A STUDY OF MUTATIONAL ANALYSIS OF HEMOGLOBIN BETA GENE (HBB) IN CHILDREN AND ADOLESCENTS WITH BETA-THALASSEMIA MAJOR

BY

Dr.K.Sharath K

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Dr.M.M.PATIL

PROFESSOR

DEPARTMENTOF PEDIATRICS

BLDE (Deemed to be University)

SHRIB.M.PATILMEDICALCOLLEGE

HOSPITAL & RESEARCH CENTRE, VIJAYAPUR

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ABBREVIATIONS

HBB	Hemoglobin beta gene
HBA	Adult Hemoglobin
HBF	Fetal Hemoglobin
GT	Guanine , thiamine
AG	Adneine ,Guanine
HBH	Hemoglobin H
PIH	Pyridoxal isonicotynol hydrazine
HBED	Hydroxy benzyl ethylenediamine
HCT	Hematopoietic cell transplantation
GVHD	Graft versus host disease
JAK2	Janus kinase 2
PCR	Polymerase chinase reaction
IVS	InterVening sequence
G>C	Guanine to Cytosine
T>G	Thymine to Guanine
T>C	Thymine to Cytosine
G>A	Guanine to Adenine
T>G	Thymine to Guanine
FRC	Frame shift codons
β –T.M	Beta thalassemia major
TDT	Transfusion dependent thalassemia
NTDT	Non Transfusion dependent thalassemia
ARMS	Amplification Refractory Mutation System
RFLP	Restriction Fragment Length Polymorphism
MCV	Mean corpuscular volume
MCHB	Mean Corpuscular Hemoglobin
UTR	Untranslated region
Etbr	Ethium bromide

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INTRODUCTION

One of the most prevalent monogenic hereditary blood disorders found worldwide is Thalassemia. Around 100,000 children are born each year with transfusion-dependent Thalassemia worldwide

It has been estimated that about 8000 to 10,000 thalassemia-affected children are born each year in India, which is about 10 % of the world figure [(1)]. The overall carrier frequency of Thalassemia in India varies from .5 % to 17 % in different states and geographical areas [(2)]

Beta thalassemia is a highly prevalent autosomal recessive disorder in which structural variation in the beta-globin gene is observed. All mutations result in either absence of synthesis of beta-globin chains (b^o-thalassemia) or a reduction in synthesis (b^+-thalassemia) [(3).] More than 300 different beta-globin gene mutations have been characterized.

The HBB gene encodes beta globin chains of Hemoglobin, an oxygen-carrying protein composed of two alpha and two beta chain subunits found within red blood cells. This gene has three exons and two introns involved in b-thalassemic pathogenesis. Mutations in the HBB gene lead to an altered b-globin chain resulting in the structural change of the protein conformation of Hemoglobin. In b-thalassemia, point mutations in the b-globin structural gene are primarily responsible for either decreased or no b-globin synthesis [(4).]

Very little data about Hbb gene mutations in this population of south India are available. Therefore, the purpose of this study was to determine the spectrum of beta-thalassemia mutation in the population of the Vijayapura district of north Karnataka

AIMS AND OBJECTIVES OF THE STUDY

1. Aim of the study is to establish a database highlighting prevailing mutations of the beta gene in this population of Karnataka.

2. These data are helpful in the future molecular screening of the population for implementation of the thalassemia prevention and control program

REVIEW OF LITERATURE

Thalassemias are a class of hemoglobinopathies brought on by changes in the genes that control the production of hemoglobin or globin chains. Abnormal globin chain production decreases the Formation of Hb tetramers, affecting the quantity and quality of Hemoglobin, resulting in hypochromia and microcytosis. However, production of the normal globin proteins takes place at the usual rate causing the accumulation of α and β subunits.

Imbalanced in α and β chains and accumulation of normal globin chains are responsible for the clinical phenotype manifested by Thalassemia. The degree of impairment of the production of the affected globin protein, altered synthesis of other globin chains, and coinheritance of other abnormal globin alleles play an important role in determining the clinical severity of the disease.

HISTORY

During 1925 and 1940, the initial descriptions of the clinical traits of the various thalassemia types were published. Initially, it was thought to be a rare disorder restricted to Mediterranean ethnicities. In 1946, it was mentioned that the cause of Thalassemia was an atypical hemoglobin structure[(9)].

Dr. Cooley suggested that the disease was hemolytic in nature. In 1960, physicians treating thalassemia children started to transfuse them with fresh RBCs monthly [(9)]. This improved most of the symptoms and drastically enhanced the survival of these patients. To this day, blood transfusion is the mainstay of treatment in thalassemia patients. Many scholars have investigated thalassemias extensively since the availability of molecular biology.

It is a significant public health issue in many nations. Prevention is regarded as a critical component of management. Many of these nations now have extensive screening and education

programs in place to identify carriers as a result. The proportion of newborns with both homozygous and heterozygous forms of the disease has considerably decreased.

GLOBAL LOAD AND EPIDEMIOLOGY OF THALASSEMIA:

Thalassemias are prevalent in a wide region, reaching out from the Mediterranean region and Africa throughout the Middle East, the Indian subcontinent, Southeast Asia, and Melanesia to the Pacific Islands [(10)].

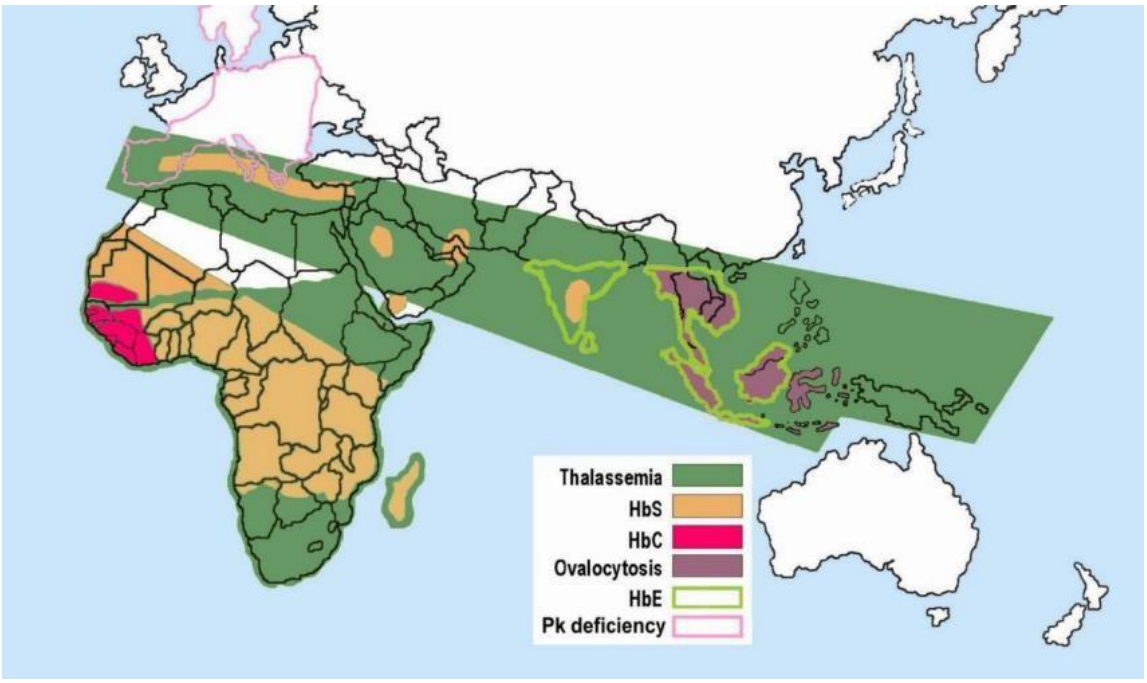

Figure 1: Global thalassemia distribution

Around 1-20 % of the population in these regions are β thalassemia carriers.[(10)]. Milder types are prevalent in 10-20 % of the people in sub-Saharan Africa, to 40% or more in some Middle Eastern and Indian populations, to as high as 80% in northern Papua New Guinea and some parts of upper eastern India [(10)].

Presently there are about 270 million thalassemia carriers in the world. Of these, 80 million are β-thalassemia carriers. Some studies estimate that 300,000 and 400,000 infants are born each year with a significant Hb disease (23,000 with major -thalassemia), and up to 90% of these births occur in low- or middle-income countries. [10,11,12].

About 10,000 babies in India are born each year with Thalassemia Major. This represents roughly 10% of all children born annually around the world. β-thalassemia is common in certain groups in India, like Sindhis, Gujratis, Punjabis, and Bengalis. Occurrence fluctuates from 1 to 17%.

The Indian Red Cross Society and the International Thalassaemic Federation play a critical role in organizing volunteer blood donations and assisting with the improvement of thalassemic patient care. Many states offer free chelation therapy and blood transfusions. Additionally, several centers have facilities for cord blood stem cell storage and bone marrow transplantation, which enhances the quality of care for these patients.

HEMOGLOBIN STRUCTURE AND FUNCTION:

In 1960 Dr. Max Perutz demonstrated the 3-dimensional molecular structure of Hemoglobin using X-ray crystallography, for which he received the Nobel Prize in 1962. Hb is a globular protein comprised of four subunits. Each of these subunits has a polypeptide chain called globin and a heme group

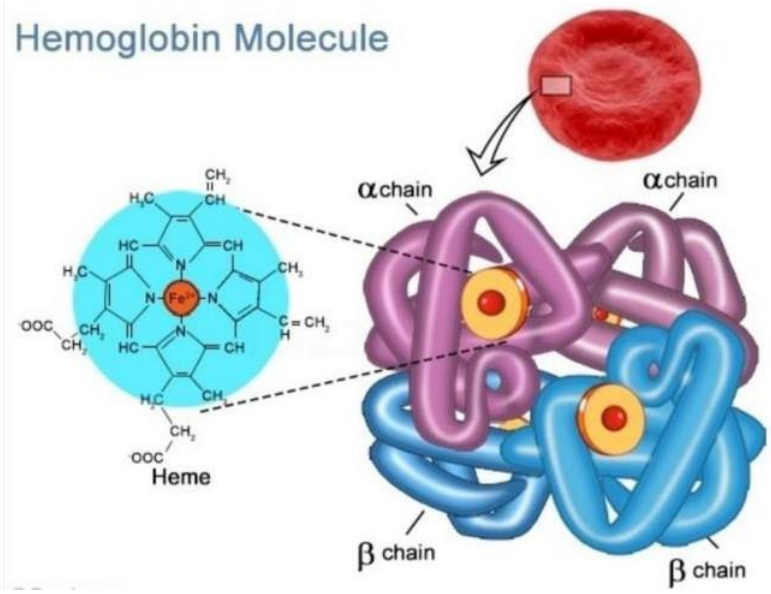

Figure 2: Structure of hemoglobin molecule

Different hemoglobins are produced during different phases of life- embryonic, fetal, and adult. Each of these types is composed of a tetramer of globin protein chains- two α chains (141 amino acids) and two β chains (146 amino acids). In adults, HbA (α2 β2) is the major Hemoglobin, while HbA2 (α2δ2) is present in minor amounts.[3] HbF(α2γ2) is majorly fetal Hemoglobin [11].

Two alpha globin genes on chromosome 16 produce the alpha globin chain. A single beta-globin chain on chromosome 11 encodes for the beta-globin chain[3]. Each globin protein chain encases one heme moiety, comprised of a protoporphyrin IX ring complexed with one iron molecule in the ferrous state (Fe2+).

One heme moiety can bind to one Oxygen (O2) molecule; one Hb molecule can carry up to 4 O2 molecules[3]. The sequence of amino acids in different globin chains is homologous to one another.

Each of these globin chains has a profoundly helical secondary structure. Because of their globular tertiary structure, the outer surface of the globin chain is rich in polar (hydrophilic) amino acids. This enhances the solubility of the globin chain. Nonpolar groups form the inner

lining, making a hydrophobic pocket into which heme is embedded [11]. The tetrameric HbA contains two αβ dimers. α 1 β 1 links bind the α & β chains together. The entire tetramer is held together by interfaces (i.e., α 1 β 2 contacts) between the non-α chain of the one dimer and the α-like chain of the other dimer [11].

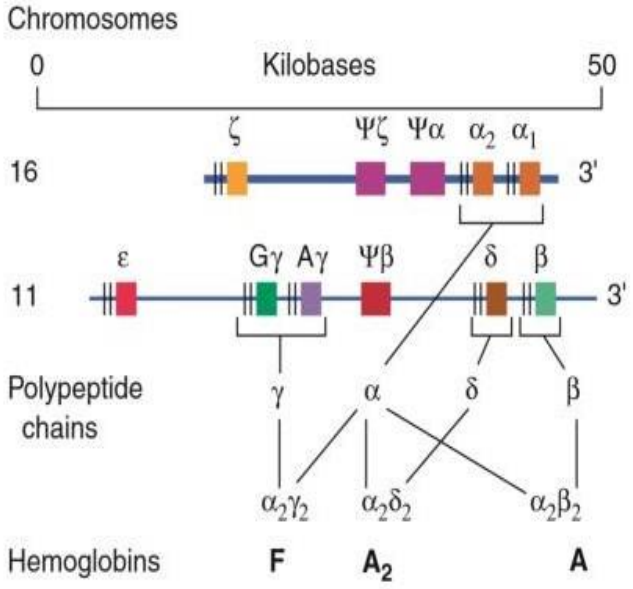

Fig 3: The globin genes. α-like genes (α,ζ) are encoded on chromosome 16; the β-like genes (β,γ,δ,ε) are encoded on chromosome 11. ζ and ε genes encode embryonic globins.[5]

The Molecular Basis of Beta Thalassemia:

There are more than 200 mutations linked to beta-thalassemia [53].For the majority of the beta thalassemia determinants, there are about 20 alleles. Globin chain synthesis is not present in the β0 mutation. Reduced globin chain synthesis is linked to the β + mutation.

The 3' end of the beta gene is deleted by the Indian 619 bp deletion, but the 5' end is retained. A β0-thalassemia is present in homozygotes with this deletion.

Hemoglobin A2 and HbF levels are higher in individuals who are heterozygotes for the Indian deletion. The dinucleotides G.T. at 5' and AG at 3' serve as constant markers for the borders of introns and exons. Any of these splice junctions can be affected by a single base substitution, which will disrupt the RNA's normal splicing and cause the phenotype known as β0-thalassemia.

A nonsense mutation is caused by base substitution and the codon for the amino acid changes to a chain termination codon. It causes 0-thalassemia and stops the translation of mRNA.

Shift mutations occur when nucleotides are added or removed from the beta-globin gene's coding region. The two most frequent mutations among Asian Indians are the insertion of one nucleotide between codons 8 and 9 and the deletion of four nucleotides in codons 41 and 42.(54)

Exon 3 mutation of the beta-globin gene is most affected

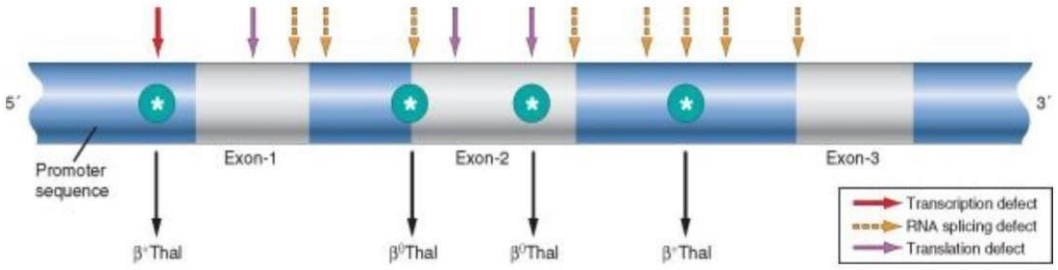

Figure 4: Exons in the beta-globin gene

The hemoglobin tetramer is highly soluble, but individual globin chains are insoluble. The unpaired globin chain precipitates and forms inclusions. These inclusions damage the red blood cell (RBC) membrane. Typically globin chain production is balanced. Each newly synthesized α or non-α globin chain has a partner with which it pairs. Reversible O2 binding of Hb and solubility are the main properties deranged in most hemoglobinopathies. These properties depend mainly on the hydrophilic surface of amino acids.

Hb plays a vital role in ensuring O2 supply to all cells in the body. The primary function of Hb is O2 transport from the lungs to the peripheral tissues. It also helps in the transport of carbon dioxide released from the peripheral tissues to the lungs. [(12)]The various steps in O2 and carbon dioxide (CO2) transport are as follows:

- O2 pickup: In the lungs, inhaled O2 diffuses through the alveolar membrane and capillaries and binds to Hb in the RBCs. Since one Hb has four heme groups, it can carry 4 O2 molecules

- O2 delivery: When Hb reaches the peripheral tissues, due to the low pH encountered, there is a decrease in affinity for oxygen, and oxygen is released which is taken up by the tissue. This phenomenon is also known as the Bohr effect.

