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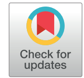
Review Article

Exploring the Impact of Iron Overload on Mitochondrial DNA in β-Thalassemia: A Comprehensive Review



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Abstract

Iron overload is a significant complication commonly observed in individuals with β-thalassemia, resulting from enhanced iron absorption due to ineffective erythropoiesis and frequent blood transfusions. Iron overload can lead to severe tissue damage and organ dysfunction, significantly impacting the quality of life for those affected. Additionally, recent research indicates that iron overload may also adversely impact mitochondrial function, further exacerbating the pathophysiology of this disease. Excessive iron accumulation in mitochondria can impair the electron transport chain, reduce adenosine tri phosphate synthesis, and increase the generation of reactive oxygen species, resulting in elevated tissue damage and clinical complications. Emerging evidence suggests that specific mitochondrial DNA (mtDNA) mutations may further contribute to the severity of iron overload in β-thalassemia patients. Currently, the clinical management of iron overload in patients with β-thalassemia primarily relies on conventional iron chelation therapies, aiming to reduce iron burden and prevent tissue damage. However, cases involving mtDNA mutations introduce additional complexities, necessitating personalized treatment approaches. Advances in gene therapy and mitochondrial replacement strategies offer promising avenues for potential targeted interventions. This review provides a comprehensive overview of the mechanisms underlying iron overload in β-thalassemia and its association with mtDNA mutations. It discusses the clinical manifestations, diagnostic challenges, and current treatment options for managing iron overload, while also highlighting emerging research directions and potential therapeutic targets for improved patient care. Ultimately, a better understanding of the complex interplay between iron overload and mtDNA mutations in β-thalassemia will pave the way for innovative strategies to alleviate the disease burden.

Keywords: Erythropoiesis; Iron overload; β-thalassemia; Reactive oxygen species; mtDNA; Gene therapy.

Abbreviations: AGY, wobble codon for serene (Y represents C/U); AMPK, AMP-activated protein kinase; *ATP*, adenosine tri phosphate; BMP6, bone morphogenic factor 6; *COX*, cytochrome oxidase; CREB, cyclic adenosine monophosphate response element binding protein; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats-CRISPR associated 9; CUN, wobble codon for leucine (N represents any nucleotide); cyt, cytochrome C; DFO, deferoxamine; DFP, deferiprone; DFX, deferasirox; D-loop, displacement loop; DMT1, divalent metallic ion transporters 1; DNA, deoxyribonucleic acid; EPO, erythropoietin; ERFE, erythroferrone; Erk, extracellular signal-regulated kinase; Fe-S cluster, iron sulfur clusters; FPN, ferroportin; GDF15, growth differentiation factor 15; HAMP, hepcidin antimicrobial peptide; Hb, hemoglobin; Hfe, hemochromatosis gene; HIF, hypoxia-induced factor; HJV, hemojuvelin; h-mtRNAP, human mitochondrial RNA polymerase; HSP, heavy strand promoter; IL-6, interleukin 6; JAK-STAT, Janus kinase/signal transducer and activator of transcription; LIC, liver iron concentration; LIP, labile iron pool; LSP, light strand promoter; LTCC, L-type calcium channel; MAPK, mitogen-activated protein kinase; mrfn2, mitoferrin2; MRI, magnetic resonance imaging; mRNA, messenger ribonucleic acid; MRT, mitochondrial replacement therapy; mtDNA, mitochondrial deoxyribonucleic acid; NTBI, non-transfusion bound iron; NTDT, non-transfusion dependent thalassemia; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; rRNA, ribosomal ribonucleic acid; sHJV, soluble hemojuvelin; shRNA, Short homologous RNA; siRNA, small interference ribonucleic acid; SMAD, mothers against decapentaplegic; STEAP3, six-transmembrane epithelial antigen of the prostate 3; TDT, transfusion dependent thalassemia; TERM, termination; TFAM, mitochondrial transcription factor A; TFB2M, mitochondrial transcription factor B 2; TfR, transferrin receptor; TfR1/2, Transferrin receptor; TMPRSS6, transmembrane serine protease 6; tRNA, transfer ribonucleic acid; TTCC, T type calcium channel; TWSG1, twisted gastrulation factor 1.

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Introduction

β-thalassemia is an autosomal recessive genetic disorder characterized by impaired erythropoiesis due to point mutations in the splice site or promoter sites of the *β-globin* genes on chromosome 11. These mutations result in either the absence or reduced production of β-globin chains.[1] While the babies are born healthy, the clinical

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manifestations typically manifest at approximately six months of age when the fetal hemoglobin (Hb) is replaced by adult Hb. This disorder is prevalent in Asian, Mediterranean, and African populations, affecting both males and females, with an estimated global prevalence of 1.5%.[2] The clinical manifestations vary according to the zygosity of the mutation and are classified into three categories: Thalassemia minor, characterized by a single-point mutation and reduced production of β chains, exhibits mild symptoms of anemia. Thalassemia major, often referred to as Cooley's anemia, is characterized by severe anemia due to two-point mutations in the *β-globin* gene. Thalassemia intermedia falls between the two extremes, exhibiting mild to moderate symptoms. Excess unpaired alpha chains precipitate as hemochromes, leading to the premature death of erythroid precursor cells and impaired erythropoiesis. This underlies the anemic manifestations of the disease, necessitating repeated blood transfusions. A recent classification introduces the terms "transfusion-dependent thalassemia" (TDT) and "non-transfusion dependent thalassemia" (NTDT) to describe the frequency of blood transfusions, with TDT requiring frequent transfusions and NTDT requiring fewer.[3]

Iron is an essential micronutrient crucial for various cellular activities, including cell survival, proliferation, and the function of numerous enzymes. It constitutes a significant component of hemoglobin, acts as a cofactor for multiple enzymes, serves as a redox site of the respiratory complexes, forming the electron transport chain of mitochondria, and is involved in processes such as ferroptosis. Heme accounts for the majority of iron in the body, followed by hepatocytes and macrophages. With no excretory mechanism available to eliminate excess iron from the body, a fine-tuned iron homeostasis exists to monitor cellular iron levels. Any disturbances to this equilibrium, either deficiency or overload, are associated with tissue and subsequent organ damage.

Iron absorption primarily occurs in the intestinal lumen, facilitated by the conversion of ferric iron to its ferrous state by the duodenal ferric reductase cytochrome B, which is present in the luminal cells of the intestine. This ferrous iron is transported into the cytoplasm through divalent metal ion transporters (DMT1) on the cell membrane of the enterocytes.[4] The systemic circulation of iron is maintained through ferroportin 1 (FPN1) transporters expressed on the basolateral membrane of enterocytes. Within the circulation, iron is oxidized to its ferric form by ferroxidases to facilitate binding to the blood carrier, transferrin. Transferrin delivers the metal ions to various tissues expressing transferrin receptors (TfR1 and TfR2).[5,6] A small portion of non-transferrin-bound iron (NTBI), bound to citrate or acetate, can also be found in plasma and is transported with the assistance of zinc and T-type calcium channels. Iron overload, as observed in β-thalassemia, occurs when transferrin becomes saturated, leading to the accumulation of NTBI.

The iron regulatory circuit employs the hepcidin FPN axis. Hepcidin serves as the principal regulator of iron homeostasis, binding to FPN1 and triggering its internalization, ubiquitination, and lysosomal degradation, thereby affecting iron mobilization within tissues.[7,8] Hepcidin secretion is inhibited by factors such as erythropoiesis, hypoxia, anemia, and iron deficiency, while infection, inflammation, and iron overload stimulate its expression.[9–13] Erythropoietin (EPO) regulates hepcidin secretion through the hemojuvelin/ bone morphogenic factor 6 (BMP6) pathway.[14–16] Impaired erythropoiesis, as in β-thalassemia, suppresses hepcidin secretion, promoting iron absorption from intestinal cells, and ultimately leading to iron overload.

