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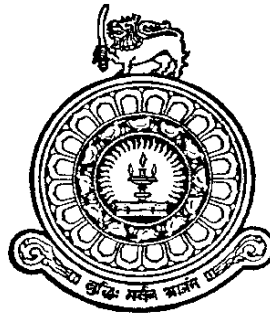
BY

ARACHCHIRALALE GEDARA PADMAPANI MEGALATHA PADENIYA, M.B.B.S

HUMAN GENETICS UNIT

FACULTY OF MEDICINE

UNIVERSITY OF COLOMBO

DISSERTATION SUBMITTED TO THE UNIVERSITY OF COLOMBO, SRI LANKA

FOR THE DEGREE OF MASTER OF SCIENCE IN CLINICAL GENETICS

July 2012

TABLE OF CONTENTS

ACKNOWLEGMENT

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 previousely described in Sri Lankan patients with β and HbE/β 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 Restiction 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; β^0 and β^+. β^0 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).
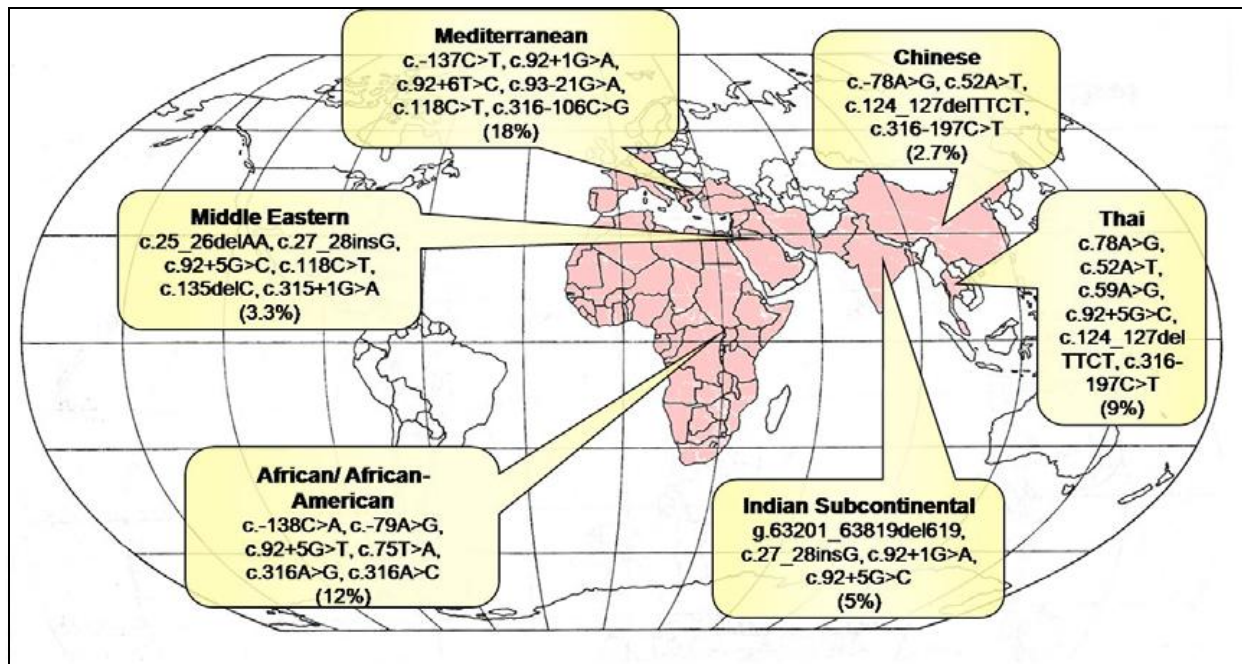
Fig: 1.1 Beta thalassaemia mutations in at risk populations. Mutations listed are those which are common and represent 91-95% of affected individuals in these regions. Listed in brackets are beta thalassaemia carrier frequencies in each region. (*P. Lahiry , S. A. A.-A. a. R. A. H. V. B. R. G., Robarts 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 thalasseamia 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 thalassaemics 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).
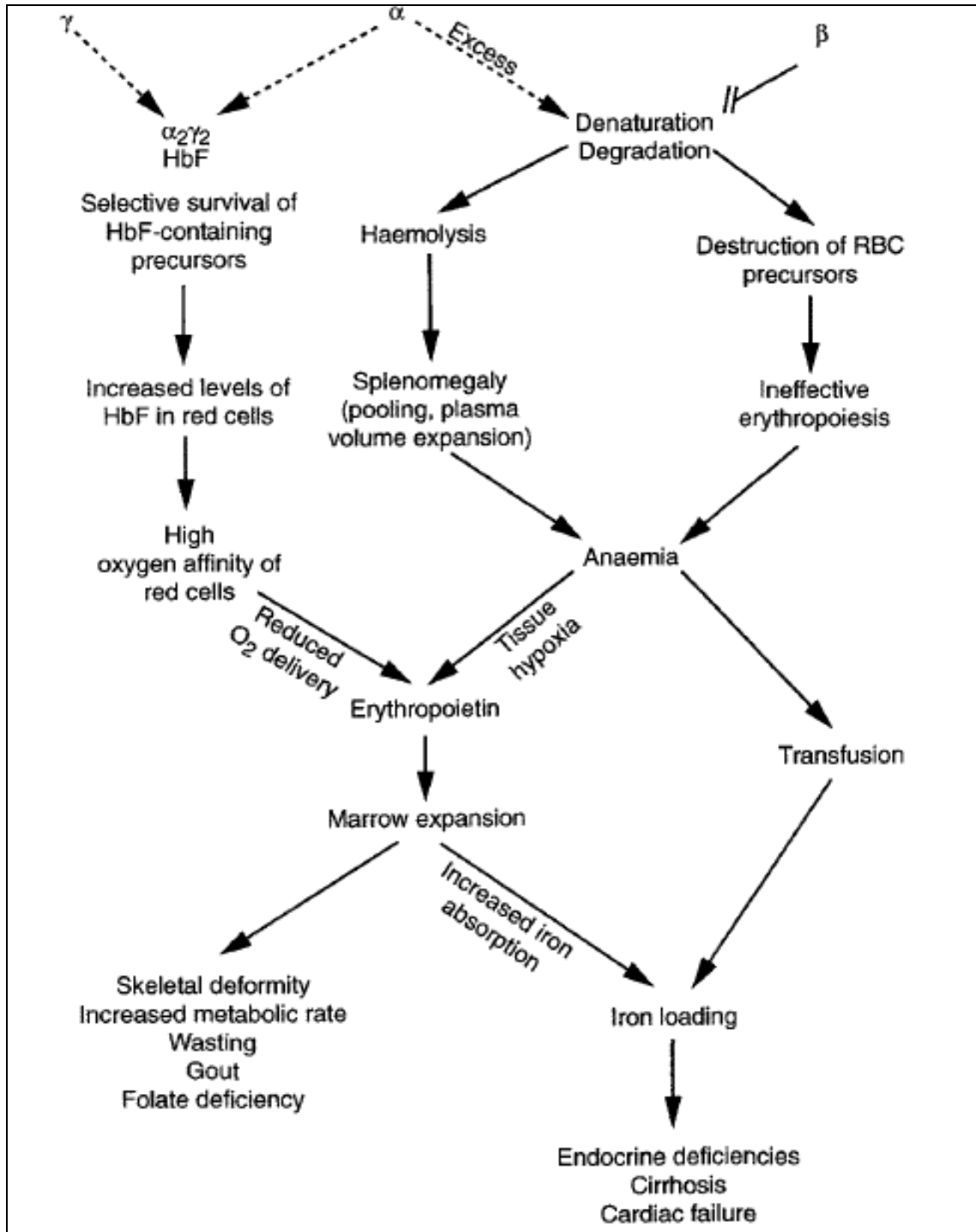
Fig: 1.2 A summary of main pathophysiological features of β thalassaemia. (*Weatherall, D. J. (1998). "Pathophysiology of thalassaemia." <u>Baillieres Clin Haematol</u> 11(1): 127-146.*)

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; $β^0$ and $β^+$ alleles. In homozygous or compound heterozygous for $β^0$ allele ($β^0β^0$), HbA is absent, the predominant type is HbF (approx. 95-98%) and HbA_2 can be variable. When there is homozygous state for $β^+$ allele ($β^+β^+$) or compound heterozygous state for $β^0$ and $β^+$ alleles ($β^0β^+$) 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

2004; P. Lahiry 2008). At time of birth, γ globin synthesis decreases and β 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 β gene become clinically apparent. Due to persistence of small amount of γ expression in adult life, all adults have small amount of fetal haemoglobin in their blood (Thein 2004).
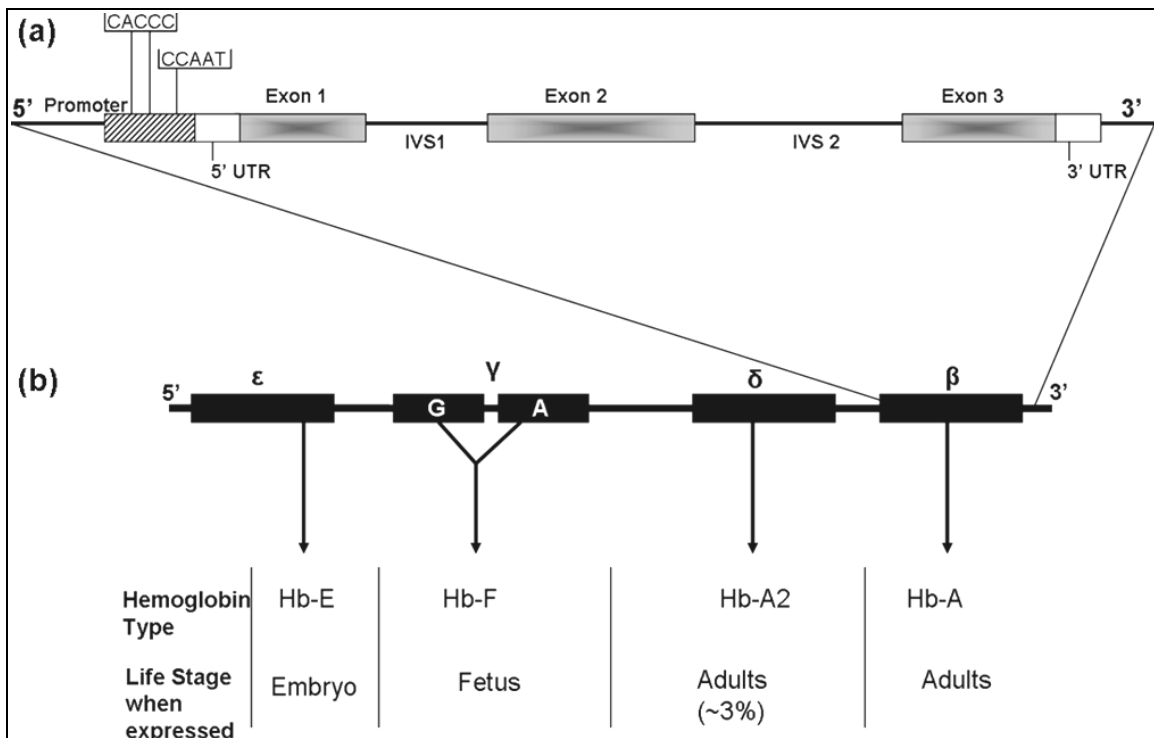
Fig: 1.3 Schematic representation of beta-globin gene cluster. (*P. Lahiry , S. A. A.-A. a. R. A. H. V. B. R. G., Robarts Research Institute and the University of Western Ontario, Canada (2008). "Understanding Beta-Thalassemia with Focus on the Indian Subcontinent*)

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, hyperbilirubinaemia, osteoporosis; are called tertiary modifiers (Weatherall 2001; Thein 2004; Thein 2005). (**Fig: 1.4).**
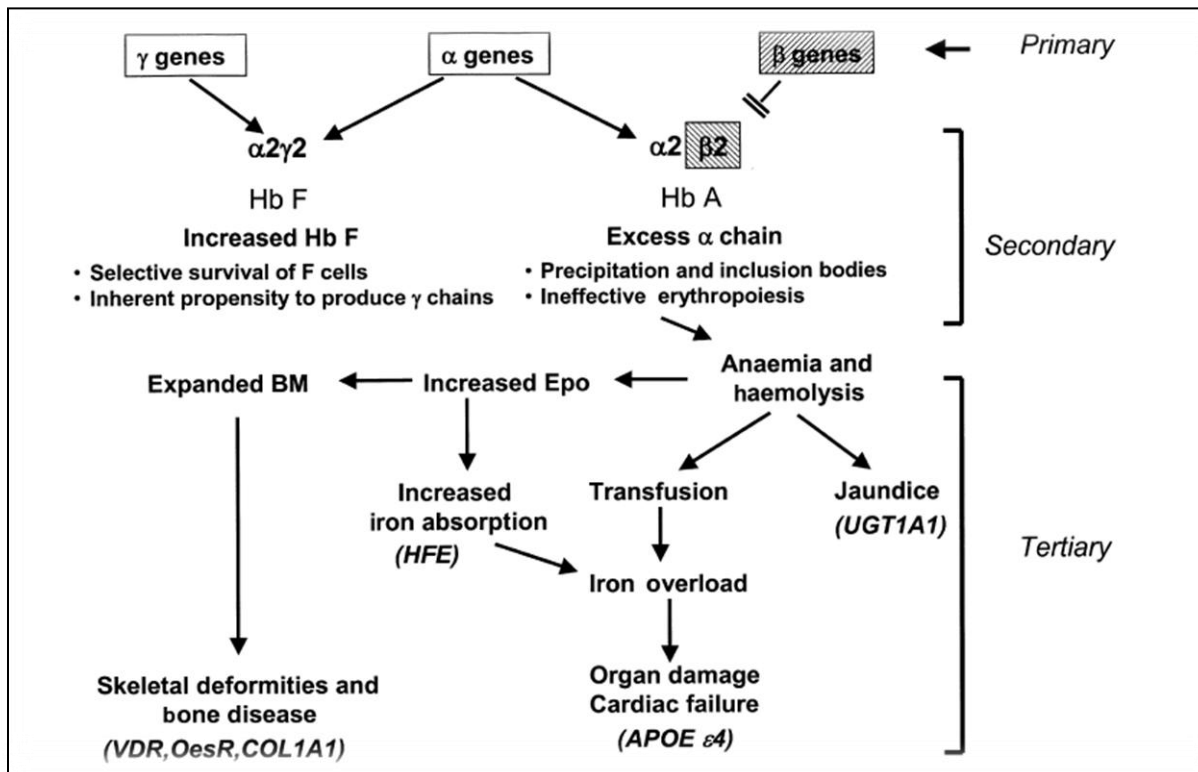
Fig: 1.4 Factors that modify the β thalassaemia phenotype at primary, secondary and tertiary level. *Thein, S. L. (2004). "Genetic insights into the clinical diversity of beta thalassaemia." Br J Haematol 124(3): 264-274.*

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) 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 hall mark 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) β^0/ β^+ / β^{++}mutations

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

Allele 1	Allele 2	Disease severity
β^0	β^0	Severe(thalassaemia major)
β^0	Severe β^+	Severe (thalassaemia major)
β^0	Mild β^+	Moderate(thalassaemia intermedia)
β^0	β^{++}	Moderate(thalassaemia intermedia)
β^+	β^+	Mild/moderate (thalassaemia intermedia)
β^+	β^{++}	Mild
β^{++}	β^{++}	Mild
β^0 or β^+	Normal	Carrier/trait/thalassaemia minor
3rd exon mutation	Normal	Dominant type of β thalassaemia

Approximately half of β thalassaemia causing mutations trigger premature termination of β chain extensions due to nonsence and frameshift mutations which introduce premature termination codons resulting in β^0 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 β^0 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 β^0 alleles (Thein 2005).

