CHAPTER 1

INTRODUCTION
1.1 Introduction

The thalassemias are a diverse group of genetic blood diseases. Thalassemia is the most common inherited single gene disorder. In which body is not able to make enough normal hemoglobin and the life of red blood cells is much shorter (10-15 days) as compared to the normal 120 days. This leads to gradual and progressive anemia that does not respond to any type of conventional treatment. The condition is diagnosed around the age of 8 to 10 months when the child looks pale, weak and lethargic.

The word thalassemia was derived from two Greek words – ‘Thalassa’ meaning the sea and ‘haima’ meaning blood. The first description was written by Dr. Thomas Benton Cooley in 1925 hence it was coined Cooley’s anemia. Cooley’s anemia was a fatal microcytic anemia of children of Mediterranean descent. Countries like Italy, Greece and Cyprus have the highest frequency of Thalassemia cases in world. These exists a thalassemic “belt” that includes the Mediterranean passing through west and central Asian countries like Turkey, Iran, Afghanistan onto Pakistan and India and passes on to the south East Asian countries like Indonesia, Burma and Thailand.

Thalassemia is caused by an abnormality of hemoglobin, the red protein that carries oxygen from the lungs to the tissues. People with thalassemia disease make an abnormal hemoglobin. Structurally hemoglobin consist of α and β chain. Chromosome 16 contains the genes for the all important α chain. The genes for β chain are on chromosome 11. Mutations in the genes that code for the alpha or beta chains potentially.

The globin moiety of haemoglobin (Hb) molecule is composed of seven differ-
ent types of polypeptide chains, varying in number and arrangement of amino acids during different stages of human intrauterine development and are designated by Greek letters alpha (α), beta (β), gamma (γ), delta (δ), epsilon (ε) and zeta (ζ). Epsilon, zeta and some alpha chains are synthesized in early embryonic life, alpha and gamma chains in foetal life and alpha, beta chains predominate in the postnatal life. The beta, gamma and delta chains of human hemoglobins have highly conserved primary structures. In earliest embryonic (foetal) life, zeta and epsilon chains combine to form Hb Gower II (α2ε2) and zeta and gamma chains form Hb Portland (ζ2γ2). The alpha and zeta chains contain 141 amino acid residues and the beta, gamma, delta and epsilon chains contain 146 amino acid residues.

Clinical features: Each major hemoglobinopathy occurs both in a heterozygous form. In the heterozygous state, two principal types of thalassemias alpha and beta are due to a reduced rate of synthesis of alpha and beta chains of hemoglobins 9,10.

Alpha-chain variant haemoglobins

Alpha chains are involved in the formation of HbA, HbA2 and HbF. Heterozygotes for alpha-chains variants produce both normal and abnormal HbA, HbF and HbA212,13. Normal subjects have two linked alpha gene loci on the short arm of chromosome 16, thus giving an alpha gene haplotype of αα and genotype of αα/αα. Thalassemia is most common due to deletions of one or more of these genes. The alpha thalassemia in the world have been reviewed by Higgs and Weatherall.

Work on molecular genetics has provided clear evidence that the alpha-thalassaemia showing a complete absence of alpha chain production results in severe form of alpha thalassaemia, called α0 thalassaemia, whereas the mild form of alpha thalassaemia having only a partial deficit of alpha chain production is called α+ thalassaemia. Both α0 and α+ thalassaemias can result from several different
molecular defects involving the alpha globin gene cluster. The α^0-thalassaemias result from a series of gene deletions which involve both alpha globin genes. The α^+ thalassaemias result from deletions of one of the linked pairs of alpha globin genes or from a series of non-deletion defects in which the alpha globin genes are present, but their output is reduced. There are common structural haemoglobin variants which are synthesized at a reduced rate and produce the clinical phenotype of α^+ thalassemia. The commonest of these are haemoglobin Constant Spring and Koya Dora which have been reported from the populations of Southeast Asia and Andhra Pradesh in India.

**Alpha-thalassemia trait**: Alpha-thalassemia trait is asymptomatic and is difficult to diagnose with certainty in adult life, except using gene-mapping studies.

**Alpha-thalassaemia 2 (α^+ thalassaemia)**: This represents the heterozygous state for α^+ thalassaemia (αα/-α). During neonatal period, affected infants may have 1-2% Hb-Bart's which they gradually lose over the ensuing months. In adult life, the haemoglobin pattern is normal, and Hb-H inclusions are not found at any stage. Haemoglobin level and blood film are normal, although the mean cell volume (MCV) and mean cell haemoglobin (MCH) may be mildly reduced.