Iron overload, often referred to as hemochromatosis, represents a secondary manifestation of β-thalassemia. Inefficient iron utilization for the synthesis of functional red blood cells, coupled with increased absorption and a lack of mechanisms to eliminate excess iron from the body, results in transferrin saturation and eventually the accumulation of NTBI in secondary sites such as the liver, cardiac tissue, and endocrine glands.[17,18] Free, unbound iron initiates the formation of toxic free radicals such as reactive oxygen species (ROS), leading to significant oxidative stress and subsequent tissue injury. Although oxidative stress is not the primary cause of β-thalassemia, it plays a prominent role in the pathogenesis of this disease.[19] Recent studies have observed that disorders resulting in impaired erythropoiesis such as sickle cell anemia and β-thalassemia, are associated with point mutations in the mitochondrial DNA (mtDNA).[20] This study included 38 TDT patients aged 4 to 53 years (median 29.5 years) and 24 healthy controls aged 19 to 46 years (median 24.5 years), who were receiving regular transfusions with no congestive heart failure during the study. Liver iron concentrations (LICs), serum ferritin and myocardial iron levels were monitored. The mtDNA/nuclear DNA ratio was 41% higher in patients compared to healthy controls. Researchers also observed a trend of increased mtDNA Δ4977 mutations among TDT patients with age, while no significant change was observed in the control group. However, 46% of the affected individuals with $T2^*>40$ ms exhibited a higher percent of mtDNA Δ4977 mutations because of oxidative damage to the mtDNA due to accumulated iron in the tissue.[21] Research suggests that hemochromatosis associated with repeated blood transfusions can generate a significant amount of ROS. The mitochondrial genome's susceptibility to ROS-induced oxidative damage, owing to the lack of histones, may contribute to organ failure in patients with β-thalassemia. Emerging research emphasizes the importance of mtDNA mutations in the clinical manifestation of this blood disorder, particularly in the context of cardiomyopathy, a major cause of morbidity among thalassemic patients. Iron overload-induced mtDNA mutations in cardiac tissue may play a role. Understanding the mechanisms of mtDNA variations associated with hemoglobinopathies and their genotype-phenotype correlations may provide new insights into the development of novel therapeutic approaches for these inherited disorders. In this comprehensive review, we aim to explore the intricate relationship between iron overload and mtDNA mutations in β-thalassemia patients, shedding light on the implications for the clinical management of this complex and multifaceted disease.

Mechanisms of iron overload in β-thalassemia

Iron overload is a prominent complication associated with β-thalassemia, contributing to numerous health challenges. Two primary contributors to iron overload have been identified in β-thalassemia. In TDT patients, recurrent blood transfusions predispose them to iron accumulation at secondary sites, including the liver, heart, and endocrine glands, thereby increasing the risk of iron toxicity. Monthly, approximately 2–4 units (0.3–0.6 mg/kg per day) of packed red blood cells are transfused, with each unit containing 200–250 mg of iron. The absence of any physiological mechanism for eliminating excess iron in human body, the deposition of iron at secondary sites can lead to lethal consequences, commonly known as hemochromatosis. Regular monitoring of serum ferritin and LIC is essential for initiating iron chelation therapy, as the onset of iron overload consequences may develop after prolonged years of transfusion. However, hemochromatosis is not limited to TDT. About 63.8% of HbE/β-thalassemia patients with

NTDT develop iron overload, characterized by elevated serum ferritin levels (200 to 400 ng/mL). Surprisingly, although dietary iron contributes to only 15% of the iron source, iron overload in NTDT seems to be fuelled by increased absorption of iron from duodenal cells.[22] Ineffective erythropoiesis and the suppression of hepcidin synthesis play key roles in driving the excessive absorption of iron from duodenal cells and its accumulation at secondary sites.[23] This can be attributed to the decreased production of β chains, leading to an imbalance between α and β globin chains, ultimately causing hemolytic anemia. NTDT is associated with increased proliferation of early erythroid precursors and premature death of erythroblasts, causing severe anemia with elevated erythropoietin (EPO) levels. Elevated serum EPO stimulates the secretion of erythroferrone (ERFE) from erythroid precursor cells, which inhibits hepcidin synthesis and promotes iron absorption from the intestinal cells. Importantly, hepcidin secretion is suppressed by EPO and ERFE, while it is induced by iron overload. However, erythropoietic signals tend to outweigh signals from the iron store regulators, perpetuating the effects of iron overload on hepcidin secretion.[24] These effects of EPO on hepcidin secretion are mediated through cytokines belonging to the transformation growth factor β family, such as growth differentiation factor 15 (GDF15) and twisted gastrulation factor. NTDT patients exhibit elevated GDF15 with suppressed hepcidin.[25] Other factors contributing to the hepcidin suppression include platelet-derived growth factor-BB, which is expressed in various cell types exposed to EPO, downregulating the transcription factors cyclic adenosine monophosphate response element binding protein (CREB) and CREB-H.[26] EPO-induced ERFE secretion mediates the repressive effect of hepcidin via the BMP6 pathway.[13] BMP6 acts as a regulator for sensing cellular iron levels, and in conjunction with the hemojuvelin receptor, mediates its effects on hepcidin synthesis. The production of BMP6 by non-parenchymal cells induces the hepatic expression of hepcidin. High levels of intracellular iron in non-parenchymal cells induce ferroportin-mediated degradation, maintaining the high levels of intracellular iron and further stimulating BMP6 production in a positive feedback loop.[27] However, hepcidin suppression during ineffective erythropoiesis impairs this feedback loop, emerging as a major contributor to iron overload in β-thalassemia. Figure 1 outlines the mechanism of regulating hepcidin expression.

Organization of mitochondrial DNA

The organization of mtDNA is distinct, lacking histones or chromatin beads, and is present in multiple copies per cell, which varies based on the cell type and energy demand. This double-stranded DNA is packaged into elongated protein structures known as mitochondrial nucleoids with a diameter of approximately 100 nm. The double strand is classified as the light strand and the heavy strand, reflecting their differences in guanine-cytosine content. Both strands contain genetic information to encode various RNA molecules, including messenger RNA, transfer RNA (tRNA), and ribosomal RNA (rRNA), which are essential for maintaining key functions of the organelle.[28] Human mtDNA is composed of 16,569 bases encoding 37 genes, including 13 genes coding for peptides forming the oxidative phosphorylation (OXPHOS) system, 2 ribosomal RNA genes (12S and 16S), and 22 tRNAs (Figure 2). The remaining proteins essential for mitochondrial function, as well as those required for the maintenance and expression of mtDNA, are encoded by the genomic DNA and synthesized in the cytosol. These proteins are then targeted and sorted to the mitochondria through protein translocators, guided by mitochondrial peptide signals.[29–31] The mtDNA forms histone-free protein-DNA complexes, referred to as nucleoids, with 6–8 nucleoids tethered together forming foci. A typical cell may harbor an average of 450 foci, tethered directly or indirectly through mitochondrial membranal kinesin. Human mtDNA is apparently four times smaller than genomic DNA and exhibits less complex packaging compared to the nuclear genome. Mitochondrial transcription factor A (TFAM) facilitates the packaging of mtDNA into nucleoids and regulates its transcription and replication processes. TFAM consists of two high mobility group box domains that intercalate with the minor groove of the DNA. A small positively charged linker connects these two domains and interacts with the DNA backbone, facilitating the tight packing of mtDNA. A single nucleoid is found to be bound with approximately 1,000 TFAM molecules, and the abundance of the TFAM molecules determines the transcriptional accessibility of the nucleoid.[32]

The only non-coding region is the 3′ terminal displacement loop (D-loop), measuring approximately 1.1 kilobases (kb) in length, formed by the displacement of two genomic strands by a third DNA strand. The D-loop region is responsible for housing the regulatory components involved in the replication and transcription of mtDNA. The 650 bp of the D-loop DNA forms the 3rd strand loop of mtDNA, and adjacent 250 bp hosts the light strand promoter (LSP) and heavy strand promoter (HSP1 and HSP2). The LSP and HSP exhibit opposite transcriptional orientations for mtDNA, with a medial 154 bp region serving as binding sites for various TFAMs that regulate the transcriptional activity from both promoters. Transcription initiation sites were identified on both the light and heavy strands, with the light strand encoding for one OXPHOS protein and eight tRNAs, while the heavy strand utilized HSP1 to facilitate the transcription of two rRNA genes, namely *12S* and *16S rRNA*. Additionally, HSP2 promotes the transcription of 12 messenger RNAs in the OXPHOS complex and 14 tRNAs. The primer required for the replication of the leading strand of mtDNA is encoded by the light strand.[33]