However Cao and his colleagues (2010) have described few β^0 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)**

Mild β-thalassaemia alleles			'Silent' β-thalassaemia alleles
−90 C→T	−30 T→A	CD 26 G→A (Hb E)	−92 C→T
−88 C→A	−30 T→C	CD 27 G→T Knossos	−101 C→T
−88 C→T	−29 A→G	IVS1 6 T→C	5' UTR +10 −T
−87 C→A	−28 A→G (mild in blacks;	3' UTR 47 C→G	5' UTR +33 C→G
−87 C→G	severe in Chinese)	PolyA AATAAA→AACAAA	IVS2 844 C→G
−87 C→T	5' UTR +22 G→A	PolyA AATAAA→AATUAA	CAP +1 A→C
−86 C→G	CD 19 A→G Malay	PolyA AATAAA→AATAGA	3' UTR +6 C→G
−31 A→G	CD 24 T→A	PolyA AATAAA→AATAAC	

Fig: 1.5 Mild and Silent β thalassaemia mutations. (UTR- untranslated region: IVS-intervening sequence (intron): CD- coding region (exon)). *Weatherall, D. J. (2001). "Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias." Nat Rev Genet 2(4): 245-255.*

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).
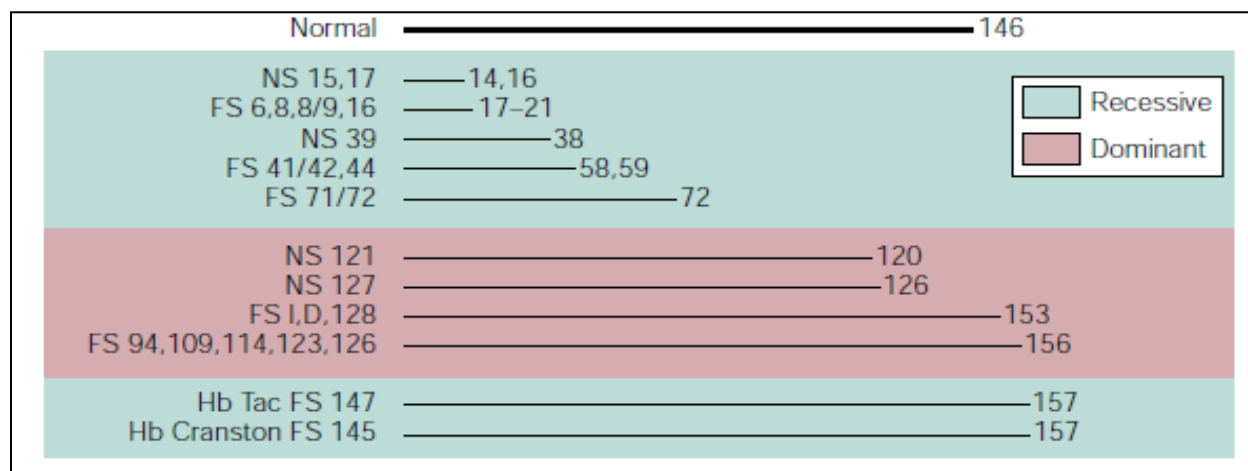
Fig: 1.6 Dominant β thalassaemia. The lengths (in amino acids) of the abnormal products are indicated, with the normal product shown at the top. Nonsence (NS) or Frameshift (FS) mutations in exon 1 and 2 of the β globin gene are recessively inherited and those that are terminating in exon 3 are dominantly 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 β^0 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/β^0 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 /β^0 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 β^0 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.*

1994). 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 (X*mn*1-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 X*mn*1-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 gastro intestinal 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.*

1997). 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 over load 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- 8grams 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, distruction 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 9.5Kb 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 hereditory haemochromatosis patients. They are c.845G>A (C282YG>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; Elmrghni *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 1.2 The estimated prevalence of haemochromatosis mutations in the general population. *Elmrghni, S., R. A. Dixon, et al. (2011). "Frequencies of HFE gene mutations associated with haemochromatosis in the population of Libya living in Benghazi."* <u>*Int J Clin Exp Med*</u> *4(3): 200-204.*

	N	H63D allele frequency	C282Y frequency	References
Europe				
North				
North Ireland	404	14.10%	9.9%	Murphy, 1998
Denmark	200	12.75%	6.75%	Steffen, 1998
Norway	94	11.20%	6.4%	Clarke, 1997
Sweden	117	12.39%	3.84%	Cardoso, 1998
South				
France	503	12.23%	1.07%	Piperno, 1998
Germany	153	14.07%	2.96%	Gottschalk, 1998
Greece	196	13.50%	1.3%	Clarke, 1997
Spain	420	20.40%	3.09%	Sanchez, 1998
Turkey	70	13.57%	0%	Clarke, 1997
Africa/Middle East				
Middle East				
Saudi Arabia	118	8.5%	0%	Clarke, 1997
North				
North Africa	171	13.2%	9.0%	Aguilar, 1997
Benghazi-Libya	100	17%	0%	Current study
South				
Gambia	39	1.3%	0%	Clarke, 1997
Senegal	130	0%	0%	Clarke, 1997
Kenya	78	1.3%	0%	Clarke, 1997
Nigeria	80	1.9%	0%	Clarke, 1997
Zambia	76	0.7%	0%	Clarke, 1997
Asia				
China	72	2.8%	0%	Clarke, 1997
Indonesia	90	2.8%	0%	Clarke, 1997
Taiwan	80	1.9%	0%	Clarke, 1997
Australasia				
Australia (aboriginals)	93	0%	0%	Clarke, 1997
Australia (vanuatuans)	90	0.6%	0%	Clarke, 1997
America				
Mexico	54	6.5%	0%	Clarke ,1997
Jamaica	90	2.2%	1.1%	Clarke, 1997
United States	5171	13.5%	5.4%	Karen, 2001

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 cycteine 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).**
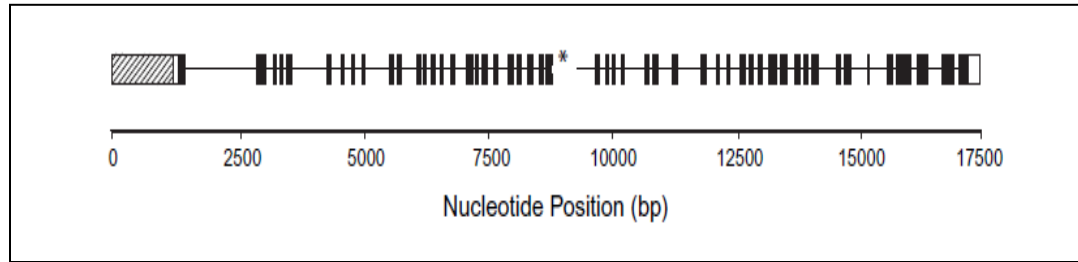
Fig: 1.7 The *COL1A1* gene locus with all coding and non-coding regions to scale. DNA sequenced region in human includes 1223bp of the promoter (striped box), 263bp of the 5' and 3' mRNA untranslated regions(white boxes), the 4392bp of coding sequence for all 51 exons (black boxes), with non coding region 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." <u>Mol Biol Evol</u> 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 *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 *et al.* 2011).

1.13.3 Mutations/ Polymorphisms

Most studies have concluded that the c.104-441G>T polymorphism in the *COL1A1* gene is significantly associated with reduced bone mass and osteoporotic fractures (Grant *et al.* 1996; MacDonald *et al.* 2001; Mann *et al.* 2001; Mann *et al.* 2003; Jin *et al.* 2009). This polymorphism was first described by Grant *et al* (1996) and it is a G>T substitution (rs 1800012) in the regulatory region of the *COL1A1* gene at a recognition site for the transcription factor Sp1 (Grant *et al.* 1996; Jin *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 *COL1A1* gene transcription and collagen protein production, hence resulting in alteration of biochemical properties of bone (Mann *et al.* 2003). The c.104-441G>T polymorphism lies within the first intron of the *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 *et al.* 2009). When this polymorphism is present it modulates Sp 1 binding site and *COL1A1* gene regulation, resulting in excess production of collagen α1 (I) chains relative to α2 (I) chains thereby altering the structural intergrity of bone leading to brittle bones (Mann *et al.* 2001; Mann *et al.* 2003).

According to Grant *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 *APOEε4* gene; Cystein -Argrnine 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 *COL1A1* 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 *COL1A1* c.104-441G>T polymorphism.

2.0 METHODS

2.*1 Ethic*al 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 vene-puncture 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 thalasaemia 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 bio chemical 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)**

Male	28	397
Female	6	159

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

Age	Male	Female
1 day-6 weeks	Up to 400	Up to 400
7 weeks – 1 year	10-95	10-95
1 year – 9 years	10-60	10-60
10 years – 18 years	10-300	10-70

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

(Lewis S.M., B. B. J., Bates I., Ed. (2006). Dacie and Lewis Practicle Haematology, Elsivier)

	Age (years)	Mean value(/mm for the 1st Hr)
Westergren	4-7	13
	8-14	10.5

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)*

Men (Age in years)	ESR in mm for the 1st hrs	Women (Age in years)	ESR in mm for the 1st hrs
17-50	10 or <	**17-50**	12 or <
51-60	12 or <	**51-60**	19 or <
61-70	14 or <	**61-70**	20 or <
>70	30 or <	**>70**	35 or <

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 vortexting 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 $MgCl_2$ and 1.5 Unit Taq. The amplification on the thermal cycler was carried out which consisted of an initial denaturation at 94^oC for 5 minutes followed by 30 cycles; each cycle consisted of denaturation at 94^oC for 1 minute, primer annealing at 65^oC for 1 minute and DNA extension at 72^oC for 2 minutes, with a final extension at 72^oC for 10 minutes.

Table 2.5 ARMS-PCR primer sequences

Primer	Sequence
c.92+1G>A	5' TTAAACCTGTCTTGTAACCTTGATACCGAT 3'
c.92+5G>C	5' CTCCTTAAACCTGTCTTGTAACCTTGTTAG 3'
c.79G>A	5' TAACCTTGATACCAACCTGCCCAGGGCGTT 3'
c.126_129delCTTT	5' GAGTGGACAGATCCCCAAAGGACTCAACCT 3'
Common_F	5' ACCTCACCCTGTGGAGCCAC 3'
CNT_R	5' GAGTCAAGGCTGAGAGATGCAGGA 3'
CNT_F	5' CAATGTATCATGCCTCTTTGCACC 3'

Table 2.6 Primers used for genotype the respective mutations.

Primer	Sequence
c.92+1G>A Wild	5' GATGAAGTTGGTGGTGAGGCCCTGGGTAGG 3'
c.92+5G>C Wild	5' CTCCTTAAACCTGTCTTGTAACCTTGTTAC 3'
c.79G>A Wild	5' TAACCTTGATACCAACCTGCCCAGGGCGTC 3'
c.126_129delCTTT Wild	5' GAGTGGACAGATCCCCAAAGGACTCAAAGA 3'
Common_F	5' ACCTCACCCTGTGGAGCCAC 3'
HBB-COMM-2	5' CCCCTTCCTATGACATGAACTTAA 3'
CNT_R	5' GAGTCAAGGCTGAGAGATGCAGGA 3'
CNT_F	5' CAATGTATCATGCCTCTTTGCACC 3'

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 $MgCl_2$ and 1 Unit Taq. The amplification of P1 region on the thermal cycler was carried out consisting of an initial denaturation at 95^oC for 5 minutes followed by 35 cycles; each cycle consisted of denaturation at 95^oC for 30 seconds, primer annealing at 58^oC for 30 seconds and DNA extension at 72^oC for 1 minute, with a final extension at 72^oC for 7 minutes.

The amplification of P2 and P3 regions on the thermal cycler was carried out consisting of an initial denaturation at 95^oC for 5 minutes followed by 35 cycles; each cycle consisted of denaturation at 95^oC for30 seconds, primer annealing at 60^oC for 30 seconds and DNA extension at 72^oC for 1 minute, with a final extension at 72^oC for 7 minutes.
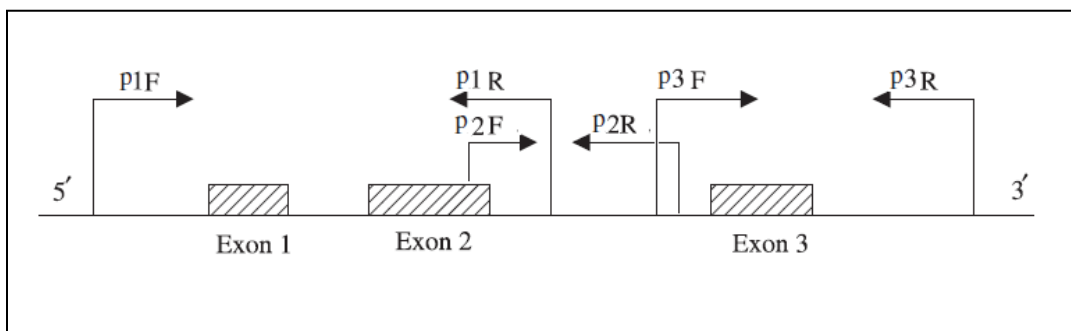
Fig: 2.1 Relative locations of the PCR primers complimentary to the *HBB* gene.

