Introduction

The thalassemias refer to a diverse group of hemoglobin disorders characterized by reduced or absent synthesis of one or more of globin chains. [1] βeta-thalassemia (β-thalassemia) is one of most common autosomal recessive disorders worldwide, high prevalence is present in populations in the Mediterranean, Middle-East, Central Asia, Indian subcontinent, and Far East; it is also relatively common in populations of African descent. The highest incidences are reported in Cyprus (14%), Sardinia (12%), and South East Asia. [2]

The high gene frequency of β-thalassemia in these regions is most likely related to the selective pressure from *Plasmodium falciparum* malaria, as it is indicated by its distribution quite similar to that of present or past malaria endemic. Carriers of β-thalassemia are indeed relatively protected against the invasion of *Plasmodium falciparum*. However, because of population migration, β-thalassemia is at present, also common in Northern Europe, North and South America, Caribbean, and Australia. [3] In Egypt, the estimated carrier rate ranges from 5.3 to 10 % [4] with a gene frequency of 0.03. [5]

Pathophysiology of β -thalassemia

β -thalassemia occurs when there is a quantitative reduction of β globin chains that are usually structurally normal. They are caused by mutations that nearly all affect the β globin locus and are extremely heterogeneous. Almost every possible defect affecting gene expression at transcription or post-transcriptional level, including translation, has been identified in β-thalassemia. These genetic defects lead to a variable reduction in β globin output ranging from a minimal deficit (mild β^+ thalassemia alleles) to complete absence (β^0 thalassemia). [3]

In β-thalassemia, the synthesis of normal α globin chains from the unaffected α globin gene continues as normal, resulting in the accumulation within the erythroid precursors of excess unmatched α globin. The free α globin chains are not able to form viable tetramers and instead precipitate in the red cell precursors in the bone marrow forming inclusion bodies. They are responsible for the extensive intramedullary destruction of the erythroid precursors and hence the ineffective erythropoiesis that underlies all β-thalassemia. [6]

Anemia in β-thalassemia thus results from a combination of ineffective erythropoiesis, peripheral hemolysis, and an overall reduction in hemoglobin synthesis. The severity of disease in β-thalassemia correlates well with the degree of imbalance between α and non-α globin chains and the size of the free α chain pool. Thus, factors that reduce the degree of chain imbalance and the magnitude of α chain excess in the red cell precursors will have an impact on the phenotype.[7]

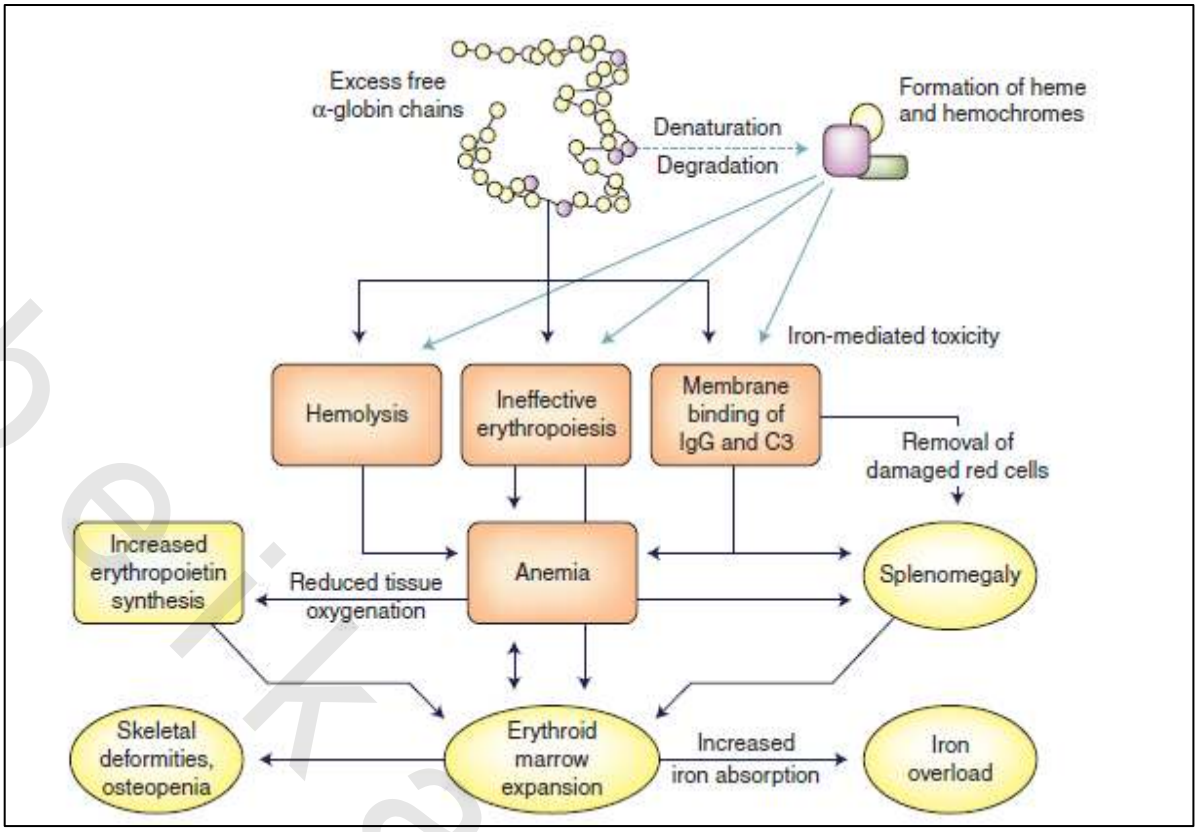


Figure 1. Pathophysiology of β-thalassemia. [6]

Because of the imbalance in chain synthesis, an excess of free α globin chains accumulates within erythroid cells. Aggregation, denaturation, and degradation of these chains lead to the formation of insoluble precipitates which damage cell membranes. Membrane damage leads to ineffective erythropoiesis within the bone marrow, hemolysis of red cells within the circulation, and binding of immunoglobulin and complement components to red cell membranes, triggering loss of red cells in the spleen. The resulting anemia leads to diminished tissue oxygenation, an increase in erythropoietin levels, and further stimulation of the bone marrow. Bone marrow expansion causes skeletal deformities and osteopenia. Substances released from degenerating red cells increase iron absorption, which contributes to iron overload.

Phenotypic diversity of β-thalassemia

The β-thalassemia have extremely diverse clinical phenotypes, at the severe end of the spectrum, many homozygous or compound heterozygous states are characterized by profound anemia from early life that, if not treated with regular blood transfusions, it leads to death in the first year, a condition known as β-thalassemia major (βTM). Conversely, many patients with the same disease have a milder illness that ranges from being only slightly less severe than the major form, through a spectrum of decreasing severity of anemia, to one which is symptomless and is ascertained only by routine examination of the blood. This diverse collection of β-thalassemia of varying severity constitutes the β-thalassemia intermedia. [8]

Even the heterozygous states for β-thalassemia show wide phenotypic diversity. Typically, the inheritance of a single β-thalassemia allele is associated with mild anemia and characteristic morphological changes of the red cells. However, in some cases, the effect of a single β-thalassemia allele can be completely silent with no definable hematological abnormalities, whereas in others it might cause a phenotype as severe as the major forms of the illness — that is, a dominantly inherited form of β-thalassemia.[9]

Mechanisms underlying phenotypic diversity

The remarkable variability in clinical severity of the β-thalassemia reflects both genetic and environmental factors. It is becoming apparent that the genetic element involves many loci, some of which are directly involved with the basic defect in globin synthesis, whereas others, which modify the variable complications of the disease, have nothing to do with globin. For this reason, it is convenient to classify these genetic modifiers into the following groups: Primary, the many different mutations of the β-globin genes that underlie β-thalassemia. Secondary, loci that are also involved in globin synthesis. Tertiary, loci that are not involved in globin production but that might modify the complications of the disease in many different ways. The latter group includes the many different polymorphisms that have been co-selected with the thalassemia and that might further modify their phenotypes. Complications acquired as the result of the primary defect in β-globin synthesis can have a profound effect on the phenotype. Similarly, it is becoming increasingly clear that for at least some forms of the disease, environmental and social factors might also have an important role in modifying individual responses to the different forms of thalassemia. [10]

Primary modifiers

Over 200 different mutations have been identified in the β-globin genes of patients with β-thalassemia. With the exception of a few deletions, the bulk of them consist of point mutations or the loss of one or two bases, which interferes with gene function either at the transcriptional, translational or post-translational levels. There are two main varieties of β-thalassemia alleles, β^0-thalassemia in which the β-globin chains are absent and β^+-thalassemia in which β-globin chain production is variably reduced. The resulting phenotypes reflect the effects of the β^0-thalassemia, and the β^+ or β^{++}-thalassemia, respectively .Some of the clinical heterogeneity of the β thalassemia can be explained by the differing severity of particular alleles. [11]

Clearly, the β^0-thalassemia is associated with a severe phenotype, while β^+ and β^{++} thalassemia alleles are remarkably diverse in their effect on the output of β-globin chains. They are most easily described by their phenotypic effects in heterozygotes. A few β-thalassemia mutations are completely silent, they have no demonstrable effects in carriers and have usually been ascertained by finding individuals with intermediate forms of β-thalassemia in whom one parent has typical β-thalassemia traits and the other seems to be normal. [12] Overall, they are uncommon except for the –101 C→T mutation, which has been observed frequently in the Mediterranean region. There, it interacts with a variety of more severe β-thalassemia alleles to produce mild forms of β-thalassemia intermedia.[13] The majority result from mutations in the promoter elements of the β-globin genes or in the poly(A) cleavage sites; a few involve mutations at cryptic splice sites in exons or consensus sequences in introns. [11]

Phenotypically, they all result in milder, although clearly definable, changes in the red cells in heterozygotes, and disorders of intermediate severity in homozygotes. Overall, their interactions with severe alleles result in transfusion-dependent disorders or intermediate forms of β-thalassemia at the more severe end of the spectrum. [7]

Secondary modifiers

Family studies have shown that there is wide phenotypic diversity even among individuals with the same β-thalassemia genotype. There are two particularly striking examples. First, not every homozygote or compound heterozygote for β^0-thalassemia, in which there is no output of β-chains, is severely affected.[14] Second, studies of compound heterozygotes of Indian or Southeast Asian origin, who have inherited an HbE allele from one parent and the same β-thalassemia allele from the other, show wide phenotypic variability in the resulting disorder, HbE β-thalassaemia.[15] For example, individuals with this condition who carry identical β-thalassemia mutations have phenotypes that range from transfusion-dependent anemia in early life to a clinically 'silent' condition that is ascertained by chance in middle age. At least some of this remarkable phenotypic diversity can be explained by the action of the products of other loci involved in globin synthesis. Because the severity of the anemia of β-thalassemia reflects defective β-globin chain production, which leads to excess α-chains and their deleterious effects on red-cell production and survival, it follows that anything that modifies the magnitude of the surfeit of α-chains should have an important effect on the phenotype. Variation at two loci that mediate this effect has been identified — the α- and γ-globin loci. [16]

- *Co-inheritance of α- thalassemia*:

Because α-thalassemia coexists with β-thalassemia at a high frequency in many populations, it is not uncommon to inherit both conditions. So, homozygotes or compound heterozygotes for severe β-thalassemia alleles might also be heterozygous or homozygous for α^+-thalassemia, or heterozygous for α^o-thalassemia. [16]

The co-inheritance of different α-thalassemia alleles might reduce the severity of the homozygous or compound heterozygous states for β^0-thalassemia to some degree and can convert the severe forms of β^+- thalassemia into milder non-transfusion-dependent conditions, and provides clear evidence that the chief pathophysiological mechanism in the β-thalassemia is imbalanced globin chain production rather than the under-production of hemoglobin. So, although the red cells of individuals who have inherited both types of thalassemia might be grossly under-hemoglobinized, the anemia is less severe and consequently the phenotype is milder. [17]

- *Variation in fetal hemoglobin production*:

It became clear that some homozygous β^0-thalassemics have a mild clinical phenotype and are able to maintain a relatively high hemoglobin level, all of which is fetal hemoglobin (HbF); it seemed likely that an unusual propensity for the production of HbF after birth might be an important factor in modifying the clinical course of β-thalassemia. Normal children and adults produce small amounts of HbF that seem to be confined to particular red-cell populations called F cells. [18]

In a patient with β-thalassemia, the γ-chains bind some of the excess α-chains to produce HbF, and so red-cell precursors that are synthesizing γ-chains come under intense selection, a mechanism that accounts for much of the increased HbF in the blood of β-thalassemics. Because the number of F cells in normal individuals is under genetic control, it is not surprising that there is a variable propensity for producing HbF in patients with β-thalassemia. However, it is now clear that many different genes must be involved, some in the β-globin gene cluster, others on different chromosomes. [19]

There are several determinants within the β-globin gene cluster that are involved in setting the level of HbF in β-thalassemia. The conditions that constitute hereditary persistence of fetal hemoglobin result from deletions that involve the β-globin gene cluster or point mutations in the promoters of one or other of the duplicated γ-globin genes. They are all characterized by the persistent production of high levels of HbF into adult life. However, these genetic variants are rare and, play a relatively small part in the modification of the β-thalassemia phenotype. [20]

By contrast, there is a relatively common polymorphism at position −158 in the Gγ-gene, which involves a C→T change. Although this seems to have little effect in normal people, there is good evidence that homozygous individuals have an increased propensity to produce HbF under conditions of haemopoietic stress, and that this can have the effect of rising fetal hemoglobin levels in patients with β-thalassemia. As this polymorphism is widespread, it is an important factor in the modification of β-thalassemia phenotypes, particularly those with β^0-thalassemia of the intermediate variety. [21]

In addition, some β-thalassemia alleles might themselves favor a higher output of HbF. This is certainly true in the case of promoter mutations, or deletions that involve the promoter elements of the β-globin gene, an observation that might reflect competition between γ- and β-globin gene promoters for rate-limiting regulatory proteins or for interaction with the Locus control regions (LCR). [22]

There are also genetic determinants responsible for increasing the output of HbF in some patients with β- thalassemia that are not encoded in the β-globin gene cluster. For example, in families with milder forms of β- thalassemia owing to increased HbF production, unusually high levels of HbF are sometimes found in one of the heterozygous parents, or one or more unaffected relatives have slightly increased levels of HbF. [9] In studies of several generations of a large family in which a gene of this type segregated independently from the β-globin gene cluster, the locus involved has been assigned to chromosome 6. [23]

However, analyses of similar families indicate that there are genetic determinants involved in increased HbF production in β-thalassemia that are not linked to the β-globin gene cluster or chromosome. There is also a locus on the X chromosome that seems to have an effect on the numbers of F cells in adults, although its role, if any, in determining the level of HbF in β-thalassemia is not yet clear. [24] In short, it is apparent that here are several genes that are not linked to the β-globin gene complex that can fine tune the level of HbF, both in normal adults and in those with β-thalassemia. Presumably they encode transcription factors that are involved with the activation or repression of γ-chain synthesis or in modulating the kinetics of haemopoietic cell development to make γ-chain synthesis more likely in conditions of haemopoietic expansion. [25]

- *Increasing the severity of β-thalassemia*:

Just as the α- thalassemia, or a genetically determined increase in HbF production, can ameliorate the phenotype of β- thalassemia, variability at the α-chain loci can also have the opposite effect. Instead of the duplicated α-globin gene arrangement, αα, some individuals are heterozygous or even homozygous for triplicated or quadruplicated α-globin gene arrangements, ααα, αααα. [26] They are found in most populations, although the frequency of chromosomes that contain additional α-globin genes is not known in detail. They have no phenotypic effect in normal people presumably the small excess of α-chains that is synthesized can be dealt with by proteolysis but this is not the case in individuals with β-thalassemia. The consequences on the phenotype of β-thalassemia of the inheritance of additional numbers of α-globin genes have been best defined in heterozygotes. For example, β-thalassemia carriers who

are heterozygous or homozygous for triplicated α- globin gene arrangements have a β-thalassemia disorder of intermediate severity ,[27] a similar effect is seen in those who have inherited a chromosome with the quadruplicated α-globin gene arrangement .[28]

Tertiary modifiers

The clinical phenotype of homozygous β-thalassemia may also be modified by the coinheritance of other genetic factors mapping outside the β-globin gene cluster and affecting some disease complications. Among these factors the ones best delineated so far are those affecting bilirubin, iron, bone metabolisms. Although so far there are only limited data about these tertiary modifying genes, it seems likely that they will become of increasing importance, particularly if the polymorphisms that affect their function are common in populations in which β-thalassemia occurs at a high frequency.[29]

- *Bilirubin metabolism*:

Because of the rapid turnover of red-cell precursors in patients with β-thalassemia and the resulting breakdown of haem products, many of those with more severe forms of the disease are mildly jaundiced and have a propensity to gall stone formation and gall bladder disease. It has been found that the level of bilirubin in β-thalassemia heterozygotes is related to a polymorphism in the promoter of the gene that is involved in the hepatic glucuronidation of bilirubin, uridine diphosphate-glucoronyltransferase IA (UGTIA) . In normal individuals, the UGTIA promoter has a run of six TA repeats in the TATA box $(TA)_6$. Individuals who are homozygous for an additional repeat, $(TA)_7$, tend to have mild hyperbilirubinaemia; β-thalassemia heterozygotes with the $(TA)_7$ arrangement can have more persistent jaundice .[30,31]

- *Iron metabolism*:

Cardiac disease, hepatic disease and diabetes are important complications of β-thalassemia that reflect tissue damage from iron loading, not only from transfusion but also from increased intestinal absorption. Although there have been few studies to date, preliminary data indicate that the common mutation of HFE that causes hereditary hemochromatosis, C282Y, might be involved in the variability of iron loading in some patients with the intermediate forms of β-thalassemia.[32]

Furthermore, there is recent evidence that the β-thalassemia trait favors higher rates of iron loading in C282Y homozygotes.[33] However, this mutation is rare in parts of the world in which β-thalassemia is common and so it will probably have only a small role in iron loading in the more severe forms of β-thalassemia. By contrast, the HFE polymorphism H63D occurs commonly throughout many of the populations affected by β-thalassemia. Further study of genetic variability in the rate of iron loading in the thalassemia will be of considerable importance, because polymorphisms that result in more effective iron absorption are likely to have had a selective value in the past, and because there are now so many candidate genes that are involved in iron homeostasis. [34]

- *Bone disease*:

Progressive osteoporosis is common problem in young adults with β-thalassemia, associated with bone pain and fractures that might, in part, be related to secondary hypogonadism due to iron-mediated damage to the hypothalamic–pituitary axis. There is increasing evidence that this complication might be modified by polymorphisms at loci that are

involved in bone metabolism, including the genes for the vitamin D receptor (VDR), collagen, and the estrogen receptor. [35]

- *Phenotypic variation due to co-selection*:

There is strong evidence that the high frequency of the α-thalassemia, and almost certainly the β-thalassemia, is a reflection of heterozygote advantage against *Plasmodium* falciparum . [36, 37] The fact that, every population has its own unique set of β-thalassemia mutations indicates that, in evolutionary terms, the exposure of these high-frequency populations to malaria might have been fairly recent. In other words, individual mutations have arisen locally and have been expanded by heterozygote advantage. Studies indicate that exposure to malaria has not simply expanded mutations at the hemoglobin loci, but that varying susceptibility to malaria is also reflected by polymorphisms at many other loci, including the major histocompatibility complex loci HLA-DR[38],tumor-necrosis factor- α (TNF) [39],intercellular adhesion molecule-1(ICAM-1) .[40]

Just as in the case of the thalassemia, the malaria-related polymorphisms of these systems vary greatly between different racial groups, again reflecting fairly recent exposure to malaria. Because these systems have an important role in defense mechanisms against many infectious agents, it follows that children with thalassemia from different parts of the world might have quite different responses to infection, an important complication in this disease. [41]

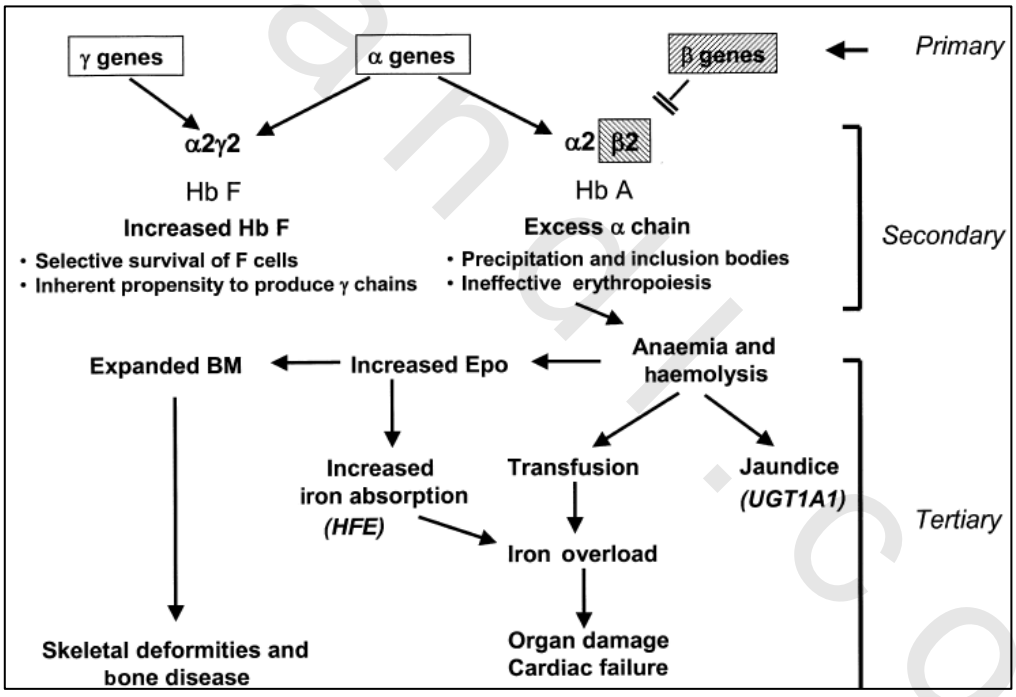


Fig 2. Factors that modify the β thalassaemia. [29]

