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Iron as Therapeutic Targets in Human Diseases

Edited by

Paolo Arosio, Maura Poli and Raffaella Gozzelino

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About the Special Issue Editors

Paolo Arosio is full professor in Molecular Biology. He graduated in Milan, then as a post-doc worked at Tufts University in Boston, US. Then he moved to Italy, at the University of Milan and eventually at the University of Brescia in the medical school. He then retired in 2017. He has been working on proteins of iron metabolism, with particular attention to the ferritin structure, about which he wrote numerous research papers and also review articles of success. He then studied the role of ferritin in iron homeostasis, ferritin-linked disorders, some regulatory aspects of iron metabolism and the use of heparin as inhibitor of hepcidin expression. He directed a laboratory on iron metabolism first in San Raffaele Institute of Milan, and then in University of Brescia. He organized congresses of the European Iron Club and of the International Society of Bioiron. He has been guest editor of an issue of *Frontiers in Pharmacology* (2014) titled “The importance of Iron in Pathophysiological Conditions”, of *IUBMB Life* (2016) titled “Iron in Biology”. He is coauthor in about 230 papers on international peer review journals that have been cited more than 15.000 times. He has an H-index \geq 65.

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Iron as Therapeutic Target in Human Diseases

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Iron is essential for almost all organisms, being involved in oxygen transport, DNA synthesis, and respiration; however, it is also potentially toxic via the formation of free radicals. Thus, iron homeostasis is tightly controlled by mechanisms that have been partially elucidated. Numerous disorders have recently been linked to deregulation of iron homeostasis, leading iron metabolism to become an interesting therapeutic target for novel pharmacological treatments against these diseases. The targeting includes the hepcidin/ferroportin axis for the regulation of systemic iron homeostasis, the cytosolic machinery for the regulation of intracellular iron status and oxidative damage, proteins of iron metabolism such as ferritin and transferrin receptor, and the recently described form of programmed cell death named ferroptosis. Because of its tight link with anemia, iron metabolism has been mainly of interest in terms of the hematological pathologies, but, recently, clinicians have become aware of the importance of iron in non-hematological disorders, suggesting that iron could be a therapeutic target for various conditions. To verify whether this is the case and to try to gather together all the novel information in this developing field, we launched this issue in *Pharmaceuticals*, and we were glad to find that this attracted the attention of the 49 research groups that proposed papers. This result confirms the increasing importance of iron in various disorders.

From the articles received, it became clear that one of the fields that started to be highly investigated is the role of iron in the brain. Indeed, the recent evidence that excess iron occurs in the brains of subjects with neurodegenerative disorders suggested the therapeutic potential of iron targeting in this organ. In this issue, Crichton et al. [1] reviewed studies on iron chelators, which have been successful at removing excess iron from liver, spleen, heart, and pituitary gland in various iron-loading disorders. Oral deferiprone has been used to chelate brain iron overload in Parkinson's disease and Friedreich's ataxia with encouraging results, and new and safer chelators are under study. The authors also suggested that the presence of inflammation might reduce the efficacy of these chelators, which is in agreement with other literature findings. Similarly, Nunez et al. [2] focused on the properties required for an optimal iron chelator to treat neurodegenerative diseases involving brain iron accumulation, a group of disorders that in addition to the already cited Parkinson's disease and Friedreich's ataxia, also includes pantothenate-kinase-associated neurodegeneration, Huntington's, and Alzheimer's disease (AD). While the ideal chelator should target the mitochondria, quench free radicals, have micromolar-iron-binding affinity, and be selective for neuronal cells, the current situation on available chelators was discussed by the authors in their manuscript. Studies on specific brain pathologies have also been included in this issue, as the work by Alsina et al. [3], which focused on the role of iron in Friedreich's ataxia, a genetic disorder caused by trinucleotide GAA expansions in the first intron of the frataxin gene that decrease its expression. In addition to discuss the proposed functions of this mitochondrial protein and how it is related to iron homeostasis, they also reported the beneficial effects of iron chelators as therapeutic agents for this disease. The role of iron in AD was reviewed by Masaldan et al. [4], who pointed out iron dyshomeostasis as a critical feature of this disease, given the participation of iron in the generation of free radicals and in the interaction with proteins known

to cause of AD pathology. The hypothesis described by the authors is that iron accumulation might derive from an age-associated increase of senescent cells that drive inflammation, which predisposes to oxidative stress, cellular dysfunction and iron-dependent death. Elevated brain iron is associated with AD progression and cognitive decline, and it may be reduced pharmacologically. Nnah et al. [5] considered that microglia activation and secretion of proinflammatory cytokines are hallmarks of neurodegenerative disorders, including AD. However, it is still unclear whether increased brain iron augments the inflammatory responses of microglia and how these cells accumulate, store, and utilize intracellular iron to carry out their functions under normal and pathological conditions. This review describes known and emerging mechanisms involved in microglial cell iron transport and metabolism, as well as the inflammatory responses affecting the brain, in the context of AD. Neurodegeneration with brain iron accumulation (NBIA) was reviewed by Levi and Tiranti [6]. NBIA is a group of rare monogenetic diseases that are heterogeneous in onset and symptoms but characterized by specific brain iron deposition in the region of the basal ganglia. Fifteen NBIA genes have been described so far, but only two of these code for iron proteins. The review reports the recent data on new models of these disorders aimed at understanding the pathogenesis of iron deposition. The impairment of mitochondrial function, which also contributes to cause neurodegeneration, is discussed in the review published by Fiorito et al. [7], who proposed a new perspective regarding the impact of heme. Heme is synthesized in the mitochondria and its metabolism plays a central role in organelle function. Since some evidence indicates that alterations of heme metabolism are associated with neurodegenerative disorders, these studies may open new therapeutic avenues in the struggle against these disorders.

The importance of iron in the development of human brain was also described in this issue, in which Markova et al. [8] reviewed the recent findings on the effects of gestational and lactational iron deficiency on the correct formation of the central nervous system. In early embryonic life, iron is needed for the developing brain, which is characterized by a widespread expression of transferrin receptors. If iron deficiency occurs at this stage, the brain may not fully develop, weight less and present an impaired myelin formation, which result in chronic and irreversible damage affecting individual cognitive, memory, and motor skills. Studies on humans and animal models suggest the possibility to reverse these effects with iron substitution therapy. Rockfield et al. [9] argued that the maintenance of iron and lipid homeostasis is critical also to the brain. Since this is the fattiest organ in the body, many evidences point out the existence of a cross-talk between these pathways. In this article, the authors discuss human diseases involving iron and lipid alterations, with special emphasis on neurodegenerative disorders, and the therapeutic potential of iron reduction techniques for these patients. The mechanisms of iron action in the brain was also described by Ferreira et al. [10], who took a broader approach exploring in particular the cognitive and behavioral implications of disruption of iron homeostasis on the onset and progression of psychosocial disorders. In this review, the authors also discuss the links between iron and the biological, psychological, and social dimensions that contribute to the development of a diverse set of neuro-pathologies. Potential avenues of research are also outlined. Genetic diseases with brain iron accumulation were reported to also cause retinal degeneration, as discussed by Shu and Dunaief [11]. Iron dysregulation in the eye might also occur upon dietary or parenteral supplementation, which has been reported to elevate iron levels in the retinal pigment epithelium (RPE) and to promote retinal degeneration. While studies in mice and humans suggest that iron toxicity might contribute to the pathogenesis of age-related macular degeneration, iron chelators were found capable to protect photoreceptors and RPE in mouse models. So, their therapeutic potential is currently under investigation.

Although hereditary hemochromatosis (HH) is the first iron overload disorder to be studied, there are still many aspects to be investigated, some of which considered in this issue. Loreal et al. [12] reviewed works on HH, which are mainly related to the C282Y mutation in the *HFE* gene. This mutation causes hepcidin deficiency and iron accumulation in liver, pancreas, heart, and bone. Treatment mainly consists of venesection for the removal of iron contained in red blood cells, which seems to be effective. Nevertheless, new approaches targeting hepcidin levels could be useful to better control

iron parameters and especially some symptoms of this disease, like arthritis. The work by Porto et al. [13] described a 20 year follow up of three siblings, diagnosed with HH in their childhood, who were homozygous for the C282Y mutation of *HFE*. These patients were assessed yearly for the determination of iron indices and lymphocyte counts, and the analyses revealed an important transition of this disease from childhood to adult life. Emphasis on the tight link between iron status and the activation of immune cells was given by the authors. HH with dominant transmission, which is also known as ferroportin disease, was reviewed by Vlasveld et al. [14]. The authors reported phenotypes ranging from a loss of ferroportin function (LOF) to a gain of function (GOF) of this gene, with hepcidin resistance. The analyses of 359 patients with 60 ferroportin variants allowed the authors to conclude that the phenotypes of hepcidin-resistant GOF variants were indistinguishable from the other types of HH. While these can be categorized as ferroportin-associated HH, ferroportin disease may be confined to the LOF variants. Although many proteins are involved in the regulation of iron homeostasis, Katsarou and Pantoupolos [15] considered hepcidin as an interesting therapeutic target. Genetic defects of hepcidin expression lead to “hepcidinopathies”, a series of pathologies ranging from HH to iron-refractory iron deficiency anemia. Indeed, dysregulation of hepcidin is a cofactor in iron-loading anemias with ineffective erythropoiesis and anemia of inflammation. Hence, this review summarized the state of the art on hepcidin agonists and antagonists, as well as inducers and inhibitors of hepcidin expression. The interaction between hepcidin and ferroportin in the regulation of systemic iron homeostasis was also the focus of the study published by Hawula et al. [16]. Indeed, this axis can be affected by various stimuli and its deregulation can lead to a variety of disorders, which include HH. The treatment options for regulating iron levels in patients are limited, and efforts are being made to uncover approaches restoring hepcidin and ferroportin expression. In this review, the authors examined the current status of hepcidin and ferroportin agonists and antagonists, as well as inducers and inhibitors of these proteins and their regulatory pathways. Investigating the types of cellular events occurring in the liver during iron overload conditions, Tangudu et al. [17] examined the hepatic signaling pathways underlying acquired and genetic iron overload disorders. The authors found an association between these pathologies and the decline in the activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (Erk) kinase (Mek1/2) pathway, which selectively affects the phosphorylation of Erk1/2. The uncoupling of this signaling from iron-Bmp-Smad-mediated hepcidin induction indicates that it may contribute to a number of liver pathologies. A new approach to study the hepcidin-binding proteins was proposed by Diepeveen et al. [18], who observed that this method, in the serum, might influence hormone function and quantification. The authors used peritoneal dialysis to measure freely circulating solutes in blood and peritoneal fluid of patients undergoing a peritoneal equilibration test. The protein-bound fraction of hepcidin was calculated to be 40% ($\pm 23\%$), which led the authors to conclude that a substantial proportion of hepcidin is freely circulating.

Other genetic disorders associated with dysregulated iron metabolism are those characterized by new mutations in the ferritin L gene, as described by Cadenas et al. [19]. These pathologies cause dominant L-ferritin deficiencies and hereditary hyperferritinemia cataract syndrome (HHCS). An accurate diagnosis is needed for the appropriate treatment of the multiple phenotypes caused by FTL gene mutations, and a novel diagnostic algorithm was proposed in this study for that purpose. As discussed by Chiou and Connor [20], ferritin is the main iron cellular storage molecule in the body, able to store a large amount of iron within its mineral core. Recently, ferritin was shown to have a range of abilities that go well beyond iron storage. This review aims at discussing novel functions and biomedical uses of ferritin in the processes of iron delivery, delivery of biologicals such as chemotherapies and contrast agents, and the utility of ferritin as a biomarker in a number of neurological diseases. The autophagic degradation of ferritin (known as “ferritinophagy”) is necessary to maintain intracellular iron homeostasis and is mediated by the nuclear receptor coactivator 4 (NCOA4), as described by Santana-Codina and Mancias [21]. In this review, the authors related the biochemical regulation of NCOA4, its contribution to physiological processes, and its role in

disease. Its potential to activate or inhibit ferritinophagy and ferroptosis for therapeutic purposes was also addressed. In line, Bou-Abdallah et al. [22] reviewed our current understanding of iron mobilization from ferritin by various reducing agents. The authors reported recent results supporting a mechanism that involves a one-electron transfer through the protein shell to the iron mineral core. The physiological significance of the iron-reductive mobilization from ferritin by the non-enzymatic FMN/NAD(P)H system is also discussed. Sha et al. [23] studied plant ferritin, a novel form of iron supplement, the absorption of which can be affected by phenolic acids of the plant. The authors found that cinnamic acid derivatives induce the release of iron from soybean ferritin, thus having a negative effect on iron stability. The authors also pointed out that the iron chelating activity and reducibility of these compounds may affect the iron availability of soybeans. Regulation of iron homeostasis is also provided by the role exerted by the transferrin receptor 2 (Tfr2), as described by Roetto et al. [24]. Tfr2 is one of the hepcidin regulators and mutations of this gene cause type 3 HH. While this review summarized the data on Tfr2 extrahepatic role, including the importance of the two main isoforms, Tfr2 α and Tfr2 β , the use of Tfr2 as therapeutic target for hepcidin control is also discussed. Finally, the systemic iron regulation, achieved by controlling heme metabolism, was described by Montecinos et al. [25], who reviewed the present knowledge on the heme-binding protein hemopexin (HPX) and provided information on its biochemistry. HPX prevents the toxicity induced by hemoglobin (Hb)-derived heme, which occurs in hemolytic conditions and can be triggered by the activation of the immune system. The review highlights some newly identified actions of heme and HPX, engaged especially when normal processes fail to maintain heme and iron homeostasis. The authors also presented data showing that the cytokine IL-6 cross talks with activation of the c-Jun N-terminal kinase pathway in response to heme–hemopexin in models of hepatocytes.

Recent studies have shown the importance of iron in kidney diseases. This particular topic was discussed by Vera-Aviles et al. [26], who reviewed evidence for iron-induced toxicity in chronic kidney disease (CKD) and the mechanisms by which histidine exerts cytoprotective functions. In fact, CKD is often associated with iron and histidine deficiency. This amino acid, which is essential for erythropoiesis and to enhance iron dietary absorption, was shown to have antioxidant properties capable to improve the oxidative stress in CKD. Balla et al. [27] discussed some of the most important findings relating to the role of iron and ferritin heavy chain in the context of kidney-related diseases and, in particular, in vascular calcification. The authors provided evidence that the ferroxidase activity of ferritin prevents this frequent complication of CKD. Nuhu and Bhandari [28] reviewed a specific cardio-renal morbidity in CKD, providing an understanding of the pathophysiology and impact of uremic toxins, inflammation and anemia, on oxidative stress. Anemia in CKD increases the risk of left ventricular hypertrophy and oxidative stress, thereby magnifying the deleterious consequences of uremic cardiomyopathy. This enhances its progression and increases the risk of sudden cardiac death.

Iron toxicity also affect the lungs. The continuous exposure of this organ to oxygen turns it highly sensitive to oxidative damage, which is enhanced in the presence of excess iron. This was discussed by Zhang et al. [29], who provided an overview of systemic and local lung iron regulation. The authors described the role of this metal in the development of lung infections, airway disease, and lung injury, offering important foundations for the development of therapeutic applications. Also Neves et al. [30] reviewed the current knowledge on the regulation of pulmonary iron homeostasis. In addition to report the functional importance of iron and its link in the development of lung disorders, the authors provide a better understanding of the association between pulmonary iron deregulations and the frequently correlated chronic obstructive pulmonary disease and lung cancer. Possible improvement of these pathologies with iron-related therapeutic strategies has also been described.

The interplay between iron and inflammation, elicited in the course of the infection, has long been observed. Petzer et al. [31] reviewed the association between anemia and chronic inflammatory diseases, addressing how iron levels could be improved with the resolution of the disease, supplementation and redistribution strategies. Moreover, investigations referring to the key role of hepcidin in these forms of anemia encouraged the development of novel therapeutic approaches, which were discussed in

this review along with the current guidelines of iron replacement therapies. In chronic inflammatory diseases, these refer to oral versus parenteral iron supplementation. The authors also reported the emerging potential of hepcidin-antagonizing drugs, which are currently under preclinical and clinical investigations. Hepcidin regulation has been also reported as beneficial in patients with rheumatoid arthritis (RA), who are often treated with an anti-IL-6-receptor (anti-IL-6R) monoclonal antibody (tocilizumab) that inevitably influences iron metabolism. Ribeiro et al. [32] studied a cohort of patients under this treatment, identifying the association between higher serum iron and transferrin saturation, induced by the drug, and the risk of infection. Their study strongly indicates the need to monitor iron indices in RA patients on anti-IL-6R therapy to prevent infections. The most recent literature on infection and iron metabolism has been reviewed by Gomes et al. [33]. Special emphasis was given to iron changes induced by pathogens invasion, which culminate with the development of anemia, and to potential therapeutic approaches that, modulating iron metabolism, correct iron levels and control the infection. One of the most studied infectious diseases involving disruption of iron homeostasis is malaria. The increase in hepcidin during the development of this disease has been reported by Muriuki and Atkinson [34], along with the higher levels of tumor necrosis factor- α , as the cause that leads to poor iron absorption and recycling. While this may be an important driving factor of iron deficiency, its prevalence in children increases over a malaria season and decreases when it is interrupted, as described by the authors. Once the link between malaria and iron deficiency will be formally demonstrated, it would aid readjusting priorities for programs towards prevention and treatment of iron deficiency, which will benefit malaria control. Armitage and Moretti [35] reviewed the demand and supply of iron during early childhood, addressing its importance in aspects that refer to the physiology and development of young children coming from low- and middle-income countries, in particular. Thus, discussing the implications for interventions to improve iron status whilst minimizing infection-related risks is of utmost importance, since strategies should be adapted according to iron deficiency, inflammation status, and infection risk. It is known that macrophages play a central role in regulating iron homeostasis, especially during infections, like but not restricted to malaria. In their manuscript, Recalcati et al. [36] described how macrophages control iron levels and how this determines, in turn, their plasticity. Changes in the expression of genes coding for major proteins of iron metabolism may result in different iron content availabilities for the macrophage itself and other cells present in the microenvironment. This review also discussed the role of macrophages in immunometabolism, which cross-talks with erythropoiesis as reported by Sukhbaatar and Weichhart [37]. Coordination between spleen, liver, and bone marrow is essential for macrophages ensure proper iron recycling and erythroblast differentiation. This article also focuses on the role of distinct macrophage populations to maintain iron metabolism, describing the cellular and systemic mechanisms involved in iron-regulating processes. Macrophages are also the main target of *Mycobacteria Tuberculosis*, a pathogen that requires iron to proliferate, as pointed out by Agoro and Mura [38], who reassessed that *Mycobacteria*-infected hosts use systemic iron restriction and cellular iron distribution as defense mechanism against infection. The authors reviewed the importance of iron availability to elicit an immune response against *Mycobacteria*, which then dictates host susceptibility. Hence, the need for future therapeutic directions capable to prevent this disease were also pointed out. At this regards, specific chelators against infections caused by *Mycobacterium avium* have been reviewed by Rangel et al. [39], who described in particular a selected class of the 3-hydroxy-4-pyridinone ligand, which could be functionalized with the addition of fluorophores. This was shown to improve antimycobacterial activity and the affinity of chelators to biological membranes, thus indicating that “to label means to change”. The authors further discuss the need of combined therapeutic approaches and the use of rhodamine B conjugates to target bacterial resistance and biofilm production.

More evidences point at iron deficiency as critically involved in the pathogenesis of different conditions. An example is provided by Lakhali-Littelton [40], who described the prevalence of iron deficiency in patients with cardiovascular disease and associated it with worse outcomes. Although, the mechanisms by which iron deficiency affects cardiovascular function are still unclear, this review

discusses the benefits of therapeutic strategies aimed at restoring cellular iron homeostasis rather than approaches based on iron supplementation. These have been described in particular on two diseases: chronic heart failure and pulmonary arterial hypertension. Another compartment also affected by iron deficiency is the bone. As explained by Balogh et al. [41] bone homeostasis is based on the regulation between osteoclasts' function, which resorb the bone, and osteoblasts', which produce new bone. Both iron deficiency and iron overload disrupt this delicate balance, influencing skeletal health and emphasizing the need to develop novel therapeutic approaches to inhibit the pathological effects of altered iron levels in this tissue.

An important aspect of restoring iron homeostasis is how to supplement this metal in case of deficiency, since possible side effects of oxidative damage and changes in intestinal microbiota have been pointed out. The effects of oral treatments on iron deficiency were reviewed by Ginanjar et al. [42], who also considered the potential toxicity of plasma non-transferrin-bound iron (NTBI). The authors found that FeSO₄ is more absorbed than NaFeEDTA, although causes a remarkable increase of NTBI. In a double-blind, randomized trial, they showed that a low dose of NaFeEDTA (6.5 mg), given with a meal, was highly effective for the treatment of iron deficiency, maintaining normal levels of NTBI. Bhandari et al. [43] discussed the inefficacy of oral iron replacement therapies in the treatment of some patients with iron deficiency. In these cases, replacement with intravenous (IV) iron therapies, now in their third generation, could increase iron levels without causing toxic effects. This review described the properties of different IV irons, and how differences in formulations might impact the current and future clinical practice. Novel innovative oral iron formulations were described by Gomez-Ramirez et al. [44]. Sucrosomial®iron (SI), in which ferric pyrophosphate is protected by a phospholipid bilayer plus a sucrose matrix (sucrosome) and absorbed via para-cellular and trans-cellular routes (M cells), was shown to increase iron bioavailability while having excellent gastrointestinal tolerance. An important concern, though, needs to be raised from a human nutritional point of view. The genetic selection for large litter sizes and high birth weights makes piglets severely iron-deficient, as described by Szudzik et al. [45]. In need for iron supplementation, these animals receive intramuscular injection of a large amount of iron dextran, which if from one side corrects the iron deficiency of the animal, on the other it may generate toxic effects. Whether this might also affect, in long term, the human population eating pork meat is not known. Therefore, new iron supplements need to be considered, turning iron-deficient piglets as a convenient animal model for pre-clinical studies. The influence of food compounds on iron absorption was also the focus of Lesjak and Srail's review [46], which discussed how iron homeostasis is affected by several dietary factors, such as flavonoids. Their ability to modulate the expression and activity of proteins involved in the systemic regulation of iron metabolism and uptake turn flavonoids clinically relevant for the potential treatment of both anemia and iron overload diseases. The influence of dietary iron absorption in the gut was discussed by Yilmaz and Li [47], who described the dynamic modulation of intestinal microbiota induced by different iron levels. The authors reviewed the current understanding of the effects of luminal iron on host–microbe interactions in human health and disease. The side effects induced by the excessive amount of unabsorbed iron at the interactive host–microbe interface of the human gastrointestinal tract was particularly described.

The involvement of iron in the development of tumors has long been studied, and Busti et al. [48] consider that anemia in cancer is multifactorial, and iron deficiency (ID) is a major contributor. Since the treatment of functional iron deficiency is complex and still controversial, this work discusses the possible approaches for the management of ID in cancer patients, in different clinical settings. Current guidelines and recommendations were also reported to emphasize the need for further research in the field. In agreement, one article in this issue studied the activity of the anticancer drug didox, which is thought to act by inhibiting ribonucleotide reductase, the rate-limiting enzyme for dNTP synthesis that is highly expressed in aggressive tumor cells. Asperti et al. [49] showed that didox cell killing was suppressed by iron supplementation, and capable to reduce iron availability by acting as

an iron chelator. The authors indicated that this property might contribute to its antitumor activity by sequestering iron to enzymes, as the ribonucleotide reductase.

Altogether, this issue, which was published in *Pharmaceuticals*, provides an interesting overview on the complexity of the role of iron in health and disease conditions, emphasizing the need to control iron homeostasis. This is achieved by supplying iron in sufficient amount, when deficient, and removing it, when in excess. Regulating iron distribution among various tissues and compartment is also essential to prevent dysregulated levels of this metal and the occurrence of disorders like HH, neurodegenerative and cardiovascular diseases, cancer, and infections.

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Review

The Efficacy of Iron Chelators for Removing Iron from Specific Brain Regions and the Pituitary—Ironing out the Brain

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Abstract: Iron chelation therapy, either subcutaneous or orally administered, has been used successfully in various clinical conditions. The removal of excess iron from various tissues, e.g., the liver spleen, heart, and the pituitary, in beta thalassemia patients, has become an essential therapy to prolong life. More recently, the use of deferiprone to chelate iron from various brain regions in Parkinson's Disease and Friederich's Ataxia has yielded encouraging results, although the side effects, in <2% of Parkinson's Disease(PD) patients, have limited its long-term use. A new class of hydroxypyridinones has recently been synthesised, which showed no adverse effects in preliminary trials. A vital question remaining is whether inflammation may influence chelation efficacy, with a recent study suggesting that high levels of inflammation may diminish the ability of the chelator to bind the excess iron.