Impact of iron overload on mitochondrial function

The mitochondria play a pivotal role in cellular iron utilization, particularly in heme synthesis and the formation of mitochondrial redox clusters known as iron-sulfur clusters (Fe-S clusters). These clusters are crucial components of the electron transport chain, highlighting the fundamental role of mitochondria in cellular energy production. Cellular iron homeostasis is intricately linked to the biogenesis of Fe-S clusters, underscoring the importance of proper iron management within these organelles.[34] Disruptions in mitochondrial iron transport can have profound consequences for cellular health. Iron transport across the mitochondrial membrane is a finely tuned process essential for meeting the mitochondrial iron requirements while maintaining the balance with the cytosolic labile iron pool.[35] Furthermore, excess labile iron within the mitochondria can induce oxidative stress by generating ROS, which can be detrimental to mitochondrial function. This highlights the necessity for stringent regulation of iron trafficking across the mitochondrial membrane. Iron is transported into mitochondria predominantly through passive mechanisms driven by membrane potential and fusion events. Iron can exist as free labile ferric ions or be bound to chaperone proteins for delivery. In the erythroid cells, endosomal delivery is the primary mode of ferric ion transport.[36] Numerous transporters serve as carrier proteins facilitating iron transportation through the outer mitochondrial membrane. For example, mitoferrin has been identified as a high-affinity iron

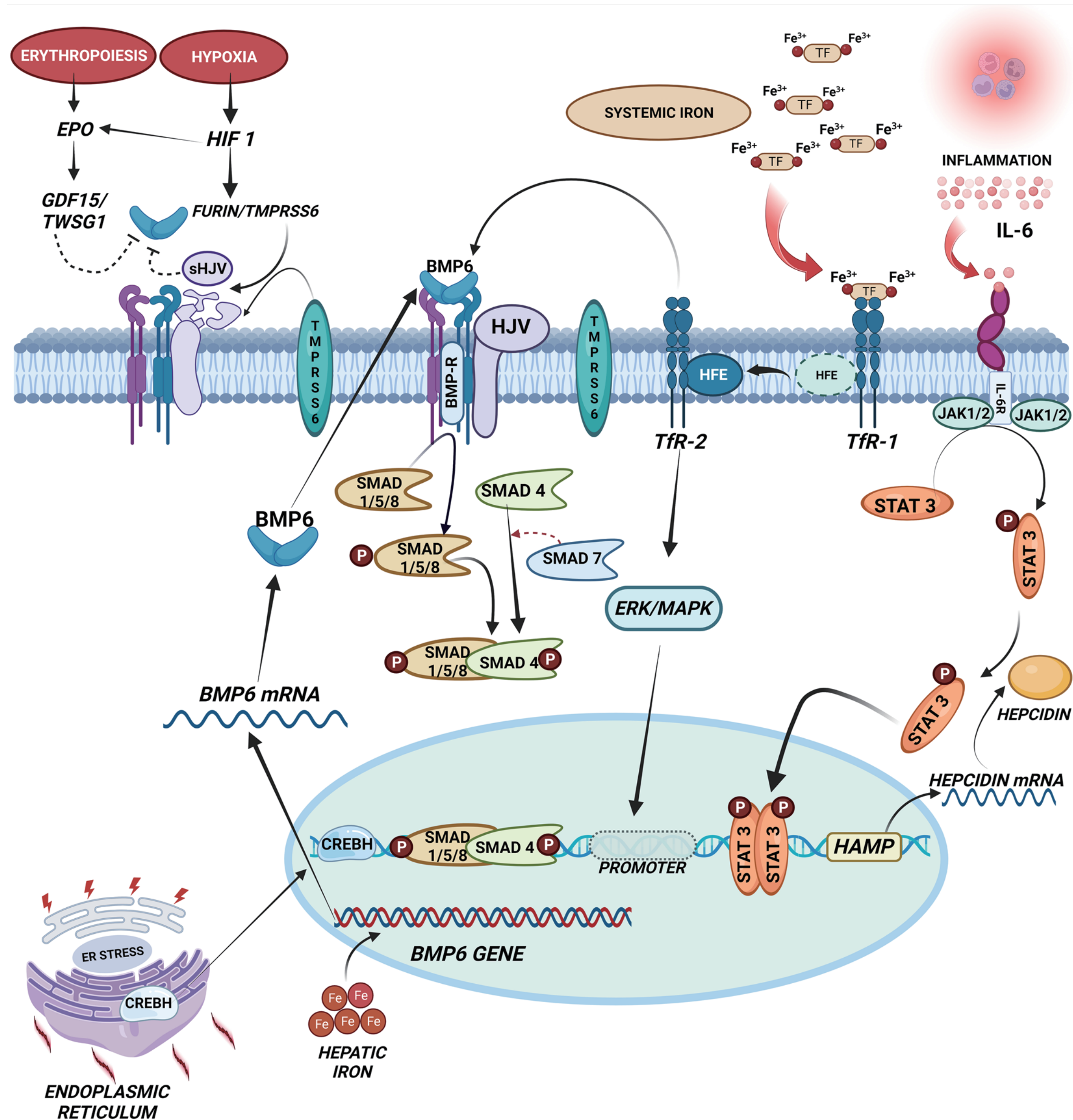

Fig. 1. Increased systemic iron levels induce the expression of hepcidin via TfR/Hfe. Saturation of TfR1 leads to the dissociation of Hfe and the binding of the alter to the TfR2, which in turn influences the binding of BMP6 to its receptors on the membrane. Increased liver iron concentration induces the expression of the *BMP6* gene, forming BMP6 proteins, which bind to the BMP receptors (ALK2). When bound to BMP6, the receptors recruit SMAD proteins and phosphorylation of SMAD1/5/8-SMAD4 complex mobilizes to the nucleus, activating the expression of hepcidin. Inflammatory signals via IL-6 through the JAK-STAT cascade induce the expression of the Hepcidin gene (*HAMP*). Higher EPO and HIF lead to the secretion of ERFE from erythroid precursor cells, which attenuates the BMP6 signaling cascade via GDF15/TWSG1. In the absence of BMP6, TMPRSS6 binds to ALK2, inhibiting the hepcidin signaling cascade. BMP6, bone morphogenic protein 6; EPO, erythropoietin; ERFE, erythroferrone; Erk, extracellular signal-regulated kinase; GDF15, growth differentiation factor15, HAMP, hepcidin antimicrobial peptide; Hfe, hemochromatosis gene; HIF, hypoxia-induced factor; IL-6, interleukin 6; JAK-STAT, Janus kinase/signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; sHJV, soluble hemojuvelin ; SMAD, mothers against decapentaplegic; TfR1/2, Transferrin receptor; TMPRSS6, transmembrane serine protease 6; TWSG1, twisted gastrulation factor1.

transporter in the hematopoietic tissues of mice, playing a crucial role in iron transport across the outer mitochondrial membrane.[37] Within the mitochondrial matrix, free labile iron is chelated by the mitochondrial chaperone protein frataxin. This interaction not only facilitates the transport of iron for the synthesis of heme and Fe-S cluster synthesis but also prevents potential oxidative damage caused by free ferrous iron.[38] Additionally, iron can be stored in the matrix in the form of mitochondrial ferritin. Figure 3 summarizes the mechanism of iron accumulation and its effects on mitochondrial function, resulting in hepcidin suppression in β-thalassemia.

Any disruption in the proper processing of iron inside the mitochondria can lead to mitochondrial dysfunction, with a wide range of cellular consequences. This includes impaired energy production, increased oxidative stress, and compromised cellular health, highlighting its critical role in understanding the pathophysiology of iron overload disorders like β-thalassemia.

