DISSERTATION

On

A STUDY OF IRON OVERLOAD USING ORAL EXFOLIATIVE CYTOLOGY IN BETA THALASSEMIA MAJOR PATIENTS

submitted in partial fulfillment of

requirements for

MD DEGREE EXAMINATION

BRANCH-III PATHOLOGY

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SALEM

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Thalassemia major or Cooleys anemia causes severe transfusion dependent anemia3. The heterozygous state knows as beta thalassemia trait,

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thalassemia major) causes severe, transfusion-dependent anemia. In the heterozygous state, the beta thalassemia trait (

causes milder form of disease. Thalassemia intermedia presents as milder form than beta thalassemia major but more severe than the beta thalassemia trait4. Beta thalassemia major is characterized by severe anemia, hepatosplenomegaly, growth retardation, endocrine dysfunction and skeletal changes due to ineffective erythropoiesis which leads to bone marrow expansion. Many patients with beta thalassemia requires regular packed red blood cell transfusion to overcome the effects of anemia3. Transfusion therapy reduces compensatory bone marrow expansion and permitting normal or near normal growth and development6.

These patients undergoes repeated blood transfusions results in iron overload, which is a common complication encountered in these patients who do not receive effective iron chelation therapy. It is mainly because of increased absorption of iron in gastrointestinal tract and the body has no effective means of excreting the excess iron7. Iron is an essential nutrient for every human cells. It plays role in cellular metabolism and erythropoietic function and becomes highly toxic in excess8. Normally, an individual requires 1-2 mg/ L of elemental iron physiologically, and it is available in daily diet. On each unit of packed red cell contain 200-300mg iron. On repeated blood transfusion leads to increased accumulation of iron in the tissue parenchyma causing multiorgan failure and signs of iron overload usually seen after 10 -12 transfusions7. The iron overload on the body can be estimated in the blood by measuring serum ferritin, serum iron and total iron binding capacity. Liver biopsy is a widely followed definitive test for assessing the parenchymal iron overload. Bone marrow biopsy is indicated in iron overload associated with reticuloendothelial system. These procedures are invasive and may not be feasible in every occasions9. A clear clinical need is evident for

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CONTENTS

S.NO:	TOPICS	Page No.
1.	INTRODUCTION	1
2.	AIM AND OBJECTIVES	4
3.	REVIEW OF LITERATURE	5
4.	MATERIALS AND METHODS	54
5.	OBSERVATION AND RESULTS	59
6.	DISCUSSION	69
7.	SUMMARY	75
8.	CONCLUSION	77
9.	COLOUR PLATES	
	BIBLIOGRAPHY	
	ANNEXURES	
	MASTER CHART	

LIST OF FIGURES

F.No.	TITLE	PAGE.NO.
1.	Genetic control of Hemoglobin synthesis	11
2.	Mutations in Beta Thalassemia	13
3.	Splicing mutation in Beta Thalassemia	14
4.	Pathogenesis of Beta Thalassemia	18
5.	Imaging feature of Beta thalassemia	24
6.	Normal Iron metabolism	27
7.	Pathogenesis of iron overload in Beta Thalassemia	29
8.9	Peripheral smear of Beta Thalassemia major	38
10.	Acid Elution Test	40
11.	Naked Eye Single Tube Red cell Osmotic Fragility TEST	41
12.	Haemoglobin Electrophoresis of Beta Thalassemia major	42
13.	High performance Liquid Chromatography of Beta Thalassemia major	43
`14-19	Grading of iron deposition in buccal mucosa	51-53

LIST OF TABLES

S.No.	TITLE	PAGE.NO.
1.	Comparison of age with groups	61
2.	Gender distribution	62
3.	Group wise gender distribution	63
4.	Comparison between Perl stain test with groups	66
5.	Comparison of serum ferritin with groups	67
6.	Comparison of Perls stain positivity with similar studies	72
7.	Comparison of mean serum ferritin level with similar studies	73

LIST OF CHARTS

S.No.	TITLE	PAGE.NO.
1.	Distribution of study group	59
2.	Age distribution in study group	60
3.	Comparison of age with groups	61
4.	Gender distribution	62
5.	Group wise gender distribution	63
6.	Distribution of frequency of blood transfusion	64
7.	Agewise distribution of blood transfusion among study group	65
8.	Comparison between Perl stain test with groups	66
9.	Comparison of serum ferritin with groups	67
10.	Agewise distribution of mean serum ferritin level among study group	68

LIST OF COLOUR PLATES

IMAGE.No.	TITLE
1.2.3	Oral exfoliative squamous cells showing positive staining for Perls Prussian blue
4.	Method of obtaining buccal smear
5.	Method of smearing of the material in the microscopic glass slide
6.	Materials used in Perls Prussian blue stain

ABBREVATIONS

CGH	-	Comparative genomic hybridisation
COL1A1	-	Collagen Type 1 gene
GDF15	-	Growth differentiation factor 15
HPLC	-	High performance liquid chromatography
IGF1	-	Insulin like growth factor 1
LCR	-	Locus control region
LIC	-	Liver iron concentration
LPI	-	Labile plasma iron
LVDCC	-	L Type voltage dependent calcium 2+channel
MRI	-	Magnetic Resonance Imaging
NESTROFT	-	Naked eye single tube red cell osmotic fragility test
NTBI	-	Non transferrin bound iron
PCR	-	Polymerase chain reaction
ROS	-	Reactive oxygen species
SQUID	-	Super conducting quantum interference device
TWSG1	-	Twisted Gastrulation 1

A STUDY OF IRON OVERLOAD USING ORAL EXFOLIATIVE CYTOLOGY IN BETA THALASSEMIA MAJOR PATIENTS

INTRODUCTION

Thalassemia are group of inherited disorder of haemoglobin synthesis caused by genetic mutations that reduce or prevent the synthesis of either alpha or beta globin chain of haemoglobin[1]. Most forms are inherited in a autosomal recessive fashion from asymptomatic parents who are the gene carriers and also have one in four chances of having an affected child. The disease spectrum has variations from silent asymptomatic carriers to transfusion dependency for survival[2].

Thalassemia affects approximately 4 % of every 10,000 live birth throughout the world[4]. It is about 1.5 % of global population are carriers of beta thalassemia .

In the homozygous state of beta thalassemia gene results in severe or total suppression of beta chain synthesis, clinically characterized as Thalassemia major or Cooleys anemia causes severe transfusion dependent anemia[3]. The heterozygous state knows as beta thalassemia trait, causes milder form of disease. Thalassemia intermedia presents as milder form than beta thalassemia major but more severe than the beta thalassemia trait[4].

Beta thalassemia major is characterized by severe anemia, hepatosplenomegaly, growth retardation, endocrine dysfunction and skeletal changes due to ineffective erythropoiesis which leads to bone marrow expansion. Many patients with beta thalassemia requires regular packed red blood cell transfusion to overcome the effects of anemia[3].

Transfusion therapy reduces compensatory bone marrow expansion and permitting normal or near normal growth and development[6].

These patients undergoes repeated blood transfusions results in iron overload, which is a common complication encountered in these patients who do not receive effective iron chelation therapy. It is mainly because of increased absorption of iron in gastrointestinal tract and the body has no effective means of excreting the excess iron[7].

Iron is an essential nutrient for every human cells. It plays role in cellular metabolism and erythropoietic function and becomes highly toxic in excess[8].

Normally, an individual requires 1-2 mg/ L of elemental iron physiologically, and it is available in daily diet. On each unit of packed red cell contain 200-300mg iron. On repeated blood transfusion leads to increased accumulation of iron in the tissue parenchyma causing multiorgan failure and signs of iron overload usually seen after 10 -12 transfusions[7].

The iron overload on the body can be estimated in the blood by measuring serum ferritin, serum iron and total iron binding capacity. Liver biopsy is a widely followed definitive test for assessing the parenchymal iron overload. Bone marrow biopsy is indicated in iron overload associated with reticuloendothelial system. These procedures are invasive and may not be feasible in every occasions[9].

A clear clinical need is evident for quantitative, noninvasive, safe, accurate, and readily available means of measuring body storage iron to improve the diagnosis and management of patients with iron overload from such disorders as hereditary

hemochromatosis, thalassaemia major, sickle cell disease, aplastic anemia, and myelodysplasia among others[10].

A Non invasive test to asses iron overload is exfoliative cytology and the smears from buccal mucosa is stained with Perls Prussian blue. It is a simple, painless, bloodless and quick procedure.

This study is being carried out to demonstrate iron overload in patients with β-thalassemia major undergoing blood transfusions using Perl's Prussian blue reaction and compared with serum ferritin levels.

The staining kit consists of potassium ferrocyanide, which reacts with ferritin in the cells to form a blue colored compound. This blue colored compound is visible under light microscopic study as blue granules.

Hence, this study can be used to assess iron overload as a very simple, non-invasive procedure.

AIM AND OBJECTIVES

Aim:

To correlate iron overload in exfoliative squamous cells from buccal mucosa with serum ferritin level among Beta Thalassemia Major patient.

Objective :

- To analyze iron overload by examining exfoliated squamous cells from buccal mucosa using Perls Prussian blue method.
- To analyze serum ferritin level

REVIEW OF LITERATURE

CLASSIFICATION OF THALASSEMIA

β–Thalassemias		
GENOTYPE	**ABNORMAL HEMOGLOBIN**	**CLINICAL SEVERITY**
β-Thalassemia major	Homozygous β-thalassemia (β0/β0, β+/β+, β0/β+)	Severe requires blood transfusions
β-Thalassemia intermedia	(β0/β+, β+/β+, β0/β, β+/β)	Severe but does not require regular blood transfusions
β-Thalassemia minor	Heterozygous β-thalassemia (β0/β, β+/β)	Asymptomatic with mild or absent anemia red cell abnormalities seen
δβ0/thalassaemia	Homozygous δβ0/δβ0	Similar to Thalassaemia intermedia
δβ0/thalassaemia	Heterozygous δβ0/δβ	Similar to Thalassaemia minor 5-20%
HbLepore	Homozygous Lepore/Lepore	Similar to Thalassaemia major
HbLepore	HeterozygousHbLepore /β	Similar to Thalassaemia minor
α-Thalassemias		
Silent carrier	–/α α/α	Asymptomatic no red cell abnormality
α-Thalassemia trait	–/– α/α OR–/α –/α	Asymptomatic like β-thalassemia minor
HbH Disease	–/– – /α	Severe resembles *β* thalassemia
Hydropes fetalis	–/– –/–	Lethal in utero without transfusion

BASED ON REQUIREMENT OF BLOOD TRANSFUSION CLASSIFIED INTO

I.NON TRANSFUSION DEPENDENT THALASSEMIA

1. Occasional transfusion required –
   - β Thalassemia Intermedia
   - β Thalassemia Trait
   - α Thalassemia Trait
   - HbC/ β Thalassemia
   - Mild HbE/β Thalassemia
   - HbH with β Thalassemia Trait
   - Homozygous HbE/C Trait
2. Intermittent transfusion required –
   - β Thalassemia Intermedia
   - Deletional HbH
   - Non deletional HbH
   - Moderate HbE/β Thalassemia

II.TRANSFUSION DEPENDENT THALASSEMIA

- Thalassemia Major
- Non deletional HbH
- Survived Hb Barts hydrops
- Severe HbE/β Thalassemia

HISTORY

In olden era, a disease known as anemia splenic infuntum that included several conditions but could not well distinguished from one another . These children were born normal and grew well until 6 month of age and developed abdominal distention, pallor and facial bone deformities giving the children a Mongolian appearance.

- In 1925, Cooley and Lee observed the disease in Italian and Greek childrens who were coming from malarial area[11].
- In 1932, George H Whipple and William L Bradford published the pathological finding in this disease[4].
- In 1940, Wintrobe reported the presence of familial hematopoietic disorders in adults of Italian origin[12].
- In 1943 & 1947 – Silvestroni and Bianco defined the hematologic, clinical and epidermiologic characteristics of thalassemia minor and its relationship with thalassemia major[12]
- In 1949, J.B.S. Haldane suggested that thalassemia had reached its high frequency in tropical regions because heterozygotes are protected against malaria.
- In 1968, Cligg discussed the unbalanced chain synthesis of thalassemia[16].
- In 1977, R.B Thampson reported that β Thalassemia are due to genetically determined defect of β chain synthesis[17].

The word thalassemia comes from Greek word thalasea means " sea " are endemic in the Mediterranean basin, Middle east, Tropical African, Indian subcontinents and Asia[13].

The Thalassemia syndrome are heterogenous group of disorders of adult haemoglobin only in 1930 and 1940 it become apparent that this disease first described with severe anemia, bone changes and splenomegaly results from a defective synthesis of either alpha globin or beta globin chain of haemoglobin[14].

The Beta thalassemia disorders are due to mutations that impair the normal process of beta globin chain production. Normally two globin chains are encoded by a single beta globin gene on chromosome 11[15].

In the homozygous state (β^+ / β^+ ,β^+/ β^0, β^0/ β^0) cause severe transfusion dependent anemia called β thalassemia major . In the heterozygous state (β^+/ β^0) causes mild to moderate microcytic anemia called as β thalassemia trait. Patients with heterozygosity are some what resistant to malaria.

EPIDERMIOLOGY

The inheritance of thalassemic mutations represents a significant public health problem in a majority of nations (71% of 229 countries). It is estimated that upto 1.5% of the entire population may carry a genetic mutation affecting hemoglobin production and about 270 million persons and approximately 1% of couples worldwide are at risk for having children with an haemoglobin disorder.

β – Thalassemia are widely distributed in Mediterranean population, Middle east, part of India, Pakistan and Southeast Asia. The disease is common in Tajikistan,

Turkmenistan, Kyrgyzstan, and the People's Republic of China. Because of extensive migration from areas of high gene frequency such as Mediterranean regions, Africa , Asia to America. The alpha and beta thalassemia genes and the clinical disease are relatively common in North and South America[4].

β-thalassemia occurs sporadically in all racial groups and has been observed in the homozygous state in persons of pure Anglo-Saxon heritage and thus the patient's racial background does not hinders the diagnosis.

The *δβ*-thalassemias have been observed sporadically in many racial groups although no high-frequency populations have been seen. Similarly the hemoglobin Lepore syndromes have been found in many populations but with the exceptions of central Italy, Western Europe, and parts of Spain and Portugal.

Thalassemia affects approximately 4 % of every 10,000 live birth throughout the world[4]. It is about 1.5 % of global population are carriers of beta thalassemia [18].

The estimated annual worldwide number of births is 22 989 for *β*-thalassemia major, 9568 for HbH disease (the intermediate form of α-thalassemia), and 19,128 for HbE/*β*-thalassemia,5183 for Hb Bart hydrops fetalis syndrome.

β thalassemia prevalence in America(0- 3%), Eastern Mediterranean (2 -18 %), Europe (0- 19%), South East Asia (0 -11%), Sub Saharan African(0-12 %), Pakistan (5 -7%), Iran 7% . The highest incidence are seen in Cyprus 14 % , Sardinia 12%, South East Asia and the prevalence varies among different countries around the world[4] .

The thalassemia genes may have resulted from drift, independent mutation and provide the overall basis for their world distribution.

The high gene frequency of beta thalassemia in these region shows evidence for protection of individuals with beta thalassemia against Plasmodium falciparum malaria[4].

PREVALENCE OF BETA THALASSEMIA IN INDIA

Beta thalassemia prevalence across India varies between 5 – 15%. There are 25% chances of developing homozygous β thalassemia child for a β thalassemia carrier couple[19].

Ten percent of the total world thalassemia are born in India every year. Around 180 different mutations which leads to beta thalassemia have been identified .

In India the frequency of thalassemia is increased due to the consanguinity and endogamous mating, it is assumed that the tribal communities in India are facing these problem in large scale[20].

In India carrier frequency of β thalassemia varies from 4.05 to 20% and birth incidence of β thalassemia homozygote to be 11,316 per year in India. Northern, Western and Eastern parts of India have a greater frequency, Gujarat has highest frequency of beta thalassemia trait (10 – 15%), followed by Calcutta(10%), Punjab (6.5%),Delhi(5.5%), Tamilnadu(4%), Kerala (0.6%).

Based on the population distribution the higher number of frequency of beta thalassemia is seen in the group of people Bhanishali(15%), Lohana(13%), Sindh(8%), Assam(5%), Bangalan(3.7%) and Ahan (1%).

GENETIC AND MOLECULAR PATHOLOGY

GENETIC CONTROL AND HEMOGLOBIN SYNTHESIS

The normal haemoglobin molecule is a tetramer of two alpha-like chains (alpha or beta) with two beta-like chains (beta, delta, gamma, or epsilon). Combinations of these chains produce six normal hemoglobins. Three are embryonic hemoglobins (Hb gower 1, Hb gower 2, Hb Portland 1&2) and two adult hemoglobins (Hb A, Hb A2). The alpha-like globin gene is located on chromosome16, and beta-like globin gene cluster is on chromosome11[1].

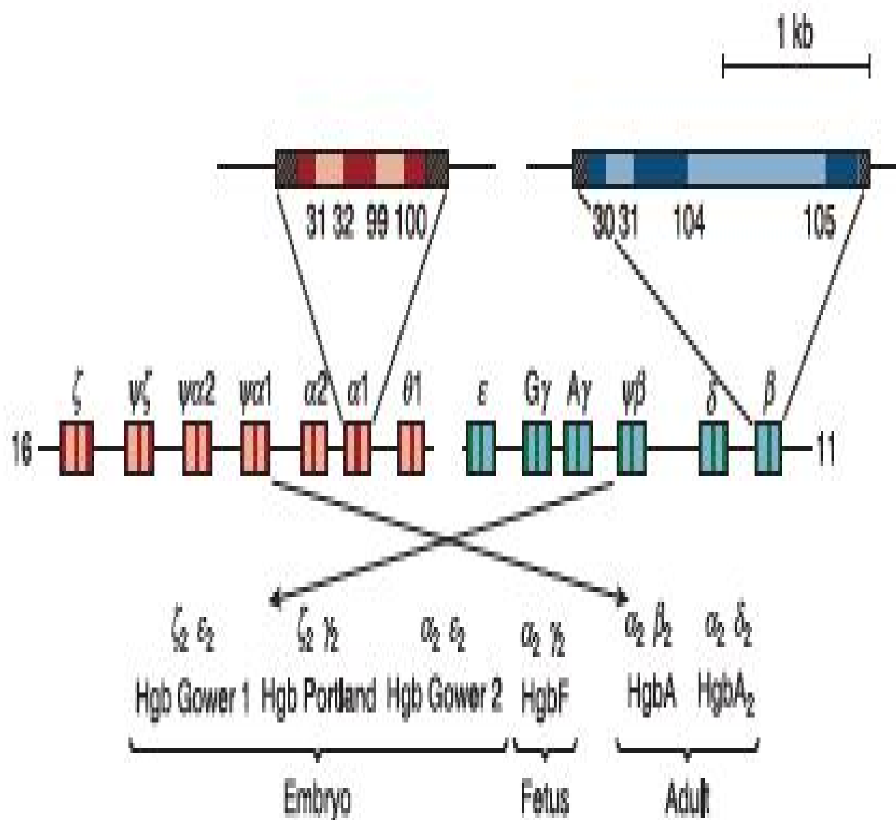

Figure 1 – Genetic control of Haemoglobin synthesis

The embryonic switch to fetal globin genes (*α* to *γ* and ζ to *α*) will start in the early pregnancy and is completed at 10 weeks of gestation then the fetal switch to adult globin genes (*γ* to *β*), which occurs during the perinatal period and these globin gene switches results in changes in the Hemoglobin composition [21].

When an individual inherits one cluster of five functional genes on chromosome 11 from the each parent ,genotype for normal *β* chain synthesis is designated as *β* / *β*. When two *α* -globin genes (*α* 1 and *α2*) are inherited on each chromosome 16,the normal genotype is designated as *αα* /*α α*[1].

CHANGES IN GLOBIN GENE EXPRESSION

The series of developmental switches is synchronized throughout the developing hematopoietic tissues. Some form of "time clock" is built into the hematopoietic stem cell at the chromosomal level, and the regulation appears to occur in a complex manner involving both developmental stage-specific *trans*-activating factors and the relative proximity of the different genes of the *β*-globin gene cluster to LCR(locus control region). KLF1 (erythroid Kruppel like factor), BCL11A and MYB a developmental stage–enriched protein, activates human *β*-globin gene expression and is involved in human *γ*- to *β*-globin gene switching[22].

GENETIC MUTATIONS IN THALASSEMIA

Nearly 200 different mutations have been described in patients with β-Thalassemia and related disorders[19]. Genetic defects that cause a decrease or absent production of a particular globin chain include single nucleotide or point mutations, small insertions or deletions or large deletions[1].

All mutations result in either the absence of the synthesis of β-globin chains [β0-thalassaemia] or a reduction in synthesis [β+-Thalassemia].

THALASSEMIA MUTATIONS IN INDIA

MUTATIONS	FREQUENCY
IVS 1-5(G → C)	48% - Commonest
619 bp deletion	18% - Sindhis, Gujaratis
IVS 1-1 (G → T)	9%
FR 41/42 (TCTT)	9%
FR 8/9 (+G)	5%
CODON 15 (G → A)	6%

The most common five mutations found in Indian subcontinent are IVSI-5 (G→C), Del 619,IVSI-1 (G→T),Fr 41-42(-TTCT) and Fr 8-9 (+G)[23]. In the Sind and Gujarati populations of Pakistan and India the common gene deletion is 619-bp deletion at the 3′ end of the *β* gene and it accounts for approximately 50 percent of *β*-thalassemia alleles[24,25].