Body iron pathways

In humans total body iron stores is maintained normally within the range of 200-1500 mg by adequate adjustment of intestinal iron absorption, since there is no known regulated mechanism for iron excretion. [42] Approximately 65–75% is found in the hemoglobin of erythrocytes in the form of heme. The liver stores ~10–20% in the form of ferritin, which can be readily mobilized for utilization when needed. About 3–4% of the body's iron is in heme-bound myoglobin in striated muscle, the rest is distributed in other tissues. [43]

From a quantitative point of view, the most important pathway of iron metabolism is the unidirectional recycling of iron from senescent red blood cells to the erythroid bone marrow through macrophages. [44] These hematopoietic sub compartments are composed of erythroblasts surrounding a central macrophage. It was suggested that the macrophage functions as a "nurse" cell, providing iron to developing erythroblasts for heme. The second pathway is the cycling of iron from hepatocytes to the blood and vice versa, according to body needs and the third is iron absorption through duodenal and upper jejunum that balances the 1-2 mg daily iron loss occurring through cellular exfoliation. [45]

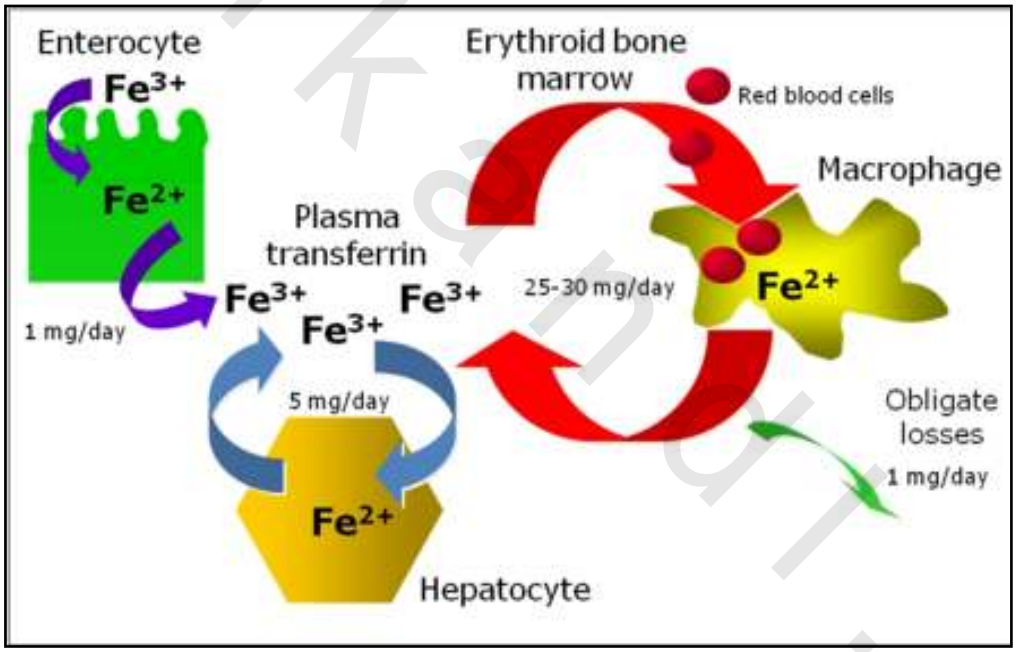


Figure 3. Body iron pathways. [43]

Iron transport and utilization

Systemic transporter of iron in the body is transferrin (TF), an abundant, high affinity iron-binding protein. Under normal circumstances, TF carries nearly all serum iron collected from duodenal absorptive epithelium, macrophages and hepatocytes, and dampens its reactivity. Very small amounts of iron may be loosely associated with albumin or small molecules. In normal human subjects, iron occupies approximately 30% of the iron-binding sites on plasma TF. [46]

The saturation of TF by iron shows diurnal fluctuation exhibiting a morning peak and an evening nadir. It is likely to be higher in the portal circulation, where recently absorbed iron from the intestine enters the circulation and passes through the liver. This first-pass effect may explain the periportal iron accumulation in the hepatic lobule observed in iron overload

disorders associated with inappropriately increased intestinal iron absorption.[47]Conversely, TF saturation is likely lower than average in plasma leaving the erythroid bone marrow, where most of the iron present is extracted for use by erythroid precursor cells. The erythroid bone marrow is the largest consumer of iron. Normally, two-thirds of the body iron endowment is found in developing erythroid precursors and mature red blood cells. [48]

Erythroid precursors express cell surface transferrin receptors (TfR1) that take up iron from TF by receptor-mediated endocytosis. [49] Targeted disruption of the murine TfR1 gene causes embryonic lethality, attributable to severe anemia. Spontaneous mutations disrupting the TF gene demonstrate the importance of TF in animals and humans. [50]

Lack of TF results in severe iron deficient microcytic anemia. However, iron deficiency occurs only in hematopoietic cells whereas other tissues develop massive iron overload. This underscores the importance of the TF-TfR1 endocytic cycle in erythropoiesis. [49] Hepcidin synthesis is markedly depressed and intestinal iron absorption is increased in congenital hypo-transferrinemia, apparently to try to compensate for the lack of iron available to erythroid precursors. This is compelling evidence for a signaling mechanism that allows the erythroid bone marrow to communicate its iron needs to the intestine's absorptive epithelium. [51]

Iron absorption:

Iron in food is present as ferric iron or as heme. Heme is a biologically important iron containing compound and a key source of dietary iron but the mechanism by which the enterocyte takes up heme and catabolizes it to utilize the iron is still poorly understood. Currently, there are two prevailing hypotheses explaining the mechanisms of this process; first, heme is taken up by receptor mediated endocytosis; secondly, the recent discovery of a heme transporter (PCFT/HCP1) that may have the capability of transferring heme from the small intestinal lumen directly into the cytoplasm. [52]

In recent years, two mammalian heme transporters have been discovered, namely PCFT/HCP1 and FLVCR possibly acting at the apical and basolateral site of the small intestinal enterocyte, respectively. PCFT/HCP1 has been independently characterized as a folate/proton symporter and appears to play a key role in intestinal folate absorption. Interestingly, the folate transport capabilities of PCFT/HCP1 are at least an order of magnitude higher than that observed for heme, suggesting that folate may be the more physiologically relevant target of this transport protein. [53] FLVCR is a heme exporter relevant for erythropoietic activity that acts as an overflow valve for excess manufactured heme that would otherwise result in cellular toxicity. [54]

Much more knowledge has been developed on non-haem iron absorption. Non-haem iron requires to be converted in ferrous iron by the apical ferric reductase duodenal cytochrome B although the physiological significance of this pathway is the subject of continued debate. Following the reduction, iron crosses into the cytoplasm via an apical iron transporter Divalent Metal Transporter1 (DMT 1). [55]

Cells regulating body iron homeostasis:

Besides enterocytes other cell lines, namely macrophages, hepatocytes in adults and placental cells during fetal life, have core functions within iron metabolism that is to acquire iron from different sources (senescent erythrocytes and holotransferrin) and to deliver it to the rest of the body according to its needs. To do this important function these cells have a specialized mechanism for exporting iron to plasma, that is the iron exporter ferroportin. [56]

Ferroportin needs copper-ferroxidases to release iron to plasma transferrin, namely hephaestin in duodenal cells and ceruloplasmin in hepatocytes, and macrophages. When defective, these proteins induce cellular iron retention in specific cell types as shown in hephaestin deficient mice [57] and in humans with aceruloplasminemia. [58]

Ferroportin acts under the control of hepcidin and this interaction can explain the systemic regulation of iron metabolism, Interestingly, it has been shown that hepcidin-induced internalization of ferroportin requires binding and cooperative interaction with Janus kinase (JAK).[59] Hepcidin binding to ferroportin results in the phosphorylation of ferroportin, a step necessary for its internalization by clathrin-coated pits and the kinase responsible for the phosphorylation is Jak2.[60]

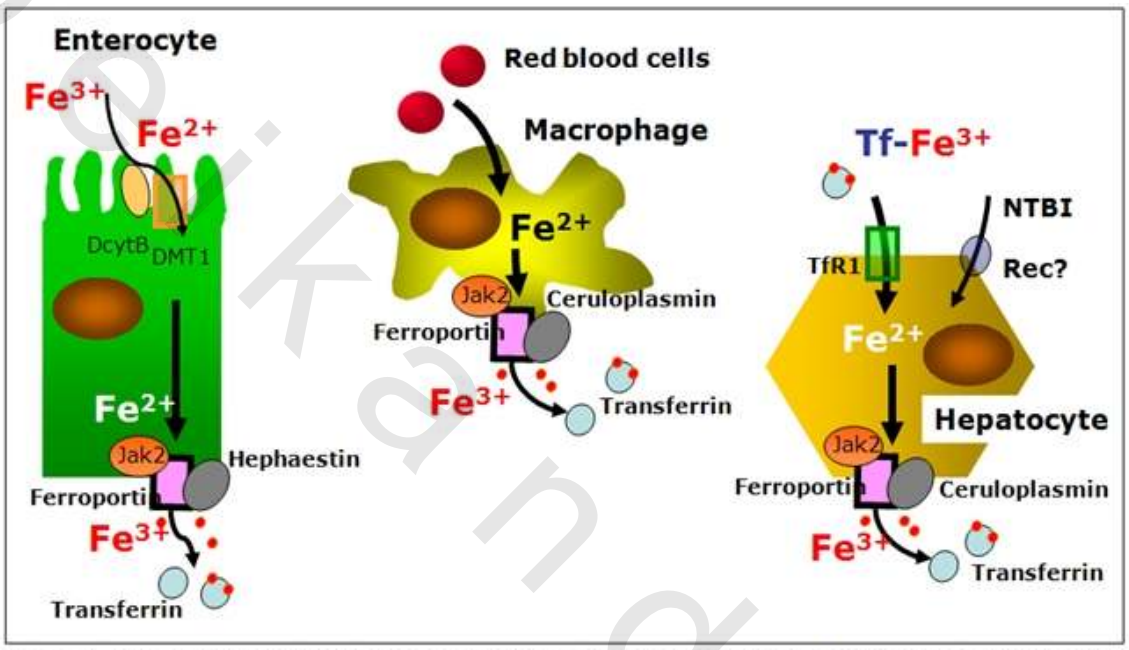


Figure 4. Cells regulating body iron homeostasis. [56]

Iron storage:

Following phagocytosis of old and damaged erythrocytes, tissue macrophages, particularly in the spleen, lyse cells and catabolize hemoglobin presumably by heme oxygenase, to liberate iron. Some iron remains stored in macrophages, although some is exported to plasma TF. Ferroportin is critical for macrophage iron export and can be regulated to change the ratio between stored and released iron. [61]

Hepatocytes represent the main depot for iron storage in normal conditions and in non-transfusional iron overload. Although the TF cycle may be involved in hepatocyte iron acquisition to some extent, non–transferrin-bound iron (NTBI) uptake pathways become particularly important when serum iron levels exceed TF binding capacity.[62] The identity of the hepatocyte NTBI uptake system is not known, but DMT1 is unlikely to be involved, because hepatocytes can accumulate iron in the absence of DMT1.[63] Candidates for the NTBI transporter include Zip14, a member of the SLC39A zinc transporter family, which can mediate the uptake of zinc and NTBI in hepatocytes. [64]

Hepatocytes have a large capacity to store excess iron. Most storage iron is probably in the form of ferritin, which can be mobilized when needed elsewhere in the body. Eventually, however, massive iron overload results in hepatotoxicity; hepatitis leads to fibrosis and cirrhosis. The liver is probably exposed to more NTBI than are other tissues because of the first-pass effect of the portal circulation. However, other tissues have NTBI uptake activities and load iron when NTBI is present in the plasma. The heart and endocrine tissues are particularly susceptible; cardiomyopathy and endocrinopathies are the predominant non hepatic complications of iron overload. L-type calcium channels [65] can mediate the uptake of NTBI in the myocardium, whereas other candidates, such as transient receptor potential canonical protein (TRPC6), need further confirmation. [66]

Regulation of iron homeostasis.