**Alpha-thalassaemia 1 (α^0 thalassaemia)**: This represents the heterozygous state for α^0 (-/αα) or the homozygous state for α^+ thalassaemia (-α/-α). During neonatal period, 5-6% Hb-Bart's is found, but the haemoglobin pattern is normal in later life. Hb-H inclusions are usually present in very small numbers. The haemoglobin level is normal or mildly reduced, but the red cells are usually mildly hypochromic and microcytic, and the MCV and MCH are reduced. HbA2 is reduced in some cases.

**Haemoglobin-H disease**: Interaction of the α^+ (-α/) and the αα (-/-) determinants gives rise to this form of alpha-thalassaemia (-α/-). Clinically, Hb-H disease is
characterized by a moderate anaemia with a haemoglobin level of 8–9 g/dl, mild jaundice and physical findings similar to that of beta-thalassaemia major. The reticulocytosis ranges between 5 and 10%. After incubation of red cells with brilliant cresyl blue, many ragged inclusion bodies form due to the redox action of the dye, causing precipitation of HbH. The haemoglobin pattern consists of Hbs A, H and A2 with variable amounts of Hb Bart’s, the A2 being reduced to 1.5–2%. It is very unstable and likely to be precipitated at room temperature. On haemoglobin electrophoresis, Hb-H and Bart’s migrate more rapidly than HbA at an alkaline pH.

The severity of anaemia fluctuates during pregnancy or intercurrent infection, etc. Splenomegaly is present in 85% of patients and cholelithiasis is also common. Blood film shows marked red cell morphological changes, including severe hypochromia, microcytosis and target cell formation. Red cells are nucleated. There is a mild reticulocytosis. Numerous Hb-H inclusions with brilliant cresyl blue stain and large Heinz body-like inclusions are also present after the splenectomy. The haemoglobin pattern consists of 2–40% Hb-H, the remainder being HbA, HbA2 (which is reduced) and HbF. HbH disease has a small amount of an alpha-chain variant, Hb Constant Spring. Neonates with Hb-H disease have 25% Hb-Bart’s. Haemoglobin Constant Spring occurs at extremely low levels in heterozygous carriers, usually less than 1% of the total haemoglobin. It migrates more slowly than HbA2 on alkaline haemoglobin electrophoresis and tends to break up into 2–3 separate bands. Family studies show the alpha thalassaemia trait in one parent and the Hb Constant Spring trait in the other. Individuals homozygous for Hb Constant Spring have slightly hypochromic red cells with normal MCV.

The earliest case of Hb-H was recorded in a Bengaliac subject from Calcutta. Subsequently, five more such instances have been found in Bengalites (4%).
The proportion of Hb-H in these varied from 12 to 25%. In none of the parents of these index patients was the anomaly detected, while Hb-H thalassaemia was found in the siblings\textsuperscript{25,26,27,28}.

Hb-H was reported by Swarup et al. in a 19-year old Bengalee Hindu from India. Similar cases (1%) were reported from Mumbai \textsuperscript{15,16}. Saha and Banerjee reported two cases of Hb-H traits among Malayalis, and one each among Tamils, Gujaratis and Sindhis in Singapore. Mishra et al. reported 15% haemoglobin Constant Spring (both heterozygotes and homozygotes) among the coastal people of Orissa.

\textit{Haemoglobin-Bart’s hydrops foetalis:} The most severe manifestation of the alpha-thalassaemia gene is haemoglobin Bart’s hydrops foetalis. The compound heterozygous state for both $\alpha^0$ thalassaemia and $\alpha^+\text{thalassaemia}$, or $\alpha^0\alpha^+\text{thalassaemia}$ and Hb Constant Spring, results in Hb-H disease. The homozygous state for $\alpha^+\text{thalassaemia}$ causes a very mild anaemia with hypochromic red cells and no change in the haemoglobin pattern\textsuperscript{19}. The homozygous state for Hb Constant Spring is characterized by a mild haemolytic anaemia with splenomegaly. Affected infants are homozygous for the $\alpha^0$ determinant (\(-/-\)) , both parents having heterozygous $\alpha^0\alpha^0$-thalassaemia. There is total suppression of alpha-chain synthesis with a gross excess of gamma chains. The gamma-chain tetramer, Hb-Bart’s, has a high oxygen affinity, and results in severe tissue hypoxia. Affected infants are either born dead (stillborn between 28 and 40 weeks gestation) or die within a few hours of birth\textsuperscript{30,31}. They are underweight, pale (haemoglobin values in the range of 6–8 g/dl), mildly jaundiced, grossly edematous and have hepatosplenomegaly and ascites. The haemoglobin is around 6 g/dl, the blood film is grossly abnormal with
anisopoikilocytosis, hypochromia, target cells, polychromasia and a large number of nucleated red cells. The reticulocyte count is high and serum bilirubin elevated. The haemoglobin pattern consists of 80–90% Hb-Bart's, with a small amount of Hb-H and Hb-Portland. There is usually no HbA, HbA2 or HbE. In West Bengal, four out of 100 cord blood samples obtained from newborns showed Hb Bart's. In one isolated case with Hb Barts at birth, Hb-H thalassaemia was recorded at the age of 2 years. Incidence of Hb Bart's in 2% of cord blood samples was reported by Chouhan et al.15 and 4.2% by Vora et al.21 from Mumbai. Mishra reported 7.7% haemoglobin Bart's in the cord blood samples among the people of north-western Orissa and, sub-sequently, 12.6% among the people of coastal Orissa.