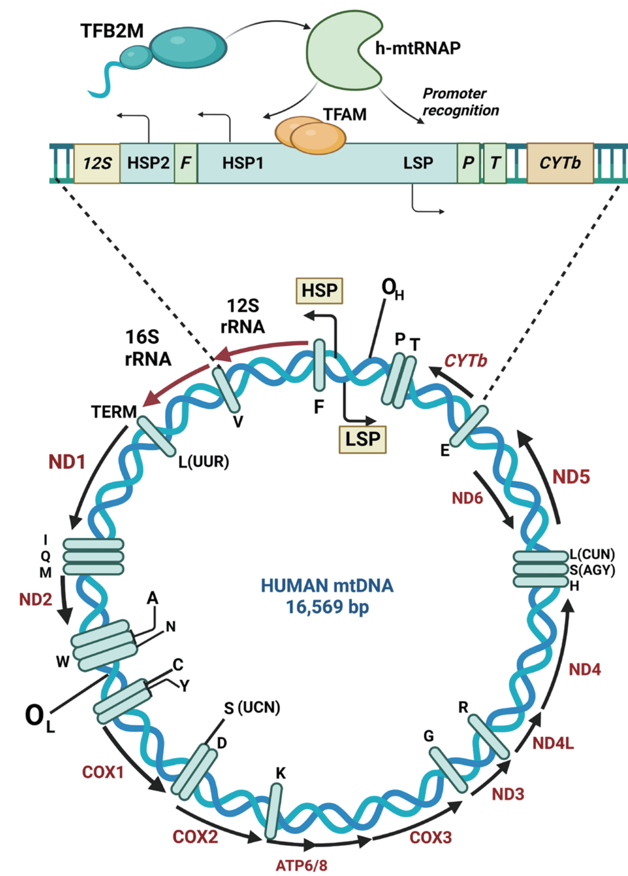

Fig. 2. Structural organization of human mitochondrial DNA. Human mitochondrial DNA is composed of 16,569 bp. The DNA strands are categorized into heavy (H-strand) and light (L-strand) strands based on their G: C content. The mitochondrial genome encodes 37 genes, including 13 genes coding for peptides forming the OXPHOS system, 2 ribosomal RNA genes (12S and 16S), and 22 tRNAs. O_H and O_L represent the origins of H and L strands, respectively. The promoter sites for HSP andLSP are depicted, and TERM represents the binding site for the mitochondrial transcription terminator. *CYTb* encodes apocytochrome b of ubiquinol: ferricytochrome c oxidoreductase *ND*, *COX* and *ATP* refer to genes coding for subunits of NADH: ubiquinone oxidoreductase, ferrocytochrome *c*: oxygen oxidoreductase (cytochrome *c* oxidase) and F_1F_0-ATP synthase, respectively. The single letter codes represent the amino acids of *tRNA* genes (L(CUN) and S(AGY) represent the iso acceptors for leucine and serine respectively. AGY, wobble codon for serene (Y represents C/U); *ATP*, adenosine triphosphate; *COX*, cytochrome oxidase; CUN, wobble codon for leucine (N represents any nucleotide); h-mtRNAP, human mitochondrial RNA polymerase; HSP, heavy strand promoter; LSP, light strand promoter; mRNA, messenger RNA; ND, nicotinamide dehydrogenase; OXPHOS, oxidative phosphorylation; rRNA, ribosomal ribonucleic acid; TERM, termination; TFAM, Mitochondrial transcription factor A; TFB2M, mitochondrial transcription factor B 2; tRNA, transfer RNA. UUR, wobble codon for leucine, LSH, leucine, serine, histidine

Effects of iron overload on mitochondrial functions

Although mitochondria are the major consumers of iron, excess iron in the mitochondrial matrix can result in the generation of ROS and subsequent oxidative damage to the mitochondrial membrane. Notably, during iron overload conditions, mitochondria exhibit an increased rate of iron uptake, resulting in mitochondrial iron accumulation. This heightened iron uptake is facilitated by the failure of iron chelators to inhibit iron transport across the mitochondrial membrane, ultimately leading to mitochondrial dysfunction.[39] The disruption in iron homeostasis has a significant impact on the pathology observed in mitochondrial disorders, such as Leigh syndrome, characterized by neurodegeneration and brain inflammation. Interestingly, malfunction or loss of mitochondrial complex I can disrupt iron metabolism, leading to hepatic iron overload and neurological dysfunction.[40] In mouse studies, the administration of ferric citrate to liver cells resulted in lipid peroxidation of the mitochondrial membrane and significant DNA fragmentation. Lipid peroxidation generates malondialdehyde, a biomarker for oxidative stress-mediated membrane damage. However, conflicting studies have reported varying levels of malondialdehyde in thalassemia patients following blood transfusion, suggesting that the process of ROS-mediated lipid peroxidation is influenced by multiple factors.[41] Iron overload can also impact mitochondrial function by affecting the expression of key proteins. Proteomic analysis of erythroid precursor cells from thalassemic patients revealed altered expression patterns of mitochondrial chaperones, including heat shock protein 60 (HSP60) and prohibitin2. Prohibitin2 downregulation and HSP60 upregulation were associated with apoptotic cell death in erythroid precursors, highlighting the link between mitochondrial iron status and apoptosis in thalassemic patients.[42] Studies have shown that in the presence of excess serum iron, as observed in conditions such as hereditary hemochromatosis (caused by the loss of the *Hfe* gene), iron accumulates excessively in secondary sites, particularly in the liver and cardiac tissue.[43] Cardiac arrhythmias are a common consequence of this iron buildup, contributing significantly to morbidity in affected individuals. Chronic iron overload is associated with cumulative oxidative damage to mtDNA and disrupts the synthesis of electron transport chain complexes, resulting in respiratory distress. It is important to note that despite this decrease, however, the cytoplasmic reactive iron concentration remains unchanged. This imbalance can result in cardiac arrhythmia and myocardiopathy, particularly in individuals with conditions like β-thalassemia.[44]

Recent findings have shed light on how iron overload affects cardiac mitochondrial function, leading to reduced mitochondrial respiration, increased synthesis of ROS, mitochondrial membrane depolarization, and mitochondrial swelling, which affect the mitochondrial fission and fusion process.[41] A study conducted on a cohort of 100 patients diagnosed with TDT, revealed that patients with homologous myocardial iron overload had a significantly greater likelihood of experiencing cardiac complications compared to those without any myocardial iron accumulation.[45] Interestingly, left ventricular dysfunction tends to be more prevalent than right ventricular dysfunction in these cases. This preference for left ventricular iron accumulation is thought to be a key factor in ventricular dysfunction, and is attributed to the toxic impact of ROS induced by labile iron deposited in cardiac tissue.[46,47]

Inhibition of mitochondrial calcium uniporter can effectively alleviate the detrimental impact of iron overload on mitochondria by mitigating iron-mediated mitochondrial swelling and depolarization.[48,49] Administration of iron chelation therapy resulted in several positive outcomes in thalassemic mice subjected to a high-iron diet. These benefits included attenuating the effects of cardiac overload, improving mitochondrial function, and enhancing left ventricular function. Notably, these effects were not associated with any alteration in cardiac mitochondrial biogenesis.[45] Mesenchymal stromal cells affected by iron overload exhibit an excessive degree of mitochondrial fragmentation, accompanied by a decrease in ATP levels. This phenomenon can be attributed to elevated levels of ROS and decreased electron transport chain activity. Research conducted