The liver peptide hepcidin regulates intestinal iron absorption and iron release from storage cells by binding ferroportin causing its internalization and degradation, thus exerting a general inhibitory effect on iron release in the body.[59,67] Animal models clarified the role of hepcidin as hepcidin knock-out mice developed massive iron overload [68] and hepcidin over-expression induced iron deficiency.[69] In physiological conditions, hepcidin production is tightly regulated in response to signals released from other organs, prevalently from bone marrow (erythroid regulator) and the iron stores (store regulator). Hepcidin levels increase in iron overload in order to limit iron absorption and are reduced up to undetectable levels in iron deficient erythropoiesis, either dependent from decreased iron supply or increased erythroid iron requirement, to allow iron acquisition. [70]

Studies on genetic disorders of iron metabolism and of corresponding animal models have identified the hemochromatosis proteins (HFE, TFR2 and HJV) as the iron-dependent regulators of hepcidin expression. Patients affected by hemochromatosis have a defective synthesis of hepcidin that is absent in JH, reduced in type 3 HH due to TFR2 mutation or inadequate to the amount of iron overload in classical HH.[67] Such difference is related to the role of each protein in hepcidin regulation. HFE, TFR2 and HJV are all positive regulators and represent only a part of the complex regulation of hepcidin transcription, where mediators and signaling pathways of iron- inflammatory- and erythroid- dependent regulation of hepcidin are reported. Recent studies suggest a role for HFE as a component of an iron-sensing complex that involves interactions with diferric transferrin, TFR1 and TfR2 at the plasma membrane of hepatocytes. [71]

Defective HFE or TFR2 prevents formation of a functional iron sensor and signal transduction effector complex leading to dysregulated hepcidin expression as observed in hereditary hemochromatosis. Indeed, double heterozygotes for HFE and TFR2 mutations develop hemochromatosis [72], whereas this does not occur in carriers of single allelic mutations in both HFE and HJV [73], suggesting they behave to different pathways of hepcidin regulation.

The main activator of hepcidin in iron overload is HJV, the protein mutated in type 2a juvenile hemochromatosis: HJV-deficient patients and mice have undetectable levels of hepcidin.[74] HJV is a GPI linked protein that activate hepcidin as a co-receptor for Bone morphogenetic proteins (BMPs), a family of cytokines that signal through SMAD proteins as a second messenger.[75] Several BMPs may activate hepcidin in vitro but the relevant BMP in vivo is BMP6, since knockout mice for this BMP have no developmental defect but develop severe iron overload.[76] That the BMPs pathway is involved in iron homeostasis through hepcidin regulation is further demonstrated by the severe iron overload and very low hepcidin expression reported in Smad4 liver conditional knockout.[77]

Several other signals regulate hepcidin expression. Infection and inflammation markedly increase hepcidin synthesis through the IL-6/IL-6 receptor and STAT3 pathway, a mechanism largely implicated in the pathogenesis of anemia of chronic diseases. [78, 79]

Hepcidin inhibition occurs in iron deficiency, hypoxia and erythropoiesis expansion in order to increase iron export to plasma. Several inhibitors of hepcidin have been proposed: HIF-1a that is stabilized in hypoxia/iron deficiency, reduces hepcidin transcription by binding a HIF responsive element of hepcidin promoter [80], the soluble variant of HJV down regulates hepcidin in vitro by competing with mHJV for the BMP ligand [81], and matriptase 2 is a serine protease recently identified as a strong hepcidin inhibitor by cleaving membrane-HJV.[82] Accordingly, mutations in the gene coding matriptase 2 cause the rare form of iron refractory iron deficiency anemia in mice and humans. [83]

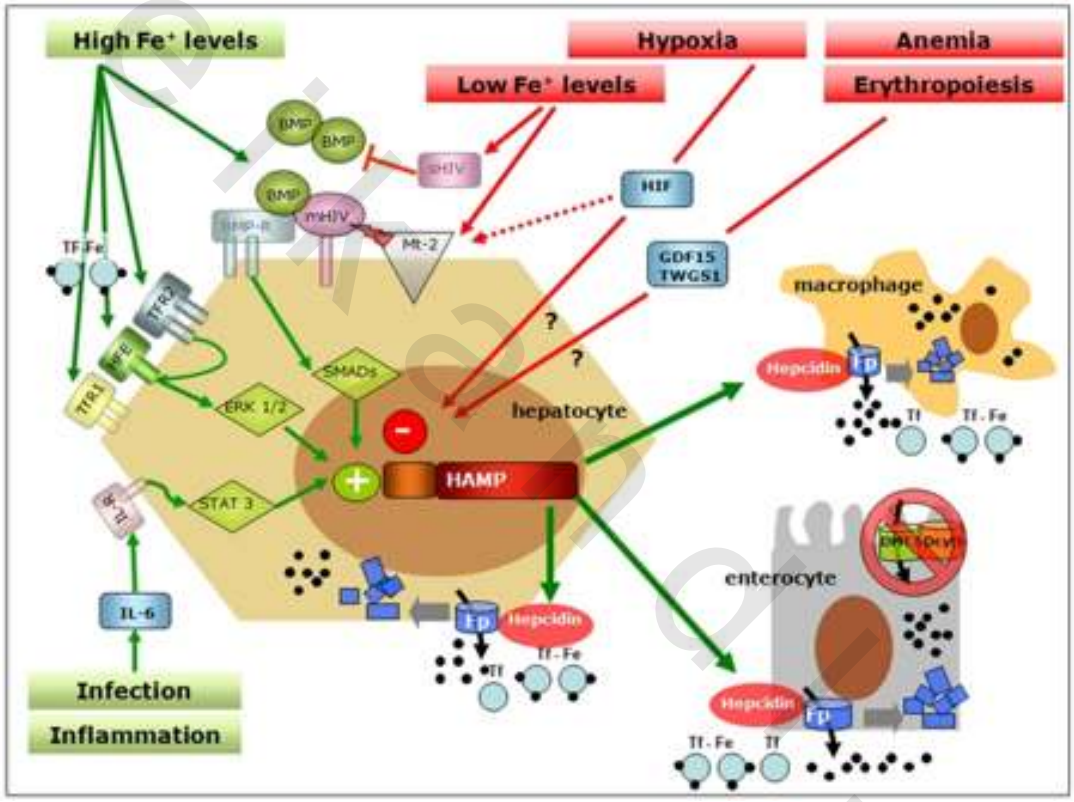


Figure 5. Signal pathways in systemic regulation of hepcidin. (70)

<u>Erythroid-dependent regulation of hepcidin</u>:

It was demonstrated that erythropoietin suppresses hepcidin synthesis, but this effect was lost by pharmacologic inhibition of erythropoiesis. [84] Similarly it showed a dramatic increase in hepcidin expression in mice when erythropoiesis was inhibited by irradiation or post transfusion polycythemia.[85] The nature of the erythropoietic regulator of hepcidin is now debated, but may include one or more proteins secreted by developing erythrocytes. When HepG2 cells were treated with sera from β-thalassemia patients, in which erythropoietic drive is greatly elevated, or with sera from HFE-hemochromatosis or control subjects, only β-thalassemia sera decreased hepcidin mRNA. [86] Another study treating HepG2 cells with sera from patients with iron-deficiency anemia or β-thalassemia showed similar results. [87]

Regulation of hepcidin by erythropoietic activity is of particular importance in iron-loading anemias such as β-thalassemia and dyserythropoietic anemias. Studies in animal models of thalassemia indicate that in conditions of extreme ineffective erythropoiesis, there is relatively little peripheral destruction of red cells and iron accumulates more rapidly in the liver than in the spleen, consistent with the interpretation that iron loading results primarily from increased intestinal absorption.[86,88] Thus, very high erythropoietic activity and increased iron requirement from the bone marrow generates a signal that can override hepcidin regulation by iron, leading to increased iron absorption, but the marrow signal that modulates hepcidin expression is debated.

One proposed hepcidin inhibitor in these conditions is growth differentiation factor, (GDF15), a cytokine member of the TGF-β superfamily which is strongly expressed in hemoglobinized erythroblasts at the final stages of human erythropoiesis. GDF15 is hyperexpressed in thalassemia.[89]

Very recently, twisted gastrulation 1 (TWSG1) was identified as a novel erythroid regulator of hepcidin expression in murine and human cells. [90] TWSG1 suppressed hepcidin in human hepatocytes through a BMP-dependent mechanism. It is proposed that TWSG1 and GDF15 act together to inappropriately inhibit hepcidin expression in thalassemia. Due to the expansion of erythropoiesis, TWSG1 expressed during the early stages of erythropoiesis acts indirectly by inhibiting BMP-mediated expression of hepcidin. [90] In thalassemia, over-expression of TWSG1 would inhibit the host's ability to sense and respond to iron loading. It is also hypothesized that the increased expression of TWSG1 may also impact erythropoiesis in thalassemia by inhibiting the BMP4 dependent expansion of stress erythroblasts, which in turn would exacerbate the anemia.

The role of hypoxia:

Anemia generates tissue hypoxia and activates the cellular mediator of biological response to hypoxia, hypoxia inducible transcription factors: HIFs, which induce a signaling cascade involving hundreds of genes. Seminal studies established an association between oxygen and iron regulation by showing that hypoxia results in higher iron absorption in mice and rats. Accordingly, both hypoxia and anemia induce the synthesis of erythropoietin (EPO) and are the two main signals that increase iron absorption independently of iron stores. [91] In addition, hypoxia was found to increase the expression of transferrin, transferrin receptor, ceruloplasmin and hemoxygenase-1 which are hypoxia-inducible HIF-1 target genes, thus enabling iron transport to erythroid tissue and cellular uptake, and iron recycling and release from stores.