1.2 Pathophysiology

A decrease in the rate of production of certain globin chains (alpha, beta, gamma, delta) impedes Hb synthesis and creates an imbalance with the other, normally produced globin chains. Since two types of chains pair with each other to form normal Hbs, an excess of the normally produced type is present and accumulates in the cell as an unstable product leading to the destruction of the cell. This imbalance is the hallmark of all forms of thalassemia. For this reason, most thalassemias are not considered hemoglobinopathies, because the globin chains are normal in structure, and the defect is limited to a decreased rate of production of these normal chains. The type of thalassemia usually carries the name of the underproduced chains. The reduction varies from a slight decrease to complete absence of production. When beta chains are produced at a lower rate, the thalassemia is termed beta+, whereas beta-0 thalassemia indicates a complete absence of production of beta chains from involved allele. The consequences of impaired production of globin chains ulti-
mately result in less Hb being deposited in each RBC, leading to hypochromasia. The Hb deficiency causes RBCs to be smaller, leading to the classic hypochromic and microcytic picture of thalassemia. This is true in almost all anemias caused by impairment in production of either of the two main components of Hb, heme or globin. However, this does not occur in the silent carrier state, since both Hb level and RBC indices remain normal\textsuperscript{43}.

In the most common type of beta thalassemia trait, the level of A2 Hb (delta2/alpha2) usually is elevated. This is due to the increased utilization of delta chains by the excessive free alpha chains resulting from lack of adequate beta chains with which to pair. The delta gene, unlike beta and alpha genes, is known to have a physiologic limitation in its ability to produce adequate delta chains; by pairing with the alpha chains, delta chains produce Hb A2, some but not all of the excessive alpha chains are used to form Hb A2 with the delta chains, while the remaining alpha chains precipitate in the cells, reacting with cell membranes, intervening with normal cell division, acting as foreign bodies, leading to destruction of RBCs. In other types of beta thalassemia traits, the mutation is not limited to the beta gene but extends to the adjacent delta gene; thus, no elevation of Hb A2 is expected, and instead, gamma chains are activated, resulting in elevation of Hb F. Less common are the types associated with elevation of both Hbs A2 and F. In the severe forms, such as beta thalassemia major of Cooley anemia, the same pathophysiology applies with significant exaggeration. The significant excess of free alpha chains brought about by the deficiency of beta chains causes destruction of the RBC precursors in the bone marrow i.e. ineffective erythropoiesis\textsuperscript{46,47,48}. To understand the genetic changes that result in thalassemia, it should be very necessary to understand the physiologic process of globin chain production in the healthy individual. The globin chain as a
unit is a major building block for Hb: together with heme, it produces the Hb molecule. Two different pairs of globin chains form a tetrameric structure with a heme moiety in the center. All normal Hbs are formed from two alpha chains and two non-alpha chains. Various types of Hb are formed, depending on the types of chains pairing together. Such Hbs exhibit different oxygen binding characteristics, normally related to the oxygen delivery requirement at different developmental stages of life. In embryonic life, zeta chains combine with gamma chains to produce Hb Portland (zeta2/gamma2) and with epsilon chains to produce Hb Gower-1 (zeta2/epsilon2). Subsequently, when alpha chains are produced, they form Hb Gower-2 pairing with epsilon chains (alpha2/epsilon2). Fetal Hb is composed of alpha2/gamma2 and the primary adult Hb of alpha2/beta2. A third physiologic Hb, known as Hb A2, is formed by alpha2/delta2 chains.

