The thalassemias are diverse group of inherited disorders of hemoglobin synthesis and most common of all single-gene disorders in the world population. There are over 21 million carriers of β-thalassemia gene in India [1]. On the basis of absence or reduction in the synthesis of a particular type of polypeptide chain, thalassemia is classified into α, β, γ and δ variants and its subtypes [2]. β-thalassemia and its combination with abnormal hemoglobins is widespread (incidence about 3%) in the Indian subcontinent [3,4] and appears to show predominance in certain communities, where the incidence recorded about 6-10 percent.

β-thalassemia is the most common single gene disorder but is markedly heterogeneous at the molecular level. Over 180 different mutations that cause β-thalassemia have been reported till now [5]. Different populations have their own spectrum of mutations. In India 28 mutations have been identified of which 5 are common and account for about 90 percent cases [6]. Hemoglobin E disease is widely prevalent in South-East Asia [7]. In India, there is a high prevalence of this gene in the North-Eastern region [8].

The incidence of these genetic disorders can be reduced markedly by genetic counselling and prenatal diagnosis. For effective genetic counselling the population at risk needs to be identified. An accurate and reliable diagnosis of β-thalassemia in both heterozygous and homozygous states is an absolute requirement for prevention and adequate management of this disorder. Determination of red cell indices, hemoglobin electrophoresis and HbA2 level estimation can be used for identification of β-thalassemia heterozygote [9].

Future prospects for treatment of thalassemia major includes bone marrow transplantation, which has already been tried in a few patients and is curative if the marrow transplant is successfully carried out. Other approaches, which are still in experimental stage, include gene therapy, in which a normal β-globin gene is transferred into the bone marrow of an individual with β-thalassemia major. This can only be feasible when high-level expression of β-globin can be achieved [10].

In normal situation, equal amounts of α and β globin chains are produced, so they are able to combine stoichiometrically generating appropriate hemoglobin tetramers. But in thalassemia there is relative deficiency or total absence of either α or...
β globin chains. Alpha thalassemia is characterized by relative deficiency of α-globin chains when production of β-globin chains remains unperturbed.

In β-thalassemia, β-globin chains are deficient. Under these circumstances, α-globin chain is in excess and is also capable of forming homotetramers. These tetramers, however, are highly insoluble and precipitate within red blood cells leading to their premature destruction in the bone marrow and marked trapping in the spleen.

The genetics of β-thalassemia is complicated by large number of mutations that can result in decreased or absent function of β-globin gene. β-thalassemia is markedly heterogeneous at the molecular level [3]. The anemia in beta-thalassemia is caused by combination of ineffective erythropoiesis and hemolysis of adult RBC in the peripheral circulation but premature removal of RBC is yet to be understood [11].

Pathology of the disease arises due to the presence of free α chains, unpaired with β chains, which are unstable and oxidize. They bind to membrane and induce oxidation of lipids, proteins and ultimately lead to molecular cross-linking [12-14].

In normal individuals, free α chains are present in red blood cells in a very small amount, (0.067 ± 0.017%) of Hb. In β-thalassemic trait, α chain pool is only slightly increased in spite of an unequal globin chain synthesis (α/β ratio = 2) [15] suggesting a very efficient removal of soluble α chains by proteolysis in heterozygous β-thalassemia [16,17], than by precipitation because of the absence of significant amounts of insoluble α chains associated with ghosts. But in β-thalassemia major, traces of soluble α-chains able to combine with β-chains to form HbH which have been detected. The pool of soluble α chains observed was (0.26 to 1.30)%, highly different from one patient to another [18].

HbE/β-thalassemia, one of the commonest forms of β-thalassemia worldwide, is characterized by a remarkable and largely unexplained variation in its clinical phenotype, and hence both transfusion dependent and untransfused patients with similar genotypes are available [19,20]. The pattern of globin chain binding to the red blood cell membrane in HbE/ β-thalassemia is similar to thalassemia major, with only α-globin present, and has been confirmed by electron microscopic immuno-cytochemical studies [21].

Oxidative damage to β-thalassemia red blood cells is a major factor in thalassemia RBC Pathology [22,23]. It was shown that unpaired α-hemoglobin chains...
might denature and bind to the cell membrane, thus giving rise to cytoskeleton alterations and lipid peroxidation [24,25].