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_000007.3) 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

Two non-allele specific primers	Two allele specific primers
F1: AAGCAGCCAATGGATGCCAAG	Rw: CCTGGGTGCTCCACCTGGC
R1: CCACTGATGACTCCAATGACTA	Fm: GGGAAGAGCAGAGATATACGTA

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 TCGTGTTCTATGATC

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^{0}C for 11 minutes followed by 35 cycles of denaturation at 95^{0}C for 30 seconds, annealing at 64^{0}C for 30 seconds and extension at 72^{0}C for 30 seconds with a final extension at 72^{0}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 $MgCl_2$ and 1 Unit Taq. The first round of nested-PCR was carried out with the following primer set: 5′- TAACTTCTGGACTATTTGCGGACTTTTGG- 3′ (p1) and 5′ - GGGCGAGGGAGGAGAGGAA- 3′ (p3), which amplifies a 283-bp region surrounding the polymorphic site in the first intron of *COL1A1*. The thermal cycling protocol was: 95^oC for 2 minutes, 58^oC for 1 minute (one cycle), 95^oC for 50 seconds, 58^oC for 1 minute (13 cycles); 95^oC for 50 seconds, 58^oC for 1 minute, 72^oC 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′ - GTCCAGCCCTCATCCTGGCC - 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^oC for 3 minutes, 62^oC for 10 seconds, ramping at 1^oC per 10 seconds to 72^oC, 72^oC 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 for10 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 Mscl 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

- Anonymous (2008) *Chromas LITE Version 2.01.* Available from: www.technelysium.com.au.

- Anonymous (2011) *EMBOSS Needle - Pairwise Sequence Alignment.* European Bioinformatics Institute 2011.

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.
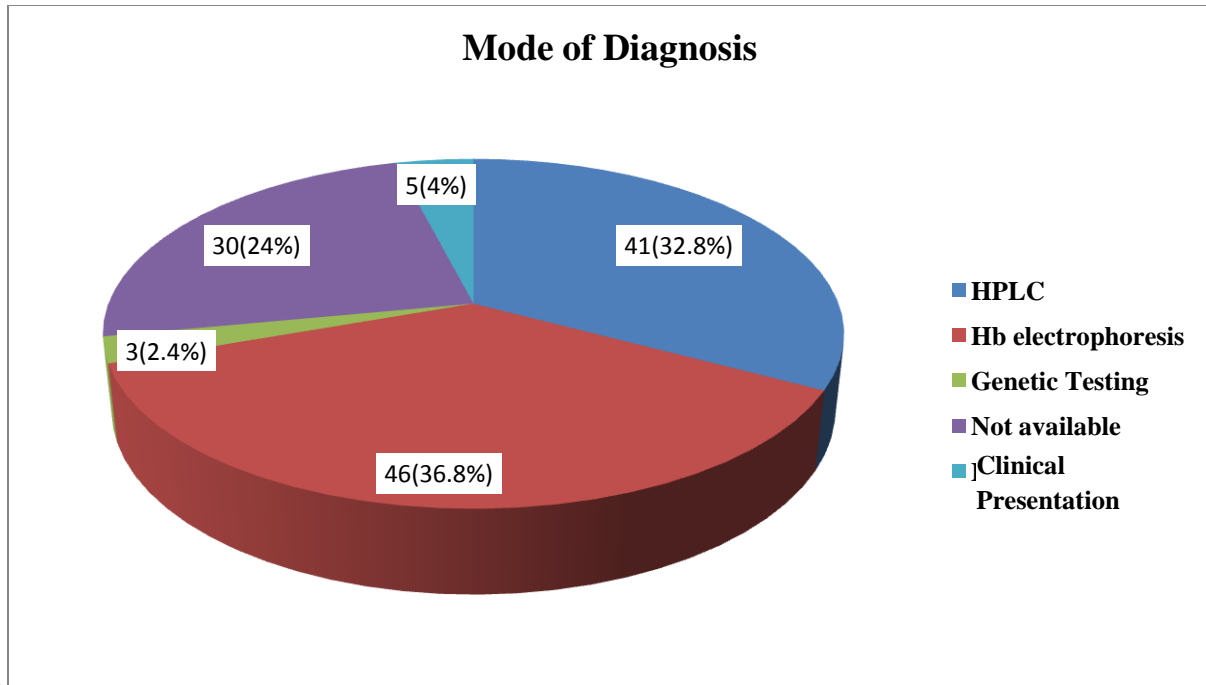
Figure 3.1: Modes of diagnosis of β thalassaemia patients in the study group.

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 cholecyctectomy 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 Bio chemical investigations, ESR and genotyping of *HBB*, *HFE* and *COL1A1* genes.

Thal Number	Serum Ferritin (ng/ml)	CRP (mg/l)	ESR (mm/ 1st hr)	SGPT (U/L)	*HBB* mutations	Type of allele; β^+/ β^0	c.845G>A	c.187C >G	c.104-441G>T
Thal 001	7130.00	7.90	139.00	46.40	c.92+1G>A /-	β^0/ -	GG	CG	GG
Thal 002	5810.00	0.90	31.00	126.60	c.92+5G>C HOMO	β^0/ β^0	GG	CC	
Thal 003	3310.00	0.50	13.00	42.50	c.92+5G>C HOMO	β^0/ β^0	GG	CC	
Thal 004	1900.00	10.00	0.70	59.70	c.92+5G>C HOMO	β^0/ β^0	GG	CC	
Thal 005	7670.00	1.00	35.00	36.20	c.92+5G>C HOMO	β^0/ β^0	GG	CC	GT
Thal 006	5070.00	0.50	16.00	201.70	c.92+5G>C HOMO	β^0/ β^0	GG	CC	GG
Thal 007	4190.00	1.90	21.00	111.00	c.92+5G>C HOMO	β^0/ β^0	GG	CG	GG
Thal 008	1930.00	0.20	8.00	97.30	c.92+5G>C; c.126_129delCTTT	β^0/ β^0	GG	CC	GG
Thal 009	1650.00	0.40	21.00	26.80	c.92+5G>C /-	β^0/ -	GG	CG	GT
Thal 010	3340.00	2.20	4.00	40.90	c.126_129delCTTT /-	β^0/ -	GG	CG	GG
Thal 011	2180.00	1.10	5.00	14.10	c.92+5G>C /-	β^0/ -	GG	CC	GG
Thal 012	907.00	0.60	25.00	12.50	c.51delC HOMO	β^0/ β^0	GG	CC	
Thal 013	6230.00	7.30	52.00	52.00	c.92+5G>C HOMO	β^0/ β^0	GG	CC	GG
Thal 014	2060.00	1.40		78.50	c.92+5G>C HOMO	β^0/ β^0	GG	CC	GG
Thal 015					c.126_129delCTTT HOMO	β^0/ β^0	GG	CC	GG
Thal 016	2770.00	0.20	16.00	143.60	c.92+1G>A /-	β^0/ -	GG	CC	GG
Thal 017	4800.00	1.30		138.90	c.92+5G>C HOMO	β^0/ β^0	GG	CC	GG
Thal 018	3190.00	1.80	45.00	58.40	c.92+5G>C HOMO	β^0/ β^0	GG	CC	GG
Thal 019	1497.00	1.70	22.00	33.70	c.92+5G>C HOMO	β^0/ β^0	GG	CC	GG
Thal 020	1770.00	0.60	13.00	91.20	c.92+5G>C /-	β^0/ -	GG	CC	GG

Thal 021	9530.00	15.00	103.90	65.20	c.92+5G>C HOMO	β^0/β^0	GG	CG	GG
Thal 022	4290.00	0.50	55.00	49.60	c.92+5G>C HOMO	β^0/β^0	GG	CC	GG
Thal 023	3410.00	0.30	16.00	18.44	c.92+5G>C HOMO	β^0/β^0	GG	CC	GG
Thal 024	2120.00	0.40	36.00	12.60	c.92+5G>C HOMO	β^0/β^0	GG	CC	GG
Thal 025	10760.00	0.30	65.00	61.80	c.92+5G>C HOMO	β^0/β^0	GG	CC	
Thal 026	2390.00	0.30	4.00	51.50	c.92+5G>C HOMO	β^0/β^0	GG	CC	GT
Thal 027	7920.00	0.20	46.00	144.90	c.92+5G>C; c.126_129delCTTT	β^0/β^0	GG	CC	GG
Thal 028	6280.00	1.10	16.00	67.40	c.92+5G>C /-	$\beta^0/$ -	GG	CC	
Thal 029	2310.00	1.30	15.00	54.60	c.92+5G>C HOMO	β^0/β^0	GG	CG	
Thal 030	3300.00	1.00	34.00	101.10	c.92+5G>C HOMO	β^0/β^0	GG	CC	
Thal 031	3260.00		17.00		c.92+5G>C HOMO	β^0/β^0	GG	CG	
Thal 032	4000.00	1.80	15.00	31.00	c.92+5G>C HOMO	β^0/β^0	GG	CC	
Thal 033	2180.00	5.70	12.00	27.00	c.27_28insG HOMO	β^0/β^0	GG	CG	
Thal 034	5030.00	2.50	15.00	202.00	c.92+5G>C /-	$\beta^0/$ -	GG	CC	
Thal 035	6400.00	1.30	7.00	203.80	c.92+5G>C /-	$\beta^0/$ -	GG	CC	
Thal 036	3740.00	0.30	10.00	64.40	c.92+5G>C /-	$\beta^0/$ -	GG	CC	
Thal 037	4730.00	1.80	10.00	74.30	c.92+5G>C HOMO	β^0/β^0	GG	CC	
Thal 038	12450.00	0.80	5.00	60.60	c.92+1G>A /-	$\beta^0/$ -	GG	CC	
Thal 039			23.00		c.92+5G>C HOMO	β^0/β^0	GG	CC	
Thal 040	4830.00	1.20	32.00	115.10	c.92+5G>C /-	$\beta^0/$ -	GG	CG	GG
Thal 041	5410.00	1.40	52.00	73.60	c.92+5G>C HOMO	β^0/β^0	GG	CC	GG
Thal 042	4810.00	1.00	17.00	62.80	c.92+5G>C HOMO	β^0/β^0	GG	CG	GT
Thal 043	8770.00	1.80	17.00	150.90	c.92+5G>C HOMO	β^0/β^0	GG	CC	GG
Thal 044	5330.00	3.50	38.00	173.20	c.126_129delCTTT /-	$\beta^0/$ -	GG	CG	GG
Thal 045	5290.00	1.90	4.00		c.92+5G>C HOMO	β^0/β^0	GG	CC	GG
Thal 046	6730.00	0.60	11.00	92.00	c.92+5G>C /-	$\beta^0/$ -	GG	CC	GG
Thal 047	2600.00	1.40	26.00	19.80	c.92+5G>C HOMO	β^0/β^0	GG	CC	GG
Thal	3500.00	0.70	12.00	126.00			GG	CC	GG

048									
Thal 049	9110.00	1.50	23.00	72.40	c.92+5G>C /-	β⁰/ -	GG	CG	GG
Thal 050	4410.00	1.40	8.00	42.10	c.92+5G>C /-	β⁰/ -	GG	CC	GG
Thal 051	9870.00	1.10	10.00	218.30	c.92+5G>C; c.126_129delCTTT	β⁰/ β⁰	GG	CC	GG
Thal 052	4620.00	2.00	90.00	263.40	c.92+5G>C; NG_000007.3:g.71609_72227del619	β⁰/ β⁰	GG	CC	GG
Thal 053	4540.00	0.60	8.00	41.30	c.92+5G>C; c.92+1G>A	β⁰/ β⁰	GG	CC	GG
Thal 054	3830.00	0.30	9.00	76.00	IVS1-1 G>A HOMO	β⁰/ β⁰	GG	CC	
Thal 055	3280.00	1.40	10.00	62.30	c.92+5G>C /-	β⁰/ -	GG	CC	
Thal 056	3200.00	0.90	5.00	34.50	c.92+5G>C HOMO	β⁰/ β⁰	GG	CC	GG
Thal 057	1810.00	0.10	19.00	44.20	c.92+5G>C /-	β⁰/ -	GG	CG	
Thal 058	5260.00	3.10	21.00	88.30	c.92+5G>C /-	β⁰/ -	GG	CC	GT
Thal 059	868.00	1.70	9.00	16.80	c.92+5G>C HOMO	β⁰/ β⁰	GG	CC	GG
Thal 060	813.00	0.60	4.00	22.00	c.92+5G>C /-	β⁰/ -	GG	CC	GG
Thal 061	3540.00	1.00	15.00	35.20	c.92+5G>C /-	β⁰/ -	GG	CC	GG
Thal 062	2980.00	1.20	8.00	12.70	c.92+5G>C HOMO	β⁰/ β⁰	GG	CC	GT
Thal 063	12470.00	0.80	44.00	175.30	c.92+5G>C; CD41/42	β⁰/ β⁰	GG	CC	GG
Thal 064	3760.00	1.00	15.00	71.60	c.92+5G>C HOMO	β⁰/ β⁰	GG	CC	
Thal 066	5960.00	0.80	6.00	78.30	c.92+5G>C /-	β⁰/ -	GG	CC	GG
Thal 067	11540.00	2.10	4.00	581.50			GG	CC	GG
Thal 068	157.00	0.30	8.00	13.80	c.92+5G>C /-	β⁰/ -	GG	CC	
Thal 069			33.00		c.92+5G>C HOMO	β⁰/ β⁰	GG	CC	GG
Thal 070	950.00	0.80	4.00	22.90	c.92+5G>C /C46delT	β⁰/ β⁰	GG	CG	
Thal 071	4530.00	1.20	20.00	79.60	c.92+5G>C; c.92+1G>A	β⁰/ β⁰	GG	CG	
Thal 073	2920.00	34.70	12.00	91.20	c.92+5G>C HOMO	β⁰/ β⁰	GG	CC	
Thal 074	4210.00	1.00	23.00	51.90	c.92+5G>C HOMO	β⁰/ β⁰	GG	CC	
Thal 075	6750.00	0.30	10.00	48.00	c.92+1G>A HOMO	β⁰/ β⁰	GG	CG	
Thal 076	5920.00	5.80	35.00	25.10	c.92+5G>C; c.92+1G>A	β⁰/ β⁰	GG	CC	