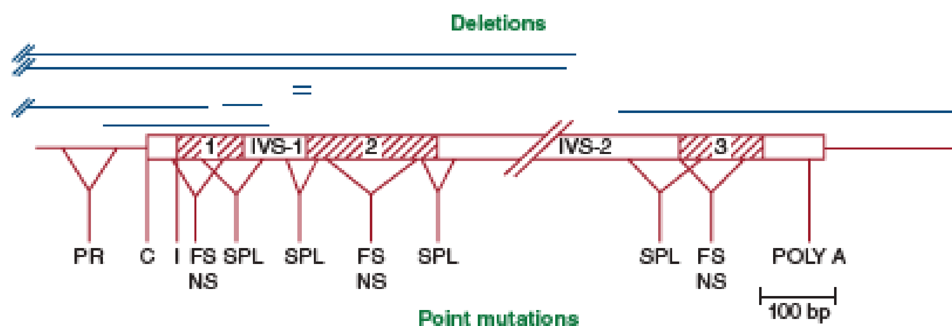

Figure 2: Mutations in Beta Thalassemia

In the Indian 619-bp deletion removes the 3′ end of the β gene but leaves the 5′ end intact. Many of the other deletions remove the 5′ end of the gene and leave the δ gene intact. Homozygotes for these deletions have $\beta 0$-thalassemia.

Heterozygotes for the Indian deletion have increased hemoglobin A2 and F levels similar to those seen in heterozygotes for the other common forms of β-thalassemia[4].

SPLICING MUTATIONS.

These are the most common cause of β^+-thalassemia. Most of these mutations are within introns, and few are located within exons[13].GT at the 5′ (donor) and AG at the 3′ (receptor) sites[4].Some of these mutations destroy the normal RNA splice junctions and completely prevent the production of normal β-globin mRNA, resulting in β^0-thalassemia.

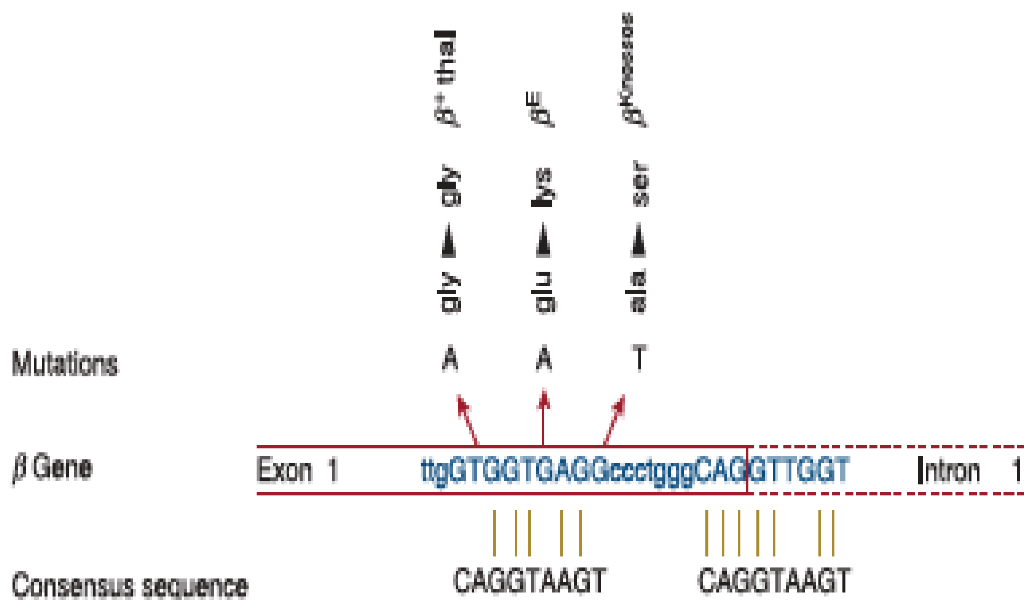

Figure 3 : Splicing mutation in Beta Thalassemia

In some "ectopic" splice site within an intron which leads to the flanking normal splice sites and both normal and abnormal splicing occurs and some normal β-globin mRNA is made, resulting in β^{+}-thalassemia[13].

Different varieties of *β*-thalassemia involve single-base substitutions within the consensus sequence of the IVS-1 donor site[4]. The substitution of the G in position 5 of IVS-1 by C or T results in severe *β*+-thalassemia[26].

Mutation IVS I-1 (G–T) causes the change in splice junction results in ineffective RNA processing. IVS I-5 (G–C) destroys consensus sequences around the splice junction which are important for splicing. Mutation at codon 15 (G–A) causes a nonsense codon, terminating the synthesis of beta-globin at a premature stage[23].

Another class of processing mutations involves the polyadenylation signal site AAUAAA in the 3′ untranslated region of *β*-globin mRNA, a T→C substitution in this sequence leads to one-tenth the normal amount of *β*-globin mRNA which results in severe *β*+-thalassemia phenotype[27].

PROMOTER REGION MUTATIONS

Several mutations are in or around the 5′flanking sequence of *β*-globin genes, they reduce binding of RNA polymerase reducing the rate of mRNA transcription to 20% to 30% and causes moderate decrease in *β*-globin chain causing *β*+-thalassemia phenotype[28].

5′-UTR Mutations

In the 50-nucleotide region, numerous mutations of single-base substitution and minor deletions have causing mild effects on gene transcription. Heterozygotes have normal or borderline red cell indices and HbA2 and compound heterozygotes, with severe β-thalassemia alleles usually have a mild phenotype.

In mutation at the β-globin gene mRNA capsite (Cap +1 ASC) in the homozygotes shows hematologic values consistent with thalassemia trait[29].

CHAIN TERMINATOR MUTATIONS.

These are the most common cause of β^0-thalassemia.There are two subtype mutations. In the most common type - stop codon within an exon and the other type, small insertions or deletions that shift the mRNA reading frames (frameshift mutation). Both block translation and prevent the synthesis of any functional β-globin[13].

Base substitutions that change an amino acid codon into a chain termination codon(nonsense mutations) prevent translation of the mRNA which result in β^0 thalassemia[30,31]. The codon 17 mutation is common in Southeast Asia[32,33]

The insertion or deletion of one, two, or four nucleotides in the coding region of the β-globin gene which disrupts the normal reading frame and results in translation of the mRNA, in the addition of anomalous amino acids until a termination codon is reached in the new readingframe.[28,29] Two mutations—the insertion of one nucleotide between codons 8 and9 and a deletion of four nucleotides in codons 41 and 42—are common in Asian Indians[34]

PATHOGENESIS

In beta thalassemia reduced or absent production of beta globin chain which diminishes hemoglobin synthesis and produces microcytic, hypochromic RBCs and unequal production of the alpha - or beta-globin chains causing an imbalance in the α/β chain ratio which leads to decreased survival of RBCs and their precursors[35,36].

The unpaired excess alpha chains forms as inclusion bodies and gets precipitated in the developing RBCs and this causes oxidative stress and damage to cellular membranes[37]. Excess α-chains oxidation results in the formation of hemichromes, whose basic structure composed of covalent binding of distal histidine E7 to the sixth coordination site of the heme iron.

Denatured α-chains and irreversible hemichromes precipitates as inclusion bodies which occurs during early differentiation and throughout erythroid maturation[38]. In the red cell membrane, α-Chain precipitation causes structural and functional alterations which causes changes in stability, deformability and red cell hydration.

Protein 4.1, a major component of the cytoskeleton, undergoes partial oxidation in β-thalassemia which results in defective capability in formation of the spectrin–protein 4.1–actin complex, which is essential in maintaining the cytoskeleton stability[39].

PATHOGENESIS OF BETA THALASSEMIA

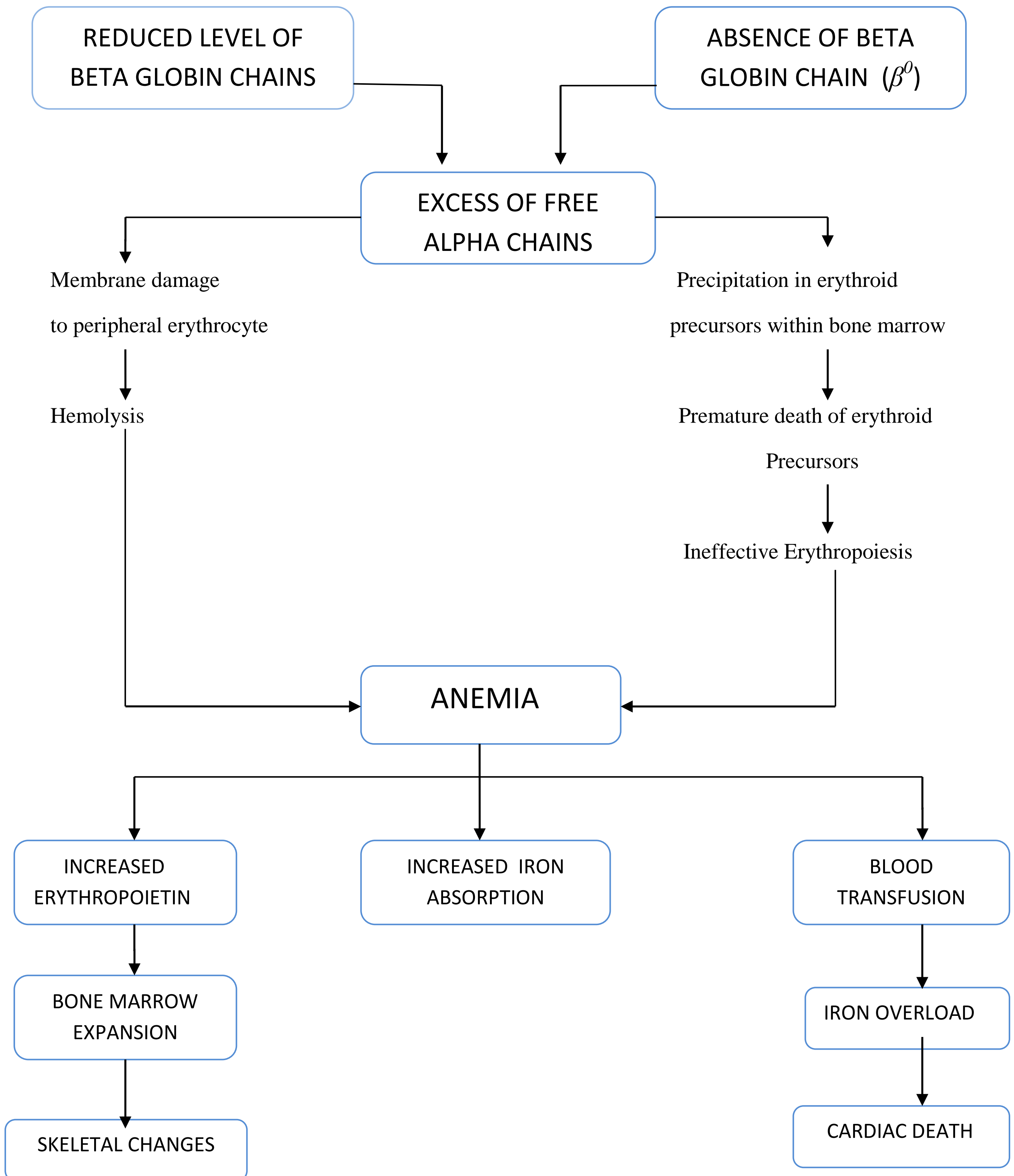

Figure 4 : Pathogenesis of Beta Thalassemia

In β-thalassemia, red blood cells loses K+, and stores Ca2+ and are dehydrated, resulting in altered deformability. The free α - chains are subjected to degradation and the formation of denatured α-globin protein, heme and free iron, which play a role in damaging erythroid precursors and red cell membranes.

Through Fenton reaction, the free iron generates reactive oxygen species, which causes lipid and protein peroxidation and results in damage of red cell membranes and intracellular organelles[40]. In the membrane of β-thalassemic red cells, elevated levels of iron are associated with denatured Hemoglobin and are thought to decrease red cell survival.[41] These features triggers the apoptosis and the apoptotic RBC precursors are subsequently phagocytized and destroyed in the bone marrow by activated macrophages. The premature death of RBC precursors in the bone marrow is called ineffective erythropoiesis[37].

Due to ineffective erythropoiesis the bone marrow attempts to produce RBCs but could not able to release sufficient viable cells into the circulation. The cells that are released into the periphery are laden with inclusion bodies and are rapidly sequestered and destroyed by macrophages in the spleen (extravascular hemolysis). This results in ineffective production and increased destruction of RBCs.

In beta-thalassemia major, due to severe uncompensated anemia stimulates an increase in erythropoietin production by the kidney and results in massive marrow erythroid hyperplasia and extra medullary hematopoiesis[36]. The expanding mass of red cell precursors erodes the bony cortex, impairs bone growth, and produces skeletal abnormalities.

Extramedullary hematopoiesis involves the liver, spleen, and lymph nodes, and in extreme cases produces extraosseous masses in the thorax, abdomen, and pelvis and as an serious complication of ineffective erythropoiesis causes increased absorption of iron in the gut by suppression of hepcidin level will results in secondary injury to parenchymal damage[13].

CLINICAL FEATURES

In beta-thalassemia, the child during fetal life and upto 6 months of age does not show any clinical manifestation because of Hb F is the predominant circulating hemoglobin. The disease manifest between 6 and 24 months of age, after completion of the gamma to beta switch.

To compensate for the decreased expression of the beta-globin gene, the gamma- and/or delta-globin genes are usually upregulated, but in beta-thalassemia major this increase is insufficient to correct the α/β chain imbalance[35,36].

Initial symptoms in beta thalassemia are child becomes progressively anemic and have feeding problems with recurrent diarrhoea, irritability, fever, hepatosplenomegaly and failure to thrive[42].

MAIN CLINICAL FEATURES ARE

- Bone deformities
- Osteoporosis
- Cholelithiasis
- Thromboembolic complications
- Jaundice

- Long term blood transfusion – causing iron overload and transfusion induced infections.

BONE DEFORMITIES

Inadequate red cell transfusions lead to the development of typical bone abnormalities, caused by increased erythropoiesis and expansion of the bonemarrow . The skull is large and deformed by frontal and posterior bossing with increase thickness in diploe. The outer and inner tables are thin and the trabeculae arranged in vertical striations resulting in a"hair-on-end" appearance[43].

There will be prominence of

- Malar eminence
- Mangoloid slant of eye
- Hypertrophy of maxilla leading to exposure of upper teeth[44].

Delayed pneumatization of the sinuses, depressed base of nose. Severe malocclusion with a rodent-like appearance is due to overgrowth of the maxilla. Due to the consequence of increased erythropoiesis causes expansion of metacarpal and metatarsal bones. The ribs are broad often with a "rib-within-rib" appearance and the vertebral bodies are square. The trabeculation of the medullary space gives the bones a mosaic pattern.

Premature fusion of the humeral and femoral epiphyseal lines due to shortening of long bones which resulting in causing stunned growth[45]. Extramedullary erythropoiesis gives rise to masses that protrude from bones where red marrow persists.

Overgrowth of vertebral bodies can cause cord compression and paraparesis. As a consequence of extramedullary marrow growing in the middle ear causes audiologic impairment and compressive optic neuropathy causes progressive visual loss [46].

OSTEOPOROSIS

The thalassemia patients have reduced bone mineral density and more susceptibility to fractures. The males are more commonly affected than the females, this represents an important complication causing morbidity in both sexes of adult patients.

Varietions in the genetic and acquired factors plays the role in the pathogenesis of osteoporosis in thalassemia major and it is multifactorial. Severe osteoporosis is associated with the polymorphism at the Sp1 site of the collagen type I gene(*COLIA1*).

Other factors that contributing to osteoporosis is ineffective hematopoiesis with progressive bone marrow expansion and exogenous factors such as endocrine dysfunction, iron overload and chelation therapy, vitamin deficiencies, and decreased physical activity.

Defective osteoblastic activity is the major pathogenetic mechanism for osteoporosis and there is evidence of increased osteoclast activation. It is suggests that the receptor activator of nuclear factor Ob ligand (RANKL)/osteoprotegerin (OPG) pathway mediates osteoclast proliferation in thalassemia and contributes to osteoporosis.

Bone pain of varying severity is a common complaint among adult patients and it is due to the expanded bone marrow with consequent pressure on the cortical bone. Back pain is sometimes associated with compression fractures and intervertebral disc degeneration. Osteoporosis is a progressive disease thus early detection, prevention, and treatment are essential for effective control of this potentially debilitating condition.

CHOLELITHIASIS

Gallstones may occur in thalassemia but the frequency is variable, depending on the transfusion regimens and amount of inefficient erythropoiesis, hemolysis, timing of splenectomy. It is associated with presence of the (TA)7 promoter mutation of uridine diphosphoglucuronosyl transferase gene.

Regular ultrasonography of the gallbladder should be done. cholecystectomy should be performed ,if gallstones are present at the time of splenectomy,.

THROMBOEMBOLIC COMPLICATIONS

Thalassemia patients have an increased risk to develop thromboembolic manifestation. The increased tendency of thromboembolic manifestations was found to be 10% in patients with thalassemia intermedia and 4% in patients with thalassemia major . In female, history of splenectomy and degree of anemia were thombophilic risk factors.

The presence of a chronic hypercoagulable state in the thalassemia is due to procoagulant effect of the anionic phospholipids exposed on the surface of damaged

circulating red cells and to endothelial derangement the inflammatory state associated with thalassemia[47].

Concomitant prothrombotic conditions in thalassemia patients after the first decade of life frequently include insulin-dependent diabetes, estrogen therapy, atrial fibrillation, and postsplenectomy thrombocytosis[47].

JAUNDICE

Due to the excessive destruction of RBCs and their precursors leads to release of hemoglobin and causes increase in the level of plasma indirect bilirubin. The bilirubin can diffuse into the tissues causing jaundice[1].

IMAGING FEATURES IN BETA THALASSEMIA

Radiologically, the skeletal changes in thalassaemia are most striking in the skull there is extreme thickening of the diploe, the inner and outer plates become poorly defined and trabeculae between the plates become elongated, producing a bristle like ***crew cut or hair-on-end*** appearance of the surface of the skull.

In rib shows a features of ***rib-within rib appearance*** and is noted particularly in the middle and anterior portions of the ribs. This findings consist of a long linear density within or overlapping the medullary space of the rib and running parallel to its long axis.

The proliferation of marrow within the frontal and facial bones impedes pneumatization of the paranasal sinuses. This results in hypertrophy of osseous structures and a consequent prominence of lateral margins of the malar eminences

together with anterior and medial displacement of developing teeth and ethmoidal sinuses are not involved, a factor attributable to the absence of red marrow in the sinus walls.

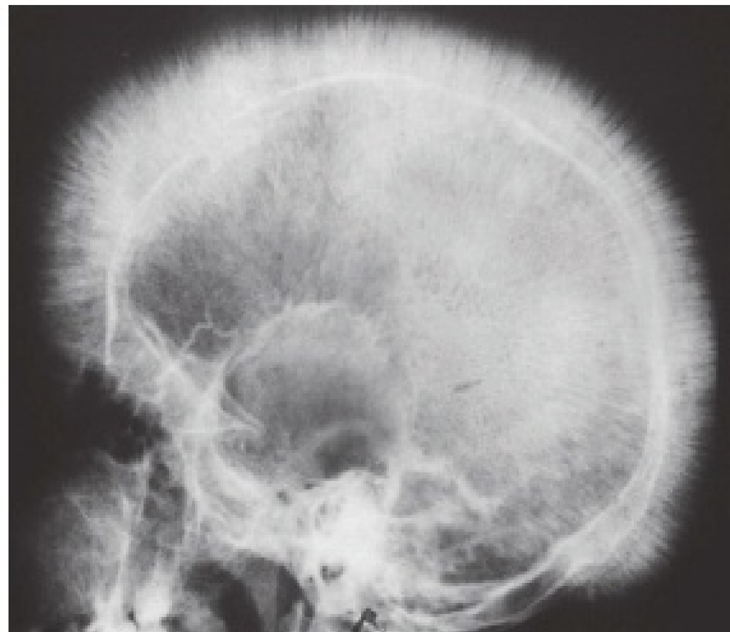

Figure 5.X ray showing Hair on end appearence

Intra-oral roentgenograms in some cases reveal a peculiar trabecular pattern of the maxilla and mandible, characterized by an apparent coarsening of some trabeculae and the blurring and disappearance of others, resulting in a ***salt and pepper*** effect[48].

Magnetic resonance imaging (MRI) in the thalassemia cases, there is a replacement of fatty marrow by the hypercellular area. Microradiography and x-rays detects the reduced and irregular mineralization of the bone [47].

Ultrasound imaging and evaluation may reveal gall bladder with pigment stones and splenomegaly.

IRON OVERLOAD IN BETA THALASSEMIA

NORMAL IRON METABOLISM

Iron is an essential micronutrient required for adequate erythropoietic function, oxidative metabolism and cellular immune response. The human body has no effective means of excretion of iron and on increased level of iron, it is highly toxic and hence needs regulation of absorption of dietary iron from the proximal duodenum is essential in iron homeostasis.