- CO2 pickup: Since Hb has a higher affinity for CO2 than O2, CO2 molecules bind to deoxyhemoglobin. This phenomenon is known as the Haldane effect.

- CO2 delivery: Dissociation of CO2 occurs in the lungs in the presence of O2. Hb is now free for O2 pickup again

Classification of thalassemia syndromes:

Thalassemia syndromes are mainly classified into three types

1. **β Thalassemia**- This is the most common type all over the world.

The different variants are:

- Thalassemia major
- Thalassemia intermedia
- Thalassemia trait
- Thalassemia minima

2. α Thalassemia

This type is mainly seen in South East Asian countries, China, the Middle East, Europe, and the Indian subcontinent. The different variants are:

- Hydrops fetalis
- Hb H disease
- α Thalassemia trait

Table 1: **Beta Thalassemia Types**

Beta thalassemias (reduction in beta globin chains) ¶			
Major (transfusion-dependent)	β^0 / β^0 or β^0 / β^+	Severe microcytic anemia with target cells (typical Hb 3 to 4 g/dL)	HbA_2 (5% or more); HbF (up to 95%); no HbA
Intermedia (non-transfusion-dependent)	β^+ / β^+	Moderate microcytic anemia	HbA_2 (4% or more); HbF (up to 50%)
Minor (also called trait or carrier)	β / β^0 or β / β^+	Mild microcytic anemia	HbA_2 (4% or more); HbF (up to 5%)

Miscellaneous Thalassemic syndromes-

These thalassemic syndromes result from multiple combinations of β and α genes with other structurally abnormal Hb. These are usually asymptomatic and self-limiting.

These are:

- Hb S- Thalassemia
- Hb E- Thalassemia

- Hb D- Thalassemia
- δ – β- Thalassemia
- HPFH- Hereditary persistence of fetal Hemoglobin
- γ- Thalassemia
- δ- Thalassemia

Hemolysis reduced HbA synthesis, and poor erythropoiesis all contribute to anemia. Anemia can range widely in severity depending on how many genes are affected and which genes have been changed. Hypoxia caused by anemia is occasionally exacerbated by the presence of aberrant hemoglobins with a high oxygen affinity (HbH and Hb Bart's). These hemoglobins do not readily transport oxygen to the tissues.

Numerous negative outcomes result from chronic hemolysis. Because extravascular hemolysis takes place mostly in the spleen, splenomegaly is commonly seen. Functional hyposplenism can occur when the spleen is overworked due to the breakdown of erythrocytes. In this situation, the spleen's role as a secondary lymphoid tissue is disrupted, which raises the risk of infections. Gallstone development is another side effect of chronic hemolysis.

The bone marrow increases erythropoiesis to satisfy the demand, which results in erythroid hyperplasia . Chronic demand for erythrocytes also has negative effects. Patients consequently experience pathologic fractures and skeletal deformities.

A higher need for iron promotes erythropoietic activity, which in turn causes the gastrointestinal tract to absorb more iron (GIT). As a result of this extra iron is ineffectively incorporated into Hemoglobin, which builds up in bone marrow, liver, and spleen macrophages.

Iron gradually builds up in the parenchymal cells of different organs as this process goes on, negatively affecting how well they function. Organs such as the liver, pituitary, heart, and bone are frequently impacted by iron deposition. This could lead to pathologic fractures, cirrhosis, hypogonadism, growth failure, arrhythmias, and cardiomyopathies. (13)

Extramedullary erythropoiesis in the liver and spleen can occur in conjunction with inefficient erythropoiesis in the bone marrow. Extramedullary erythropoiesis may result in masses that are large enough to result in compression symptoms. Thalassemia pregnant women have physiological demands that influence the developing fetus more than the mother.

If the woman's O2 concentration falls below 70 mmHg, reduced growth, preterm birth, and even intrauterine mortality can occur in a developing fetus.

To prevent iron overload brought on by the combination of transfusion therapy and increased iron absorption, pregnant women should receive deferoxamine both during and after the transfusion (14)

Clinical findings

Anemia:

Microcytic hypochromic anemia and a higher RBC count will both be present. Due to the ongoing erythropoietic stress, they are vulnerable to medications, nutritional deficiencies, and infections. Examples include the aplastic crisis caused by parvovirus B19 and the hypoplastic crisis by various viruses.

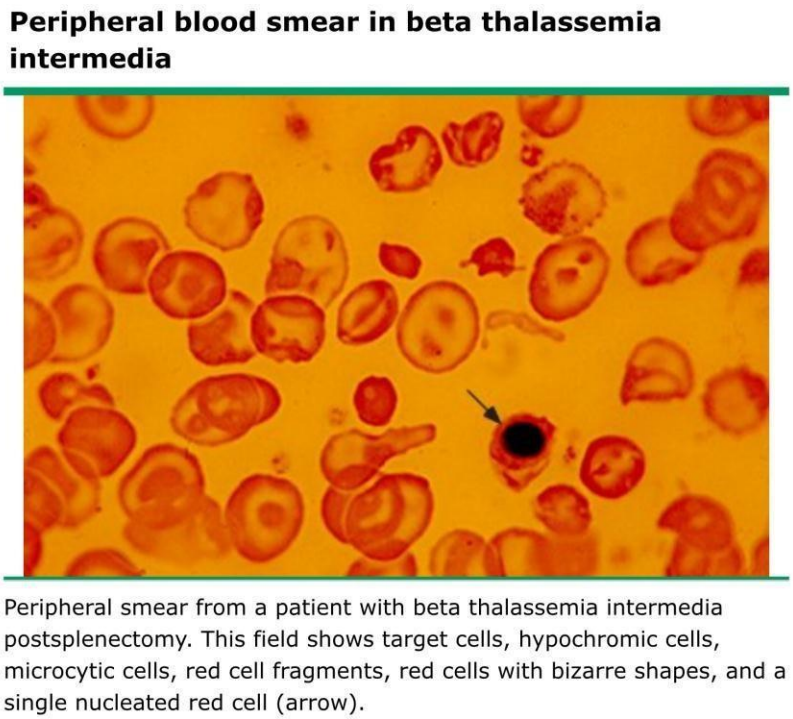

Figure 5: Peripheral blood smear in Beta thalassemia Intermedia

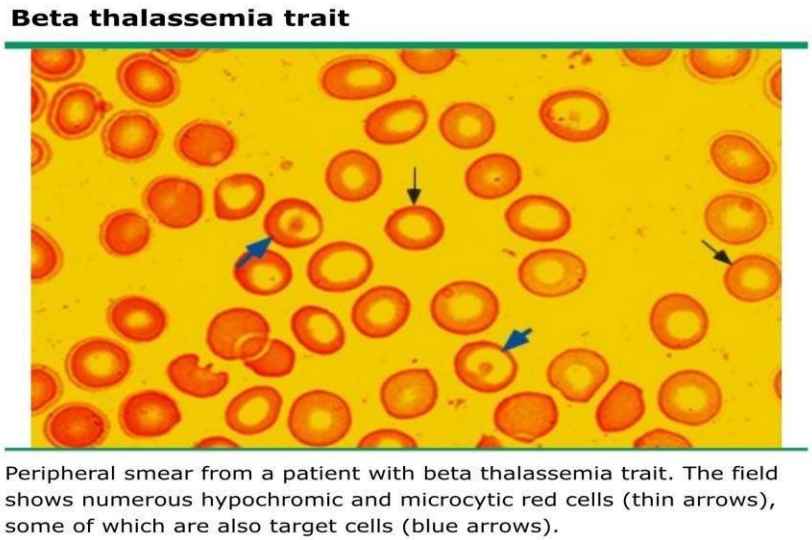

Figure 6: Peripheral blood smear in Beta thalassemia Trait

Biliary gallstones with jaundice:

Chronic hemolysis causes pigment gallstones and biliary tract inflammation in children with beta-thalassemia major.

Skeletal Deformities :

Due to ineffective erythropoiesis and increased medullary hematopoiesis, skeletal deformities in thalassemia major patients and many intermedia patients are more evident.

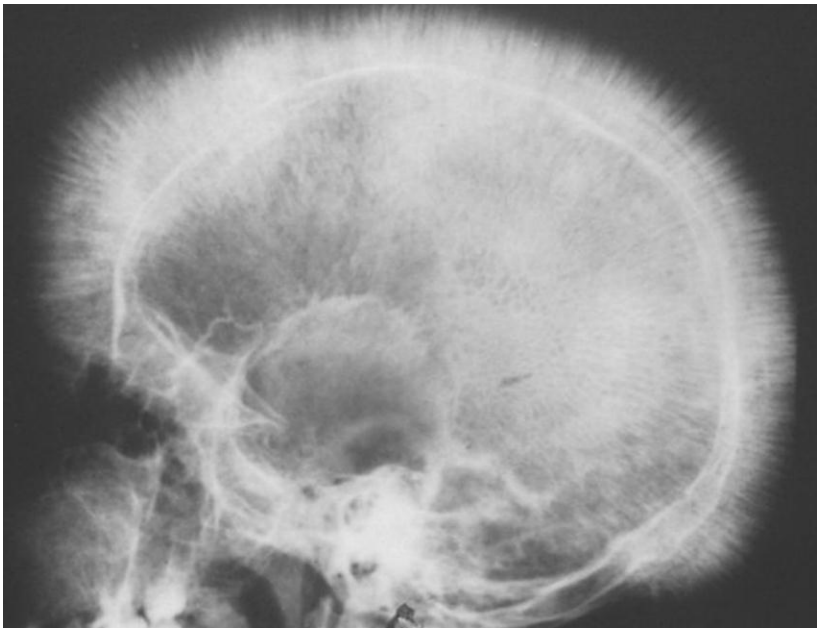

Figure 7: Skeletal changes in Thalassemia major child

- **Facial changes:** The typical chipmunk facies is characterized by delayed sinus pneumatization, bossing of the frontal bone, maxillary overgrowth, increased malar prominence.
- **Modifications in skeletal structures:** The extremities' bones gradually assume a convex form and take on a box-like structure. Premature epiphysis closure will result in shortened limbs, especially the arms.

- Due to the enlargement of the bone marrow gaps, **osteopenia and osteoporosis** are highly common [56]. The widening of the diploid gaps in the skull is what gives the skull x-ray its "hair on end" appearance. If osteoporosis is left untreated, it can result in vertebral fractures, back pain, and deformities of the spine, as well as bone fractures.
- **Bony changes:** The erythroid bone marrow invades the bony cortex, penetrates the bone, and establishes ectopic erythroid colonies in the sinuses and thoracic and pelvic cavities. The tumor-like behavior of the growing masses causes compression of the spinal cord

Hypertransfusion therapy in the early years of life can help to delay or even reverse these skeletal changes.

Growth changes :

These patients' growth and development are compromised for the reasons listed below.

- Chronic Anemia
- Hypermetabolic state caused by inefficient erythropoiesis.
- The hypermetabolic condition that causes dietary deficiencies
- Endocrinopathies are brought on by excessive iron deposition and the toxicity associated with iron chelation therapy.

Hepatomegaly and splenomegaly:

Hepatosplenomegaly is caused by both extramedullary hematopoiesis and persistent hemolysis. Viral hepatitis can potentially harm the liver when routine testing of blood products is not done. Due to viral infection and extra iron, these patients have an increased chance of developing hepatocellular carcinoma.

Thalassemia Minor patients have spleens that are larger than average, but they are not palpable.

Metabolic and Endocrine abnormalities:

Due to the iron excess, it is frequent in both Thalassemia major and minor.

Hypogonadism: It is a result of the pituitary's accumulation of extra iron. Both girls and boys have delayed development of primary and secondary sexual traits [57]

In these cases, menarche is delayed, and oligomenorrhea or amenorrhea are highly common. Boys' libido will decline, and they lack or have minimal facial and body hair.

Hypothyroidism in these patients results from iron deposition in the thyroid gland and pituitary gland.

Diabetes and insulin resistance: Glucose intolerance and diabetes are caused by improper carbohydrate metabolism and iron accumulation in the pancreatic islets.

Diabetes is a major independent risk factor for cardiac complications in thalassemia patients. [58]

Arrhythmias and cardiac complications:

The main causes of death in these patients are heart failure and severe arrhythmias.

The cardiac iron deposition is a contributor to heart failure, restrictive cardiomyopathy, supraventricular and ventricular arrhythmias, and sterile pericarditis.

The most important signs of excessive iron deposition in the heart are repolarization changes and bradycardia (The left shift of the T wave axis and Q.T. interval prolongation).[59]

The American Heart Association stressed in 2013 the significance of MRI to determine the iron load in the heart in Thalassemia patients [60]

Iron chelation therapy is to be started in those with elevated cardiac iron causing Myocardial fibrosis

Pulmonary hypertension and Abnormalities

Smaller airway obstruction, restrictive patterns, hyperinflation, and some changes in pulmonary function testing.

Table 2: Clinical and Laboratory Findings in Thalassemia

Clinical Finding	Pathophysiology Laboratory	Finding
Anemia/hypoxia	↓ hemoglobin production/erythropoiesis Ineffective erythropoiesis Presence of high-affinity hemoglobins (HbH and Hb Bart's) ↑ extravascular hemolysis	↓ nRBC count, ↓ hemoglobin, ↓ hematocrit Microcytic / hypochromic RBCs ↓ MCV, ↓ MCH, ↓ MCHC Increased Reticulocyte count Anisocytosis and poikilocytosis Target cells, basophilic stippling, nRBCs, Bone marrow erythroid hyperplasia, ↑ RDW, Abnormal hemoglobin electrophoresis
Splenomegaly/ hemolysis	Splenic removal of abnormal erythrocytes Ineffective erythropoiesis	↑ Bilirubin ↓ Haptoglobin
Gallstones	↑ intravascular and extravascular hemolysis	↑ Bilirubin
Skeletal abnormalities	Expansion of bone marrow	Bone marrow erythroid hyperplasia
Pathologic fractures	Thinning of calcified bone	
Iron toxicity	Iron overload Multiple transfusions, Increased iron absorption	↑ Prussian blue staining in Bone marrow ↑ Serum iron/ferritin and ↓ TIBC

PATHOPHYSIOLOGY OF THALASSEMIA:

Normally equal amounts of α-chains and β-chains are synthesized by the maturing erythrocyte. In α and β thalassemia, synthesis of one of these chains is decreased or absent, resulting in excess of the other chain. [7] If the α-chain is affected, there is an excess of β-chain 14 and vice versa. This imbalance in the synthesis of globin chains has several effects, all contributing to anemia in Thalassemia. [15]

Some of these effects are:

(1) Decreased total erythrocyte hemoglobin production.

(2) ineffective erythropoiesis.

(3) chronic hemolysis.

Excess α-chains are unstable and precipitate within the cell. These residues stick to the RBC membrane, damaging the membrane and reducing the deformability of the RBC. The bone marrow precipitate-filled erythrocytes are destroyed by macrophages, which impairs erythropoiesis. The spleen pits and removes precipitated circulating erythrocytes, resulting in chronic extravascular hemolysis. [(15)].

Excess β-chains can combine to form Hb molecules with four β-chains known as HbH. This Hb has a high O2 affinity and is also unstable. Thus, it is a poor transporter of O2.

In infants, when α-chains are decreased, excess γ-chains combine to form Hb molecules with four γ-chains. This is known as Hb Bart's. This Hemoglobin also has a very high oxygen affinity. Thalassemia-like conditions (e.g., HbE) having structural Hb variants can result in decreased synthesis of globin chains, giving the clinical picture of Thalassemia[(15)].

Anemia in Thalassemia is brought on by shortened RBC survival and ineffective erythropoiesis. Ineffective erythropoiesis is the main cause of anemia, according toferrokinetic and erythropoietic studies, as it severely harms the erythroid precursors in the bone marrow [(9)]

Hemolysis:

When evaluating the severity of anemia in thalassemia patients, the destruction of aberrant RBCs does not matter as much as inefficient erythropoiesis. In various studies using Ashby or 51Cr-labelling methods, it has been observed that the survival time of RBCs of thalassemias ranged from 7 to 22 days [(16-22)].

There are two types of populations of RBCs. One is rapidly destroyed. [18,24]. A few studies have mentioned that RBCs rich in HbF have longer life spans while RBC populations containing mostly HbA or α-chain precipitates are destroyed early [.9].

The buildup of extra globin chains at the RBC membrane surface results in variations in the stability, deformability, and cellular dehydration of the RBCs.[10,32,33].