Thal 077	5620.00	1.20	9.00	71.70				GG	CC
Thal 078	7630.00	1.50	36.00	85.20	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 079	3930.00	0.40	30.00	46.70	c.92+5G>C HOMO	β^0/β^0		GG	CG
Thal 080	4430.00	2.50	12.00	168.70	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 081	4600.00	0.60	7.00	126.10	c.92+1G>A /-	$\beta^0/$ -		GG	CC
Thal 082	5140.00	1.30	40.00	76.20	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 083	770.00	1.40	3.00	23.40	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 085	4710.00	1.50	32.00	80.00	c.92+5G>C; CD41/42	β^0/β^0		GG	CC
Thal 087	1680.00	0.60	4.00	13.30	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 088	3950.00	0.70	6.00	29.90	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 089	5320.00	5.60	37.00	168.90	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 090	4090.00	0.60	35.00	20.90	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 091	3170.00	5.40	14.00	42.10	c.92+1G>A HOMO	β^0/β^0		GG	CC
Thal 092	5690.00	1.10	65.00	151.20	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 093	3000.00	1.20	6.00	96.90	c.92+1G>A /-	$\beta^0/$ -		GG	CC
Thal 095	1443.00	0.90	57.00	131.30	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 097	1475.00	0.60	12.00	156.50	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 098	11020.00	1.50	20.00	94.40				GG	CG
Thal 099	6530.00	29.00	10.00	268.80	c.92+5G>C HOMO	β^0/β^0		GG	CG
Thal 100	2440.00	0.60	13.00	94.60	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 101	5780.00	0.10	19.00	232.90	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 102	3710.00	1.50	9.00	18.60	c.92+1G>A HOMO	β^0/β^0		GG	CC
Thal 103	4830.00	1.30	10.00	23.80	c.92+1G>A HOMO	β^0/β^0		GG	CC
Thal 104	4420.00	0.90	12.00	124.40	c.92+5G>C /C46delT	β^0/β^0		GG	CG
Thal 105	3350.00	0.60	8.00	226.00	c.92+5G>C HOMO	β^0/β^0		GG	CC
Thal 106	5120.00	1.20	9.00	59.00	c.92+5G>C; c.92+1G>A	β^0/β^0		GG	CC
Thal 107	6190.00	0.60	20.00	58.70	c.92+5G>C; c.79G>A	β^0/β^+		GG	CC
Thal	11670.00	0.90	15.00	32.00	c.92+5G>C	β^0/β^0		GG	CC

Thal 108					HOMO			
Thal 112	5150.00	0.90	10.00	210.00	c.92+5G>C /-	β⁰/-	GG	CC
Thal 113					c.92+5G>C; c.79G>A	β^0/β^+	GG	CC
Thal 115	5100.00	2.00	36.00	165.80	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 116	5200.00	1.60	27.00	108.90	c.92+5G>C /-	β⁰/-	GG	CC
Thal 118	4130.00	2.00	10.00	22.50	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 120	3040.00	0.90	45.00	83.20	c.92+5G>C HOMO	β^0/β^0	GG	CG
Thal 122	6260.00	1.60	5.00	83.90	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 123	4340.00	0.60	30.00	103.90	c.92+5G>C HOMO	β^0/β^0	GG	CG
Thal 125	6350.00	0.30	21.00	55.80	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 126	2170.00	0.90	3.00	28.80	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 128	5740.00	1.90	18.00	198.30	c.92+5G>C HOMO	β^0/β^0	GG	CG
Thal 129	4490.00	22.00	1.30	62.20	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 131	2130.00	1.30	10.00	77.90	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 133	4120.00	1.30	72.00	13.00	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 134	9200.00	0.50	10.00	328.10	c.92+5G>C; c.92+1G>A	β^0/β^0	GG	CC
Thal 135	2210.00	0.90	8.00	48.80	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 137	2800.00	1.00	11.00	93.10	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 138	10230.00	0.60	25.00	40.60	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 139	3370.00	1.00	10.00	39.90	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 140	8450.00	0.60	37.00	332.60	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 141	3050.00				c.92+5G>C /-	β⁰/-	GG	CC
Thal 142	1970.00	0.80	13.00	34.60	c.92+5G>C HOMO	β^0/β^0	GG	CC
Thal 146	5400.00	0.30	20.00	109.40	c.92+5G>C HOMO	β^0/β^0	GG	CC

.

3.2.1 Results of the *HBB* gene mutation analysis

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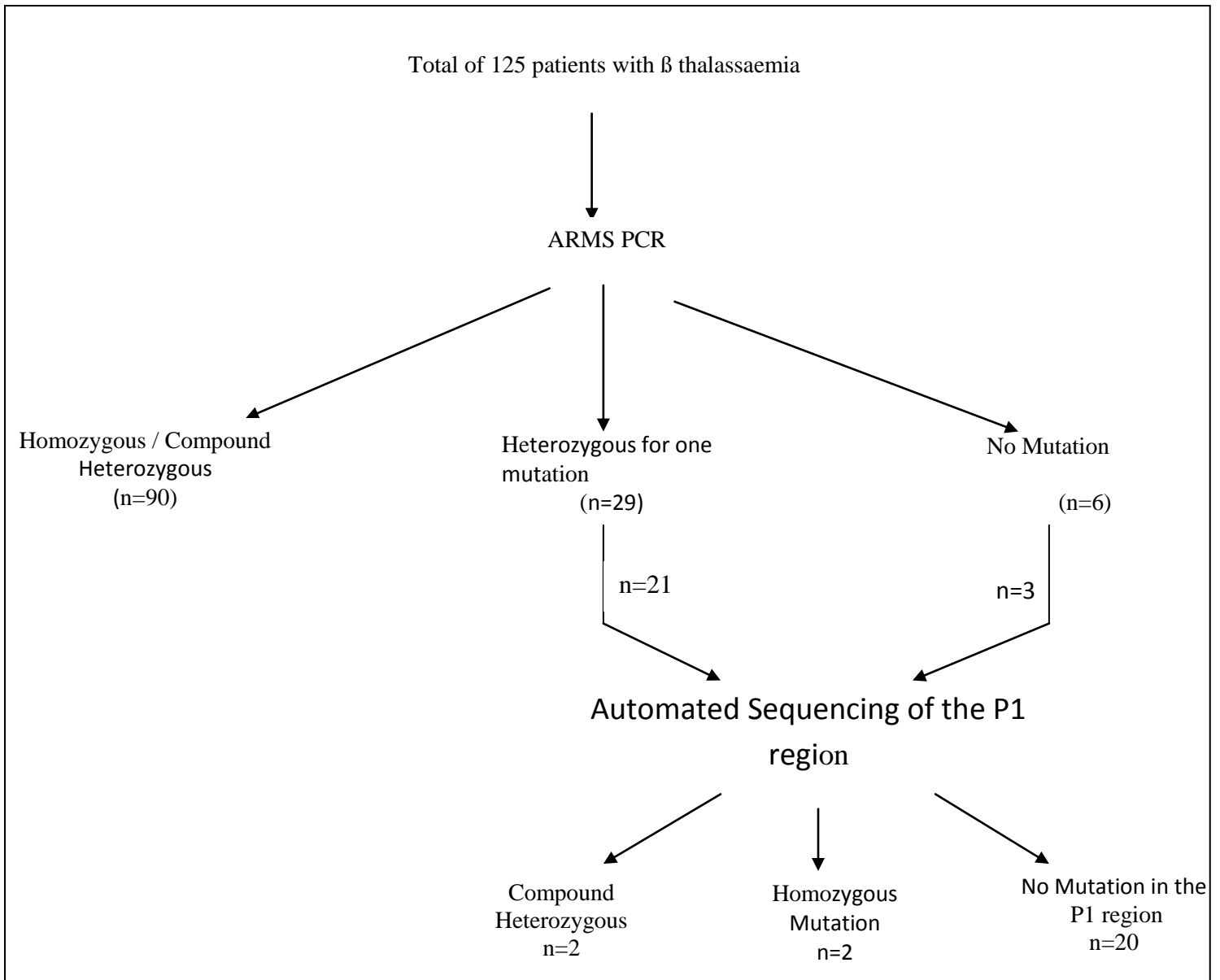
Fig: 3.2 *HBB* gene analysis and results

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.
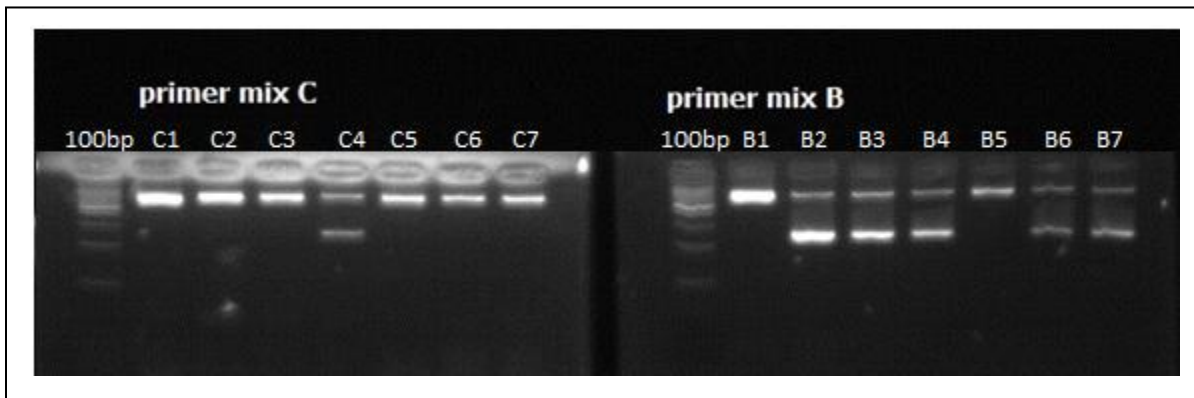
Fig: 3.3 Gel image showing ARMS PCR for the detection of *HBB* gene mutations; Primer mix B contained c.92+5G>C mutation specific primer. Primer mix C contained c.92+1G>A, c.126_129delCTTT mutation specific primers. Each mixture (B and C) contained control primers to detect NG_000007.3:g.71609_72227del619 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.
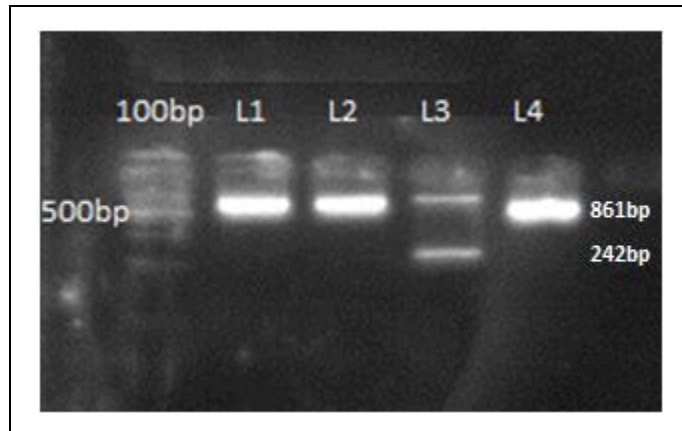
Fig: 3.4 Gel image of g.71609_72227del619 mutation, only control primers were used (861bp). 100bp ladder, L1- Thal 134 (–ve) , L2- Thal 106 (–ve), L3- Thal 52 (+ve), L4- (-ve) control
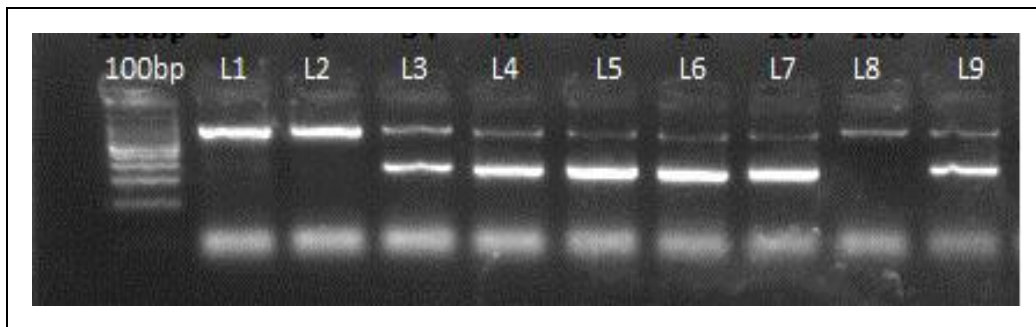
Fig: 3.5 Gel image showing genotyping for (+ve) c.92+5G>C samples. L1, L2- Homozygous for c.92+5G>C, L3-L7- Heterozygous for c.92+5G>C, L8- (+ve) control, L9- (-ve) control

Table 3.2 Pathogenic mutations detected in 119 patients with β thalassaemia major by ARMS PCR method.