Normally total body iron content in men is around 6 gm and 2.5gm in women, and it is divided into functional and storage compartment. It is limited that 1-2mg of loss of iron every day through sloughing of intestinal mucosal cells, menstruation and other blood loss.

About 80% of functional iron is found in haemoglobin, myoglobin and iron containing enzymes. The main site for iron storage is in liver and mononuclear phagocytes. The intracellular storage of iron in the form of ferritin and hemosiderin and it circulates in plasma in bounded form to transferrin[13].

Dietary iron is found in Haem(10%) and non Haem(90%) forms. The non haem forms which will be in ferric state (Fe^{3+}) will be reduced to ferrous state by ferrireductases and then transported and their get absorbed at the apical surface of duodenal enterocyte through divalent iron transporter. Iron is exported across the basolateral membrane of enterocyte by means of ferroprotin and hephestin in contact with blood, where it bind to transferrin and transported to circulation.

Transferrin bound iron enters the targeted cells mainly erythroid cells, immune and hepatic cells through a receptor mediated endocytosis. The senescent erythrocytes undergoes phagocytosis by reticuloendothelial system macrophages and the haem is metabolised by hemeoxygenase and stored as ferritin. The level of plasma ferritin correlates with the body stores.

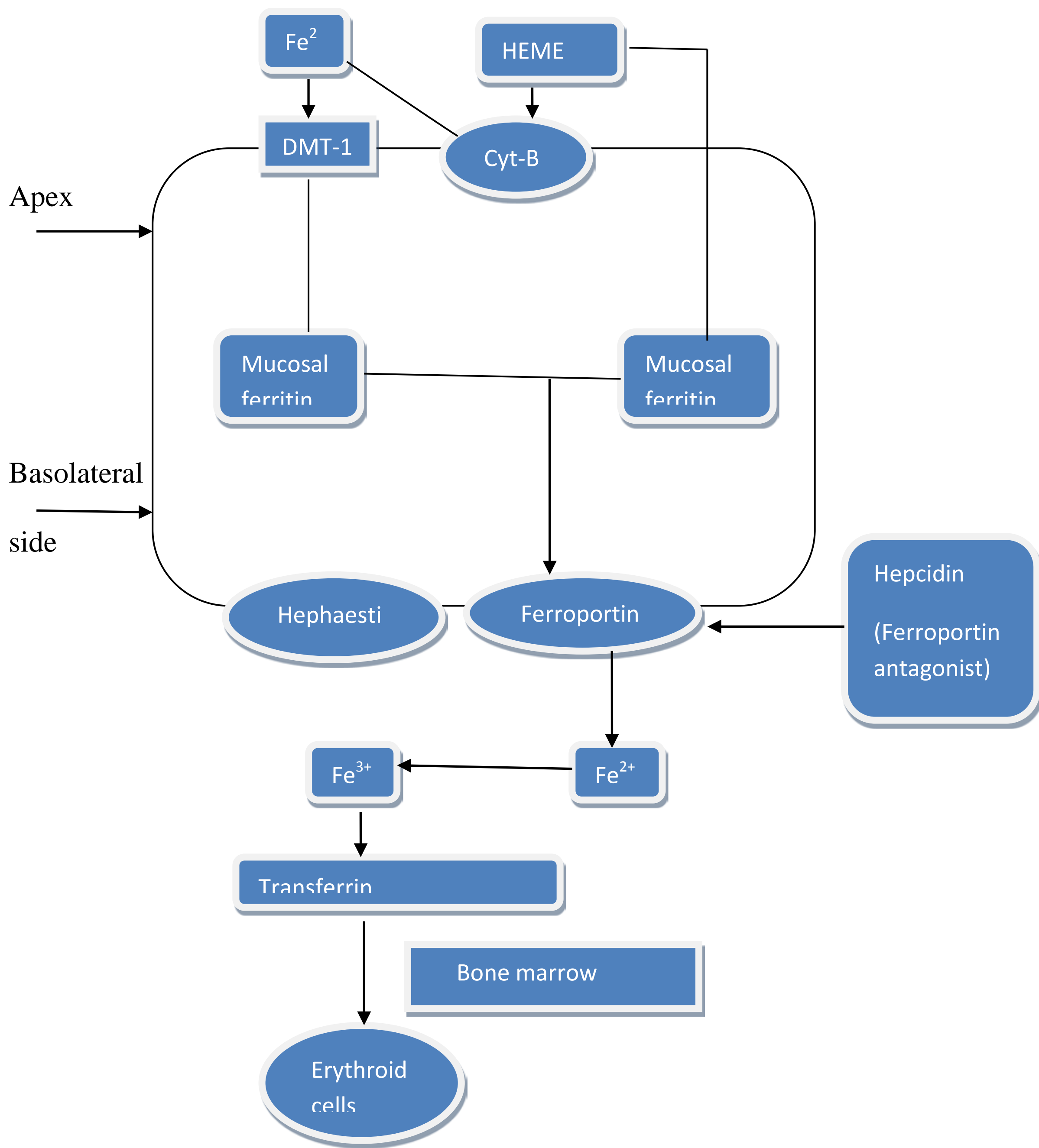

Figure 6. Normal iron metabolism

Ferritin is a ubiquitous protein-iron complex and in the liver most ferritin is stored within the parenchymal cells and in other tissues, such as the spleen and the bone marrow it is found mainly in macrophages. Intracellular ferritin is located in the cytosol and in lysosomes, in which partially degraded protein shells of ferritin aggregate into hemosiderin granules. The iron is later released from macrophages and bound to transferrin and transported to bone marrow[49].

IRON REGULATION BY HEPCIDIN

Iron absorption is regulated by hepcidin, a small circulating peptide that is synthesized and released from the liver in response to increases in intrahepatic iron levels. Transferrin receptors (TfR1 and TfR2) with the membrane protein hereditary hemochromatosis protein (HFE) regulates the hepcidin production by sensing the iron levels in the body and stimulates the hepcidin. Hepcidin inhibits iron transfer from the enterocyte to plasma by binding to ferroportin and causing it to be endocytosed and degraded. As a result, as hepcidin levels rises iron becomes trapped within duodenal cells in the form of mucosal ferritin and is lost as these cells are sloughed[13].

PATHOGENESIS OF IRON OVERLOAD IN BETA THALASSEMIA

In cases of beta-thalassemia require regular blood transfusion to reduce the chronic anemia and multiple blood transfusions causes increased gastrointestinal iron absorption lead to iron overload . A patient who receives more than 10 units of blood per year, accumulates 3 g of iron each year in the absence of any iron chelation therapy. Excess iron is toxic and causes serious and irreversible organ damage such

as cirrhosis, diabetes, heart disease, and hypogonadism which lead to significant morbidity and mortality if untreated.

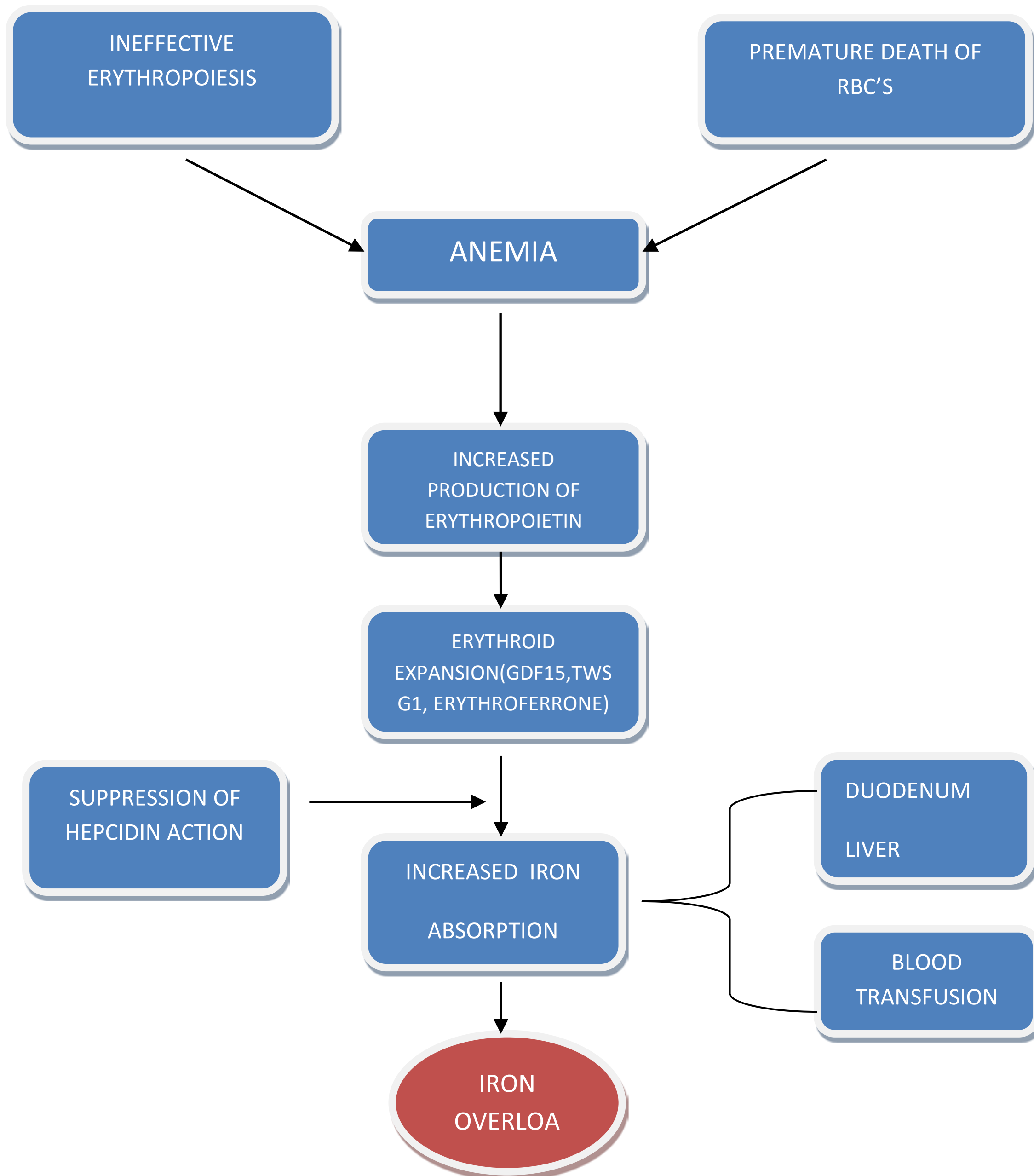

Figure 7. Pathogenesis of iron overload in beta thalassemia

The excess iron in beta-thalassemia patients saturates the ability of the transferrin iron transport system and leading to non transferrin bound iron (NTBI) and labile plasma iron (LPI) starting to circulate in plasma and gets deposited inside susceptible cells. NTBI enters cells by other cellular channels including L-type voltage-dependent Ca2+ channel(LVDCC), a divalent cation transporter and Zip14, a member of the SLC39A zinc transporter family.

Long-term uptake and accumulation of NTBI and LIP, its redox active component leads to increased levels of storage iron and labile cellular iron. Tissues susceptible to iron accumulation by this mechanism include the liver, endocrine system and myocardium.

When the cellular labile iron pool exceeds the capacity of the cell to synthesize new ferritin molecules, a critical concentration is reached that can generate reactive oxygen species (ROS). The Reactive oxygen species like hydroxyl radicals, increase lipid peroxidation and organelle damage leading to cell death and fibrogenesis.

Iron overload can also contribute to ineffective erythropoiesis to an extent depending on the disorder. It is suggested that the production of growth differentiation factor 15 (GDF15) which is derived from cytokine family and inhibition of hepcidin synthesis due to the protein called twisted-gastrulation 1 (TWSG1) and it is leading to the increased iron absorption in beta-thalassemia patients.

It is also suggested that on increased erythropoiesis, bone marrow and spleen erythroblasts increasingly produce erythroferrone, which directly acts on the liver to

inhibit hepcidin production and causes increased iron mobilization and absorption during periods of erythropoietic stress.

Hepcidin inhibition by the liver serine protease TMPRSS6 has also been seen in beta thalassemia patients. Serum levels of cytokine (GDF15) are elevated in patients with homozygous beta thalassemia, while intermediate levels are found in carriers of alpha-thalassemia and in beta-thalassemia trait carriers[50].

CLINICAL MANIFESTATION OF IRON OVERLOAD IN ORGANS

IRON OVERLOAD IN HEART

70% of the deaths of patients is due cardiac complications and which are the most common complication of iron overload resulting in heart failure and arrhythmias. The prevalence of heart failure is significantly higher in males than in females.

MECHANISM

Heart disease caused by iron overload is mainly by injury to the phospholipids of mitochondria and lysosomes is mediated through the labile iron-mediated peroxidation. In the absence of chelation, subclinical dysfunction appears in the second decade of life or when approximately 20 g of iron have been accumulated.

However cardiomegaly and left ventricular function deterioration progress to congestive heart failure and sudden death from arrhythmias can occur. In asymptomatic thalassemia patients with normal myocardial mass the early manifestation to occur is diastolic dysfunction where the systolic function is mildly impaired.

The **classic picture of end-stage iron-induced cardiomyopathy** combination of

- **Left ventricular diastolic dysfunction**
- **Pulmonary hypertension**
- **Right ventricular dilatation.**[36]

Although chelation therapy plays a crucial role in preventing or correcting the cardiac damage in thalassemia patients but iron accumulation may occur in patients with good compliance and low ferritin and liver iron concentration. The prognosis for thalassemic patients with heart failure has always poor.

IRON OVERLOAD IN LIVER

Liver disease is a major complication of thalassemia major and is caused by the damage produced by iron overload and the effects of transfusion-transmitted viral infections. In the absence of chelation hepatic siderosis is start to appear from the early stages of iron loading and progresses to fibrosis and cirrhosis.

The mechanisms of the effect of iron is suggested that peroxidation of intracellular organelles and membrane components by reactive oxygen species is the major cause of tissue toxicity and organ damage. Iron mediated oxidation and the consequent loss of integrity of RNA is considered an important contributing factor to disease development.[36]

IRON OVERLOD IN ENDOCRINE GLANDS

Iron deposition in the endocrine glands occurs either directly or through the hypothalamic–pituitary axis. High ferritin levels, poor compliance with chelation, and splenectomy increase the risk of endocrinopathies. Intensive iron chelation can normalize the iron load and prevent or even reverse endocrine dysfunction.

GROWTH RETARDATION

Stunted growth is common in thalassemia and is characterized by normal growth during childhood, decreased growth velocity at the end of the first decade of life.

The causative factors for growth failure which includes

- Iron overload free radical toxicity
- Desferrioxamine toxicity
- Zinc deficiency anaemia
- Delayed puberty
- Primary hypothyroidism
- Liver cirrhosis
- Defect in the Growth Hormone-Insulin- like Growth Factor-1 (GH-IGF-1) axis.

In Thalassaemic patients, there will be lower serum IGF-1 and normal GH reserve which shows the relative GH resistance but improves growth with GH therapy. The linear growth in childhood is disturbed due to anaemia, ineffective

erythropoiesis, high ferritin levels & desferrioxamine treatment. Due to desferrioxamine therapy and iron loading may have effects on local IGF-1 production and paracrine growth regulation which affects the growth plate results in stunted growth.[36]

HYPOGONADISM AND PUBERTY

Sexual immaturity is a severe complication of severe thalassemia and primary gonadal failure due to gonadal iron deposition. Iron deposition on gonadotrophic cells of the pituitary gland which results in secondary hypogonadism due to impaired response of FSH and LH to GnRH stimulation.

Iron toxicity on adipose tissue has shown to cause impaired synthesis of Leptin and leading to delay in sexual maturation.

Leptin is a polypeptide hormone produced by adipose cells due to expression of the ob gene and acts as a signal to initiate puberty. Gross iron overload in the pituitary hypothalamus and gonads is progressive even with chelation therapy.

Delayed onset of menarche, oligomenorrhoea, secondary amennorhoea, breast size at Tanner Stage 2 or 3. Attenuated testicular size ,decreased fertility in men with thalassemia major are attributed to lower sperm count and sperm motility, and proportion of sperm with normal morphology is significantly lower are common manifestations of significantly elevated serum iron and ferritin levels[51].

HYPOTHYROIDISM

Hypothyroidism is the second most common endocrine disorder after hypogonadism. The majority of patients have subclinical or mild forms. Central hypothyroidism is less frequently encountered.

Regular assessment of free thyroxine and thyroid-stimulating hormone is recommended after the first decade of life. Thyroid ultrasonography may show an irregular echogenic pattern with thickening of the capsule.

Abnormal thyroid function has been reversed to with intensive chelation therapy with DFO or in combination with DFP.

HYPOPARATHYROIDISM.

Hypoparathyroidism affects 3% to 10% of thalassemia patients and is attributed to iron deposition in the parathyroids. Males are more affected than females.

Early detection requires periodic estimation of calcium homeostasis. Symptoms are paresthesias, muscle pain. When severe, tetany and even convulsions occurs.

Extreme hypocalcemia is a late event. Intracranial calcifications have been reported in 40% of patients with hypoparathyroidism in the absence of symptoms.[36]In mild cases, normalization of calcium and phosphate with calcitriol and careful monitoring of the serum calcium are warranted.

LABORATORY INVESTIGATIONS

The following investigations used in the diagnosis of beta thalassemia.

- Complete Haemogram
- Peripheral smear
- Reticulocyte count
- Bone marrow examination
- Biochemical test
- Kleihauers Test (Acid Elution test)
- Hemoglobin Electrophoresis
- Naked eye single tube red cell osmotic fragility test (NESTROFT)
- High performance liquid chromatography(HPLC)
- DNA molecular analysis
- Globin chain synthesis
- Amplification refractory mutation system (ARMS) – PCR for point mutation
- Polymerase chain reaction(PCR) and Multiplex PCR
- Minisequencing
- Reverse DOT Blot analysis

COMPLETE BLOOD COUNT

The laboratory results can vary from borderline abnormal to markedly abnormal. This depends on the type and number of globin gene mutations. The haemoglobin value range from 3-7g/dl and hematocrit are decreased but the RBC count can be disproportionately high relative to the degree of anemia, which can lead to very low MCV-60 -70fl and mean cell haemoglobin (MCH) -12-18pg . The mean cell hemoglobin concentration (MCHC) is also decreased. The RBC distribution width (RDW) is elevated reflecting anisocytosis in untreated beta-thalassemia major but it is normal in beta-thalassemia minor[1].

PERIPHERAL SMEAR

The blood film shows

- Moderate to severe hypochromia with severe anisopoikilocytosis .
- Fragment RBCs, teardrop cells, target cells also may be seen.
- Basophilic stippling, Pappenheimer bodies may be noted.
- Numerous nucleated red blood cells seen as a result of defective hemoglobinization and dyserythropoietic features.
- In children with massive splenomegaly the anemia becomes severe with neutropenia and thrombocytopenia[52].

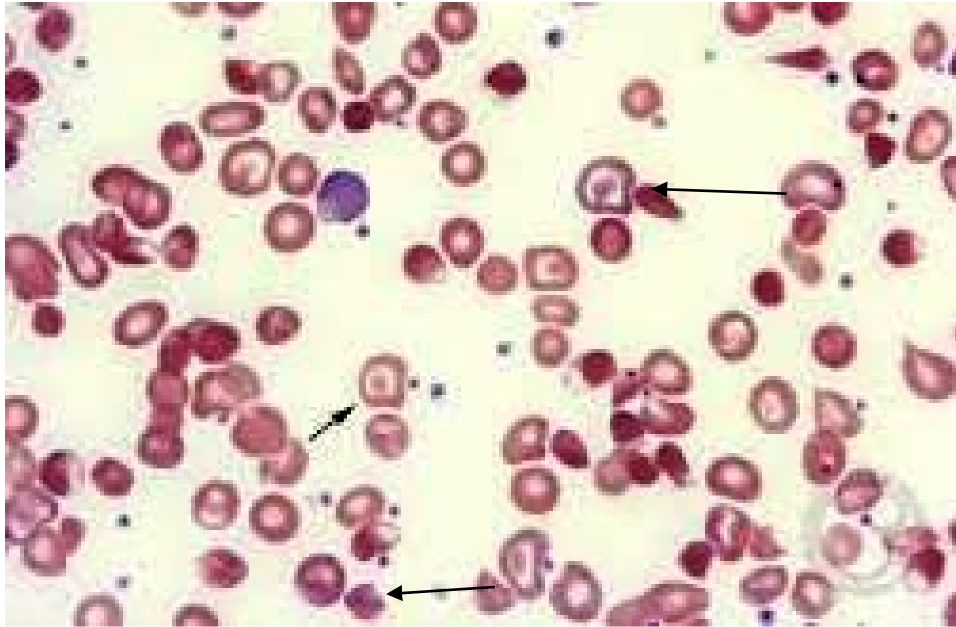

Figure 8:Peripheral smear showing moderate hypochromasia with fragmented red blood cells and target cells

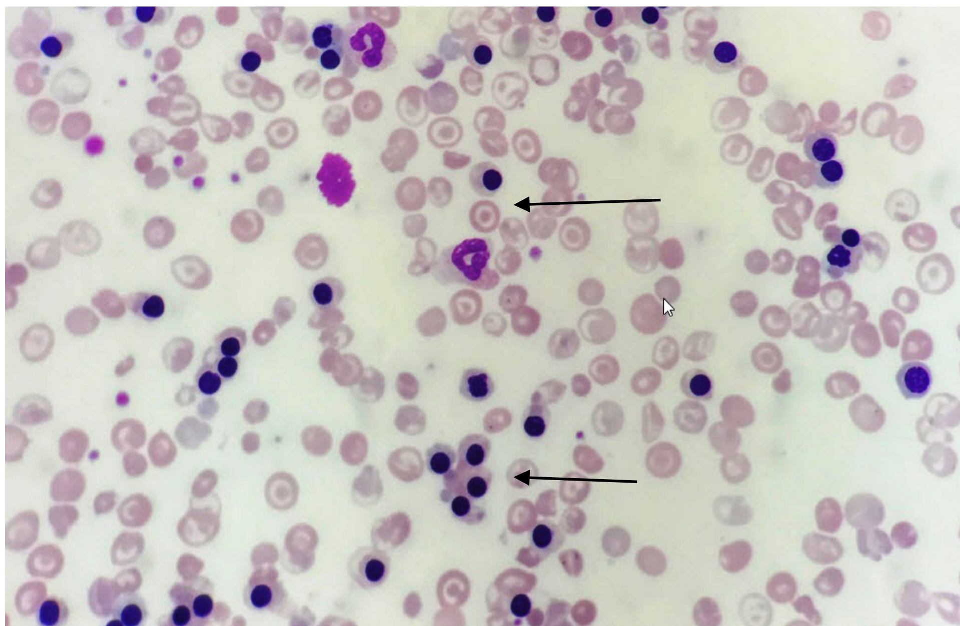

Figure 9 : Peripheral smear showing numerous nucleated red blood cells and target cells

RETICULOCYTE COUNT

The reticulocyte count is elevated, which indicates that the bone marrow is responding to a hemolytic process. In homozygous beta-thalassemia, it is typically 2% to 8%, disproportionately low relative to the degree of anemia and inadequate reticulocytosis reflects the ineffective erythropoiesis. The absolute reticulocyte count is rarely high, although it tends to increase after splenectomy[1].