Reduced spectrin/band 3 ratios, partial oxidation, and faulty band 4.1 function were observed[9,23]

Anemic response:

Profound anemia causes an increase in erythropoietin synthesis in response to persistent hypoxia. In early studies, thalassemia patients with Hb of 7.0 g/dl or less had a significant increase in erythropoietin levels in their blood and urine. [9]

Expansion of Erythroid:

Ineffective erythropoiesis, which causes up to 10–30 times the usual amount of erythroid growth, is a feature of Thalassemia [9,24,25]. The majority of the clinical characteristics of Thalassemia are caused by this unchecked growth of erythroid mass. Particularly important are bone deformities and the Formation of extramedullary tumor masses in a few [9].

Poor muscle development is seen, and body weight and body fat are decreased. [9] The shunting of blood through the greatly enlarged marrow in the presence of splenomegaly causes the anemia to worsen [26]. Children with profound thalassemic anemia are in a high output stage. In certain people, this could result in cardiomegaly. Urate and serum uric acid levels are also raised relative to normal levels due to increased RBC precursor oxidation.

Splenomegaly and Hypersplenism

The exact mechanism of splenomegaly in Thalassemia is not clear. It has been mentioned that exposure of the splenic reticuloendothelial elements to abnormal RBCs in β thalassemia patients is the cause of progressive enlargement of the spleen [9]. The finding that patients with regular blood transfusions starting at a young age will not have more aberrant red blood cells in circulation is evidence in favor of this theory. Therefore, these patients don't experience substantial splenomegaly.[9] In 1963, it was discovered that splenectomy was the only way for RBCs with inclusions to appear in peripheral blood. This highlights how crucial the spleen is to the pathophysiology of anemia in Thalassemia. ([10,27]).

Additionally, extramedullary hemopoiesis might be a factor in splenomegaly. Pancytopenia is brought on by splenomegaly, which traps all of the blood's produced components (thrombocytopenia, neutropenia, and anemia). The splenic sinusoids get a considerable portion of the RBC mass, which dilutes the blood and raises plasma volume. Expanding plasma volume brought on by splenomegaly makes anemia worse and puts a greater strain on the heart. Plasma volume is increased by the vascular shunt mechanism across the greatly larger bone marrow.

According to a study by Blendis et al.[20] , the splenic pool contained 9 to 40% of the total RBCs.

The spleen displayed significant extramedullary hematopoiesis. Interestingly, they also suggested that a child's development spurt could be the reason for splenomegaly in Thalassemia patients. Thalassemia's enlarged spleen can have a variety of negative repercussions. [.9,20]

Hypercoagulability state:

In those with beta-thalassemia, thromboembolism risk is enhanced. However, nothing is known about the mechanisms. The outer leaflet's increased phosphatidylserine may encourage thrombosis in a manner similar to how it stimulates the coagulation cascade on the surface of activated platelets [55]

Excess Iron overload:

Excess Iron deposition in tissues and various organs, as well as overall iron overload, are some of the most well-known side effects of Thalassemia [18,28-31]. Excess iron is absorbed into the body system by erroneous absorption of iron from the GIT and frequent blood transfusions. Increased absorption from the gastrointestinal tract is thought to be the main cause of iron overload in patients who get insufficient transfusions[9].The latter process, however, predominates as the main reason for iron overload in individuals who have not had enough transfusions. [9]

Mechanisms of iron overloading: The body's iron storage and the level of erythropoiesis are the two main variables that influence how much iron is absorbed from the stomach. About 200 milligrams of iron are present in one unit of blood. Because iron cannot be removed from the body by metabolic means, frequent blood transfusions significantly increase the body's iron reserves [9]. Additionally, due to inefficient erythropoiesis, the rate of increase in intestinal iron absorption is exponential [9]. Blood transfusion has numerous difficulties even though it saves the lives of thalassemia patients. Iron overload is, therefore, the most important difficulty out of all of them.

Although the body needs iron for numerous physiological processes, too much of it damages tissues severely by producing damaging free radicals.

Usually, proteins that store or transport iron are strongly attached to them. For instance, binding plasma iron to transferrin can limit the catalytic effect of iron in the generation of free radicals. [30] However, plasma iron that isn't linked to transferrin is detected in the blood and produces toxicity when the transferrin becomes saturated with rising levels of iron overload[31,34,35] . Iron is detected in the serum and other bodily tissues of patients with iron excess [31]. Generally speaking, transferrin, ferritin, and Hemoglobin are all strongly associated with iron. If iron-containing proteins are subjected to oxidative stress, free iron may be liberated. Iron excess has significant pathogenic effects on the liver, cardiac system, and endocrine system [9]

IRON OVERLOAD CONSEQUENCES

The majority of thalassemia problems are brought on by iron overload. Commonly afflicted organs include the heart, liver, and several endocrine glands. Affected organs frequently include the thyroid, parathyroid, pituitary, pancreas, and gonads. Investigations for early detection are necessary because they are occasionally not clinically visible. All thalassemia patients should periodically be monitored for iron overload, and the proper treatment should be administered. Initial thyroid and parathyroid gland dysfunction may be asymptomatic. Therefore, it is important to regularly check serum calcium (Sr. Ca), thyroid function, and blood sugar.

Numerous factors, such as excessive iron accumulation and recurrent blood-borne infections, can harm the liver, so liver function tests are to be done every six months. Nearly all thalassemia youngsters in our nation have growth failure. The average age at which sexual maturity is attained is likewise later. Numerous factors can contribute to retardation in growth, such as regular blood transfusions, insufficient chelation, a lack of growth hormone production due to pituitary iron overload (hemosiderosis), and chronic hypoxia brought on by anemia.

It is debated whether subclinical hypothyroidism requires medication. When treatment is unnecessary, patients must be closely monitored. Treatment with L-thyroxine is an option in cases with overt hypothyroidism, defined by low levels of T4 and symptoms like constipation, weight gain, and physical exertion. Early on, aberrant thyroid functioning can be reversed with rigorous chelation therapy.

Heart problems in Thalassemia, such as cardiac failure and arrhythmias, account for 70% of fatalities. Extra iron accumulates in the heart, particularly in the conduction system and ventricle walls. When free iron accumulates in cardiac tissue as a result of lipid peroxidation and lysosomal rupture, it causes damage to cells. Some of the cardiac issues that newborns with thalassemia experience include overt cardiomyopathy left atrial dilatation, and aortic root dilatation.

T2 weighted cardiac MRI is the best tool for determining the seriousness of a cardiac examination, however, it is only available in a few centers in India.

Organs affected:

It is caused by the combined consequences of a chronic inflammatory condition, iron overload, chronic hypoxia, and anemia.

1. **Renal**: The excess medullary erythropoiesis that affects the kidneys is the cause. Elevated uric acid levels and other metabolic consequences of enhanced hematopoietic turnover similarly effect the kidney. The kidney is directly effected by iron excess. The iron-chelating substance deferasirox is harmful to the kidneys.

2. **Cardiac**: Cardiomyopathy and pulmonary hypertension are two forms of cardiac illness. There are many different causes. The malfunction of the heart is significantly effected by iron deposition. Other factors include lung disease, endocrinopathy, vascular abnormalities, chronic anemia, hemolysis, and blood loss.

3. **Endocrine**: In addition to other endocrine and metabolic problems, diabetes will result from iron buildup and subsequent oxidative damage to the pancreas cells.

MANAGEMENT IN THALASSEMIA MAJOR PATIENTS

- Packet cell transfusions are used to cure anemia
- Chelation therapy is used to remove excess iron.
- Stem cell transplantation is used to treat cancer.
- Gene therapy may be used in the future.
- Genetic counseling, prenatal diagnostics, and preimplantation genetics can all be used to avoid disease.

Dietary Advice and Supplements

Patients with beta-thalassemia are advised to take folic acid supplements to make up for the increased needs brought on by the rapid RBC turnover. If a patient does not exhibit indications of a zinc shortage, such as poor taste or smell, or has a known zinc deficiency, zinc supplementation is not administered. Iron-rich foods are to be avoided.

Transfusion

The purpose of blood transfusion therapy is to treat anemia and reduce inefficient erythropoiesis. Currently, the mainstay of treatment is routinely packed cell transfusion.

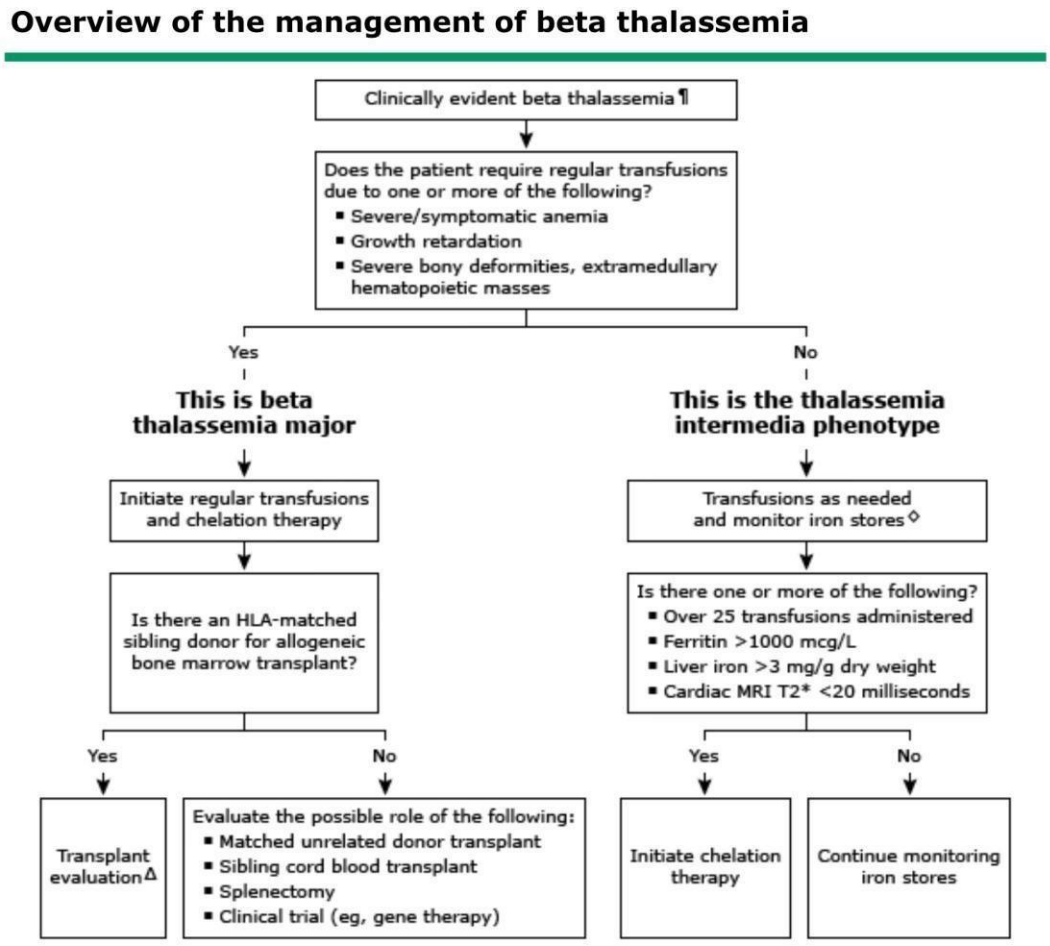

Table 3: Transfusion regimen types according to pretransfusion Hb

Type of transfusion	Pre transfusion Hb	Mean Hb maintained
Palliative	<7g%	<8.5 g%
Hyper transfusion	>10g%	>12 g%
Super transfusion	>12 g%	>14 g%
Moderate transfusion	9 -10.5 g%	>12g%

Regular blood transfusion:

Thalassemia major patients should receive regular transfusions. Hyper transfusion is the term used to describe chronic transfusion in these patients. For these patients, a pretransfusion hemoglobin level of 9.5 to 10 gm/dL is advised. This strategy aims to strike the ideal balance between reducing iron overload and stifling hematopoiesis. More than two units of PRBC cannot be transfused at once due to practical considerations. The time between two transfusions is therefore modified to keep Hemoglobin at the proper amount. Hemoglobin levels post-transfusion should range from 12 to 13 g/dL and not exceed 15 g/dL.

In their third and fourth decades of life, many people with thalassemia intermedia will become transfusion-dependent.

Table 4: Calculation of the amount of blood to transfuse based on the hematocrit of PRBCs and the desire in hemoglobin level

Desired increase in hemoglobin	Hematocrit of donor red cells			
	50%	60%	75%	80%
2 g/dL	12 mL/kg	10 mL/kg	8 mL/kg	7.5 mL/kg
3 g/dL	18 mL/kg	15 mL/kg	12 mL/kg	11.2 mL/kg
4 g/dL	24 mL/kg	20 mL/kg	16 mL/kg	15 mL/kg

Assessment of iron stores and initiation of iron chelation

Serial measurement of serum ferritin levels is advised.In these cases, a baseline MRI is obtained. If serum ferritin levels are incongruent, liver and heart iron estimates rely on MRI scans.

Cardiac iron is often measured using an MRI at 8 to 10 years of age or older. Serum ferritin will therefore be useful in assessing the patient's overall iron excess [(61)]. Features of iron overload develop at 10 to 15 years of age

When one or more of the following occur, iron chelation is initiated.

- serum ferritin exceeding 1000ngm/dL
- The liver has more than 3 milligrams of iron per gram of dry weight.
- Twenty to twenty-five units of packed cells have been transfused.
- when a chronic transfusion program first begins.

Keeping the mean post-transfusion hemoglobin levels at 12 gm% is currently advised. Patients with Hb levels between 9 and 10.5 g% are advised to have a moderate transfusion. Hemoglobin levels after transfusion shouldn't exceed 15–16 g%.

Table 5: Complications of blood transfusion

Iron overloading	
Infections	- Viral infections (HIV, HCV, HBV, HTLV1, West Nile virus) - Bacterial infections - Parasitic infections - Creutzfeld – Jacob disease
Hemolytic reactions	-Acute hemolytic reactions - Delayed hemolytic reactions. - Autoimmune hemolytic anemia
Non hemolytic reactions	- Allergic and anphylactic rections - Febrile non hemolytic reactions - Transfusion-related acute lung injury - Graft versus host disease - Circulatory overload - Transfusion purpura

Chelation treatment:

The fundamental issue in the treatment of Thalassemia is iron excess. Iron chelators are the sole means to eliminate excess iron because there are no efficient mechanisms for excreting extra iron from the organs. Iron chelators primarily work to keep the body's iron storage levels low. Currently, desferrioxamine, Deferiprone, and deferasirox are utilized as medications.

Desferrioxamine

30 to 40 mg/kg/day of the dose is administered subcutaneously for 8 to 10 hours six nights per week. A more recent variation of chelation therapy is depot desferrioxamine. In cases of anuria or severe renal illness, it is contraindicated. There is no need to change the dosage if the liver is impaired. Following a brief I.V. infusion of the medication, urticaria, hypotension, skin flushing, and shock may ensue.

With the use of this medication, urine may become pink, crimson, or orange. Dysplasia, thrombocytopenia, and leukopenia are further side effects. The patients are more vulnerable to yersinia and mucormycosis infections. Desferrioxamine has been linked to ARDS, particularly when given at larger doses.

Ascorbic acid and deferoxamine together will affect heart functioning. Ascorbic acid is to be started after deferoxamine treatment .The amount of iron that is lowered by Deferiprone and deferoxamine does not differ noticeably.

Deferiprone:

The dosage is 75–100 mg/kg/day, administered orally in divided doses. It efficiently lowers serum ferritin and tissue iron overload and is 70–100% as effective as desferrioxamine. It can result in agranulocytosis, which can cause fatal infections and other serious health problems. The absolute neutrophil count is determined before starting the medication and then every week after that. There is no need to change the dosage when there is a renal and hepatic failure.

Consequences related to the gastrointestinal tract include vomiting, diarrhea, and weight gain. Increases in ALT values have also been noted. If the enzyme levels continue to rise, treatment must be stopped.Additionally, recorded hypersensitivity events include urticaria, periorbital edema with skin rashes, and Henoch-Schönlein purpura. It is teratogenic, so patients who are considering getting pregnant should avoid it, according to reports.

Deferasirox:

The dose is which is 20–40 mg once daily

In animal trials, it was discovered to be over five times more efficient than sc desferrioxamine and ten times more effective than Deferiprone

Renal function tests and liver function tests should be done before starting Deferasirox treatment

It can result in gastrointestinal hemorrhages, which can even be life-threatening in older individuals with advanced hematological illnesses and low platelet counts.

Deferasirox use may lead to hearing abnormalities like high-frequency hearing loss. There is little information available on its use in expectant women.