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

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

Mutation	N (%)
c.92+5G>C	69.6%
c.92+1G>A	9.2%
c.126_129delCTTT	3.6%
g.71609_72227del619	0.4%
c.79G>A	0.8%

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

Genotype/Mutation	N (%)
Homozygosity	
c.51delC	1(0.8%)
c.27_28insG	1(0.8%)
Compound Heterozygosity	
c.92+5G>C ; C46delT	2 (1.6%)

.
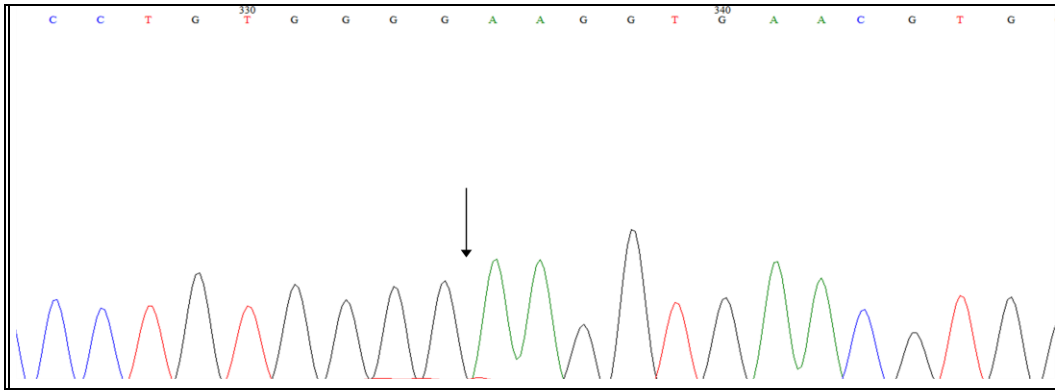
Fig: 3.6 Chromatogram view showing homozygous c.51delC mutation (Thal 012).
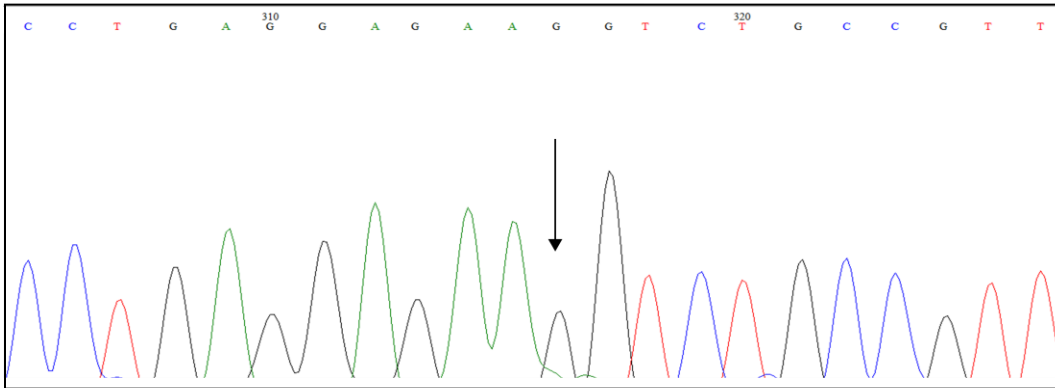
Fig: 3.7 Chromatogram view showing Homozygous c.27_28insG mutation (Thal 033).
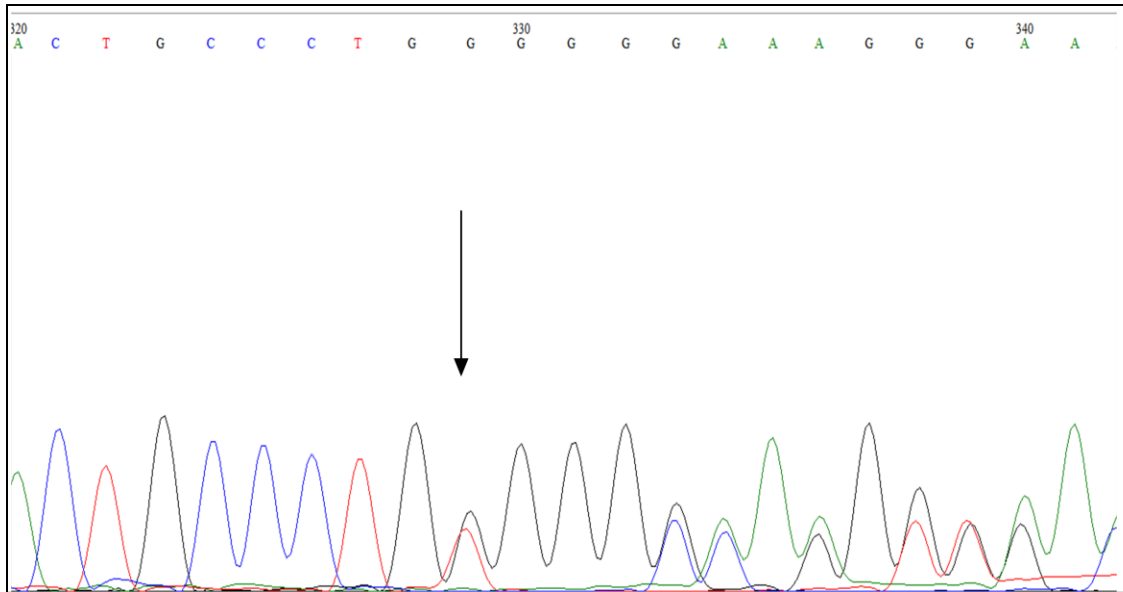
Fig: 3.8 Chromatogram view showing Heterozygous C46delT mutation (Thal 070 and Thal 104).

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)
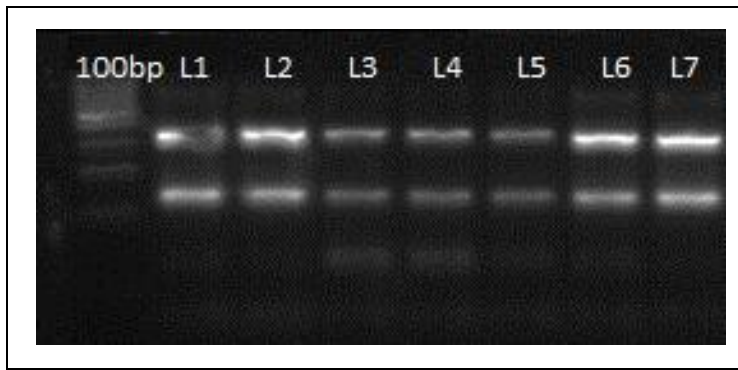
Fig: 3.9 Gel image showing the c.845G>A mutation: Lane1-L6 – patient samples for homozygous wild type variant (GG), Lane 7-Standard (–ve) control
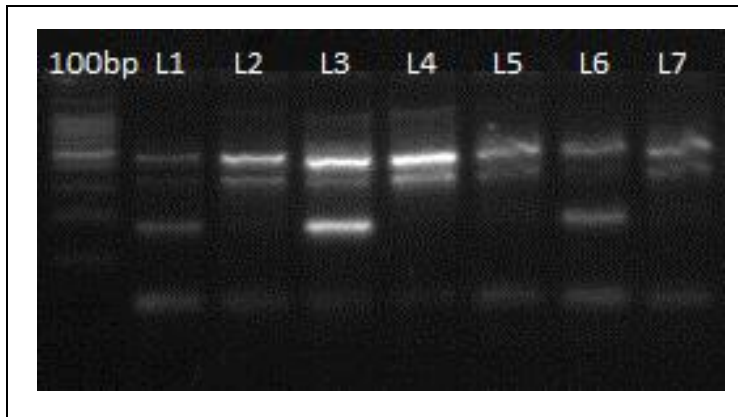
Fig: 3.10 Gel Image showing the c.187C>G mutation: 100bp ladder, Lane1- heterozygous for H63D mutation (CG). Lane2- CC, Lane3- CG, Lane4- CC, Lane5 CC, Lane6- (+ve) control, Lane7- (-ve) control

Table 3.5 Prevalence of the c.187C>G and the c.845G>A mutations in the study group (N/N – wild type)

c.187C>G genotype	Number of patients	% of the population
c.187C>G/N	23	18.4%
c.187C>G/ c.187C>G	0	0%
c.845G>A/ c.187C>G	0	0%
N/N	102	81.6%

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-homozygouse for the wild type, CG- heterozygouse for the variant allele.

C>G mutation	Number of patients	Mean ferritin level (ng/ml)	SEM
CC	98	4571	265
CG	23	4987	541

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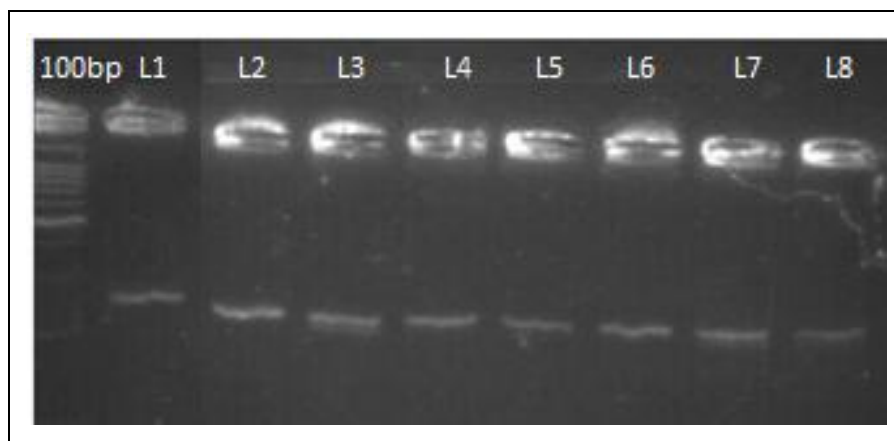
**Fig: 3.11 Gel image showing *COL1A1* gene c.104-441G>T polymorphism: L1- Thal 001-
GG, L2 Thal 008- GG, L3 Thal 009- GT, L4 Thal 010- GG, L5 Thal 011- GG, L6 Thal 012
GG, L7 (+ve) control , L8 (-ve) control.**

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

Mutation	N (%)
GG	40 (32%)
GT	6 (4.8%)

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.
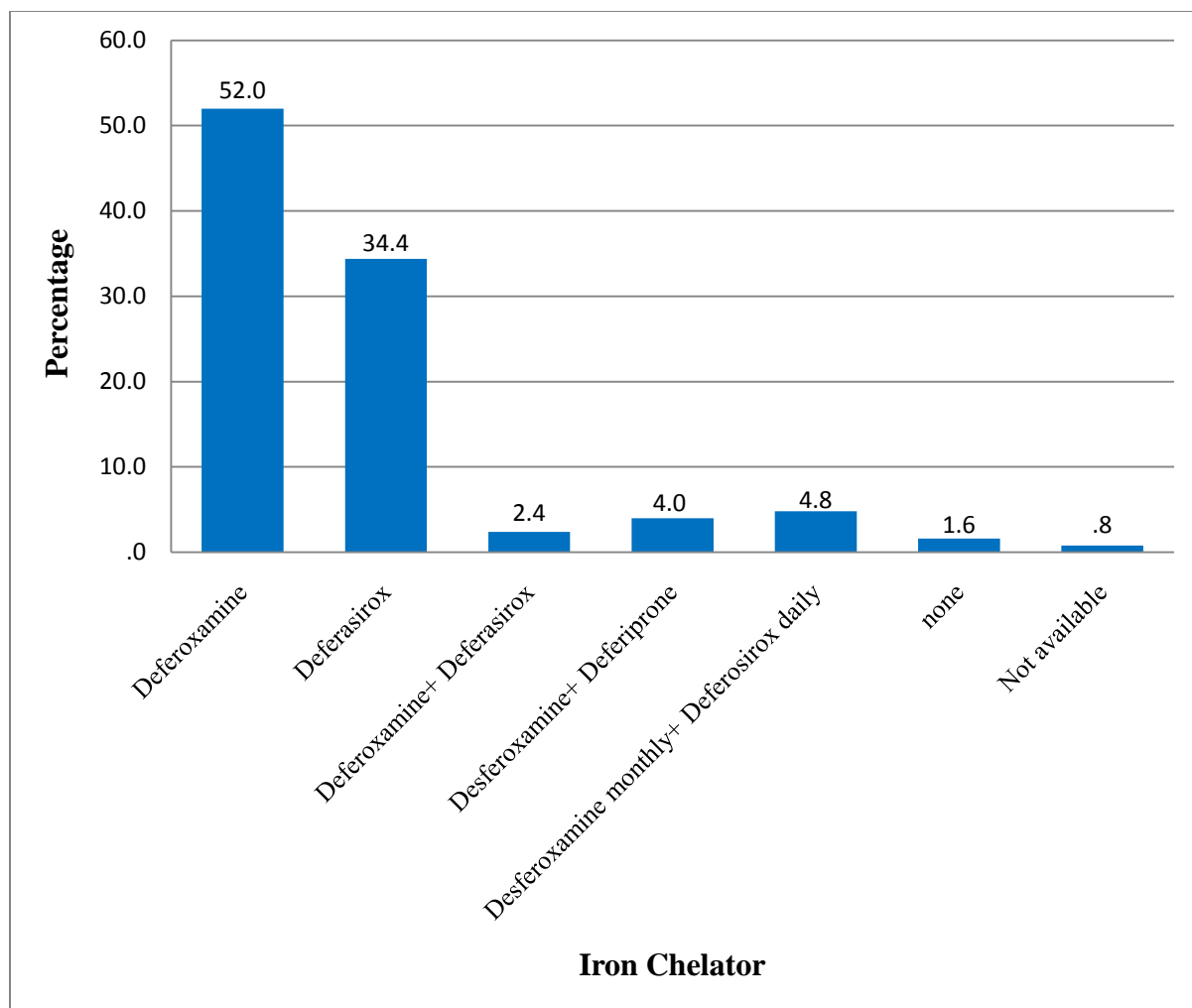
Fig: 3.12 Modes of iron chelation and the percentages of patients.

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).

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

Frequency of transfusions	Mean serum ferritin (ng/ml)	Number of patients	SEM
1	5700	5	1502
2	4527	36	454
3	4611	78	289
4	5725	2	625

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.

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

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 thalasaemia 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 $\beta^+\beta^0$ 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 $\beta^0\beta^+$. 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 β^0 allele, which completely abolishes β globin chain synthesis (Patrinos 2004). Therefore a patient who is homozygous for this mutation has β^0/β^0 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 β^0 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 β^0/β^0 allele composition hence β 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)->-GG causing a frameshift mutation, hence resulting β^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 β^0 functional allele. So the two patients with c.92+5G>C; c.46delT mutations had the same functional allele composition; β^0/β^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 β^0 except the c.79G>A mutation which was β^+. Therefore all the 90 patients detected by ARMS PCR had β^0/β^0 allele composition except the two patients who were compound heterozygous for c.92+5G>C; c.79G>A mutations whose allele composition was β^0/β^+. β^0/β^0 patients belong to thalassaemia major phenotype whereas β^0/β^+ 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 β^0 alleles. Therefore after automated sequencing, the allele composition of those 4 patients was confirmed as β^0/β^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

Study Author	Number of alleles studied	c.845G>A allele frequency	c.187C>G allele frequency
Merryweather-Clarke et al (1997)	260	0%	9.2%
Rochette et al (1999)	218	0.08%	10.8%
Current Study	250	0%	9.2%

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 coinheritance 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.