After splenectomy, the usual features of hyposplenism are present such as Howell–Jolly bodies, target cells, lymphocytosis, thrombocytosis and giant platelets. Pappenheimer bodies are very prominent and nucleated red cells are markedly increased.

BONE MARROW EXAMINATION

The bone marrow aspirate shows gross erythroid hyperplasia. There is severe dyserythropoiesis with nuclear lobulation and fragmentation, basophilic stippling, defective hemoglobinization and the presence of alpha chain precipitates. Actively phagocytic macrophages are prominent and pseudo-Gaucher cells are present. Iron stores are increased[49].

BIOCHEMICAL TESTS

Biochemical tests show elevated levels of total bilirubin, elevated urinary urobilinogen levels and hyperuricaemia. Decreased or absent haptoglobin levels. In the plasma ,there may be detectable free methemalbumin and haemoglobin may be present[52]

KLEIHAUERS TEST(ACID ELUTION TEST)

PRINCIPLE

The acid elution technique is based on the differential elution of fetal and adult haemoglobin from ethanol fixed red blood cells at acidic PH.

In homozygous beta thalassemia has higher HbF level of 30 -90% and in this cytochemical test shows that all red cells containing HbF are stained pink as HbF are resistant to acid elution of hemoglobin. The red cells which are appearing as ghost cells indicating the presence of HbA as it is not resistant to elution by acid[53].

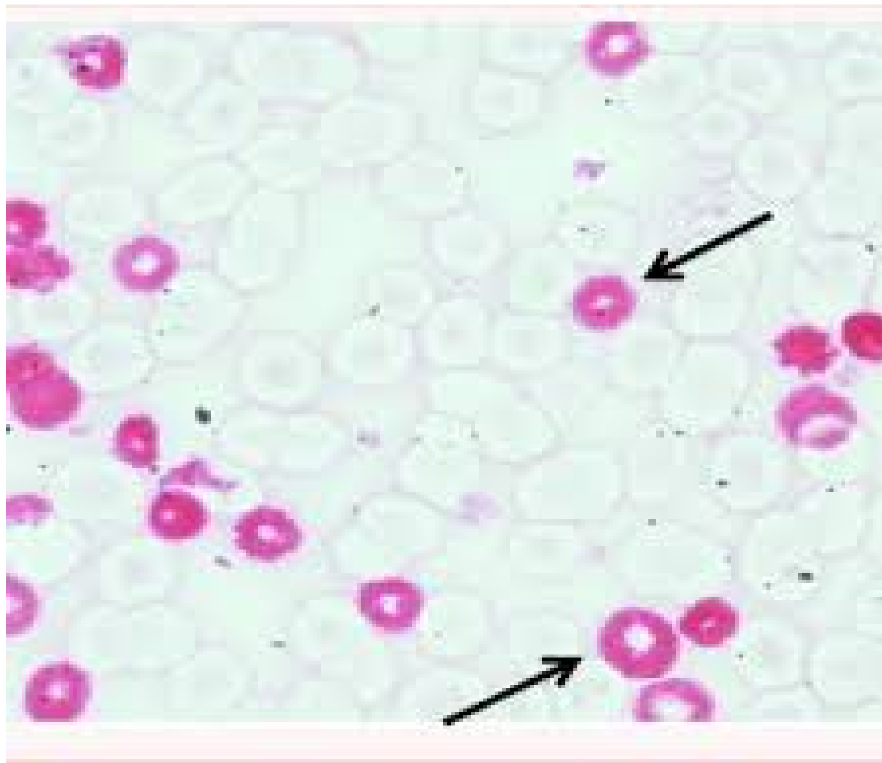

Figure 10 : Acid elution test showing red cells with HbF stains pink and HbA as Ghost cell

NAKED EYE SINGLE TUBE RED CELL OSMOTIC FRAGILITY TEST

It is used to measure erythrocyte resistance to hemolysis when exposed to hyposaline solution. In a hypotonic solution, water enters the red blood cell (RBC) resulting in swelling and eventually RBC lysis. The susceptibility of this osmotic hemolysis is a function of surface area to volume ratio. In thalassemia, RBCs have a low cellular haemoglobin resulting in an increase in osmotic resistance compared with a normal RBC.

PROCEDURE:

- 20 mL of fresh whole blood is pipetted and mixed with buffered 0.36% NaCl solution .
- The mixture is left at room temperature for15 to 30 minutes
- Degree of hemolysis is assessed by visual inspection .
- A clear solution is interpreted as a negative result and a cloudy appearance as a positive result[54].

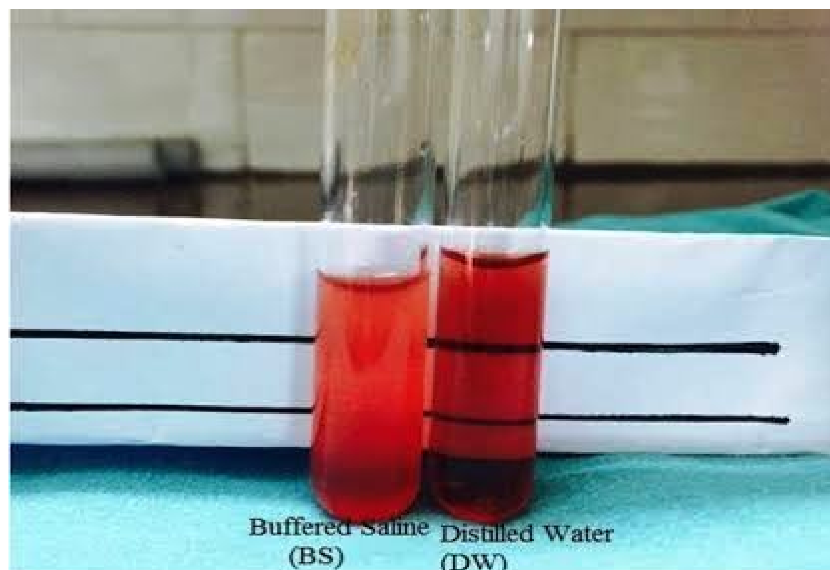

Figure 11 : Showing Positive NESTROF TEST

HEMOGLOBIN ELECTROPHORESIS

The Hemoglobin electrophoresis is used as a screening test in the diagnosis of thalassemia.

PRINCIPLE

It is used to detect the different type of haemoglobin. Different haemoglobin have different charges and according to those charges and the amount of haemoglobin moves at different speed in alkaline or acid gel.

In Hb electrophoresis on starch – agarose / cellulose acetate membrane demonstrates bands of both HbA and HbF in heterozygous beta thalassemia. In homozygous beta thalassemia, band shows more than >90% of HbF[53].

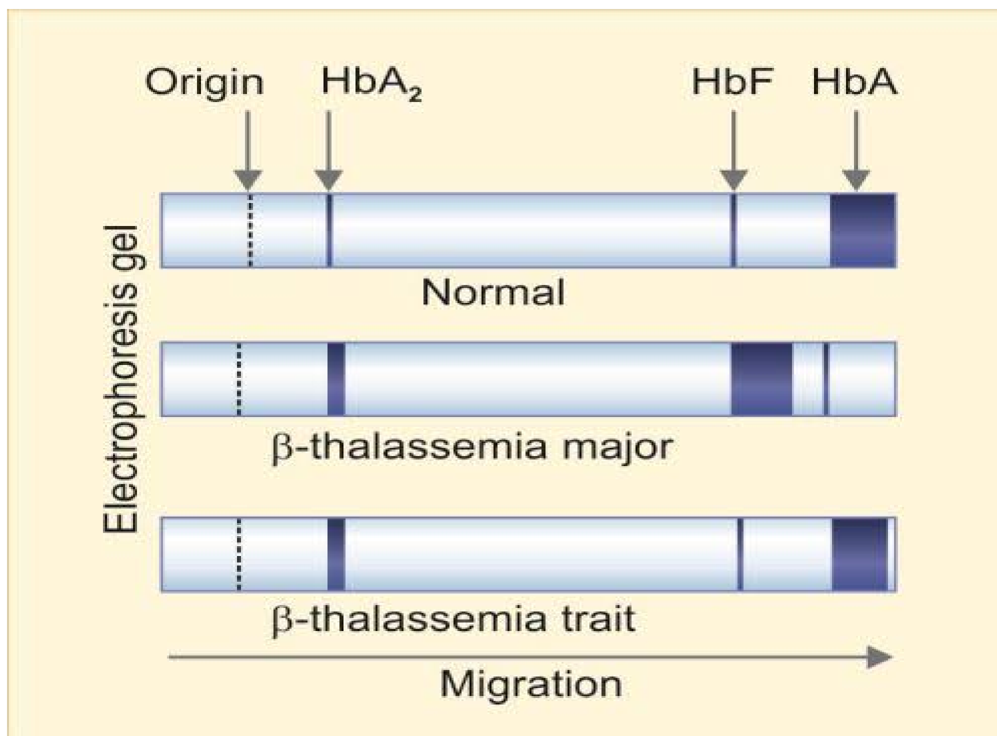

Figure 12: Hemoglobin Electrophoresis of Beta Thalassemia

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The high performance liquid chromatography is used as diagnostic method for thalassemia. In the case of homozygotes or compound heterozygotes for β^0 Thalassemia (β^0 β^0), techniques such as hemoglobin electrophoresis and High performance liquid chromatography (HPLC) show only hemoglobin F and hemoglobin A2. In these cases HbA is absent, HbF is 95– 98% and HbA2 is 2–5% When there is homozygosity for B+ thalassemia (B+ B+) or compound heterozygosity for $\beta^0$and β^+thalassemia ($\beta^+\beta^0$), hemoglobin A is also present. Such cases show HbA between 10 and 30%, HbF between 70–90% and HbA2 between 2–5%.

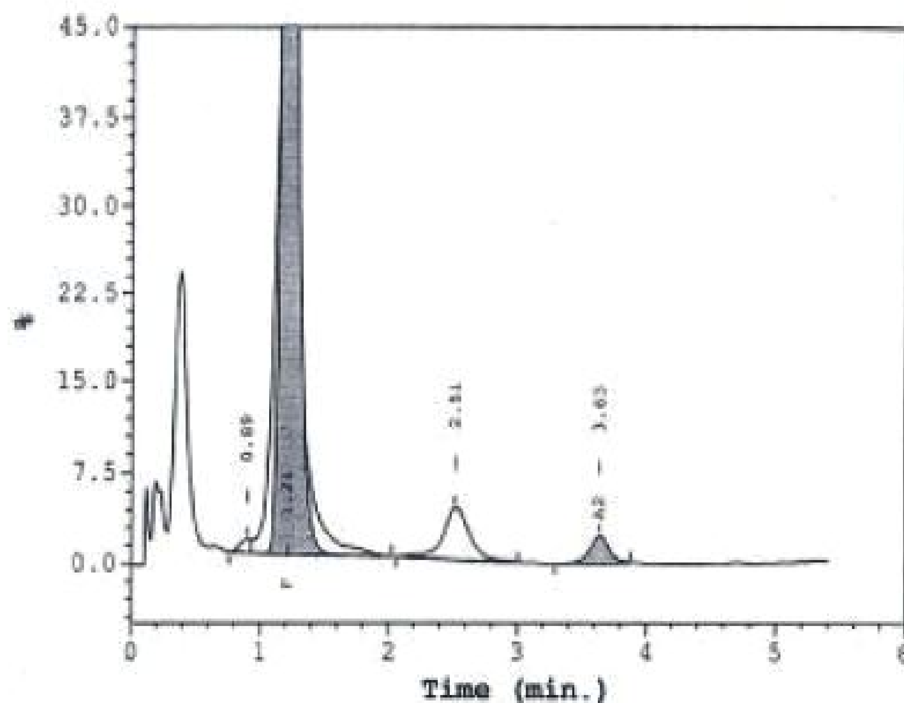

Figure 13 : High performance liquid chromatography of beta thalassemia major HbF – 70 -90%,HbA2 -2-5%

DNA MOLECULAR ANALYSIS

Because thalassemia and hemoglobinopathy are mainly caused by mutations in globin genes, the molecular analysis of DNA sequences is the most definitive diagnostic modality for such conditions. There are several measures to study the molecular basis of globin disorders. The molecular studies of thalassemia divided into 2 main categories: Mutation-specific detection and Genome scanning .

Mutation-specific detection makes use the information from any given population on their common profiles of both alpha-globin and beta-globin mutations(deletions, point mutations, or gene rearrangements) to generate a panel of mutation detection, and uses different polymerase chain reaction (PCR)-based methods to detect these known mutations.

There are several molecular techniques used to detect known mutations, including GAP-PCR using conventionalor real-time detection for gene deletions or insertions, allele-related mutations specific PCR, reverse dot blot hybridization or array-based detection, mismatched-PCR restriction fragment length polymorphism, and analyses of a high-resolution melting curve for point or small nucleotide changes.

Though this approaches can provide a robust, cost-effective, and more rapid measure to identify causative mutations it have its own limitations that it cannot detect unknown or rare variations which might not be included into the panels.

In such situations, genome scanning by denaturing gradient gel electrophoresis, denaturing HPLC or single strand conformation polymorphism and direct sequencing of the whole globin genes would be useful.

For genome deletions or rearrangement, a multiple ligation probe amplification assay has been used as because it can be used to scan the globin clusters first to determine the possibility of gene deletions, duplications or rearrangement. However there would be a need for further confirmation by breakpoint identification using a CGH array. Although this approach can detect almost all possible globin mutations causing thalassemia, it is costly and requires a high level of expertise and it is not widely validated, and the availability only in limited laboratories in the world.

These mutation analyses would be critical for the confirmation of thalassemia diagnoses in only a few selected cases for whom the basic hematology and hemoglobin analysis described could not provide a conclusive diagnosis. These molecular analyses would be indispensable in a program for the prevention and control of thalassemia syndromes because the mutation data would be required for genetic counselling, genetic risk calculation in the offspring, prenatal and preimplantation genetic diagnosis. In addition DNA analysis could help in predicting the clinical severity and guiding clinical management[54].

EVALUATION OF IRON OVERLOAD

Evaluation of iron levels in the body assumes significance in assessing the prognosis of iron overload patients. Various methods are employed to estimate body iron status

- Non invasive method
    - Serum iron concentration
    - Transferrin saturation
    - Serum ferritin

➢ Invasive methods

- Liver biopsy [stainable parenchymal iron/hepatic iron concentration]
- Bone marrow biopsy and Histochemical demonstration.

SERUM FERRITIN

The serum ferritin is the mostly commonly used to monitor iron overload and iron chelation therapy. Serum ferritin measurements are inexpensive and generally correlate with both total body iron stores and clinical outcomes.

Normal Serum ferritin levels

- New borns – 25 – 200 ng/mL
- Infants aged 2-5 month – 50 – 200ng/mL
- Children aged 6 month – 15 years – 7-140ng/mL
- Adult male – 20 -250ng/mL
- Adult female – 10 -120ng/mL

Serum ferritin of 1000 ng/mL represents a significant risk threshold in thalassemia syndromes. The main disadvantage is that the serum ferritin is an acute phase reactant and the levels sharply rises in response to inflammatory stimuli, liver diseases, ascorbate deficiency[55].

HEPATIC IRON EVALUATED BY LIVER BIOPSY

The liver is the major site of iron storage in iron overload, containing more than 70% of body iron stores. The measurement of hepatic iron stores provides the most quantitative means of assessing the body iron burden in patients with

thalassaemia major and may be considered the reference method for comparison with other techniques. The liver iron concentration (LIC) is obtained by biopsy. Although it is an invasive procedure the complication rate is low in experienced hands.

The measurement of the LIC [liver iron concentration] by needle biopsy has an acceptable co-efficient of variation [cv] at 6.6% on duplicate measurements, if the sample size is more than 1mg and in the absence of fibrinosis.

In beta-thalassemia patients on iron chelation, the LIC has shown to have prognostic value. It is found that the threshold LIC at 80 mol/g wet weight (ww) (about 270 mol/g dry wt) in patients with thalassemia major on iron chelation treatment with desferrioxamine (DFO). LICs values exceeding more than the threshold were associated with increased risk of cardiac disease and early death[56].

NON-INVASIVE ESTIMATION OF HEPATIC LIVER IRON CONCENTRATION

In percutaneous liver biopsies are often not feasible, because of the risk of complications and not suitable for multiple repeated measurements.

COMPUTED TOMOGRAPHY (CT)

It can detect the increase in X-ray density caused by the greater electron density of iron compared with normal liver tissue constituents. But the method has not been useful because of wide variations .

MAGNETIC SUSCEPTIBILITY MEASUREMENT OF HEPATIC IRON

The paramagnetic response of iron in ferritin and haemosiderin is detected by use of a **superconducting quantum interference device (SQUID)**. SQUID provides the most precise, calibrated and validated, non-invasive method for quantitative estimation of the LIC. The usefulness of the method has been demonstrated in patients with transfusional iron overload and also in the evaluation of the long-term efficacy of iron chelation therapy with DFO.

MAGNETIC RESONANCE IMAGING

It measures the tissue iron concentration indirectly by detecting the paramagnetic effect produced by the presence of storage iron on the proton resonance behavior of tissue water. This effect can be assessed by calculating the longitudinal and transversal nuclear magnetic relaxation times of nearby solvent water protons[56].

EVALUATION OF IRON OVERLOAD IN LIVER BIOPSY – HISTOCHEMICAL PERLS STAIN

The siderosis was best demonstrated by the Perls stain using acid ferrocyanide which gives the Prussian blue reaction with the ferric compounds, ferritin and hemosiderin. Ferritin dispersed in the hyaloplasm gives a bluishtint to the cell's cytoplasm, whereas intense blue granules correspond to ferritin and hemosiderin packed together within siderosomes or iron laden lysosomes.

Evaluation of the Perls stain requires attention to both the extent [grade/amount] of stainable iron and its distribution in different cell types of portal

tract and lobule. Iron distribution may vary according to the cause of siderosis. The siderosis is predominantly parenchymal in hemochromatosis and neonatal iron overload. Both hepatocytes and Kupffer cells store iron in thalassaemia, whereas exogenous siderosis loads Kupffer cells in the first instance.

Semiquantitative assessment of stored tissue iron can be achieved in different ways. The simplest system grades from 1 [minimal] to 4 [massive deposits], with grades 2 and 3 indicating intermediate amounts. They also reported that the estimated iron in hepatocytes, mesenchymal cells, cholangiocytes, blood vessels, and connective tissue; generate a score between 0 and 60[57].

EVALUATION OF IRON OVERLOAD IN EXFOLIATED BUCCAL CELL BY PERLS STAIN

The semi-quantitative assessment of iron overload by Perls Prussian blue staining method. Perls Prussian blue reaction is considered to be the classical histochemical reaction[58].

PRINCIPLE OF THE METHOD

When the tissue is treated with acid ferrocyanide solution results in the unmasking of ferric iron in hemosiderin in the form of ferric hydroxide by diluted HCL then ferric iron then react with diluted potassium ferrocyanide solution to produce an insoluble blue compound ferric ferrocynaide(Prussian blue).

The excess iron can get accumulated in various tissue depends on various factors such as iron storage pool. The amount of ferritin formed in exfoliated buccal

cells may vary as well as variation in the amount of hemosiderin formation may invariably affects the perls positivity.

Although Ferritin may present in the cells but cannot be visualized under light microscope but Hemosiderin because of its larger size can be visualized under light microscope as blue coloured granules in the cytoplasm of the cells.