Keywords: iron; chelation; neurodegenerative diseases; pituitary; brain

1. Introduction

Iron is one of the essential elements in the body, the concentration of which is tightly regulated to prevent toxicity. Iron overload, particularly in the liver and spleen, is known to occur in several conditions, such as hereditary hemochromatosis, African iron overload, sickle cell disease, beta thalassemia, sideroblastic anaemia, enzyme deficiency (pyruvate kinase, glucose-6-phosphate dehydrogenase), and rare disorders of proteins involved in iron transport (atransferrinaemia, aceruloplasminemia). In certain conditions, e.g., sickle cell anaemia, beta thalassemia, and myelodysplasia, regular blood transfusions are an essential part of the therapy, thereby contributing to the increased iron stores, as the body is unable to excrete iron to any great extent. However, little focus has been directed at the brain iron concentrations in these conditions. Furthermore, it is also apparent that aging and neurodegenerative diseases, such as Parkinson's and Alzheimer's Disease, as well as Friederich's Ataxia, are associated with an increasing brain iron accumulation [1]. With the advent of several magnetic resonance imaging (MRI) techniques over the past 10 years it is now possible to measure pituitary and brain iron in specific brain regions and to monitor changes in its concentration after iron chelation therapy. Iron deposits are not assayed directly, but their effects on water protons as they diffuse in the magnetically inhomogeneous environment induced by iron deposition are assessed. The scanner transmits energy into the body as microwaves, followed by a waiting period, after which the energy is recalled, to be received by an antennae or coil. This process is known as relaxation and is characterised by relaxation rates R2 and R2* (measured in Hz), which are mathematically inverse of the characteristic relaxation times T2 and T2* (measured in ms).

2. Iron chelation

The chelation of excessive amounts of iron from the liver and spleen and, more recently, from the pituitary gland and the brain, has been utilised as a therapeutic approach. In what follows, we will outline the development of the most important therapeutic application of iron chelation therapy to date, namely in the treatment of thalassemia [2–5] and neurodegenerative diseases [1].

Iron Chelators in Current Clinical Use

The hexadentate chelator, deferoxamine (DFO) (Figure 1), a bacterial hydroxamate siderophore, was introduced in the early 1970s, and initially gave poor results because it was not active by oral administration, and had a short half-life (20–30 min). However, the development of continuous subcutaneous infusion of DFO by a portable pump [6] and the establishment of sensible schedules for the optimal use of the pump [7] meant that by the 1990s markedly prolonged cardiac disease-free survival in patients, who faithfully followed the Propper and Pippard regime, could be demonstrated in three independent studies [8–10]. However, sadly, half of the patients, who could not or would not comply, developed cardiac failure or arrhythmia much more rapidly [11]. What was urgently required was a chelator which could be used more easily, thereby improving compliance, and which was both effective and orally active.

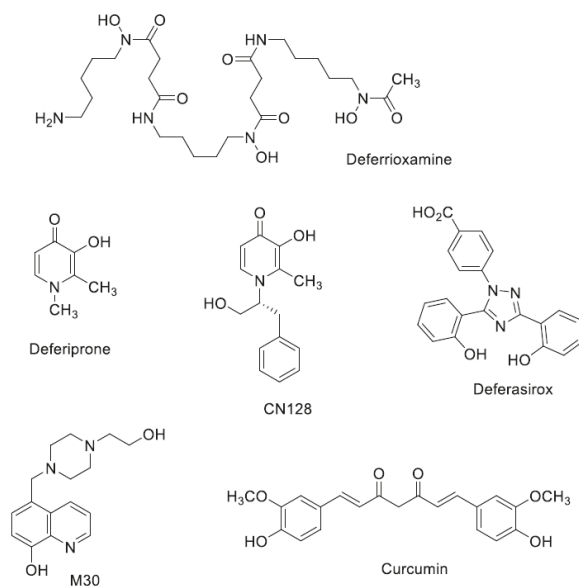


Figure 1. Chemical structures of iron chelators.

Deferiprone (DFP) a small, lipophilic bidentate chelator of the 3-hydroxypyridin-4-one family (Figure 1), was introduced into clinical practice in the 1980s [12]. Although it is orally active, its half-life is 3–4 h, which means that it must be administered three times a day at doses of 75 mg/kg/day to maintain sufficient negative iron balance equivalent to 50 mg/kg/day of DFO [13,14]. DFP has good bioavailability, but its clearance is accelerated by rapid biotransformation: approximately 85% of the drug is metabolised to a nonchelating 3-O-glucuronide conjugate [15]. The most important side effects of deferiprone are agranulocytosis and milder forms of neutropenia, which require appropriate monitoring [16]. However, DFP enters cells and can access intracellular chelateable iron more readily than DFO [17]. DFP possesses cardioprotective effects [18], and a multi-centre prospective comparison

showed that a combined DFP + DFO regimen was more effective in removing cardiac iron than DFO, and was superior in clearing hepatic iron than either DFO or DFP monotherapy [19].

In the years that followed the introduction of DFP, the search for other orally active chelators intensified [20]. The microbial tridentate chelator, desferriethiocin, discovered in 1980, was shown to be orally active and very effective in mobilising hepatic iron in a rat model with hepatic iron loading induced by a ferrocene derivative [21], and in iron-loaded monkeys [22,23]. However, ferrithiocin itself proved to be toxic in animals [24], and although many desferriethiocin derivatives were tested and found to be more effective than desferriethiocin, toxicity remained associated with this class of chelators [25]. The clinical development of Deferasirox (DFX) (ICL670) represents an investment, the magnitude of which has no precedent in the history of chelator research. In the search for a safe tridentate chelator, a completely new chemical class of iron chelators, the bis-hydroxyphenyltriazoles, was discovered. More than forty derivatives of the triazole series were synthesised at Novartis, and evaluated, together with more than 700 chelators from other chemical classes. The tridentate chelator 4-[(3,5-Bis-(2-hydroxyphenyl)-1,2,4 triazol-1-yl)-benzoic acid (ICL670, Figure 1) emerged as the chelator which best combined high oral potency and tolerability in animals [20]. DFX has a plasma half-life of 12–16 h, which allows it to be taken once per day, effectively eliminating non-transferrin bound iron (NTBI) from the circulation [26]. In clinical studies, DFX has proven to be as effective as DFO at doses of 20–30 mg/kg [27], and in an extensive study involving 1744 patients, it was shown that fixed starting doses of DFX, based on transfusional iron intake, with dose titration guided by serum ferritin trends and safety markers, provide clinically acceptable chelation in patients with transfusional hemosiderosis from various types of anaemia [28]. Side effects are minimal, although the use of DFX is associated with rash formation and renal toxicity [29].

3. Thalassaemia, Sickle-Cell Anaemia, and Haemoglobinopathies

Thalassaemia, sickle-cell disease, and other hereditary disorders of haemoglobin biosynthesis are the most prevalent monogenic diseases worldwide [5]. Thalassaemia results from an inherited defect in the rate of synthesis of one of the two polypeptide chains of haemoglobin, resulting in imbalance in the α , β -globin chain ratio, ineffective erythropoiesis, chronic haemolytic anaemia, and increased intestinal iron absorption. In the 1960s and 1970s, the only effective treatment for thalassaemia was blood transfusion. Since humans cannot increase their iron excretion to compensate for iron loading, the transfusional iron overload, aggravated by increased intestinal iron absorption, results in massive and progressive iron overload. Each unit of transfused blood contains approximately 200 mg of iron, and since the mean transfusional loading in thalassaemia major is 0.4 mg/kg/day, it comes as no surprise that most patients faced certain death before the age of 20 years from heart failure. However, the incidence of heart failure was not directly related to the cardiac iron overload, but to the level of iron loading in the liver. The explanation for this lies in the demonstration by Chaim Hershko [30] of the presence in the serum of thalassaemic patients of toxic non-transferrin-bound iron (NTBI) which was responsible for the cardiac damage. When the liver iron load attains a critical level, iron is released into the circulation, and once its concentration exceeds the binding capacity of the transferrin pool, NTBI is formed. NTBI is the main source of iron accumulation in iron loading in both thalassaemia and hereditary hemochromatosis, notably in the heart, pancreas, and liver [31], as well as in the brain. The influx of NTBI seems to be mediated by the ZIP14 transporter in the liver [32,33], whereas in cardiomyocytes, two calcium channels transport Fe^{2+} with an affinity similar to that of Ca^{2+} [34,35], and their expression is insensitive to cardiac iron overload.

3.1. Thalassaemia-Iron Accumulation in the Pituitary

Clinical hypogonadism is common in thalassaemia patients (83%) with detectable cardiac iron and is independently associated with pituitary iron deposition and gland shrinkage [36]. The pituitary gland maintains its anatomical and functional connections with the brain, yet sits outside the blood–brain barrier. Iron deposition in the anterior pituitary continues to pose a serious problem in homozygous

beta thalassemia patients, particularly in terms of gonadal function. Magnetic resonance imaging (MRI) measurements are able to estimate the amount of iron in the pituitary, which can then be correlated with gonadal function. The anterior pituitary gland is particularly sensitive to free radicals produced by oxidative stress, such that exposure to these radicals injures the gland. MRI has shown that even a modest increase in iron deposition in the anterior pituitary can lead to dysfunction, such as abnormalities in the hypothalamic–pituitary growth hormone axis and growth hormone neurosecretory dysfunction, and low insulin-like growth-factor-1 levels. Abnormalities in the hypothalamic–pituitary–gonad axis will induce lower follicle stimulating hormone (FSH) and luteinizing hormone (LH) secretion, low LH/FSH response, and low sex steroid secretion from the gonads, i.e., testosterone (reviewed [37]). The spleen plays an important role in destroying abnormal red cells, sequesters 30–40% of the circulating platelet pool, and plays a role in the regulation of plasma volume. In thalassaemic patients, the spleen may become enlarged, which can result in an increased propensity to recurrent infection, such that a splenectomy is required. Following splenectomy, the pituitary iron loading increases [38]. In early studies of four thalassaemia patients, (Hb-E thalassaemia and thalassaemia major), the pituitary iron content, as assessed by MRI, was increased in all four patients compared to controls [39]. A subsequent MRI study of 84 patients with β -thalassaemia established a correlation between pituitary iron overload, (evaluated by T2*) and both hepatic and cardiac iron load. In addition, pituitary MRI values correlated with serum ferritin and patient age, but not with height of the pituitary [38]. A study of 30 children and young adult thalassaemic patients (13 females and 17 male patients) confirmed iron accumulation in the liver, myocardium, and the pituitary, a linear regression between pituitary iron and age in patients > 14 y, while MRI values between the pituitary and liver, and liver and myocardia, were only moderately correlated, ($r = 0.34$ and 0.42 , respectively). However, no correlation was evident between pituitary and myocardial MRI results, which were of interest, since it might be considered that NTBI would be taken up by both of these organs [40]. It is clear that a therapeutic approach based on the use of iron chelators offers a solution to many of these dilemmas, associated with systemic iron overload, and their development has resulted in innumerable lives being saved, and, just as importantly, has led to an enormous improvement in the quality of life of thalassaemia patients all over the world [5]. Investigations have now been extended to monitor whether excessive amounts of iron in specific brain regions and the pituitary can be removed by iron chelators.

3.2. Iron Chelation from the Pituitary Gland

Iron chelators, such as deferoxamine, deferiprone, or deferasirox, could be used alone or in combination to induce negative iron balance and reverse hypogonadism and endocrine complications in severely iron-overloaded thalassaemic patients. Therefore, it is of interest to ascertain whether iron chelation can reduce iron overload in the pituitary as well as the liver, heart, and spleen. Berkovitch et al. [41] investigated 33 patients >15 years old with transfusion-dependent homozygous beta thalassaemia, all of whom had received DFO. Anterior pituitary function (gonadotrophin releasing hormone, GnRH, stimulating test) correlated well with the MRI results for iron deposition in the anterior pituitary, although there was no correlation between the MRI measurements, the GnRH stimulation test, and the clinical status of the patients. Interestingly, 28 out of the 33 patients achieved normal puberty. The deficit in endocrine function in thalassaemic patients was confirmed in a study of 78 male thalassaemic patients (4–11 years) treated with frequent transfusions and long term DFO therapy, who were compared with 30 age- and sex-matched control children. The prevalence of hypogonadotropic hypogonadism in the thalassaemic patients was 76.2%, with a significant increase in serum ferritin ($\times 7$), and significantly reduced the serum level of cortisol, growth hormone, stimulating hormones and testosterone [42].

Using the new oral chelator DFX for a two-year period, Wood et al. [43] investigated the pituitary iron content and volume in 31 chronically-transfused patients (28 thalassaemia major and three Blackfan Diamond). MRI measurements were taken at the baseline, one, and two years. Twenty-six patients completed the study, 10 were prepubertal, 12 had achieved normal puberty, and four were hypogonadal

at base line. Interestingly, the decreased pituitary volume at baseline returned to normal values after two years of DFX therapy, while pituitary iron content as assayed by R2* showed no net change. This latter result was of interest, as it was expected that with increasing age, pituitary iron would increase in parallel (predicted to be a 0.4 increase) [36].

Such studies indicate that iron chelators are able to reduce pituitary iron content as well as improve its function. It is clearly of interest to know whether brain regions are vulnerable to increased iron deposition in β -thalassemic patients. There have been three main studies which investigated other regions of the brain by quantitative MRI. In the studies of Duprez et al. [44] and Hasiloglu et al. [45], various MRI methodologies were utilised to identify an increase in iron in the choroid plexus of β -thalassemic patients. Qiu et al. [46] investigated the distribution of iron in different brain regions of 31 thalassaemic patients, aged 25.3 ± 5.9 years, and 33 age matched healthy volunteers. Of the 31 patients, 27 exhibited abnormal iron deposition in one of the brain regions investigated by quantitative susceptibility, showing significantly lower susceptibility values in the globus pallidus and substantia nigra and significantly higher susceptibility in the red nucleus and choroid plexus. In the control subjects, there was a positive age effect on susceptibility value in the putamen, dentate nucleus, substantia nigra, and red nucleus. However, more information is needed with respect to the liver and heart iron deposition and the extent of chelation in each of the thalassaemic patients for complete interpretation of these results. Surprisingly, there have been no studies on iron distribution in specific brain regions of thalassaemic patients, before or after iron chelation. Whether continuous iron chelation in these patients will preclude such iron accumulation is unknown, nor whether there is preferential iron accumulation in specific brain regions. Further studies are clearly required.

4. Parkinson's Disease—Iron Accumulation in the Substantia Nigra

Parkinson's disease is the second most common form of motor system degeneration, and is characterised by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta in the ventral midbrain. There is an increased accumulation of iron in the substantia nigra (SN) [47], with a smaller accumulation of iron in other brain regions, such as the red nuclei, globus pallidus, and the cortex of Parkinson's Disease (PD) patients. As the severity of the disease increases the total iron content increases in the SN, which correlates with motor disability [48] and microgliosis [49]. Semi-quantitative histochemical studies of the SN show that iron deposits are present within the neurons and glia of the substantia nigra, putamen, and globus pallidus, with an increase in ferritin-loaded microglia cells in the substantia nigra [50]. The increase in iron in the SN of PD patients is associated with increased ferritin and neuromelanin iron loading [50,51], as well as increased expression of divalent metal transporter 1, which may contribute to PD pathogenesis via its capacity to transport ferrous iron [52]. Intense microgliosis occurs around extra neuronal neuromelanin (released by dying neurons) in the substantia nigra of patients with Parkinson's disease [53,54], which could be an important factor in inducing iron accumulation within the microglia [49].

Preliminary reports suggested that identification of neuromelanin, NM, (rather than iron) by MRI might be feasible in patients with Parkinson's disease [55,56]. Indeed, the loss of neuromelanin in the locus coeruleus and substantia nigra, together with the associated loss in the ability to sequester iron, might be a characteristic sign of Parkinson's disease [56]. Recent studies have been able to discriminate neuromelanin in PD brains. For example, three-dimensional (3D) neuromelanin-sensitive ^{31}M MRI [57] showed that signal densities and contrast ratios were significantly lower in the SN of PD patients compared to controls. Another study of 13 late stage PD patients (LSPD) and 12 de novo PD patients (2–5 years duration) showed that the signal was significantly decreased in LSPD compared to de novo PD, while in the lateral SN region, a decrease in the contrast ratios was detected in all PD groups compared to controls [58]. Interestingly, in this study, the NM signal area was significantly correlated with Hoehn Yahr Stage and Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) part II, while a weak correlation was found with MDS-UPDRS part III. Such results

may indicate that measurement of the neuromelanin content in the brain by MRI techniques could be an important diagnostic approach.

Chelation of Iron in the Brains of Parkinson's Disease Patients

Chelation of iron from the SN of PD brains was proposed many years ago. In our initial animal studies over 15 years ago [59,60], we showed that iron chelators (DFO and DFP) already in clinical use for the treatment of iron loading systemic diseases were able to cross the blood brain barrier (BBB), reduce the iron content in various brain regions, and induce neuroprotection in an animal model of PD. In 2010, Kwiatkowski et al. [61] reported the beneficial use of DFP in one PD patient. After one year's administration of DFP, at 30 mg/kg/day, there was an improvement in the UPDRS score and a decrease in iron accumulation in the bilateral dentate nuclei, as well as the SN [61]. It is of interest that the time period to observe a beneficial action was approximately one year. Subsequent clinical trials in 2011 and 2013 confirmed that it was necessary to administer DFP for at least nine months at a comparable dose to observe a beneficial effect, reflected by changes in the UPDRS score. Both studies [62,63] identified the beneficial effects of DFP. In the study of Devos et al. [62], R2* sequences were used to assess iron content in the SN, while UPDRS scores were acquired at various intervals to assess clinical parameters, and serum ferritin was assayed as a marker of iron stores. After six to nine months, decreases in SN iron content were quantitated by MRI, and there were improvements in both motor and UPDRS motor scores. In the second study, of six months duration [63], decreases in SN iron content were assayed by MRI T2* in patients receiving 30 mg/kg/day, and there were indications of an improvement in UPDRS. Interestingly, PD patients exhibiting high inflammatory markers in the blood, e.g., IL-6, did not respond well to iron chelation, leading to the suggestion that chelatable iron was not freely available for chelation. There was no evidence that the chelation therapy had an adverse effect on haematological parameters. Interestingly, in the study of Bastida et al. [63], the chelation of iron from other brain regions was also assessed. After three months, the caudate nucleus showed decreased iron content, while the dentate nucleus showed an iron decrease at six months. At this time there was also evidence for a decrease in substantia nigra iron content, which took up to nine months in the Davos study. Clearly, this indicates that there is a selective removal of iron from different brain regions. The major disadvantage of the use of DFP was the incidence of neutropenia and agranulocytosis, which occurred in 8% of the patients. This side effect resolved rapidly with cessation of the oral therapy. Such a side effect required that all PD patients entering the clinical trial undertook a weekly white cell count. These positive results from the clinical trials have confirmed that iron chelation therapy has potential as a therapeutic option for the treatment of PD; if the side effect could be eradicated, it would become a much more universal treatment. In addition, it remains unclear whether the long-term use of such chelators might alter oligodendrocyte function, since they have a large requirement for iron. It is therefore suggested that clinical studies that use iron chelation therapy for diseases associated with iron deposition should monitor white matter (WM) changes. A number of these diseases already report apparent hypomyelination, and further decline of WM integrity will impact the functional outcomes, since myelin integrity impacts movement and cognition. Currently, a multi-centre phase 2 clinical trial involving 300 patients from all over Europe is in place, centered at the University of Lille, France.

Over the past decade, work has been directed towards the design of hydroxyypyridinones, which lack an effect on white blood count in rodents and primates. A lead compound, CN128 (Figure 1), has been identified, which possesses all of the therapeutic properties of DFP, including penetration of the blood–brain barrier, but lacks the side effects of induction of neutropenia and agranulocytosis in primates after prolonged exposure (nine months) [64]. CN128 is currently undergoing tests in a range of Parkinson's disease models.

5. Alzheimer's Disease

Alzheimer's disease (AD) is a fatal age-related neurodegenerative disease which results in cognitive decline, memory loss, and psychosis. Clinically, AD is characterised by progressive dementia. Initial symptoms are short-term memory loss, which is followed by extensive neuronal loss in the hippocampus and selected cortical and subcortical area. There is abnormal protein processing, with accumulation of the β -amyloid peptide, which is deposited extracellularly and manifests itself as neuritic amyloid plaques, and of the hyperphosphorylated tau protein, in the form of neurofibrillary tangles. Both of these abnormal protein aggregates are definitive markers of the disease in post mortem material.

There is considerable evidence that there is defective homeostasis of iron in the AD brain. Increases in the iron content of AD have been reported for over 50 years. In a recent paper, which made a comprehensive systematic meta-analysis and review of over 2556 publications, 43 eligible studies were identified where the iron content in AD serum, cerebrospinal fluid (CSF), or brain tissue had been studied. In nineteen studies, the iron content in 12 selected brain regions was analyzed by separated meta-analysis, and it was concluded that eight specific brain regions (the temporal, parietal, and frontal lobes) had higher iron concentrations, which correlated with the clinical diagnosis of AD [65]. A recent laser ablation inductively coupled mass spectroscopy study also identified increases in iron in the frontal cortex [66].

Alzheimer's Disease—Iron Chelation from the Cortex and Hippocampus

Current therapies for Alzheimer disease (AD), such as the acetylcholinesterase inhibitors and the NMDA receptor inhibitors, may provide moderate symptomatic delay at various stages of the disease, but do not arrest the disease progression or induce meaningful remission. The confirmation of altered iron homeostasis in the brain of AD patients has opened the possibility of using iron chelators as a new therapeutic approach. Various animal models of AD have been utilised to investigate the therapeutic action of iron chelators. For example, the iron chelator (-) epigallocatechin-3-gallate and M-30 (Figure 1) reduced amyloid precursor protein (APP) expression in cultured cells [67,68]. Although it is known that there is an increase in iron in various brain regions in AD patients, there has been no clinical evidence which supports the use of chelating agents as an adjunctive treatment for AD. Tea polyphenols and curcumin (Figure 1) have been advocated as metal chelators for the treatment of AD, although the efficacy of such natural products awaits further investigation.

In 1991, McLachlan showed that DFO significantly reduced the behavioral and cognitive decline of AD patients [69]. However, since this ground-breaking study it is only now that further studies are being undertaken to investigate the therapeutic efficacy of iron chelators in AD. Currently, a phase 2, randomised, placebo-controlled, multi-centre study to investigate the safety and efficacy of DFP has started recruiting. Approximately 171 participants with Prodromal Alzheimer's disease and mild Alzheimer's disease will be recruited for the study. Controlled release DFP will be used in an attempt to prevent the unwanted side effects of neutropenia and agranulocytosis. The aim of this study is to ascertain whether DFP (15 mg/kg BID orally) slows cognitive decline in Alzheimer's patients. MRI will ascertain the iron content of brain regions during the period of the study (Neuroscience Trials Australia—Clinical Trial Deferiprone to Delay orallyDementia).

Other compounds are in development for the treatment of AD, which have multitargeting properties. They include a series of (3-hydroxy-4-pyridinone)-benzofuran hybrids [70] which mimic donepezil, an inhibitor of acetylcholinesterase, and possess additional properties, such as metal chelation, radical scavenging, and the inhibition of amyloid peptide aggregation. In addition, a series of thiosemicarbazones, (pyridoxal 4-N-(1-benzylpiperidin-4-yl) thiosemicarbazone compounds exhibit very low anti-proliferative activity, substantial iron chelation efficacy, inhibition of copper-mediated amyloid- β aggregation, inhibition of oxidative stress, moderate acetylcholinesterase inhibitory activity, and autophagic induction [71]. Although such compounds show neuroprotective effects in vitro,

it remains to be investigated whether they have a beneficial effect in either animal models of AD or AD patients.

6. Friedreich's Ataxia

Friedreich's ataxia (FRDA) is one of a number of neurological disorders caused by the anomalous expansion of unstable nucleotide repeats in which, unlike Huntington's disease, the nucleotide expansion occurs in the non-coding part of the gene (the introns). FRDA is characterised by a progressive degeneration of large sensory neurons and cardiomyopathies [72]. Although rare, FRDA is the most frequent inherited ataxia, with an estimated prevalence of two to four people in 100,000 individuals. Most patients carry homozygous GAA expansions in the first intron of the frataxin gene on chromosome 9. Whereas the critical pathologic triplet repeat threshold is 66 repeats, the expansion can be as many as 890 GAA repeats. This results in the partial silencing of frataxin, a small mitochondrial protein which plays an essential role in iron–sulfur cluster (ISC) biogenesis, an essential metabolic pathway found in all organisms [73,74]. Frataxin interacts directly with the two central components of the ISC biogenesis machine, the NFS1/IscU complex [75]. Correct ISC synthesis in mitochondria is closely linked to cellular iron homeostasis [76], and a lack of frataxin therefore causes dysregulation of iron metabolism. As a consequence, failure to assemble mitochondrial Fe–S proteins results in increased cellular iron acquisition, mitochondrial iron overload [77], and mitochondrial iron deposits in some FRDA patients [78].

Iron Chelation from the Dentate Nucleus in Friedreich's Ataxia Patients

In one clinical trial, deferiprone (10–15 mg/kg) was administered twice daily to FRDA patients in a small clinical trial over a six-month period to nine adolescent patients with no overt cardiomyopathy. Brain iron was reduced significantly in dentate nuclei. The chelator treatment caused no apparent hematologic or neurologic side effects, while reducing neuropathy and ataxic gait in the youngest patients [79]. In a second clinical trial, DFP (10 mg/kg) was administered in combination with idebenone (20 mg/kg) to 20 FRDA patients. No significant differences were observed in the total international cooperative ataxia rating score (ICARS) scores when comparing baseline status and the end of the study in the whole group of patients. Echocardiography data showed a significant reduction of the interventricular septum thickness and in the left ventricular mass index. After 11 months of treatment, iron was reduced in the dentate nuclei. However, there was a worsening of posture and gait compared to baseline [80].