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

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 *COL1A1* gene

The c.104-441G>T polymorphism in *COL1A1* 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 *HBB* gene in a group of patients with transfusion dependent β or HbE/ β thalassaemia and to analyze two tertiary modifier genes; *HFE* and *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 goegraphical 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 *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 *COL1A1* gene is polymorphic in the study population and needs further study including correlation of the genotyping data with the Bone Mineral Density.

References

Agarwal, S., M. Naveed, et al. (1994). Characterization of beta-thalassaemia mutations in 57 beta-thalassaemia families seen at Lucknow. *Indian J Med Res* **100**: 106-110.

Angastiniotis, M. and B. Modell (1998). Global epidemiology of hemoglobin disorders. *Ann N Y Acad Sci* **850**: 251-269.

Bain, B. J. (2006). Haemoglobinopathy Diagnosis, Blackwell Publishing.

Beutler, E. (1997). The significance of the 187G (H63D) mutation in hemochromatosis. *Am J Hum Genet* **61**(3): 762-764.

Beutler, E., A. V. Hoffbrand, et al. (2003). Iron deficiency and overload. *Hematology Am Soc Hematol Educ Program*: 40-61.

Bosma, P. J., J. R. Chowdhury, et al. (1995). The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med* **333**(18): 1171-1175.

Brittenham, G. M., A. R. Cohen, et al. (1993). Hepatic iron stores and plasma ferritin concentration in patients with sickle cell anemia and thalassemia major. *Am J Hematol* **42**(1): 81-85.

Burt, M. J., P. M. George, et al. (1998). The significance of haemochromatosis gene mutations in the general population: implications for screening. *Gut* **43**(6): 830-836.

Camaschella, C., A. C. Kattamis, et al. (1997). Different hematological phenotypes caused by the interaction of triplicated alpha-globin genes and heterozygous beta-thalassemia. *Am J Hematol* **55**(2): 83-88.

Cao, A. and R. Galanello (2010). Beta-thalassemia. *Genet Med* **12**(2): 61-76.

Cao, A., P. Moi, et al. (2011). Recent advances in beta-thalassemias. *Pediatr Rep* **3**(2): e17.

Cappellini, M. D., S. R. Fargion, et al. (1998). Nontransfusional iron overload in thalassemia intermedia: role of the hemochromatosis allele. *Blood* **92**(11): 4479-4480.

Chui, D. H. (2005). Alpha-thalassemia: Hb H disease and Hb Barts hydrops fetalis. *Ann N Y Acad Sci* **1054**: 25-32.

De Silva, C. C. and C. E. Weeratunge (1951). Cooley's anaemia in Sinhalese children. *Arch Dis Child* **26**(127): 224-230.

de Silva, S., C. A. Fisher, et al. (2000). Thalassaemia in Sri Lanka: implications for the future health burden of Asian populations. Sri Lanka Thalassaemia Study Group. *Lancet* **355**(9206): 786-791.

Dora Bachir, F. G. (2004) "Hemoglobin E." Orphanet Encyclopedia.

Drakesmith, H., E. Sweetland, et al. (2002). The hemochromatosis protein HFE inhibits iron export from macrophages. *Proc Natl Acad Sci U S A* **99**(24): 15602-15607.

Dresner Pollack, R., E. Rachmilewitz, et al. (2000). Bone mineral metabolism in adults with beta-thalassaemia major and intermedia. *Br J Haematol* **111**(3): 902-907.

Economou-Petersen, E., A. Aessopos, et al. (1998). Apolipoprotein E epsilon4 allele as a genetic risk factor for left ventricular failure in homozygous beta-thalassemia. *Blood* **92**(9): 3455-3459.

Eldor, A. and E. A. Rachmilewitz (2002). The hypercoagulable state in thalassemia. *Blood* **99**(1): 36-43.

Elmrghni, S., R. A. Dixon, et al. (2011). Frequencies of HFE gene mutations associated with haemochromatosis in the population of Libya living in Benghazi. *Int J Clin Exp Med* **4**(3): 200-204.

Estevao, I. F., P. Peitl Junior, et al. (2011). Serum ferritin and transferrin saturation levels in beta(0) and beta(+) thalassemia patients. *Genet Mol Res* **10**(2): 632-639.

Fairbanks, V. F., D. J. Brandhagen, et al. (1998). H63D is an haemochromatosis associated allele. *Gut* **43**(3): 441-442.

Faustino, P., L. Osorio-Almeida, et al. (1998). Dominantly transmitted beta-thalassemia arising from the production of several aberrant mRNA species and one abnormal peptide. *Blood* **91**(2): 685-690.

Feder, J. N., A. Gnirke, et al. (1996). A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* **13**(4): 399-408.

Feder, J. N., D. M. Penny, et al. (1998). The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proc Natl Acad Sci U S A* **95**(4): 1472-1477.

Ferrara, M., S. M. Matarese, et al. (2002). Effect of VDR polymorphisms on growth and bone mineral density in homozygous beta thalassaemia. *Br J Haematol* **117**(2): 436-440.

Fisher, C. A., A. Premawardhena, et al. (2003). The molecular basis for the thalassaemias in Sri Lanka. *Br J Haematol* **121**(4): 662-671.

Galanello, R., S. Piras, et al. (2001). Cholelithiasis and Gilbert's syndrome in homozygous beta-thalassaemia. *Br J Haematol* **115**(4): 926-928.

Gennari, L., D. Merlotti, et al. (2005). Estrogen receptor gene polymorphisms and the genetics of osteoporosis: a HuGE review. *Am J Epidemiol* **161**(4): 307-320.

Grant, S. F., D. M. Reid, et al. (1996). Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I alpha 1 gene. *Nat Genet* **14**(2): 203-205.

Hash, R. B. (2001). Hereditary hemochromatosis. *J Am Board Fam Pract* **14**(4): 266-273.

He, Z., Y. Hu, et al. (2006). Polymorphisms in the HBB gene relate to individual cardiorespiratory adaptation in response to endurance training. *Br J Sports Med* **40**(12): 998-1002.

Ho, P. J., G. W. Hall, et al. (1998). Beta-thalassaemia intermedia: is it possible consistently to predict phenotype from genotype? *Br J Haematol* **100**(1): 70-78.

Ho, P. J., S. N. Wickramasinghe, et al. (1997). Erythroblastic inclusions in dominantly inherited beta thalassemias. *Blood* **89**(1): 322-328.

Hoffbrand, A. V., Catovsky, D., Tuddenham, E.G.D., Ed. (2005). Postgraduate Haematology, Blackwell Publisher.

Hoffman R., B. E. J., Shattil S.J., Furie B., Cohen H.J., Silberstein L.E, McGlave P., Ed. (2005). Hematology Basic Principles and Practice, ELSEVIER.

Huisman, T. H. J., Carver, M.F.H. & Baysal, E., Ed. (1997). A Syllabus of

Thalassemia Mutations., The Sickle Cell Anemia Foundation, Augusta. 309.

Jin, H., R. J. van't Hof, et al. (2009). Promoter and intron 1 polymorphisms of COL1A1 interact to regulate transcription and susceptibility to osteoporosis. *Hum Mol Genet* **18**(15): 2729-2738.

Karimi, M., M. Yavarian, et al. (2004). Spectrum and haplotypes of the HFE hemochromatosis gene in Iran: H63D in beta-thalassemia major and the first E277K homozygous. *Hematol J* **5**(6): 524-527.

Kaur, G., C. C. RAPTHAP, et al. (2003). Distribution of C282Y and H63D mutations in the HFE gene in healthy Asian Indians and patients with thalassaemia major. *THE NATIONAL MEDICAL JOURNAL OF INDIA* **16**.

Kulkarni, G. D., S. S. Kulkarni, et al. (2012). Molecular basis of beta-thalassemia in Karnataka, India. *Genet Test Mol Biomarkers* **16**(2): 138-141.

Lewis S.M., B. B. J., Bates I., Ed. (2006). Dacie and Lewis Practicle Haematology, Elsivier.

Lok, C. Y., A. T. Merryweather-Clarke, et al. (2009). Iron overload in the Asian community. *Blood* **114**(1): 20-25.

Longo, F., G. Zecchina, et al. (1999). The influence of hemochromatosis mutations on iron overload of thalassemia major. *Haematologica* **84**(9): 799-803.

MacDonald, H. M., F. A. McGuigan, et al. (2001). COL1A1 Sp1 polymorphism predicts perimenopausal and early postmenopausal spinal bone loss. *J Bone Miner Res* **16**(9): 1634-1641.

Mann, V., E. E. Hobson, et al. (2001). A COL1A1 Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality. *J Clin Invest* **107**(7): 899-907.

Mann, V. and S. H. Ralston (2003). Meta-analysis of COL1A1 Sp1 polymorphism in relation to bone mineral density and osteoporotic fracture. *Bone* **32**(6): 711-717.

Marengo-Rowe, A. J. (2006). Structure-function relations of human hemoglobins. *Proc (Bayl Univ Med Cent)* **19**(3): 239-245.

Mathias, L. A., T. C. Fisher, et al. (2000). Ineffective erythropoiesis in beta-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. *Exp Hematol* **28**(12): 1343-1353.

Melis, M. A., M. Cau, et al. (2002). H63D mutation in the HFE gene increases iron overload in beta-thalassemia carriers. *Haematologica* **87**(3): 242-245.

Merryweather-Clarke, A. T., J. J. Pointon, et al. (1997). Global prevalence of putative haemochromatosis mutations. *J Med Genet* **34**(4): 275-278.

Musallam, K. M., M. D. Cappellini, et al. (2011). Elevated liver iron concentration is a marker of increased morbidity in patients with beta thalassemia intermedia. *Haematologica* **96**(11): 1605-1612.

Newton, C. R., A. Graham, et al. (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res* **17**(7): 2503-2516.

Old, J. M., S. N. Khan, et al. (2001). A multi-center study in order to further define the molecular basis of beta-thalassemia in Thailand, Pakistan, Sri Lanka, Mauritius, Syria, and India, and to develop a simple molecular diagnostic strategy by amplification refractory mutation system-polymerase chain reaction. *Hemoglobin* **25**(4): 397-407.

Oliveira, T. M., F. P. Souza, et al. (2006). HFE gene mutations in Brazilian thalassemic patients. *Braz J Med Biol Res* **39**(12): 1575-1580.

Origa, R., E. Fiumana, et al. (2005). Osteoporosis in beta-thalassemia: Clinical and genetic aspects. *Ann N Y Acad Sci* **1054**: 451-456.

Origa, R., R. Galanello, et al. (2007). Liver iron concentrations and urinary hepcidin in beta-thalassemia. *Haematologica* **92**(5): 583-588.

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

and the Middle East *The Open Hematology Journal* **2**: 5-13.

Papanikolaou, G., M. Tzilianos, et al. (2005). Hepcidin in iron overload disorders. *Blood* **105**(10): 4103-4105.

Patrinos, G. P., B. Giardine, C. Riemer, W. Miller, D.H.K. Chui, N.P. Anagnou, H. Wajcman, and R.C. Hardison (2004). A database of Human Hemoglobin Variants and Thalassemias.

Perrotta, S., M. D. Cappellini, et al. (2000). Osteoporosis in beta-thalassaemia major patients: analysis of the genetic background. *Br J Haematol* **111**(2): 461-466.

Piperno, A., R. Mariani, et al. (2000). Haemochromatosis in patients with beta-thalassaemia trait. *Br J Haematol* **111**(3): 908-914.

Premawardhena, A., S. De Silva, et al. (2004). Thalassemia in Sri Lanka: a progress report. *Hum Mol Genet* **13 Spec No 2**: R203-206.

Premawardhena, A., C. A. Fisher, et al. (2005). Haemoglobin E beta thalassaemia in Sri Lanka. *Lancet* **366**(9495): 1467-1470.

Rees, D. C., L. Y. Luo, et al. (1997). Nontransfusional iron overload in thalassemia: association with hereditary hemochromatosis. *Blood* **90**(8): 3234-3236.

Rochette, J., J. J. Pointon, et al. (1999). Multicentric origin of hemochromatosis gene (HFE) mutations. *Am J Hum Genet* **64**(4): 1056-1062.

Rossi, E. and G. P. Jeffrey (2004). Clinical penetrance of C282Y homozygous HFE haemochromatosis. *Clin Biochem Rev* **25**(3): 183-190.

Roy, C. N. and N. C. Andrews (2001). Recent advances in disorders of iron metabolism: mutations, mechanisms and modifiers. *Hum Mol Genet* **10**(20): 2181-2186.

Rund, D. and E. Rachmilewitz (2005). Beta-thalassemia. *N Engl J Med* **353**(11): 1135-1146.

Santiago Rodriguez, T. R. G., Ian N. M. Day (2009) "Hardy-Weinberg Equilibrium Testing of Biological Ascertainment for Mendelian Randomization Studies." American Journal of Epidemiology DOI: 10.1093/aje/kwn359.

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.

Swinkels, D. W., M. C. Janssen, et al. (2006). Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. *Clin Chem* **52**(6): 950-968.

Takeuchi, T., H. Soejima, et al. (1997). Efficient large-scale screening for the hemochromatosis susceptibility gene mutation. *Blood* **90**(7): 2848-2849.

Technelysium Pty Ltd. (2008). "Chromas LITE Version 2.01." Retrieved September 20, 2011, from www.technelysium.com.au.

Thein, S. L. (2004). Genetic insights into the clinical diversity of beta thalassaemia. *Br J Haematol* **124**(3): 264-274.

Thein, S. L. (2005). Genetic modifiers of beta-thalassemia. *Haematologica* **90**(5): 649-660.

Thein, S. L., J. S. Wainscoat, et al. (1987). Association of thalassaemia intermedia with a beta-globin gene haplotype. *Br J Haematol* **65**(3): 367-373.

Totaro, A., A. Roetto, et al. (1998). Generation of a transcription map of a 1 Mbase region containing the HFE gene (6p22). *Eur J Hum Genet* **6**(2): 105-113.

van Dijk, F. S., J. M. Cobben, et al. (2011). Osteogenesis Imperfecta: A Review with Clinical Examples. *Mol Syndromol* **2**(1): 1-20.