Chelation therapy should typically be begun in Thalassemia major patients after 10 to 20 transfusions or when serum ferritin levels exceed 1000 g/dl. [31] Both prospective and retrospective investigations have demonstrated that Deferiprone monotherapy is more successful at reducing cardiac siderosis [36-38]

Iron chelator therapy enhances thyroid function, glucose intolerance, and other iron excess adverse effects.[39,40,41]

Future perspectives:

The effectiveness and low toxicity of more recent medications like pyridoxal isonicotynoyl hydrazine(PIH), hydroxy benzyl ethylenediamine (HBED), and dimethyl HBED make them more promising. It has been attempted to boost the generation of fetal Hemoglobin (HbF)and stop the Formation of unpaired Hemoglobin chains using pharmacological gene manipulation[42]

Splenectomy indications :

- When the yearly need for a packed cell transfusion doubles or exceeds the minimal minimum. That is, roughly 220–250 ml per kg.
- Reduced platelet count - This hypersplenism sign is often late to appear.
- The patients' rapid increase in transfusion needs
- Splenomegaly producing early satiety and abdominal pain
- Pancytopenia due to hypersplenism
- Splenic infarction
- Severe anemia
- Splenic vein thrombosis

Meningococcal, pneumococcal, and H. influenza type B vaccinations are given in all patients six weeks before splenectomy

These vaccines should be avoided in children under the age of five.

Reduction of problems and alloimmunization:

10 to 50 percent of thalassemia patients are thought to have alloimmunization. Additionally, these patients have a higher chance of allergic reactions, circulatory overload brought on by transfusions, fever, non-hemolytic reactions, and acute lung injury

Allogeneic hematopoietic cell transplantation:

It is a treatment that may be curative for Thalassemia sufferers. The resources and availability of a qualified donor are the main obstacles to this procedure. Even for the finest candidates, mortality and toxicity associated with transplants are quite serious issues.

These patients are placed in risk classifications according to the Pesaro approach based on iron excess.

A child under the age of 14 who receives frequent blood transfusions, receives iron chelation, and has a sibling donor who has an HLA type is the ideal candidate for HCT. In these individuals, HCT has a success rate of about 90% and a mortality rate of about 4%. Before HCT, postpubertal boys should bank their sperm. For the girls, oocyte preservation might be tried.

To lower the chance of developing chronic graft versus host illness in these individuals, bone marrow stem cells are preferable over peripheral blood stem cells. Busulfan, cyclophosphamide, and fludarabine are the main ingredients of myeloablative conditioning regimens. To stop secondary cancers from developing and to get rid of radiation's harmful effect on growth, radiation is avoided.

Investigational disease-modifying approaches:

Luspatercept and sotatercept

As part of the family of transforming growth factor beta molecules, which is crucial for the maturation of RBCs, luspatercept sequesters activin A and other members of the group. It acts subcutaneously through an unclear mechanism, stimulates bone development and erythropoiesis in individuals with Thalassemia**.** [(62)]

Decitabine

In patients with beta-thalassemia, a hypomethylating drug is found to raise fetal hemoglobin levels.

Histone deacetylase inhibitors

HbF is raised in these patients by substances such as sodium phenylbutyrate and arginine butyrate [63]

JAK 2 inhibitor

Ruxolitinib has been observed to lessen these patients' need for transfusions.

specific situations

During Pregnancy

The American College of Obstetricians and Gynecologists advises thalassemia major patients to conceive only if they have a healthy heart.

Thalassemia-related anemia in these people will get worse during pregnancy due to physiological anemia. In these patients, the hemoglobin level must be maintained at 10 g/dL. These drugs are often stopped because there is no information on the safety of using iron-chelating agents during pregnancy**.**

Concerns about Anaesthesia and Surgery

In these patients, a pretreatment hemoglobin level of 10 to 11 gm/dL is advised. Due to the deformities of the fascial structures, these patients may have difficulty managing their airways.

GENETIC COUNSELING AND PRENATAL DIAGNOSIS

The main methods for preventing Thalassemia are carrier identification, genetic counseling, and prenatal diagnosis. Genetic counseling should be obtained if both partners are carriers because they run the risk of siring a thalassemic kid. They should be made aware of the possibility of passing hereditary diseases to their offspring as well as the available medical alternatives.

The prenatal analysis is available in high-risk pregnancies by analyzing the DNA of fetal cells acquired during amniocentesis or chorionic villi sampling. Before prenatal testing is conducted, it is crucial to determine whether the parents have a mutation that causes a disease. Numerous investigations are being conducted right now to check for paternal alterations in fetal cells and DNA in maternal blood. In families with known disease-causing mutations, preimplantation genetic analysis can be performed.

Preimplantation Genetic Diagnosis

The preimplantation diagnosis that PCR is capable of achieving would be a more recent, real technique of illness prevention. 1-2 blastomeres from embryos are isolated for this process. A polar body can also be aspirated from the oocytes as an alternative. If the mutation causing the problem is eliminated, the leftover blastomeres can ideally be transplanted into the mother's womb to facilitate normal embryonic development. [43]

The possibility of making available a thorough genetic screening of fertilized embryos created in vitro in the future is anticipated. Therefore, by manipulating the gamete with the use of a biopsy collected from the embryo and the PCR technology, Thalassemia may be reduced or eliminated [44]

Thalassemia prevention:

Only 10 to 15% of Indian children suffering from Thalassemia get optimum treatment. Its costs around 10,00,00 yearly for the treatment of thalassemic children. The majority of patients cannot afford to have a bone marrow transplant, and the child is put through a lot of mental and emotional pain, and the same is true for the family and the country's economy.

So, the goal has to shift from merely treating the patient to the preventing such births

The only efficient option is genetic counseling and prenatal diagnosis.

Mass population screening has been carried out using a variety of screening tests. Mentzer's index, Naked Eye Single Tube Red Cell Osmotic Fragility Test(NESTROFT), and others are some examples. However, no one can accurately predict the HbA2 confirmation estimate for the detection of beta-thalassemia carriers

The fractions of Hb A, A2, F, H, E, and other variants are measured by Hb electrophoresis and High-performance liquid chromatography. Tests for partners should be recommended for those who have the thalassemia trait. When both tests yield positive results, the patient should be counseled on prenatal diagnosis. This can be carried out by chorionic villi sampling in the first trimester and amniocentesis in the second trimester.

Therefore, prenatal testing should be done when two thalassemia carriers conceive a child, and if the fetus is affected, termination should be suggested. The fractions of Hb A, A2, F, H, E, and other variants are measured by Hb electrophoresis and High-performance liquid chromatography.

A study conducted by Gururaj D . Kulkarni, Suyamindra s.kulkarni, Gurushantappa s. Kadakol et al. included thirty-six (36) beta-thalassemia children from JJM Medical College in Davanagere and KIMS Hubli from January 2008 to February 2010. Thirty-six samples were examined; thirteen of them revealed IVSII-16 G > C, ten of IVSI-5 G > C, eight of IVSII-74 T > G, seven of codon 3 (T > C), three Poly A site (T > C), and one each of codons six (-C.T.), codon sixteen (G > A), codon thirty-one (G > C), codon thirty-one (G > A), and IVSII-837 T > G.

A unique frameshift deletion (-C.T.) at codon 6 has been identified in the current investigation [Codon 6 (-C.T.)]. The commonest mutation is IVSII16 G > C (36%), followed by IVSI5 G > C (28%), IVSII-74 T > G (22%), codon 3 (T > C) (19%), and Poly A site (T > C) (8%). This shows that b-thalassemia-causing molecular abnormalities are becoming more prevalent in Indian people. Population screening in conjunction with genetic counseling is advantageous because it enables at-risk families to make educated decisions about their reproductive options[5]

A study conducted by Ajay F.Christopher, Anita kumara, sunali Chaudhary et .al., included 48 unrelated people from western Uttar Pradesh, whose ages ranged from 2 to 16, made up the study's sample (32 were male and 16 were females). The study's participants were all dependent on blood transfusions. With the parent's consent, the patient's records were reviewed to determine the parents' ages, sexual preferences, family history, and consanguinity. As young as six months old was the age of presentation of the condition.

Most of these patients had transfusions 14–16 times per year, although only a small number of them did so at sporadic intervals because of financial and other limitations. The most frequent thalassemia mutation, according to molecular analysis, was IVS1-5 (G-C) (46%) followed by Fr8/9 (+G), which was present in 21% of cases. This analysis identified the IVS mutation as

the most prevalent mutation (46%) in the Indian population, which is consistent with earlier findings. Mutation Fr 8/9 was found to be the second most common mutation in this study, with a frequency of 21%.

According to previously published data, it was the fourth most prevalent mutation in India. This discovery is in opposition to that information. This region (including the mutation for globin) lacks some mutations that are common in other locations. The Western U.P. can therefore claim that these discoveries are unique [(6)]

A study conducted by Manisha Shrivastava, Rashmi Bathri, and Nirupama Chatterjee included sixty-two transfusion-dependent patients. Information about the recruited children was acquired, including their splenectomy, age, frequency of transfusions, and age at which they had their first transfusion.. 42 males and 20 females out of 62 transfusion-dependent patients were enrolled. The mutation with the highest frequency in this analysis was G-C substitution at IVSI-5, which is of Asian-Indian origin.

The second common mutation, Mediterranean mutation IVS I-1 (G-T), was discovered in 15 loci (12%), followed by the 619 bp Deletion, which was discovered at 11 loci (9%). Codon 8/9 (+G) and CD44/42 (TCTT), the other two frequently occurring Indian mutations, were found at roughly 7% and 4% of the loci, respectively. The two uncommon variants, 88C/T and capsite A/G, made for 2.4% and 1.6% of all the loci analyzed, respectively. This research aids in determining the mutational spectrum of the area and provides the framework for the establishment of a prenatal diagnosis facility there.

The data reinforces the government of India’s commitment to preventing and controlling hemoglobinopathies and places a strong emphasis on prevention in order to lower mortality, morbidity, and the economic, psychological, and social costs of Thalassemia on society.

A national strategy for the prevention and management of hemoglobinopathies has been developed, with a focus on the importance of prioritizing these hereditary diseases. It is necessary to take appropriate control measures, such as identifying carriers and preventing the birth of affected children through prenatal diagnostics, given the severity of the issue and the management's financial ramifications [7]

A study conducted by Ali Bazi MSc, and Ebrahim Miri-Moghaddam PhDshowed that The most prevalent severe phenotype of Thalassemia among Iranians is -thalassemia major (TM). Recent years have seen a big potential in terms of diagnostic problems due to molecular understanding of the etiology of Thalassemia Major. The development of thorough molecular databases enables the development of prenatal diagnosis (PND) molecular screening assays and very sensitive diagnostic tools for Thalassemia Major

Despite a sizable amount of work on the molecular basis of Thalassemia Major, there aren't many review studies that take a broad perspective on the distribution of Thalassemia Major mutations in Iran.

Common genetic abnormalities discovered in Iranian individuals with TM from 2005 to 2014 have been reported in the current review. Additionally, the distributional patterns and prevalences of known mutations were reviewed. IVSII-1 (G>A) and IVSI-5 (G>C) were discovered to be by far the most prevalent mutations observed in Iranian patients. FSC 8/9 (+G), IVS I-110 (G>A), FSC 36/37 (- T), IVSI-1 (G>A), IVSI (-25bp), and codon 44(-C) were among the other frequently reported mutations The molecular profile of T.M. among various Iranian groups was discovered to be highly diverse; in particular, ethnicity and intra-migration may be the most significant contributing variables in dictating distributional patterns. [8].

A study by Sajan Sinha, Paramita Bhattacharya, Mrinal Kanti Das, Atanu Kumar Dutta, et al. included 103 children aged up to 12 years. This study was conducted in a tertiary care facility in West Bengal, Eastern India. The same hospital conducted genetic testing. A total of 103 people participated in the study, of which 61 were male, 42 were females, seventy-four were TDT, and twenty-nine were NTDT.

The HbE mutation in the HBB gene and five common mutations for Thalassemia were assessed. The IVS 1-5/1-5 mutation was present in 38 of the 54 TDT patients. IVS 1–5 showed a high association between the severity of phenotype and genotype. Twenty of the 45 patients with E thalassemia had TDT, and 25 had NTDT. In this study, only two mutations, IVS 1–5 and Cd41–42, are highlighted. The HbE mutation research detected symptomatic Thalassemia in 79.6% of the study population, which is much higher than the detection rate with the conventional five mutations study in other regions of India.

IVS 1–5 mutations emerged as the most prevalent mutation in this analysis and were found to be a predictor of clinical severity in symptomatic β/β Thalassemia but not in E β Thalassemia. The current investigation supported the findings that IVS 1-5 (G>C) is the commonest mutation in this eastern region of India and that genotype and phenotype correlation was established in β/β Thalassemia but not in E β Thalassemia.

The importance of genetic mutation analysis as a supplement to Hemoglobin HPLC was highlighted in this study. This was due to the fact that those patients began receiving monthly blood transfusions extremely early in the first year and remote places. Studies on genetic mutations can close this gap and possibly validate the diagnosis in the antenatal stage.

The present study confirmed that IVS 1-5 (G>C) is the commonest mutation of the allele in this eastern part of India and that genotype and phenotype correlation was found in β/β

Thalassemia but not in E β Thalassemia. Further research is required to define the genetic modification which interacts with Thalassemia to provide a better understanding of the disease's progression. (46)

A study conducted by Parth S Shah, Nidhi D Shah, Hari Shankar P Ray et al. included 75 referral cases of both sexes with ages ranging from 6 months to 38 years were gathered to test for the beta thalassemia mutation. The samples from various regions of Gujarat, Rajasthan, Maharashtra, Assam, and West Bengal were examined for thalassemia mutation analysis

Sanger Gene Sequencing, together with PCR, The data showed that 92+5 G>C (IVS-1-5) is the commonest mutation in cases (60.29%) in Rajasthan and Gujarat, followed by deletion 619 bp. Western India's Rajasthan, Gujarat, and Maharashtra had a greater incidence of the 92+5G>C mutation (26+10+2) and subsequent deletion 619 bp (3+4+2) than Eastern India's West Bengal and Assam

This needs to be explained in more detail. The variation in these mutations' frequency is influenced by racial diversity, migration, genetics, and other lifestyle factors. We, therefore, urge that before using assisted reproductive and preimplantation technologies in India, these populations be provided with access to mass screening, prenatal diagnostic techniques, genetic counseling, transfusion programs, and clinical treatment (45)

A study conducted by Mahmoud M. Sirdah, Jürgen Sievertsen, Mansour S. Al-Yazji, et al. included 264 unrelated Palestinian individuals living in the Gaza Strip. They were divided into three groups: group (I) 49 transfusion-dependent -thalassemia subjects, group (ii) 176 thalassemia carriers and group (iii) 39 microcytic and/or hypochromic participants (MCV80 fl or MCHb 26 pg. The IVS-I-1 G>A, IVS-I-6 T>C, and the IVS-I-110 G> Codons 106/107+/G, A, Codon 37 G>A, Codon 39 C>T, and Codons 106/107+/G, which were previously believed to be common among the Arabic and Mediterranean population were analyzed for sequencing

Additionally, the polymorphisms IVS-II-16 and IVS-II-74, two genetic variants without disease associations, and the new variants 50 (G), 43 (C>T), and IVS-II-26 (T>G) with unknown disease associations were discovered. Six of the 39 microcytic and hypochromic suspect carriers—those whose HbA2 levels were below the diagnostic threshold for -thalassemia carriers (HbA23.5%)—showed HBB mutations, including IVS-I-6 (T>C), Codon 39 (C>T), and 3′ UTR +101 (G>C).

This analysis supported previous studies that IVS-I-110 was the commonest mutation in the Gaza Strip[47,48), in contrast to other Palestinian communities from the West Bank or mountainous regions. IVS-I-110 G>A was the commonest mutation among the 15 distinct HBB variants detected, found in 34% of the chromosomes of thalassemic patients, then followed by IVS-I-1 G>A, IVS-I-6 T>C, Codon 39 C>T, and Codon 37 G>A. By directly sequencing the gene, three new HBB variants were identified: IVS-II-26 T>G, 5′ UTR-43 C>T, and 5′ UTR-50 (/G).

The information provided might encourage the adoption of molecular testing in the Gaza Strip's premarital screening program for -Thalassemia among Palestinians, which would enhance the screening process and genetic counseling going forward [49]

A study by Ali Fettah, Cengiz Bayram, Nese Yarali, Pamir Isik, et al. included 106 thalassemia patients from Ankara Children's Hematology and Oncology Research Hospital, Department of Hematology. Ankara, Turkey. Among 106 individuals with a mean age of 11.2 years (52.8% were female, and 47.2% were male). The remaining 22 individuals were found to have intermediate Thalassemia, whereas 84 patients had significant Thalassemia.

. IVS I.110 (G>A) was the mutation that was found the most frequently (35.3%), followed by Codon 8 del-AA (10.4%), IVS II.1 (G>A) (8%), IVS I.1 (G>A) (7.5%), Codon 39 (C>T) (7.1%), and Codon 5 (-C.T.)