The following 3 criteria for grading :

1. Evenly dispersed cells with minimal overlapping.
2. Minimum 20 squamous cells and maximum 30 cells per high power field .
3. Presence/Absence of blue intracytoplasmic granules.

GRADING:

- Grade 0—No granules
- Grade I—<5 granules/high power field
- Grade II—5–10 granules/high power field
- Grade III—10–20 granules/high power field
- Grade IV—Clumps seen in<3 high power field
- Grade V—Coarse granules or clumps seen in 3/>3 high power field[58].

GRADING OF IRON DEPOSITION IN BUCCAL MUCOSA

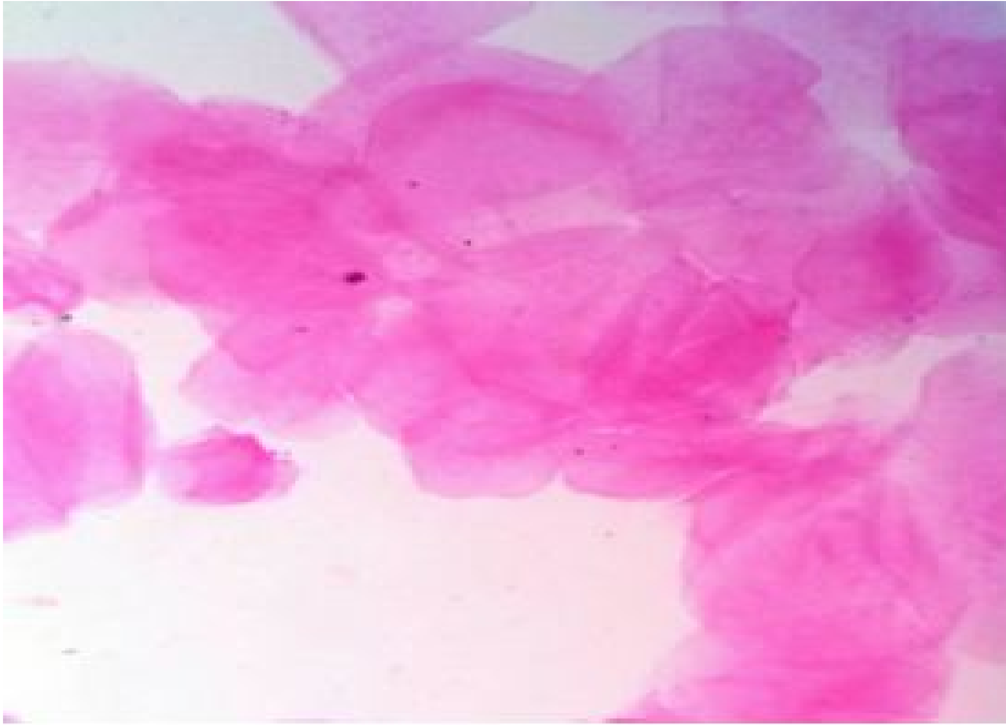

Figure 14 : Showing Grade 0—No granules

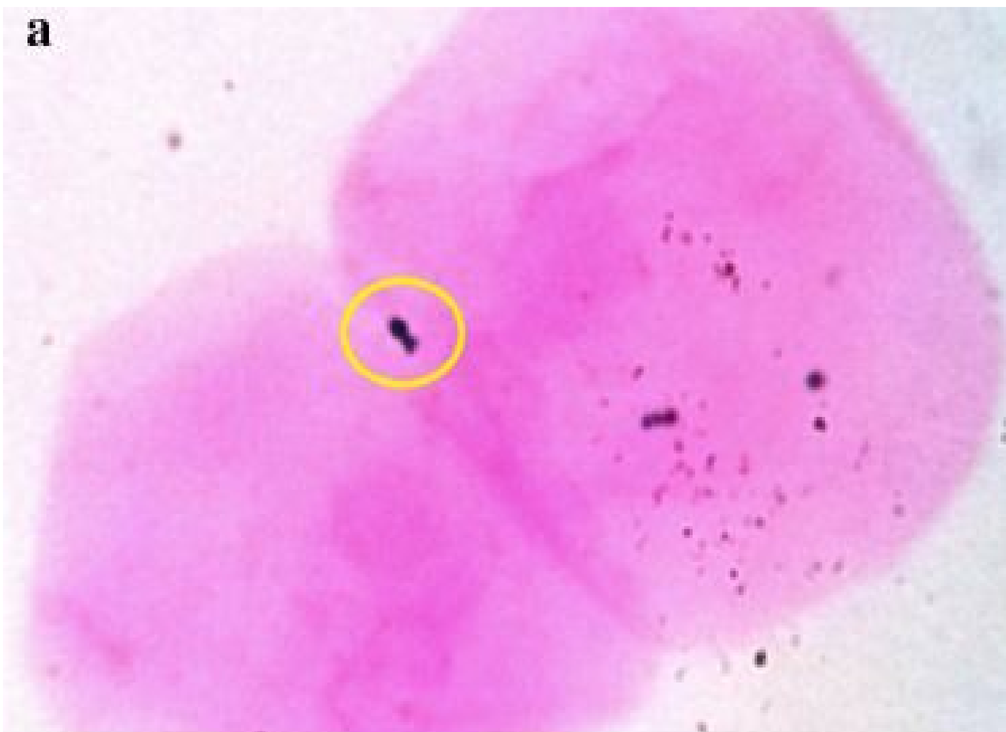

Figure 15 : Showing Grade I - < 5 granules/ High power field

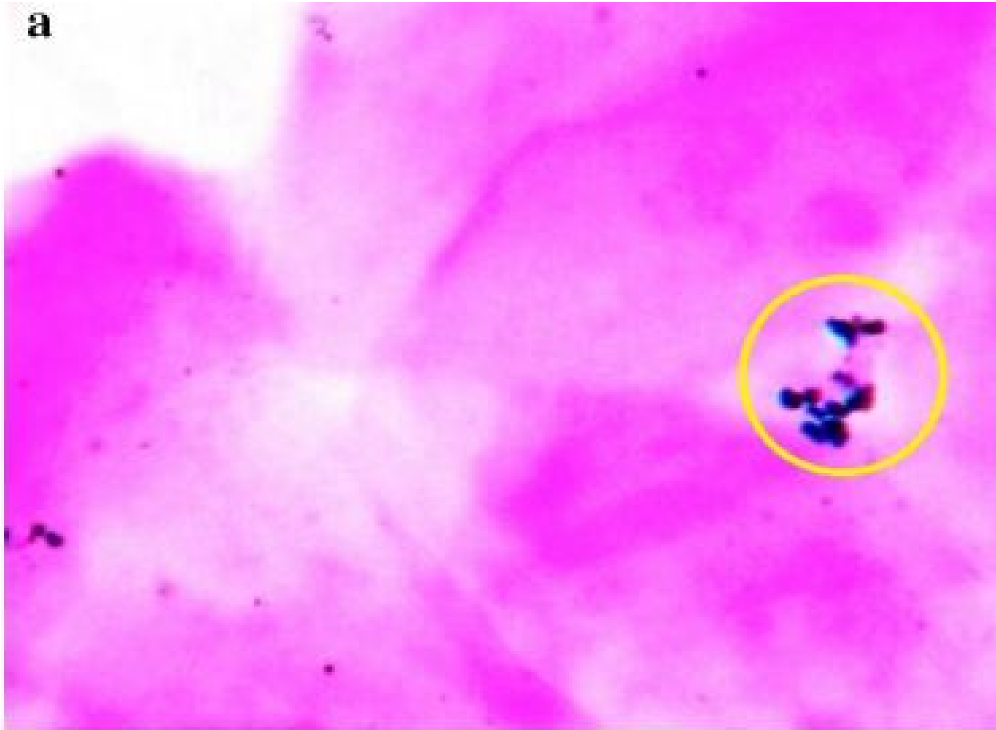

Figure 16 : Showing Grade II -5-10 granules/High power field

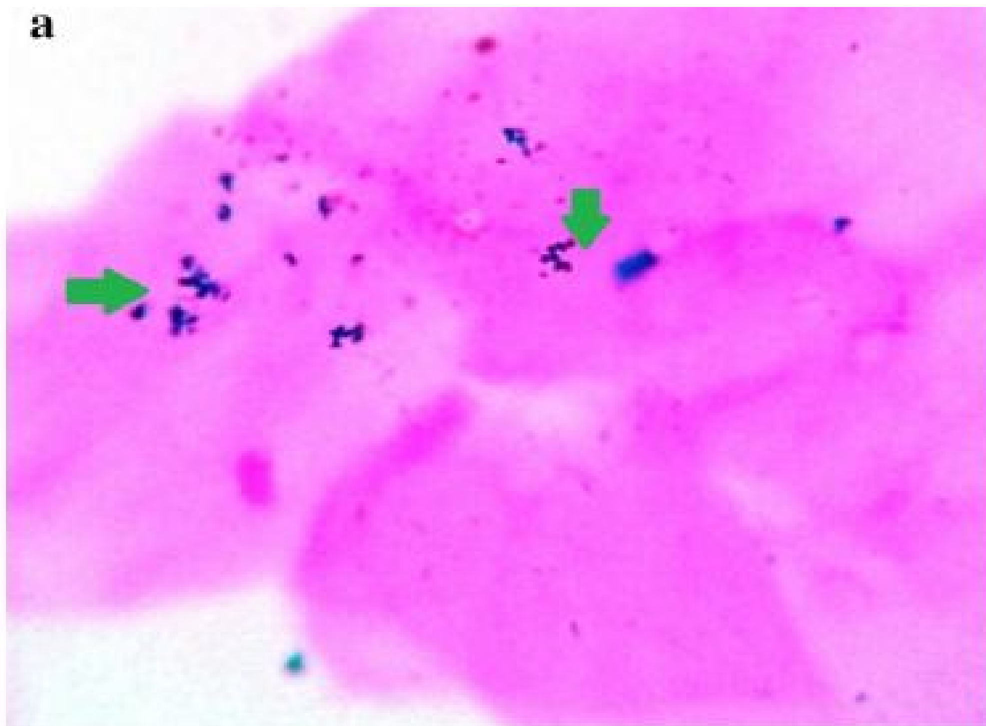

Figure 17 : Showing Grade III -10-20 granules/High power field

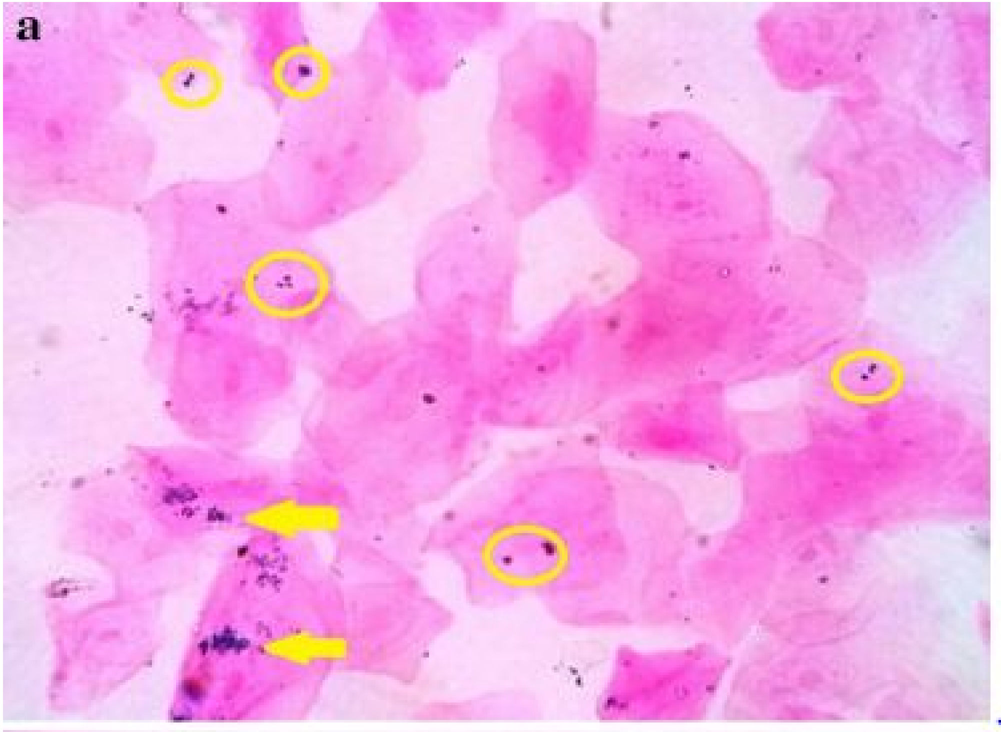

Figure18 : Showing Grade IV – Clumps seen in <3 High power field

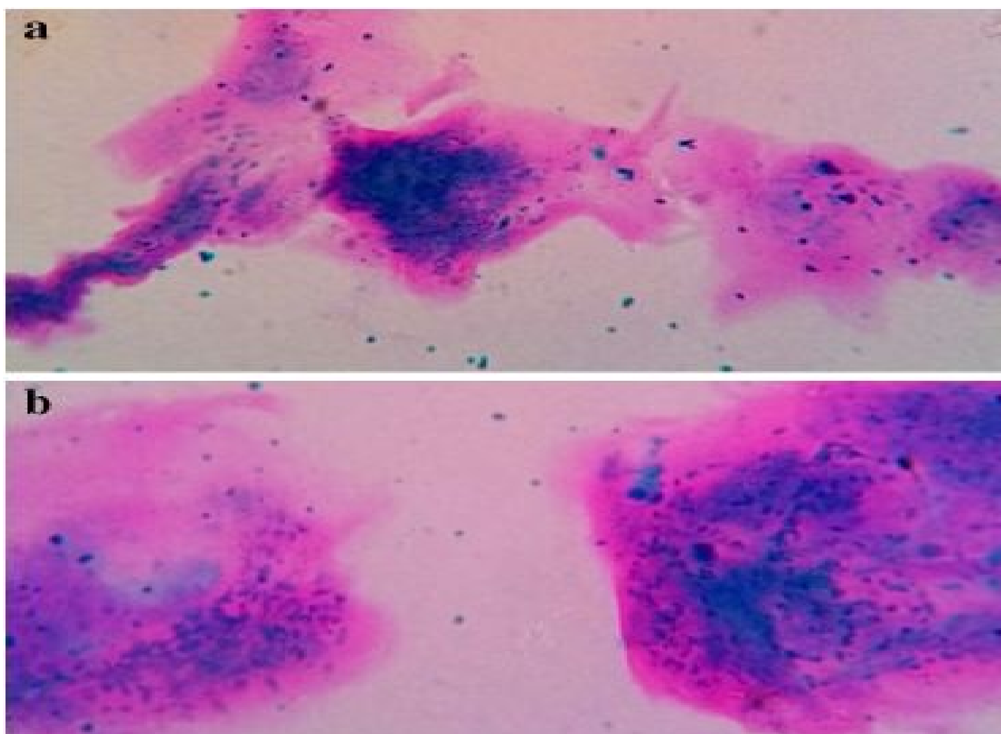

Figure 19 : Showing Grade V –Coarse granules or clumps seen in> 3 high power field

MATERIAL AND METHODS

STUDY PLACE

Pediatric ward and General medicine ward in Government Mohan Kumaramangalam Medical College, Salem.

STUDY PERIOD

November 2018 to June 2020

STUDY DESIGN

Cross sectional study

SAMPLE SIZE

Sample size – 100

SELECTION OF STUDY POPULATION

INCLUSION CRITERIA

- Age group from 1 – 20 yrs
- Study group with beta thalassemia who are previously confirmed by high performance liquid chromatography or hemoglobin electrophoresis.
- Subject who received minimum of 10 regular blood transfusions over a period of time.

EXCLUSION CRITERIA

- Subject who are newly diagnosed case yet to receive blood transfusion
- Patients with iron deficiency anemia, megaloblastic anemia, chronic liver disease, malignancy, Hepatitis and other co morbid conditions
- Patients with intraoral lesions

STUDY GROUP

- Study group of 50 beta thalassemia major patients
- Control group of 50 healthy individuals of same age group

STUDY MATERIAL:

Exfoliated epithelial cells taken

- From the buccal mucosa of β thalassemia patients
- From the buccal mucosa of normal subjects of same age group (control group).

STUDY METHOD:

1. Mouth mirror
2. Microscopic glass slides [dimensions 76mm x 22mm x 1.35mm]
3. Coverslips
4. Wooden spatula
5. Fixative solution (70% ethanol)
6. Coplin Jar
7. Perl's staining kit
8. Mounting medium
9. Microscope with Oil immersion Lens

PROCEDURE

- The procedure is explained to the patients and consent taken from them.
- The patients from the study group and the control group were asked to gargle their mouth with distilled water.
- The buccal mucosa of the patients were scraped with a sterile wet wooden spatula and smeared onto microscopic glass slides.
- The smear was fixed immediately in 70% ethanol for one hour
- Stained with Perl's staining kit, which consists of potassium ferrocyanide, which reacts with the ferritin in the cells to form a blue colored compound. This blue colored compound is visible under the light microscope as blue granules. This staining reaction is referred to as Perl's Prussian blue reaction.
- Neutral red or eosin was used as the nuclear stain.
- The stained smear was examined under the light microscope through the transmitted light at 10x, 40x and 100x magnifications to study the presence or absence of blue colored granules in the cells.

STAINING PROCEDURE

FIXATION:

- The smears were fixed in 70% Ethanol.

FERROCYANIDE SOLUTION:

- 1% aqueous potassium ferrocyanide
- 2ml of 2% aqueous hydrochloric acid

METHOD:

1. The slides were kept in 70% ethanol for 45 minutes.
2. Smears treated with freshly prepared acid ferrocyanide solution for 10-45minutes.
3. Slides washed well in distilled water.
4. Counter stain the nuclei with 0.5% aqueous neutral red for30 seconds.

5 Washed in distilled water.

6. Smears are dehydrated, cleared and mounted with DPX synthetic resin.

ESTIMATION OF SERUM FERRITIN

- 3 ml of blood sample taken from both study and control group in clot activator tube

- The blood is allowed to clot
- Serum was separated and stored at -20^0 C
- Ferritin level were performed by Indirect enzyme linked immunosorbate assay (ELISA) method.

OBSERVATION AND RESULTS

During the study period from November 2018 to June 2020 patients who are diagnosed as beta thalassemia major undergoing blood transfusion were taken for the study for evaluation of iron overload. In our study, the study group of 50 patients are with beta thalassemia major compared with 50 healthy subjects of same age group as a control group