A number of experimental findings suggested that activated oxygen species might be involved in these reactions. Isolated α or β chains of Hb rapidly autooxidized with concomitant generation of superoxide (O₂⁻). The membrane protein alterations found in β-thalassemia RBC may be stimulated by exposing normal RBC to free radical generating system in vitro [26].

Oxidative damage induced by free globin chains have been implicated in membrane abnormalities, alteration of membrane transport, such as increased K⁺-Cl⁻ cotransport, Na⁺-Li⁺ counter transport and reduced Na⁺-K⁺-Cl⁻ cotransport. The Na-K pump was increased in severe beta thalassemia cells. The increased K⁺-Cl⁻ cotransport activity has been observed in light and dense fractions of beta thalassemia cells. Thus, oxidative damage represents an important factor in the increased activity of the K⁺-Cl⁻ cotransport observed in thalassemias, and the K⁺ loss observed in beta-thalassemic erythrocyte [27].

Generally the presence of unpaired excess globin chains is the primary circumstance leading to membrane alterations as oxidation of phospholipids, modification of cytoskeletal proteins and their interactions, reduced membrane associated ATPase activities, and enhanced permeability of cations. Such perturbations in turn result in the exposure of outer surface neoantigens, enhanced binding of autoantibodies and complement fixation to the outer red cell surface. These factors contribute to the observed distinctive morphologies, increased rigidity and decreased deformability of the thalassemic red cells [28].

It is really difficult to investigate the relationship between excess alpha globin chains and in vivo oxidation in red blood cells from individuals with beta thalassemia disease, many investigators attempted to understand these mechanisms in ‘model’ beta-thalassemic cells. This ‘model’ beta-thalassemia is the entrapment of unpaired purified alpha-hemoglobin chains within normal erythrocytes. These ‘model’ beta-thalassemic cells generated significantly (P < 0.001) greater amounts of methemoglobin and intracellular hydrogen peroxide than did control cells. This resulted in significant time dependent reduction in protein concentrations and decreased thiol content of spectrin and ankyrin. These abnormalities are correlated with rate of alpha-hemoglobin chain...
autoxidation and appearance of membrane-bound globin. In addition, alpha-hemoglobin chain loading resulted in a direct decrease (38.5%) in catalase activity. Membrane bound heme and iron were also significantly elevated (P < 0.001) in the alpha-hemoglobin chain-loaded cells and lipid peroxidation could be partially inhibited by entrapment of an iron chelator. In contrast, chemical inhibition of cellular catalase activity enhanced the detrimental effects of entrapped alpha-hemoglobin chain [25].

In presence of pro-oxidant drug primaquine, β-thalassemic RBC from patient's peripheral blood produced twice more O₂⁻ than normal RBC [29]. Excess of O₂⁻ may reduce some oxidized iron in the membrane, thus initiating Fenton-type reactions resulting in OH formation [30]. β-thalassemic RBCs are capable of producing increased amounts of hydroxyl radical as compared to normal RBC [31]. Hydroxyl radical generations in the biological systems are suggested to be induced by O₂⁻ and H₂O₂ interaction with iron or copper. It is known that low-molecular weight complexes of reduced iron catalyze lipid peroxidation [32]. In line with that, an increased generation of hydroxyl radical (•OH) and lipid peroxidation were found in sickle RBC [33] which contained much more membrane bound iron than normal RBC but still less than thalassemic RBC [34].

Iron is released in free form when erythrocytes are incubated with a number of oxidizing agents, such as phenylhydrazine [35,36]. Iron is released from hemoglobin [37], the release being accompanied by methemoglobin (Met-Hb) formation under conditions of glutathione (GSH) depletion, lipid peroxidation and hemolysis. A similar release of iron also occurs during erythrocyte aging, experimentally induced by aerobic incubation of calf or human erythrocytes in buffer for 24-60 hour, a model of rapid in vitro aging [38]. Iron release is accompanied by oxidative alterations of membrane proteins as well as by appearance of senescent antigen, as measured by autologous IgG binding. Intracellular chelation of the released iron by chelators, which enter erythrocytes, prevents both membrane protein alterations and senescent antigen formation. Increased amount of IgG with antiband 3 specificity has been found in sickle and hemoglobin Kohn erythrocyte [39] and probably even in β-thalassemic erythrocyte. Both thalassemic and normal red blood cell are found to release the iron when incubated aerobically for 24 hours. However iron release from thalassemic erythrocyte is greater than normal [41].
Iron release and *in vitro* aging are proportionally related [37]. It represents that the mechanism of formation of senescent cell antigen or any other membrane event occurs through free iron release in greater extent and is responsible for the early removal of β-thalassemic erythrocytes from blood stream [41].