The most frequent mutation found in patients with thalassemia major was IVS I.110 (G>A) homozygous (39.2%), followed by Codon 8 del -A.A. homozygous (8.3%), IVS I.1 (G>A) homozygous (7.1%), Codon 5 (-C.T.) homozygous (7.1%), and Codon 39 (C>T) homozygous (4.9%). The most common mutation found, IVS I.110 (G>A), was reported in this study.[50]

A study by Sandeep B. Satpute, Mangesh P. Bankar, and Abdulrahaman A. Momin included 126 beta-thalassemia carrier individuals in a western Indian population, mainly from southwestern Maharashtra. The six most prevalent b-thalassemia mutations, IVS I-5 (G-C), IVS I-1 (G-T), codons 8–9 (+G), codons 41–42 (-TCTT), codon 15 (G-A), and a 619 bp deletion at the 3' end of the b-globin gene, were found. In 126 individuals who were b-thalassemia carriers, these mutations made up 93.66% of the cases, whereas 6.34% were yet unidentified.

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The pattern of b-thalassemia mutations from southwest Maharashtra is provided by this study, which will aid in preventing b-thalassemia by prenatal detection and appropriate counseling.

Because there is no effective screening program in place, the high-risk couple often consults the doctor for a prenatal diagnosis at an advanced stage of pregnancy, which leaves less time for the mutational workup. From the perspective of prenatal diagnosis, this study's accurate depiction of the range of b-thalassemia mutations in its individuals is essential [(64)]

A study by Sultana GNN, Begum R, and Akhter H, included 70 patients with β-thalassemia major who visited Thalassemia Hospital of Bangladesh. From the 1,045 cases in our previous study, samples were chosen at random. Among them, 51 had HbE-thalassemia, and 19 had beta–thalassemia major

In this study, 70 Bangladeshi individuals with Thalassemia were found to have nine mutations in the HBB gene (-90 C>T, Codon 1 T>A, Codon 2 C>A, Codon 2 T>C, HbE/Codon 26 G>A, Codon 30 G>C, IVS-I-5 G > C, IVS-2-16 G>C, and IVS-2-81 C>T).

IVS-I-5 G>C (81.4%) was the commonest mutation in this group. This finding is consistent with earlier research that identified IVS-I-5 G > C as the commonest mutation in - thalassemia patients from Bangladesh. HbE/Codon 26 G>A was the second most frequent mutation (72.8%), followed by Codon 2 T>C (57.1%) and IVS-2-16 G>C (57.1%).

The current study shows that Bangladesh has a variety of -thalassemia cases. Three mutations, Codon 1 T,>A, Codon 2 C>A, and IVS-2-81 C>T were discovered for the first time in Bangladeshi patients and were not seen in any other geographically comparable countries. IVS-I-5 G> C was commonest mutation in Bangladeshi patients, accounting for 81.4 percent of all cases.

Most notably, the discovered mutations in this study were situated within HBB: c-92 to IVS-2-81, which indicates that the 42 bp promoter, 5'UTR, exon 1, intravenous sequence 1, Exon 2, and 81 bp of intravenous sequence 2 of the HBB gene were all covered.

This region may therefore be used as a biomarker for genetic counseling, carrier screening, and the creation of an extensive allele-specific prenatal testing kit for thalassemia identification in Bangladesh [51]

A study by Waseem Chauhan, Mohammad Afzal, and Zeeba Zaka-ur-Rab et al.included children from two thalassemia centers in the major hospitals (LLRMC Meerut and JNMC, Aligarh) in the Western Uttar Pradesh.The seven different types of mutations were reported in this study for the first time in Western Uttar Pradesh, India. The loss of 4 nucleotides from codon 66/67 (-AAAG) in the exon 2 region, a novel frameshift mutation, is described for the first time.

The most commonly reported mutations are IVS 1-5 (G>C) and Codon 41/42 (-CTTT). The molecular spectrum for these two cases consisted of 44 and 42 alleles out of 108 alleles analyzed from 46 homozygous and 31 compound heterozygous patients, respectively. The participants were drawn from 20 districts in Western Uttar Pradesh to discover the three commonest HBB gene variants, namely IVS I-5 (G>C), Codon 41/42 (-CTTT), Codon 3 (T>C), and the confirmation of Codon 66/67 (- AAAG) (novel mutation)

Sequence examination of the HBB gene in the populations revealed that seven distinct HBB gene mutations and some common alterations in 108 alleles, two deletions, and five point mutations were identified. Exon-2 had one new mutation [Codon 66/67 (-AAAG)].

Three of the seven alterations (Codon 3 (T>C), Codon 41/42 (-CTTT), and Codon 66/67 (-AAAG)) were found in the exon area, while the remaining four were found in the intron region.

The most common mutations in -thalassemia patients' genotypes were Codon 41/42 (-CTTT) and IVS I-5 (G>C). Codon 3 (T>C) has the second most prevalent mutation

For the first time, this study describes seven new types of mutations in Western Uttar Pradesh, India, including a unique frameshift mutation (deletion of four nucleotides (Codon 66/67 (-AAAG)) in the exon-2 region. The most common reported mutations are IVS 1-5 (G>C) and Codon 41/42 (-CTTT) [52]

Materials and methods

Source of data

All children and adolescents enrolled in the thalassemia clinic at Shri B M Patil Medical College and Hospital were included in this study

Type of Study: Cross-sectional study (Mutation analysis study)

Duration of Study : JANUARY 2021 to JUNE 2022

During above mentioned period we have collected total 47 samples, clinically diagnosed with beta-thalassemia major

Method of collection of Data (including sampling procedures, if any)

The Institutional Review Board provided ethical approval, and all participants provided informed written consent before blood samples were collected..

SELECTION CRITERIA

Inclusion criteria:

Children and Adolescents diagnosed with beta-thalassemia major between the age group of six months to eighteen years, who were registered in the pediatric department of Shri BM Patil Medical College and Hospital were included in this study

Exclusion criteria:

Patients unwilling to give consent for providing blood samples

Other hemoglobinopathies

Data analysis

Determination of sample size (n)

With the anticipated Proportion of Mutations observed in β thalassemia patients 8%[(ref)], the study would require a sample size of 47

patients with 95**%** level of confidence and 10% absolute precision**(5)**

Formula used

- $\mathbf{n=\frac{z^2\ p*q}{d^2}}$

Where Z= Z statistic at α level of significance

d^2= Absolute error

P= Proportion rate

q= 100-p

Statistical Analysis

- The collected data will be entered into a Microsoft Excel spreadsheet, and statistical analysis will be performed using a social science statistical tool (Version 20).
- Results will be presented as Mean (Median) ±SD, counts and percentages, and diagrams.

Clinical Sample (Blood) Collection

Children who were enrolled in the study gave their consent. 1 ml of Peripheral blood was drawn after receiving consent and stored at 4°C in EDTA-coated vacutainers (BD367863).

Isolation of Genomic DNA and Quantification

A commercial DNA isolation kit was used to isolate genomic DNA from 300µl of peripheral blood (Bangalore Genei, India).

Brief Genomic DNA Isolation Protocol:

1. In a 1.5 ml EDTA-coated vial 300 µl of peripheral blood was collected.
2. By adding1 ml of 1 X solution A (provided by the kit) RBC cells were lysed.
3. At room temperature the vials were centrifuged for 5 min at 8000 RPM.
4. Until a clear white WBC pellet was obtained the above step was repeated.
5. 600µl of solution B was added (provided by the kit) to the WBC and mixed gently for clear lysis.
6. It was centrifuged at room temperature for 10 min at 10,000 RPM.
7. The Supernatant was collected and 0.9 ml absolute cold ethanol was added to it and mixed.
8. Centrifuged at 4ºCfor 20 min, at 10,000 RPM.
9. Precipitate DNA was washed with 0.5 ml of 75% ethanol.
10. Centrifuged for 5 min at 10,000 RPM.
11. 100 µl of solution C was added (provided by the kit) after air drying the DNA pellet.
12. The vial was incubated at 55ºC for 10 min.
13. To remove any insoluble materials it was centrifuged at 10,000 RPM for 2 min.
14. The DNA thus obtained was stored at -20ºC until further use.

The quality of the isolated DNA was checked under gel electrophoresis. 100 ml of 1% agarose gel was prepared (1 gm of Agarose + 100 ml of 1X TAE buffer). The same isolated DNA was quantified under “Nanodrop” (Quawell) and the quantity and quality of the DNA were reported.

Primer designing:

The web-based freely available program "Primer3" which is widely accepted was used, (http://frodo.wi.mit.edu/ primer3/ input. Html) for designing PCR primers. Primer 3 is a Bioinformatics tool that helps in designing the primers for the target region in the given nucleotide sequence as per the requirement of the user or applications. The designed primers using Primer 3 were reconfirmed for the specificity of its binding site using the web-based bioinformatics tool "Genome Build 36" (https://genome.ucsc.edu/FAQ/FAQreleases.html), and for its Insilco amplification on "Insilco PCR" (http://insilico.ehu.es/PCR/). All the designed primers for our target genes or region are tabulated in table No. 1 along with the annealing temperature and amplicon size. A commercial oligo synthesiser is used to create primers (MWG Biotech, India).

Table 6. Primer sequences and the annealing temperatures used for the amplification of exon 1 of Hbb gene.

primer	Sequence	Amplicon Size(Base Pairs)	Annealing Temperature
TH1F	AGGGTTGGCCAATCTACTCC	**287bp**	**59.5°C**
TH1R	GTCTCCACATGCCCAGTTTC		

Polymerase Chain Reaction (PCR):

A 20-liter reaction volume was used for the PCR amplification, which contained 0.5 litres of genomic DNA (75 ng/l to 150 ng/l), 0.5 litres of each primer (5 pmol), 0.4 litres of dNTP (10 pmol), 0.2 litres of Taq DNA polymerases (3 units/l), and 4 litres of Taq Buffer (5X) (all from BioRad, USA). In a Master cycler gradient (Eppendorf, Germany), amplification was carried out under the following conditions: a 10-second denaturation at 980°C, followed by 35 cycles at 980°C for 10 seconds (cycle denaturation).

The primer annealing temperature was determined based on the primer annealing temperature (Table-1) for 10sec 720C for 15sec (primer extension) and a final extension at 720C for 5 min. Gel electrophoresis with a conventional 100bp ladder validated the amplicon size of the PCR results. The following were the PCR cycling conditions: Initial denaturation is 980C for 10 seconds, Denaturation is 980C for 10 seconds, Annealing is primer dependent for 10 seconds, Elongation is 720C for 5 minutes, and Hold at 400 degrees Celsius.

Agarose Gel Electrophoresis

Gel electrophoresis is one of the molecular biology techniques used to separate DNA and RNA depending on the length of fragments. It is a widely used and accepted method, to estimate the size of DNA and RNA fragments or to separate proteins by charge. Nucleic acid molecules are separated based on an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving.

DNA Sequencing (Capillary Based)

PCR products were subjected for capillary based Big-Dye terminator sequencing. Prior to sequencing, the PCR products were subjected to cycle sequencing and plate processing

Cycle Sequencing

As per the Sanger Sequencing protocol, Big-Dye labeling and chain termination were carried out by the cycle sequencing method. To label each base, the PCR amplicon was subjected to a cycle sequencing reaction with a single primer. Big-Dye TM terminator v3.1was used for cycle sequencing (Applied Biosystems, USA) following the manufacturer's guidelines. Cycle sequencing of the PCR products was carried out according to the annealing temperature of the primers.

Table 7. Standardised master mix conditions for sequencing

SL. No.	Constituents	Quantity
1	Molecular Biology grade water	6.3 µL
2	Big Dye Buffer (5X)	1.3 µL
3	Big Dye	1.0 µL
4	Template (PCR product)	1.0 µL
5	Forward Primer	0.2 µL
6	Reverse Primer	0.2 µL
Total		**10 µL**

Note: Only one of the primers i.e either forward or reverse primer was used during cycle sequencing

Table 8. The cycle sequencing conditions

Process	Temperature (°C)	Time
Initial. Denaturation	98	10sec
Denaturation	98	10sec
Annealing	Primer Dependent	10sec
Elongation	72	5min
Hold	4	

Note: The annealing temperature is primer dependant and varies for each primer

Sequencing Clean-up (Plate Processing)

To remove the unbounded florescent DNTPs from the terminator sequencing reaction, 2µl of 3M sodium acetate, and 50µl of 100% ethyl alcohol were added to each sample and incubated at room temperature for 15 minutes to precipitate the DNA. The samples were centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatant was discarded and the reaction plate was centrifuged in a reverse manner at 300 rpm for 20 seconds. 100µl of 75% alcohol was added to each sample and centrifuged at 4000rpm for15 minutes at 25°C. The supernatant was discarded and the plate was centrifuged in a reverse manner at 300 rpm for 20 seconds to remove the alcohol completely. The plate was dried at room temperature until the last drop of alcohol dripped off

10µl of Hi-Di Formamide was added to each well of the sample plate. The samples were heated to 96°C for 5 minutes and immediately cooled to 4°C to denature and linearise the cycle sequencing products. The processed products were loaded in the sequencer for sequencing.

Sequencing Run

Sample information sheets which contain analysis protocols along with the sample details were prepared and imported into the data collection software. Prepared samples were analyzed on ABI 3730 genetic analyzer (Applied Biosystems, USA) to generate DNA sequences or electropherograms. After completion of the sequencing reaction, the quality of generated sequence was checked by using Sequencing Analysis v5.4software (Applied Biosystems, USA)

Sequence Alignment

The generated sequences were aligned to their respective reference sequences with the use of Variant reporter software (ABI v1.1). The variant reporter is one of the compatible software of Applied Biosystems designed for automated sequence data analysis. It performs sequence comparisons for novel mutations, known variants, insertions, and deletions. It allows analysis of the resequenced data, comparing the consensus sequences to a known reference sequence. The results of the variant reporter were tabulated in PDF format as the default program of the software.

Here, we used this technique to check the isolated genomic DNA from whole blood. EtBr (ethium bromide stain) stain was used to stain the DNA fragments. In all the 47 samples from thalassemic children in figure 8 and 9,Which confirmed the presence of genomic DNA and the same samples were taken for quantification based on Nanodrop technique

Figure 8.Agarose gel image(1) of genomic DNA of thalassemia children samples

Figure 9 .Agarose gel image(2) of genomic DNA of thalassemia children samples

Quantification of Genomic DNA

We used Tecon multimode reader for the quantification of genomic DNA. Tecon multimode reader is a micro-volume UV spectrophotometer specifically designed for the measurement of nucleic acids and purified proteins. Its unique technology holds 0.5-2.5 ul samples between upper and lower measurement surfaces without the use of a cuvette. Tecon multimode reader measures the samples in less than 2 seconds with a high degree of accuracy and reproducibility.

The Tecon multimode reader works on the principle, "Nucleic acids absorb light at a wavelength of 260 nm and when 260 nm light source shines on a sample, the amount of light that passes through the sample can be measured, and the amount of light absorbed by the sample can be inferred. For doublestranded DNA, an Optical Density (OD) of 1 at 260 nm correlates to a DNA concentration of 50 ng/µl, so that DNA concentration can be easily calculated from OD measurements" as shown in Table no. 6

Table 9: Quantification of Thalassemia Samples

Sl. No. of DNA samples	OD at 260/280	Concentration in ηg/µl
1	1.86	54
2	1.75	65
3	1.40	44
4	1.90	70
5	1.57	136
6	1.98	64
7	1.84	82
8	1.92	73
9	1.65	68
10	1.79	111
11	1.85	64
12	1.81	66
13	1.75	53
14	2.02	65
15	2.15	82
16	1.51	94
17	1.88	49
18	2.09	39

19	1.93	46
20	2.04	100
21	2.6	51.5
22	2.35	85.5
23	1.96	73.9
24	3.05	57
25	2.01	81
26	2.24	125
27	2.09	137
28	1.76	104
29	1.96	92
30	1.58	93
31	1.81	53
32	1.72	66
33	1.63	42
34	1.69	68
35	1.75	126
36	1.71	66
37	1.65	73
38	2.02	63
39	2.25	76
40	1.41	101
41	1.58	64
42	2.10	56
43	1.73	53
44	1.63	55
45	1.65	72
46	1.30	65
47	1.70	56

Polymerase Chain Reaction (PCR)

In PCR, the DNA polymerase's capacity to produce new DNA strands that are complementary to the provided template strand is utilised. DNA polymerase needs a primer to which it can add the first nucleotide since it can only add a nucleotide to an already produced 3'-OH group. The researcher might specify a particular portion of the template sequence to amplify using this criterion. At the conclusion of the PCR process, billions of copies of the exact sequence will have been produced (amplicons).We used HBB gene exon 1 specific primers as

given in table 1 and carried out the PCR reactions. After PCR, the products were subjected to Gel electrophoresis, and results were documented for thalassemia samples. In all the products we observed bright specific amplicons in comparison with the 100bp ladder, which confirmed the primer-specific amplification in the PCR reaction.