1.3 Molecular biology

All the genes that control the production of globin chains lie within 1 of 2 clusters located on different chromosomes. Chromosome 11 is the site of five functional betalike globin genes arranged in a link cluster over 60 kilobases. From left to right (5' – 3') they are epsilon/gamma-G/gamma-A/delta/beta. Gamma-G and gamma-A differ by only one amino acid (alanine vs glycine). A critical control region of the delta globin gene i.e. promoter region is known to be defective; it inhibits messenger RNA processing, resulting in only a small amount of Hb A2 (alpha2/delta2) production, which thus accounts for less than 3% of total Hb in adult RBCs. The alphalike globin gene cluster is located on chromosome 16. It consists of three functional genes. Form left to right (5' – 3'), they are zeta/alpha2/alpha1.

Each globin gene consists of a string of nucleotide bases divided into 3 cod-
ing sequences termed exons and 2 noncoding regions known as introns or intervening sequences. Three other regions, known as regulatory regions, also exist in the 5 noncoding or flanking region of each globin gene. The first is the promoter, which plays a major role in the transcription of the structural genes. The second region is the enhancer, which has an important role in promoting erythroid specific gene expression, as well as in coordinating the changes in globin gene activity at different stages of development (embryonal, fetal, adult). Enhancers are able to influence gene expression, despite being located some distance away from the gene itself and, unlike the promoter, they can stimulate transcription irrespective of their orientation relative to the transcription start site. Finally, master regulatory sequences known as locus control regions and HbS40 (in the alpha gene complex) are responsible for activating the genes in erythroid cells.

Each of these regulatory sequences has a modular structure, consisting of short nucleotide motifs that act as binding sites for transcriptional activator or suppressor molecules. Such molecules activate or suppress gene expression in different cell types at different stages of development. Transcription of a certain gene is achieved by an initiation complex formed of certain proteins and a number of transcription factors, which interact with binding sites on the promoters and other regulatory sequences of the relevant genes. When a gene is transcribed, mRNA is synthesized form one of the gene’s DNA strands by the action of RNA polymerase. The initial product is a large mRNA precursor, the introns ultimately are subsequently eliminated, and the exons are spliced together in the nucleus. At this stage, the mRNA, which also has been modified at both 5’ and 3’ ends, moves to the cytoplasm to act as a template for the production of globin chains.

Mutations resulting in beta or alpha thalassemia are similar in principle but
different in their patterns. More than 150 different mutations are known to result in various types of beta thalassemia. Major deletions are unusual in contrast to alpha thalassemia, and most of the encountered mutations are either single base changes, small deletions, or insertions of 1-2 bases at a critical site along the gene. These mutations occur in both exons and introns. This leads to the production of short, nonviable beta chains.\textsuperscript{73,74,75,76,77,78} Conversely, in the frame shift mutation, one or more bases on the exon are lost or inserted, resulting in a change in the reading frame of the genetic code or the production of a new stop codon. RNA splicing mutations are fairly common and represent a large portion of all mutations resulting in beta thalassemia. These mutations corrupt the splicing process. The importance of precise splicing in the quantitative production of stable functional mRNA cannot be overemphasized. Slippage by even one nucleotide changes the reading frame of the mRNA. Specific consensus sequences exist at both ends of the RNA introns specifically at the junction with the exons, these motifs include GT in the 5' consensus sequences are obligatory for correct splicing, and a single substitution at the invariant GT or AG sequences prevents splicing altogether and results in beta-0 or alpha-0 thalassemia. Mutations in the other member of the consensus sequences, even though still highly conserved, result in variable degrees of ineffective beta globin production, causing milder types of beta thalassemia.

Mutations in exon sequences may activate a "criptic" splice site. In exon 1 of the beta globin gene, a consensus sequences that resembles a sequence in IVS-1 has been identified as the site for several distinct mutations, resulting in a gene that carries the features of both thalassemia and hemoglobinopathy at the same time. This type of mutations represents a clear link between the thalassemias and the hemoglobinopathies, and accordingly, these are labeled "thalassemic hemoglobin-
pathies" Thus, mutations at codon 19 (A to G), 26 (G to A), and 27 (G to T) all in exon 1, result in reduced production of mRNA (thalassemia), due to inefficient splicing, and an amino acid substitution, encoded by the mRNA that is spliced and translated into protein.\textsuperscript{10,40,81,82,83}