7. Conclusions

The current longevity of the population will result in increasing iron loading in various brain regions, although this is not normally associated with toxicity. Exactly why and how the iron which accumulates in specific brain regions in neurodegenerative diseases is highly toxic is unclear. However, new studies of activated microglia indicate that these cells may, in part, be the source of the iron loading. Over the past 50 years chelation therapy has progressed from subcutaneous administration to oral administration, and it is hoped that toxicity that has been associated with the oral chelators will be eliminated with new formulation of the hydroxypyridinones.

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Abbreviations

AD	Alzheimer’s Disease
Ca ⁺⁺	Calcium
CSF	Cerebrospinal fluid
DFO	Desferrioxamine
DFP	Deferiprone
DFX	Deferasirox
Fe ²⁺⁺	Ferrous
FDRA	Friederich’s Ataxia
FSH	Follicle stimulating hormone
GAA	Trinucleotide repeat
GnRH	Gonadotropin-releasing hormone
GnRH stimulation	Gonadotropin-releasing hormone test.
ICARS	International Co-operative Ataxia Rating Scale
IL-6	Interleukin-6
ISC	Iron sulphur protein
LH	Luteinizing hormone
LSPD	Late stage Parkinson’s Disease
MDS-UPDRS	Movement Disorder Society Unified Parkinson’s Disease Rating Scale
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartate
NTBI	Non transferrin bound iron
PD	Parkinson’s Disease
SN	Substantia nigra
WM	White matter
ZIP14	Zinc transporter

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Review

New Perspectives in Iron Chelation Therapy for the Treatment of Neurodegenerative Diseases

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Abstract: Iron chelation has been introduced as a new therapeutic concept for the treatment of neurodegenerative diseases with features of iron overload. At difference with iron chelators used in systemic diseases, effective chelators for the treatment of neurodegenerative diseases must cross the blood–brain barrier. Given the promissory but still inconclusive results obtained in clinical trials of iron chelation therapy, it is reasonable to postulate that new compounds with properties that extend beyond chelation should significantly improve these results. Desirable properties of a new generation of chelators include mitochondrial destination, the center of iron-reactive oxygen species interaction, and the ability to quench free radicals produced by the Fenton reaction. In addition, these chelators should have moderate iron binding affinity, sufficient to chelate excessive increments of the labile iron pool, estimated in the micromolar range, but not high enough to disrupt physiological iron homeostasis. Moreover, candidate chelators should have selectivity for the targeted neuronal type, to lessen unwanted secondary effects during long-term treatment. Here, on the basis of a number of clinical trials, we discuss critically the current situation of iron chelation therapy for the treatment of neurodegenerative diseases with an iron accumulation component. The list includes Parkinson’s disease, Friedreich’s ataxia, pantothenate kinase-associated neurodegeneration, Huntington disease and Alzheimer’s disease. We also review the upsurge of new multifunctional iron chelators that in the future may replace the conventional types as therapeutic agents for the treatment of neurodegenerative diseases.

Keywords: neurodegeneration with brain iron accumulation; iron chelation therapy; multifunctional iron chelators

1. Introduction

Iron content increases with age in several regions of the brain. Particularly, high levels of non-heme iron are found in the globus pallidus, the red nucleus, substantia nigra, cortex and putamen; in contrast, the iron content of the medulla oblongata does not change with age whereas the iron content of the thalamus decreases from age 30 to 90 [1–3]. The causes underlying the increase in brain iron with age remain elusive. It is unclear whether this increase is a reflection of total body iron, since a report shows that non-heme iron in the liver does not change with age [1], although body stores of iron, as determined by circulating ferritin levels, seem to increase with age [4].

Abundant evidence suggests that disturbed iron homeostasis and mitochondrial dysfunction play important roles in the development of an increasing number of neurodegenerative diseases [3,5–9]. The occurrence of high iron content in brain areas susceptible to neurodegeneration, in conjunction with the known ability of iron to generate reactive oxygen species (ROS) and induce the formation of protein aggregates, provides a relevant seed mechanism for downstream events leading to the death of affected neurons. It has been postulated that the high iron–ROS–mitochondrial dysfunction events

undergo a positive feedback loop that further fosters oxidative damage, protein aggregation and cell death [3,6,8].

The use of iron chelators for the treatment of systemic diseases such as thalassemia major and hemochromatosis is already a proven therapeutic approach [10]. As a norm, chelator treatment in iron overload patients induces substantial iron excretion and a negative iron balance [11–15]. On the basis of this experience, strategies to stop or slow neurodegenerative process with an iron accumulation component are now being tested in therapeutic trials.

A review on the current evidence of the benefits and drawbacks of iron chelation therapy, and the analysis of new compounds that could be used for the treatment of neurodegenerative diseases, follows.

2. Neurodegenerative Diseases with an Iron Accumulation Component

A wide variety of neurological diseases are characterized by the accumulation of iron in different areas of the central nervous system; these diseases include Parkinson's disease (PD) and other parkinsonisms such as Lewy bodies dementia, progressive supranuclear palsy, corticobasal degeneration [16–21], the Westfal variant of Huntington disease [22], Alzheimer's disease (AD) [23–27], Friedreich's ataxia [28], pantothenate kinase-associated neurodegeneration [29–31] and other neuropathologies associated with brain iron accumulation [32–34]. From the pathophysiological standpoint, different mechanisms are observed, so the clinical and therapeutic implications of iron accumulation may be different for each individual disease process [2,35].

There is ample evidence linking iron to the pathology of idiopathic PD. A good review on this subject can be found in the article by Moreau et al. [36]. Iron is particularly abundant in dopaminergic neurons, where it is needed for dopamine synthesis [37] and the production of energy through the electron transport chain [38,39]. In dopaminergic neurons, iron behaves as a double-edged sword since it also participates in the production of the noxious hydroxyl radical during dopamine auto-oxidation. Moreover, the nonenzymatic oxidation of dopamine produces the leukoaminochrome o-semiquinone radical, which reacts with oxygen to generate superoxide anion [40–42]. Since under physiological conditions iron reacts with superoxide and hydrogen peroxide [6], it is possible that iron dyshomeostasis plays a fundamental role in mediating oxidative damage in dopaminergic neurons. Indeed, the hydroxyl radical, the most reactive ROS in living matter, is formed by the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{HO}^\bullet$), a nonenzymatic reaction that obeys mass action law. Hence, there is a direct relationship between the concentration of redox-active iron and the production of hydroxyl radical.

The mechanisms of iron homeostasis that go awry in neurodegeneration form a cutting-edge question in metalloneurobiology [43]. Highly relevant to this point is the mitochondria-iron connection. The iron homeostasis regulator iron regulatory protein 1 (IRP1) is activated in idiopathic PD. Postmortem brain tissue from PD patients displays increased IRP1 activity when compared to tissue from control individuals [44]. Increased IRP1 activity was found also in the ipsilateral ventral mesencephalon of 6-OHDA-treated rats [44]. Studies performed in our laboratory showed that in SH-SY5Y cells, loss of mitochondrial function caused by inhibition of complex I results in decreased Fe-S cluster synthesis and increased IRP1 binding activity, accompanied by increased intracellular iron levels [45]. Further studies revealed that complex I inhibition is associated with increased levels of iron uptake proteins, and decreased levels of the iron efflux transporter ferroportin 1 [46]. Complex I inhibition also results in increased oxidative modifications and increased cysteine oxidation, while IRP1 silencing abolishes the increase in ^{55}Fe uptake activity and protects cells from death induced by complex I inhibition [46]. Thus, mitochondrial dysfunction initiates a positive feedback loop that also comprises increasing iron uptake and increased oxidative damage. In this view, iron accumulation, more than a primary cause, seems to be a consequence of other initiation factors.

An attractive hypothesis for the genesis of diseases with a redox-active metal accumulation component is the metal-based neurodegeneration hypothesis [3]. According to this hypothesis, redox-active metal ions like iron and copper generate ROS that cause the peroxidation of membrane

phospholipids, which in turn leads to the formation of reactive aldehydes. Reactive aldehydes, together with other ROS, interact with proteins inducing their aggregation, which overwhelms the cellular protein degradation systems, resulting in their accumulation within intracellular inclusion bodies. Accordingly, this hypothesis suggests that protein aggregation occurs downstream of iron or copper dyshomeostasis.

In the specific case of idiopathic PD, α -synuclein aggregation has been proposed to be a consequence of mitochondrial dysfunction/ROS production [47–49] or, inversely, mitochondrial dysfunction has been proposed to be a consequence of α -synuclein aggregation [50–53]. Thus, it is possible that mitochondrial dysfunction, oxidative stress and α -synuclein aggregation jointly establish a positive feedback cycle that taxes energy production and overloads the protein degradation systems [53–55]. This positive feedback concept is further augmented by the observation that iron induces α -synuclein aggregation (see above). Since mitochondrial dysfunction increases iron accumulation, another positive feedback cycle could be formed that includes mitochondrial dysfunction, iron dyshomeostasis and α -synuclein aggregation (Figure 1). It follows that any of the components of these two cycles (mitochondrial dysfunction, oxidative stress, iron dyshomeostasis and α -synuclein aggregation) could initiate these processes. From the therapeutic stand point, multiple-task strategies targeting these events should provide more effective treatment to stop the progression of this disease.

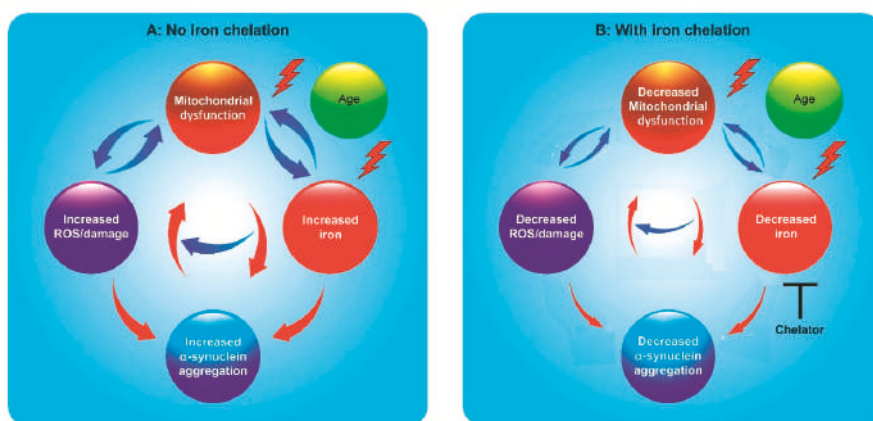


Figure 1. Self-feeding cycles in Parkinson's neurodegeneration. (A) Blue arrows: Mitochondrial dysfunction, caused by internal or external toxins, or derived from genetic factors, results in increased oxidative stress and in decreased synthesis of iron–sulfur clusters, which in turn results in the spurious activation of iron regulatory protein IRP1 and increased iron uptake. Both increased ROS and increased iron produce additional mitochondrial dysfunction through the generation of the hydroxyl radical. Red arrows: Increased ROS and increased iron levels induce α -synuclein aggregation, establishing a positive feedback cycle including mitochondrial dysfunction, further taxing energy production. (B) Iron chelation decreases redox-active iron and the production of damaging ROS. The decrease in ROS and redox-active iron results in decreased α -synuclein aggregation. The decrease in ROS and α -synuclein aggregation also results in improved mitochondrial function. Thus, decreasing redox-active iron by chelation slows or stops the process of neuronal death. In this scheme, age is a neurodegeneration factor not influenced by iron chelation.

3. Clinical Trials Using Iron Chelation

Overwhelming evidence shows that iron accumulation in the brain may contribute to neurodegenerative processes, as shown in recent reviews [7,35,43,56,57]. A tempering view states that

cellular iron homeostasis mechanisms are sufficient to limit its toxicity [58]. Nevertheless, excessive iron levels will increase hydroxyl radical generation, for which there are no cellular mechanisms to counteract its noxious effects.

Successful experiences support the use of iron chelation therapy for the treatment of systemic diseases with an iron accumulation component, such as thalassemia major, sickle cell disease and cardiomyopathy associated with hereditary hemochromatosis [59–67]. The chelators used in these therapies are deferoxamine, deferasirox, deferiprone and PBT2. Adherence to treatment by patients treated with deferoxamine is low since, as it does not permeate the intestinal barrier, it must be injected. Oral chelators such as deferasirox and deferiprone have better treatment compliance. Deferasirox (Exjade[®], Novartis Pharma AG, Basel, Switzerland) was the first oral chelator approved for human use in 2005, while Deferiprone (Ferriprox[®], Apotex Inc., Toronto, ON, Canada) was approved in 2011.

Recently, iron chelation has been introduced as a new therapeutic concept for the treatment of neurodegenerative diseases that have a component of metal ion accumulation [56,68–70]. Essentially, the iron chelator should be able to penetrate the cell membranes, as well as the blood–brain barrier, and should have the capacity to extract the chelated iron from the accumulation site and to transfer it to other biological acceptors such as circulating transferrin [68,71]. In addition, small doses of chelators must be used in order to minimize side effects [72,73].

A search in <https://clinicaltrials.gov> indicated 12 ongoing or finished trials of iron chelation for the treatment of neurodegenerative diseases: four trials for Parkinson’s disease, three for Friedreich’s ataxia, two for amyotrophic lateral sclerosis, one for mild Alzheimer’s disease, one for pantothenate kinase-associated neurodegeneration and one for neurodegeneration with brain iron accumulation. A review on the results of finished trials with published results follows. A number of single-case reports that lack the appropriate controls will not be mentioned here (reviewed by Dusek et al. [70]).

3.1. Parkinson’s Disease

A randomized pilot clinical trial tested 40 patients with early-stage PD, treated with deferiprone versus placebo. A dose of 30 mg/kg/day, during a period of six months, produced a decrease in the iron content of the substantia nigra, evaluated by T3 magnetic resonance [69]. A significant improvement of the motor indicators of the progression of the disease was found. Nevertheless, once the treatment was suspended, iron accumulation reappeared, suggesting a reversal to the pathological state. In a second report of this same study, the usefulness of ceruloplasmin (CP) as a biomarker was emphasized, associating the low activity of this enzyme in Parkinson’s disease with iron overload in the substantia nigra [72]. It was found that after six to 12 months of deferiprone treatment, greater reductions in substantia nigra iron levels and Unified Parkinson’s Disease Rating Scale (UPDRS) motor scores were obtained in patients with higher serum and cerebrospinal fluid levels of CP-ferroxidase activity. A second stage of this project, under the acronym FAIRPARK, intends to enroll 338 participants (<https://clinicaltrials.gov/ct2/show/NCT02655315?term=chelation&cond=Parkinson+Disease&draw=2&rank=1>).

In another series reported in Clinical Trials by researchers from the Imperial College London, good tolerance to deferiprone was reported in patients with Parkinson’s disease, removing excess iron in dentate and caudate nucleus but with minimal effects on the symptoms of the disease [73].

In summary, the reported results on chelator treatment of Parkinson’s disease discussed above are encouraging in terms of a possible slowdown of the disease progression, granting the development of further long-term trials.

3.2. Friedreich’s Ataxia

This genetic disease presents a disorder of iron metabolism associated with chronic inflammation and iron accumulation at the level of the central nervous system, the peripheral system, the myocardium and the endocrine system [74]. It was suggested earlier that a reduction of iron accumulation could be a therapeutic alternative for Friedreich’s ataxia patients [75–77]. Initial

pilot studies in young patients with no overt cardiomyopathy showed that treatment with 20 to 30 mg/kg/day of deferiprone significantly decreased iron accumulation in the dentate nucleus in the cerebellum, while reducing neuropathy and ataxic gait. Nevertheless, the effects of this chelation therapy on neurological symptoms remained controversial [78]. In a follow-up of this study, a one-year open-label extension, it was determined that deferiprone dosed at 40 mg/kg/day worsened ataxia, as indicated by a four-point mean increase in International Cooperative Ataxia Rating Scale (ICARS) scores in this group (unpublished data, compiled in [79]). As in the first study, results using a dose of 20 mg/kg/day were inconclusive. Nevertheless, a significant reduction in cardiac hypertrophy was an interesting side effect of deferiprone treatment [79].

A study of co-administration of deferiprone and the coenzyme Q10 analog idebenone reported improvement in heart hypertrophy parameters and iron deposits in the dentate nucleus, but no improvements in ICARS [80]. Another study that used deferiprone in association with idebenone also failed to find significant improvement in neurological function. Nevertheless, an improvement in heart hypertrophy was reported as possible [81].

The largest series reported for the treatment of Friedreich's ataxia enrolled 72 patients, who were treated with varied doses of deferiprone, 20, 40, or 60 mg/kg/day in a six-month Phase-II placebo-controlled trial. The results confirmed the safety of deferiprone at doses lower than 20 mg/kg/day, while the 60 mg/kg/day dose was discontinued due to worsening of ataxia in two patients [82]. Patients receiving 20 mg/kg/day of deferiprone showed a decline in the left ventricular mass index but did not present changes in ICARS scores [82]. The decrease in cardiomyopathy correlated with the decrease of iron in the cardiac muscle.

In a study with 13 Friedreich's ataxia patients, a triple therapy with deferiprone plus idebenone and riboflavin (both antioxidants) resulted in four patients discontinued due to adverse effects after 15–39 months of therapy. Other parameters, like the annual worsening rate scale, the scale for the assessment and rating of ataxia scores and cardiac function did not present significant changes [83]. The authors concluded that the benefits of this triple therapy are uncertain.

In summary, it has been established that therapeutic doses of deferiprone, 20 mg/kg/day, appear to be safe for long-term use for the treatment of Friedreich's ataxia. In most trials, this treatment produced some improvement in heart function, but no improvement of the neurological symptoms were apparent. In addition, doses higher than 40 mg/kg/day seemed to worsen the disease. The effects of long-term treatment at low doses on, for example, slowing or stopping disease progression, need to be evaluated.

3.3. Neurodegeneration with Brain Iron Accumulation (NBIA) Disorders

Within the spectrum of iron deposition disorders there is a group of genetic diseases that have in common a syndrome of NBIA [32,84–86]. These disorders include pantothenate kinase-associated neurodegeneration (PKAN, previously known as Hallervorden-Spatz syndrome) [87–90], PLA2G6 calcium-independent phospholipase A2 (PLAN) [91,92], infantile neuroaxonal dystrophy (INAD) [93,94], mitochondrial membrane protein-associated neurodegeneration (MPAN) [95–98], beta-propeller protein-associated neurodegeneration (BPAN) [99,100], CoA synthase protein-associated neurodegeneration (CoPAN) [101–103], fatty acid-2 hydroxylase-associated neurodegeneration (FAHN) [104,105], Kufor-Rakeb disease [106–108], aceruloplasminemia [109,110] and neuroferritinopathy [111,112]. Of these diseases, iron chelation therapy has been tried in PKAN patients.

The PKAN neurodegenerative condition is characterized by the presence of iron deposits at the level of the basal ganglia, currently detected by MRI [86,113,114]. There have been several trials oriented to the use of deferiprone for the treatment of PKAN patients [115–118]. In all trials, deferiprone treatment decreased iron accumulation in the ganglia, in addition to an improvement in the Unified Parkinson's Disease Rating Scale [115–118]. Although the series is still too small to establish definitive conclusions, iron chelation may be a therapeutic option for the treatment of PKAN. Additionally, in

a downside, treatment of a single BPAN patient with deferiprone had to be interrupted because of worsening of the parkinsonian symptoms [116].

3.4. Huntington Disease

In a study with early/mid-stage Huntington disease patients, patients were subjected to daily doses (250 mg or 100 mg) of 5,7-dichloro-2-[(dimethylamino)methyl]quinolin-8-ol (PBT2) or placebo [119]. After treatment for 26 weeks with this iron chelator, none or marginal improvements were found in cognitive tests. The authors concluded that PBT2 was generally safe and well tolerated, but the evaluation of potential benefits remains uncertain and in need of further studies.

3.5. Alzheimer's Disease

In an initial Phase-II trial with Alzheimer's disease patients, treatment with the iron-copper chelator clioquinol resulted in stabilization of Alzheimer's Disease Assessment Scale scores, compared to placebo-treated controls. In addition, plasma A β 1–42 levels declined in the clioquinol-treated group [120]. In a subsequent Phase-II double-blind trial, it was found that patients treated with doses of 250 mg PBT2 exhibited a significant reduction in cerebrospinal fluid A β 1–42 concentration compared with those treated with placebo [121]. In addition, PBT2-treated patients showed significant improvement in executive function and cognition tests. No serious secondary effects were reported by patients receiving PBT2. The authors concluded that the findings of this study support larger-scale testing of PBT2 in Alzheimer's disease patients.

4. Potential Risks of Iron Chelation Therapy

Iron is vital to life, participating as an essential cofactor in essential cellular processes that include oxygen transport, energy production, DNA synthesis, and a myriad of hydrolysis and redox reactions [122]. Thus, therapeutic iron chelation may affect not only the intended target but also processes that are essential for cell function, generating cases in which “the cure is worse than the disease”. Indeed, excessive iron depletion could result in cognitive decline, by decreasing the activity of enzymes and iron-containing complexes [122,123] and the synthesis of neurotransmitters, such as dopamine, norepinephrine and serotonin [37,124–126]. Fortunately, cognitive decline has not been reported in clinical chelation trials for neurodegenerative diseases using therapeutic concentrations of deferasirox, deferiprone, PBT1 or PBT2 [115–119,121,127,128].

In thalassemia patients treated with high concentrations of deferiprone (75–99 mg/kg/day [129]), the most frequent adverse effects were arthritis, nausea and, more critically, agranulocytosis and neutropenia [130–133], whereas deferasirox was generally well tolerated, with mild gastrointestinal adverse effects [12,134]. A recent meta-analysis, which analyzed results from 34 studies with a total of 2040 young patients with hemoglobinopathies, found increased transaminase adverse effects (between 3.9% and 31.3% in the different studies) and gastrointestinal complaints for both deferiprone (3.7–18.4%) and deferasirox (5.8–18.8%) [135]. Other effects included arthritis, nausea and, most seriously, agranulocytosis in 0.6% to 4% of patients. This work concluded that there may be few but serious adverse reactions in performing iron chelation therapy with these chelators.

Another potential problem with iron chelation therapy is the depletion of other essential metal ions, in particular Zn and Cu [131,136,137]. Accordingly, in a clinical trial of thalassemia with deferiprone, four of eight patients who received treatment for one year showed increased excretion of Zn in the urine and subnormal values of zinc in the serum, associated with areas of dry skin and itching. These symptoms were resolved with zinc supplementation [136].

There are no reports in the literature indicating that iron chelation therapy causes copper depletion. This is possibly because intracellular copper is not free but it is strongly bound to chaperones [138,139].

From the previous analysis, it is clear that the concentration of the iron chelator should be finely tuned to achieve maximal effectiveness in removing excess redox-active iron, and at the same time avoiding toxicity and other side effects.

5. New Multifunctional Iron/Copper Chelators with Therapeutic Capacity

The limited success of metal chelation therapy trials using deferiprone raises the need for novel multifunctional agents, which in addition to decreasing iron accumulation, will have the capacity to interfere with two or more symptoms of a given disease, thus improving the possibilities of stopping, and eventually reversing, the neurodegenerative process.

The question arises on what are the characteristics of an ideal compound for the therapeutic treatment of the vicious cycle of mitochondrial dysfunction–iron accumulation–oxidative damage–protein aggregation in neurodegenerative diseases (Figure 1). These basal properties include: (1) effectiveness via oral administration, which implies crossing the intestinal and blood–brain barrier without modification of its therapeutic properties. Compliance of treatment is better with oral drugs than those injected on a daily basis during long-term treatments [140]; (2) low molecular weight and high membrane permeability. The compound must fulfill the rule of 5 of Lipinski, an empirical model that allows one to evaluate qualitatively how a chemical compound could be absorbed once it is orally ingested as a medicine [141]. Desirable additional properties include (3) free radical quenching capacity independent of its iron chelation capacity. Free radical quenching should decrease lipid peroxidation, thus decreasing ferroptosis events [142]; (4) to undergo mitochondrial accumulation. The reason for this last property is that both high concentrations of ROS and high concentrations of iron coexist in the secluded space of the mitochondrion, which makes this organelle particularly prone to oxidative damage [57,143,144]; (5) to have intermediate (μM) affinity and high specificity for iron, consequent with chelation of the redox-active (labile) iron pool, estimated to be in the micromolar range [134,135]; and (6) in addition, the chelator must have “shuttle” capacity, that is, the capacity to deliver excessive intracellular iron to innocuous acceptors such as transferrin or ferritin [145].

During the last two decades, several multifunctional agents (MFAs) have been reported to be effective in experimental models of Alzheimer’s or Parkinson’s disease. These agents are in an experimental phase, with no clinical trials associated with them. Table 1 shows the basic characteristics of these agents, including their metal chelation characteristics, their capacity to act as antioxidants/free-radical scavengers, their route of administration and blood-brain barrier permeability. We will review next the characteristics of MFAs that could become effective therapeutic agents.

Table 1. Iron chelators with multifunctional characteristics.