Primers (HBB) specific amplification results are shown in figure 4. After the PCR amplification, the amplicons were run through 1% agarose gel electrophoresis and the DNA bands were observed in gel documentation (Figure 4).

The PCR product of 287bp

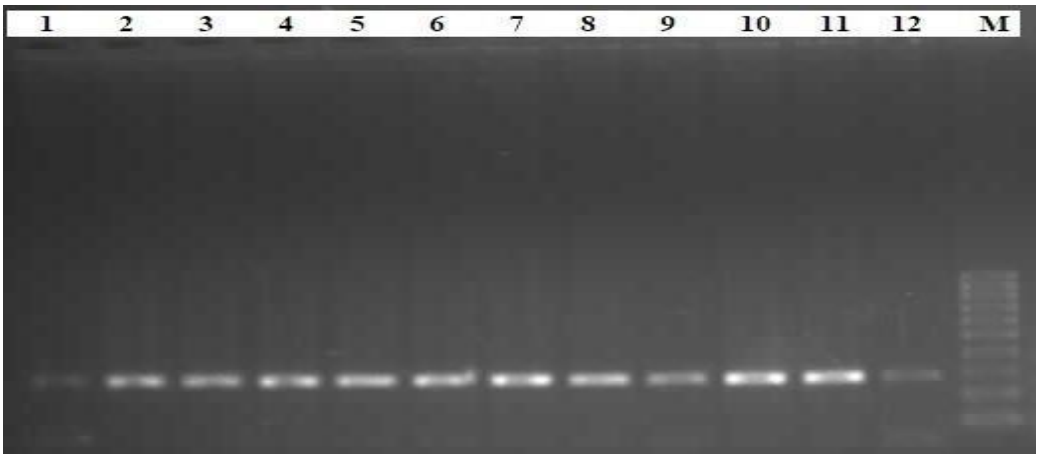


Figure 10 : Agarose gel electrophoresis image of amplified products of exon1 of HBB gene. Lane No: 1-12 thalassemia samples. M: 100bp marker

Figure 11 : PCR products (1)

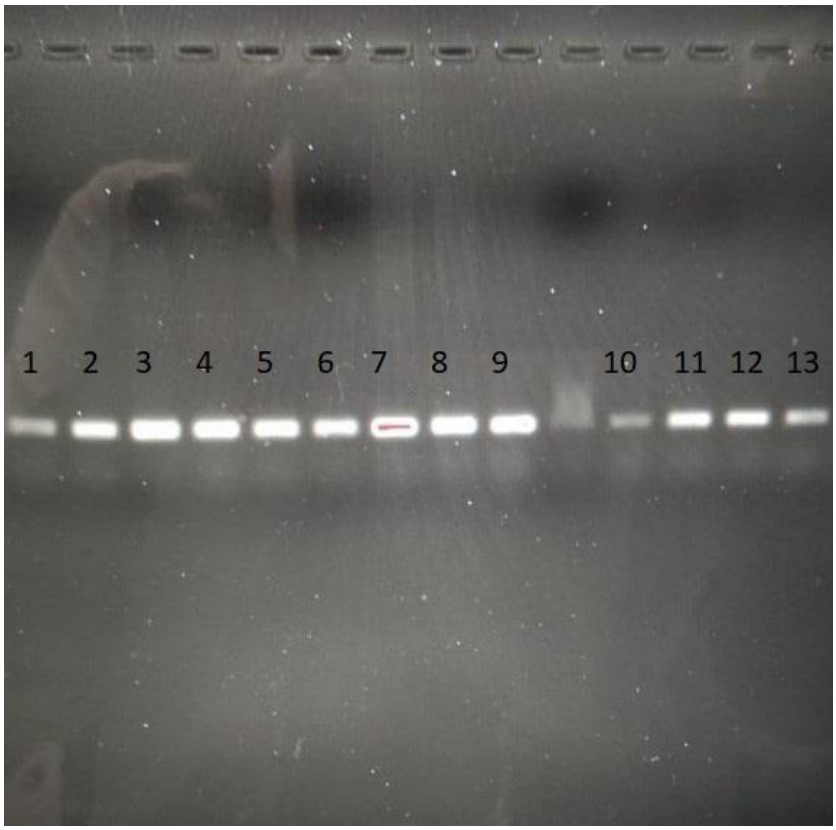
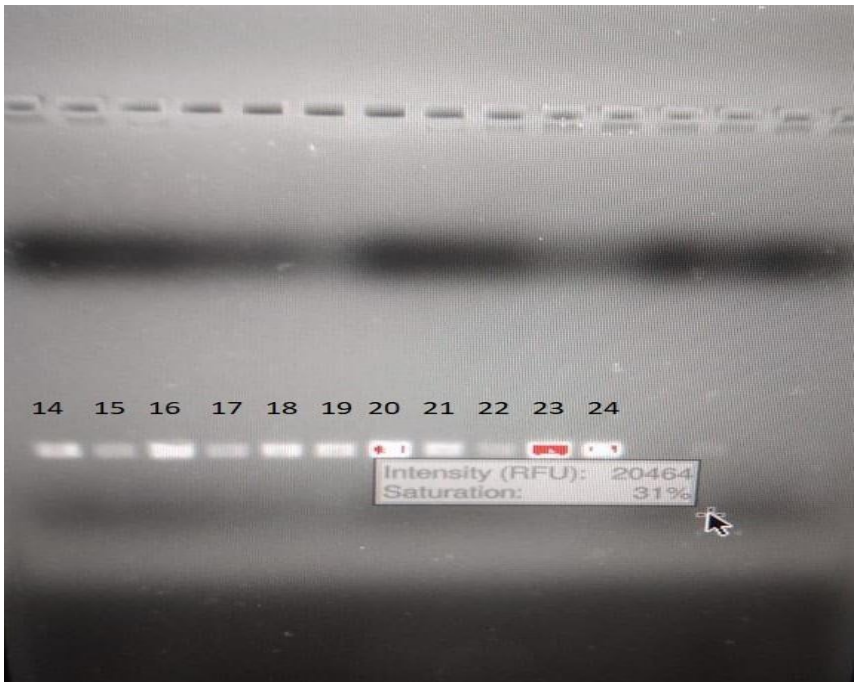


Figure 2 : PCR products (2)



RESULTS

This study was done in Department of Pediatrics, B.L.D.E (Deemed to be University), Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka where thalassemia patients were being transfused. Mutational analysis of HBB gene was done in the Genetic research lab , Department of Anatomy .47 transfusion-dependent thalassemia patients were included in the present study for the mutational analysis of Hbb gene. All 47 patients were found to be known cases of Thalassemia major

TABLE 11 : Age Distribution (n=47)

Age(Years)	No. of children	Percentage
≤5	24	51
6 - 10	14	29
≥11	12	25
Total	47	100.0

In the present study, the Age of the children was within the range of 1 to 20 years , with most children under less than 5 years amounting to 51%

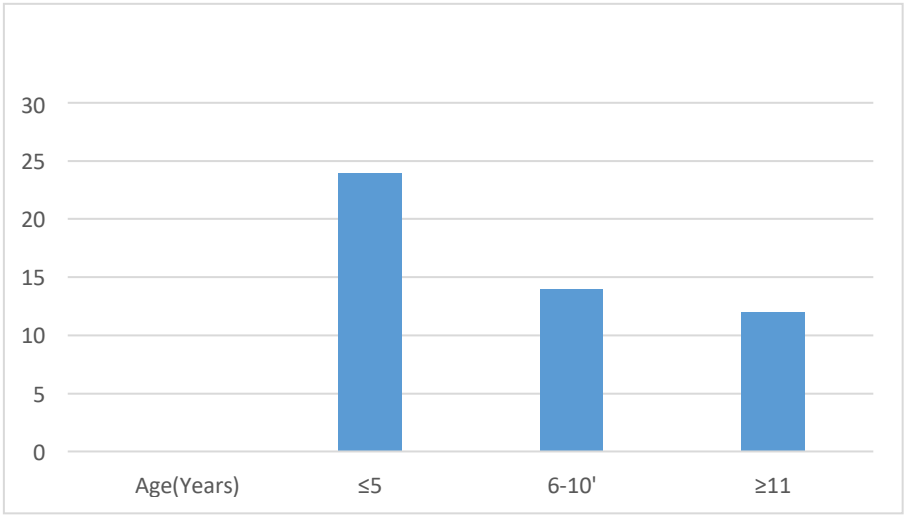


Figure 13 : Age Distribution

Table 12 : Gender Distribution (n=47)

Gender	No of Patients	Percentage
Male	30	63.8
Female	17	36.2
Total	47	100

In this study, the maximum number of children were males (30 cases) amounting to 63.8 %, with females (17) amounting to 36.2% ,The male to female ratio is 1.7:1

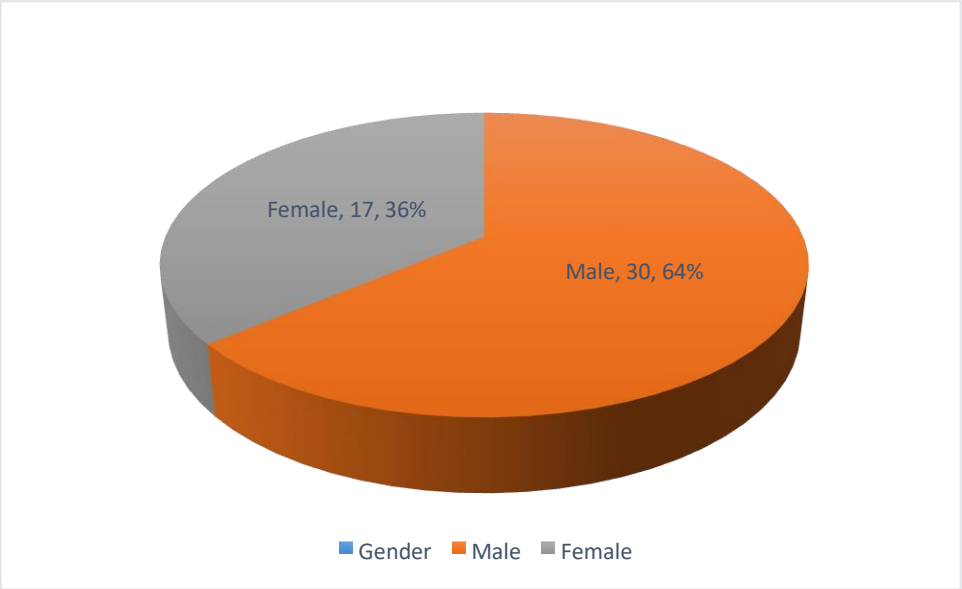


Figure 14 : Gender Distribution

Table 13: Consanguineous status (n=47)

Consanguineous history	No of Patients	Percentage
YES	29	61.70
NO	18	38.2
Total	47	100

In the present study ,Consanguinous history was present in 29 thalassemia children amounting to 61.7%

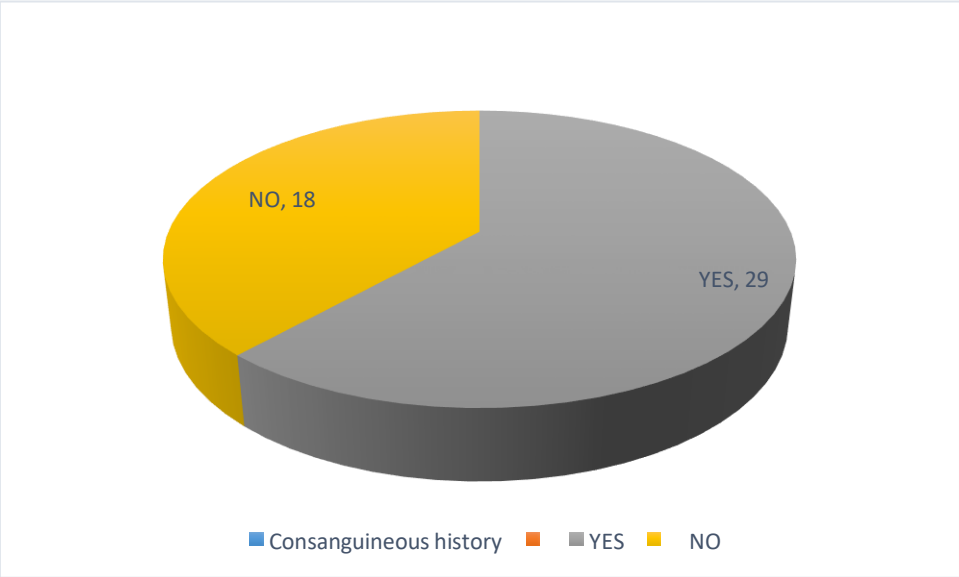


Figure 15 : Consanguineous status

Height for Age	No of children	Percentage
<3rd centile	18	38.2
3rd -50th centile	20	42
50th – 75th centile	9	19
Total	47	100

Table 14 : Height for age Distribution (n=47)

In the present study Height for Age was with in the range of 3rd -50th centile in most children (18) amounting to 42%

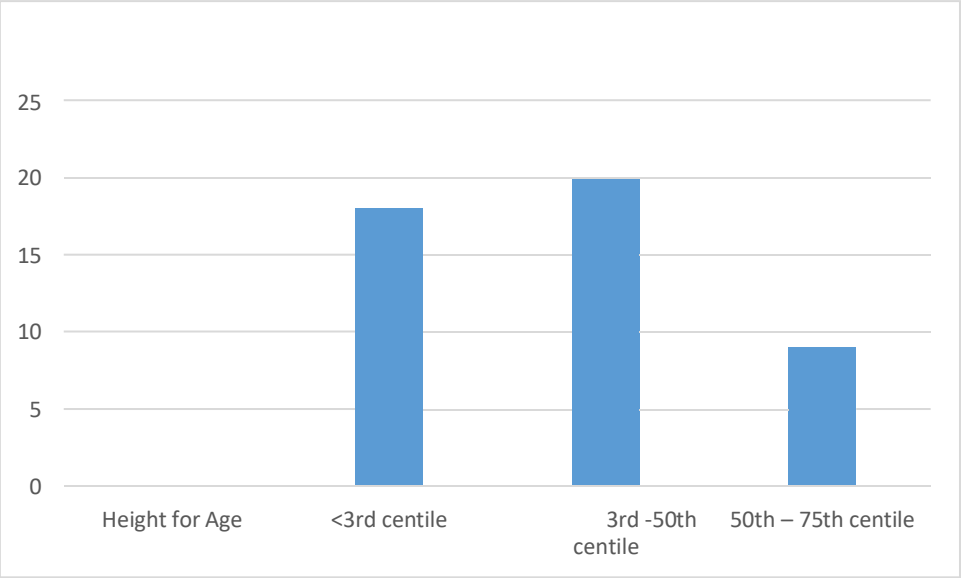


Figure 16 : Height for Age Distribution

Table 15 : Weight for Age Distribution (n=47)

Weight for Age	No of children	Percentage
<3rd centile	12	25.5
3rd -50th centile	31	66
50th – 75th centile	4	8.5
Total	47	100

In the present study weight for Age was with in the range of 3rd -50th centile in most children (31) amounting to 66%

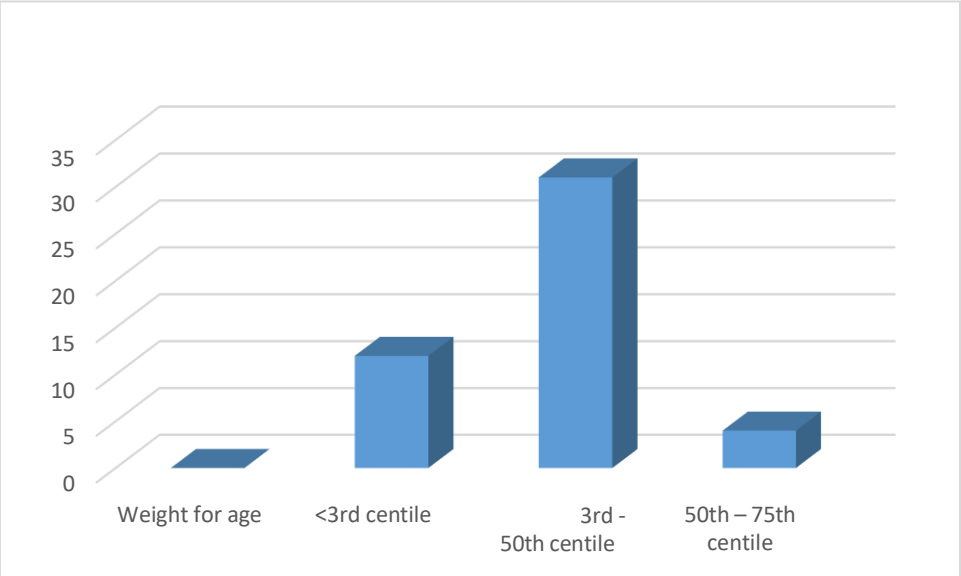


Figure 17 : Weight for Age Distribution

Table 16 : Grades of splenic enlargement (n=47)