Hepcidin is suppressed in human cultured hepatoma cells exposed to hypoxia and HIF-1 has been implicated in hypoxia-mediated hepcidin regulation[92] , HIF-1 binds to the hepcidin promoter in vitro and decreases its transactivation and stabilization of HIF in hepatocytes, down-regulates hepcidin and increases ferroportin expression in mice. Thus, HIF-1 acts as a regulator of iron homeostasis, but recent studies also suggest a potential role of HIF-1α in iron sensing in the liver. In fact, iron deficiency increases HIF-1α levels in mice liver. In addition, one recent study demonstrates a role for HIF as a transcription factor that regulates the expression of DMT-1 and apical ferric reductase duodenal cytochrome b (Dcyt-b) genes, which are regulators of intestinal iron absorption.[93] Thus, HIF may act both as an iron sensor and iron regulator, and be an essential link between iron and oxygen homeostasis in vivo that through coordinate gene regulation mobilizes iron to support erythrocyte production. Other experimental studies further support the iron-oxygen connection, that involves iron deficient and hypoxia-dependent production of soluble-HJV, GDF15 and Matriptase-2 in hepatocytes.[94]

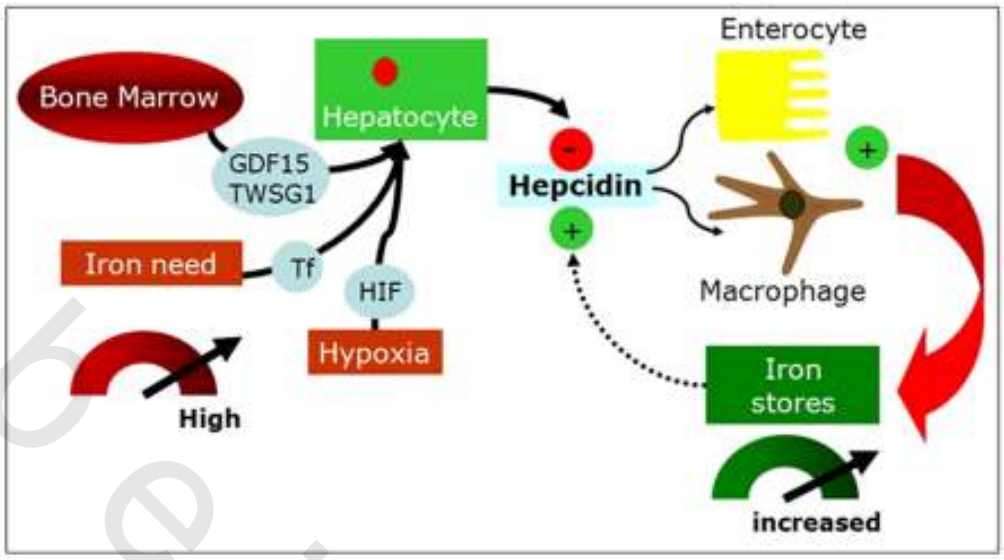


Figure 6. Hepcidin regulation by erythroid -iron and hypoxia related signals. [91]

Iron metabolism in thalassemia

Patients affected by the most severe forms of thalassemia require chronic blood transfusions to sustain life and chelation therapy to prevent iron overload. A unit of red blood cells transfused contains approximately 250 mg of iron, while the body cannot excrete more than 1 mg of iron per day. Those affected by β-thalassemia intermedia do not require chronic blood transfusions but eventually develop elevated body iron loads due to ineffective erythropoiesis and hypoxia dependent hepcidin down regulation that, in turn, induces increased gastrointestinal iron absorption. [88, 95]

Iron absorption studies in patients affected by beta thalassemia intermedia show that the rate of iron loading from the gastrointestinal tract is approximately three to four times greater than normal.[96] In non-transfused patients with severe thalassemia, abnormal dietary iron absorption results in an increased body iron burden between 2 and 5 g per year depending on the severity of erythroid expansion.[97] If regular transfusions are required, as in β-thalassemia major patients, this doubles the rate of iron accumulation. In addition to the transfusion-related iron overload, increased iron absorption also plays a role in β-thalassemia major, in which its importance is inversely related to Hb levels. [98] As loading continues, the capacity of transferrin, the main transport protein of iron, to bind and detoxify this essential metal may be exceeded. The resulting NTBI fraction within plasma may promote the generation of reactive oxygen species (ROS), propagators of oxygen-related damage.[99]

Thalassemia intermedia and major are the most studied human models of hepcidin modulation by ineffective erythropoiesis alone and the combined and opposite effect of both ineffective erythropoiesis and transfusion dependent iron overload, respectively. Regular transfusions, in fact, induce massive iron loading but also inhibit erythropoietic drive. Accordingly, hepcidin production is higher in thalassemia major than in thalassemia intermedia although still inappropriate to the massive transfusional iron loading that partially counteracts the erythropoietic-dependent hepcidin down regulation.[100,101] Transfusions strongly influence hepcidin production as shown by the significant post-transfusion increase of urinary hepcidin levels in thalassemia patients, probably related to transfusion dependent suppression of erythropoiesis. [102]

Oxidative stress in βThalassemia

Oxidative stress was documented in various types of thalassemia as well as in other hereditary and acquired hemolytic anemia. [103] The main cause of oxidative stress in thalassemia is iron overload, which results from increased iron absorption in the gastrointestinal tract and multiple blood transfusions as well as from intracellular denaturation of Hb subunits and eventual release of iron from heme. [104] When the access incoming iron surpasses the binding potential of transferrin, it accumulates as a NTBI and its redox-active form labile plasma iron (LPI) in the plasma and as labile iron pool (LIP) in the cells. [105]

The free-iron species participate in chemical reactions and generate oxygen radicals that affect various cell components, particularly the cell membrane, damaging vital organs (heart, liver, and the endocrine system) as well as the hematopoietic system where the final outcome is hemolysis and ineffective erythropoiesis. The former is due to short survival of mature red blood cells (RBCs) as a result of enhanced susceptibility to undergo extravascular phagocytosis by macrophages in the spleen and the marrow. The latter is due to enhanced apoptosis of developing erythroid precursors in the bone marrow and extra medullary sites, with the end result of severe chronic anemia. [106]

The oxidative status of cells is determined by the balance between pro-oxidants and antioxidants. The pro-oxidants, referred to as reactive oxygen species (ROS), are classified into radicals and non radicals. Radicals are defined as molecules or molecular fragments containing at least one unpaired electron in the shells around the nucleus [depicted as a superscripted dot (R•)]. The presence of unpaired electrons usually confers a considerable degree of reactivity upon a free radical. Radicals in biological systems include superoxide ion radical (O_2 $^{\bullet -}$), hydroxyl radical (OH•), peroxyl (ROO•), alkoxyl radicals (RO•) and a single oxygen ($^1O_2$). The non radical ROS include the hypochlorous acid (HClO), hydrogen peroxide (H2O2), organic peroxides, aldehydes, ozone (O_3), and O_2. [107]

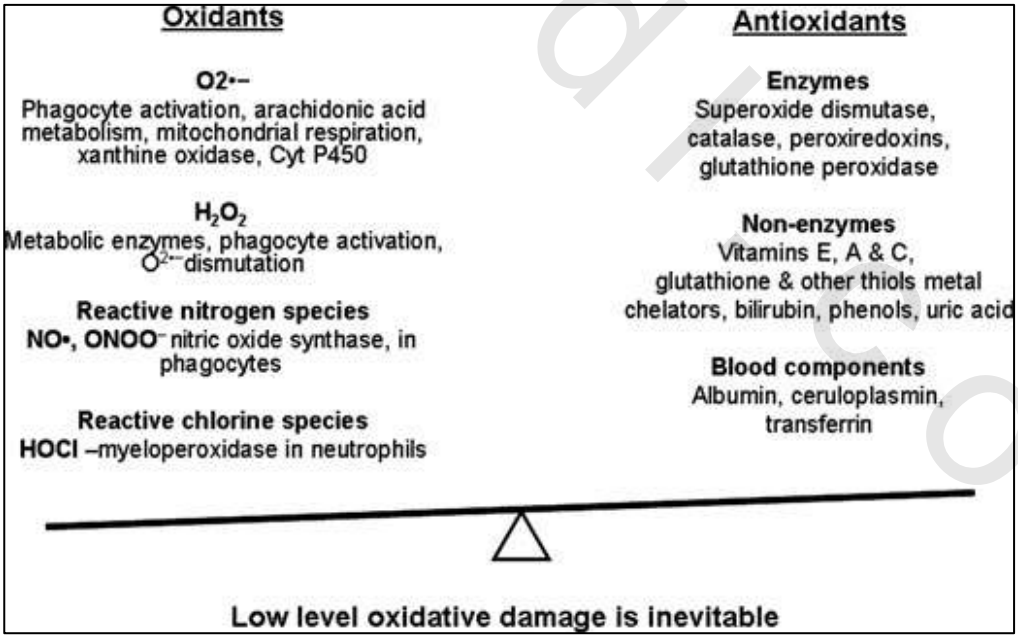


Figure 7. The oxidant/antioxidant balance. (103)

The generation of ROS occurs in most cells mainly during energy production, approximately 2% of the total mitochondria $^1O_2$ consumption results in $O_2\bullet^-$ production. Although $O_2\bullet^-$ is not particularly reactive, it can act as a reductant toward divalent metal ions, and can react with itself by spontaneous or enzymatic (e.g., superoxide dismutase, SOD) dismutation to form H2O2. The latter is a mild oxidant, but in the presence of divalent metals (e.g., iron) it can generate the reactive hydroxyl radical**.** In addition to mitochondrial production, ROS are generated by enzymatic reactions; for example, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases produce ROS by reduction of O_2 to O_2^- that is rapidly converted to H_2O_2. [108]

The generation of various free radicals is closely linked with the participation of redox-active metals. The redox state of the cell is largely linked to an iron redox couple and is maintained within strict physiological limits. Iron-mediated formation of ROS leading to DNA and lipid damage appears to result from an exaggeration of the normal function of iron, which is to transport oxygen to tissues. Labile or "free" iron can convert relatively stable oxidants into powerful radicals. Iron concealed in proteins, as in catalytic sites of enzymes or stored in ferritin, is not exposed to oxygen radicals and cannot participate in this chemistry. [109]

At physiological pH, most of the iron is bound to biological chelates in its oxidized form, Fe^{3+}. In order to take part in the generation of OH• (Haber–Weiss reaction), the iron must undergo reduction into its reduced form, Fe^{2+} by superoxide radicals (Fenton reaction). Fe^{2+} can interact with H_2O_2, produced from the spontaneous or enzymatic dismutation of superoxide radicals, to yield OH•. The final result of these two reactions is the production of hydroxyl radicals:

$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$ (Haber-Weiss reaction)

$Fe^{2+} + H2O2 \rightarrow Fe^{3+} + OH^- + \bullet OH$ (Fenton reaction)

Net reaction: $\bullet O_2^- \rightarrow + H_2O_2 \rightarrow \bullet OH + HO^- + O_2$

Fig 8: Reactions associated with the generation of reactive oxygen species. [103]

Free radicals can interact and damage various cellular components, including DNA, proteins, and lipids. Peroxidation of membrane lipids represents a primary consequence of cellular oxidative stress**.** [110] Lipid peroxidation refers to the addition of oxygen to unsaturated fatty acids to form organic hydroperoxides (ROOH). Organic ROO• and O_2-dependent peroxidation of lipids occurs during the initiation of the radicals that can also produce alkoxyl radicals (RO•) in metal-catalyzed reactions. The oxidation of phospholipids in the plasma membrane and internal organelle membranes such as the mitochondria interferes with their function. Moreover, lipid peroxidation yields additional reactive species, (e.g., 4-hydroxynonenal and malonaldehyde), which may contribute to toxicity. [111]