DISTRIBUTION OF STUDY GROUP

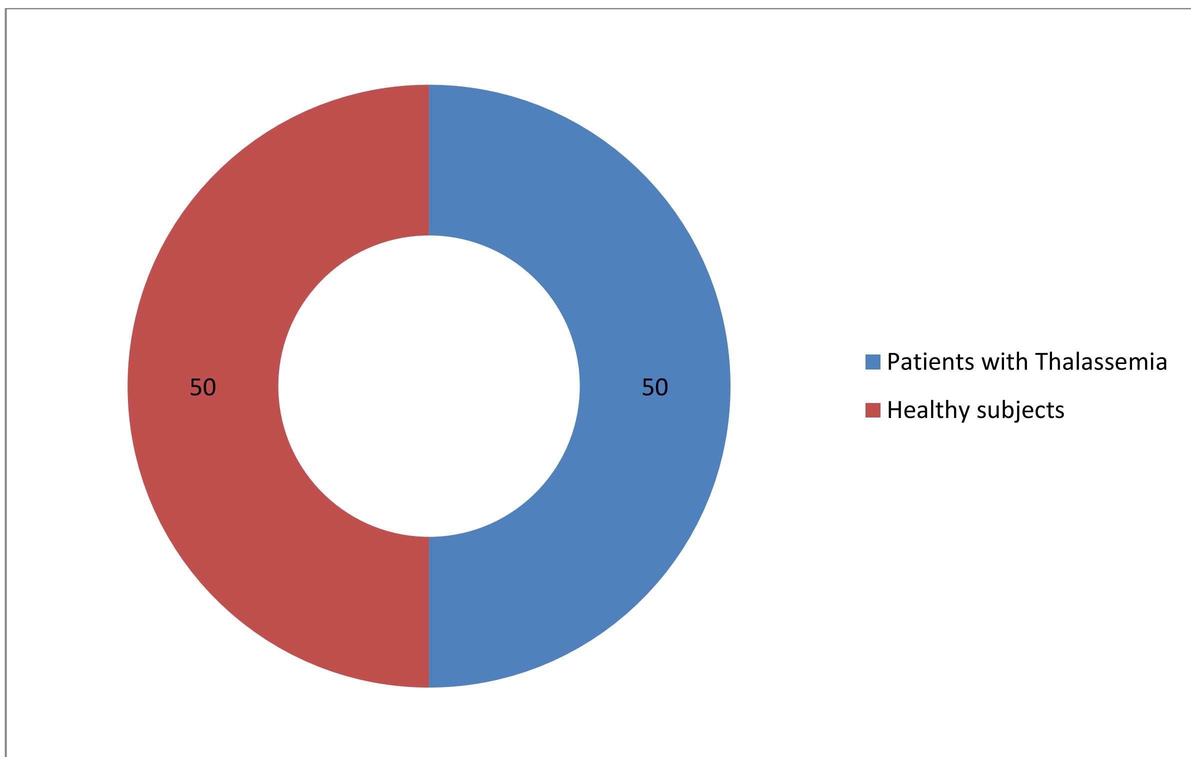

Chart No. 1

AGE DISTRIBUTION IN STUDY GROUP

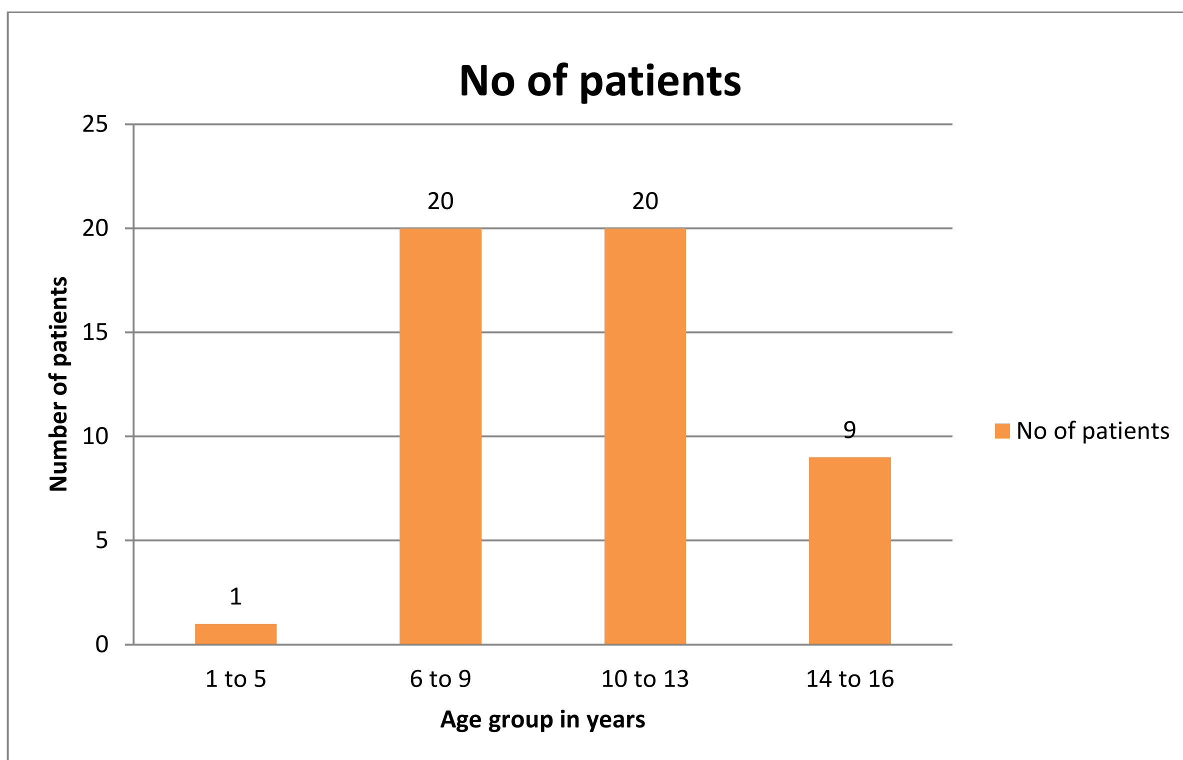

Chart No. 2

The above chart shows the distribution of study participants based on age group. The majority number of studied subjects of about 20 participants belong to the age group of 6 to 9 years and 10 to 13 years. The least number of studied subjects of about 1 participant belong to the age group of 1 to 5 years.

Table 1: COMPARISON OF AGE WITH GROUPS

Variable	Groups	N	Mean	S.D	t-value	p-value
Age	Cases	50	10	3	0.182	0.856 #
	Controls	50	10	3		
# No Statistical Significance at p > 0.05 level						

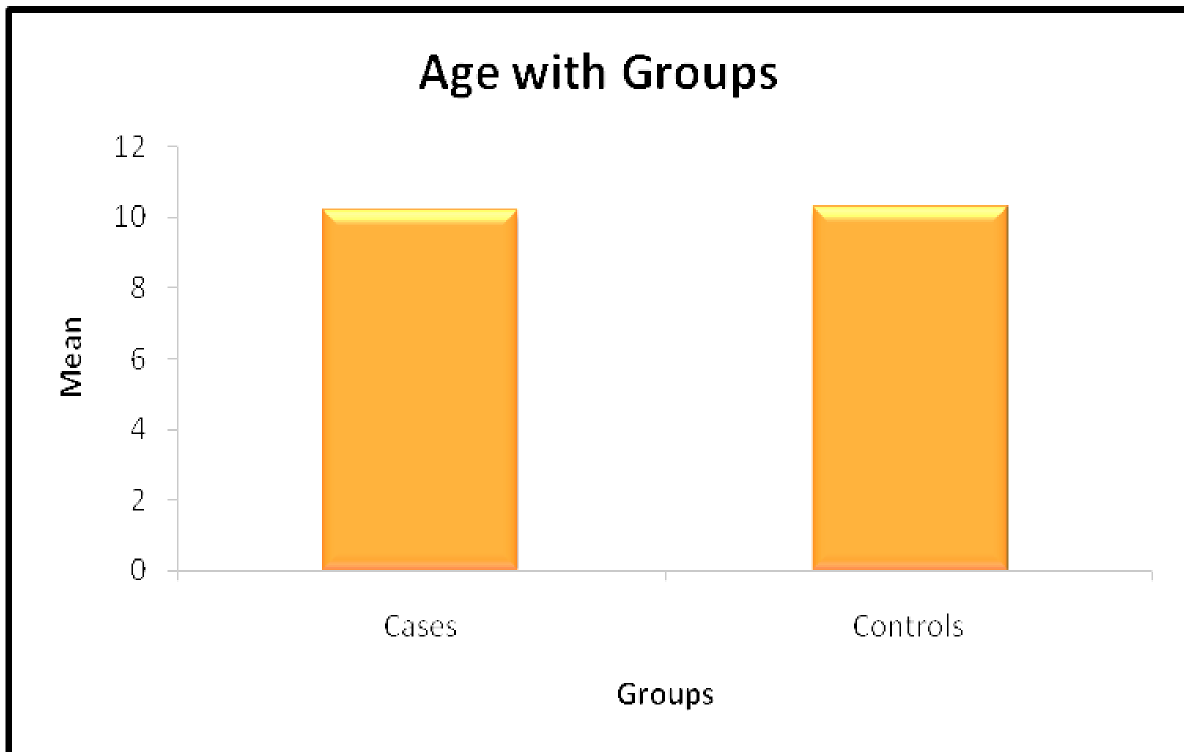

Chart No. 3

The above table shows comparison of Age with Groups shows the mean age of 10 for both the groups .

Table 2: GENDER DISTRIBUTION

Gender	Frequency	Percent
Female	48	48.0
Male	52	52.0
Total	100	100.0

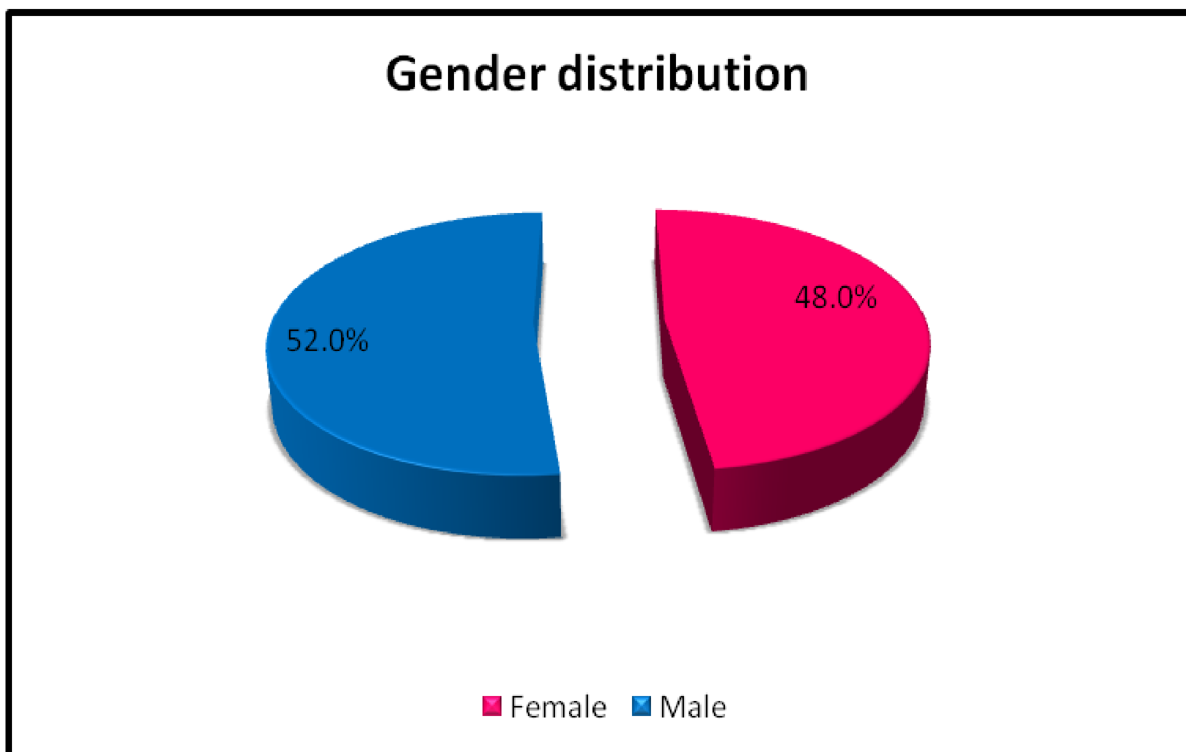

Chart 4

In our study group , 48.0% are Female , 52.0% are Male.

Table 3: GROUP WISE GENDER DISTRIBUTION

			Groups		Total	□ 2 - value	p-value
			Cases	Controls			
Gender	Female	Count	22	26	48	0.641	0.423 #
		%	44.0%	52.0%	48.0%		
	Male	Count	28	24	52		
		%	56.0%	48.0%	52.0%		
Total		Count	50	50	100		
		%	100.0%	100.0%	100.0%		
# No Statistical Significance at $p > 0.05$ level							

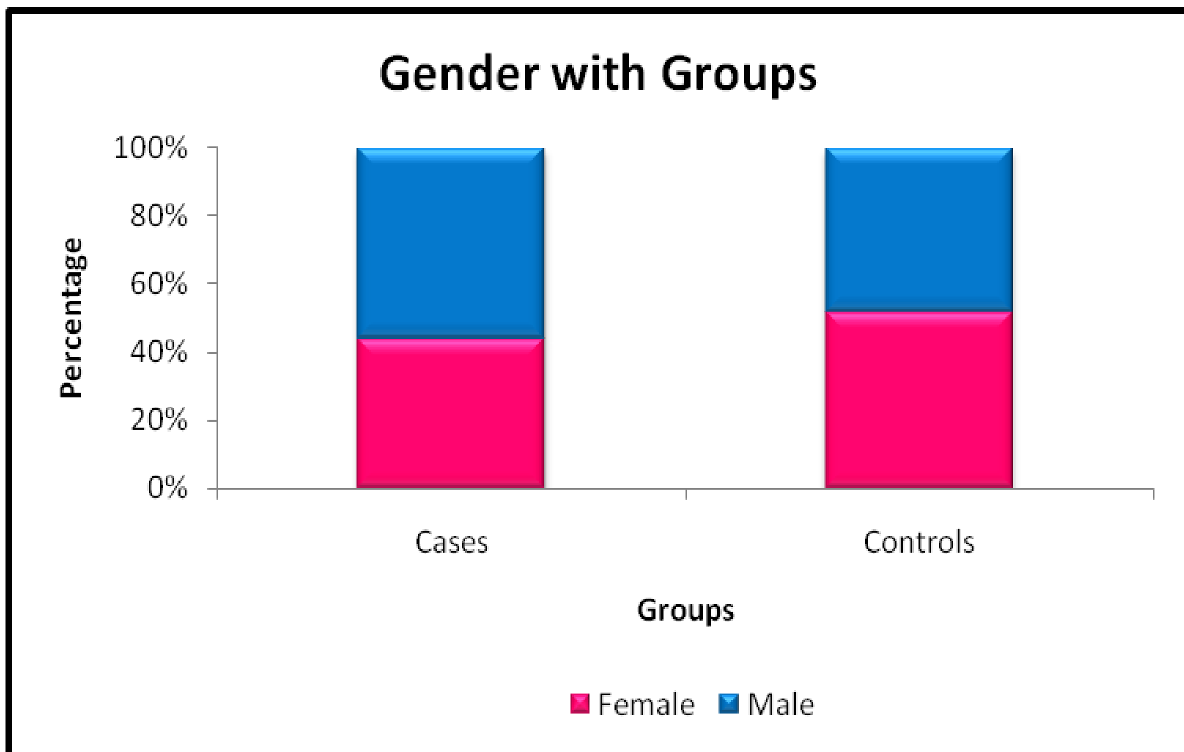

Chart No. 5

The above table shows comparison between gender with groups which shows no statistical significant association

DISTRIBUTION OF FREQUENCY OF BLOOD TRANSFUSION

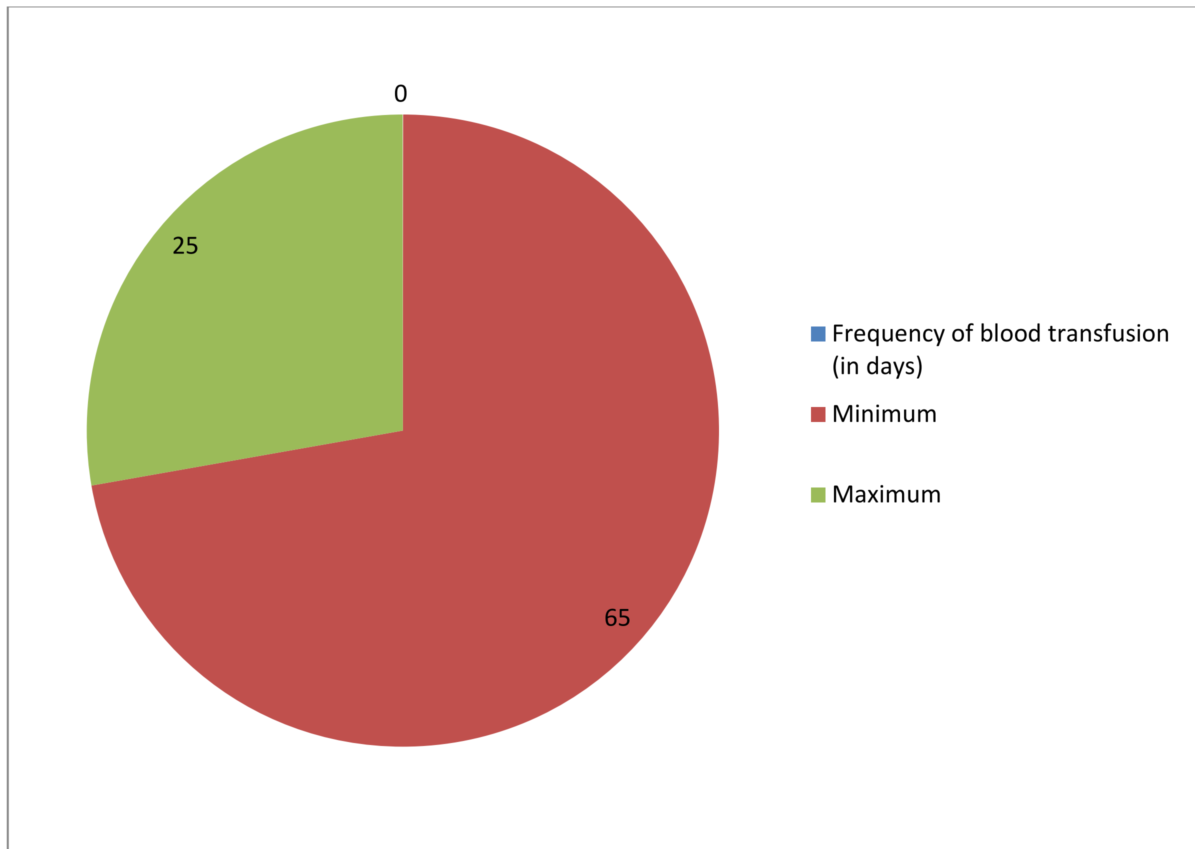

Chart No. 6

The above chart depicts the maximum number of frequency of blood transfusion was done once in 25 days and the minimum number of frequency of blood transfusion was done once in 65 days.

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AGEWISE DISTRIBUTION OF BLOOD TRANSFUSION AMONG STUDY GROUP

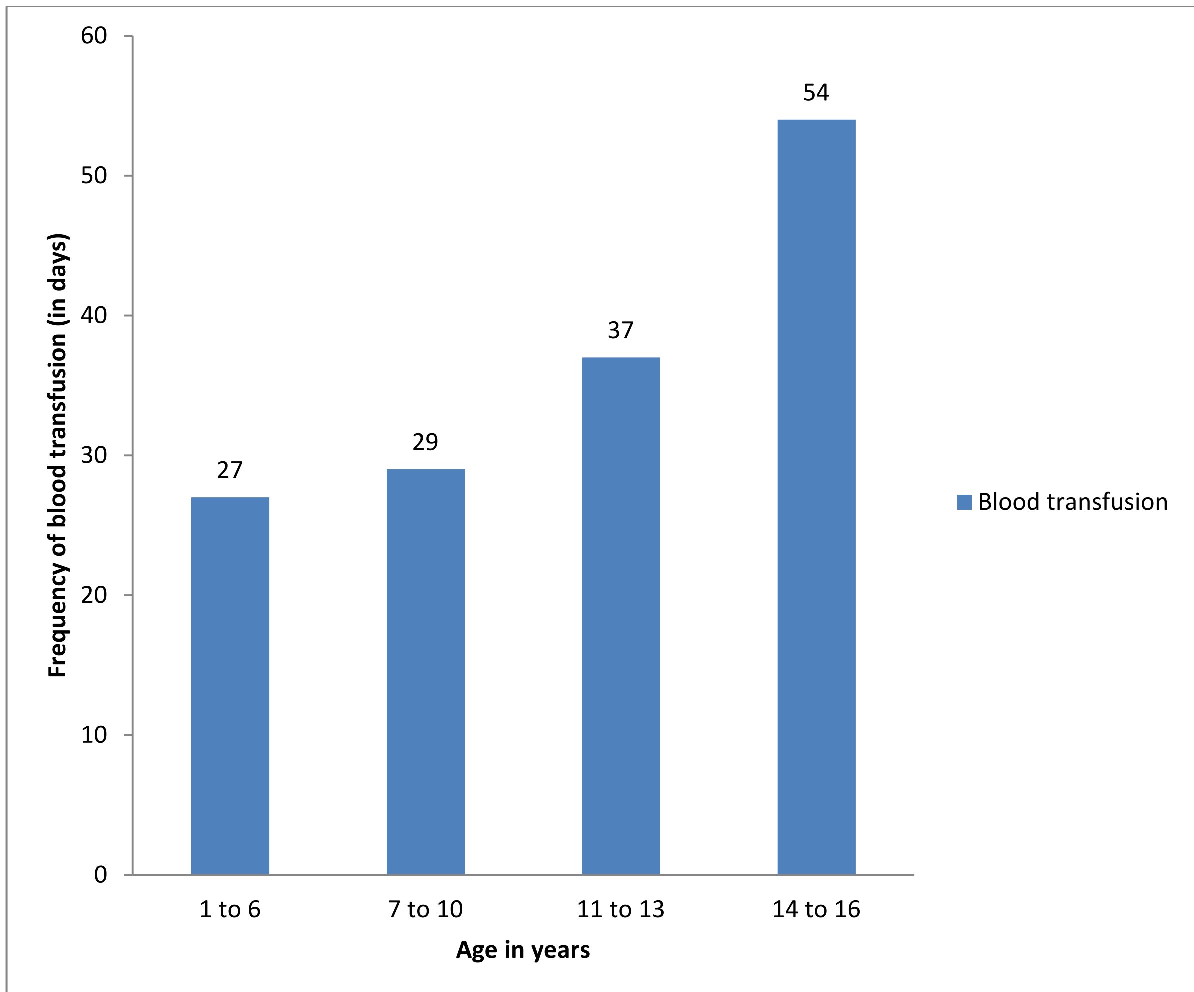

Chart No. 7

The above chart depicts the agewise distribution of blood transfusion among study group. The maximum number of blood transfusion about 54 days was done among the age group of 14 to 16 years. Whereas minimum number of blood transfusion for about 27 days was done among the age group of 1 to 6 years.