In addition to these well-described mechanical abnormalities, β-thalassemic RBCs are phagocytosed by murine monocytes/macrophages at a rate 20 times of normal [42] and by human macrophages at a rate of 2 to 3 times of normal [43]. Several mechanisms have been implicated in the enhanced recognition and phagocytosis of thalassemic RBCs. One hypothesis is that the lower sialic acid level of β-thalassemic RBCs leads to enhanced recognition and phagocytosis [42]. Another observation is that RBCs from β-thalassemia intermedia patients show aggregated clusters of hemichrome and band 3, presumably as a result of oxidant injury. Immunoglobulin (perhaps autologous antibodies) and complement components localize at the membrane exoface over these clusters [40]. Macrophages recognize immunoglobulin as well as complement components and act to remove these damaged cells [43]. Loss of phospholipid (PL) asymmetry has also been implicated in the recognition of RBC. In particular phosphatidyl serine (PS), which in normal cells is strictly confined to the inner half of the membrane phospholipid bilayer, is recognized as a first step before removal *in vitro* [44] and *in vivo* [45]. It has been proposed that PS exposure might play a role both in the removal of β-thalassemic RBCs and the hypercoagulable state thought to exist in severe β-thalassemia intermedia [46]. β-thalassemic red cells induced increased thrombin formation *in vitro* when incubated with purified prothrombinase factors, indicating that a PS-containing surface is available [47].

Recently, a method has been developed to determine the exposure of PS in individual red cells, using fluorescently labelled annexin V (AV) [48] and this approaches suggest that normally maintained asymmetry of the PL bilayer can be disrupted locally. However, based on the rapid, diffusion rates of phospholipids in the plane of bilayer it was hypothesized these domains in case of restricted movement of PS on the surface of the cell. Few data suggests that in some cases PS was rich on the outer surface in areas of α-globin chain accumulation. It has been recently reported that endogenous red cell AV is found in regions where Heinz bodies attach to the plasma membrane [49] thereby suggesting that PS was enriched in these areas in the inner monolayer. Membrane skeletal proteins including spectrin [50] and a band 4.1 [51,52]
have been shown to interact with PS. A change in the distribution or lipid/protein interaction of these proteins could be involved in the local accumulation of PS. However, the underlying mechanism is not known at present.

Free radicals are highly reactive chemical species that are characterized by unpaired electrons. Free radicals react with a variety of substrates, especially with polyunsaturated fatty acids, in a chemical process involving three stages: initiation, propagation, and termination [53].

Most free radical reactions involve the reduction of molecular oxygen, which leads to the formation of highly reactive oxygen species such as superoxide anion (O$_{2}^-$), hydroxyl radical (HO·), hydrogen peroxide (H$_2$O$_2$), and singlet oxygen ($^{1}$O$_2$).

Circulating red cells are extremely susceptible to peroxidation. Conditions that favour peroxidation are seemingly optimal in red cells: the red cell membrane is rich in polyunsaturated fatty acids, the cells are continuously exposed to high oxygen tensions and the cells contain high concentrations of hemoglobin, a powerful catalyst for the initiation of peroxidative reactions [54]. Because red cells are regularly exposed to oxidative stress, it is not surprising that they are equipped with a delicate and complex antioxidant system to defend themselves against toxic oxygen species. This antioxidant system is, in part, a reflection of the presence of protective enzymes and biologic antioxidant [55] that include superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione. These protective enzymes and free radical scavengers work synergistically and are complementary to each other with respect to their function as antioxidants.

If the red cell cannot defend itself adequately against free radicals because of either a defect in its defense system or an overwhelming oxidative insult, it is damaged and prematurely removed from the circulation. In vitro experiments also indicate that certain pathologic red cells are particularly susceptible to free-radical attack.