Compound	Properties/Characteristics	Metal Specificity	In-Vivo Testing	Route of Administration	Brain Permeability	Disease Model	References
EGCG	Metal chelation; antioxidant; neuroprotective; activation of cell survival genes.	Cu^{2+} ; Fe^{3+} ; Al^{3+} ; Mn^{2+}	Yes	Intraperitoneal; Oral	Yes	PD, AD	[146–150]
Hydroxyquinoline-propargyl hybrids M30, VAR10303	Metal chelation; MAO-B inhibition; antiapoptotic; activation of cell survival genes; neuroprotective; neurotogenic.	$\text{Fe}^{3+} > \text{Cu}^{2+} > \text{Zn}^{2+}$	Yes	Oral	Yes	PD, AD, amyotrophic lateral sclerosis	[151–154]
Hydroxypyridinone glycoconjugates H_2GL^1 , H_2GL^2	Metal chelation; reduction of amyloid-beta aggregation	$\text{Cu}^{2+} > \text{Zn}^{2+}$	No	Not tested	Not tested; probably yes	AD	[155]
Bis-tacrine hybrids	Metal chelation; AChE inhibition; reduction of amyloid-beta aggregation	Cu^{2+}	No	Not tested	Not tested	AD	[156]

Table 1. Cont.

Compound	Properties/Characteristics	Metal Specificity	In-Vivo Testing	Route of Administration	Brain Permeability	Disease Model	References
8-OH-Quinoline-tacrine hybrids	Metal chelation; AChE inhibition	Cu ²⁺	No	Not tested	Probably yes	AD	[157]
Benzylamine-tacrine hybrids	Metal chelation; AChE inhibition; inhibition of amyloid-beta aggregation; moderate antioxidant activity	Cu ²⁺ ; Fe ²⁺ ; Zn ²⁺	No	Not tested	Not tested	AD	[158]
Phenyl-benzimidazole-tacrine hybrid	AChE inhibition; metal chelation; inhibition of Cu-induced amyloid-beta aggregation; free radical scavenger	Cu ²⁺ ; other metals not tested	No	Not tested	Not tested	AD	[159]
Coumarin-tacrine hybrid	Metal chelation; AChE inhibition; inhibition of amyloid-beta aggregation; free radical scavenger	Cu ²⁺ ; other metals not tested	No	Not tested	Not tested	AD	[160]
Piperazine-8-OH-quinolone hybrids	Metal chelation; dopamine D2/D3 receptor agonists; hydroxyl radical scavenger	Fe ²⁺ ; Fe ³⁺	Yes	Subcutaneous	Yes	PD	[161]
Dipyridyl-D2R/D3R agonist hybrids	Metal chelation; dopamine D2/D3 receptor agonist; antioxidant; neuroprotective	Fe ²⁺ >>> Fe ³⁺	Yes	Intraperitoneal	Yes	PD	[162,163]
Curcumin hybrids	Metal chelation; antioxidant activity; reduction of amyloid-beta aggregation	Cu ²⁺ ; Fe ²⁺	No	Not tested	Not tested	AD	[164]
Benzyl-indanone hybrid compound 41	Metal chelation; antioxidant activity; AChE inhibition; inhibition of amyloid-beta aggregation	Cu ²⁺	No	Not tested	Not tested	AD	[165]
Benzothiazole-linker-pyridinone hybrids	Metal chelation; antioxidant activity; AChE inhibition; inhibition of amyloid-beta aggregation	Fe ³⁺	No	Not tested	Probably yes	AD	[166]
Clioquinol-selegiline hybrids	MAO-B inhibition; metal chelation; antioxidant activity	Cu ²⁺ ; Fe ²⁺ ; Zn ²⁺	No	Not tested	Probably yes	PD	[167]
Deferiprone-H3 receptor antagonist hybrid C5	H3R inhibition; metal chelation; antioxidant activity; reduction of amyloid-beta aggregation	Cu ²⁺ ~ Fe ²⁺ >>> Zn ²⁺	Yes	Intraperitoneal	Yes	AD	[168]
7,8-Dihydroxycoumarin derivative DHC12	Metal chelation; MAO-B inhibition; mitochondriotropic; free radical scavenger; neuroprotective	Cu ²⁺ ~ Fe ²⁺ > Zn ²⁺ > Fe ³⁺	Yes	Oral	Yes	PD	[169]
Coumarin-tris hybrid CT51	Metal chelation; impedes Fe ²⁺ /Fe ³⁺ cycling; antioxidant; mitochondriotropic; calcium upsurge blocker	Fe ²⁺ > Fe ³⁺	No	Not tested	Not tested	PD	[170]

5.1. Epigallocatechin-3-Gallate (EGCG)

The pioneering work of Youdim's group at Technion resulted in two of the best-characterized MFAs: EGCG, a component of green tea, and M30, an *N*-propargyl-8-hydroxyquinoline hybrid.

Epidemiological reports associate tea consumption with positive effects in the central nervous system function, such as reduced dementia incidence, delayed PD onset and diminished cognitive impairment in the elderly (reviewed in [171]). Additional studies have shown the neuroprotective effect of EGCG in the MPTP model of PD [146,149,150,172,173]. However, this neuroprotective effect may be secondary to the inhibition of the dopamine transporter (DAT) by EGCG [148,174]. Indeed, MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP⁺) by MAO-B in astrocytes; after release from astrocytes, MPP⁺ is transported into dopaminergic neurons via DAT [175,176]. Thus, ablation, or inhibition, of DAT results in neuroprotection against MPTP/MPP⁺ [177,178].

The protection exerted by EGCG probably involves direct scavenging of ROS such as superoxide, hydrogen peroxide and nitric oxide [179–181]. Nevertheless, the EGCG antioxidant effect is observed only at low concentrations. In-vitro studies show that high concentrations (10–100 μM) of EGCG actually prompt pro-oxidant effects [182–186]. In rat hippocampal neurons, EGCG causes elevation of intracellular calcium and ROS in a dose-dependent way [185,187]. Downstream, high calcium/ROS levels were associated with reduction in the Bcl-2/Bax expression ratio, reduction of the mitochondrial membrane potential and apoptotic cell death [185,188].

In addition, EGCG has anti-inflammatory properties that may result in neuroprotection [189–191]. The administration of EGCG to rats subjected to restraint-induced stress improved open-field and step-through behavioral tests, through a mechanism that involves the restoration of PKC α and ERK1/2 expression, which were diminished by stress [192]. Furthermore, EGCG supplementation restores the production of ATP and the expression of the peroxisome proliferators-activated receptor- γ coactivator-1 α (PGC-1 α), a key regulator of energy production. The authors concluded that EGCG-mediated protection against stress-induced neural injuries is mediated by a PKC α and ERK1/2 signaling pathway linked to PGC-1 α -mediated ATP production.

As shown in Table 1, EGCG is a rather promiscuous metal ion chelator, with affinity towards Cu, Fe, Al and Mn. Since Fe, Cu and Mn are redox-active, EGCG should decrease their redox cycling, thus decreasing the production of ROS.

In summary, EGCG has demonstrated antioxidant, anti-inflammatory and metal-binding properties that may be useful in the prevention of neuronal death. However, EGCG metal ion selectivity is poor, and high concentrations of EGCG promote oxidative damage and induce calcium upsurges that may end up in apoptotic cell death. The sum of these characteristics advises against the use of EGCG as an MFA in disease treatment.

5.2. MAO-B Inhibitor Hybrids

M30 was one of the first chelators designed with the purpose of multifunctionality, by linking the Fe³⁺ chelator 8-hydroxyquinoline to a propargyl group with MAO-B inhibition activity through a methyl–amino–methyl bridge [151]. M30 has been used successfully as a neuroprotective agent in models of PD, AD and ALS. In vivo, it is effective when given orally so most probably it crosses the intestinal and blood–brain barriers without loss of activity.

Multiple reports have shown the neuroprotective capacity of M30 in the MPTP model of PD. Since MPTP is metabolized to its active form MPP⁺ by MAO-B, and M30 inhibits the activity of MAO-B, an important caveat for the neuroprotection effect of M30 in the MPTP model is that it could be a reflection of deficient MPP⁺ formation.

Numerous effects, from antiapoptotic to neuritogenic, can be ascribed to the iron chelation capacity of M30 [193,194]. A major breakthrough was the finding that M30 activates the transcription factor HIF-1 α signaling pathway [195]. In fact, M30 stabilizes HIF-1 α , by inactivating the prolyl hydroxylase that initiates its degradation, the activity of which depends on oxygen and Fe [196]. The stabilization of HIF1 α results in the increased transcription of HIF-1 α -dependent genes, including vascular endothelial growth factor, erythropoietin, enolase-1, p21 and tyrosine hydroxylase. In addition, M30 also increases the expression levels of the transcripts of BDNF, GDNF and GAP-43 [195], and induces the expression of the antioxidant enzymes catalase, superoxide dismutase 1 (SOD1) and gGPx [152]. Immunoblot studies showed that M30 also enhances the phosphorylation of PKC, MAPK/ERK kinase, PKB/Akt and GSK-3 β [152]. VAR10303, a derivative of M30, demonstrated properties similar to M30 [154], and was successfully tested in the SOD1(G93A) mice model of amyotrophic lateral sclerosis [197,198].

Discarding an effect of the propargyl moiety of M30 on gene expression, it is possible that the remarkable properties of M30 should be common to many iron chelators with the capacity to reach the brain at therapeutic doses. Importantly, the activation of HIF1 α by iron chelators opens a wide field of expectations for iron chelation therapy, evolving from merely inhibition of ROS production, which still holds, to the putative activation of neuroregenerative pathways.

5.3. Glucose Hybrids

A novel approach in the design of MFAs was the association of metal chelator moieties to glucose, under the consideration that these agents would be preferentially incorporated into the brain given the high density of hexose transporters (GLUTs) at the blood–brain barrier [199,200]. Once in the brain, these hybrids should be taken up by neurons and astrocytes, considered the cells of major energy consumption. Since astrocytes display preferential transport and metabolism of glucose compared

to neurons [201], it would be expected that metal-hexose MFAs should preferentially accumulate in astrocytes. The hydroxypyridinone glycoconjugates H2GL¹ and H2GL² demonstrated substantial affinity for Cu²⁺ and Zn²⁺, and both agents decreased A β 1–40 aggregation induced by Cu and Zn. They also demonstrated antioxidant activity, probably through their phenolic moieties, capable of quenching free radicals by a hydroquinone/quinone conversion [155]. No in-vivo testing of these agents was reported, so their putative Fe³⁺-chelating and blood–brain barrier permeability properties remain undetermined.

5.4. Acetyl Cholinesterase Inhibitor Hybrids

Under the concept that inhibition of acetylcholinesterase (AChE) increases acetylcholine at cholinergic synapses, thus reducing the cognitive deficit [202,203], an approach for the putative treatment of AD is the design and synthesis of MFAs with AChE inhibition activity. This particular strategy is common for MFAs with a tacrine (a cholinesterase inhibitor) component [156–159] (Table 1). None of these compounds have been tested for brain permeability, although tacrine derivatives, without an iron chelator component, are brain-effective under intravenous and intranasal administrations [204]. At this stage, tests of effectiveness in animal models of AD are needed for further evaluation of their putative usefulness.

5.5. Dopamine Receptor Agonist Hybrids

19a, 19b and D-607 are dopamine D2/D3 receptor agonists and metal chelator hybrids, designed under the notion that D2/D3 receptor agonists have been used for the treatment of both motor and psychiatric syndromes in PD [179]. The three compounds demonstrated iron chelation and antioxidant capacity in vitro [180,181]. In addition, 19b partly reversed hypolocomotion in reserpinized rats, and reduced the rotational activity in a 6-OHDA/apomorphine model, thus demonstrating in-vivo neuroprotective activity [181]. D-607 was the product of a further D2/D3 agonist/chelator development by the same research group. The chelator moiety of D-607 was changed from 8-OH quinolone, a Fe³⁺ chelator, to bipyridyl, a Fe²⁺ chelator. D-607 was shown to suppress retinal degeneration in a *Drosophila melanogaster* PD model that expresses α -synuclein A30P, a PD-causing variant of the protein [180]. In addition, D-607 was shown to confer significant neuroprotection in the mouse MPTP PD model under chronic MPTP administration [180]. Overall, the published evidence points to D-607 as a putative candidate for the pharmacological treatment of PD. A drawback is that D-607 is administered intraperitoneally, and not orally, which may decrease hypothetical patient compliance.

5.6. Curcumin Hybrids

Curcumin analogs are proposed as potential anti-AD drugs, based on the radical scavenger [182,183] and metal chelator [184,185] properties of curcumin. Curcumin analogs A1–A10 were tested for their radical quenching activity and their ability to reduce metal-induced amyloid-beta aggregation [186]. A1, A2, A3 and A4 presented good radical quenching capacity in SH-SY5Y cells while the capacity of A5, A6, A7, A8, A9 and A10 was weak. All analogs, with the exception of A7, A8 and A10, presented the capacity to diminish amyloid beta self-aggregation at IC₅₀s similar to curcumin. The authors reported Fe and Cu chelation capacity by A4 based on the red shift of absorbance peaks at 267 nm and 427 nm. Nevertheless, A4 lacks the two adjacent ketone groups responsible for metal binding by coumarin [184] and it does not have putative metal binding groups in its structure, so the mechanism of iron binding is not apparent. No in-vivo studies were reported for these curcumin analogs.

5.7. Benzothiazole–3-Hydroxy-4-Pyridine Hybrids

Another approach for MFAs is the design of constructs of benzothiazole and 3-hydroxy-4-pyridine connected by a variable linker [83]. Given its hydrophobicity, benzothiazole has strong affinity for

amyloid plaques [84,85] while the 3-hydroxy-4-pyridine moiety (deferiprone) has strong Fe^{3+} chelation capacity. The linker between these two moieties was modelled in order to obtain AChE inhibitory capacity [83]. Of the tested compounds, 2a and 2d formed Fe^{3+} chelates with affinities similar to deferiprone. The chelators displayed significant antioxidant properties, with compounds 1a, 1b, 1c and 2d having significant AChE inhibitory activity. Accordingly, these same hybrids presented good inhibitory capacity towards $\text{A}\beta_{42}$ self-aggregation, mostly above 40%. In addition, hybrid 2d inhibited zinc-induced $\text{A}\beta_{1-42}$ aggregation [83]. Overall, the hydroxypyridinone-spacer-benzothiazole hybrids appear as good candidate drugs for the treatment of AD, but in-vivo testing is needed before further development.

5.8. MAO-B Inhibitors

On the basis of its moderate success in AD treatment [187], the MAO-B inhibitor selegiline has been used for the design of MFAs directed to the treatment of AD [188,189]. The most promising compounds are selegiline–cloquinol hybrids, which combine MAO-B inhibition with metal chelation capacity. Selegiline–cloquinol hybrids tested in vitro demonstrated inhibition of Cu-induced $\text{A}\beta_{1-42}$ aggregation, antioxidant activity and Cu^{2+} , Fe^{2+} and Zn^{2+} chelation capacity [189]. Like other MFAs in a proof-of-concept step, demonstration of in-vivo effectiveness is needed.

5.9. Histamine H3 Receptor Antagonists

A different approach in the design of MFAs was the design of a histamine H3 receptor antagonist, 1-phenyl-3-hydroxy-4-pyridinone, and the 3-hydroxy-4-pyridinone iron chelator moiety of deferiprone [190], under the rationale that blocking the action of presynaptic H3 receptors produces increased secretion of histamine and other excitatory neurotransmitters [191]. H3 antagonist treatment results in modest effects on cognitive function [192]. The most promising compound, 5c, displayed H3 receptor antagonistic activity, free radical scavenging capacity, copper and iron chelation, and inhibition of self- and Cu^{2+} -induced $\text{A}\beta_{1-42}$ aggregation [190]. After intraperitoneal administration to Sprague Dawley rats, compound 5c demonstrated good blood–brain barrier penetration. In conclusion, the histamine H3 receptor antagonist–iron chelator hybrid 5c is brain-permeant and possesses four functions applicable for the treatment of AD, which makes it a good therapeutic candidate.

5.10. Coumarin Hybrids

Two coumarin derivatives have been proposed as candidate drugs for the treatment of neurodegenerative conditions with an iron accumulation component [193,194], based on the known qualities of hydroxycoumarins as free radical quenchers [195] and metal chelator [196,197] agents. DHC12 is a 7,8-dihydroxycoumarin with an amino substituent group at position four of the coumarin ring [193]. The molecule is small and quite simple; nevertheless, it has interesting neuroprotective features. DHC12 exhibited metal binding capacity for Fe^{2+} and Cu^{2+} . DHC12 distributed to mitochondria, where it chelated the mitochondrial and cytoplasmic labile iron pool. In a cell model of PD, DHC12 protected cells from plasma membrane and mitochondrial oxidative damage. Oral administration of DHC12 protected substantia nigra neurons in the MPTP model of PD. On the whole, DHC12 emerges as a good candidate for further development as a PD treatment drug.

CT51 is a hybrid of 7-hydroxycoumarin linked through an acetomethyl group to tris(hydroxymethyl) aminomethane (tris). The hydroxyl group in the coumarin ring quenches free radicals [195] while the three hydroxyl residues of tris provide metal binding capacity [198]. In vitro, CT51 exhibited selective Fe^{2+} and Fe^{3+} binding with no apparent interaction with Cu^{2+} , Zn^{2+} and other divalent cations. It also demonstrated free radical quenching capacity superior to Trolox. Interestingly, cyclic voltammetry analysis revealed irreversible binding of Fe^{3+} to CT51, an important finding since stopping $\text{Fe}^{2+}/\text{Fe}^{3+}$ cycling in cells should prevent hydroxyl radical production fostered by oxygen and intracellular reductants [8]. In SH-SY5Y cells, CT51 distributed to both mitochondria and cytoplasm bound iron reversibly and protected against rotenone-induced oxidative damage, while in primary hippocampal

neurons, CT51 largely prevented the increase in intracellular calcium levels produced by an agonist of redox-sensitive RyR channels [194]. These capacities so-far demonstrated make CT51 a good therapeutic candidate for the treatment of PD, although in-vivo efficacy needs to be demonstrated.

6. Conclusions

The aging of the world population introduces an ever-increasing burden of neurodegenerative diseases on public health systems worldwide. Among these diseases, Parkinson's, Alzheimer's and other diseases with an iron accumulation component are at the top of the list. Based on initial trials using the iron chelators deferiprone and PBT2, the metalloneurobiology community has reached the conclusion that therapies targeted to decrease the iron content in specific areas of the brain is a viable course of action to slow or stop the progress of these diseases.

Given the multifactoriality of the neurodegenerative process, the use of multifunctional iron chelators is a promising developmental avenue. As discussed in the text, additional properties of future iron chelator drugs should comprise high selectivity for iron, free radical quenching capacity, mitochondrial distribution and the capacity to block protein aggregation. Several of the compounds now in experimental stages have one or more of these additional characteristics. Let us hope that further research will provide treatments that are both effective and affordable for public health systems.

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Review

Iron in Friedreich Ataxia: A Central Role in the Pathophysiology or an Epiphenomenon?

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Abstract: Friedreich ataxia is a neurodegenerative disease with an autosomal recessive inheritance. In most patients, the disease is caused by the presence of trinucleotide GAA expansions in the first intron of the frataxin gene. These expansions cause the decreased expression of this mitochondrial protein. Many evidences indicate that frataxin deficiency causes the deregulation of cellular iron homeostasis. In this review, we will discuss several hypotheses proposed for frataxin function, their caveats, and how they could provide an explanation for the deregulation of iron homeostasis found in frataxin-deficient cells. We will also focus on the potential mechanisms causing cellular dysfunction in Friedreich Ataxia and on the potential use of the iron chelator deferiprone as a therapeutic agent for this disease.

Keywords: Iron-sulfur; Friedreich Ataxia; Oxidative stress; Iron chelators

1. The Disease

Friedreich's Ataxia (FRDA) is a neurodegenerative disease described at the end of the 19th century by the German physician Nikolaus Friedreich from whom acquired the name. Friedreich observed in a group of patients that during the puberty a characteristic symptomatology began to manifest, specifically: ataxia, dysarthria, loss of sensitivity, muscle weakness, scoliosis, pes cavus, and heart symptoms. Later, a greater incidence of diabetes mellitus in patients than in the rest of the population was also reported [1]. It is considered a rare disease that follows a pattern of autosomal recessive inheritance. The frequency of carriers oscillates, depending on the area, between 1:50 and 1:100, while those affected by the disease are approximately 1:50,000, which makes this disease the most common form of hereditary ataxia [2].

The mutation responsible for the disease is an expansion of GAA trinucleotides in the first intron of the FXN or X25 gene (located in chromosome 9), which codes for the mitochondrial protein frataxin [3]. This expansion of triplets, which in patients can reach up to more than 1000 copies, results in a marked decrease in protein frataxin levels (below 5%–30% of normal levels) [4]. The number of GAA expansions has an inversely proportional relation to the age at which the first symptoms of the disease begin to manifest and it also determines their severity [5–7]. Besides GAA expansions, epigenetic modifications might also contribute to the variability in the onset and disease progression. Sarsero and collaborators reported differences in DNA methylation patterns between patients upstream and downstream the GAA expansion. Such differences caused variations in frataxin gene expression [8]. Finally, a small percentage of patients, around 4%, are compound heterozygous for a GAA expansion and a frataxin (FXN) point mutation or deletion [9].

2. Frataxin, an Ancestral Conserved Protein

Frataxin is a highly conserved protein throughout evolution and homologues can be found in most species. Its structure is formed by two helix alpha joined by a series of antiparallel beta sheets

and is highly conserved (Figure 1). Despite the high degree of conservation in the three-dimensional structure, the stability of this protein varies significantly between species. One of the factors that are responsible for the differences in the stability of the protein is the C-terminal endpoint. While in Yfh1 (the yeast's homologue), this region is virtually nonexistent, in the human protein this fragment is found inserted between the two alpha helices. This protects the hydrophobic nucleus of the protein and increases its stability [10].

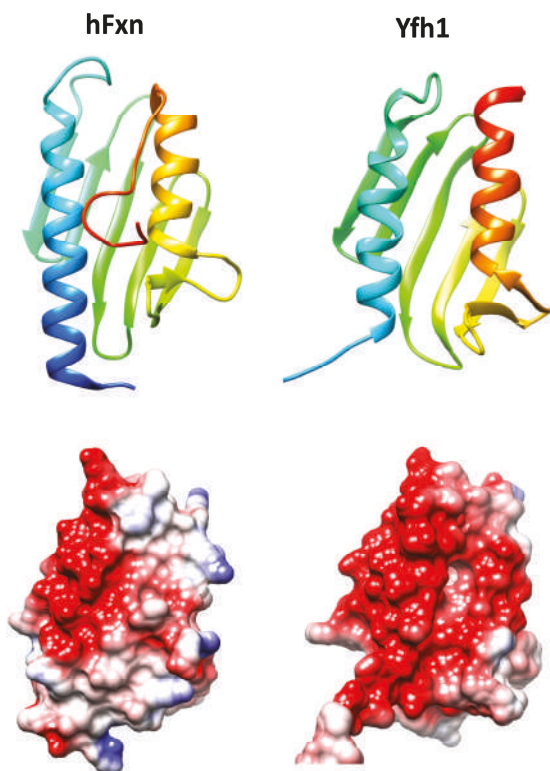


Figure 1. Structures of human frataxin (hFxn, pdb code 3s4m) and Yeast Frataxin Homologue 1 (Yfh1, pdb code 2fqj). Top, ribbons representations showing the conserved alpha-beta-alpha structure. Structures are colored according to sequence, from dark blue (N-terminal) to red (C-terminal). In human frataxin the C-terminal region folds over the hydrophobic cavity formed between both alpha helices. Below, coulombic surface coloring of the same structures. The red color indicates the presence of a marked acidic ridge, which may be involved in iron binding. Molecular graphics and analyses were performed with the UCSF Chimera package [11].

Frataxin is a mitochondrial protein, and as such, it has a signal peptide at its N-terminal end. It was soon identified, both for Yfh1 and for mouse frataxin, that Mitochondrial Processing Peptidase interacted with frataxin and was responsible for its processing [12,13]. This processing involves two sequential cleavages, which first produce an intermediate form of frataxin and subsequently the mature form. For human frataxin, the cleavage positions described are between amino acids 41–42 (intermediate form) and between amino acids 80–81 (mature form), although other less abundant forms that correspond to alternative cleavage sites (for instance, positions 55–56) have also been identified [14,15]. In these works, it was shown that the mature form of frataxin corresponds to FXN^{81–210}. This form is fully active and capable of improving the survival and phenotypes of frataxin

deficient cells. Despite the demonstration that the FXN^{81–210} form is the dominant form and that it is active, there is some debate over the role that could be developed by the intermediate forms, as these forms can also be detected in certain tissues (although at low levels) [16]. It was shown that processing of the intermediate form was slower than that of the precursor form, which suggested that this could be a mechanism for controlling the levels of the different frataxin forms [17]. More recently, it has been suggested that the different forms of frataxin might play different roles. This point will be discussed in the next section. In addition to post-translational processing, alternative splicing mechanisms have also been observed that may generate different isoforms of frataxin. Work by Xia and collaborators suggested that these alternative forms could be tissue-specific and could have different functions and locations [18]. However, there are not additional evidences about the nature and relevance of these alternative forms.