Table 4: COMPARISON BETWEEN PERL STAIN TEST WITH GROUPS

			Groups		Total	χ^2 - value	p-value
			Cases	Controls			
Perl stain test	Negative	Count	17	50	67	49.254	0.0005 **
		%	34.0%	100.0%	67.0%		
	Positive	Count	33	0	33		
		%	66.0%	0.0%	33.0%		
Total		Count	50	50	100		
		%	100.0%	100.0%	100.0%		
** HIghly Statistical Significance at p < 0.01 level							

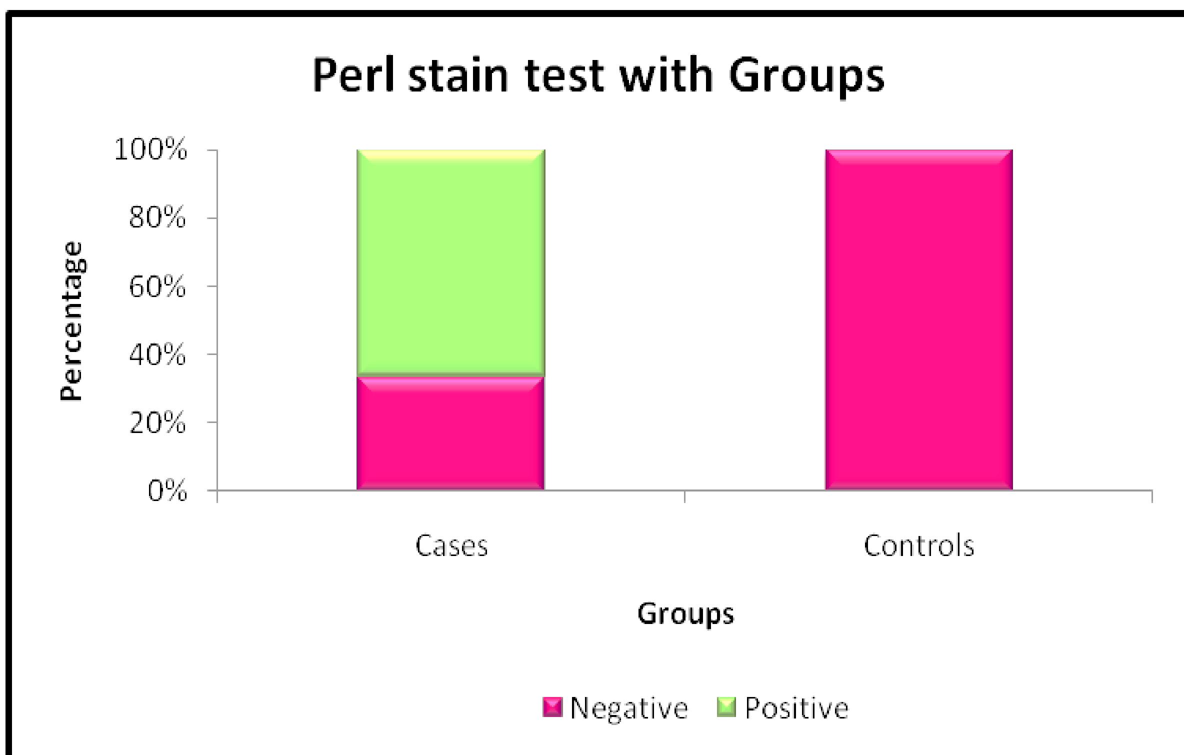

Chart No. 8

The above table shows comparison between Perl stain test with Groups by Pearson's chi-squared test shows p value =0.0005<0.01 which shows highly statistical significant association between Perl stain test and Groups.

Table 5:COMPARISON OF SERUM FERRITIN WITH GROUPS

Variable	Groups	N	Mean	S.D	t-value	p-value
Serum Ferritin	Cases	50	1952.7	677.4	20.133	0.0005 **
	Controls	50	73.6	10.8		
** Highly Statistical Significance at $p < 0.01$ level						

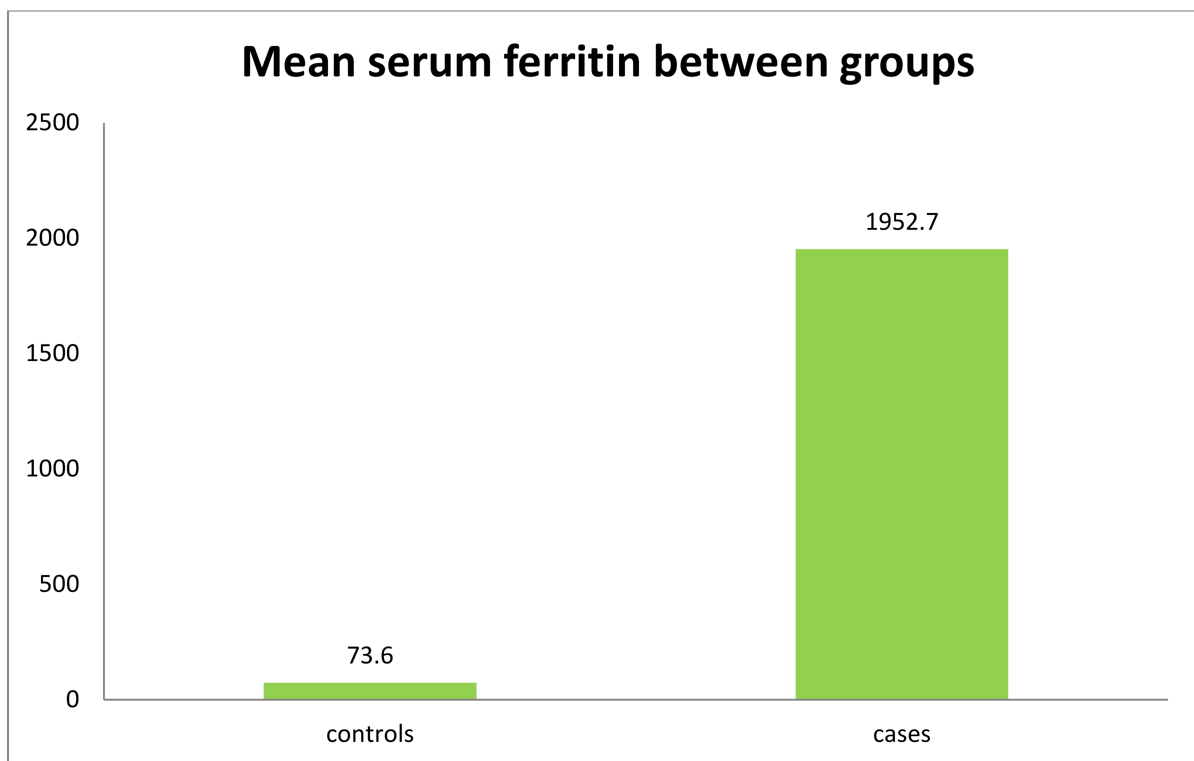

Chart No. 9

The above table shows comparison of Serum Ferritin with Groups by Unpaired t-test were t-value=20.133, p value=0.0005<0.01 which shows highly statistical significant difference between Serum Ferritin and Groups.

AGEWISE DISTRIBUTION OF MEAN SERUM FERRITIN LEVEL AMONG STUDY GROUP.

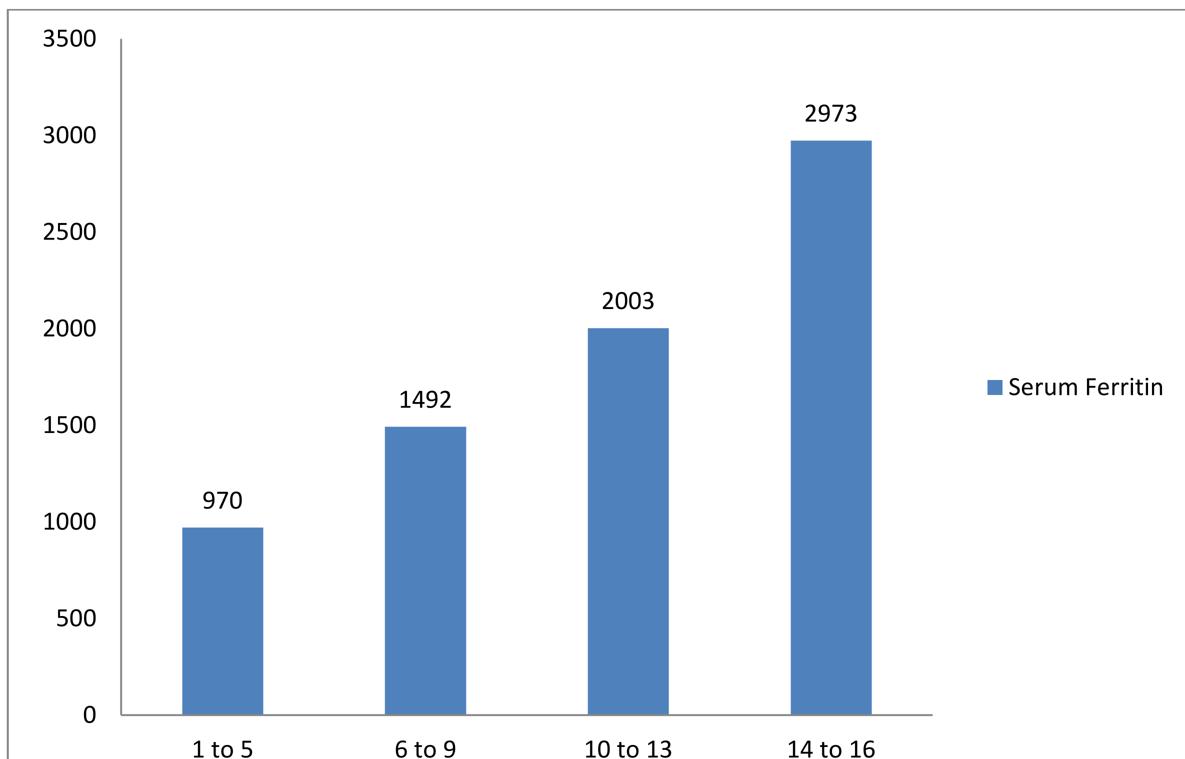

Chart No. 10

The above chart depicts the agewise distribution of mean serum ferritin level among study group. The highest mean serum ferritin level of 2973 ng/ml was found among the age group of 14 to 16 years. The lowest mean serum ferritin level of 970 ng/ml was found among the age group of 1 to 5 years.

DISCUSSION

Thalassemia are group of inherited disorder of haemoglobin synthesis caused by genetic mutation that reduce the synthesis of either alpha or beta chain of haemoglobin.

In our study mainly focuses on patients with β-thalassemia major. The β-thalassemias are widespread throughout the Mediterranean region, Africa, the Middle east, the Indian subcontinent and Burma, Southeast Asia, Southern China, the Malay peninsula, and Indonesia[4].

Thalassemia affects approximately 4 % of every 10,000 live birth throughout the world[4]. It is about 1.5 % of global population are carriers of beta thalassemia [18]

β-Thalassemia syndromes are a group of hereditary disorders characterized by a genetic deficiency in the synthesis of β-globin chains. In the homozygous state, β-Thalassemia major causes severe transfusion dependent anemia. Due to imbalance of globin chain synthesis and excess unpaired alpha chain aggregate to form precipitates that damage red cell membranes, resulting in intravascular hemolysis.

Premature destruction of erythroid precursors result in intramedullary death and ineffective erythropoiesis. The profound anemia is typically associated with erythroid hyperplasia and extramedullary hematopoiesis. Hence to overcome the effects of severe anemia, patients with beta thalassemia major undergoes regular red blood cell transfusion.

With multiple blood transfusion leading to progressive iron overload in the parenchyma is mainly due to the consequences of increased gastrointestinal absorption

of iron, ineffective erythropoiesis and lack of physiological mechanism to excrete the excess iron from the body[7].

Normally, an individual requires 1-2 mg/ L of elemental iron physiologically, and it is available in daily diet. On each unit of packed red cell contain 200-300mg iron. On repeated blood transfusion leads to increased accumulation of iron in the parenchyma causing multiorgan failure and signs of iron overload usually seen after 10 -12 transfusion[7].

As iron overload is an unavoidable complications of beta thalassemia major and transfusional hemosiderosis is the major cause of morbidity and late mortality in this patients.

In healthy normal person the storage iron gets distributed equally in the reticuloendothelial cells(spleen, liver, bone marrow) but in the transfused iron is first gets deposited within the reticuloendothelial cells before the parenchymal overload. The storage iron occur in two forms as ferritin and hemosiderin.

Normally ferritin predominates over the hemosiderin. As the hemosiderin is more stable storage pool and less mobilized for haemoglobin formation and gets stored in hepatocytes. But in iron overload state the hemosiderin increases to a greater degree than ferritin[3].

Effective management of iron overload requires evalution of iron storage frequently. The iron status of body in iron overload can be assessed by different methods

- Invasive methods –As liver is the major site for iron overload accounting 70% of body iron content. Liver iron correlates with total body iron in transfusional iron overload. Estimation of liver iron concentration by liver biopsy gives a accurate of estimating iron overload.
- Non invasive methods – serum ferritin level, serum transferritin, total serum iron concentration, other imaging method like computed tomography, MRI.

Though estimation of serum iron concentration is an easy and non-invasive procedure when compared to biopsy, serum iron in ascorbic acid deficiency iron overloaded patients is relatively low as compared to tissue iron stores.

In our study, exfoliated cells from the buccal mucosa of 50 β- Thalassaemia major patients comprising the study group who had undergone a minimum of 10 transfusions and a control group containing 50 normal individuals who had not have any disease like acute and chronic liver damage, malignancy, megaloblastic anemia and the smears obtained were stained with Perls Prussian blue stain.

Perls Prussian blue reaction is considered to be the classical histochemical reaction. When the tissue is treated with acid ferrocyanide solution results in the unmasking of ferric iron in hemosiderin in the form of ferric hydroxide by diluted HCL. Then ferric iron then react with diluted potassium ferrocyanide solution to produce an insoluble blue compound ferric ferrocyanide(Prussian blue).

Although ferritin may present in the cells but cannot be visualized under light microscope but hemosiderin because of its larger size can be visualized under light microscope as blue coloured granules in the cytoplasm of the cells[9].

In our study exfoliated cells from buccal mucosa of 33 (66%)out of 50 beta thalassemia major patients group revealed positivity for Perls Prussian blue reaction and none of our control group showed positivity for Perls Prussian blue stain. Pearson's chi-squared test were applied showed p value<0.01 which shows highly statistical significant association between Perl stain test and Groups.

Table 6 : Comparison of Perls stain positivity with similar studies

S.NO	STUDIES	POSTIVE CASES	% POSITIVITY
1	Gururaj and Sundaram et al	10/10	100%
2	Nandprasad et al	65/100	65%
3	Atul A Bhat et al	43/60	71.7%
4	Ajit Singh Rathore et al	29/35	82.9%
5	Present study	33/50	66%

Detection of serum ferritin is most commonly used method for assessment of iron overload and monitoring the response of treatment and it is an indirect measurement of iron burden in the body. But the level of serum ferritin can be influenced by others conditions like infections, inflammation, ascorbate deficiency

and hence it requires serial measurements and interpretation with other investigations of iron overload[3].

In our study serum ferritin level of study group of 50 beta thalassemia major patients was increased markedly than the control group. The mean ferritin level of our study group is 1952ng/mL. The comparison of serum ferritin level with the study group and control group by Unpaired t-test showed a t-value=20.133, p value =0.0005<0.01 which shows highly statistical significant difference between the Groups.

Table 7: Comparison of mean serum ferritin level with similar studies

S.NO	STUDIES	MEAN FERRITIN LEVEL
1	Atul A Bhat et al[3]	4544ng/mL
2	Ajit Singh Rathore et al[7]	3714 ng/mL
3	Sonam gupta et al	586 ng/mL
4	Present study	1952 ng/mL

Similar study conducted by Atul A Bhat et al in the year 2013 who have reported mean ferritin level of 4544ng/mL. In the study conducted by Ajit Singh Rathore et al in the year 2016 who reported mean ferritin level of 3714ng/mL. In the study conducted by Sonam gupta et al in the year 2014 who reported mean ferritin level of 586ng/mL

FACTORS AFFECTING THE IRON OVERLOAD IN THE STUDY GROUP ARE DEPEND UPON THE

- Number of blood transfusions
- Age of intiation of iron chelation therapy/ regular therapy
- Socioeconomic status
- Nutritional deficiencies
- Other comorbidities.

SUMMARY

The present study titled as" A **Study of iron overload using oral exfoliative cytology in beta thalassemia major patients"** was undertaken during the period of November 2018 to June 2020 in Government Mohan Kumaramangalam Medical College and Hospital, Salem, Tamilnadu.

- Out of 100 cases in the study, 52 were male and 48 are female.
- The maximum percentage of patients fall between the age group of 6-13 yrs.
- The agewise distribution of blood transfusion among study group. The maximum number of blood transfusion about 54 days was done among the age group of 14 to 16 years. Whereas minimum number of blood transfusion for about 27 days was done among the age group of 1 to 6 years
- The maximum number of frequency of blood transfusion was done once in 25 days and the minimum number of frequency of blood transfusion was done once in 65 days.
- The agewise distribution of mean serum ferritin level among study group. The highest mean serum ferritin level of 2973 ng/ml was found among the age group of 14 to 16 years. The lowest mean serum ferritin level of 970 ng/ml was found among the age group of 1 to 5 years
- In this study serum ferritin level of study group of 50 beta thalassemia major patients was increased markedly than the control group. The mean ferritin level of in the study group is 1952ng/mL.

- In this study exfoliated cells from buccal mucosa of 33 (66%) out of 50 beta thalassemia major patients group revealed positivity for Perls Prussian blue reaction and none of our control group showed positivity for Perls Prussian blue stain.

CONCLUSION

The objective of this study was to establish iron overload using oral exfoliative cytology as an screening test in β-thalassemia major patients undergoing repeated blood transfusions in comparison with serum ferritin level. The presence of iron in exfoliated squamous cells in patients with iron overload has been demonstrated qualitatively and not quantitatively. Serum ferritin levels could not be correlated to the amount of Perls Prussian blue reaction in oral exfoliated cells to demonstrate iron overload.

By considering the simplicity and non invasive method and further studies on correlating Perls Prussian blue reaction with serum ferritin levels and MRI images can be used as an ideal non invasive screening method in all the patients who are undergoing repeated blood transfusions and also use to assess future complications associated with iron overload and thereby ensure proper management.

COLOUR PLATES

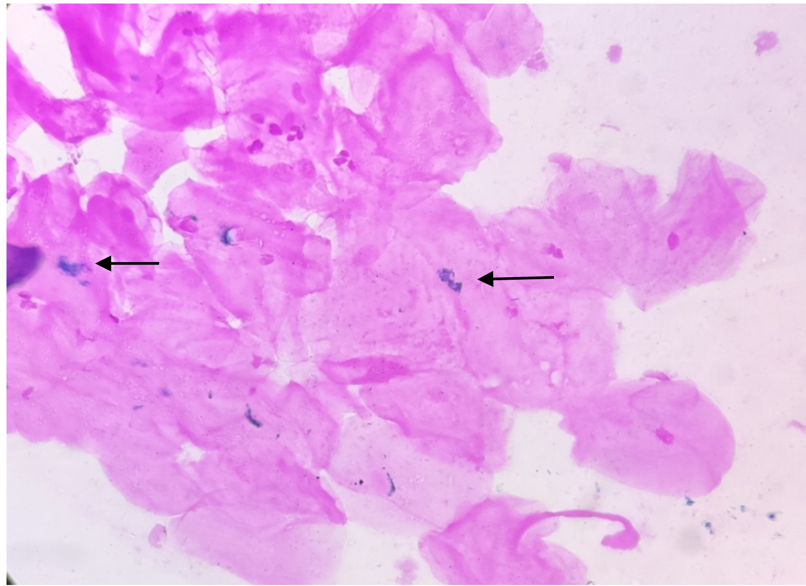

Image 1

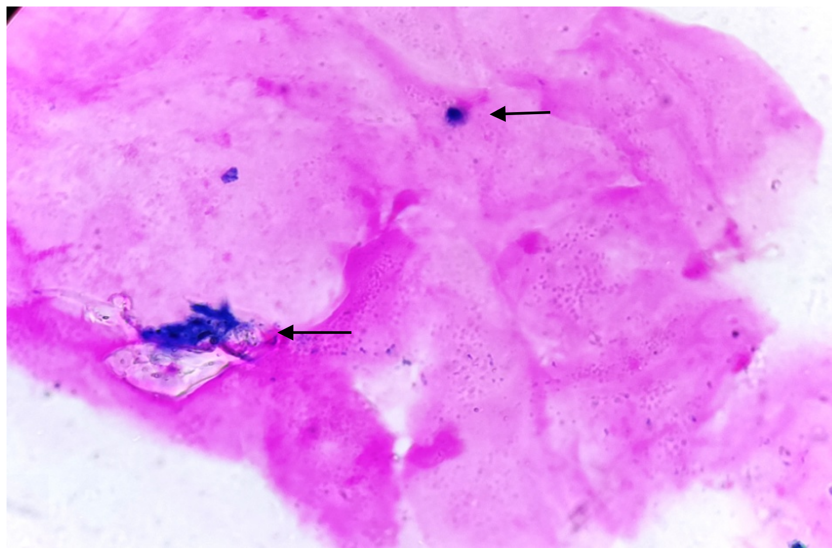

Image 2

Both the Image 1 & 2 : Oral exfoliative sqaumous cells showing positive staining for Perls Prussian blue(40 X)