Vichinsky, E. (2007). Hemoglobin E Syndromes. *Hematology*.

Voskaridou, E. and E. Terpos (2004). New insights into the pathophysiology and management of osteoporosis in patients with beta thalassaemia. *Br J Haematol* **127**(2): 127-139.

Weatherall, D. J. (1998). Pathophysiology of thalassaemia. *Baillieres Clin Haematol* **11**(1): 127-146.

Weatherall, D. J. (1998). Thalassemia in the next millennium. Keynote address. *Ann N Y Acad Sci* **850**: 1-9.

Weatherall, D. J. (2001). Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. *Nat Rev Genet* **2**(4): 245-255.

Weatherall, D. J. and J. B. Clegg (1996). Thalassemia--a global public health problem. *Nat Med* **2**(8): 847-849.

Weatherall, D. J. C., J.B, Ed. (2001). The Thalassaemia Syndromes, Blackwell Science, Oxford.

Wonke, B., C. Jensen, et al. (1998). Genetic and acquired predisposing factors and treatment of osteoporosis in thalassaemia major. *J Pediatr Endocrinol Metab* **11 Suppl 3**: 795-801.

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 the 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 the 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   Prof VajiraDissanayake

MSc Student   Senior Lecturer

Human Genetics Unit   Human Genetics Unit

Faculty of Medicine   Faculty of Medicine

Colombo   Colombo

0112689545   0112689545

තොරතුරු පත්‍රිකාව

බීටා තැලසීමීයා රෝගය සහ එහි රෝග ලක්ෂණ සඳහා බලපාන අනෙකුත් ජාන අධ්‍යයනය-: ලාංකීය රෝගීන්ගේ එම ජාන වල විවිධත්වය සහ රෝග ලක්ෂණ අතර සම්බන්ධතාවය අධ්‍යයනය කිරීම.

කොළඹ වෛද්‍ය පීයේ මානව ප්‍රවේණි විද්‍යා අංශයේ පශ්චාත් උපාධියක් හදාරන වෛද්‍ය පද්මපානි පාදෙනිය වන මම ඇතුළු අනෙකුත් පර්යේෂණ සමාජිකයින් ඉහත සඳහන් පර්යේෂණයට සහභාගී වීම සඳහා ඔබ හට ආරාධනා කරමු.

අපගේ පර්යේෂණ කණ්ඩායමේ සමාජිකයින් වනුයේ

- මහාචාර්ය වජිර එච්. ඩබ්ලිව්. දිසානායක (ප්‍රධාන අධීක්ෂක) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීයේ කාය ව්‍යවච්ඡේද ඒකකයට සම්බන්ධ මහාචාර්ය වරයෙකි.
- මාහාචාර්ය රෝහාන් ඩබ්ලිව්. ජයසේකර (අධීක්ෂක) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීයේ මානව ප්‍රවේණි විද්‍යා ඒකකයට සම්බන්ධ මහාචාර්ය වරයෙකි.
- විශේෂඥ වෛද්‍ය නිලධාරීනී හේමමාලී ගුණසේකර (සායනික අධීක්ෂක)- කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීයේ කාය ව්‍යවච්ඡේද ඒකකයට සම්බන්ධ රක්ත වේදී විශේෂඥ වරියකි.
- වෛද්‍ය පද්මාණි පාදෙනිය (ප්‍රධාන පර්යේෂකවරිය)

1. **මෙම අධ්‍යයනයේ අරමුණු**

   මෙම පර්යේෂණයේ ප්‍රධාන අරමුණ වනුයේ බීටා තැලසීමීයා රෝගීන්ගේ සායනික රෝග ලක්ෂණ සහ එම සායනික රෝග ලක්ෂණ වල විවිධත්වය කෙරෙහි බලපාන අනෙකුත් ජාන අතර සම්බන්ධතාවයක් තිබේදැයි පරීක්ෂා කිරීම.

2. **ස්වේච්ඡා සහභාගීත්වය**

   මෙම අධ්‍යයනය සඳහා ඔබගේ සහභාගීත්වය ඔබගේ කැමැත්තෙන්ම සිදුකරන්නකි. මෙම අධ්‍යයනය සඳහා සහභාගී නොවීමට ඔබට පූර්ණ අයිතිය ඇති අතර සහභාගී වීමට කලින් කැමැත්ත ප්‍රකාශ කර තිබුණද මින් ඕනෑම අවස්ථාවක අපහට දැනුම් දී ඔබට අධ්‍යයනයෙන් ඉවත් වීමට පූර්ණ අයිතිය ඇත. සහභාගී නොවීම නිසා ඔබට ලැබිය යුතු වෛද්‍ය ප්‍රතිකාර හා සේවාවන්හි කිසිදු වෙනසක් සිදු නොවනු ඇත.

3. **කාල සීමාව, ක්‍රියා පිළිවෙල සහ සහභාගී වන්නන්ගේ වගකීම**

   කොළඹ වෛද්‍ය පීයේ මානව ප්‍රවේණි විද්‍යා අංශය වෙත යොමු කරන සියලුම බීටා තැලසීමීයා රෝගීන් මේ සඳහා සහභාගී කරගනු ලැබේ. ඔබ මෙම අධ්‍යයනයට සහභාගී වීමට කැමති නම් ඔබගේ දරුවාගේ වෛද්‍ය වාර්තා සහ සායනික විස්තර සටහන් කර ගැනීමට ඔබගේ අනුමැතිය අපට අවශ්‍ය වේ. තවද මෙම අධ්‍යයනය අවසානයේ එහි ප්‍රතිඵල විද්‍යාත්මක සඟරාවක පල කිරීමට බලාපොරොත්තු වන බැවින් ඒ සඳහා ද ඔබගේ අනුමැතිය අපට අවශ්‍ය වේ. එහිදී ඔබගේ දරුවාගේ අනන්‍යතාවය සුරකින බවට අපි සහතික වෙමු. මීට අමතරව ඔබගේ දරුවාගේ බීටා තැලසීමීයා රෝගය පිළිබඳ ජාන පරීක්ෂණය සඳහා රුධිරය මි.ලී. 10ක් ලබා ගැනීමටත් අස්ථි දිරාපත් වී ඇති දැයි (ඔස්ටියෝපොරෝසිස්) දැන ගැනීම සඳහා කරන "DEXA ස්කෑන්" පරීක්ෂණයටද අනුමැතිය අවශ්‍යය. අධ්‍යයනය සඳහා අවශ්‍ය අනෙකුත් රුධිර පරීක්ෂණ වාර්තාවන් ඔබගේ දරුවාගේ පැරණි සායනික විස්තර මඟින් ලබා ගැනේ.

4. **මින් ලද හැකි ප්‍රතිලාභ**

   ඔබ මෙම අධ්‍යයනයට සහභාගී වීම නිසා බීටා තැලසීමියාවේ ජානමය වර්ගීකරණය පිළිබඳ රෝග විනිශ්චයක් ඔබ වෙත ලැබේ. ශ්‍රී ලංකාවේ මෙවැනි රෝග විනිශ්චයක් නොමිලේ ලබා ගත නොහැකි නිසා එය ඔබට ඉමහත් ප්‍රයෝජනයක් වේ. තවද මෙම අධ්‍යයනයේ අවසාන ප්‍රතිඵල මගින් ඔබගේ ළමා රෝග විශේෂඥ වරයාට / වරියට හෝ කාය විශේෂඥ වරයාට / වරියට ඔබගේ රෝගය පිළිබඳවත් රෝගී තත්ත්වය පාලනය කිරීම පිළිබඳවත් වැඩි අවබෝධයක් ලැබේ. මීට අමතරව ඔබට අප ආයතනය මගින් ඔබගේ රෝගය පිළිබඳ ජාන උපදේශනයක් නොමිලේ ලැබේ.

5. **අවධානම් අනතුරු සහ අපහසුතා**

   බීටා තැලසීමියා රෝගයේ ජාන වර්ගීකරණය සඳහා මි.ලී. 5ක පමණ රුධිර සාම්පලයක් ලබා ගැනේ. එහිදී ඇතිවිය හැකි අවධානම් අනතුරු හා අපහසුතා විය හැක්කේ රුධිරය ලබා ගත් ස්ථානයේ වේදනාව, ඉදිමුම සහ කලාතුරකින් ඇතිවිය හැකි ආසාත්මිකතාවන්ය. මෙම තත්ත්වයන් අවම කර ගැනීම සඳහා රුධිර සාම්පලය ලබා ගැනීම සියලුම ආරක්ෂිත තත්ත්වයන් යටතේ පළපුරුදු හෙද නිලධාරීනියක් මගින් සිදු කරනු ලැබේ.

6. **දීමනා**

   ඔබ මෙම අධ්‍යයනයට සහභාගී වීම නිසා ඔබට දීමනාවක් නොලැබේ. එහෙත් ඔබට බීටා තැලසීමියා රෝගයේ ජානමය වර්ගීකරණය පිළිබඳ වාර්තාවක් ලැබේ.

7. **රහසිගත බව**

   සියලුම තොරතුරු සහිත වාර්තාවන් සහ අධ්‍යයනය මගින් ලබා ගන්නා දත්තයන්ගේ රහස්‍යභාවය තහවුරු කරන අතර ඔබගේ අනන්‍යතාවය හඳුනාගත හැකි ආකාරයේ කිසිවක් ඔබගේ කැමැත්තකින් තොරව හෙලිකිරීමක්, ඉදිරිපත් කිරීමක් හෝ ප්‍රකාශයට පත් කිරීමක් සිදු කරනු නොලැබේ.

8. **අධ්‍යයනයට සහභාගීවීම නැවැත්වීම**

   අධ්‍යයනයට සහභාගීවීමට දුන් කැමැත්ත ඉවත් කර ගැනීම අධ්‍යයනයේ කුමන හෝ අදියරකදී සිදු කිරීමට ඔබට හැක. එසේ සිදු කරන්නේ නම් එම තීරණය ගත් විගසම ඒ බව අපහට කරුණාකර දැනුම් දෙන්න. නමුත් එකතු කර ගන්නා ලද දත්ත ප්‍රකාශයට පත් කිරීමෙන් පසුව ඔබට අධ්‍යයනයෙන් ඉවත් වීමට නොහැක.

9. **වැඩිදුර තොරතුරු**

   ඔබට මේ ක්‍රියා පටිපාටින් පිළිබඳ කිසියම් ප්‍රශ්ණයක් ඇත්නම් හෝ වැඩිදුර තොරතුරු අවශ්‍ය නම් කරුණාකර පහත සඳහන් වෛද්‍ය වරුන් අමතන්න.

වෛද්‍ය පද්මාණි පාදෙනිය   මහාචාර්ය වජිර එච්. ඩබ්ලිව්. දිසානායක.
පශ්චාත් උපාධි අපේක්ෂිකා   මානව ප්‍රවේණි විද්‍යා අංශය
මානව ප්‍රවේණි විද්‍යා අංශය   වෛද්‍ය පීඨය
වෛද්‍ය පීඨය   කොළඹ
කොළඹ   දු.ක 011-2689545

දු.ක 011-2689545

<u>தகவல் படிவம்</u>

β தவசீமியாவுடைய பரம்பரையியல் மாற்றங்கள்

இலங்கையிலிருந்து பெற்ற மாதிரிகளின்

தோற்றவமைப்புக்கும் மரபிடயல் அமைப்புக்கும்

இடையிலான தொடர்பு

பத்மபானி பாதெனிய (Msc மாணவி) ஆகிய நான் தற்போது மனித பரம்பரை அலகுகள் பிரிவு, மருத்துவபீடம், கொழும்பு பல்கலைக்கழகத்துடன் (Msc – மருத்துவ பரம்பரையலசியல்) இணைக்கப்பட்டுள்ளேன்.

எனது ஆய்வின் ஒன்றிணைத்து பங்குபற்றுவதற்காக உங்கள் அனைவரையும் வரவேற்கிறேன். இவ் ஆய்வானது பேராசிரியர் வஜிர திஸநாயக்கா, பேராசிரியர் றொஹான் ஜயசேகர மற்றும் வைத்தியர் ஹெமாலி குணசேகர (மனித பரம்பரை அலகுகள் பிரிவு, கொழும்பு மருத்துவபீடம்) ஆகியோரின் மேற்பார்வையின் கீழ் நடைபெறும்.

1.   ஆய்வின் நோக்கம்

β தவசீமியா நோயினுடைய மாற்றக் காரணிகள் நோயாளிகளில் ஏற்படுத்தும் மாற்றங்களை அவதானித்தல்.

பரம்பரையியல் மாற்றக் காரணிகளுக்கும் தோற்ற அமைப்புகளுக்குமிடையே ஏதாவது தொடர்புகள் உண்டா என கணித்தல்.

2.   சுயவிருப்பின் பேரில் பங்கேற்றல்

இவ் ஆய்வில் உங்கள் பங்கானது உங்கள் சுயவிருப்பம் தொடர்பானது

நீங்கள் ஆய்வில் பங்குபற்ற விரும்பாத பட்சத்தில் எந்த நேரத்திலும் இவ் ஆய்விலிருந்து விலகிக் கொள்வது உங்கள் சுயவிருப்புடையது.

இவ் ஆய்வில் நீங்கள் பங்குபற்றுவது உங்கள் நோய் தொடர்பான மருத்துவ கவனிப்பு அல்லது உங்களுக்கு கிடைக்கக்கூடிய மருத்துவ சிகிச்சையில் எந்தவித பாதிப்பையும் ஏற்படுத்த மாட்டாது.

நீங்கள் தற்போது ஒப்புதல் அளித்து பின்னர் ஆய்விலிருந்து விலக விரும்பினால் எந்த நேரமும் ஆய்விலிருந்து விலக்கப்படுவீர்கள். இருப்பின் இதை எமக்கு முன்னதாக தெரியப்படுத்தவும்.

3. ஆய்வுகாலம், செய்முறை மற்றும் பங்குபற்றுபவர்களின் பொறுப்புக்கள்

β தவசீமியா மூலக்கூற்று பரம்பரையில் பரிசோதனைக்காக நோயாளர்கள் மனித பரம்பரை அலகு பிரிவு, மருத்துவபீடத்துக்கு அழைக்கப்படுவார்கள்.