3. Frataxin Function

Although very early after the discovery of frataxin as the gene that was responsible for Friedreich's Ataxia, it was established that iron homeostasis was altered by frataxin deficiency, the specific function of this protein remains controversial. Over the years, different functions have been proposed for frataxin, most of them related to iron metabolism and the control of oxidative stress in mitochondria.

3.1. Frataxin, an Iron Binding and Storage Protein

It has been proposed that frataxin could work like a metallochaperone and iron storage protein. In several studies with purified protein, it has been observed that frataxin has the ability to interact with metal ions, but the coordination environment of these metal binding sites has not been properly defined. There is also uncertainty on the number of metal ions that are bound per frataxin monomer. It has been described that frataxin can bind divalent metal ions using a group of exposed acidic residues. These amino acids are located in a specific area of the protein forming an acidic ridge, quite conserved, different from the canonical iron binding motifs where cysteine and/or histidine amino acids are usually found. This acidic zone results in a weak and non-specific electrostatic bonding of iron, which also allows the coordination of other divalent metals [19]. The estimated Kd for Fe²⁺ and Fe³⁺ of this region was calculated on the micromolar range [20]. Gentry and collaborators reported the potential presence of a high affinity iron binding site. They showed that three metal ions could be bound by each frataxin monomer, and that His86 was required for one of these binding sites. This residue is located in the disordered N-terminal tail and had not been previously reported to be involved in metal coordination. They calculated that this site would have an affinity for Fe²⁺ higher than the iron chelator ferrozine, while the remaining two sites would have lower affinities [21]. His86 is not included in most of the frataxin structures that are found in the protein data bank nor is conserved in yeast and bacterial homologues. More recently, while using NMR to investigate iron binding, it was also proposed that frataxin tightly binds a single Fe²⁺ but not Fe³⁺ [22].

Isaya and collaborators noticed that iron binding to yeast and bacterial frataxin promoted its oligomerization to complexes of high molecular weight (850–1100 kDa) [23–25]. These oligomeric forms resembled those that were formed by ferritin, the main protein responsible for iron storage in eukaryotes. Indeed, oligomeric frataxin was shown to use a ferrooxidation reaction to build a ferrihydrite mineral core inside the particles. Therefore it was proposed that frataxin could act as a mitochondrial ferritin. Although this function would be redundant in higher eukaryotes due to the presence of a mitochondrial ferritin, this hypothesis acquired some strength when subsequent studies demonstrated that the expression of mitochondrial ferritin in frataxin deficient yeast was able to partially recover some of the observed phenotypes. Specifically, the heterologous expression of mitochondrial ferritin partially prevented the accumulation of iron, the cells were more resistant to oxidizing agents and they partially recovered the activities of enzymes with iron-sulfur centers (which is a common described consequence of frataxin deficiency) [26]. Regarding human frataxin, it has been claimed that the mature form (FXN^{81–210}) does not form aggregates [27] and that only the intermediate

forms FXN^{42–210} and FXN^{56–210} would be assembled into larger structures. Based on this observation, it has been suggested that different frataxin proteoforms would perform different functions. The mature form would be monomeric and involved in iron binding and delivery to biochemical pathways requiring this metal, while FXN^{56–210} and FXN^{42–210} would be able to oligomerize and store iron. A caveat to these hypotheses is that the long frataxin isoforms are not observed in most tissues by western blot. Mass spectrometry data also suggests that these long frataxin isoforms may be present at very low concentrations: data collected in the PeptideAtlas repository indicates that the theoretically likely frataxin peptides between positions 42 and 81 have never been observed, while those from the FXN^{81–210} have been observed at least 400 times. (PeptideAtlas is a publicly accessible compendium of peptides identified in mass spectrometry proteomics experiments) [28]. Recently, it has been shown that FXN^{81–210} can also undergo oligomerization under certain conditions, although the stability of these oligomers would be lower than that of bacterial frataxin [29].

Nevertheless, there are other caveats on the ferritin-like hypothesis. It has been argued that physiological conditions of calcium and magnesium stabilize the monomeric frataxin form and consequently frataxin would not oligomerize *in vivo* [27]. It has also been shown that mitochondrial iron in yeast strains expressing different Yfh1 concentrations, presented nearly identical chemical and biochemical characteristics [30]. Another point to take into account is which could be the contribution of frataxin to mitochondrial iron storage or trafficking from a quantitative point of view. Most iron that is present in mitochondria is found at the active sites of proteins. Despite that, non-proteinaceous labile metal iron pools have also been detected within cells. These pools are thought to be involved in cellular trafficking, regulation, signaling, and/or storage of metal ions. Due to their lability (and their presence at low concentrations), their structure and functions are not completely understood. For instance, mitochondria contain 0.7–2 mM Fe, but the proportion of labile iron is not completely known, with estimates ranging from 1 to 100 μM [31,32]. These differences in the estimates may be due to real differences between the models or experimental conditions used, but also on the methodological approaches used to analyze this elusive iron pool. Also, the nature of these nonproteinaceous metal complexes is not known. Based on the abundance of GSH within the mitochondria it has been hypothesized that could be an FeII (GSH) adduct. Citrate, which is also present in the mitochondrial matrix at high concentrations, has also been considered as a potential ligand for non-proteinaceous iron complexes (reviewed in [32]). In yeast, by using Mössbauer spectroscopy, it has been shown that the proportion and nature of these labile iron pools may vary depending on the metabolic state of the cell. Respiring mitochondria were estimated to contain $\sim 15 \mu\text{M}$ labile non-heme high spin Fe²⁺, while this pool in fermenting mitochondria increased to $\sim 150 \mu\text{M}$. The concentration of yeast frataxin in mitochondria has been estimated to be three orders of magnitude lower, around 35 nM [33]. Therefore, as monomeric frataxin has been claimed to bind three iron atoms, it cannot contribute significantly to store iron in a non-reactive easy deliverable form. However, it could play a role in iron trafficking as a temporary carrier or catalyzing its speciation between different forms. For instance, it could bind Fe²⁺ and promote its controlled oxidation to Fe³⁺, which would be then stored in the form of Fe³⁺-phosphate nanoparticles. Regarding oligomeric frataxin, the mineralization process would allow for this protein to bind much more iron atoms per subunit. The yeast 48 subunit oligomer can store $\sim 50\text{--}75$ iron atoms per subunit in 1–2 nm cores [34]. This raises the iron potentially bound by frataxin up to the μM range, but still this amount may not be a significant contribution to the whole mitochondrial iron pool. In comparison, around 35% of mitochondrial iron in fermenting mitochondria may be stored in the form of Fe³⁺ nanoparticles [33]. That said, the contribution of mitochondrial ferritin to iron storage in mitochondria is also intriguing, as the concentration of this protein according to the PaxDb database is much lower than that of frataxin (PaxDB is a database that contains protein abundance information across organisms and tissues) [35].

3.2. Frataxin in the Biosynthesis of Iron Containing Proteins

It has also been proposed that frataxin could participate in the biosynthesis of both heme groups and of iron-sulfur centers. This hypothesis has several fundamentals: (a) frataxin deficiency leads to a loss in proteins which contain iron-sulfur centers or heme groups; (b) frataxin has the ability to bind iron atoms; and, (c) several studies have shown the ability of frataxin to interact with proteins that are involved in heme or iron-sulfur biosynthesis.

3.2.1. Biosynthesis of Heme Groups

The incorporation of the iron atom into protoporphyrin IX is the last step in the biosynthesis of heme groups. This step is catalyzed by ferrochelatase, but it is not known how iron is provided to this enzyme. In *in vitro* studies, it was shown that there was a physical interaction between frataxin and ferrochelatase with a 1 to 2 stoichiometry, which seems logical, since ferrochelatase functions as a dimer. In addition, this interaction resulted in the formation of heme groups [36]. A study in which NMR spectroscopy was used to analyze the binding between both of the proteins suggested that ferrochelatase interacted with frataxin in a manner that included its iron-binding interface [37]. More recently, Söderberg and collaborators presented a model of the interaction of trimeric Yfh1 (yeast frataxin) and ferrochelatase in which one of the subunits of the Yfh1 trimer interacted with one subunit of the ferrochelatase dimer, whereas another trimer subunit was positioned for iron delivery [38]. These results support the hypothesis of frataxin acting as an iron donor in the biosynthesis of heme groups. However, heme deficiency is not always observed in frataxin-deficient cells and anemia has not been shown to be a symptom of FRDA. Indeed, no alterations were observed in heme synthesis in erythroid progenitor stem cells that were obtained from FRDA patients [39]. Moreover, experiments using either conditional frataxin-deficient T-Rex-293 cells or yeasts have shown that heme deficiency is a late consequence of frataxin deficiency [40,41]. These observations suggest that heme deficiency may be an epiphenomenon observed in certain frataxin-deficient cells which could be caused by poor iron availability or by metabolic remodeling. In this regard, we have shown in frataxin-deficient yeast that heme deficiency could be caused by the induction of Cth2, a protein induced in response to iron limitation, which promotes the degradation of mRNAs from iron-containing proteins [42].

3.2.2. Biosynthesis of Iron-Sulfur Centers

In addition to interacting with ferrochelatase, frataxin also interacts with the proteins that form the central biosynthesis machinery of iron-sulfur centers: IscU (Isu1 in yeast), Nfs1, and Isd11 [43,44]. Several authors have shown that this interaction facilitates the formation of an iron-sulfur center into IscU, the scaffold protein where these cofactors are first assembled. It was initially suggested that frataxin would act as an iron donor in the biosynthesis of these centers [45]. More recently, it has been suggested that frataxin would participate in the biosynthesis of iron-sulfur centers as an allosteric regulator and not as an iron donor. In works that were carried out *in vitro* with the CyaY protein (bacterial homologue of frataxin), it was shown that this protein had an inhibitory effect on the production of iron-sulfur centers. As this effect was increased in response to iron concentration, the authors suggested that frataxin could adapt the production of iron-sulfur to the number of final acceptor proteins [46]. Surprisingly, studies with human proteins demonstrated that eukaryotic frataxins would have a contrary effect. In this case, they stimulated the production of iron-sulfur centers by favoring the desulfurase activity of Nfs1 [47–49].

Today, despite the intense debate that is generated around the function or functions of frataxin, this non-essential activity in the metabolism of iron as an allosteric regulator of the biosynthesis of iron-sulfur centers has strong support from *in vitro* biochemical data and is the most accepted hypothesis. A detailed explanation of this complex biochemical process can be found in recent reviews [50,51]. However, this hypothesis also presents caveats when exposed to *in vivo* biological data. Remarkably, iron-sulfur cluster deficiency is not observed in several models of frataxin deficiency,

such as flies [52], patient fibroblasts [53], or rat cardiac myocytes [54]. Moreover, detailed analysis of the cellular events that are caused by frataxin deficiency in yeast, have shown that iron-sulfur deficiency is an epiphenomenon that is caused by a metabolic remodeling program activated in response to disrupted iron homeostasis [41,55]. These observations question the role of frataxin in iron-sulfur biogenesis or at least suggest the possibility of additional functions for frataxin beyond iron-sulfur biogenesis. From these observations, it also becomes obvious that frataxin is not essential for iron-sulfur biogenesis.

3.3. Control of Oxidative Stress and the Generation of Ros

One of the phenotypes most consistently observed in frataxin-deficient cells is sensitivity to oxidizing agents [56,57]. Some authors have linked such sensitivity to oxidative stress to impaired biosynthesis of iron-sulfur centers. This hypothesis suggests that a vicious cycle would be created in which the deficient formation of iron-sulfur clusters would increase mitochondrial free iron that would increase the production of reactive oxygen species (ROS) through Fenton reaction. Then, ROS would further damage iron-sulfur clusters and promote the formation of more free iron. In fact, increased presence of labile iron has been reported in frataxin deficient yeast [58], T-Rex-293 cells [40] and in a mouse model of hepatic FXN deficiency [59]. However, no differences were observed between the mitochondrial iron pools from human lymphoblasts and fibroblasts that were obtained from either controls or FRDA patients [31]. Nevertheless, some observations suggest that oxidative stress could be the cause (and not the consequence) of iron-sulfur deficiency. For instance, in fibroblasts or lymphocytes from patients [53], or in frataxin-deficient cardiac myocytes [54], oxidative stress could be observed while the activities of iron-sulfur proteins remained unaltered. Moreover, there are evidences that iron-sulfur deficiency can be modulated by oxygen concentration or antioxidant treatment. In this regard, frataxin is not required for iron-sulfur biogenesis in yeasts grown at low oxygen tensions [60,61]. In some fly models, iron-sulfur deficiency is only observed under hyperoxic conditions [52], while in other models it can be prevented by antioxidants [62].

Which could be the origin of oxidative stress? Since frataxin has the ability to bind iron, a redox active metal, and oxidize it to Fe^{3+} , it has been proposed that frataxin could prevent oxidative stress by limiting the presence of free Fe^{2+} through its binding and the subsequent controlled oxidation to Fe^{3+} . This reaction would prevent the formation of reactive oxygen species through the reaction of Fe^{2+} with oxygen [63,64]. Therefore, the vicious cycle would have its origin in free iron than would then generate oxidative stress that would damage iron-sulfur clusters and generate more free iron. Another potential source of reactive oxygen species in frataxin-deficient cells could be the OXPHOS system. In this regard, an interaction was described between frataxin and mitochondrial electron transport chain proteins [65]. Also, decreased activity of the mitochondrial electron transport chain has been observed in several biological models of frataxin deficiency [53,66,67]. Any alteration in the OXPHOS system that was caused by frataxin deficiency could increase electron leakage and thus generate ROS [68].

Rustin and collaborators observed that frataxin-deficient cells could not properly activate the NRF2 signaling pathway in response to oxidative damage and in consequence they had a deficient response to oxidative insults and hypersensitivity to oxidative stress. They hypothesized that this impairment was related to actin remodeling [69]. This phenomenon has also been described in frataxin-deficient motor neurons [70], and in the frataxin-deficient YG8R mouse model where transcriptomic analysis showed a downregulation of NRF2-dependent antioxidant enzymes [71].

4. Evidences of Iron Accumulation and Its Relation to Pathophysiology in FRDA

Iron accumulation in a frataxin deficient cell model was first described in yeast *yfh1* mutants [72]. This early observation has been subsequently confirmed by several other researchers. Using Mössbauer spectroscopic analysis, Dancis and collaborators showed that in *Dyfh1* mitochondria iron was present as amorphous nano-particles of ferric phosphate [73]. Iron accumulation is caused by increased iron uptake due to activation of the iron sensor Aft1 [58], but the mechanisms leading to such activation are

not completely understood. It has been assumed that it would be caused by iron-sulfur cluster loss, as Aft1 is known to be regulated by the presence of these cofactors. However, previous research from our group using conditional Yfh1 mutants provided two observations that challenged this hypothesis: (i) activation of Aft1 was observed earlier than iron-sulfur loss [41]; and, (ii) loss of iron-sulfur containing proteins in Yfh1 deficient yeasts was not observed in *cth2* cells. Therefore iron-sulfur loss was an epiphenomenon mainly caused by Cth2, which is an Aft1 target that binds to mRNAs from iron-containing proteins and promotes its degradation [42]. Moreover, we have also observed that nitric oxide can prevent Aft1 activation in Yfh1-deficient cells, but not in cells that are deficient in iron-sulfur biogenesis [74]. This observation also indicates that in Yfh1 deficient yeast Aft1 may be activated by a mechanism different than iron-sulfur cluster deficiency. Besides yeast, iron deposits or accumulation have also been clearly observed in frataxin deficient flies [75,76] and in cardiac muscles from frataxin deficient mice [77] and FRDA patients [78]. Iron in the heart from cardiac KO conditional mouse (the MCK mutant) was found in mitochondrial aggregates 100–400 nm in diameter, markedly different from those observed for mammalian ferritin. Energy-dispersive X-ray analysis showed that, in addition to iron, phosphorus and sulfur were present in these aggregates. Mössbauer spectra also confirmed that these aggregates were different than mammalian ferritin. The absorption profile observed was consistent with paramagnetic high-spin Fe(III) [79]. These observations are consistent with those that were obtained in frataxin-deficient yeast, and suggest that iron could be in the form of ferric-phosphate nanoparticles in both models. In other tissues or mammalian cell types, iron accumulation is not consistently observed. For instance, in fibroblasts or lymphoblasts from patients, there are no evidences of iron accumulation [31], while some authors have observed it in the nervous system [80]. Changes in the iron-responsive proteins, ferritin, divalent metal transporter 1 (DMT1), transferrin receptor 1 (TfR1), and ferroportin have been reported in the dentate nucleus of affected individuals [81]. Similar to yeast, iron deregulation in mammals might be caused by Iron-responding protein 1 (IRP1) activation [82,83], but the mechanisms causing this activation are not completely understood. It could be caused by deficiencies in iron supply to mitochondrial iron-dependent pathways that would activate the mechanisms of response to iron deficiency [59]. Frataxin has also been shown to interact with IRP1 and modulate the switch between its aconitase and RNA-binding forms. This function would be carried on by a cytosolic form of frataxin [84]. However, some authors are skeptical about the existence of an extra mitochondrial form of frataxin, and therefore question the physiological relevance of the observed interaction between IRP1 and frataxin.

As indicated above, many evidences support that frataxin deficiency causes a dysregulation in iron homeostasis, and it has also been shown in several models that the modulation of iron homeostasis ameliorates several phenotypes caused by frataxin deficiency [74,85]. However, the contribution of iron accumulation to the pathophysiology of FRDA has not been clearly determined. In this regard, several hypotheses have been formulated. It has been proposed that iron accumulation would be toxic and could be contributing to the formation of reactive oxygen species through the Fenton reaction. Iron overload could be also inducing the synthesis of sphingolipids, which, through the Pdk1/Mef2 pathway, would trigger neurodegeneration [76,80]. Iron toxicity could be also related to the formation of iron-phosphate aggregates that would compromise phosphate availability [86]. Nevertheless, it has also been suggested that iron accumulation would not be toxic per se, and that pathological consequences of frataxin deficiency would be mostly related to deficient iron supply to iron-dependent proteins. In this regard, it has been shown that IRP1 activation has a protective effect in a mouse model of hepatic FXN deficiency, as it contributes to sustain mitochondrial iron needs and mitochondrial function in these mice [59]. It has also been observed that dietary iron supplementation limits cardiac hypertrophy in MCK mutant mice [79].

These contradictory observations suggest that the pathological mechanisms could be more complex and specific for different models and tissues. For instance, in yeast, we have observed that activation of Aft1 causes the overexpression of Cth2, an mRNA binding protein that downregulates the expression of most iron-binding proteins that are required for aerobic growth. Thus, the activation

of such pathway has a strong contribution to the alterations observed in yeast [42]. Beyond yeast, there are several evidences that frataxin deficiency may be causing perturbations in signaling pathways that could contribute to pathology. For instance, as mentioned before, neurodegeneration has been linked to the activation of the Pdk1/Mef2 pathway [76,80]. Cardiac hypertrophy could be related to the activation of the NFAT/calcineurin pathway, which has been observed in rat frataxin deficient cardiac myocytes [87]. Therefore, pathophysiology could be related to the pathways activated in different cells and tissues in response to the perturbations caused by frataxin deficiency.

Besides iron, some authors have reported deregulation of the homeostasis of other metals as a consequence of frataxin deficiency. In frataxin deficient yeast, we observed a decrease in manganese content and limited copper availability [58,88]. Subsequent studies using a conditional frataxin mutant indicated that manganese deficiency was caused by downregulation of Smf2, a Mn transporter that was degraded in response to iron accumulation [41]. In frataxin deficient flies it was found that the levels of zinc, copper, and manganese were increased, and that copper and zinc chelation improved the impaired motor performance of these flies [89]. In Dorsal Root Ganglia from FRDA patients, zinc and iron related proteins displayed major shifts in their cellular localization [90]. Alterations in calcium homeostasis have also been reported in several models of FRDA [87,91]. These alterations are mostly considered to be consequences of the deregulation of iron homeostasis, which may impact other metals. Nevertheless, frataxin is known to have also the capacity to chelate metals that are different than iron, such as manganese or copper [92]. The biological significance of these interactions has not been explored yet.

5. Targeting Iron as a Therapeutic Approach in FRDA

There is currently no cure for FRDA but several therapeutic approaches are being investigated. Some drugs have already entered clinical trials. Briefly, therapeutic approaches can be divided into compounds that improve mitochondrial function and reduce oxidative stress, drugs that modulate the altered metabolic pathways, and strategies to increase the expression or content of frataxin, either by promoting its expression, by supplying it through gene therapy (reviewed in [93]) or by protein replacement strategies [94].

Due to the alterations that were observed in iron homeostasis in different models and patients of FRDA, the use of iron chelators as a treatment to eliminate the excess iron accumulating in mitochondria was proposed many years ago. Deferoxamine was not considered to be a suitable iron chelator for depleting the intracellular iron deposits found in FRDA, as it does not cross the blood brain barrier and poorly penetrates biological membranes. It also has a high affinity for iron, which could compromise iron availability. The proposed alternative was deferiprone, an orally administered, lipidsoluble iron chelator that had been previously used to treat iron overload in polytransfused individuals with hemoglobinopathies. This compound can easily cross the blood–brain barrier and cellular membranes and therefore reach intracellular (or mitochondrial) iron deposits. In addition, since its affinity for iron is lower than that of transferrin, it has been shown that it can redistribute iron from intracellular iron deposits to this protein [95]. Indeed, the cellular properties that are affected by frataxin deficiency in HEK-293 cells were corrected by deferiprone treatment [96].

Several clinical trials have been performed with deferiprone in FRDA patients. In summary, these studies suggested that low doses of deferiprone would be beneficial on cardiac parameters. Higher doses of the drug worsened the condition and could result in agranulocytosis (reviewed in [97]). We can speculate that this dose dependent effect could be a consequence of different pathological mechanisms that are exerted by frataxin deficiency. Some of them would be caused by iron accumulation, while others would be caused by deficient iron availability. Therefore, low doses of the chelator would partially prevent the toxic effects that are caused by iron accumulation, while higher doses of deferiprone would compromise iron availability, and therefore worsen those pathological conditions caused by inefficient iron availability. Nevertheless, it has been proposed that deferiprone at low doses could be combined with other drugs. A pilot study with five FRDA patients suggested

that combined therapy of deferiprone and idebenone (a Q10 analogue) was relatively safe and it could provide some benefits on neurological function and heart hypertrophy [98].

6. Concluding Remarks

Many evidences indicate that the lack of frataxin leads to alterations in iron cellular homeostasis. However, the precise mechanism(s) causing iron deregulation in frataxin-deficient cells are not completely understood. Several hypotheses have been formulated, but although most of them are well supported by *in vitro* data, all of them present caveats when exposed to biological data. In Figure 2, we have summarized two potential mechanisms which in our opinion could explain iron accumulation and oxidative stress: (1) the iron-sulfur hypothesis proposes that frataxin contributes to iron-sulfur biogenesis and its deficiency activates cellular iron sensors that would promote iron uptake; and, (2) the iron toxicity hypothesis assumes that frataxin would be involved in controlled iron ferrooxidation, and its deficiency would lead to ROS generation and the increased formation of ferric-phosphate nanoparticles. The iron-sulfur hypothesis is well supported by *in vitro* data, but its major caveat is the absence of iron-sulfur deficiency in many models of frataxin deficiency. On the other hand, the iron toxicity hypothesis does not provide a clear explanation for the activation of iron sensors.

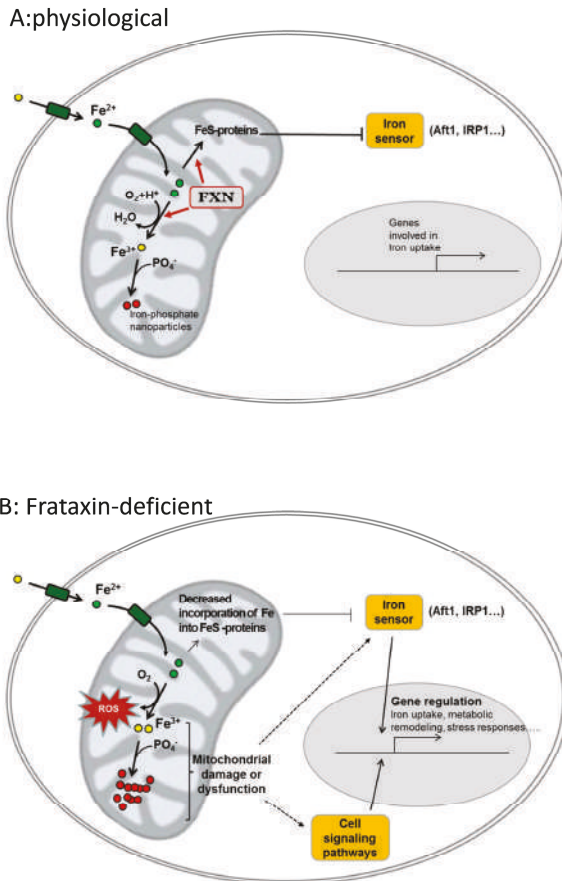


Figure 2. Potential contribution of frataxin to iron homeostasis and cellular consequences of its deficiency. (A), physiological: frataxin (FXN) binds Fe²⁺ and contributes to its controlled oxidation to

Fe³⁺ and/or to incorporate it into Fe-containing proteins. These Fe-containing proteins (notably FeS proteins) keep the iron sensor inactive and genes involved in iron uptake are not expressed. Oxidized iron (Fe³⁺) is stored in the form of ferric-phosphate nanoparticles. (B), frataxin-deficient: loss of frataxin leads to decreased incorporation of iron into Fe-proteins and/or uncontrolled oxidation of Fe²⁺ by O₂. Such events lead to reactive oxygen species (ROS) generation, decreased phosphate availability, and mitochondrial dysfunction. Iron sensors and other cell signaling pathways are activated and regulate the expression of genes involved in iron uptake and/or other cell-specific pathways involved on metabolic remodeling, hypertrophy or neurodegeneration.