To protect against the deleterious effects of ROS, cells maintain an effective antioxidant system consisting of water- or lipid-soluble antioxidants and enzymes that remove ROS by metabolic conversion. The major cellular antioxidant is the reduced thiol glutathione (GSH), which maintains sulfhydryl buffering capacity. The mitochondrial, cytoplasmic, and extracellular SODs catalyze the conversion of O_2^- to H_2O_2, which in turn is converted to water andO_2 by catalase. Glutathione peroxidases degrade organic peroxides

at the expense of GSH. The GSH/GSH reductase and thioredoxin/thioredoxin reductase systems regenerate cellular GSH or reduced thioredoxin, respectively, at the expense of NADPH. [112] In addition, secondary antioxidant repair defenses remove or replace oxidative modified molecules. These include proteases that degrade oxidative modified proteins as well as DNA repair or lipid repair enzymes. Antioxidants are defined on the basis of their capacity to prevent the pro-oxidation processes of ROS and their damage. A host of plant-derived flavonoid and polyphenolic compounds constitutes a dietary source of antioxidants. Among these include water soluble (e.g., ascorbate) or lipid-phase antioxidants (e.g., vitamin E). [103]

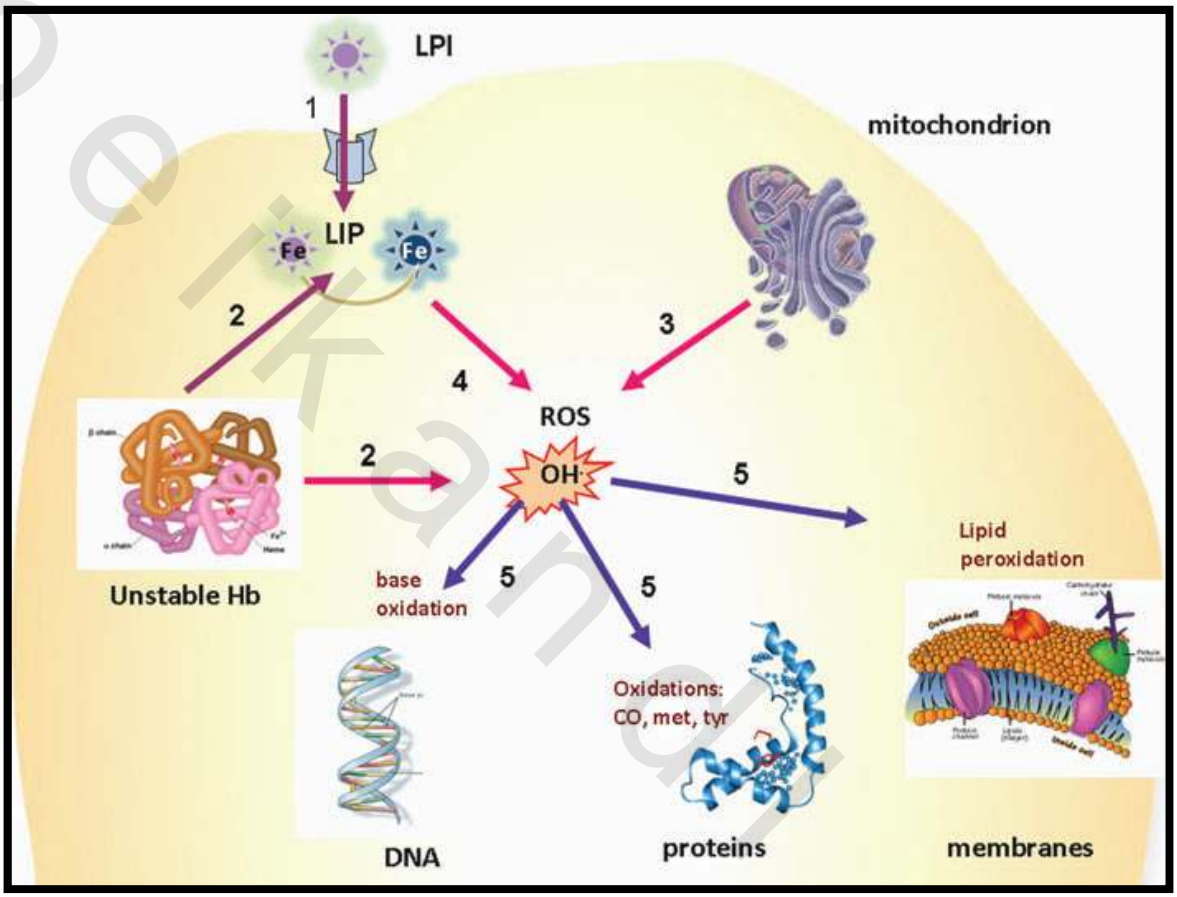


Figure 9: Role of free-iron species in generation of oxidative stress and cell damage in thalassemia. [103]

(1) The labile plasma iron (LPI), present in blood of iron overloaded thalassemic patients, enters into cells and accumulates as the labile iron pool (LIP).
(2)Unstable Hb contributes to LIP accumulation and reactive oxygen species (ROS) generation in erythroid cells.
(3) Normally, most cellular ROS is generated during energy production in the mitochondria, but (4) in thalassemia, the increased LIP accelerates ROS generation.
(5) ROS, and particularly the highly reactive OH• radicals, modify cellular DNA, proteins, and lipids.

Haptoglobin

Haptoglobin (Hp), is an α 2-sialoglycoprotein synthesized mainly in hepatocytes in response to the secretion of cytokines such as interleukin (IL)-6, IL-1 and tumor necrosis factor (TNF). [113,114] The intravascular destruction of erythrocytes, which accounts for ~10%-20% of the normal destruction of erythrocytes, releases free Hb into the general circulation. The primary function of Hp is to bind to this Hb, thereby preventing the renal excretion of iron and protecting blood vessels from the oxidative effects of this protein,[115]

During intravascular hemolysis, cell-free plasma hemoglobin may overwhelm homeostatic systems in place to remove it. Hemolytic conditions with substantial intravascular hemolysis include paroxysmal nocturnal hemoglobinuria (PNH), sickle-cell disease (SCD), thalassemias, hereditary spherocytosis and stomatocytosis, microangiopathic hemolytic anemias, pyruvate kinase deficiency, ABO mismatch transfusion reaction, paroxysmal cold hemoglobinuria, severe idiopathic autoimmune hemolytic anemia, infection-induced anemia, malaria, cardiopulmonary bypass, mechanical heart valve–induced anemia, and chemical-induced anemias. [116]

The serum concentration of Hp is influenced by age and is generally measurable from three months onwards, with a gradual increase until adult concentrations (30-200 mg /dl) are reached at 20 years of age, [116] when not bound to Hb, Hp is cleared from the plasma in ~3.5-5 days, but when bound to Hb, the average time for removal of the complex (mainly by hepatocytes) is ~20 min. [117]

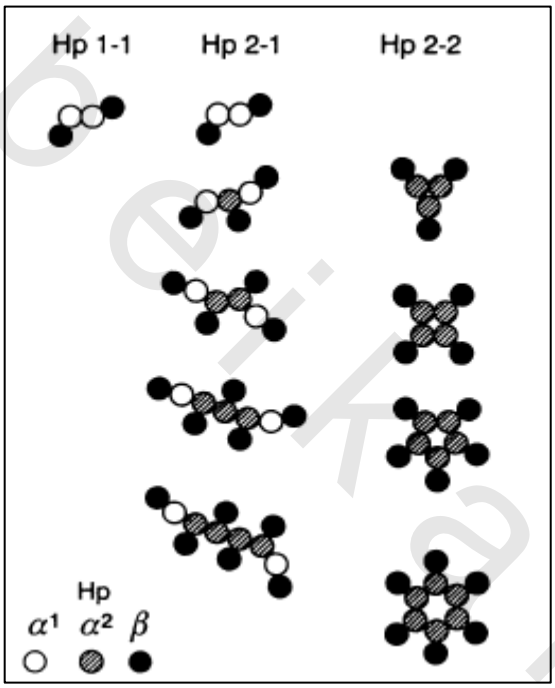
Measurement of the circulating Hp concentration can be used to determine whether there have been recent episodes of hemolysis since increased Hp consumption during these episodes leads to reduced plasma levels of this protein. [118] Because Hp is also a positive acute-phase protein with immunomodulatory properties that may inhibit or stimulate the immune response, the concentration of this protein is elevated in inflammatory and infectious processes and in malignancies. [119]

Protein Structure

Hp is a tetrameric protein that structurally resembles certain immunoglobulins because it has two light chains (α) and two heavy chains (β) covalently bound to each other by disulphide bridges (S-S).[120] Although present in all vertebrates, in humans Hp is characterized by molecular heterogeneity caused by genetic polymorphism, Hp1-1, Hp2-1 and Hp2-2 phenotypes are controlled by two autosomal co-dominant alleles identified as Hp1 and Hp2.The β-chain of Hp has a molecular mass of 40 kDa (245 amino acids) and is not polymorphic.[121]

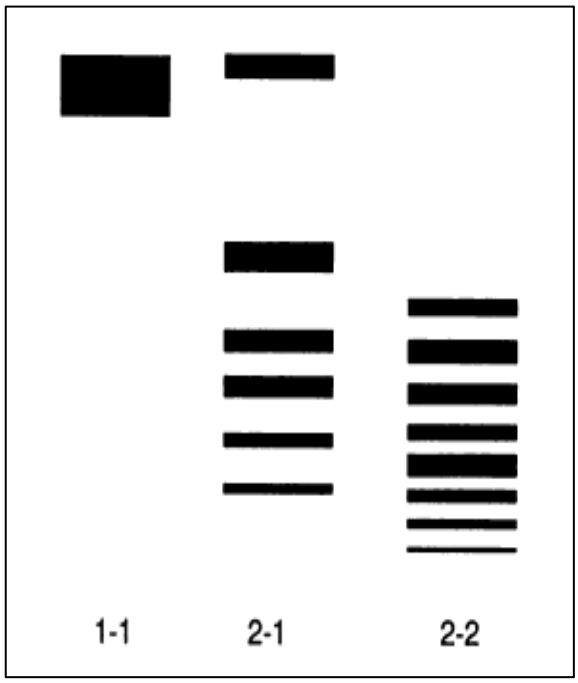
Haptoglobin polymorphism reflects inherited variations in the α-chain (the smallest chain) of Hp that result from differences between the α1-chain (with 83 amino acids) and the α2-chain(with 142 amino acids).[122] The α1-chain can be further classified into α1S (slow) or α1F (fast), depending on the electrophoretic mobility. The difference between these chains lies in the amino acids at positions 52 and 53, which are asparagine and glutamic acid in α1S and aspartic acid and lysine in α1F, respectively. [123]