உங்கள் குழந்தையை இவ்வாய்வில் பங்குபடுத்த விரும்பினால் நாங்கள் உங்கள் நேர்காணவும். உங்கள் குழந்தையின் மருத்துவ அறிக்கைகளைப் பார்வையிடவும் பரிசோதிக்கவும் அனுமதி கேட்கிறோம்.

பரிசோதனையின் முடிவுகளை விஞ்ஞான சஞ்சிகையில் பிரசுரிப்பதற்காக உங்கள் அனுமதியை கேட்கின்றோம். இருப்பினும் உங்கள் சம்பந்தமான உங்களை அடையாளப்படுத்தக்கூடிய எந்தத் தகவலும் பிரசுரிப்பில் இடம் பெறமாட்டாது.

இவ்வாய்வில் பங்குபற்றுவதற்கு உங்களிடமிருந்து 5ml குருதி மாதிரி எடுக்கப்படும். இதற்காக உங்கள் அனுமதியை கேட்டு நிற்கிறோம்.

உங்கள் என்புக்கனிய அளவை அறிவதற்காக "DEXA SCAN" எனும்

பரிசோதனை மேற்கொள்ளப்படல் வேண்டும். இது scan உபகரணத்தை உங்கள் குழந்தையின் உடலில் பொருத்துவதன் மூலம் பரிசோதிக்கப்படும். இதற்கு உங்கள் அனுமதியை வேண்டி நிற்கிறோம். உங்கள் குழந்தையின் முன்னைய உயிர் இரசாயன அறிக்கைகளும் பரிசோதிக்கப்படும்.

4.   சாத்திய கூறுடைய நன்மைகள்

இவ் மரபியல் பரிசோதனையானது இவ் ஆய்வில் பங்கு பற்றுபவர்களுக்கு இலவசமாக நடாத்தப்படும். இலங்கையில் இவ் மரபியல் பரிசோதனையானது இலவசமாக நடாத்தப்படுவதில்லை. எனவே நோயாளர்களுக்கு இது ஒர் வரப்பிரசாதமாக அமையும்.

வேறுபட்ட மரபியல் விகாரங்களின் மாற்றங்கள், அவற்றின் தோற்ற அமைப்பு தொடர்பான அறிவு; குழந்தை மருத்துவ நிபுணர்களை இவ் மருத்துவ தோற்ற அமைப்புகள் தொடர்பாக மேலும் அறிந்து கொள்ள உதவும்.

அத்துடன் இவ் ஆய்வானது சமூகம் தொடர்பாக மிகவும் பயனள்ளதாக அமையும், ஏனெனில் இவ்வாய்வு இந்நோய் தொடர்பாக ஒர் பொது அறிவினை மக்கள் பெற்றுக் கொள்வதற்கும் பங்களிக்கிறது.

5.   ஆபத்து, இடையூறுகள் மற்றும் அசௌகரியங்கள்

β தவசீமியா நோயினுடைய மூலக்கூற்று நோய் அறுதி விளக்கப்பதிவுக்காகவும், சாத்தியக்கூறுடைய பரம்பரையியல் மாற்ற காரணிகளை கண்டறிவதற்காகவும் 10ml குருதியானது எடுக்கப்படும்.

ஊசி ஏற்படும் போது கன்றல், வலி ஏற்படுவதையும், தொற்றல் ஏற்படுவதையும் தவிர்ப்பதற்காக மிகவும் தகுதி வாய்ந்த தாதி பயன்படுத்தபடுவான். மேலும் குருதியானது கிருமியழிக்கப்பட்ட நிலையில் பாதுகாப்பாக எடுக்கப்படும்.

6.   மீளளிப்பு

இவ்வாய்வில் பங்குபற்றுவதற்காக உங்களுக்கு எந்தவித பண உதவியும் செய்யப்பட மாட்டாது. இருப்பினும் இவ்வாய்வின் முடிவில் மருத்துவ பரிசோதனை முடிவுகள் எழுத்து மூலமாக உங்கள் சொந்த மொழியில் வழங்கப்படும்.

7.   இரகசியம் பேணல்

உங்களுடைய அனைத்து பதிவுகளும் இரகசியமாகப் பேணப்படுவதுடன், எந்தவொரு தகவலும் வெளியிடப்படவோ பிரசுரிக்கப்படவோ மாட்டாது.

தகவல்களை சேகரிப்பதற்காக தகவல் திரட்டு புத்தகம் பேணப்படும்.

மேலும் தகவல்களைக் கொண்டிருக்கும் இலத்திரனியல் தகவல் தளமானது உங்கள் தகவல் தொடர்பான இலக்கத்தை மட்டும் கொண்டிருப்பதுடன் அத்தகவல் தளமானது இரகசிய குறியீடு மூலம் பாதுகாக்கப்படும்.

இத்தகவல்களானது உங்கள் அடையாளப்படுத்தக்கூடிய புகைப்படம் மற்றும் அந்தத் தகவல்களும் உங்களுடைய அனுமதியின்றி பிரசுரிக்கப்பட மாட்டாது என்பதை உறுதிப்படுத்துகின்றோம்.

8.   ஆய்விலிருந்து விலகிக் கொள்ளல்

இவ் ஆய்விலிருந்து எந்த நேரத்திலும் தண்டம் ஏதுவுமின்றி, நீங்கள் விலகிக் கொள்ளமுடியும். உங்களுடைய முடிவு உங்களுடைய மருத்துவ சிகிச்சையில் எந்தவித இழப்பையும் ஏற்படுத்தாது.

இருப்பினும் உங்கள் முடிவை இவை பிரசுரிக்கப்படுவதற்கு முன்னமாகத் தெரியப்படுத்த வேண்டும்.

9.   மேலதிக தகவல்களிற்கு

இவ் ஆய்வு தொடர்பாக உங்களுக்கு ஏதாவது சந்தேகங்களோ, வினாக்களோ இருப்பின் மேலதிக தகவல்களுக்கு பின்வருபவர்களைத் தொடர்பு கொள்ளவும்.

Dr. பத்மபானி பாதெனிய   பேராசிரியர் வஜிர திஸநாயக

Msc மாணவி   சிரேஷ்ட விரிவுரையாளர்

மனித பரம்பரையலகு பிரிவு   மனித பரம்பரையலகு பிரிவு

மருத்துவபீடம்   மருத்துவபீடம்

கொழும்பு   கொழும்பு

0777-334750   0777-351835

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, **YES/NO**
without having to give a reason and without affecting your future medical care?

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 **YES/NO**
give your permission for these individuals to have access to your records?

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**

Parrticipant'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)……………………………………………………………..

………………………………………………………………………………………………

කැමැත්ත ප්‍රකාශ කිරීමේ පත්‍රය

(a)   සහභාගී වන්නන් විසින් පිරවීම සඳහාය.

මෙම පත්‍රය සහභාගී වන්නන් /භාරකරුවන් විසින් සම්පූර්ණයෙන් පිරවිය යුතුය.

1. අධ්‍යයනය සම්බන්ධයෙන් තොරතුරු පත්‍රිකාවෙ පැහැදිලි කරන ලද කරුණු
    ඔබට තේරුනාද? (කරුණාකර තොරතුරු පත්‍රිකාවේ පිටපතක් ඔබ ලබාගන්න (

                                                                          ඔව්/නැහැ

2. මෙම අධ්‍යයනය සම්බන්ධව සාකච්ඡා කිරීමට හා ඒ පිළිබඳව ප්‍රශ්න
    ඇසීමට ඔබට අවස්ථාවක් ලැබුණා ද?

                                                                          ඔව්/නැහැ

3. ඔබ ඇසු ප්‍රශ්න සියල්ලටම සෑහීමකට පත්විය හැකි පිළිතුරු ලැබුණාද?

                                                                          ඔව්/නැහැ

4. මෙම අධ්‍යයනය සම්බන්ධයෙන් ප්‍රමාණවත් තොරතුරු ලැබුණාද?   ඔව්/නැහැ

5. මෙම අධ්‍යයනය සම්බන්ධයෙන් ඔබට පැහැදිලි කරන ලද්දේ කවුරුන් විසින්ද?

    ....

6. කිසිදු කරුණු දැක්වීමකින් තොරව, මෙම අධ්‍යයනයෙන් ඉවත් වීමට
    ඔබ හට ඕනෑම අවස්ථාවක හැකියාව ඇති බව පැහැදිලි වුවාද?

                                                                          ඔව්/නැහැ

7. ඔබෙ වෛද්‍ය වාර්තා සහ පර්යේෂණ දත්ත මෙම අධ්‍යයන සම්බන්ධ
    සාමාජිකයින් විසින් අධ්‍යයනය කෙරෙ. සියළු වාර්තා සහ දත්තවල
    රහස්‍යභාවය තහවුරු කෙරෙ. මෙම අධ්‍යයනය සම්බන්ධ සාමාජිකයින්ට
    තොරතුරු ලබා දීමට එකඟ වෙනවාද?   ඔව්/නැහැ

8. මෙම අධ්‍යයනයෙන් පසුව ඉතිරි වන රුධිර සාම්පලයක් ඇතොත් ඉදිරියෙ
    කෙරෙන   බීටා තැලසීමියා රෝගය සම්බන්ධ පර්යේෂණ සඳහා භාවිතා
    කිරීමට ඔබ එකඟ වෙනවාද?

                                                                          ඔව්/නැහැ

9. රුධිර සාම්පල පිටරට යැවීමට එකඟ වෙනවාද?

                                                                          ඔව්/නැහැ

10. මෙම අධ්‍යයනයට සහභාගී වීම සම්බන්ධයෙන් තීරණයකට එළඹීමට ඔබට

    ඇති තරම් කාලය ලැබුණා ද?

                                                                          ඔව්/නැහැ

11. ඔබ මෙම අධ්‍යයනයට සහභාගී වීමට එකඟ වෙනවාද?   **ඔව්/නැහැ**

සහභාගී වන්නන්ගෙ /භාරකරුවන්

අත්සන:………………………………………………………… දිනය:………………………

නම:…………………………………………………………………………………………...

(b) පර්යේෂක විසින් පිරවීම සඳහාය.

     මෙම අධ්‍යයනය සම්බන්ධ කරුණු, මා විසින් අධ්‍යයනයට ස්වේච්ඡාවෙන් සහභාගී වන්නන් හට පැහැදිලි කරන ලදී .ඔහු/ඇ විසින් මෙම අධ්‍යයනයට සහභාගී වීමට කැමැත්ත ප්‍රකාශ කරන ලදී.

පර්යේෂකගේ අත්සන:………………………………………… දිනය:………………………

නම:…………………………………………………………………………………………...

<u>**ஒப்புதல் படிவம்**</u>

பங்குபற்றுபவரால் அல்லது பாதுகாவலரால் நிரப்பப்பட வேண்டியது

பங்குபற்றுபவர் / பாதுகாவலர் தானே முழுமையாக இப்படிவத்தை நிரப்ப வேண்டும்.

1.   தகவல் படிவத்தை வாசித்து புரிந்து கொண்டீர்களா?

     (தயவு செய்து உங்களுக்கு ஒரு பிரதியை வைத்துக் கொள்ளவும்)

                                                                                          ஆம் / இல்லை

2.   இந்த ஆய்வு சம்பந்தமாக உங்களுக்கு ஏற்பட்டு இருக்கக்கூடிய சந்தேகங்களைக்
     கேட்டறிவதற்கு சந்தர்ப்பம் வழங்கப்பட்டதா?

                                                                                          ஆம் / இல்லை

3.   உங்கள்   வினாக்களுக்கு   திருப்தி   அளிக்கக்கூடிய   பதில்களைப்   பெற்றுக்
     கொண்டீர்களா?

                                                                                          ஆம் / இல்லை

4.   இவ் ஆய்வு சம்பந்தமாக போதுமான தகவல்களைப் பெற்றுக் கொண்டீர்களா?

5. இவ்வாய்வு சம்பந்தமாக உங்களுக்கு விளக்கமளித்த ஆய்வாளரின் பெயர்?

....

6. நீங்கள் எந்த நேரத்திலும் இவ் ஆய்வில் இருந்து விலகிக் கொள்ளமுடியும் என்பதையும் இது உங்கள் எதிர்கால மருத்துவ கவனிப்பில் எவ்வித மாற்றத்தையும் உண்டுபண்ண மாட்டாது என்பதையும் புரிந்து கொண்டீர்களா?

ஆம் / இல்லை

7. உங்கள் மருத்துவ அறிக்கைகளும் தகவல்களும் இவ் ஆய்வில் பங்குபற்றும் மற்றைய ஆய்வு குழுவினரால் தேவை ஏற்படின் பரிசோதிக்கப்படும் என்பதையும் உங்கள் பிரத்தியேக தகவல்கள் அனைத்தும் இரகசியமாக பேணப்படும் என்பதையும் உங்களுக்கு தெரிவிக்க விரும்புகின்றோம்.

இதற்கு உங்கள் சம்மதத்தை அளிக்கிறீர்களா?

ஆம் / இல்லை

8. உங்களுடைய குருதி மாதிரிகளை எதிர்கால β தலசீமியா தொடர்பான ஆய்வுகளுக்கு மேற்பார்வையாளரின் மேற்பார்வையின் கீழ் உபயோகப்படுத்த சம்மதிக்கிறீர்களா?

ஆம் / இல்லை

9. உங்கள் முடிவை தெரிவு செய்யவும், தெரிவிக்கவும் போதுமான கால அவகாவம் அளிக்கப்பட்டதா?

ஆம் / இல்லை

10. உங்கள் குருதி மாதிரியை வெளிநாட்டிற்கு அனுப்புவதற்கு அனுமதிக்கின்றீர்களா?

ஆம் / இல்லை

11. இவ் ஆய்வில் பங்குபற்ற சம்மதிக்கிறீர்களா?

ஆம் / இல்லை

பங்குபற்றுபவரின் / பாதுகாவலரின் கையொப்பம் ....

திகதி: ....

விசாரணையாளரால் நிரப்ப வேண்டியது

என்னால் இவ் ஆய்வு பற்றி மேற்குறிப்பிட்ட நபருக்கு விளக்கம் அளிக்கப்பட்டு, அவரால் ஆய்வில் பங்குபற்ற சம்மதம் தெரிவிக்கப்பட்டது.

பெயர் ....   திகதி ....