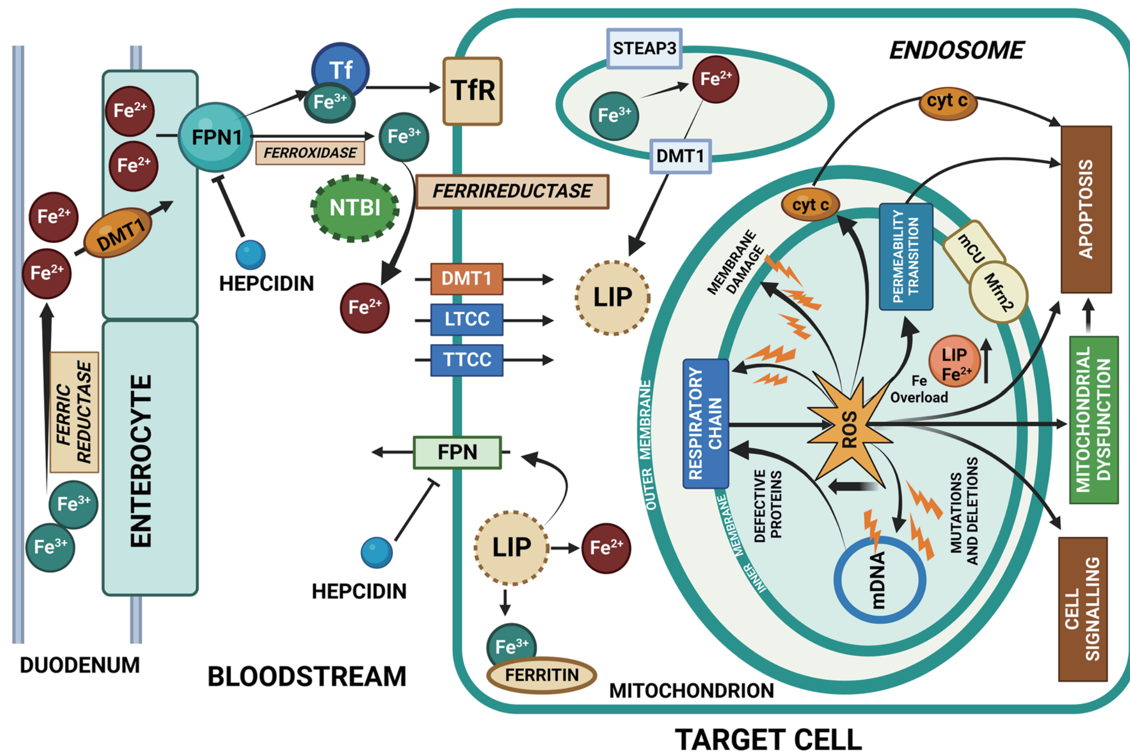

Fig. 3. Hepcidin suppresses the iron uptake from duodenal cells by sequestering the iron import through FPN transporters. Hepcidin suppression will lead to increased iron uptake by FPN transporters mediated via TfR receptors. The NTBI is converted to ferrous iron by the ferrireductases and transported through DMT1 transporters, increasing the mitochondrial LIP. Within the mitochondrial matrix, an excess of increased LIP concentration leads to ROS-mediated oxidative damage to the mitochondrial membrane and mtDNA. *cyt*, cytochrome C; DMT, divalent metal ion transporter; FPN, ferroportin; LIP, labile iron pool; LTCC, L-type calcium channel; mrfn2, mitoferrin2; mDNA, mitochondrial DNA; NTBI, non-transferrin bound iron; ROS, reactive oxygen species; STEAP3, six-transmembrane epithelial antigen of the prostate 3; TfR, transferrin receptor; TTCC, T type calcium channel.

by Zheng *et al*. revealed that mitochondrial damage mediated by ROS involves the activation of the AMP-activated protein kinase (AMPK) signaling cascade.[50] The study found that silencing the *AMPK* gene resulted in reduced cell apoptosis, enhanced cell viability, and decreased mitochondrial DNA damage.

Furthermore, the presence of ROS has been found to result in mtDNA mutations, which accumulate over time in both aging individuals and those affected by diseases. As the site of OXPHOS, mitochondria generate excess ROS in the form of superoxide free radicals, which are unstable and subsequently released into the mitochondrial matrix. Within the matrix, superoxide dismutase facilitates their reduction, resulting in the formation of hydrogen peroxide, a relatively stable compound. Hydrogen peroxide has the potential to generate other ROS members, such as hydroxyl free radicals, which possess a high capacity to inflict damage to DNA.[51] Accumulation of ROS might result in either the formation of thymine glycols or the formation of 8-oxo-dG (8-oxo, 7,8-dihydro, 2′ deoxyguanosine), leading to G>T transversions.[52] The buildup of such mutational lesions exhibits rapid and exponential escalation, ultimately leading to a significant impact on the integrity of mtDNA. Oxidative stress eventually leads to the loss of damaged mtDNA, an interesting phenomenon observed exclusively in mitochondria.[53]

Exploring the association between mitochondrial DNA mutations and iron overload

mtDNA aberrations, such as deletions, transversions, transitions, duplications, and insertions, have been identified as influential factors affecting mitochondrial function. mtDNA nucleoids, located close to the respiratory complexes, are more susceptible to oxidative damage, leading to mtDNA mutations. The limited presence of non-coding sequences in mtDNA contributes to a more pronounced phenotypic manifestation of disease states resulting from mtDNA mutations. This difference is attributed to the heteroplasmic state, wherein both mutant and wild-type DNA coexist, favoring a greater abundance of mutated DNA. Individuals with a low mutational load may experience a prolonged absence of clinical symptoms. Nevertheless, the initiation of mitochondrial diseases is subject to temporal variation across individuals and is primarily characterized by a diverse range of manifestations specific to various tissues. The mutations most frequently observed in mitotic cells are sporadically acquired, establishing as either point mutations or base deletions. These mutations arise within a single DNA molecule, which may undergo clonal expansion or loss of the gene. Clonal expansion results in the heterogeneous expression of mitochondrial diseases as individuals age.[54] Accumulation of point mutations is primarily observed in mitotic cells, whereas deletions are more prevalent in post-mitotic cells. Various hypotheses and scientific models have been proposed to explain the clonal expansion of these mutations. The majority of these theories rely on the concepts of mtDNA copy number and mtDNA turnover. The presence of multiple copies of mtDNA allows the coexistence of wild-type and mutant genes, creating a heteroplasmic condition. However, phenotypic variations are not apparent until the mutation

Table 1. Types of mitochondrial DNA mutations identified in *β-thalassemia*

Sl.no	Mutation site	Nature of mutation	Affected gene	Clinical phenotype	Reference
1	G>T	Transversion	ROS-mediated oxidative damage of mtDNA	Hereditary hemochromatosis	48,49
2	Δ4977	Deletion mutations	OXPHOS	Cardiac malfunction and atrial fibrillation	54
3	IVSI-5 (AC>AG)	Transversion	D-loop	Hereditary hemochromatosis	56
4	Codon 8/9 (CT>CC)	Transition	D-loop	Hereditary hemochromatosis	
5	Cd44 (GG>GA)	Transition	Hypervariable sites of D-Loop	Hereditary hemochromatosis	
6	16,069C>T, 16,189T>C, 16,319G>A, and 16,519T>C	Point mutations	do	B thalassemia	48,58

D-loop, displacement loop; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species.

frequency reaches the threshold level. Reported threshold levels vary depending on the specific mutation type, ranging from 50% to 60% for deletion mutations and 90% for mutations occurring in tRNA genes.[55] Indeed, the disease presentation is determined by the quantity of retained wild-type DNA within the cell. The random replication events of mtDNA affect the clonal expansion, thereby influencing the severity of mitochondrial diseases.

According to Zheng *et al*.,[56] transition mutations may arise due to errors in the function of poly γ, a protein involved in driving the replication of mtDNA. Additionally, it has been observed that spontaneous deamination of cytosine to uracil can occur, leading to the formation of a base pair between uracil and adenine, ultimately resulting in a G>A transition. The Δ4977 deletion is a frequently occurring mutation induced by ROS-mediated oxidative damage, affecting the genes encoding tRNAs and a few components of OXPHOS. This deletion mutation serves as a biomarker indicating the occurrence of oxidative damage to mtDNA.[57] Studies conducted on a group of TDT patients support the hypothesis that increased free iron leads to the formation of ROS, eventually increasing the frequency of the Δ4977 deletion mutation. The mtDNA to nuclear DNA ratio was considered a metric for assessing the impact of extrahepatic iron accumulation and associated organ dysfunction. Patients who underwent blood transfusions for a duration exceeding five years developed cardiomyopathy.[21] Furthermore, these individuals were shown to have the Δ4977 deletion mutation. This finding aligns with the increased likelihood of cardiac malfunction and atrial fibrillation reported in TDT patients. Frequent mutations are observed in non-coding regions of the D-loop, often referred to as hypervariable regions HV1 (16,024–16,569 bp) and HV2 (57–372 bp).[58] With higher mutation rates, multiple polymorphisms are observed, which can be associated with a variety of diseases, including neurological disorders and cancers. In β-thalassemia oxidative stress is induced by repeated blood transfusions, suppressed hepcidin, and subsequent iron overload, resulting in 8-oxodG mutagenic lesions on mtDNA. Heterozygous alleles in the HV2 region, characterized by nucleotide transition/transversion events at IVSI-5 (AC>AG), codon 8/9 (CT>CC), and Cd44 (GG>GA), were detected.[59] Homoplasmic D-loop polymorphisms were identified in a group of affected individuals with point mutations of 16,069C>T, 16,189T>C, 16,319G>A, and 16,519T>C. The variants 16,069C>T, 16,319G>A, and 16519T>C were more prevalent among the affected individuals compared to the healthy controls, unlike the variant 16,189T>C (Table 1).[48,49,54,56,58,60] The same single nucleotide polymorphisms have been reported in various cancers,[52] hereditary hemochromatosis and type 2 diabetes.[60,61] However, the underlying mechanisms behind these observations remain to be elucidated. The interplay between iron overload and mitochondrial dysfunction, as well as the relationship between mtDNA mutations and iron overload, is complex and multifaceted.[62] Further research is needed to fully understand the mechanisms underlying these interactions and their implications for human health.

