Date……………………

Name (BLOCK CAPITALS)………………………………………………………………}
කැමැත්  ප්රකාං ිරීතම් ප්රය

(a) සයරමි සහාරක මිලින් දිවිං විද්‍යා

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11. ම එමු ආරම්භයේ සාමාන්‍ය අනුව සිටින ගැටලුවක්?

උපදාය ආරම්භ /උපදායර

දිවය:…………………………………………………………..දිවය:…………………………

වම:…………………………………………………………………………………………

(b) ක්‍රියා සංස්කරණ කරන්නේමද.

ම එමු බැහැරින් ආරම්භ ආරම්භ, එ සංස්කරණ ආරම්භය ආරම්භයකට අයත්වා ම ආරම්භ උපදායක් පදා ආරම්භ ආරම්භය ආරම්භයක් ආරම්භයේ නිමැති අතරයි. එව පිළිතු藕ම එමු ආරම්භය ආරම්භය ආරම්භයේ නිමැති අතරයි.

දිවය:…………………………………………………………..දිවය:…………………………

වම:…………………………………………………………………………………………
சுருக்கிய பேச்சு

பதிப்புப் பலகை அல்லது பாடுகாலத்திலும் பிரிவுபட்டு சேர்த்துப் பலகை

பாலாம்பரப் பலகை / பாலாம்பர இன்ன பலகையில் பிரிவுபட்டு நடித்துப் பலகை.

1. தலைப் பார்வையில் பார்வையில் பார்வை நாளன்று கூறுவது?

(பாலையான ஒரு கூற்றுக்கு ஒரு ப்ரீதைம் தலைப் பார்வை நாளன்று)

ஆம் / இல்லாம்

2. திட்டம் ஆப்பிரா திட்டம் அல்லது ஆப்பிரா திட்டத்தின் முழுமையான சிலைகளைக் கேள்விக்க தலைப் பார்வை வழத்துப்பட்டா?

ஆம் / இல்லாம்

3. அகலவான விளக்கங்களில் மிகப் பெரிய அல்லது குறைவான பெருகுத் தலைப்புகளை கேள்விக்காணா?

ஆம் / இல்லாம்

4. இதை ஆப்பிரா திட்டத்தில் கேள்வியாக தலைப்பு வழத்து பலகை நாளன்று கூறுவது?
5. மிகவும் சம்பந்தமாக எந்தக் குற்றமையில் அப்பால்திய ரகம்?

........................................

6. நீண்டுள்ள நேரங்களிலும் இனம் அப்பால்திய இறுதி நிலைக்கு கால்நடைபூர்வமாக விளக்கம் செய்ய என்று அறியில்லை. மறுவுடியும் நேரத்தில் மதத்தைக் கொண்டு மறுக்கக்கூடமாக என்று அறியில்லை. எனவே நேரங்களில் புகழ்பெறும் வராத்தை நோக்கியதோடு நேரங்களில் புகழ்பெறல் என்று கூறியுள்ளார்.

அம்ம / மாளீ

7. ஒவ்வொரு மனிதன் அனுமதிக்கும் குறான்களும் இனம் அப்பால்திய மரபுகள் இல்லை. அப்பால்திய நேரங்களில் மரபுகள் என்று பெரும்பாக குறான்கள் அனுமதிக்கும் திரும்பும். மறுவுடியும் என்றால் குறான்களுக்கு ஓய்கிறது.

இதுக்கு ஒவ்வொரு மலராக அனுமதிக்கிறானா?

அம்ம / மாளீ
8. வாழ்க்கையை செய்வதற்கு வைத்தியல் சலிசைல் குறுக்கு நேரங்கள் அம்பாறிக்கு நேரங்களாக நேரங்களாகக் கொள்ளுமைக்கு எப்படியாலும் சலிசைல் குறுக்கு நேரங்கள் எப்படியாலும் சலிசைல் குறுக்கு நேரங்கள்?

ஆம் / வாழ்ந்தால்

9. வாழ்க்கை முழுவதும் தொழில் நம்பலும், தொழிலிங்கும் நம்பலான வாழ்வு அவசமப்பட்டா?

ஆம் / வாழ்ந்தால்

10. வாழ்க்கை ஆரம்ப வேலைநாளுக்கு அளவுபடுத்து அளவுபடுத்து எளிதில் நேரங்கள்?

ஆம் / வாழ்ந்தால்

11. இந்த ஆய்வுக்கு பார்வையும் சலிசைல் குறுக்கு நேரங்கள்?

ஆம் / வாழ்ந்தால்
பிண்ணருளியக்கின் / பரவலாறுகின் கணினிப்பம் ...........................................

திேசம்: ........................................

விளைநுண共识ஏசருவர் தின்பன் வேல்குறும

வித்தியாசங்கள் உள்ள அம்மவ பயிற்பு விளைநுணையில் புதுக்கோட்ட விளைநுண் அசைக்கப்படும், அம்மவ
அம்மவின் பக்கத்தின் இருபாகைக் கருத்தாக்கப்படும்

மப்பா ........................................

திேசம் ........................................