Since all red cells continuously undergo peroxidative reactions, it is reasonable to speculate that the cumulative effect of peroxidative damage may contribute to the senescence of red cells and the pathophysiology of certain red cell disorders such as sickle cell disease [56] and thalassemia [57]. If red cell damage secondary to peroxidative reactions can be detected in its early stages, therapeutic intervention to prevent further damage may be possible.
In erythrocytes glutathione mediated antioxidant defense system plays a major role for red cell protection. Glutathione (γ-glutamyl-cysteinyl-glycine), the widely occurring tripeptide is found in large amounts in the erythrocytes and has also potential protective role such as protection of hemoglobin and enzyme sulphhydryl groups from oxidation. It has been suggested that glutathione protects intracellular components from oxidative attack and thus plays a significant part in maintaining the viability of erythrocytes. Glutathione present in our body in two states, reduced sulphhydryl form (GSH) and the oxidized form (GSSG) but in our body it is mainly present in its reduced form. It may be oxidized to disulphide (GSSG) by the action of the enzyme glutathione peroxidase (present in the blood) preventing toxic effect of peroxides. GSSG and mixed disulphides with proteins (GSSP) may also be formed non-enzymatically in presence of other oxidants. Under normal conditions, GSSG and GSSP are reduced rapidly to GSH through the action of glutathione reductase. This reaction is coupled with reducing equivalent NADPH. In human erythrocytes the supply of NADPH by the pentose phosphate pathway is dependent on the activity of glucose-6-phosphate dehydrogenase (G6PD).

On the other hand erythrocyte contains considerable amount of superoxide dismutase (SOD), which catalyzes the reaction:

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]

and catalase, which completes the detoxification by eliminating hydrogen peroxide:

\[ 2H_2O_2 \rightarrow 2H_2O + O_2 \]

All these enzymes act in concert which successfully scavenge the superoxide or highly activated oxide or peroxide radicals and can maintain the normal physiological condition.

The erythrocyte membrane and cytoskeleton are highly specialized and are not typical of all cells. The membrane has two principal glycoproteins, glycophorin, which are 64% carbohydrate, and band-3, which is a major transmembranous protein involved in the transport of water and anions and carrier of blood group-I antigen [58]. These are the only integral membrane proteins that are exposed to the outer surface of the membrane, and they provide the erythrocyte with the complex carbohydrate surface. The other integral membrane proteins- spectrin, ankyrin and actin are exposed on the cytoplasmic side of the membrane and form the cytoskeleton of the cell. The cytoskeleton of erythrocyte constitutes a network under the plasma membrane, and...
plasma membrane binds it through the cytoplasmic segments of band-3 protein. The erythrocyte is exposed to significant stresses and its shape is distorted while circulating through the heart, blood vessels and capillaries. Loss of mechanical strength and the resulting deformed shape of erythrocytes in hereditary spherocytosis result from a genetic defect in spectrin that affects the structure of cytoskeleton [59].

The erythrocyte membrane separates extracellular fluid from the cytoplasm. This emphasizes the diversity of the lipid composition of inner and outer membrane. The main lipids in the erythrocyte membranes are phospholipids. This is true of all biologic membranes; the basic structure of membranes is a bilayer composed of phospholipids. In this arrangement the hydrocarbon chains of the phospholipid fatty acyl groups project into the center of the bilayer. The hydrophilic glyceryl-phosphoryl-base components of the phospholipids are called head groups and are located on the outside of the bilayer, where they interact with water or other polar, charged molecules. The lipid bilayer is composed of two leaflets, the outer phospholipid leaflet that faces the extracellular fluid and the inner phospholipid leaflet that faces the cytoplasm [59,60].

The membrane lipid bilayer contains a mixture of phospholipids; most of that are glycerol derivatives called phosphoglycerides. Sphingomyelin is the exception, it is a phospholipid derivative of sphingosine.

The fatty acid composition of the different phospholipids varies considerably. For example, choline phosphoglycerides are rich in palmitic (16:0) and linoleic (18:2) acids, whereas the ethanolamine and serine phosphoglycerides are rich in arachidonic acid (20:4) and the 22-carbon polyunsaturated fatty acids. By contrast, sphingomyelin is rich in saturated fatty acids and 24-carbon fatty acids. The fatty acids also are not evenly distributed between the sn-1 and sn-2 positions of the glycerophosphoric lipids. Saturated fatty acids are more prevalent in the sn-1 position and polyunsaturated fatty acids in the sn-2 position. Mono-unsaturated fatty acids tend to be more evenly distributed among both positions [61].