Also, it is not clear the contribution of iron to FRDA pathology, which could be related either to iron accumulation or to limited iron availability. Indeed, iron homeostasis deregulation could be an epiphenomenon that is not linked to pathology. In fact, many evidences suggest that the mechanisms causing cellular dysfunction could be tissue or model specific. They could also be related to the signaling pathways activated in response to the alterations that are caused by frataxin deficiency. This complexity may explain the limited effects of iron chelators on clinical trials, as these compounds would only prevent certain pathological mechanisms in a limited number of tissues.

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Review

Cellular Senescence and Iron Dyshomeostasis in Alzheimer's Disease

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Abstract: Iron dyshomeostasis is a feature of Alzheimer's disease (AD). The impact of iron on AD is attributed to its interactions with the central proteins of AD pathology (amyloid precursor protein and tau) and/or through the iron-mediated generation of prooxidant molecules (e.g., hydroxyl radicals). However, the source of iron accumulation in pathologically relevant regions of the brain and its contribution to AD remains unclear. One likely contributor to iron accumulation is the age-associated increase in tissue-resident senescent cells that drive inflammation and contribute to various pathologies associated with advanced age. Iron accumulation predisposes ageing tissue to oxidative stress that can lead to cellular dysfunction and to iron-dependent cell death modalities (e.g., ferroptosis). Further, elevated brain iron is associated with the progression of AD and cognitive decline. Elevated brain iron presents a feature of AD that may be modified pharmacologically to mitigate the effects of age/senescence-associated iron dyshomeostasis and improve disease outcome.

Keywords: Alzheimer's disease; iron homeostasis; ferroptosis; senescence; chelators

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia. Pathological hallmarks of AD are the accumulation of extracellular amyloid plaques seeded by aggregated amyloid beta peptide (A β) and intracellular neurofibrillary tangles (NFTs) composed of hyper-phosphorylated microtubule-associated protein tau. The accumulation of A β is considered a toxic component of pathology and has been a primary target of clinical strategies [1]. However, strategies that have focused on reducing A β burden, including those that have demonstrated the lowering of plaque burden to normal levels, have not been successful in slowing cognitive decline in AD patients [1–3]. A careful re-examination of the factors that may lead to AD and contribute to cognitive decline may facilitate the formulation of new therapeutic strategies to prevent or arrest disease processes. Homeostatic regulation of iron is one such pathway amenable to therapeutic targeting, and it has been observed to be perturbed in several neurodegenerative disorders in addition to AD [4].

Iron is essential for life processes and cellular functions. These include essential “housekeeping” functions such as cellular respiration, DNA synthesis, and cell division, as well as specialized cellular functions such as oxygen transport and neurotransmission [4–6]. The ability of iron to cycle through its oxidation states is fundamental to its biological utility but can lead to oxidative damage of biomolecules resulting in cellular dysfunction [4–6]. This has led to the evolution of tightly regulated homeostatic mechanisms to ensure iron availability and mitigate toxicity [4–6]. However, the brain accumulates iron with age and several neurodegenerative conditions are associated with increased iron levels in affected regions of the brain [4,5].

The cause of age-associated iron accumulation in brain regions relevant to AD and its impact on disease are relevant questions to determine the utility of brain iron redistribution as a therapeutic

strategy for AD. In this review we explore the contribution of iron to AD and describe the potential contribution of the proinflammatory senescence program to brain iron accumulation (Figure 1). Notably, while iron accumulation in AD may not be sufficiently high to result in iron toxicity [6], iron dyshomeostasis and elevated iron predisposes and enhances the susceptibility of brain tissue to oxidative dysfunction (e.g., lower glutathione, increased lipid peroxidation, and increased reactive oxygen species) and accelerates cell death modalities such as ferroptosis (reviewed in [4]). Finally, we describe the therapeutic opportunities that may be explored to alleviate AD through pharmacological chelation of iron in the brain.

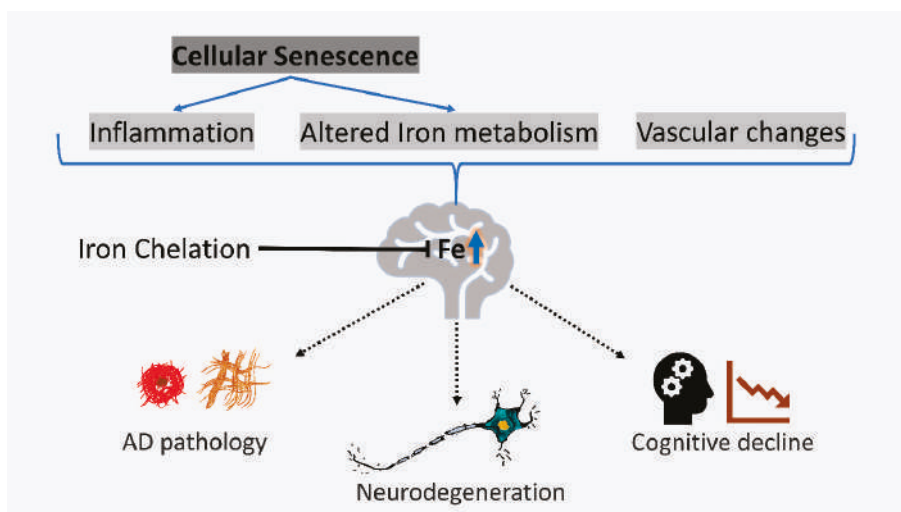


Figure 1. Cellular senescence is a potential contributor to the age-associated accumulation of brain iron. Factors that influence brain iron with age include inflammation, altered vasculature, and altered metabolism. Elevated brain iron is associated with Alzheimer’s disease (AD) pathology, cognitive decline, and may lead to neuron loss via iron-dependent oxidative cell death such as ferroptosis. Iron chelation may mitigate some of these effects and alleviate AD progression.

2. Iron Dyshomeostasis is Associated with AD

In the brain, iron accumulation is observed in regions affected by AD such as the parietal cortex, motor cortex, and hippocampus [7–15]. The intensity of iron accumulation, observed by histology, in the frontal cortex is different between the subtypes of AD. This can be used to distinguish between sporadic (late onset) and familial (early onset) AD [16] and reflects disease severity [17,18]. Overall, patients with familial AD are affected more in magnetic resonance imaging (MRI) scores compared to patients with sporadic AD, which may reflect higher iron accumulation in familial AD [16]. Further, the forms of iron observed in AD patients vary in their magnetic moment (measured via superconducting quantum interference device magnetometry) when compared with those from age- and gender-matched controls [19]. Magnetic moment is the property of a substance that determines the torque it experiences when influenced by an external magnetic field. Thus, change in magnetic moment reflects a change in the molecular state of iron and indicates possible dysregulation of iron homeostasis [19]. For example, a higher magnetic moment in AD brain tissue vs. control tissue in the absence of changes in the concentration of magnetite (an oxide of iron that can be magnetized) may indicate the larger size of magnetite particles in AD brains. This in turn suggests a dysfunction of iron storage in ferritin (iron storage protein) and/or accumulation of A β in AD tissue [19]. Further, iron levels measured post-mortem are elevated in the inferior temporal cortex only in patients diagnosed with AD during their lives, with AD pathology confirmed post-mortem [20].

Iron is associated with the pathological lesions of AD [17,21–29]. Some studies implicate iron as a direct contributor to AD pathology by promoting the aggregation and oligomerization of A β peptides [17,26,28–33]. Levels of iron and ferritin (iron storage protein) in brain tissue are associated with the amount of amyloid deposition [17,34,35]. Iron is accumulated in amyloid plaques as a mineralized magnetite species in mouse and human models of amyloid deposition [26–29]. A β may enhance iron mineralization as A β peptides can lead to the production of iron-mineral nanoparticles in vitro [36]. It is hypothesized that the oxidative damage associated with the aggregation of A β is due to redox active metals (e.g., iron and copper) to which it binds, leading to the production of oxidants such as hydrogen peroxide [37,38].

Iron may impact the production of A β by enhancing the translation and amyloidogenic processing of amyloid precursor protein (APP) [39–45]. APP, when processed through a non-amyloidogenic pathway, is cleaved by α -secretase followed by cleavage by γ -secretase. Iron availability may perturb this process through the aberrant binding of iron response proteins to putative iron response elements on APP mRNA [43–45]. Additionally, iron can mediate tau phosphorylation and aggregation [46–48]. These events can be mitigated by the chelation of iron [49]. Tau accumulation in NFTs is associated with an induction of heme oxygenase-1 which can exacerbate oxidative stress through the release of iron by the breakdown of heme [45,50–52].

Brain iron and ferritin are associated with cognitive loss in AD. Iron was strongly associated with the rate of cognitive decline 12 years prior to death in the subjects from the Memory and Ageing Project ($n = 209$) [20]. While the direct measurement of brain iron is challenging, ferritin levels in cerebrospinal fluid can be used as a proximate reporter of brain iron load. While brain iron levels are reflected in CSF (cerebrospinal fluid) ferritin, they may also be impacted by the inflammation status of the brain. Regardless, CSF ferritin can predict cognitive decline and the transition from mild cognitive impairment to AD [53]. Ferritin in the CSF predicts the rate of decline in brain metabolism (proximate indicator of neurodegeneration), as measured by fluorodeoxyglucose positron emission tomography (FDG-PET) in subjects with high amyloid pathology (high CSF t-tau/A β 42 ratio) but not in subjects with low amyloid pathology [54]. Another longitudinal study conducted over six years determined that elevated magnetic susceptibility in the hippocampus, determined by an MRI technique called quantitative susceptibility mapping (QSM), is a strong predictor for an accelerated rate of cognitive decline in amyloid positive subjects [55]. QSM may also be used to longitudinally and non-invasively monitor amyloid accumulation and iron deposition, and may serve as a diagnostic aid for AD [35]. Taken together, these studies suggest that iron is important for cognitive deterioration when there is underlying pathology.

3. Cellular Senescence is Associated with AD and Iron Dyshomeostasis

Cellular senescence is a proinflammatory cell fate associated with several age-related disorders, including AD [56–58]. The senescence phenotype is classically described in cultured cells undergoing terminal replicative arrest [59] that display enlarged cell morphology and characteristic alterations of their chromatin, secretory profile (senescence-associated secretory phenotype (SASP)), and cell cycle regulatory proteins (cyclins and cyclin-dependent kinases) [60]. Senescence in culture is typically induced through sub-culturing to replicative exhaustion, inducing DNA damage (through oxidative stress, ionizing radiation, or pharmacological agents), or aberrant oncogenic activation (e.g., overexpression of *Hras*^{V12}) [60]. The occurrence of senescent cells in tissue is now widely accepted [61–65] and several functions have been ascribed to them, including involvement in wound healing [66,67], tissue repair [68], and embryonic development (developmental senescence) [69,70]. Despite these beneficial roles, the persistent accumulation of senescence in tissue with age is associated with age-associated pathologies and functional decline. The clearance of senescent cells from tissue in mice can alleviate pathologies related to ageing [56–58,71–81]. While the overall burden of senescent cells in ageing tissue is low, these cells persistently propagate degenerative and proinflammatory conditions in their microenvironment [56–58,71–83]. In the brain the senescence program can be

triggered in astrocytes and microglia, with recent evidence suggesting that even neurons may display a senescent signature despite being post-mitotic. A more flexible definition of senescence based on the outcomes of senescent cell phenotypes (e.g., chronic inflammation) may be relevant to explain age-related pathologies *in vivo*. As the molecular changes associated with normal ageing that promote AD are yet to be fully defined, the impact of cellular senescence on age-related neuroinflammation, decline in cognition, and AD is currently unknown [75].

Evidence for the induction of senescence in cells of the brain and its links with neurodegenerative disorders is steadily increasing. In cell culture, astrocytes exposed to hydrogen peroxide (oxidative stress) or irradiation display markers of senescence, such as senescence-associated β galactosidase staining (SA β gal) (visualized by enzymatic activity assay for lysosomal β galactosidase at pH 6.0) and elevated expression levels of p16^{INK4A} and p21 (measured via quantitative PCR for the increase in transcript levels or Western blotting to reflect changes in protein abundance), and develop a proinflammatory secretory phenotype similar to the SASP observed in senescent fibroblasts [84–86]. Similarly, irradiated neuronal cells show features of senescence (SA β gal positivity) and become susceptible to the senolytic pharmacological cocktail of dasatinib (kinase inhibitor anti-cancer drug) and quercetin (plant-derived flavonoid) [58]. In human brain tissue an increase in the burden of p16^{INK4A}-expressing astrocytes is observed with age [87]. Cellular senescence in neuronal progenitor cells is implicated in the reduced remyelination observed in progressive multiple sclerosis [88]. In mouse models, senescence in the brain has been observed in response to physiological (e.g., obesity-induced [89]) and physical (e.g., traumatic brain injury following controlled cortical impact [90]) stressors.

In the context of AD, astrocytes expressing p16^{INK4A} are enriched in the frontal cortex of AD patients compared to age-matched non-AD adults [87]. Further, oligodendrocyte progenitor cells displaying a senescent phenotype (high p21 expression) are associated with amyloid plaques in the brains of human AD patients [58]. Recently, studies using mouse models that overexpress mutant tau and phenocopy aspects of AD have indicated a strong link between the induction of cellular senescence and the appearance of AD pathology [56,57]. In particular, the ablation of senescent cells from the tissue of these mice led to a reduction in the phosphorylation and aggregation of tau [56,57]. In another mouse model of AD (APP/PS1), senolytic treatment, using a combination of dasatinib and quercetin that reduced the burden of senescent cells associated with amyloid plaque, lowered A β load, reduced neuroinflammation, and reduced cognitive defects [58]. Interestingly, A β can induce senescence in cultured oligodendrocyte progenitor cells [58] and drive SASP in cultured epithelial cells and fibroblasts via CD36 [91]. This may indicate that AD pathologies can further sustain/enhance tissue-resident senescence burden. Taken together, these studies suggest that senescence induction occurs in the brain and is associated with AD.

While the clearance of senescent cells in tissues of mice has now been demonstrated to mitigate age-associated pathologies, including AD, the impact of removing them in tissues of longer-living mammals, including humans, is yet to be determined. A small ($n = 14$) first in-human clinical trial of the senolytic cocktail of dasatinib and quercetin in idiopathic pulmonary fibrosis (IPF), a fatal cellular senescence-associated disease [76], provided no conclusive evidence of senescence clearance or reduction in SASP despite achieving its primary endpoints (retention and completion rate; both 100%) and some benefit in the physical function of patients [92]. Considering the known functional benefits of senescence in wound healing and tissue repair, as well as other potential benefits that may be currently unknown, the life-long clearance of senescent cells in humans may be deleterious. Further, therapeutic strategies based on the complete clearance of senescent cells may be difficult to administer in disorders like AD where the disease develops over several decades. An alternative strategy is to target specific phenotypic features of senescent cells that are relevant in certain disease settings. Iron accumulation is one such feature that is observed in senescent cells and is of relevance in AD [82].

Senescent cells *in vitro* display aberrant iron homeostasis [82], and there are some indications that their abundance influences iron levels in ageing tissue [82,93,94]. Senescent cells display elevated

iron and a concomitant increase in ferritin and markers of oxidative stress in vitro [82,93–95]. Iron is known to promote the induction of senescence in cultured microglia [96]. Iron chelators such as deferoxamine and deferiprone can reduce and prevent the accumulation of iron and ferritin observed in cellular senescence in vitro [82]. The chelation of iron in *Caenorhabditis elegans*, a model organism of ageing that displays iron dysfunction in senescent intestinal cells [97], leads to a reduction in iron-dependent oxidation and cell death [98]. In the context of brain tissue, SASP may drive ferritin expression in neurons and glia as an acute phase response which may enhance their susceptibility to the iron-mediated cell death process, ferroptosis. The effects of iron chelation on senescence-associated iron accumulation and its impact on SASP or ferroptotic vulnerability are yet to be explored in vivo and present a therapeutic opportunity to treat AD (Figure 1).

4. Iron as a Therapeutic Target in AD

Iron neurochemistry as a modifiable feature to treat AD has generated renewed interest since clinical trials for iron chelators have shown promise recently in other neurodegenerative disorders such as Parkinson's disease (PD) and motor neuron disease (MND) [6,99–104]. The efforts towards the “iron hypothesis” of AD have also been bolstered by the unravelling of complex molecular crosstalk between iron regulatory proteins and the suspected players of AD pathology.

Historically, the first study that explored iron chelation against AD was published in 1991, which tested the effectiveness of intramuscular application of the iron chelator deferoxamine in 48 patients over two years [105]. The study showed that low-dose administration of this iron chelator slowed the clinical progression of dementia associated with AD compared with controls. A decade later, a pilot phase 2 clinical trial in patients with moderately severe AD using clioquinol (PBT1), a drug inhibiting zinc and copper ions from binding to A β , was conducted on 36 patients [106]. A positive clinical effect, corresponding to a reduction in the rate of cognitive decline, was seen in the more severely affected patients. Moreover, a biological effect corresponding to a decline in plasma A β 42 levels was observed. Currently, the safety and efficacy of deferiprone, an iron chelator that passes the blood–brain barrier, is under evaluation in a phase 2 randomized placebo-controlled clinical trial in participants with prodromal AD and mild AD (NCT03234686).

5. Conclusions

Despite the ever-increasing socioeconomic burden of AD, there is frustratingly no disease-modifying treatment for this affliction. The historic and emerging evidence that iron contributes to the clinical progression of AD should not be ignored as a potential avenue for therapy development. The reported benefits for iron chelation in other neurodegenerative diseases such as PD and MND should open the possibility of pharmacological manipulation of brain iron as an alternative therapeutic approach for AD. The perturbed iron homeostasis observed in senescent cells presents a possible cell-specific target of iron chelation therapy which may enhance the efficacy of approaches aimed at lowering age/pathology-related iron accumulation. Further, the strategy to modify specific phenotypic features (e.g., reducing iron accumulation using chelators such as deferiprone) of senescent cells that contribute to pathology may be of benefit in the human context where the complete ablation of senescent cells from tissue may be difficult or even deleterious due to a loss of beneficial effects associated with senescence (e.g., tissue repair and wound healing). The consequence of cellular senescence on the iron homeostasis of the brain requires further characterization. This may open additional avenues for the development of new classes of drugs that may provide benefits for AD patients and provide strategies for halting their decline and delaying the onset of neurodegeneration.

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Review

Brain Iron Homeostasis: A Focus on Microglial Iron

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Abstract: Iron is an essential trace element required for important brain functions including oxidative metabolism, synaptic plasticity, myelination, and the synthesis of neurotransmitters. Disruptions in brain iron homeostasis underlie many neurodegenerative diseases. Increasing evidence suggests that accumulation of brain iron and chronic neuroinflammation, characterized by microglia activation and secretion of proinflammatory cytokines, are hallmarks of neurodegenerative disorders including Alzheimer's disease. While substantial efforts have led to an increased understanding of iron metabolism and the role of microglial cells in neuroinflammation, important questions still remain unanswered. Whether or not increased brain iron augments the inflammatory responses of microglial cells, including the molecular cues that guide such responses, is still unclear. How these brain macrophages accumulate, store, and utilize intracellular iron to carry out their various functions under normal and disease conditions is incompletely understood. Here, we describe the known and emerging mechanisms involved in microglial cell iron transport and metabolism as well as inflammatory responses in the brain, with a focus on AD.

Keywords: Alzheimer's disease; neuroinflammation; neurodegeneration; cytokines; neuroimmune responses

1. Introduction

The brain is among the most metabolically active organs in the body and accounts for at least 20% of the body's energy consumption. Accordingly, an adequate supply of iron is necessary to sustain its high-energy needs [1–4]. Our understanding of the role of iron in normal brain function has improved tremendously over the last decade, with much attention directed towards deciphering the cellular and molecular cues that guide brain iron transport and metabolism. These efforts have described the essential roles of iron as a co-factor for several physiological processes including oxidative metabolism, myelination, and the biosynthesis of neurotransmitters [5–7]. However, excess iron is known to contribute to homeostatic dysregulation due to oxidative stress and has been linked to a number of neurological disorders. Being redox active, iron exists in both ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms and constantly cycles between the two states. Under aerobic conditions, this redox cycling has the potential to generate highly reactive free radicals through Fenton chemistry, resulting in oxidative stress and damage to macromolecules. Thus, the metal is directly implicated in the disease known as neurodegeneration with brain iron accumulation (NBIA), and, in addition to other trace elements implicated in neurodegeneration, including copper [8], manganese [9], and zinc [10], increasing evidence support iron's role in several other sporadic or genetic neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) [11–14].

Microglia make up 5 to 12% of the population of cells found in mouse brain and about 0.5 to 16% of those in the human brain [15,16]. These resident macrophages are largely involved in immune responses and, depending on the stimuli, they can adopt a range of pro- or anti-inflammatory states

to help maintain the integrity of the neural environment [17–19]. In addition to their roles in the neuroinflammatory response, microglia participate in neurogenesis [19,20], shaping and maintaining synaptic density and connectivity in the adult and developing central nervous system (CNS) [16,21–24], oligodendrocyte differentiation [25], synaptic pruning [26], and myelin repair [16]. Microglia require iron as a co-factor to carry out all of these varied functions [27]. Over the years, multiple studies have reported the roles these immune cells play in brain iron homeostasis [1,27,28]. This review will examine the influence of brain iron on microglial metabolism and corresponding inflammatory responses under normal and neurodegenerative conditions, with a particular focus on AD.

2. Brain Iron

Brain iron levels are tightly regulated to ensure the normal function of the CNS [29,30]. The major route of iron acquisition begins with intestinal absorption, as dietary Fe^{3+} is reduced to Fe^{2+} by duodenal cytochrome B (DcytB) at the apical surface of enterocytes [31]. Divalent metal transporter-1 (DMT1) imports Fe^{2+} into the intestinal cells, while the iron exporter ferroportin (Fpn) mediates its exit across this epithelial barrier. On the serosal side, the multicopper ferroxidases ceruloplasmin and/or hephaestin oxidize Fe^{2+} to Fe^{3+} , thereby promoting its binding to the iron carrier protein transferrin (Tf) [32]. Dietary absorption of iron is tightly regulated to respond to the body's iron needs, such that uptake is enhanced by iron deficiency but reduced under iron-loading conditions [29]. Thus, iron supplied to the brain from the diet reflects nutrient demands, while limiting the potential for excessive accumulation.

Once in the circulation, the entry of iron into the brain from the blood is controlled by the blood–brain barrier (BBB) [33]. The BBB is formed by brain microvascular endothelial cells (BMVECs), pericytes, and astrocytes [33–35]. Tf-bound iron circulating in the blood outside the CNS cannot cross the BBB directly, and, therefore, iron must enter the brain through BMVECs in a multi-step transcellular pathway. Binding of Tf to Tf receptors (TfR) at the lumen of the brain microvasculature facilitates iron uptake via receptor-mediated endocytosis [30,34,36]. The subsequent fate of the Tf–TfR complex within brain endothelial cells is not entirely clear, and exactly how iron is released to the brain remains controversial. The transcytosis model suggests that the ligand–receptor complex traverses the cell, such that Tf is released to the interstitium. However, how Tf might dissociate from its receptor at the abluminal membrane remains unexplained. An alternative model is that iron is released to the cytoplasm of BMVECs after receptor-mediated endocytosis of Tf. The endocytic uptake pathway for iron is much better understood and involves the release of Fe^{3+} from Tf in the acidic endosomal environment, its reduction to Fe^{2+} , and DMT1-mediated export from the endosome [29]. However, whether BMVECs express DMT1 or if its function is required for entry of iron into the brain is unclear, since different groups have reported conflicting data [37–42]. An alternative membrane transport mechanism could involve transient receptor potential mucolipin-1 (TRPML1) channels which function in the release of iron from endolysosomal compartments [43]. A recent study has shown that loss of TRPML1 in mice promotes dysregulation of brain homeostasis and decreased myelination, suggesting a potential role in brain iron uptake [44]. Regardless of which transporter is responsible for iron's exit from endocytic compartments, the metal would then be utilized for metabolic purposes by the endothelial cells, stored in endothelial cell ferritin (Ftn), or released to the brain via Fpn [45]. Re-oxidation of Fe^{2+} to Fe^{3+} and subsequent incorporation into apo-Tf would provide for its circulation in the brain [46–48]. It is possible that hepcidin, which is produced by the brain endothelium, plays a role in regulating this process. An in-depth review of iron uptake into BMVECs and its release has been published elsewhere [33].