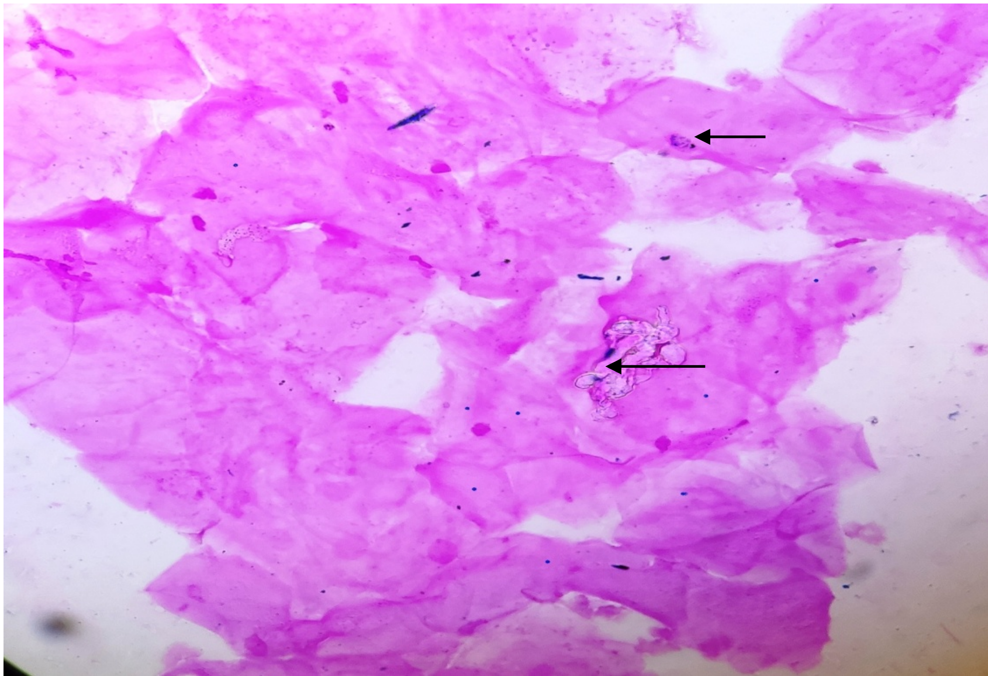

Image 3 : Oral exfoliative squamous cells showing positive staining for Perls Prussian blue(40 X)

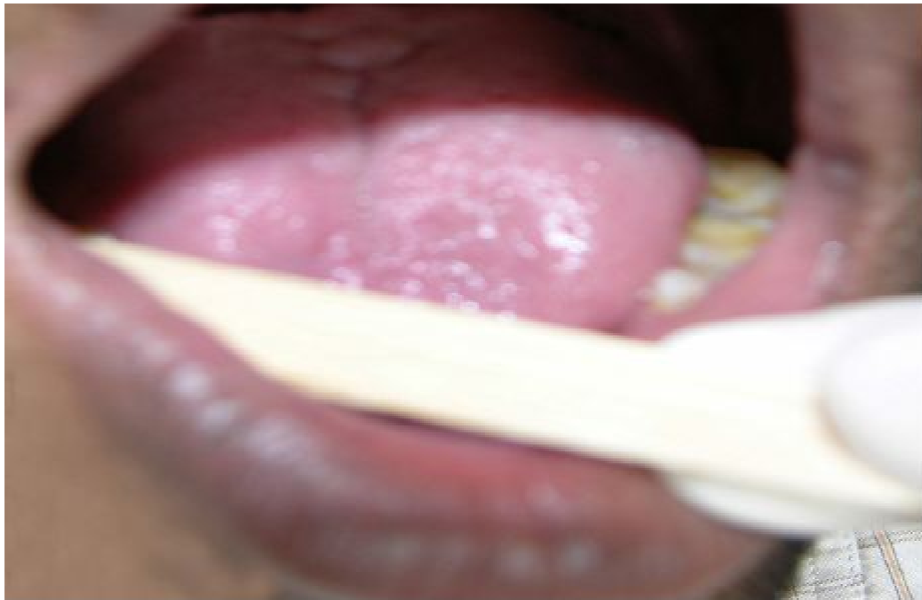

Image 4 : Showing method of obtaining buccal smear

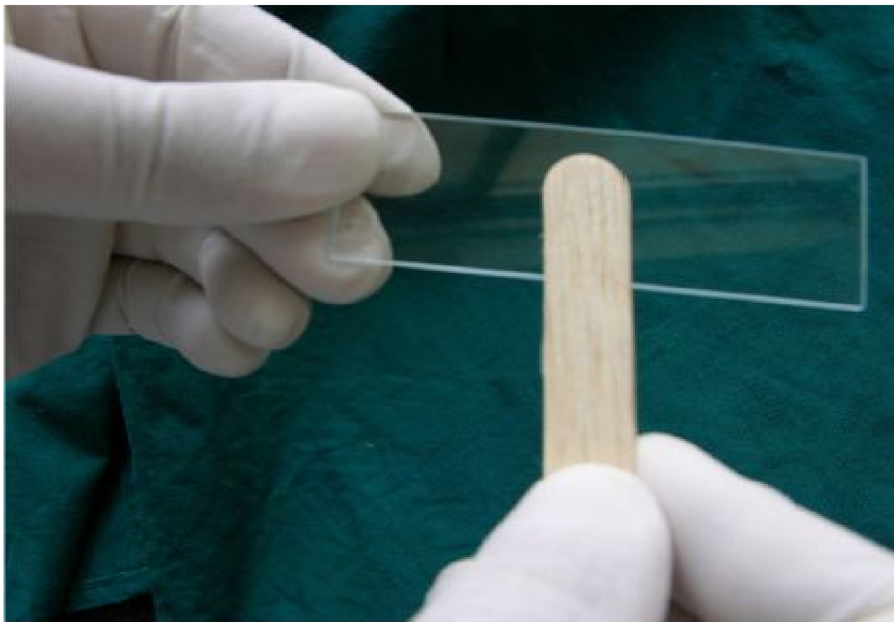

Image 5 – Showing method of smearing of the material in the microscopic glass slide

Image 6 : Materials used in Perls Prussian blue stain

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ANNEXURE I

PATIENT CONSENT FORM

STUDY TITLE:

"A STUDY OF IRON OVERLOAD USING ORAL EXFOLIATIVE CYTOLOGY IN BETA THALASSEMIA MAJOR PATIENTS".

PARTICIPANT NAME: AGE: SEX:

I.P. NO:

I confirm that I have understood the purpose of the above study. I have the opportunity to ask the question and all my questions and doubts have been answered to my satisfaction.

I have been explained about the possible complications that may occur during and after medical procedure. I understand that my participation in the study is voluntary and that I am free to withdraw at any time without giving any reason.

I understand that investigator, regulatory authorities and the ethical committee will not need my permission to look at my health records both in respect to the current study and any further research that may be conducted in relation to it, even if I withdraw from the study. I understand that my identity will not be revealed in any information released to third parties or published, unless as required under the law. I agree not to restrict the use of any data or results that arise from the study.

I hereby consent to participate in this study.

Time: Patient name;

Date:

Signature / Thumb Impression of Patient:

Place

Name and signature of the Investigator

ஆராய்ச்சி ஒப்புதல் படிவம்

பெயர்	:	தேதி	:
வயது	:	உள்நோயாளி எண்	:
பாலினம்	:	ஆய்வு சேர்க்கைஎண்	:

.

இந்த ஆய்வின் நோக்கம் மற்றும் விவரங்கள் முழுமையாக எனக்கு தெளிவாக விளக்கப்பட்டது. இவ்வாய்வில் இருந்து நான் எந்த நேரமும் பின்வாங்கலாம் என்பதையும் அதனால் எனக்கு எந்த பாதிப்பும் இல்லை என்பதையும் தெளிவாக புரிந்து கொண்டேன்.

முடிவுகளை அல்லது கருத்துகளை வெளியிடும் போதோ அல்லது ஆய்வின் போதோ என்னுடைய பெயரையோ அல்லது அடையாளங்களையோ வெளியிட மாட்டார்கள் என்பதையும் அறிந்து கொண்டேன்.

இந்த ஆய்வில் எவ்வித நிர்பந்தமும் இன்றி எனது சொந்த விருப்பத்தின் பேரில் நான் பங்கு பெறுகின்றேன்.

நான் சுயநினைவுடனும் முழு சுதந்திரத்துடனும் இந்த மருத்துவ ஆராய்ச்சியில் சேர்த்துக்கொள்ள சம்மதிக்கின்றேன்.

ஆராய்சியாளர் ஒப்பம்

பங்கேற்பாளர் ஒப்பம்

(அ)

இடது பெருவிரல் ரேகை

ANNEXURE- II

PROFORMA

GOVERNMENT MOHAN KUMARAMANGALAM MEDICAL COLLEGE

DEPARTMENT OF PATHOLOGY

SALEM

PARTICULARS OF THE PATIENT :

Name : IP/OP.No :

Age : Ward.No :

Sex : Occupation :

Address:

Presenting complaints:

Family history:

General Physical Examination:

Febrile/Afebrile:

Pallor :

Jaundice :

Lymphadenopathy:

Splenomegaly:

Serum ferritin level-

MASTER CHART

S.NO	C.NO	AGE	SEX	FREQUENCY OF BLOOD TRANSFUSION	SITE OF SCRAPPING	SERRUM FERRITIN ng/ml	PERL STAIN TEST	PERL STAIN CONTROL
1	C22/19	7	MALE CHILD	35 days	BUCCAL MUCOSA	1750	POSITIVE	POSITIVE
2	C34/19	8	FEMALE CHILD	30 days	BUCCAL MUCOSA	1620	POSITIVE	POSITIVE
3	C39/19	10	FEMALE CHILD	31 days	BUCCAL MUCOSA	2350	POSITIVE	POSITIVE
4	C40/19	6	FEMALE CHILD	28 days	BUCCAL MUCOSA	940	NEGATIVE	POSITIVE
5	C54/19	7	MALE CHILD	30 days	BUCCAL MUCOSA	1324	NEGATIVE	POSITIVE
6	C67/19	10	MALE CHILD	28 days	BUCCAL MUCOSA	1896	POSITIVE	POSITIVE
7	C88/19	11	FEMALE CHILD	35 days	BUCCAL MUCOSA	2560	POSITIVE	POSITIVE
8	C89/19	11	FEMALE CHILD	40 days	BUCCAL MUCOSA	2350	POSITIVE	POSITIVE
9	C94/19	6	MALE CHILD	28 days	BUCCAL MUCOSA	1280	NEGATIVE	POSITIVE
10	C101/19	7	FEMALE CHILD	35 days	BUCCAL MUCOSA	1360	NEGATIVE	POSITIVE
11	C121/19	12	MALE CHILD	45 days	BUCCAL MUCOSA	1780	POSITIVE	POSITIVE
12	C128/19	11	FEMALE CHILD	37 days	BUCCAL MUCOSA	1934	POSITIVE	POSITIVE
13	C138/19	8	FEMALE CHILD	30 days	BUCCAL MUCOSA	1330	NEGATIVE	POSITIVE
14	C144/19	9	MALE CHILD	40 days	BUCCAL MUCOSA	1846	POSITIVE	POSITIVE
15	C175/19	8	MALE CHILD	35 days	BUCCAL MUCOSA	1730	POSITIVE	POSITIVE
16	C194/19	11	MALE CHILD	30days	BUCCAL MUCOSA	2428	POSITIVE	POSITIVE
17	C221/19	11	FEMALE CHILD	35days	BUCCAL MUCOSA	1654	POSITIVE	POSITIVE
18	C243/19	6	MALE CHILD	28 days	BUCCAL MUCOSA	1200	NEGATIVE	POSITIVE
19	C258/19	14	FEMALE	55 days	BUCCAL MUCOSA	3230	POSITIVE	POSITIVE
20	C287/19	15	MALE	60 days	BUCCAL MUCOSA	3940	POSITIVE	POSITIVE
21	C301/19	13	MALE	35 days	BUCCAL MUCOSA	2304	POSITIVE	POSITIVE
22	C344/19	10	FEMALE CHILD	28 days	BUCCAL MUCOSA	2520	POSITIVE	POSITIVE
23	C373/19	14	MALE	48 days	BUCCAL MUCOSA	2856	POSITIVE	POSITIVE
24	C432/19	8	MALE CHILD	30 days	BUCCAL MUCOSA	1600	NEGATIVE	POSITIVE
25	C478/19	9	MALE CHILD	20 days	BUCCAL MUCOSA	2340	POSITIVE	POSITIVE

S.NO	C.NO	AGE	SEX	FREQUENCY OF BLOOD TRANSFUSION	SITE OF SCRAPPING	SERRUM FERRITIN ng/ml	PERL STAIN TEST	PERL STAIN CONTROL
26	C502/19	10	FEMALE CHILD	45 days	BUCCAL MUCOSA	1564	NEGATIVE	POSITIVE
27	C554/19	15	MALE	60 days	BUCCAL MUCOSA	3540	POSITIVE	POSITIVE
28	C645/19	7	MALE CHILD	28 days	BUCCAL MUCOSA	1354	NEGATIVE	POSITIVE
29	C689/19	16	FEMALE	65 days	BUCCAL MUCOSA	3497	POSITIVE	POSITIVE
30	C690/19	8	MALE CHILD	40 days	BUCCAL MUCOSA	1584	POSITIVE	POSITIVE
31	C754/19	11	MALE CHILD	35 days	BUCCAL MUCOSA	1800	POSITIVE	POSITIVE
32	C807/19	13	FEMALE	35 days	BUCCAL MUCOSA	2357	POSITIVE	POSITIVE
33	C842/19	10	MALE CHILD	48 days	BUCCAL MUCOSA	1758	NEGATIVE	POSITIVE
34	C878/19	15	FEMALE	60 days	BUCCAL MUCOSA	2980	POSITIVE	POSITIVE
35	C908/19	9	MALE CHILD	25 days	BUCCAL MUCOSA	1450	NEGATIVE	POSITIVE
36	C939/19	13	FEMALE	40 days	BUCCAL MUCOSA	1980	POSITIVE	POSITIVE
37	C989/19	16	FEMALE	35 days	BUCCAL MUCOSA	2897	POSITIVE	POSITIVE
38	C1006/19	10	MALE CHILD	30 days	BUCCAL MUCOSA	1746	POSITIVE	POSITIVE
39	C1107/19	8	FEMALE CHILD	28 days	BUCCAL MUCOSA	1830	NEGATIVE	POSITIVE
40	C1129/19	6	MALE CHILD	25 days	BUCCAL MUCOSA	950	NEGATIVE	POSITIVE
41	C1132/19	14	FEMALE	45 days	BUCCAL MUCOSA	1960	POSITIVE	POSITIVE
42	C1144/19	11	FEMALE CHILD	35 days	BUCCAL MUCOSA	1750	POSITIVE	POSITIVE
43	C34/20	12	MALE CHILD	50 days	BUCCAL MUCOSA	1890	POSITIVE	POSITIVE
44	C50/20	11	MALE CHILD	30 days	BUCCAL MUCOSA	1740	POSITIVE	POSITIVE
45	C62/20	7	FEMALE CHILD	25 days	BUCCAL MUCOSA	1570	NEGATIVE	POSITIVE
46	C64/20	8	MALE CHILD	30 days	BUCCAL MUCOSA	1350	NEGATIVE	POSITIVE
47	C87/20	10	MALE CHILD	48 days	BUCCAL MUCOSA	1696	POSITIVE	POSITIVE
48	C94/20	5	MALE CHILD	25 days	BUCCAL MUCOSA	970	NEGATIVE	POSITIVE
49	C102/20	15	FEMALE	60 days	BUCCAL MUCOSA	1860	POSITIVE	POSITIVE
50	C121/20	9	MALE CHILD	35 days	BUCCAL MUCOSA	1440	NEGATIVE	POSITIVE

S.NO	C.NO	AGE	SEX	SITE OF SCRAPPING	SERRUM FERRITIN ng/ml	PERL STAIN TEST	PERL STAIN CONTROL
51	C124/20	6	FEMALE CHILD	BUCCAL MUCOSA	15	NEGATIVE	POSITIVE
52	C138/20	8	MALE CHILD	BUCCAL MUCOSA	35	NEGATIVE	POSITIVE
53	C140/20	12	MALE	BUCCAL MUCOSA	65	NEGATIVE	POSITIVE
54	C143/20	7	FEMALE CHILD	BUCCAL MUCOSA	25	NEGATIVE	POSITIVE
55	C147/20	8	FEMALE CHILD	BUCCAL MUCOSA	30	NEGATIVE	POSITIVE
56	C152/20	11	MALE CHILD	BUCCAL MUCOSA	38	NEGATIVE	POSITIVE
57	C153/20	13	FEMALE CHILD	BUCCAL MUCOSA	67	NEGATIVE	POSITIVE
58	C157/20	11	MALE CHILD	BUCCAL MUCOSA	20	NEGATIVE	POSITIVE
59	C165/20	7	FEMALE CHILD	BUCCAL MUCOSA	13	NEGATIVE	POSITIVE
60	C189/20	7	FEMALE CHILD	BUCCAL MUCOSA	15	NEGATIVE	POSITIVE
61	C190/20	9	MALE CHILD	BUCCAL MUCOSA	18	NEGATIVE	POSITIVE
62	C201/20	10	FEMALE CHILD	BUCCAL MUCOSA	26	NEGATIVE	POSITIVE
63	C221/20	10	FEMALE CHILD	BUCCAL MUCOSA	13	NEGATIVE	POSITIVE
64	C223/20	9	MALE CHILD	BUCCAL MUCOSA	18	NEGATIVE	POSITIVE
65	C229/20	7	FEMALE CHILD	BUCCAL MUCOSA	15	NEGATIVE	POSITIVE
66	C267/20	14	MALE	BUCCAL MUCOSA	70	NEGATIVE	POSITIVE
67	C268/20	11	FEAMLE CHILD	BUCCAL MUCOSA	12	NEGATIVE	POSITIVE
68	C292/20	8	MALE CHILD	BUCCAL MUCOSA	26	NEGATIVE	POSITIVE
69	C294/20	13	MALE	BUCCAL MUCOSA	24	NEGATIVE	POSITIVE
70	C297/20	14	FEMALE CHILD	BUCCAL MUCOSA	50	NEGATIVE	POSITIVE
71	C302/20	13	FEMALE CHILD	BUCCAL MUCOSA	25	NEGATIVE	POSITIVE
72	C309/20	6	FEMALE CHILD	BUCCAL MUCOSA	25	NEGATIVE	POSITIVE
73	C310/20	13	MALE	BUCCAL MUCOSA	64	NEGATIVE	POSITIVE
74	C313/20	10	MALE CHILD	BUCCAL MUCOSA	14	NEGATIVE	POSITIVE
75	C323/20	9	FEMALE CHILD	BUCCAL MUCOSA	24	NEGATIVE	POSITIVE

S.NO	C.NO	AGE	SEX	SITE OF SCRAPPING	SERRUM FERRITIN ng/ml	PERL STAIN TEST	PERL STAIN CONTROL
76	C324/20	12	FEMALE CHILD	BUCCAL MUCOSA	35	NEGATIVE	POSITIVE
77	C327/20	14	MALE	BUCCAL MUCOSA	54	NEGATIVE	POSITIVE
78	C334/20	8	FEMALE CHILD	BUCCAL MUCOSA	15	NEGATIVE	POSITIVE
79	C337/20	14	FEMALE CHILD	BUCCAL MUCOSA	38	NEGATIVE	POSITIVE
80	C340/20	10	FEMALE CHILD	BUCCAL MUCOSA	11	NEGATIVE	POSITIVE
81	C342/20	12	MALE	BUCCAL MUCOSA	48	NEGATIVE	POSITIVE
82	C343/20	11	MALE CHILD	BUCCAL MUCOSA	24	NEGATIVE	POSITIVE
83	C353/20	9	FEMALE CHILD	BUCCAL MUCOSA	11	NEGATIVE	POSITIVE
84	C357/20	14	MALE CHILD	BUCCAL MUCOSA	76	NEGATIVE	POSITIVE
85	C348/20	10	MALE	BUCCAL MUCOSA	30	NEGATIVE	POSITIVE
86	C354/20	12	FEMALE CHILD	BUCCAL MUCOSA	15	NEGATIVE	POSITIVE
87	C358/20	16	MALE	BUCCAL MUCOSA	82	NEGATIVE	POSITIVE
88	C360/20	11	MALE CHILD	BUCCAL MUCOSA	18	NEGATIVE	POSITIVE
89	C362/20	10	FEMALE CHILD	BUCCAL MUCOSA	25	NEGATIVE	POSITIVE
90	C364/20	7	MALE CHILD	BUCCAL MUCOSA	39	NEGATIVE	POSITIVE
91	C365/20	12	FEMALE CHILD	BUCCAL MUCOSA	33	NEGATIVE	POSITIVE
92	C373/20	10	FEMALE CHILD	BUCCAL MUCOSA	15	NEGATIVE	POSITIVE
93	C374/20	10	MALE CHILD	BUCCAL MUCOSA	24	NEGATIVE	POSITIVE
94	C382/20	12	FEMALE CHILD	BUCCAL MUCOSA	55	NEGATIVE	POSITIVE
95	C383/20	8	MALE CHILD	BUCCAL MUCOSA	54	NEGATIVE	POSITIVE
96	C387/20	9	FEMALE CHILD	BUCCAL MUCOSA	24	NEGATIVE	POSITIVE
97	C389/20	11	MALE CHILD	BUCCAL MUCOSA	38	NEGATIVE	POSITIVE
98	C391/20	6	MALE CHILD	BUCCAL MUCOSA	22	NEGATIVE	POSITIVE
99	C394/20	14	FEMALE CHILD	BUCCAL MUCOSA	85	NEGATIVE	POSITIVE
100	C397/20	9	FEMALE CHILD	BUCCAL MUCOSA	40	NEGATIVE	POSITIVE