The amount of cholesterol contained in the various cell membranes differs considerably. All the cholesterol in the plasma membrane is in the free or non-esterified form. Cholesterol is inserted into the lipid bilayer between phospholipid molecules in both leaflets of the lipid bilayer. Its hydroxyl group is oriented towards the aqueous environment and interacts with the polar head groups of the phospholipids. The
nonpolar ring and hydrocarbon tail of cholesterol are positioned so that they interact with the hydrocarbon chains of the phospholipid fatty acyl groups. The planar ring structure of the steroid nucleus penetrates to the depth of about the first 10 carbons of the phospholipid fatty acyl chains.

The fluidity of membrane lipid bilayer is modulated by cholesterol. Cholesterol decreases the fluidity in the region of the membrane that contains many unsaturated fatty acids and increases fluidity in the region composed primarily of saturated fatty acids. Cholesterol forms clustered regions within the membrane lipid bilayer; some areas contain 1 mole of cholesterol per 1 mole of phospholipid, whereas others contain almost no cholesterol. This gives the membrane a patchy effect, with solid regions co-existing with adjacent fluid domains. In this way, areas within a membrane can have very different physical and permeability properties.

The normal red blood cell (RBC) contains approximately 20 mM iron. Under normal conditions most of this iron is within hemoglobin with little or none present as free metal (i.e. non-hemoglobin). This perfect compartmentalization breakdown in certain pathologic states. These include hemoglobin SS disease and the thalassemias; both characterized by elevated levels of free intraerythrocytic iron. Similar abnormalities in iron decompartmentalization and oxidant production have been detected in model β-thalassemic RBC created by entrapment of free α-hemoglobin chains in normal RBC via reversible osmotic lysis. These anomalous (largely membrane-associated) deposits of hemoglobin and iron have been implicated in the pathophysiology of thalassemic RBC.

In model β-thalassemic RBC, significant oxidation of intracellular hemoglobin, accompanied by release of free hemoglobin and iron has been found which could be explained by events, restricted to the entrapped α-hemoglobin chains. These observations implied that the unpaired α-hemoglobin chains destabilize the endogenous normal hemoglobin, thus stimulating its oxidation and triggering the loss of hemoglobin and subsequent release of iron.

In patients with beta-thalassemia, a secondary problem arises due to excess iron accumulation through long term blood transfusions and it results in damage to endocrine organs and heart. Several studies have highlighted the importance of sustained reduction of body iron burden as the principal determinant of clinical outcome of these disorders.
β-thalassemic patients require life long blood transfusion and chelation therapy to promote the excretion of iron accumulated from transfusions. The only iron-chelating agent available for clinical use during last 30 years was deferoxamine (DFO). Its long-term use prevents the complication of iron overload and improves survival of β-thalassemic patients [66]. But main difficulties associated with parental mode of administration (i.e. administration through transfusion pump which is very painful), have mandated a search for safe and effective therapeutic alterations which results in the invention of orally active iron chelators permitted for clinical trials.

The oral iron chelator deferiprone (L1) (1,2-dimethyl 3-hydroxy pyrid-4-one) is currently most widely investigated as an alternative to DFO. Unlike DFO, L1 is orally absorbable and less expensive. The L1 molecule has nine fold greater capacity to permeate cell membrane than DFO. Initial clinical experience with L1 has proved it to be an effective chelator, inducing increased urinary iron excretion, decreased ferritin level and decreased hepatic iron. Recently different toxicities have been reported and the drug is in clinical trial and appears to be effective with an acceptable toxicity profile [67].

All biological activity involves contact between constituent reactants. At the molecular level if one participant is smaller than its complementary partner, the former is usually called ligand and the later is receptor. Deferiprone (L1) being the ligand, extensive investigation is required regarding its interaction with several biomolecules to assess its potential toxicity. Thus to conclude this study, the present investigation has been made to understand the biochemical interaction of deferiprone with hemoglobin, the major and most important protein of red blood cell.

The observations of this present study have been described in the following chapters:

1. Studies on antioxidant defense status of red blood cells of patients with β-thalassemia and Eβ-thalassemia.
2. Studies on the effect of oral iron chelator deferiprone (L1) on membrane bound free iron in thalassemic erythrocytes.
4. Studies of the interaction of oral iron chelator deferiprone (L1) with hemoglobin, the major red blood cell protein.