This polymorphism results in Hp with different molecular masses, i.e., 86 kDa for Hp1-1, 86-300 kDa for Hp2-1 and 170-900 kDa for Hp2-2.[124] The polymeric composition of Hp is also type-dependent, with the protein product of the Hp1 allele being monovalent and that of the Hp2 allele being bivalent. Consequently, Hp occurs as a dimer in Hp1-1 homozygotes, as a linear polymer in Hp2-1 heterozygotes and as a cyclic polymer in Hp2-2 homozygous individuals, [125] these variations in shape and size form the basis of the most commonly used method for phenotyping Hp subtypes. [126]



Differences between Hp phenotypes. [124]

Figure 10



Electrophoretic patterns of Hp phenotype.[124]

Figure 11

Haptoglobin Genes

The Hp locus is located on the long arm of chromosome 16 (16q22), [127] the loci corresponding to the α and β chains are linked to each other so that a single mRNA molecule generates a large polypeptide chain that is then cleaved to yield the two Hp chains. [128]

The Hp1 allele has five exons while the Hp2 allele has seven; the 5th and 7th exons in the HP1 and Hp2 alleles, respectively, correspond to the β chain locus. The divergences between Hp1S and Hp1F are caused by base substitutions in codons 52 and 53 located in the 4th exon.[129]

In contrast, the Hp2 allele is a partially duplicated gene derived from a rare unequal crossover between the Hp1F and Hp1S alleles. This gene is 1.7 kb longer than the Hp1 alleles, and the region responsible for encoding from the 11th to the 69th residue (exons 3 and 4 of Hp1) is duplicated.[130] Combinations of these three alleles yield six distinct genotypes and their corresponding phenotypes, namely, Hp1S-1S, Hp1S-1F, Hp1F-1F, Hp2-1S, Hp2-1F and Hp2-2. [131]

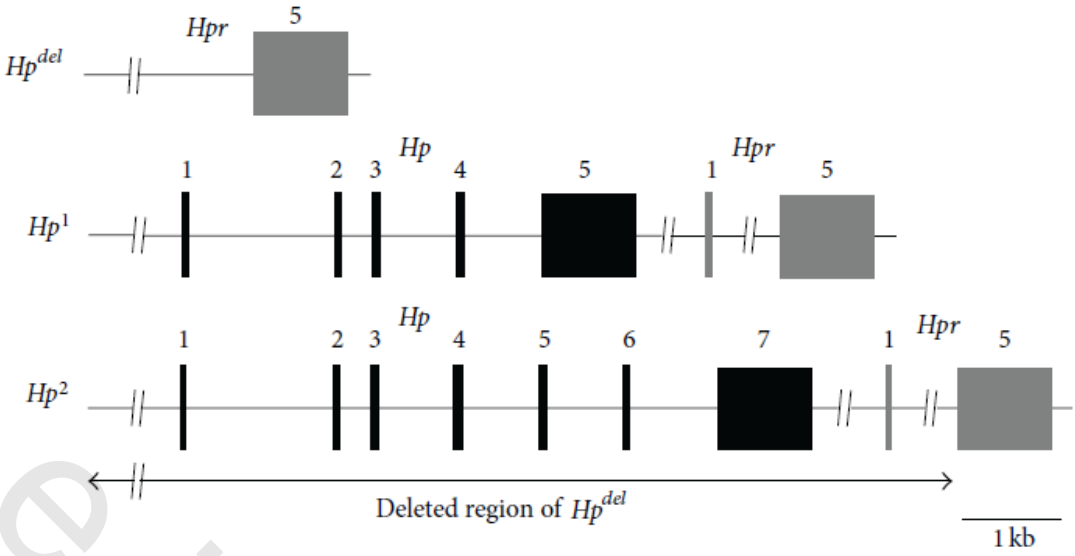


Figure 12. A schematic diagram of the genetic structure of the *Hp*1, *Hp*2, and *Hp del* alleles. [128] The *Hp*1 allele has 5 exons compared to 7 exons of the *Hp*2 allele. Exons 5 and 6 of the *Hp*2 allele are the result of an internal duplication of exon 3 and exon 4 of the *Hp*1 allele. A deletion spanning from the upstream region of exon 1 of the Hp gene to intron 4 of the Hpr gene makes the *Hp del* allele. The black box and the shaded box represent exons of the Hp gene and the Hpr gene, respectively. The number above each box designates the number of the corresponding exon .

The Haptoglobin-Related Gene

The humans Hp gene sequence is duplicated (2.2 kb downstream of the gene itself) on chromosome 16, this second gene is known as the Hp-related gene (HpR). In some individuals of African origin, multiple copies are present. The HPR gene differs from the Hp gene mainly in that it has a retrovirus- type sequence inserted into the firs intron. [132] The promoter region is active and encodes a protein called haptoglobin-related protein (Hpr), the serum concentration of which is ~5%-10% of that of Hp in healthy individuals. [133]

The majority of Hpr is associated with 2 serum complexes referred to as trypanosome lytic factors 1 (TLF1) and 2 (TLF2), which both are able to induce lysis of Trypanosoma brucei brucei, an African protozoan parasite that is transmitted to the mammalian bloodstream through the bite of infected tsetse flies and is the causative agent of the lethal disease sleeping sickness in cattle and other animals. [134] When Hpr binds to free Hb, it kills the trypanosome via oxidative damage initiated by its peroxidase activity. Since Hp is the major serum inhibitor of the TLF, the balance between the serum concentrations of Hp and Hpr determines the degree of protection against trypanocidal infection. [135]

The Hp0Phenotype

The Hp0 phenotype is characterized by the absence or reduced levels of Hp in plasma (referred to as ahaptoglobinemia and hypohaptoglobinemia, respectively).[136] This phenotype may be secondary to increased consumption or reduced production of Hp, as occurs during intravascular hemolysis and liver diseases, respectively, or may be genetically determined. [137]

In East Asian populations, genetically determined hypohaptoglobinemia results from an ~28 kb deletion, referred to as Hp^{Del}, that extends from the HP gene promoter region to exon 5 of the HPR gene.[138] The homozygous genotype (Hp^{Del}/Hp^{Del}) corresponds to the complete

absence of serum Hp, whereas the two forms of hypohaptoglobinemia ($Hp2/Hp^{Del}$ genotype and $Hp1/Hp^{Del}$ genotype) are associated with extremely low levels of Hp and levels that are approximately 50% of those observed in normal genotypes, respectively.

The Hp^{Del} gene frequencies in Japanese, Chinese and Korean populations are between 0.15 and 0.30. [139] In European and African populations, mutations in the promoter region of HP gene appear to be the primary cause of congenital ahaptoglobinemia. [140] The prevalence of the Hp0 phenotype is estimated to be 0.1% in Caucasians [124], whereas in Africans it can be as high as 40% or more. [141]The occurrence of this phenotype is influenced by acquired ahaptoglobinemia in areas where malaria is endemic and untreated. [142] In North Americans of African descent, the frequency of the Hp0 phenotype is ~2.3%. [143]

Geographic distribution of Hp alleles

The frequency of the Hp1 and Hp2 genes varies worldwide depending on racial origin: the Hp1 frequency varying from about 0.07 in parts of India to over 0.7 in parts of West Africa and South America. [144] The Hp2 allele originated in India and propagated around the world as a result of intense genetic pressure, gradually replacing the hegemony of the Hp1 allele. This suggests that the Hp2 allele may have a selective advantage over the Hp1 allele. [145]

The equilibrium of the Hp1/Hp2 polymorphism is broadly constant throughout the world. The allele frequencies in European populations are ~0.43 for the Hp1 allele and 0.57 for the Hp2 allele; in American populations, the corresponding figures are ~0.54 and 0.46.[127] Studies of populations from southern and southeastern Brazil have revealed allele frequencies of ~0.53 and 0.46 for Hp1 and 0.47 and 0.54 for Hp2, respectively. [146]

Biological functions of Hp

The Hp phenotypes have different biochemical and biophysical characteristics and functional efficiencies that account for their distinct antioxidant and immunomodulatory capacities. [147]

Haptoglobin: a hemoglobin scavenger facilitating the anti-inflammatory response

The complex formation between Hp and Hb has been studied for decades and represents one of the strongest non covalent interactions reported in plasma,[148] with only some minor variations in binding strength of the Hp phenotypes (Hp1-1 > Hp2-1 > Hp2- 2).[124] After its release into plasma, Hb rapidly dissociates from the $\alpha_2\beta_2$ tetrameric structure into αβ-dimers with the ability to bind Hp. The Hp region involved in binding these Hb (αβ)-units was recently shown by deletion analysis to include Hp1 β-chain residues 243 to 258. [149] Hence, the Hp1-1 molecule is able to bind 2 Hb αβ-dimers, whereas the Hp2-1 and Hp2-2 may be able to bind several Hb αβ-dimers. [150] Besides having a direct inhibitory effect on the toxic properties of Hb, as well as preventing peroxidative modification of Hb, Hp highly expedites Hb clearance, leading to production of anti-inflammatory metabolites. [151]

The fast removal of Hp-Hb complexes is explained by the high-affinity binding to the 130-kDa transmembrane receptor CD163 expressed in monocytes and macrophages. Hp-Hb binds to the amino terminal part of a scavenger receptor cysteine-rich domain region of CD163 and the cytoplasmic tail of the receptor conveys cellular uptake of the Hp-Hb complex by receptor-mediated endocytosis. [152]

In contrast to the Ca^2-dependent high-affinity CD163 binding elicited by Hp-Hb complex formation, Hp alone does not bind the receptor. [153] Hb, on the other hand, displays a low-affinity binding to CD163 [154] and is indeed endocytosed by CD163 in the absence of Hp, a clearance pathway that is thought to become operative if the amount of "free" Hb exceeds the binding capacity of Hp as is observed during massive hemolysis.[153]

The high-affinity interaction between Hp-Hb and CD163 critically depends on the scavenger receptor cysteine-rich domain 3 of CD163 [155] and 3 residues (glutamate 261, lysine 262, and threonine 264) of the Hp β-chain. [149] Another noteworthy aspect of the Hp-Hb-CD163 interaction is the finding that CD163 exhibits a higher functional affinity for the complexes generated between Hb and Hp2-2 than it does for the smaller Hp1-1-Hb complex.[153] After CD163-mediated internalization of Hp-Hb, the globin moieties are degraded in the lysosome, while the Hb-derived heme is converted by the cytosolic heme oxygenase-1 (HO-1) into the less toxic compounds free iron (Fe^2), carbon monoxide (CO) and biliverdin[156]

The Hp-CD163-HO-1 system for Hb clearance/metabolism and the anti-inflammatory response are linked by the metabolites generated, HO-1 is in the literature often regarded as having a protective function, but this function may be ascribed to the anti-inflammatory, anti-apoptotic, and anti-proliferative effects of one or more of its 3 products of heme catabolism, CO, biliverdin and Fe^2.[151] In particular, CO, which alone has been shown to mimic the protective effect of HO-1 in rodent disease models, is thought to contribute to these overall protective effects.[157] Biliverdin and bilirubin largely mediate their beneficial effect by their potent antioxidant properties.[158]