It is important to note that the amount of Tf in the brain interstitial fluid is thought to be much lower than the levels in the systemic circulation, while non-Tf-bound iron (NTBI) levels may be quite high [49]. Thus, although Tf is apparently involved in moving iron across the BBB, there is some evidence to suggest that Tf-iron-binding sites may become saturated in the brain, such that NTBI is a major source of iron delivery to neurons and other cells in the brain. Another alternative

source of iron is ferritin which plays an important role in brain iron homeostasis. In fact, genetic loss of ferritin function leads to brain iron dyshomeostasis [11,50–52]. The brain may acquire ferritin exogenously by transcellular transport across the BBB, or it may be produced by endothelial cells and released upon demand [53]. Other endogenous sources of brain ferritin are possible, including its synthesis by microglia [28]. The ferritin pathway of iron delivery is particularly important for mouse oligodendrocytes and their function in myelination and neuronal repair. These express the ferritin receptor Tim-2, a member of the T cell immunoglobulin and mucin domain family, and specifically take up ferritin [6,54]. In humans, the transferrin receptor may bind to and mediate the internalization of ferritin [55,56].

3. Functions of Iron in the Brain

Iron plays an indispensable role during ATP production by serving as a cofactor for cytochromes and iron–sulfur complexes of the oxidative chain [57]. The major substrate for brain energy production is glucose which becomes fully oxidized; ketone bodies can fulfill energy needs under some conditions. The brain consumes nearly 20% of the body’s energy, although representing only about 2% of its weight. About 75–80% of the energy supports neuronal activity, with the remainder utilized to maintain the “housekeeping” functions of astrocytes, oligodendrocytes, and microglia [4]. Neuronal energy needs represent both axonal and synaptic signaling, but the majority is utilized post-synaptically [58]. The mitochondrial function must provide this supply of ATP with the iron requirements to support oxidative phosphorylation, as shown in Figure 1.

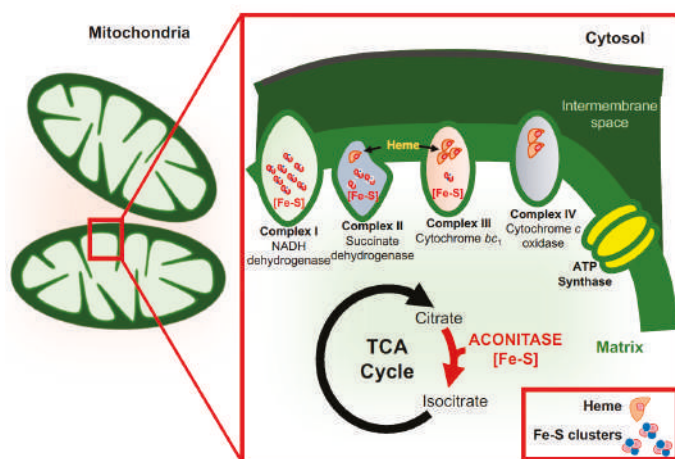


Figure 1. Iron and mitochondrial function. The mitochondrial electron transport chain contains multiple iron–sulfur clusters and heme-containing proteins necessary for ATP synthesis. NADH dehydrogenase (complex I) contains eight Fe–S clusters, succinate dehydrogenase (complex II) contains three Fe–S clusters and one heme moiety, while complex III (cytochrome bc1) contains one Fe–S cluster and several heme groups vital for its functions. Complex IV (cytochrome c oxidase) also contains two heme moieties. Aconitase, a key enzyme that catalyzes the stereo-specific isomerization of citrate to isocitrate through cis-aconitate in the tricarboxylic acid (TCA) cycle contain Fe–S clusters.

Oligodendrocytes, which are responsible for producing myelin, also require high amounts of ATP [59]. Not only do many of the enzymes involved in ATP production require a supply of iron, but also pathways for cholesterol and fatty acid synthesis necessary for myelination are iron-dependent. Some of the enzymes involved in this pathway include NADH dehydrogenase, HMG-CoA reductase, succinate dehydrogenase, dioxygenase, and glucose-6-phosphate dehydrogenase, all of which are abundant in oligodendrocytes compared to other cell types of the CNS [59]. The need for an adequate

supply of iron during myelination is reflected in the results of animal studies demonstrating that dietary iron restriction reduces the amount and composition of myelin during gestation and early post-natal periods [60,61].

Neurotransmitters serve as means of communication between neurons. The process of this communication includes biosynthesis and transport of neurotransmitters, packaging of neurotransmitters into vesicles for storage and controlled release, and binding of neurotransmitters to receptors on post-synaptic neurons with induction of cellular responses. The role of iron in each of these processes has been reviewed extensively, particularly in the case of monoamine neurotransmitters such as dopamine and serotonin that are involved in the regulation of cognitive processes including emotion and arousal behaviors [1,62–65]. For example, the synthesis of monoamine neurotransmitters depends on tyrosine hydroxylase which is an iron-requiring enzyme [66,67]. The activity of this enzyme is significantly reduced in patients suffering from PD with compromised brain iron homeostasis [67]. Iron deficiency further alters the functioning of the dopaminergic systems, with specific effects on dopamine D₁ and D₂ receptors [1,68]. Studies carried out by Youdim and colleagues demonstrated that the densities of dopamine D₂ receptors are significantly lower in the striatum of rats deficient in iron [69–71]. Also, microdialysis studies demonstrated an elevation of extracellular dopamine in the striatum of iron-deficient rats and the return to basal levels when brain iron content and iron status returned to normal [72]. In the case of serotonin, tryptophan hydroxylase carries out the rate-determining step in the synthesis of this neurotransmitter and can be inhibited by iron chelators [66,73]. Another neurotransmitter whose biosynthesis is compromised under iron-deficient conditions is gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the CNS. Iron deficiency is associated with significant reduction in the activity of glutamate dehydrogenase and GABA transaminase, key enzymes responsible for the synthesis and degradation of GABA [74,75].

4. Microglia and Iron

Microglial activation in response to pro- and anti-inflammatory stimuli is often characterized either as classical M1 or as alternative M2, similar to the nomenclature used for systemic macrophages [76,77]. M1 activation is pro-inflammatory and neurotoxic and primarily induced through the activation of toll-like receptor (TLR) and interferon gamma (IFN- γ) signaling pathway [19]. M1 microglia synthesize and secrete pro-inflammatory cytokines and chemokines such as tumor necrosis factor-alpha (TNF- α), some members of the interleukin family of cytokines interleukin-6 (IL-6), interleukin 1-beta (IL-1 β), interleukin-12 (IL-12), and C-C Motif Chemokine Ligand 2 (CCL2). In this reactive state, microglia also express inducible nitric oxide synthase (iNOS), which converts arginase into nitric oxide [19]. Accumulation of nitric oxide increases the toxic effects of glutamate and consequently potentiates N-methyl-D-aspartate (NMDA) receptor-mediated neurotoxicity [19,78,79].

In the M2 state, microglia release anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF- β). M2 microglia also induce arginase 1 to promote the conversion of arginine into polyamines [80]. These cells can secrete insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF), and neurotrophic factors including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), in the effort to resolve inflammation and promote synaptic plasticity [19].

The use of the terms “M1 versus M2” oversimplifies a complex process for microglial activity, since transcriptome studies have revealed that activation is quite variable and context-dependent [18,81]. Indeed, microglia adopt a homeostatic (M0) state under normal conditions in the CNS, and their transcriptome profile reflects their immunosurveillance activities in this state [18,81–83]. Conversely, microglia can express both neurotoxic and neuroprotective factors under disease conditions [19,81,84].

One prominent hallmark of neuroinflammation is the activation and increased acquisition of extracellular iron and subsequent downregulation of iron-interacting proteins, causing the intracellular sequestration of iron [13]. Systemically, such innate immune responses are orchestrated to deprive invading pathogens of iron, necessary for their survival [85]. This “iron withdrawal” phenomenon

could play a similar role in the brain to reduce the metal's availability. However, accumulation of intracellular iron is associated with neuronal degeneration that underlies most neurological disorders [86], and microglial secretion of the inflammatory cytokines TNF- α and IL-1 β enhances neuronal iron uptake [87]. In turn, these pro-inflammatory mediators have been shown to strongly influence microglia iron transport and metabolism [13,88–90].

Microglial cells interact with both Tf-bound-iron (TBI) and NTBI [91], and pathways for each transport substrate have been characterized [28]. For NTBI uptake, an endogenous cell surface ferrireductase reduces Fe³⁺ to Fe²⁺ for uptake by DMT1 in a pH-dependent manner at the cell surface. TBI is taken up by endocytosis of the Tf–TfR complex; after the release of iron in the acidic milieu of the endosome, it is translocated into the cytosol by DMT1 or other transporters, as described above [92].

Early studies of rat microglia raised the idea that microglial polarization and iron uptake are coordinated [89]. More recently, our group has shown that microglial iron transport pathways are differentially active in response to pro- and anti-inflammatory stimuli at both the transcript and the protein levels. Pro-inflammatory mediators increase the uptake of NTBI and expand the ferritin storage pool. These changes reflect the upregulation of both DMT1 and ferritin [28]. The uptake of NTBI by microglia would limit free extracellular iron and reduce potentially damaging reactive oxygen species (ROS) in the neural environment. In this M1 pro-inflammatory state, microglial cells also have increased glycolysis, with extracellular acidification supporting changes in the microenvironment favoring NTBI uptake by the pH-dependent transporter DMT1. Inflammatory mediators also reduce oxidative respiration, induce heme oxygenase-1, and diminish the levels of intracellular heme. These changes are associated with increased intracellular “labile iron”, suggesting that microglia can sequester both intracellular iron released by heme catabolism and extracellular iron taken up by DMT1. In contrast, anti-inflammatory IL-4 increases the expression of TfR to promote the uptake of TBI [28]. It is possible that this shift in iron transport is associated with the release of ferritin stores by M2 microglia to support the regeneration of neurons and the activity of oligodendrocytes. On the basis of these data, we propose a model by which microglia actively modify transport pathways and metabolism in response to the iron status of their environment (Figure 2).

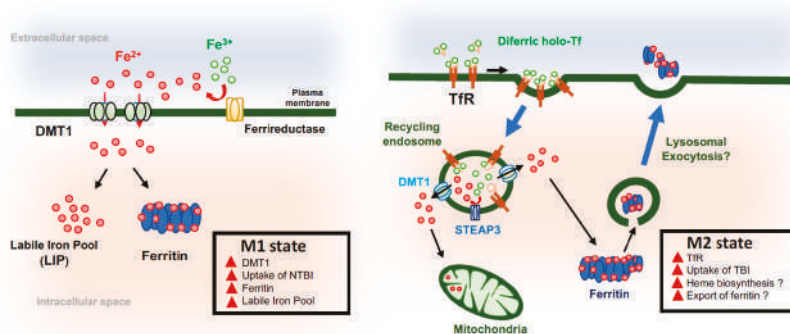


Figure 2. Iron trafficking and microglial cell polarization. **(Left)** Pro-inflammatory stimuli upregulate the expression of divalent metal transporter-1 (DMT-1) and the uptake of non-Tf-bound iron (NTBI). These effects are associated with increased labile iron and an expanded pool of ferritin. These changes reflect M1 polarization. **(Right)** Anti-inflammatory stimuli increase transferrin receptor (TfR) levels to upregulate Tf-bound iron (TBI) uptake by receptor-mediated endocytosis. In recycling endosomes, the low pH promotes the release of Fe³⁺ for ferrireduction, most likely by STEAP3. Fe²⁺ may be released into the cytosol through DMT1 or another channel for use in mitochondria to promote heme production. We speculate that, under anti-inflammatory conditions, microglia may release ferritin (Ftn)-bound iron through lysosomal exocytosis to help oligodendrocyte function and neuronal repair.

5. Microglia Activation and Alzheimer's Disease

Alzheimer's Disease (AD) is a neurodegenerative disorder and the most common form of dementia involving the progressive loss of substantive cortical and hippocampal neurons over time [93,94]. This disorder is characterized by extracellular deposition of amyloid beta ($A\beta$) in senile plaques and intraneuronal accumulation of hyperphosphorylated tau proteins. These events lead to neuronal and synaptic loss, chronic inflammation, and oxidative stress [19,89,95,96].

Genetic studies of familial Alzheimer's disease (FAD) have demonstrated that mutations in the amyloid precursor protein and in components of the gamma-secretase complex generate $A\beta$ 1-42 which can misfold and aggregate [19,93,97]. The more common sporadic form (SAD) of the disease is largely associated with aging. Although the pathophysiological mechanisms that underlie the role of aging in the onset of AD is poorly understood, accumulating evidence indicates that the onset of SAD is closely associated with brain iron and oxidative stress, both of which increase with age [95,98–103]. In AD, the observation that iron is present in local areas of neuronal cell death further supports the metal theory of dementia which proposes that iron promotes neurodegeneration [104]. Furthermore, as brain microglia are implicated in iron handling, it has been shown that iron accumulates in microglial cells that cluster around amyloid plaques in AD mouse models and post-mortem brain tissues of AD patients [105].

Iron is a key player during the induction of oxidative stress because of its function as a redox-active transition metal [13,96,106]. Indeed, the levels of damaging ROS are significantly higher in the AD brain compared to healthy control brains [96,107]. Importantly, several studies have reported that, by promoting neurotoxic oligomerization of $A\beta$ peptides and tau tangles [94,108], oxidative stress potentiates the activation of microglia [96,109,110]. Whether these events advance or hinder the disease is subject to active debate. For example, while anti-inflammatory activities of microglia would appear to be beneficial, some studies have reported that prolonged stimulation of microglia with $A\beta$ peptides provokes chronic inflammation [111].

Microglial cells express multiple receptors including CD36, TLR2, TLR4, and TLR6 [111–113], all of which can bind $A\beta$ and induce pro-inflammatory effects. The sporadic form of AD is associated with genetic variants of triggering receptor expressed on myeloid cells 2 (TREM2) [114]. TREM2 is an immune transmembrane glycoprotein receptor expressed in microglia that interacts with phospholipids, apoptotic cells, and lipoproteins [115–118]. These variants, as well as the loss-of-function mouse models of AD, appear to limit microglial proliferation around $A\beta$ plaques, causing increased plaque buildup and disease progression [119–122]. Why defective TREM2 function or expression impacts microglia responses to AD lesion is still incompletely understood. The role of TREM2 and other immune receptors identified as risk factors, including CD33, have been reviewed elsewhere [19].

SAD is also associated with the polymorphism of apolipoprotein E (apoE), a lipid-binding protein involved in lipid metabolism [123–125]. The apoE4 allele is strongly associated with an increased risk of AD, while apoE2 serves a protective role [123]. Microglia produce apoE, which has been shown to moderate the inflammatory response while enhancing the phagocytosis of $A\beta$ aggregates by microglia [124–128]. However, in carriers of apoE4, increased levels of ferritin have been reported in the cerebral spinal fluid, suggesting that iron metabolism is altered in these individuals to promote increased iron retention [129]. This evidence reinforces the concept that increased brain iron adversely affects patients with AD. In fact, patients with HFE-associated hemochromatosis are subject to earlier onset of AD [130]. Since there is clinical evidence that iron chelation is beneficial to AD patients [131,132], the relationship between microglia, iron, and neurodegeneration appears to be well worth exploring.

6. Concluding Remarks

Understanding the role of iron in chronic inflammatory responses elicited by microglia is essential for finding new therapeutic strategies to treat neurodegenerative diseases. Although a substantial amount of effort has been put into deciphering the molecular network directly involved in brain iron

metabolism, we still must pursue an in-depth understanding of how specific brain cells accumulate and use iron to carry out their various functions, both in normal and in disease conditions.

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Abbreviations

Alzheimer's disease	AD
familial Alzheimer's disease	FAD
sporadic Alzheimer's disease	SAD
neurodegeneration with brain iron accumulation	NBIA
Parkinson's disease	PD
Huntington's disease	HD
amyotrophic lateral sclerosis	ALS
multiple sclerosis	MS
amyloid beta	A β
transferrin	Tf
Tf-bound iron	TBI
transferrin receptor	TfR
non-transferrin-bound iron	NTBI
divalent metal transporter-1	DMT1
labile iron pool	LIP
central nervous system	CNS
Blood-brain barrier	BBB
brain microvascular endothelial cell	BMVEC
transient receptor potential mucolipin-1	TRPML1
ferritin	Ftn
reactive oxygen species	ROS
gamma-aminobutyric acid	GABA
inducible nitric oxidase	iNOS
N-methyl-D-aspartate	NMDA
adenosine triphosphate	ATP
3-hydroxy-3-methyl-glutaryl-coenzyme A	HMG-CoA
tricarboxylic acid	TCA
interferon gamma	IFN- γ
toll-like receptor	TLR
tumor necrosis factor-alpha	TNF- α
interleukin-6	IL-6
interleukin 1-beta	IL-1 β
interleukin-12	IL-12
interleukin-10	IL-10
C-C motif chemokine ligand-2	CCL2
transforming growth factor-beta	TGF- β
insulin-like growth factor-1	IGF-1
fibroblast growth factor	FGF
nerve growth factor	NGF
brain-derived growth factor	BDNF
toll-like receptor 2	TLR2
toll-like receptor 4	TLR4
toll-like receptor 6	TLR6
cluster of differentiation 33	CD33

cluster of differentiation 36	CD36
triggering receptor expressed on myeloid cells 2	TREM2
apolipoprotein E	apoE
apolipoprotein E4	apoE4
apolipoprotein E2	apoE2

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Review

Neurodegeneration with Brain Iron Accumulation Disorders: Valuable Models Aimed at Understanding the Pathogenesis of Iron Deposition

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Abstract: Neurodegeneration with brain iron accumulation (NBIA) is a set of neurodegenerative disorders, which includes very rare monogenetic diseases. They are heterogeneous in regard to the onset and the clinical symptoms, while they have in common a specific brain iron deposition in the region of the basal ganglia that can be visualized by radiological and histopathological examinations. Nowadays, 15 genes have been identified as causative for NBIA, of which only two code for iron-proteins, while all the other causative genes codify for proteins not involved in iron management. Thus, how iron participates to the pathogenetic mechanism of most NBIA remains unclear, essentially for the lack of experimental models that fully recapitulate the human phenotype. In this review we reported the recent data on new models of these disorders aimed at highlighting the still scarce knowledge of the pathogenesis of iron deposition.

Keywords: iron; neurodegeneration; NBIA

1. Introduction

The acronym NBIA (neurodegeneration with brain iron accumulation) designates a clinically and genetically heterogeneous group of neurodegenerative diseases, the majority of which is hallmarked by iron deposits in the brain [1]. Iron accumulation, is detected in vivo by MRI analysis, and affects mainly the basal ganglia regions, such as globus pallidus (GP) and substantia nigra (SN), although other regions including the cortex and cerebellum can be involved. These disorders are inherited as Mendelian traits (autosomal recessive, dominant, or X-linked), and typical features are extra pyramidal symptoms, namely, dystonia, parkinsonism, spasticity, variably associated with neuropsychiatric abnormalities, and optic atrophy or retinal degeneration [1]. Disorders belonging to the NBIA group are rare, between one and three per million individuals in the general population, and 15 causative genes, whose products are involved in a wide spectrum of biological activities, have been so far discovered. Although the presence of brain iron accumulation enables the diseases to be included in the NBIA group, the pathogenic mechanisms linking specific disease-genes mutations to iron metabolism are unclear [2]. *CP* (ceruloplasmin) and *FTL1* (ferritin light chain) are directly associated to iron homeostasis (Table 1). The remaining genes (*PANK2*, *COASY*, *PLA2G6*, *C19orf12*, *FA2H*, *ATP13A2*, *WDR45*, *DCAF17*, *SCP2*, *GTPBP2*, *AP4M1*, *REPS1*, and *CRAT*) are unrelated to iron metabolism, being involved in diverse metabolic pathways (Table 1).

Table 1. Molecular and clinical features of NBIA sub-types.

Gene	Disease	Inheritance	Function	Protein localization	Brain Iron and Clinical Features
Genes related to iron homeostasis					
<i>CP</i>	Acerulo-plasminaemia	AR	Iron oxidation	Plasma membrane	Iron in the basal ganglia, liver, pancreas and myocardium. Movement disorders, dementia, diabetes mellitus, retinal degeneration, dysarthria, ataxia
<i>FTL1</i>	Neuro-ferritinopathy (NF)	AD	Cellular iron storage	Cytoplasm	Iron deposition in basal ganglia, cerebellum, motor cortex; mild cerebral and cerebellar atrophy, cavitation of the putamen. Extrapyrarnidal movement disorders, dystonia, parkinsonisms, dysarthria
Genes related to Coenzyme A biosynthesis					
<i>PANK2</i>	Pantothenate kinase-associated neurodegeneration (PKAN)	AR	Pantothenate phosphorylation; Coenzyme A synthesis	Mitochondria (inner membrane space)	Iron overload in GP; "eye of the tiger sign". Dystonia, spasticity, cognitive decline, pigmentary retinopathy
<i>COASY</i>	COASY protein-associated neurodegeneration (CoPAN)	AR	4'-PP adenylyltran-ferase and dephospho-CoA kinase; Coenzyme A synthesis	Mitochondria (matrix), cytosol	Iron overload in GP. Oro-mandibular dystonia, dysarthria, spastic-dystonic paraparesis, obsessive-compulsive behavior
Genes related to lipid metabolism					
<i>PLA2G6</i>	PLA2G6-associated neurodegeneration (PLAN)	AR	Hydrolysis of ester bonds at the sn-2 position of phospho-lipids; Membrane remodeling	Mitochondria, endoplasmic reticulum, cytosol	Iron overload in GP in <50% of cases. Infantile neuroaxonal dystrophy, hypotonia, gait disturbance and cerebellar atrophy. Dystonia, spasticity and parkinsonisms in adulthood
<i>FA2H</i>	Fatty acid hydroxylase-associated neurodegeneration	AR	Hydroxylation of fatty acids; Ceramide synthesis; Myelin formation	Endoplasmic reticulum	Iron overload in GP and SN. Profound ataxia, dystonia, dysarthria, spastic quadriplegia, axial hypotonia, optic atrophy
<i>C19orf12</i>	Mitochondrial membrane protein-associated neurodegeneration (MPAN)	AR	Unknown; Lipid metabolism? Membrane remodeling?	Mitochondrial membranes, endoplasmic reticulum, MAM	Iron overload in GP and SN; abundant Lewy bodies. Global developmental delay, dystonia, parkinsonism, psychiatric symptoms, spastic paraparesis

Table 1. *Cont.*

Gene	Disease	Inheritance	Function	Protein localization	Brain Iron and Clinical Features
Genes related to lipid metabolism					
SCP2	Leukoencephalopathy with dystonia and motor neuropathy	AR	Thiolase activity; Breakdown of branched chain fatty acids	Peroxisomes	Iron deposition in thalamus; Dystonia and spasmodic torticollis; spinocerebellar ataxia, balance and gait impairment
CRAT		AR	Carnitine acetyltransferase, -oxidation	Mitochondria	Iron accumulation in GP and SN. Slowly progressive spinocerebellar degeneration. Cerebellar atrophy and posterior leukodystrophy
Genes related to autophagy					
WDR45	β -propeller-associated neurodegeneration (BPAN)	X-linked (<i>de novo</i> mutations)	Protein-protein interaction; Early autophagosome formation	Endoplasmic reticulum	Iron overload in GP and SN. Global developmental delay, neurological deterioration, dystonia, parkinsonism, cognitive decline, seizures
ATP13A2	Kufor-Rakeb disease (KRS)	AR	Lysosomal cation pump; autophagosome formation	Lysosome, mitochondria	Often no iron overload. Early onset parkinsonism, pyramidal signs, altered eye movements, dementia
AP4M1		AR	Vesicle formation	Endosome	Iron in globus pallidus reported in a single family. Early-onset developmental delay and deterioration of motor function, tetraparesis, intellectual disability
REPS1		AR	Endocytosis and vesicle transport	Cytoplasm, endosome	Iron accumulation in the globus pallidus and peduncles. Trunk hypotonia, progressive cerebellar ataxia, pyramidal syndrome. Cerebellar and cerebral atrophy.
Genes with unknown function					
DCAF17	Woodhouse-Sakati syndrome (WSS)	AR	Unknown	Nucleolus	Sometimes iron overload in GP and SN. Extrapyramidal symptoms, dystonia, cognitive impairment, hypogonadism, alopecia, diabetes mellitus
GTPBP2		AR	Unknown; mRNA/ribosome stability?	Cytoplasm	Iron overload in GP and SN; cerebellar atrophy. Mental retardation, ataxia and dystonia

PANK2 (pantothenate kinase type 2) and *COASY* (CoA synthase) code for enzymes in the crucial biochemical pathway responsible for coenzyme A (CoA) production. *PLA2G6* (phospholipase A2 group 6), *FA2H* (fatty acid hydroxylase 2), *C19orf12*, *SCP2* (sterol carrier protein type 2) and *CRAT* (carnitine acetyltransferase) genes code for proteins involved in lipid metabolism, membrane integrity and mitochondrial function. *WDR45*, *ATP13A2*, and possibly *AP4M1* are genes coding for factors involved in the autophagic process. The *GTPBP2* gene product plays a putative role in chain elongation during protein synthesis at the ribosome, *REPS1* (RALBP1-associated EPS domain-containing protein 1) is involved in endocytosis and vesicle transport, while the *DCAF17* (also known as *C2orf37*) gene encodes for a protein with still not clarified cellular localization, probably in the nucleolus, and with undefined activity (Figure 1). Around one third of the NBIA causative genes namely *PANK2*, *COASY*, *PLA2G6*, *C19orf12*, and *CRAT* are associated to mitochondria, suggesting that these organelles play a crucial role in triggering the disorders. Thus, these pathologies represent an unprecedented chance to investigate the molecular mechanisms involved in iron management in the brain and the connection with mitochondrial function and neurodegeneration. In this review, we will focus on what we have learned from available in vivo and in vitro models about iron metabolism and its connection with the different underlying genetic mutations of NBIA.