Clinical manifestations of iron overload and mitochondrial DNA mutations

The iron metabolic pathway, defined as a unidirectional process lacking intrinsic mechanisms to eliminate excess iron from the body, results in the accumulation of iron at secondary sites such as the liver, cardiac, and endocrine systems due to conditions like repeated blood transfusions and imbalances in iron homeostasis. This accumulation manifests in various clinical conditions. Patients with β-thalassemia often exhibit a spectrum of symptoms of varying severity. Common clinical manifestations include chronic fatigue, hepatosplenomegaly, irregular heart rhythm, hypogonadism, elevated blood glucose levels, hyperpigmentation (bronze skin), and depression.[63] In TDT patients, cardiac iron overload tends to occur more rapidly than liver iron accumulation, leading to myocardial failure and significant comorbidities. Conversely, NTDT patients primarily accumulate iron in hepatic cells, resulting in conditions such as liver cirrhosis and hepatocellular carcinoma.[18] The myocardial failure observed in β-thalassemia patients is attributed to the increased iron absorption and its rapid accumulation in the cardiac muscles, subsequently causing mitochondrial dysfunction, which plays a pivotal role in organ failure.

The clinical manifestations of β-thalassemia are intricate and overlap with various other medical conditions. Detecting the involvement of mtDNA mutations in these clinical presentations poses a diagnostic challenge. Many mtDNA mutations exist in heteroplasmic forms, necessitating genetic testing to confirm carriers of disease-associated mtDNA mutations. The variability in mtDNA mutations can lead to a reduction in the number of shared mtDNA molecules among offspring, a phenomenon referred to as screening by mitochondrial genetic bottleneck.[64] This genetic variation contributes to differences in the presentation of clinical phenotypes among children born to carrier females. Developing personalized therapeutic approaches is essential for managing β-thalassemia, involving the detection of mtDNA mutations and

the assessment of iron overload in tissues, serum, and liver iron levels. Genetic screening and hematological testing play crucial roles in determining disease severity and guiding treatment decisions.

Diagnosis and monitoring of iron overload

The diagnosis of iron overload, particularly in conditions such as hemochromatosis and β-thalassemia, relies on several key parameters. Serum ferritin levels are a primary indicator of iron metabolism. Elevated levels of serum ferritin, typically above 300 ng/ml in males and 150–200 ng/ml in females, indicate excess iron accumulation in the body. However, it is crucial to consider that inflammation, infection, and liver disorders can also impact serum ferritin levels. Serum ferritin levels exceeding 1,000 ng/ml necessitate the initiation of chelation therapy in TDT patients, while for NTDT patients,[63] this threshold is typically set at 800 ng/ml.[65] Assessing total iron binding capacity and serum transferrin saturation percentages, especially when exceeding 45%, can aid in diagnosing iron overload. Plasma NTBI and the labile iron pool are the major sources of extrahepatic iron accumulation. NTBI can be estimated through the chelation capture method, followed by high-performance liquid chromatography, colorimetric estimations, or indirect measurements of the oxidized fluorochrome as in the LIP assay.[66] Hepatic iron overload can be diagnosed through a liver biopsy. Percutaneous liver biopsies effectively evaluate hepatic iron overload and hepatic fibrosis, assisting in grading the extent of iron accumulation.[67] Magnetic resonance imaging (MRI) has replaced the biopsy for quantifying hepatic iron overload with its non-invasive methods, correlating the milligrams of iron per gram of liver with body iron stores. MRI T2* thresholds reflect the severity associated with organ-specific iron overload and organ damage.[68] In cases of cardiac iron overload, T2* values below 10 ms may predict a higher risk of mortality, while values between 10–20 ms can indicate a lower left ventricular ejection fraction and arrhythmias in TDT patients. MRI can be used to measure iron accumulation in various organs, such as the spleen, liver, pancreas, and heart. This approach is essential because serum ferritin and total iron concentrations do not reflect the total body iron concentration in β-thalassemia patients, necessitating organ-specific monitoring.[69]

In conjunction with clinical, biochemical, and histopathological assessments, molecular screening of mtDNA mutations provides a rational approach to understanding the onset of iron overload-related issues and their correlation with the genotype-phenotype variations among thalassemia patients. Commonly screened mtDNA mutations include Δ4977, a biomarker for deletion mutations caused by oxidative damage to mtDNA.[70] Homoplasmic D-loop polymorphisms and mutations in HV1 and HV2 regions can be assessed through RFLP or direct sequencing of the amplified mtDNA. Next-generation sequencing has enhanced the reliability and accessibility of mtDNA sequencing. Genetic screening of mtDNA mutations follows the criteria defined by DiMauro and Schon,[71] considering the presence of neutral polymorphisms, evolutionarily conserved and phenotypically distinct mutations, deletion mutations exhibiting heteroplasmy, and how the degree of heteroplasmy aligns with the severity of symptoms.

In summary, the prognosis of patients with iron overload conditions can be improved using diagnostic biomarkers such as serum ferritin and total body iron. In addition, monitoring techniques, including MRI, assist in assessing the extent of iron accumulation in different organs and guide treatment decisions. Genetic screening of mtDNA mutations provides valuable insights into the genetic factors influencing iron overload-related complications, aiding in the development of personalized therapy.

Current treatment approaches for iron overload in thalassemia

Excess iron accumulation is evidently toxic, with the potential to cause organ dysfunction and if left untreated, it can lead to fatal outcomes. Maintaining an efficient iron balance is crucial, especially in cases of repeated blood transfusions. To determine the optimal approach to iron chelation therapy, it is crucial to understand the rate at which iron accumulates from transfusion therapy. Recommended transfusion regimens suggest that individuals can accumulate an equivalent of 116–232 mg of iron/kg body weight/year, or 0.32–0.64 mg of iron/kg body weight/day with transfusion volumes of 200–400 ml per kg body weight per year. Regular blood transfusion therapy significantly increases iron stores, often surpassing normal levels, necessitating chelation treatment. Even in the case of NTDT, where iron absorption rates exceed normal levels (1–2 mg/day), the lack of extensive transfusion can contribute to 3–5 mg/day of iron absorption, representing 1–5 g of iron overload in a year.[65] Iron accumulation is influenced by various factors, including patient age, tissue-specific factors, duration of transfusion therapy, and tissue iron levels. Regular monitoring of serum ferritin levels, LIC and cardiac iron overload is essential for determining the appropriate regime of iron chelation therapy. Three iron chelators, deferoxamine (DFO), deferiprone (DFP), and deferasirox (DFX), are effectively used to facilitate excess iron excretion through either urine or feces. DFO is typically administered intravenously, while DFP and DFX can be taken orally. However, patients may experience side effects such as gastrointestinal disturbances, agranulocytosis, and transaminitis. The choice of chelation therapy may depend on the efficacy and safety of the regimen.[72] DFO is preferred for renal and hepatic clearance, while DFP is preferred for cardiac and renal clearance of accumulated iron. DFX is often prescribed for eliminating accumulated iron from the liver. DFX or DFP monotherapy is recommended over DFO monotherapy for transfusional iron overload in patients older than 2 years. Treatment typically involves regular administration often 5 times a week, with standard doses ranging from 20–40 mg/kg for children up to 50–60 mg/kg for adults, delivered via an 8–12 hour subcutaneous infusion for a minimum of 5–6 nights per week.[73] The addition of vitamin C at recommended doses can enhance iron excretion, increasing the availability of non-chelatable iron.[65] The efficacy of chelation therapy relies on strict adherence to the chelation regimen, which may be influenced by various factors, such as side effects, patient response, duration of the transfusion, and treatment cost. Novel strategies are being developed to address post-transfusion iron overload and mitochondrial dysfunction, with personalized medicine based on genetic testing at the forefront. Inducing hepcidin expression in NTDT patients through genetic manipulations is of particular interest, attracting advanced gene therapy approaches to mitigate the effects of iron overload. Several research groups are investigating strategies to induce hepcidin expression, and the emergence of CRISPR-CAS gene-editing technology has enabled proof-of-concept gene therapy for single-base pair mutations.[74] Efficient gene therapy targeting mtDNA mutations presents unique challenges.[75] Firstly, it is essential to determine whether the mutation exists in a homoplasmic or heteroplasmic state. For homoplasmic mutations, targeting and developing guide RNA and antisense therapies are efficient gene-