Fe^2, on the other hand, induces the iron-chelating protein ferritin, which mediates binding of Fe^2. The sequestering of Fe^2 by ferritin inhibits Fe^2- catalyzed generation of reactive oxygen species via the Fenton reaction and thereby protects against oxidative stress. Of interest, several therapeutic molecules, such as IL-10 and prostaglandin J2, function by activating HO-1; accordingly, it has been suggested that HO-1 may function as a so-called therapeutic funnel mediating the favorable effects of these therapeutic molecules.[157] Hp, CD163, and HO-1 are all induced by the inflammatory cytokine IL-6.[159] It thus seems likely and makes sense that these proteins are coordinately up-regulated during inflammatory conditions, thereby enhancing the capacity for Hb clearance and metabolism. Because of the anti-oxidative and anti-inflammatory effects of the heme metabolites, the overall outcome of the Hp-CD163–mediated delivery of Hb to monocytes/macrophages may be an anti-inflammatory response counteracting the inflammatory reaction. In addition, it has been suggested that intracellular signaling cascades activated by Hp-Hb binding to cell-surface CD163 may contribute even further to an anti-inflammatory response. [160]

Antibody-mediated cross-linking of CD163 at the cell surface was shown to trigger an intracellular signaling cascade resulting in Ca^2-mobilization, synthesis of inositol triphosphate, and secretion of IL-6 and granulocyte-macrophage colony-stimulating factor.[161] In analogy, the binding of Hp-Hb to cell-surface CD163 has been reported to elicit Ca^2-mobilization, synthesis of inositol triphosphate, [162] and secretion of IL-6, as well as IL-10. [163]

In view of the up-regulatory effect of IL-6 on Hp, CD163, and HO-1 [159] and the up-regulatory effect of IL-10 on CD163 and HO-1,[164] one could further speculate that IL-6 and IL-10 are involved in positive feedback mechanisms that enhance the capacity for Hb clearance and subsequent degradation of heme in response to red blood cell damage. Of interest, it was reported that secretion of IL-6 and IL-10 by macrophages was stimulated to a significantly

higher degree by Hp (1-1)-Hb than by Hp (2-2)-Hb in a process that depended on Hp-Hb binding to CD163 and casein kinase activity. [163]

Altogether, several functional differences among the Hp phenotypes in relation to its functional interaction with Hb have been reported, including the strength of the interaction with Hb [124] and CD163, [152] the rate of Hp-Hb clearance by CD163, [162] and the outcome of the signaling pathways that may perhaps be initiated by Hp-Hb binding to CD163 on the cell surface. [165] The ability to protect against Hb-mediated oxidation also appears to differ.[125]

Immunomodulation

The enhanced production of Hp during the acute phase of inflammation and infection and tumor growth suggests that this protein has additional functions. Haptoglobin has immune regulatory properties, with Hp2-2 individuals showing greater immunological reactivity than Hp1-1 and Hp2-1 individuals. [124] Haptoglobin also inhibits prostaglandin synthesis and consequently has important anti-inflammatory properties, although these are less pronounced in Hp2-2 individuals. [119]

Haptoglobin is a powerful suppressor of lymphocyte function, as shown by its ability to inhibit the mitogenic response of lymphocytes to phytohemagglutinin and concanavalin A. [166]

> Different T helper (Th) lymphocyte subtypes, known as Th1 and Th2 cells, are responsible for inducing and regulating the cellular and humoral immune response, respectively. Th1 cells produce IL-2 and interferon gamma (IFN-γ) and induce strong IgG responses, thus favoring the cellular immune response, whereas Th2 cells produce IL-4, IL-5, IL-6, IL- 10 and IL-13 and increase IgE production, thereby mediating a predominantly humoral and eosinophilic response.[167]

Hp plays an important role in modulating the balance between Th1 and Th2 lymphocytes (Th1/Th2) by promoting a predominantly Th1 cell response. These cells are more effective in protecting against infections involving intracellular parasites and inhibit the release of Th2 cytokines responsible for defense against extracellular microorganisms. [168]

More recently, it was showed that Hp1-1-Hb induces much greater IL-6 and IL-10 production than Hp2-2-Hb and that the release of these cytokines depends on the binding of these complexes to macrophage CD163 receptors and on casein kinase II (CKII) activity. The action of CKII was differentially regulated by the type of binding between the different Hp-Hb complexes and the CD163 receptor. [163]

Clinical complications in repeatedly transfused βTM

Patients with β-thalassemia major require regular transfusion therapy to sustain life. While such therapy effectively treats their anemia, the iron present in the hemoglobin of the transfused blood is retained in the body, since there is no physiological means of excreting it.[169] Iron accumulates primarily in the liver and spleen, and to a lesser extent in the heart, pancreas, and other organs.[170] which damage a variety of macromolecules and cell structures leading to hepatic cirrhosis, endocrine abnormalities, cardiac disease and eventually premature death.[171,172]

The Heart

In the absence of chelating therapy, myocardial disease remains the life-limiting complication of transfusional iron overload. As detailed over 30 years ago, irregularly transfused, unchelated children frequently developed left ventricular hypertrophy and conduction disturbances by late childhood, and ventricular arrhythmias and refractory congestive failure by the mid-teens,[173] while both chronic pulmonary hypertension and myocarditis may accelerate iron-induced cardiac failure in thalassemia.[174]

The liver

The liver is a major repository of transfused iron. Hepatic parenchymal iron accumulation, demonstrated after only 2 years of transfusion therapy, [175] may rapidly result in portal fibrosis in a significant percentage of patients. [176] In young adults with thalassemia major, in whom liver disease remains a common cause of death, viral infection [177] and alcohol ingestion [178] may act synergistically with iron in accelerating the development of liver damage.

Endocrine function and growth

The most common endocrine abnormalities in patients with thalassemia in the modern era include hypogonadotropic hypogonadism, growth hormone deficiency, and diabetes mellitus.[179] Variable incidences of hypothyroidism, hypoparathyrodism, and low levels of adrenal androgen secretion with normal glucocorticoid reserve, have been less commonly reported. [180,181]

Although normal rates of pre pubertal linear growth may be observed in patients maintained on regular transfusion programs, [182] poor pubertal growth and impaired sexual maturation have been observed in well-transfused patients, and has been attributed to iron-induced selective central hypogonadism,[183] interference of iron with the production of insulin-like growth factor (IGF-1), or both.[184]

The role of iron is supported by histologic findings of selective iron deposition in pituitary gonadotropes and by the reversibility of hypogonadism in primary hemochromatosis with intensive phlebotomy. [185]

Red cell alloimmunization

The recommended treatment for β-thalassemia major is a regular blood transfusion every 3-4 weeks. This treatment aims to correct the anemia both by significantly suppressing the hyperactive erythropoiesis and by inhibiting the excessive gastro-intestinal iron absorption. [186]

The regular blood transfusion regimen is confronted with numerous complications. In almost every patient, the transfusion requirement slowly increases over the years. Various factors which contribute towards this increased requirement include: development of hypersplenism, alloimmunization against various blood group antigens, chronic infections, folate deficiency, progressive bone marrow fibrosis as a result of toxic effect of free elemental iron, aplastic crises and hemolytic crises. [187]

Alloimmunization to erythrocyte antigens is one of the major complications of regular blood transfusions, particularly in patients who are chronically transfused. The factors for alloimmunization are complex and involve three main contributing elements: the RBC antigenic difference between the donor and the recipient, the recipient's immune status, and the immunomodulatory effect of the allogeneic blood transfusions on the recipient's immune system.[188] The development of anti-RBC antibodies (alloantibodies or autoantibodies) can significantly complicate transfusion therapy. Some alloantibodies are hemolytic and may cause various hemolytic transfusion reactions and limit the availability of further safe transfusion. Others are clinically insignificant. Erythrocyte autoantibodies appear less frequently, but they can result in clinical hemolysis and in difficulty in cross-matching blood. Patients with autoantibodies may have a higher transfusion rate and often require immunosuppressive drugs, splenectomy, or alternative treatments. [189]

A centralized system of RBC alloantibody records is available, which provides a valuable opportunity to evaluate the frequency of alloimmunization and autoimmunization to RBC antigens in multi-transfused thalassemia major patients. [190]

Iron chelators in thalassemia

Iron chelators have been shown to ameliorate oxidative damage in vivo. The mechanism of this therapeutic action is, however, complex, as desferrioxamine (DFO) has properties that can impact on oxidative damage independent of its capacity to act as an iron chelator. DFO can act as a reducing agent to remove cytotoxic ferryl myoglobin and hemoglobin and has recently been shown to prevent the formation of a highly cytotoxic heme-to-protein cross-linked derivative of myoglobin.[191] The use of chelating agents has proven to be highly effective, marked by a reduction in both morbidity and mortality. [192,193]

DFO, introduced in the 60's, was the mainstay for more than 30 years. Regular use, with improved clinical management, essentially doubled the average lifespan of patients. [194] Unfortunately, DFO must be given parenterally, the most effective regimens involving daily subcutaneous infusion over 8 to 12 hours, at doses of 40 to 60 mg/kg/day.[195] Needless to say, lifelong adherence is problematic with few patients getting the maximum benefit from their use of DFO.[196]

To overcome this hurdle, attempts to develop safe and effective oral agents have been ongoing since the mid 70's. [197] The first candidate to receive regulatory approval was deferiprone (DFP). It is generally recommended that this drug be taken at doses of 75 to 100 mg/kg/day in three divided doses, 5 to 7 days a week.[198,199] While DFP is not as effective as DFO in most patients, [200] adherence to its use is somewhat better.[201] With prolonged use, it is quite clear that body iron load is reduced and cardiac function is improved.[192,202] It does, however, have side effects that limit its usefulness. Chief among these are musculoskeletal (arthralgia, arthropathy), gastric (nausea, vomiting) and hematological (neutropenia, agranulocytosis) effects. Thus, up to 30% of patients discontinue its use for one reason or another. [203]

The approval of deferasirox (DFX) as an orally effective iron chelating drug in 2005 promised to improve the management of iron overload as this drug could be taken once daily and it exhibited few side effects. [204,205] Moreover, it proved to be non-inferior to DFO in a large, multicenter, randomized control trial involving roughly 600 patients, the doses of DFO and DFX ranging up to 60 and 30 mg/kg/day, respectively.[206] Many patients subsequently switched to DFX. Significant reductions in body iron load have been achieved with some patients showing improvement in cardiac function. [207,208] However, it is clear that DFX has its own limitations. While a wide variety of side effects have now been observed, it is primarily gastrointestinal and renal disturbances that limit its use in some patients. [209]

Adequacy of chelation

The estimation of serum ferritin levels is the most commonly employed test to evaluate iron overload in BTM. The association between serum ferritin and levels of body iron are well established and the test is easy to perform compared with other tests for iron overload. When the serum ferritin level reaches at 1000 ng/l (usually after 10th to 12th transfusion), it is generally taken as the point to initiate iron chelation therapy. [210]

Under conditions of ideal chelation, it is expected that serum ferritin levels be maintained within normal limits irrespective of the total number of transfusions. However such a uniform maintenance of serum ferritin levels was not found indicating irregular and inadequate chelation practices and/or variable response to chelation therapy. [211]

Chelation efficiency is calculated as [Iron excretion (mg/kg/day) / chelator dose (mg/kg/day)] x[molecular weight of the respective chelator /56] x n x 100. [212] where 56 is the molecular weight of iron and n = 3 with deferiprone and n = 1 with DFO.