Spleen	No of children	Percentage
No	25	53
Mild	3	6.5
Moderate	8	17
Severe	4	8.5
spleenectomy	7	15
Total	47	100

In the current study , most of the children there was no splenomegaly amounting to 53 %, splenectomy was done in 7 children amounting to 15%, severe splenomegaly was seen in 4 children amounting to 8.5 %

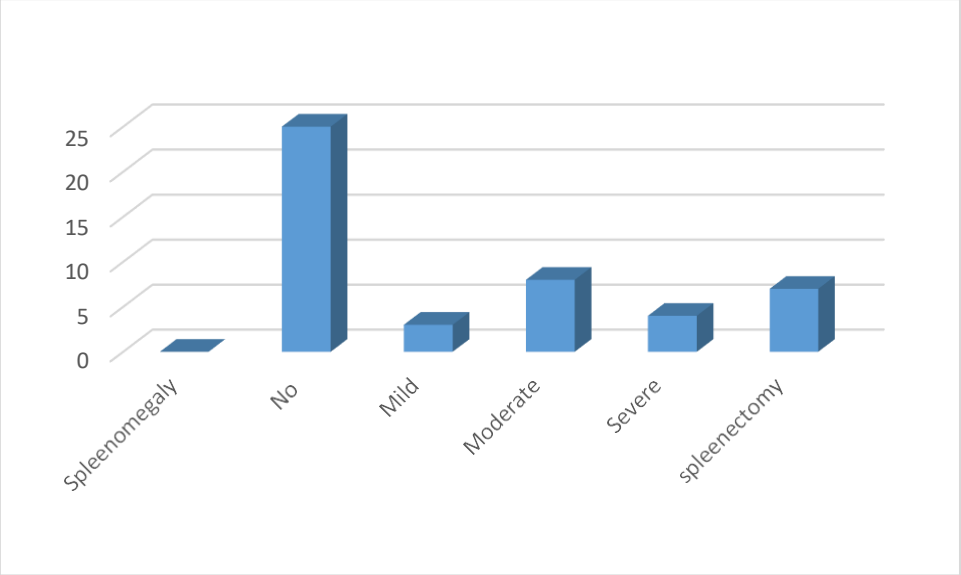


Figure 18 : Grades of Splenic enlargement

Table 17 : Interval between transfusion (n=47)

Interval between transfusion in days	No of children	Percentage
15	18	38.2
30	20	42
60	9	19
Total	47	100

In the current study , majority of the children were transfused every 30 days amounting to 42%, in every 15 days amounting to 38%

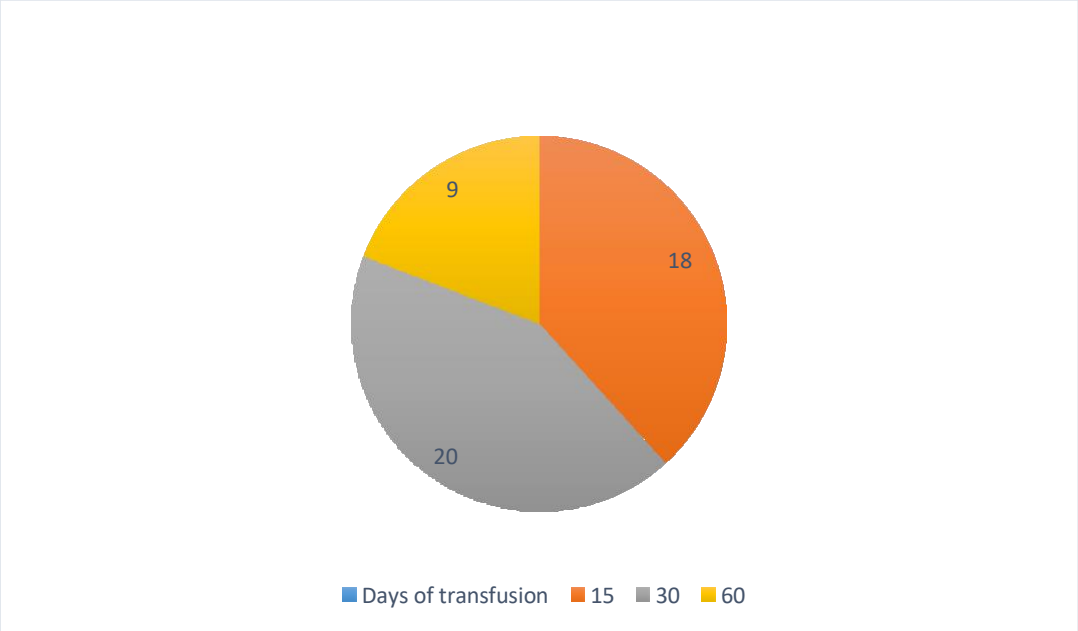


Figure 19 : Interval between transfusion

Table 18 : Percentage of children with Mean Pre-transfusion Hb (n=47)

Mean Pre transfusion Hb%	No of children	Percentage
<8 g/dl	41	87
≥ 8g/dl	6	12
Total	47	100

In the current study , majority of the children Pre- transfusion Hb was < 8g/dl in 41 children amounting to 41 %, with Hb > 8gm/dl in 6 children amounting to 12 % , Minimum Pre transfusion Hb was 3 gm/dl and maximum was 10 gm/dl with an average of 6.21 ± 1.654 (Mean ±SD)

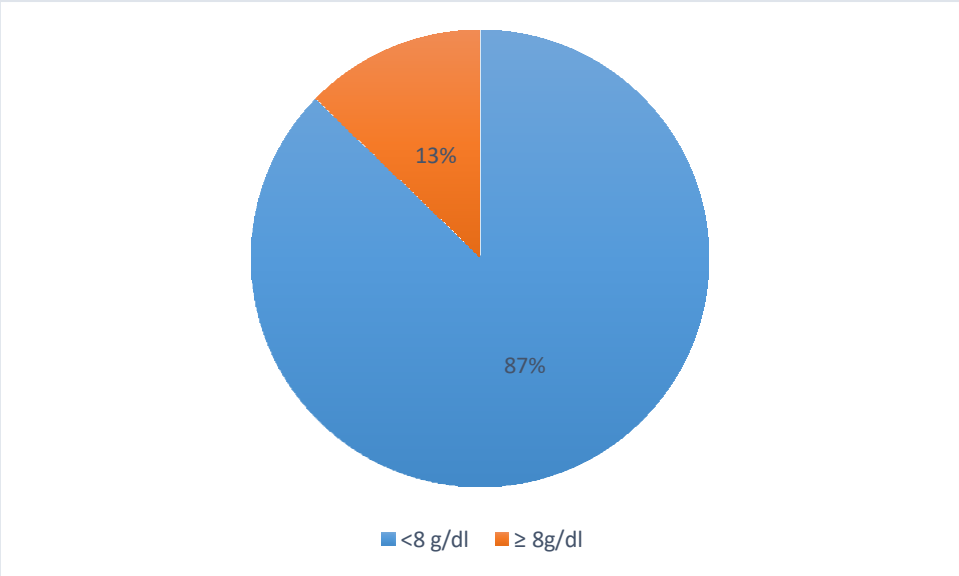


Figure 20: Percentage of children with Mean Pre-transfusion Hb

Table 19 : Age at first transfusion

Age at which first transfusion started	No of children	Percentage
<1 yr	33	70
>1yr	14	30
Total	47	100

In the present study , transfusion was started early < 1 year in 33 children amounting to 70% , > 1 year in in 14 children amounting to 30 % , Minimum Age at which Transfusion started was 3 months and maximum was 36 months with an average of 9.87±8.048 (Mean ±SD).

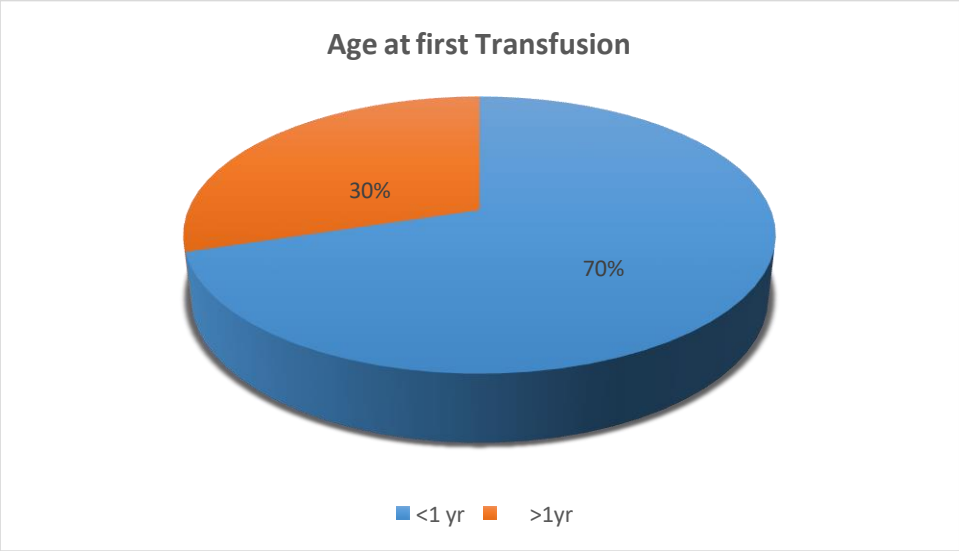


Figure 21 : Age at first Transfusion

TABLE 20: DESCRIPTIVE OF THALASSEMIC CHILDREN

Variables	Minimum	Maximum	Mean	Std. Deviation
Age(yrs)	1	20	7.85	4.809
Weight (kg)	8	45	19.09	9.004
Interval between transfusion(days)	15	60	27.13	9.710
Age at which Transfusion started (months)	3	36	9.87	8.048
HC (cm)	46	56	49.72	2.384
Length/Height (cm)	62	173	109.13	23.52
Hepatomegaly (cm)	1	10	4.41	2.085
Pre transfusion Hb (gm/dl)	3	10	6.21	1.654

On evaluation of parameters in the study group, the following findings were noted:

- Minimum Age was 1 year and maximum was 20 years with an average of 7.85 ±4.809 (Mean ±SD).
- Minimum weight was 8 kg and maximum was 45 with an average of 19.09 ±9.004 (Mean ±SD).
- Minimum Frequency of transfusions was 15 days and maximum was 60 days with an average of 27.13 ±9.710 (Mean ±SD).
- Minimum Age at which Transfusion started was 3 months and maximum was 36 months with an average of 9.87±8.048 (Mean ±SD).
- Minimum HC was 46 cm and maximum was 56 cm with an average of 49.72 ± 2.384 (Mean ±SD).

- Minimum Height was 62 cm and maximum was 173 with an average of 109.13 ±23.52(Mean ±SD). ¬
- Minimum Pre transfusion Hb was 3 gm/dl and maximum was 10 gm/dl with an average of 6.21 ± 1.654 (Mean ±SD).
- Minimum hepatomegaly was 1 cm and maximum was 10 cm with an average of 4.41 ± 1.654 (Mean ±SD)

Mutation analysis

Sequencing analysis of Exon 1 in the Hbb gene was done in 47 Thalassemia patients. Missense and synonymous mutations were found in 17 samples , Among them transversions were common followed by transition ,Among Transversions **(T-A Nucleotide change)** were most common. In the remaining samples, we did not found any mutations in the Exon1 region of the HBB gene. Here, we present an evaluation of the results of our study

Table 10 : HBB Mutation analysis

Sl.no	Sample No	Mutation Type	gDNA >NG_059281.1 (Nucleotide change)	cDNA Ref. ENST00000335295.4	ENSP00000333994.3 (AA Change)	Mutation-novel/reported	Variant (v)
1	1	Transversion	g.5342 T>A	c.162T>A	p.A54= (Alanine)	rs63751103 INDEL	Synonymous
2	1	Transversion	g.5357 T>A	c.177T>A	p.P59= (Proline)	rs63751103 INDEL	Synonymous
3	1	Transversion	g.5396 T>G	c.216T>G	p.F72L (Phenylalanine to Leucine)	rs754481448 SNP	Missense
4	2	Transversion	g.5395 T>G	c.215T>G	p.F72C (Phenylalanine to Cysteine)	rs1554917888 INDEL	Missense
5	6	Transversion	g.5357 T>A	c.177T>A	p.P59= (Proline)	rs63751103 INDEL	Synonymous

6	7	Transversion	g.5291 T>A	c.111T>A	p.V19P (Valine to Proline)	rs6375053 2 INDEL	Missense
7	9	Transition	g.5350 G>A	c.170G>A	p.G57D (Glycine to Aspartic acid)	rs6375110 3 INDEL	Missense
8	9	Transversion	g.5357 T>A	c.177 T>A	p.P59= (Proline)	rs6375110 3 INDEL	Synonymous
9	9	Transition	g.5396 T>G	c.216 T>G	p.F72L (Phenylalanin e to Leucine)	rs1554917 888 INDE L	Missense
10	10	Transversion	g.5344 T>A	c.164 T>A	p.V55D (Valine to Aspartic acid)	rs2818645 83 INDEL	Missense
11	10	Transversion	g.5345 T>A	c.165 T>A	p.V55= (Valine)	rs2818645 83 INDEL	Synonymous
12	10	Transversion	g.5361 G>C	c.181 G>C	p.V61L (Valine to leucine)	rs6375110 3 INDEL	Missense
13	10	Transversion	g.5367 G>C	c.187 G>C	p.A63P (Alanine to Phenylalanine)	rs3493345 5 SNP	Missense
14	12	Transversion	g.5362T>A	C.182T>A	p.V61D (Valine to Aspartic acid)	rs3393177 9	Missense v
15	16	Transversion	g.5091C>A	C.41C>A	p.A14D (Alanine to Aspartic acid)	rs3520374 7	Missense v
16	16	Transition	g.5430G>C	C.250G>C	p.G84R (Glycine to Arginine)	rs3393038 5	Missense v
17	17	Transversion	g.5373G>C	C.193G>C	p.G65R (Glycine to Arginine)	rs3610797 7	Missense v
18	17	Transversion	g.5394T>G	C.214T>G	p.F72V (phenylalanin e to Valine)	rs7807591 63	Missense v
19	18	Transversion	g.5374G>C	C.194G>C	p.G65D (Glycine to Aspartic acid)	rs3610797 7 INDEL	Missense v
20	18	Transversion	g.5382G>C	C.202G>C	p.V68L (Valine to Leucine)	rs3600892 2 SNP	Missense v
21	19	Transversion	g.5284T>A	C.104T>A	p.V35D (Valine to Aspartic acid)	rs1135101 SNP	Missense v

23	20	Transversion	g.5362T>A	C.182T>A	p.V61D (Valine to Aspartic acid)	rs3393177 9 SNP	Missense v
24	20	Transition	g.5375C>T	C.195C>T	p.G65= (Glycine)	CDO2325 9 DELETIO N	synonymous mutation
25	20	Transition	g.5383T>C	C.203T>C	p.V68A (Valine to Alanine)	rs3391834 3 SNP	Missense v
26	20	Transversion	g.5395T>G	C.215T>G	p.F72C (Phenylalanin e to Cysteine)	rs3436253 7 SNP	Missense v
27	20	Transversion	g.5407T>G	C.227T>G	p.L76R (Leucine to Arginine)	rs3545209 8 DEL	Missense v
28	24	Transversion	g.5334T>G	c.164T>G	p.V55G (Valine to Glycine)	rs2818645 83	Missense v
29	24	Transversion	g.5374G>C	c.194G>C	p.G65A (Glycine to Alanine)	rs3610797 7	Missense v
30	25	Transversion	g.5334T>A	c.164T>A	p.V55D (Valine to Aspartic acid)	rs2818645 83	Missense v
31	25	Transversion	g.5367G>C	c.187G>C	p.A63P (Alanine to Proline)	rs3393177 9 SNP	Missense v
32	26	Transversion	g.5347T>G	c.167T>G	p.M56R (Methionine to Arginine)	rs1564875 331 INDEL	Missense v
33	31	Transversion	g.5286T>A	c.106T>A	p.P36N (Proline to Aspargine)	rs3538989 5 DELETIO N	Missense v
34	31	Transversion	g.5342T>G	c.162T>G	p.A54= (Alanine)	rs2818645 83 INDEL	Synonymous
35	31	Transition	g.5373G>A	c.193G>A	p.G65* (Glycine)	rs3610797 7 INDEL	Missense v
36	31	Transversion	g.5378G>C	c.198G>C	p.K66N (Lysine to Aspargine)	rs3574796 1 SNP	Missense v
37	32	Transversion	g.5291T>A	c.111T>A	p.P37= (Phenylalanin e)	rs6375053 2 INDEL	Synonymous

The present study has found mutations in 17 thalassemia children of total 47 children , T>A type mutation is seen in most of our children followed by T>G, G>C, C>A and C>T in the exon 1 region of HBB gene.