Table 2. Targeted therapies for relieving iron overload and mtDNA mutations

Sl.no	Target	Gene of target	Mode of action	Effect on hepcidin expression	References
1	Free labile iron	Iron chelators DFP DFX DFO Flavonoids	Chelation of free labile iron, NTBI Free radical scavenging	–	66,73,74,77–80
2	Hepcidin expression	Hepcidin	Agonists BMP6 Mini-hepcidin	Activated hepcidin signaling Pathway Replacement therapy	83
		FPN Inhibitors	Blocking hepcidin receptor	Increased Hb in ineffective erythropoiesis	82
		DMT1*	siRNA	Prevents iron uptake by repressing FPN transporters	85
		TMPRSS6*	siRNA (Antisense RNA)	Repression of matriptase2 induces hepcidin secretion	81,84
		Hfe	CRISPR-Cas9 gene editing	Prevents binding of Hfe to TfR	75
3	Mitochondrial Iron overload	Mitoferrin	shRNA mediated inhibition	Inhibits iron trafficking to matrix	81

*In clinical trials for reducing iron overload based on mice models. BMP6, Bone Morphogenic Protein 6; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats-CRISPR associated 9; DFO, Deferoxamine; DFP, Deferiprone; DFX, deferasirox; DMT1, Divalent metal ion transporter; FPN, Ferroportin; Hb, hemoglobin; Hfe, hemochromatosis gene; mtDNA, mitochondrial DNA; NTBI, Non-transferrin bound iron; shRNA, Short homologous RNA; siRNA, small interference RNA; TfR, Transferrin receptors; TMPRRS6, trans-membrane serene protease6.

editing strategies. In contrast, heteroplasmy presents complexities in defining the mutant load and challenges in targeted therapeutic delivery. Secondly, the location of delivery must be considered, requiring the development of a large number of viral particles with specific tropisms for the target tissue, and the route of administration must be carefully selected to achieve a systemic effect. Challenges also arise in effectively delivering small non-coding RNAs across the mitochondrial membrane.[76] Thus, current treatment approaches for iron overload in thalassemia involve a comprehensive understanding of iron accumulation rates, strict adherence to chelation regimens, and ongoing research into advanced gene therapies, including those targeting mtDNA mutations. These efforts aim to provide more effective and personalized treatments for affected individuals. Table 2 summarizes the current therapeutic approaches for mitigating iron overload and mitochondrial DNA mutations.[66,73-75,77–85]

Emerging research and therapeutic avenues

With the rapid evolution of new technologies and high-throughput gene sequencing platforms, novel contributors to the onset of iron overload and associated mitochondrial DNA mutations are being identified. Considering the potential side effects and variable effectiveness of conventional chelation therapy, it is necessary to invent personalized therapeutic strategies capable of rectifying nucleotide errors at the molecular level. Current research trends focus on the discovery of small molecules that can modulate the hepcidin/ferroportin pathway, thereby influencing iron absorption and rescuing suppressed hepcidin levels in individuals with β-thalassemia. Lasjak and Srai reported that flavonoids, which have high antioxidant properties, can be used as alternatives to conventional chelators to eliminate the toxic ROS generated from iron overload,[86] thus mitigating oxidative damage to the tissues. Flavonoids, with their 6,7-dihydroxy structure, efficiently bind to labile iron and chelate it, preventing oxidative damage. The phenolic groups within flavonoids efficiently scavenge free radicals generated by labile iron and reduce the ROS load within the cell.[77] The primary mechanism of flavonoid action involves ameliorating the iron absorption pathway, increasing hepcidin expression, chelating free labile iron, and scavenging potential ROS molecules.[78,79] Manipulation of the hepcidin pathway is the current therapeutic approach in NTDT, which mainly focuses on rescuing the suppressed hepcidin expression induced by impaired erythropoiesis. Research has shown that inactivating the transmembrane serine protease matriptase-2 (TMPRSS6) can efficiently restore iron homeostasis by elevating the production of hepcidin.[80] Preclinical trials of FPN inhibitors and hepcidin analogues are currently underway to assess their efficiency in hepcidin induction.[81,82] Targeting transferrin receptors is also envisioned to help reduce iron uptake from the cells.[83] Transferrin injections and protoporphyrin IX are subjects of clinical trials to evaluate their effectiveness in inducing hepcidin expression and reducing cellular iron uptake.[87] The utilization of siRNA to mitigate gene expression and signaling is revolutionizing current therapeutic strategies. These small RNA molecules offer target-specific actions, promising high therapeutic efficacy. siRNAs designed against specific membrane transporters involved in hepcidin signaling and iron uptake are currently under investigation for their efficacy in inducing hepcidin expression and reducing iron uptake. Transfection of lipid nanoparticles carrying TMPRSS6 siRNA into murine models exhibiting hereditary hemochromatosis and β-thalassemia intermedia forms has demonstrated the potential to restore the hepcidin expression in liver cells, thereby alleviating the effects of iron overload.[88] Wang *et al*. demonstrated that oral administration of DMT1 siRNA, delivered via nanoparticle carriers, can attenuate the expression of DMT1, reducing excessive iron absorption and inhibiting tissue iron accumulation.[89] However, further clinical trials are essential to confirm the therapeutic efficacy of these siRNA-carrying nanoparticle vehicles in mitigating the consequences of iron overload associated with β-thalassemia.

Mitochondria can rapidly accumulate iron under conditions of

iron overload, leading to potentially toxic levels within the mitochondria and causing mtDNA mutations. Current strategies for treating mtDNA disorders have primarily focused on primary mitochondrial diseases, while iron-induced mtDNA disorders remain less explored. A promising approach to treating mtDNA disorders involves the direct identification of mutant sequences through next-generation sequencing platforms and the identification of single nucleotide polymorphisms contributing to these disorders. Gene therapy offers a viable option for addressing mtDNA mutations. However, the existence of heteroplasmy among different tissues poses a challenge to therapeutic strategies. Blood samples from patients can be processed for mtDNA sequencing to identify disease-causing variants and assess heteroplasmy levels. Despite progress, prenatal diagnosis remains challenging. Carrier women with low heteroplasmic loads may remain asymptomatic, making it difficult to employ traditional gene therapy for such mtDNA variants. In such cases, mitochondrial replacement therapy (MRT) has proven effective as a precautionary measure to prevent the inheritance of mtDNA mutations.[84] MRT involves the physical transfer of the nuclear genome from a mother carrying a deleterious mtDNA mutation into an enucleated oocyte from another healthy female with no mtDNA mutations. MRT can be achieved through various methods, including polar body transfusions, pronuclear transfusions, and/or spindle-chromosome complex transfusions, before or after fertilization, resulting in offspring genetically related to the parents carrying donor mtDNA and thus preventing the onset of the disease. Recent research has also explored the utility and challenges of utilizing CRISPR-Cas gene editing systems to correct mtDNA mutations. Jo *et al*. investigated the efficiency of this three-enzyme system in effectively rectifying mutagenic errors in mtDNA. The exact mechanism remains unclear,[85] but conjugating the mitochondrial target sequence to the guide RNA on Cas9 could facilitate targeted delivery to the mitochondria. Another approach to addressing mtDNA mutations in their heteroplasmic state is to exploit the action of restriction endonucleases to digest the mutant mtDNA, leaving the wild-type DNA intact. Transcription activator-like effector nucleases and zinc finger nucleases are potential tools for this approach, as they do not require guide RNA and have demonstrated the capability to alter mtDNA heteroplasmy experimentally.[90,91] However, gene editing approaches remain effective in treating inherent mtDNA mutations, while the ROS-induced sporadic and clonally expanding mtDNA mutations observed in β-thalassemia and related hemoglobinopathies present challenges for current gene editing therapeutic strategies.[92] Emerging research is exploring innovative therapeutic avenues to address iron overload and associated mitochondrial DNA mutations.