The flanking regions of the beta globin gene are also sites for various mutations. A single base substitution involving the promoter element, if there is down-regulation of beta globin gene transcription, resulting in a mild form of beta thalassemia. Conversely, a mutation affecting the 3' end of the beta globin mRNA can interfere with its processing, resulting in a severe form of beta thalassemia. Many different beta thalassemia mutations exist, and compound heterozygosity frequently is encountered. A person who manifests symptoms of beta thalassemia major without an elevated Hb A2, the explanation for such a situation is often co-inheritance of beta and delta thalassemia. Delta/beta thalassemia further is divided into delta/beta + or delta/beta-0. In the first type, a misalignment in the delta-beta genes during meiosis results in the production of fused delta/beta genes, a process responsible for the production of an Hb variant termed Hb Lepore.\textsuperscript{84,85,86} The fused delta/beta gene is under the control of a delta-globin gene promoter region. Since the delta gene promoter carries mutations that lead to ineffective transcription, the fused delta/beta chains are produced in limited amounts, resulting in thalassemia. Conversely, in delta/beta-0 thalassemia, a large deletion takes place in the beta globin gene cluster, removing both the delta and the beta genes, which also can extend to involve all globin genes on chromosome 11, thus producing epsilon, gamma, delta, and beta-0 thalassemia.
1.4 Cellular pathophysiology

The basic defect in all types of thalassemia is imbalanced globin chain synthesis. However, the consequences of accumulation of the excessive globin chains in the various types of thalassemia are quite different. In beta thalassemia, excessive alpha chains, unable to form Hb tetramers, precipitate in the RBC precursors and, in one way or another, produce most of the manifestations encountered in all of the beta thalassemia syndromes. This is not the situation in alpha thalassemia. The excessive chains in alpha thalassemia are gamma chains earlier in life and beta chains later on. Both are able to form homotetramers that, although relatively unstable, nevertheless remain viable and able to produce soluble Hb molecules such as Bart and H. These basic differences in the 2 main types of thalassemia are responsible for the major differences in their clinical manifestations and severity. Alpha chains accumulating in the red cell precursors are insoluble, precipitate in the cell, interact with the membrane causing significant damage, and interfere with cell division. This leads to excessive intramedullary destruction of the red cell precursors. In addition, the surviving cells arriving in the peripheral blood with intracellular inclusion bodies (excess chains) are subject to hemolysis. This means that both hemolysis and ineffective erythropoiesis caused anemia in person with beta thalassemia. The ability of some red cells to maintain the production of gamma chains, which are capable of pairing with some of the excessive alpha chains to produce Hb F, is advantageous. Binding some of the excess alpha chains undoubtedly reduces the symptoms of the disease 87,88,89,90.

Increased production of Hb F in response to severe anemia adds another mechanism to protect the RBCs in patients with beta thalassemia. The elevated Hb F increases oxygen affinity leading to hypoxia, which, together with the profound
anemia, stimulates the production of erythropoietin. As a result, severe expansion of
the ineffective erythroid mass leads to severe bone expansion and deformities. Both
iron absorption and metabolic rate increase, adding more symptoms to the clinical
and laboratory manifestations of the disease. The large number of abnormal red
cells processed by the spleen, together with its hematopoietic response to the ane-
mia if untreated, results in massive splenomegaly, leading to manifestations of hy-
persplenism.

1.5 Purpose of the work
Thalassemia is not a disease of “just a few people or communities or countries”. It is
world wide problem. In India, near about 10,000 children are born with thalassemia
major. In terms of cost of ideal maintenance of these children a staggering Rs. 150
crores is required to be spent every year. By now we are aware about the cause,
epidemiology, the implication and the treatment of thalassemia. Fortunately the dis-
order can be controlled very effectively with relative ease. Countries around the
Mediterranean – Italy, Cyprus have been able to prevent birth of children with thalas-
semia major in the preceding decade by sustained efforts by government as well as
NGOs. In our country what is required is a National Programme of public aware-
ness to begin with. Aim of the present study if for the awareness can be followed by
ways to prevent birth of children with thalassemia major carrying out blood screen-
ing test to detect “carriers”. In Amravati region, need arises for some communities
to screen there population. Because in previous study undertaken by different re-
searchers was not detected any heterogeneity from different communities placed in
Amravati. Communities from this region should take definite steps to get their
younger members screened for detecting “carriers”. Couples with thalassemic chil-
dren may need to know if their other siblings may suffer from the disorder. Present work is a small effort taken in this direction to screen the population from Amravati region as well as to prevent birth of children with thalassemia major and to find out the most affected community from Amravati region.