Among 17 thalassemia children who have mutations in there Hbb gene , 10 were males and 7 were females . Consaguinous history was present in 11 children, 7 patients had massive splenomegaly and splenectomy was done in two children. Pretransfusion Hb was less than 5gm/dl in all children

Hepatomegaly was present in all children.Ejection systolic murmur was seen in 2 children and stunting was seen in 7 children .

All children had hemolytic facies picture and were on iron chelation therapy

DISCUSSION

Although several causative mutations have been linked to be associated with beta thalassemia major , the spectrum of mutations and their frequency in most populations usually consist of a few number of common variants. Several mutations were found in the HBB gene exon 1 region of the sequences analysed.

The majority of them were Missense mutations. Among these mutations Transversions were more common followed by transitions.T >A type mutations were common followed by T>G type mutation. T>A type mutations were observed at positions 177,164, and111 in most sample sequences. T>G type mutations at positions 215 and 216, The low frequencies of C>A and C>T allelic forms were observed, however the high frequency of T>A type allelic form was found to be equally distributed throughout all of the analysed subject sequences .In this study T>A Type missense mutations were common compared to other studies , where G>C Type Mutations were common

A Study conducted by Monalisha *et al.* [67] showed that six mutations account for 90-94% of beta thalassemia mutations in India which includes 619 bp deletion at the 3' end of the -globin gene, IVS 1-5 (G->C), IVS 1-1 (G-T), Codon 8/9 and Codon 41/42, and nonsense codon 15. The most prevalent mutation is IVS-1-5. In our study we observed missense mutations T>A type were observed at positions 177,164, and111 in most sample sequences, T>G type mutations at positions 215 and 216 in the exon 1 of HBB gene.

A study by Panigrahi I *et.al.,*[65] IVS 1-5 (G >C) is the most prevalent mutation in the Indian population, with a high frequency of IVS 1-5 (G->C) (72%) reported in the Eastern part of India, followed by an 11% prevalence of codon 41/42 mutation.

Sinha S. *et.al.,*[66] reported that the prevalence of IVS 1-5 (G->C) ranged from 44.8% in the North to 71.4% in the East region of India, where the prevalence rate of IVS 1-5 (G->C) was 54.7% and the prevalence rate of codon 41/42 was 6.1%.We observed T>A type mutation in the most of our cases followed by T>G, G>C, C>A and C>T in the exon 1.

Research work done by Kulkarni GD *et.al,* [5] got 11 β-thalassemia variants, the most prevalent are IVSII-16 G>C, IVSI-5 G>C, IVSII-74 T>G, codon 3 (T>C), and Poly A site (T>C).We found five type mutations which are in the coding region, which are going to effect protein structural changes T>A, T>G, G>C, C>A and C>T. The present study does not targeted the intronic variant that was the limitation of our study

The present study focussed on Exon 1 region of the Hbb gene , unlike other studies which focussed on full length of Hbb gene.This study does not targeted the intronic variant that was the limitation of our study

CONCLUSION

Mutational analysis of Exon 1 in the HBB gene revealed some common mutations. Among these mutations , missense mutations were more common .Transversions (T-A Nucleotide change) were found in high percentage .

Mutations can bring about a change in codon sequences which altered the protein production. Studies suggested that there is a need to maintain a primary prevention program to analyze mutation, and sequence variations at the molecular level. It can help to overcome many genetic disorders.

By conducting awareness programs , carrier screening and beta thalassaemia screening in high risk couples, it is possible to reduce the occurrence of this inherited disease.

Children with inherited conditions like beta thalassemia major are a burden to the family and society, it is crucial to reduce the prevalence of such diseases.

Genetic counselling and prenatal beta thalassaemia diagnosis might be successfully established with the use of mutational pattern research, which would lessen the burden of this disease on society.

With some preliminary important actions and measures, the identification of a mutation in the HBB gene will reduce health disparities in an already vulnerable population.

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ANNEXURE – I

ETHICAL CLEARANCE CERTIFICATE

B.L.D.E. (DEEMED TO BE UNIVERSITY) IEC/00.09/2021 Date-22/01/2021

(Declared vide notification No. F.9-37/2007-U.3 (A) Dated. 29-2-2008 of the MHRD, Government of India under Section 3 of the UGC Act, 1956)

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Institutional ethical committee of this college met on 11-01-2021 at 11-00 am to scrutinize the synopsis of Postgraduate students of this college from Ethical Clearance point of view. After scrutiny the following original/corrected and revised version synopsis of the Thesis has been accorded Ethical Clearance

Title: A study of Mutational analysis of Hemoglobin beta gene (HBB) in children and adolescents with Beta Thalassemia major.

Name of PG student: Dr K.Sharath Kumar , Department of Paediatrics

Name of Guide/Co-investigator: Dr M.M.Patil, Professor of Paediatrics

Co/Guide : Dr R S Bulagouda, Professor & HOD of Anatomy

DR .S.V.PATIL
CHAIRMAN, IEC
Institutional Ethical Committee
B L D E (Deemed to be University)
Shri B.M. Patil Medical College,
VIJAYAPUR-586103 (Karnataka)

Following documents were placed before Ethical Committee for Scrutinization:

1. Copy of Synopsis / Research project
2. Copy of informed consent form
3. Any other relevant documents.

10

ANNEXURE 2

RESEARCH INFORMED CONSENT FORM

BLDE(DEEMED TO BE UNIVERSITY) Shri B.M PATIL Medical

College, Hospital & Research Centre,

Vijayapur-586103.

TITLE OF THE PROJECT : A STUDY OF MUTATIONAL ANALYSIS OF HEMOGLOBIN BETA GENE (HBB) IN CHILDREN AND ADOLESCENTS WITH BETA THALASSEMIA MAJOR

GUIDE : DR M.M PATIL , MD
PROFESSOR
DEPARTMENT OF PEDIATRICS.

PG STUDENT : DR K SHARATH KUMAR

RISK AND DISCOMFORTS:

None

BENEFITS:

I understand that my participation in the study will have no direct benefit to me other than the potential benefit of the research and education.

CONFIDENTIALITY:

I understand that the medical information produced by this study will become a part of hospital records and will be subject to the confidentiality. Information of sensitive personal nature will not be part of the medical record, but will be stored in the investigations research file.

If the data are used for publication in the medical literature or for teaching purpose, no name will be used and other identifiers such as photographs will be used only with special written permission. I understand that I may see the photograph before giving the permission.

REQUEST FOR MORE INFORMATION:

I understand that I may ask more questions about the study at any time; Dr.K SHARATH KUMAR at the department of Pediatrics is available to answer my questions or concerns. I understand that I will be informed of any significant new findings discovered during the course of the study, which might influence my continued participation. A copy of this consent form will be given to me to keep for careful reading.

REFUSAL FOR WITHDRAWAL OF PARTICIPATION:

I understand that my participation is voluntary and that I may refuse to participate or may withdraw consent and discontinue participation in the study at any time without prejudice. I also understand that Dr. M.M PATIL may terminate my

participation in the study after he has explained the reasons for doing so.

INJURY STATEMENT:

I understand that in the unlikely event of injury to child resulting directly from childs participation in this study, if such injury were reported promptly, the appropriate treatment would be available to the child . But, no further compensation would be provided by the hospital. I understand that by my agreements to participate in this study and not waiving any of my legal rights.

I have explained to_____the purpose of the research, the procedures required and the possible risks to the best of my ability.

_____ _____

Dr. K SHARATH KUMAR Date

(Investigator)

PARENTS / GUARDIAN CONSENT STATEMENT:

We confirm that DR K SHARATH KUMAR is doing "**A STUDY OF MUTATIONAL ANALYSIS OF HEMOGLOBIN BETA GENE (HBB) IN CHILDREN AND ADOLESCENTS WITH BETA THALASSEMIA MAJOR "** Dr.K SHARATH KUMAR, has explained to us the purpose of research and the study procedure. We are willing to give as much as information required for the study and consent for investigations and the possible discomforts as well as benefits. We have been explained all the above in detail in our own language and we understand the same. Therefore we agree to give consent for child's participate as a subject in this research project.

_____ _____

(Parents / Guardian) Date

_____ _____

(Witness to signature) Date

ANNEXURE – III

PROFORMA

Name –

IP no –

Age-

DOB -

weight -

Sex –

Address –

Frequency of transfusion:

Age at which transfusion started :

Months of transfusion :

Total number of transfusions :

GENERAL PHYSICAL EXAMINATION:

weight ..... Kg

HR :

RR :

BP:

HC :

LENGTH/HEIGHT:

HEIGHT FOR AGE:

WEIGHT FOR HEIGHT:

SYSTEMIC EXAMINATION:

CVS :

RESPIRATORY SYSTEM:

GASTRO – INTESTINAL SYSTEM:

SPLEENOMEGALY:

HEPATOMEGALY:

CNS:

Pre transfusion Hb :

Desirox : YES/NO

MUTATION PRESENT - YES/ NO

Master chart

	Name	Age (yrs)	Sex	Weight (kg)	Consanguineous stat	Interval between T	Age at which transfusion started (years)	Total Number of Tran	HC (cm)	Lengthheight (cm)	Height for Age (centi	weight for age (centile	CYS (MURMURS)	Spleen (Grades)	Hepatomegaly (cm)	Pre transfusion Hb (c	On deferasirox	Mutations
2	Kushi	5	F	15	YES	1	1	45	52	109	50th	10th	No	Mild	4	7.5	YES	Missense
3	multana	8	M	18	YES	1	3	60	51	106	<3rd	3rd	No	Massive	6	6.2	YES	Nc
4	Baby archana	6	F	15	NO	0.5	0.8	40	48	103	10th-25th	3rd-10th	No	No		6.7	YES	Missense
5	Dhaneshvari	15	F	35	YES	0.5	2	144	52	143	3rd-10th	3rd-10th	No	Spleenectomy	3	6.7	YES	Nc
6	Master Samarth	8	M	22	NO	1	0.6	50	52	111	3rd	25th	No	No		6.5	YES	Nc
7	Suraksha	9	F	17	YES	1	2.5	76	52	111	<3rd	10th-25th	Ejection systolic	Spleenectomy	5	3.1	YES	Nc
8	shilpa	11	F	24	YES	1	0.8	120	56	122	,3rd	3rd	Ejection systolic	No		4.8	YES	Nc
9	ASMITA	5	F	17	YES	1	0.3	48	49	106	25th-50th	10th-25th	No	No	2	6.3	YES	Missense
10	Manjunath	3	M	14	YES	0.5	0.3	26	51	95	50th	50th-75th	No	No	1	8.3	YES	Nc
11	Baby Nijanagouda	3	M	14	NO	1	0.3	24	48	90	5th-10th	50th-75th	No	No	7	6.9	YES	Missense
12	VEERESH	2	M	10	YES	2	0.3	20	48	86	75th	25th-75th	No	No	1	6.8	YES	Nc
13	pramod	4	M	15	YES	2	3	12	47	90	50th-75th	25th-50th	No	Moderate	5	7.2	YES	Nc
14	Paigambar	10	M	20	NO	1	0.3	108	47	124	10th-25th	<3rd	Ejection systolic	No	5	3.3	YES	Nc
15	Varsha	10	F	15	YES	1	6	50	49	109	<3rd	<3rd	No	No	10	6	YES	Nc
16	VIJAY KUMAR	15	M	30	YES	0.5	2	144	52	142	<3rd	3rd-10th	No	Spleenectomy	8	6.2	YES	Nc
17	SUNIL	10	M	25	NO	1	0.4	108	50	128	10th-25th	25th-50th	No	Spleenectomy	10	10	YES	Nc
18	MARIYAM	10	F	10	NO	1	0.8	24	46	80	<3rd	<3rd	No	No	3	10	No	Nc
19	satish kumar	13	M	25	YES	0.5	0.5	121	52	133	<3rd	3rd	No	Spleenectomy	5	6.3	YES	Nc
20	AFSANA	10	F	20	NO	1	1.6	96	50	117	<3rd	3rd-10th	Ejection systolic	Massive	5	2.9	YES	Missense
21	sinchana	3	F	14	YES	1	0.5	24	49	90	3rd-10th	50th-75th	No	No	4	8	YES	Nc
22	vasudeva	6	M	15	YES	1	0.4	60	49	104	3rd	3rd-10th	No	No	2	6.2	YES	Missense
23	Mallikarjun	2	M	10	YES	1	0.9	10	48	80	<3rd	3r-10th	No	No		8.6	No	Nc
24	SHIVANANDA	5	M	15	YES	1	0.9	48	48.5	109	50th-75th	10th	No	No	2	8.6	YES	Missense
25	mahboob	19	M	42	NO	0.5	0.8	200	52	155	10th-25th	25th-50th	No	Moderate	5	6.7	YES	Nc
26	Sanjana	15	F	40	NO	0.5	1.8	156	54	152	25th-50th	10th-25th	No	Moderate	3	6.7	YES	Synonymous
27	NOORJAN	5	F	14	YES	0.5	0.5	48	50	62	<3rd	10th	No	No	3	6.2	YES	Nc
28	Ganesh Madar	3	M	10	NO	1	0.6	24	46	82	<3rd	<3rd	No	Mild	6	6.8	YES	Missense
29	PREETAM	11	M	22	YES	0.5	0.5	120	46	120	<3rd	<3rd	No	Severe	4	5.9	YES	Nc
30	MASTER SREYAS N	5	M	13	NO	1	1	40	48	90	<3rd	<3rd	Ejection systolic	Massive	5	3	YES	Missense
31	YUVRAJ CHAVAN	5	M	15	NO	1	1	40	48	100	3rd - 10 th	25th	Ejection systolic	No	5	4.1	YES	Missense
32	AKASH PRAKASH	20	M	45	NO	0.5	0.7	200	52	173	90th	25th	No	Spleenectomy	6	8.1	YES	Nc
33	SAKSHI	4	F	13	YES	0.5	1	36	46	90	<3rd	<3rd	No	No		6.1	YES	Nc
34	Tippanna	9	M	16	YES	1	0.5	90	48	114	<3rd	<3rd	No	No	5	6.2	YES	Nc
35	siddharth	5	M	17	YES	1	0.6	53	49	100	3rd-10th	50th	No	Moderate		6.5	YES	Missense
36	Bhagesh	6	M	15	YES	1	0.6	55	50	117	50th-75th	3rd-10th	No	No	4	5.9	YES	Nc
37	Akash	1	M	8	NO	1	0.6	6	48	70	50th-75th	25th-50th	No	Mild	2	5.2	No	Nc
38	LOKESH	6	M	15	NO	1	0.6	50	47	112	25th	3rd-10th	No	Moderate	4	7.9	YES	Nc
39	Bhumi Dundappa	10	F	26	YES	0.5	0.3	100	52	126	10th-25th	25th-50th	No	Moderate	5	5.4	YES	Nc
40	Yogini	3	F	12	YES	1	0.6	28	47	95	50th	3rd-10th	No	No	2	4.6	No	Missense
41	Sangayya	4	M	10	YES	1	0.6	36	52	80	<3rd	<3rd	No	No	4	4.2	YES	Missense
42	Kashmisab	12	M	25	NO	0.5	1	100	52	125	3rd	3rd	No	Moderate	4	6.2	YES	Nc
43	Danesh	11	M	25	YES	1	0.8	100	51	128	3rd-10th	10th	No	Moderate	5	5.5	YES	Missense
44	Ashvini	4	F	12	YES	1	0.8	36	50	90	3rd-10th	<3rd	No	No	3	5.5	YES	Synonymous
45	Mahek	9	F	20	NO	1	1	90	54	112	3rd	<3rd	No	No	3	5.8	YES	Nc
46	sukadev	4	M	12	NO	1	0.8	36	48	95	3rd-10th	<3rd	Ejection systolic	No	3	4.9	YES	Missense
47	Vinay	5	M	15	YES	1	0.7	48	49	100	3rd	10th	No	No	4	5.2	YES	Nc
48	Mass Irfan	20	M	40	YES	1	1	200	52	155	<3rd	3rd-10th	No	Spleenectomy	8	5.5	YES	Nc