Clinical management and patient care

With significant advancements in understanding the prognosis of secondary iron overload and its consequences in β-thalassemia, substantial progress has been made in managing iron overload-related issues. If left untreated, these concerns can lead to fatal outcomes. While iron chelation therapy, gene therapy and bone marrow transplants hold promise for reducing the risk of comorbidities, they do not completely eliminate the high-risk factors associated with the manifestation of the disease. Increasing awareness of the disease progression and closely monitoring serum ferritin levels, liver iron levels, and tissue deposition of iron through MRI have prompted clinicians to design more effective therapeutic strategies. Although it can be challenging to specify the age of onset, regular monitoring facilitates the initiation of iron chelation therapy and the adoption of personalized therapeutic approaches. It is evident that sub-optimally treated patients may experience early endocrine dysfunction and an increased risk of organ failure in the future. Therefore, it is crucial to counsel patients and their parents regarding the manifestation of the disease and its consequences. Disease effects can vary among different age groups. For instance, early childhood may manifest endocrine malfunctions such as hypothyroidism or hyperthyroidism, along with cardiac arrhythmias; whereas adults may experience liver disorders and cardiac malfunction, potentially leading to the development of endocrine abnormalities, or vice versa. Early optimization of iron chelation therapy and regular monitoring of tissue iron accumulation, in addition to serum and liver iron levels, can enable early detection of comorbidity symptoms and the implementation of appropriate therapeutic measures to ensure patient survival. Parameters to be monitored at each transfusion include iron intake, serum ferritin, liver iron concentration, cardiac T2*, liver status, the onset of puberty as a measure of sexual development, endocrine assays, and most importantly, the psychological status of the patient and family members.[93] It is imperative to closely monitor the onset of symptoms of mitochondrial disease, such as poor growth, neurological problems, vision and hearing issues, muscle weakness, learning disabilities, and developmental delays. It is crucial to correlate these symptoms with serum ferritin levels, liver iron pool measurements, and specific tissue iron accumulation. By closely monitoring these symptoms and their relationship with iron overload, healthcare providers can take proactive measures to address mitochondrial dysfunction in β-thalassemia patients, thereby improving their overall quality of life and clinical outcomes.

Hussain *et al*. emphasized that assessing health-related quality of lifeis essential. Thalassemia patients often suffer from severe physical, clinical, social, and psychological problems.[94] The patients are often advised to follow healthy dietary patterns, undergo regular check-ups, and cultivate social relationships. Timely vaccinations can prevent serious illnesses, particularly in splenectomised thalassemia patients, who are considered at high risk for certain infections. Consuming a nutritious diet rich in fruits and vegetables, combined with moderate exercise, can improve the quality of life and positively impact treatment outcomes. Maintaining healthy relationships is crucial for managing mental and physical stress, especially during and post-transfusion. Pattanashetti *et al*. reported that thalassemia patients may exhibit poor socializing behaviour, including low self-esteem, depression, anxiety, poor communication, and inadequate schooling.[95] A healthy and warm relationship with supportive friends, co-workers, and family members will ensure the patient's mental stability and enhance their overall well-being. In addition to maintaining a proper healthy lifestyle, repeated blood transfusions, and associated complications can affect mental and family stability. In such cases, genetic counselling is essential to clarify the assumptions about the disease and provide comprehensive information about the diagnosis, cause, available treatment plans, and the risk of disease transmission to offspring. Genetic counselling is used to address reproductive issues and comprehensive care is offered for patients and parents through pedigree analysis. It assesses the risk of thalassemia and associated mtDNA disorders among family members, identifies risk factors that may affect treatment plans, incorporates psychological information that can impact the family system, and assists patients in maintaining their well-being. Counselling is necessary at different stages of the disease onset, including during diagnosis, adolescence, transfusion, before and after genetic testing, prior to pregnancy, and during regular follow-ups to reinforce patient education.

Future perspectives

Over the years, research has made significant progress in elucidating the mechanism of iron overload and the management of associated disorders in β-thalassemia. Free labile iron, contributing to ROS generation and oxidative stress-mediated tissue damage, plays a primary role in the co-morbidities observed in thalassemia. Studies have demonstrated that cardiac dysfunction and irregular arrhythmias observed in patients with TDT and NTDT result from mtDNA mutations induced by ROS within the mitochondrial matrix. Therefore, effective management of iron overload is crucial for preventing organ damage in thalassemia patients. Numerous research groups are exploring methods to mitigate iron overload-associated tissue damage, with a key focus on rescuing hepcidin expression to limit iron absorption by cells. In addition to conventional agents, many iron chelators have been screened for their efficiency, but targeted delivery of these small molecules remains an underexplored area. Several plant extract derivatives are in clinical trials to evaluate their efficiency in scavenging toxic ROS and limiting oxidative stress damage. However, further research is needed to determine the optimal dosage and administration route for effective iron chelators. Innovative strategies, such as combining small molecules within nanoparticles coated with mitochondrial target sequences, provide a novel approach for targeted delivery to mitochondria. However, the lack of clinical trial data limits the practical applications of these methods. Next-generation sequencing technology has revolutionized molecular genetics by enabling the identification of new molecules that regulate hepcidin expression and direct sequencing of mtDNA to identify disease-causing variations. Site-specific restriction endonucleases show promise in rectifying single nucleotide polymorphisms;, However, their use in rectifying mtDNA mutations, especially in the presence of heteroplasmy requires further exploration. The role of siRNA-mediated gene silencing offers an efficient approach for modulating hepcidin expression. Current research is dedicated to understanding the mechanisms, routes of administration, and their efficiency. The utilization of the highly appreciated CRISPR-Cas9 gene editing tool for correcting mtDNA variations is a topic of extensive discussion. A major challenge in exploiting CRISPR-Cas9 to edit mtDNA mutations is the requirement for three enzymes to be synchronously expressed in the mitochondrial matrix for gene editing. Achieving universal expression of viral vectors at the same rate is challenging because it may lead to immunological issues in non-targeted tissues. Delivering the three molecules cloned into the same vector to reach the mitochondrial matrix is quite challenging and requires further research. Considering the variability of mtDNA mutations in terms of nature and extent of iron overload, personalized treatment strategies are necessary to address the iron overload-associated mtDNA disorders among β-thalassemia patients. Patients should be thoroughly informed about available treatment methods for their disease management.

Conclusions

Iron overload is a pervasive complication in thalassemia syndrome, resulting from repeated transfusions in TDT and excessive iron absorption accompanied by suppressed hepcidin levels in NTDT patients. With limited mechanisms to eliminate excess iron from the body, iron accumulates in secondary sites such as the liver, heart, and cardiac muscles. While the clinical presentations of TDT and NTDT patients may differ in terms of the site of iron accumulation and associated organ dysfunction, the core molecular mechanism remains consistent, with ROS-induced tissue damage. Regular monitoring of blood intake, along with iron overload biomarkers such as serum ferritin levels, liver iron concentration, and advanced imaging techniques to assess iron tissue deposition, provides a crucial avenue for early detection and timely initiation of iron chelation therapy. Next-generation sequencing technologies offer hope by enabling direct sequencing of mtDNA and detecting disease-causing gene variants responsible for ROS-mediated mutant lesions on mtDNA. This breakthrough has the potential to revolutionize the diagnosis and management of mitochondrial disorders in thalassemia patients. Despite the integration of iron chelation therapy alongside blood transfusions, its efficacy depends on the patient's adherence and compliance with the chelating agent. Side effects and the cost of the chelation therapy limit adherence, and eventually result in iron overload. The prospect of replacing conventional iron chelators with novel siRNAs targeting hepcidin suppressors and tissue iron transporters holds promise for reducing excess free iron levels in tissue with limited side effects. However, a lack of clinical trials limits the practical applications of this technology. Future developments in targeted therapy must address the route of administration and optimal siRNA dosages. Personalized therapy is emerging as a central focus, recognizing that each patient exhibits unique responses to therapeutic agents. Current research trends are harnessing advanced gene editing techniques, such as site-specific recombinases and CRISPR-Cas9, to mitigate mtDNA mutations despite the existence of mtDNA heteroplasmy. The future landscape holds the promise of expanded genetic screening, paving the way for novel therapeutic approaches to editing mtDNA variations, mitigating ROS-induced tissue damage, and alleviating the burdens of iron overload in individuals with β-thalassemia. As research and clinical trials progress, personalized and highly effective treatments for thalassemia-related iron overload and associated mitochondrial disorders are needed, as these treatments are expected to improve patient outcomes and quality of life.

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Conflict of interest

The authors declare no conflict of interests related to this publication.

Author contributions

JMN, KS, DPK, AP conceived the idea and scope of the review article, JMN, PG, NMJ conducted extensive literature review, searching and selecting relevant articles, JMN, PG, AP drafted the manuscript, DPK, PSN, PV critically revised the manuscript by providing important intellectual content, PV and PSN also pro-

vided administrative and technical support. All authors have made a significant contribution to this study and have approved the final manuscript.

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