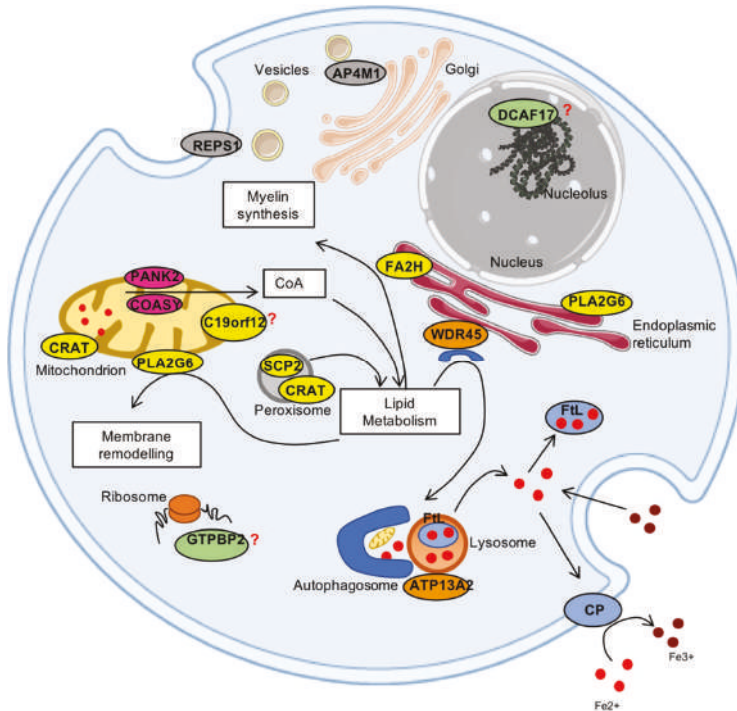


Figure 1. Scheme of the proteins associated to NBIA disorders and their cellular localization. The iron proteins (CP and FTL) are represented in light blue; in pink are the proteins (PANK2 and COASY) are involved in CoA synthesis; in yellow are the proteins related to lipid metabolism (PLA2G6, FA2H, SCP2, CRAT, C19orf12); in orange are the proteins (WDR45, ATP13A2) involved in autophagy; in grey are the proteins (REPS1 and AP4M1) associated to vesicle trafficking; and the proteins (DCAF17 and GTPBP2) in green still have unknown functions.

1.1. Iron Homeostasis in Diseases Caused by Mutations in Iron-Related Genes

1.1.1. CP (OMIM*117700)

Aceruloplasminemia (OMIM #604290) is the first disease in which the alteration of an iron-related protein has been linked to neurodegeneration. It is inherited as an autosomal recessive trait and was originally described in a female from Japan [3] where the prevalence of mutations is around one in 2,000,000 individuals. The genetic analysis of patients affected by aceruloplasminemia revealed about 50 distinct causative mutations (listed in <http://www.hgmd.cf.ac.uk/ac/index.php>) scattered along the gene and identified in more than 60 families [4,5]. *CP* gene is located on chromosome 3q21-24 and encodes ceruloplasmin (Cp), a plasma membrane glycoprotein with ferro-oxidase function containing many copper atoms, which facilitates ferroportin-mediated cellular iron export [6].

Onset of symptoms is in adulthood and the clinical features include neurological signs like blepharospasm, grimacing, facial and neck dystonia, tremors, chorea and ataxia, and cognitive dysfunction. Additional signs, generally preceding neurological symptoms are diabetes mellitus, retinal degeneration and microcytic anemia [5]. The disease is also hallmarked by alteration of hematological parameters such as: very low or undetectable serum ceruloplasmin, high levels of serum ferritin and low levels of iron [7]. Iron depositions affect the striatum, thalamus, and dentate nucleus as showed by abnormal low intensities on T1 and T2 weighted images obtained by MRI analysis [8]. Neuropathology investigations demonstrate aberrant basal ganglia and dentate nucleus, with consistent iron accumulation in neuronal and glial cells [9–12]. Characteristic neuropathological findings are enlarged or deformed astrocytes and spheroid-like globular structures caused by iron accumulation localized to terminal astrocytic processes [11]. Iron overload was also detected in the cerebral cortex, retina, and cerebellum, and in non-cerebral tissues, such as the pancreas and myocardium [12].

Mutations of *CP* determine structural modifications of the protein (Cp) which are responsible for retention of Cp in the ER, miss-incorporation of copper atoms into apo-ceruloplasmin or defective ferro-oxidase activity [13–15]. Overall, this malfunctioning causes impairment of iron export from the cell, which in the end is responsible for iron overload.

Investigations of the pathogenetic mechanisms underlining aceruloplasminemia were performed by analyzing the effect of Cp mutants' expression in mammalian cell culture [13–15] and by characterizing various aceruloplasminaemia mouse models [16–18]. Overall these data indicated that, in the presence of an aberrant ceruloplasmin, iron entering the CNS as ferrous iron could not be oxidized. As a consequence, cells can internalize large quantities of ferrous iron through non-regulated internalization pathways of non-transferrin-bound iron [19]. Accumulation of iron inside astrocytes observed in the pathology is due not only to the excess of import, but also to the inability of the cells to export iron through ferroportin, in the absence of ceruloplasmin. In this way iron would remain "locked" in the astrocytes and could not reach the neurons, which would paradoxically die from iron deficiency and from toxic compounds released by degenerating astrocytes. Additionally, it has been proposed that some mutated Cp forms could also accumulate in aggregates leading to death of astrocytes through an iron-independent pathway [20].

The specific degeneration of astrocytes and neurons is explained by their strict dependence on ceruloplasmin ferro-oxidase activity. On the contrary, other cells in the CNS, including oligodendrocytes, depend on the action of hephaestin, a protein similar in structure and function to ceruloplasmin. In fact, only the simultaneous ablation of *CP* and hephaestin (*HEPH*) genes in mice is able to recapitulate symptoms consistent with those shown by aceruloplasminemia patients, suggesting that hephaestin can compensate the lack of CP in mice, but not in humans [21,22]. This double knock-out mouse model showed iron accumulation in gray and white matter oligodendrocytes [22], macular degeneration, iron overload, and increased oxidative stress in the retina [23]. Treatment of these mice orally with DFP showed to be protective against oxidative stress, retinal degeneration and accumulation of lipofuscin, and was able to reduce ataxia and increase life span [24]. Despite the good

results obtained in mice, iron chelation therapy in patients was disappointing since it only partially reduced systemic iron deposition in liver and pancreas [25], but it was ineffective in reducing the brain iron accumulation and thus in controlling neurodegeneration [12,26].

Another proposed therapeutic approach is based on enzyme replacement therapy (ERT) with ceruloplasmin. Recently, it was demonstrated that the parental administration of human ceruloplasmin to *CP*^{-/-} mice was able to restore normal levels of the protein in the brain, as well as its ferro-oxidase activity [27]. Interestingly, in treated mice, the reduced brain iron deposition led to motor coordination improvement, suggesting ERT as a possible therapeutic option for aceruloplasminemia [27].

1.1.2. *FTL* (OMIM*134790)

Neuroferritinopathy (NF) is an extremely rare autosomal dominant disease described for the first time in 2001 [28] and linked to mutations of *FTL1*, the gene coding for L-ferritin subunit that, together with the H-ferritin subunit, forms the major iron storage protein.

The most frequent mutation is the 460InsA (or c.460dupA) described in 41 cases [29] presenting with focal onset chorea or focal dystonia and history of movement disorders. Serum ferritin level was generally low. MRI was abnormal in all reported cases and showed lesions in the globus pallidus, putamen, and dentate nuclei. Few cases of NF presented with the “eye of the tiger” sign (typical of PKAN), a hypointense MRI signal, which is pathognomonic for iron accumulation, surrounding a central region of hyperintensity, which is due to gliosis and/or necrosis, in the anteriomedial globus pallidus on the T2* sequence [8]. A typical pathological feature found in astrocytes and oligodendroglia in the grey and white matter, was the presence of intranuclear and intracytoplasmic bodies containing ferritin and iron in insoluble form. The same bodies were also present in fibroblasts, tubular epithelium of the kidney, and endothelial cells of muscle capillaries. Neurons and glia also showed swollen or vacuolated nuclei containing iron and ferritin [30].

Biochemical studies performed by overexpression of mutants 460InsA and 498InsTC (or c.497_498dupTC) in human cells, demonstrated instability of mutant proteins, a different solubility and a lower capacity to incorporate iron as compared to wild-type ferritin [31–35]. These cellular models showed that the expression of these ferritin mutants not only increased cellular free iron pool, but also induced oxidative damage and the impairment of proteasome activity [33,34].

Brain iron accumulation and increased production of reactive oxygen species, are present in mouse models generated by transgene expression of *FTL1* cDNAs coding for variants of the human ferritin protein. These mice showed features of neurological deterioration, decreased motor activity and coordination, reduced lifespan together with the presence of ferritin aggregates in the nucleus and cytoplasm [36–39].

Specifically, mice overexpressing the human mutant c.497_498dupTC recapitulate features typical of the human disease, including abnormalities of brain iron homeostasis [36,37]. Further investigation of post-natal hippocampal neurons in transgenic mice with a different genetic background, revealed high susceptibility to chronic iron overload and/or acute oxidative stress in comparison to wild-type neurons [37]. Moreover, ultrastructural analyses of specific brain regions such as cerebellum and striatum, showed an accumulation of lipofuscin granules associated with iron deposits, particularly evident in aged mice [37]. Altogether, these data indicate that the combination of increased oxidative stress and lipofuscin accumulation play a key role in the etiopathogenesis of human NF. The only data available on human naïve fibroblasts derived from a patient carrying the c.497_498dupTC, demonstrated a consistent increase of intracellular iron, altered iron management, ferritin accumulation and the presence of oxidative stress markers, as previously demonstrated in patients and transgenic mice [40].

From the overall data, the emerging hypothesis suggests that the decreased ability of ferritin to sequester iron excess has two major consequences: (i) the unbound metal precipitating on proteins promotes their aggregation [31]; (ii) the higher free iron-dependent oxidative damage required the need for the cell to increase degradation of oxidized molecules. Both these effects cause the engulfing

of the cellular recycling systems (i.e., proteasome and lysosome), and in the long-term leads to cellular death. These events occur despite the regulation system of iron entry into the cell seeming to work properly [33]; in fact, the level of the main iron-importer protein, the transferrin receptor, appears strongly reduced in different experimental models [32–34]. Thus, apparently, iron may be efficiently internalized by transferrin/transferrin-receptor independent pathway in neuronal compartment. This pathogenetic mechanism seems to be harmful mainly in non-dividing cells, partly explaining the specificity of the symptomatology. Interestingly, since the rate of lipofuscin accumulation is known to correlate negatively with longevity, the NF pathogenesis may replicate the cascade of events that occur during the physiological process of aging.

The most simple and rational way to reduce iron accumulation is the utilization of iron-chelating compounds such as desferrioxamine (DFO) or deferiprone (DFP). These treatments were tested both in animal models and in patients, with comparable results. DFP treatment in mice was effective in reducing iron accumulation in the brain [41] and had significant effects on systemic iron homeostasis [42]. As already discussed for the aceruloplasminemia, also in this case the outcome of the treatment with iron chelators is not as effective in humans. Iron-chelating based therapies in NF patients had severe detrimental effects on systemic iron level causing iron depletion without improving clinical conditions [29,43]. This may be due to a lower penetrability of the iron chelator in the basal ganglia of human and/or to a late treatment in humans, which develop symptoms when the brain region has already degenerated.

1.2. Iron Homeostasis in Inborn Errors of Coenzyme A Biosynthesis

PANK2 (OMIM*606157) and *COASY* (OMIM*609855)

Mutations in genes coding for the first (*PANK2*) and the last (*COASY*) enzyme of the biosynthetic pathway that produces Coenzyme-A (CoA) cause NBIA subtypes known as pantothenate kinase-associated neurodegeneration (PKAN) and CoA synthase protein-associated neurodegeneration (CoPAN), respectively.

PKAN accounts for a large fraction of NBIA cases [44] while CoPAN appears more rare, being so far identified in few individuals worldwide [45–48]. These two disorders display an impressively similar phenotype, presenting with early-onset spastic-dystonic paraparesis with a later appearance of parkinsonian features, cognitive impairment, obsessive-compulsive disorder, and brain iron accumulation [45]. PKAN typically manifests in early childhood with gait disturbances and rapidly progresses to a severe movement deficit with dystonia, dysarthria and dysphagia [49]. The hallmark of PKAN is the “eye-of-the-tiger” signal in the globus pallidus on T2-weighted magnetic resonance imaging, which reflects the focal accumulation of iron in this area [50]. CoPAN also accumulates iron in *GP*. The *SN* is as well affected in CoPAN and to a lesser extent in PKAN. The iron accumulation correlates with neural damage. It is often associated with axonal expansions (spheroids), but it also appears as a granular form in perivascular location [51]. Although iron accumulation hallmarks PKAN and CoPAN, its relationship with CoA dysfunctional biosynthesis is not clear.

Despite several efforts have been made during the last years to understand the connection between iron homeostasis and mutations in *PANK2* or *COASY*, no models, either in vitro or in vivo, have been so far able to recapitulate the presence of iron deposits. *Pank2* null mice showed growth reduction, azoospermia [52] and impaired mitochondrial function [53], but did not show signs of neurodegeneration or iron accumulation and did not display movement disorders. Only by increasing the fat content in the diet of these mice it was possible to unravel a neurodegenerative phenotype characterized by motor dysfunction, bioenergetic failure, and brain cytoplasmic accumulation of abnormal ubiquitinated proteins [54], features observed in the brains of PKAN patients [51]. The simultaneous knockout of *Pank1/Pank2* led to severe reduction of CoA levels in the brain but iron accumulation was not reported [55]. The combined ablation of *Pank2/Pank3* or *Pank1/Pank3* in mice is associated with a lethal phenotype [55]. Altogether, these experiments imply that, in mice, *Pank2*

loss is counterbalanced by the other *Pank* genes, but the simultaneous elimination of *Pank3* with either *Pank2* or *Pank1* is incompatible with life.

Drosophila possesses only one pantothenate kinase isoform (*fumble*) and its ablation determines brain vacuolization with defective neurological functions but without brain iron overload [56], resulting in flies with severe motor impairment [57]. The down-regulation of *pank2* in Zebrafish induced defects in neuronal development and an aberrant organization of the vascular system [58].

PKAN patients' fibroblasts have been helpful to reveal some defects in mitochondrial activity and iron metabolism associated with *PANK2* deficiency [59], however, the pathological neurodegenerative processes cannot be appreciated in these cells.

Insight on the neurodegenerative process started emerging thanks to the generation of iPSC-derived glutamatergic neurons from PKAN patients and from healthy subjects. Their compared analysis showed alteration of mitochondria functionality in PKAN patients. The altered phenotype included impairment of mitochondrial iron-dependent biosynthesis that caused iron dys-homeostasis and consequent enhanced ROS production, leading to major membrane excitability defects. In particular, the cells inability to maintain Fe/S biosynthesis simulated a cellular iron deficient phenotype that promoted cellular iron uptake increasing the translation of the TfR1 [60].

A second paper reported data on iPSCs derived cortical neuronal cells obtained from fibroblasts of an atypical PKAN patient [61], in which the *PANK2* protein was not completely absent but reduced as compared to control cells. In this case, CoA homeostasis and cellular iron handling were normal, but mitochondrial functionality resulted affected, with increased production of reactive oxygen species and lipid peroxidation [61].

Neither glutamatergic nor cortical neurons displayed iron deposits and iron chelation in cortical neurons exacerbated the mitochondrial phenotype in both control and patient neuronal cells [61]. Thus, both these investigations on human neurons agree to conclude that mitochondrial impairment is an early feature of the disease process and the consequent ROS formation a main actor in the pathogenesis, while iron chelation treatment did not seem a good option. Data on efficacy and safety of deferiprone for the treatment of PKAN were available through clinical trials on small cohorts of patients and showed controversial results [50,62].

Data on the efficacy of DFP treatment collected in a wide clinical trial involving around 100 PKAN patients in the world are still under analysis and will be available shortly (<https://tircon.eu>). While DFP efficacy is still under investigation, the work performed in vitro on glutamatergic neurons demonstrated that CoA supplementation prevented neuronal death and reduced ROS formation by restoring mitochondrial and neuronal functionality, suggesting CoA treatment as a possible therapeutic intervention [60].

Very recently, an allosteric *PANK* activator has been identified by compounds library screening. The compound denominated PZ-2891 crosses the blood brain barrier and oral administration to wild-type mice increases coenzyme A levels in the brain and liver. Moreover, PZ-2891 treatment of a knockout mouse model of brain CoA deficiency presenting with weight loss, severe locomotor impairment and early death, was able to rescue the weight, to improve locomotor activity and to extend life span [63].

Analysis of fibroblasts derived from CoPAN patients, showed impaired, but not completely abolished, de-novo synthesis of CoA and dephospho-CoA. This would suggest the presence of alternative routes for CoA production, or the preservation of a residual catalytic activity of the mutant CoA synthase protein [45]. More recently, mutations of *COASY* associated with the complete absence of the protein were reported in two cases of pontocerebellar hypoplasia, microcephaly, and arthrogyriposis [48] with an invariable lethal phenotype, probably due to the complete absence of CoA.

In the yeast *Saccaromyces cerevisiae*, CoA synthesis is carried out by five sequential enzymes (CAB1 to CAB5) deletion of which, leads to a lethal phenotype. Re-expression of wild-type yeast CAB5 or human *COASY* protein was able to rescue the lethal phenotype while mutant CAB5 or

COASY expression determined a phenotype characterized by reduced growth, auxotrophy for pantothenate, and decreased amount of CoA in isolated mitochondria [45]. Additional features included mitochondrial dysfunctions characterized by impairment of the respiratory chain and revealed iron accumulation and impaired lipid content [64].

In zebrafish the complete down-regulation of *Coasy*, obtained by morpholino-mediated approach, was associated with impaired development and premature death, while a partial down-regulation gave rise to a milder phenotype [65]. Main phenotypic features of morphants included: reduced size of the brain with less defined brain structures, disorganization of the vascular system in the brain and trunk, and reduced levels of CoA in the fish embryos. Rescue of these features was obtained by adding CoA to the fish water or by re-expression of the human wild-type gene, indicating a causative role of impaired CoA synthesis in determining the pathological phenotypes.

Hence, the connection between COASY dysfunction and iron metabolism needs to be clarified although a potential link was highlighted by the identification of an iron responsive element (IRE) in the 3' region of this gene that could stabilize the COASY mRNA in the presence of iron [2].

Mutations in *PPCS* encoding for the second enzyme in the CoA biosynthetic pathway converting 4'-Phosphopantothenate into phosphopantothenoylcysteine, have been described in two unrelated families showing a very severe pediatric phenotype characterized by dilated cardiomyopathy associated with reduced level of CoA [66]. In the affected individuals neurodegeneration and iron accumulation were not investigated or not present because of a premature death of some patients. At the moment, it is not clear why *PPCS* mutations lead to cardiac alteration instead of neurodegeneration and further investigation will be required. We have decided to mention this gene for the sake of completeness despite that *PPCS* cannot be included in the NBIA disease genes, at least from the knowledge available at the time of writing.

1.3. Iron Homeostasis and Mutations in Lipid Metabolism Related Genes

Globally these sub-types of NBIA disorders typically begin in childhood with a combination of clinical signs, which are also present in spastic paraplegia complex forms and in several cases the genetic cause of the disease is shared by the two category of diseases. The accumulation of iron is not a consistent finding and is often documented in advanced disease stages suggesting that it could contribute to disease progression but is not the primary cause [67].

1.3.1. *PLA2G6* (OMIM*603604)

PLA2G6-associated neurodegeneration (PLAN) is an autosomal recessive heterogeneous group of related neurodegenerative conditions comprising infantile neuroaxonal dystrophy (INAD, OMIM #256600) and atypical neuroaxonal dystrophy (ANAD, OMIM #610217) caused by mutations in *PLA2G6*, mapping on human chromosome 22q13.1 and encoding for calcium-independent phospholipase A2 group VI (iPLA2 β or iPLA2VI) [68].

Onset of disease is typically in childhood and presents as neuroaxonal dystrophy with psychomotor regression and axial hypotonia, accompanied by neuropathy with spheroidal bodies in the peripheral nerves, detectable by analysis of sural nerve biopsy. Cerebellar atrophy is detected in the majority of the cases by brain MRI while iron accumulation is present in only half of the affected individuals. *PLA2G6* protein is ubiquitously expressed and is localized in the cytosol and mitochondria, playing a role in phospholipids metabolism and membrane remodelling. Malfunctioning of the protein may lead to alteration of neuronal membranes integrity and fluidity [69] determining neuronal suffering.

A *Pla2g6* knock-out mouse model recapitulate in a precise way the human disease and showed cerebellar atrophy, loss of Purkinje cells, pronounced neuroinflammation, age-dependent accumulation of spheroids but not iron accumulation, which is not, however, a consistent finding neither in the affected patients [69].

This mouse model was also characterized by the presence of ultrastructural alteration of mitochondria in the brain with a consistent accumulation of cardiolipin, a phospholipid formed from two phosphatidic groups connected by a glycerol molecule [70], which is highly enriched in the mitochondrial inner membrane. In fact, first pathological signs in neurons of *Pla2g6*-null mice is the disorganization of the cristae in the mitochondrial inner membrane with the consequent destruction of the mitochondrion, the release of cytochrome c, and the formation of swollen and degenerated axons as the disease progresses. Presynaptic membranes are altered in this animal model, demonstrating the role of PLA2G6 may be to remodel cardiolipin and eliminate fatty acids damaged by oxidative stress [71]. Recently, an age-dependent iron accumulation in *SN*, *ST*, and *GP* brain regions was reported on *Pla2g6* *-/-* mice. These mice showed an increased level of iron-proteins (TfR1, DMT1 and IRPs), decreased FPN1, enhanced lipid peroxidation and mitochondrial dysfunction. These alterations precede the sign of neuronal death, suggesting that iron-dependent ROS formation might be a primary cause of neurodegeneration [71].

The similarity between PKAN and PLAN phenotypes can be traced back to the fact that PANK2 and PLA2G6 enzymes would take part to cardiolipin synthesis or remodelling in the inner mitochondrial membrane [72].

1.3.2. *FA2H* (OMIM*611026)

Fatty acid hydroxylase-associated neurodegeneration (FAHN) (OMIM #612319) was first described in two consanguineous families, of Italian and Albanian origin respectively and is a sub-type of neurodegeneration with brain iron accumulation transmitted as an autosomal recessive trait [73]. The gene responsible for the disorder is the fatty acid hydroxylase-2 (*FA2H*), that maps on human chromosome 16q23.1 and codes for a NADPH-dependent mono-oxygenase localized on ER membranes [73,74]. The same gene is also mutated in cases of leukodystrophy and a form of spastic paraplegia [75]. Main clinical features of the disease are spasticity, ataxia, dystonia, optic atrophy, and oculomotor abnormalities followed by cognitive disorders and epilepsy. Magnetic resonance studies in patients have shown iron overload in the globus pallidus, substantia nigra, and subcortical and periventricular regions [76], as well as abnormalities of the white matter distribution, cerebellar atrophy, and thinning of the corpus callosum.

FA2H enzymatic activity is responsible for the hydroxylation of fatty acids, which are incorporated in the sphingolipids and its impairment causes myelination defects with the consequent impairment of the axonal function [77]. Mice lacking the *Fa2h* gene, either constitutively or limited to the glia cells, showed degeneration and loss of myelin sheath in the spinal cord and in sciatic nerves [77,78].

Finally, in the N-terminus of *FA2H* is present a cytochrome b5-like heme binding domain and a fatty acid hydroxylase domain with an active site for the binding of non-heme di-iron. The presence of these domains has been suggested to allow a direct interaction with iron-containing moieties, thus indicating a possible link between gene function and iron accumulation [73], although this hypothesis is not supported by experimental evidences. A recent in silico protein modelling report demonstrated that the FAHN causative missense mutations alter the heme-binding site or disrupt the hydroxylase domain impairing the enzyme catalytic activity. However, the mechanism by which reduced *FA2H* activity generates the iron deposition was not investigated [79].

1.3.3. *C19orf12* (OMIM*614297)

Mutations in *C19orf12* cause a rare form of NBIA, transmitted as an autosomal recessive trait, denominated mitochondrial membrane protein-associated neurodegeneration (MPAN) (OMIM #624298). *C19orf12* maps on human chromosome 19 and encodes for a mitochondrial protein associated with the membranes and with a putative role in lipid metabolism [80,81].

Mutations in *C19orf12* cause other neurodegenerative disorders such as: pallido-pyramidal syndrome [82], hereditary spastic paraplegia phenotype 43 (SPG43) [83] and ALS [84]. The main clinical features of MPAN include: progressive spastic para/tetraparesis, dystonia, motor axonal

neuropathy, parkinsonisms, psychiatric symptoms, retinal abnormalities, and optic atrophy [80,85,86]. Iron accumulation is present in the globus pallidus and substantia nigra, as highlighted by MRI analysis, showing T2-weighted hypo-intensity [87]. Neuropathological analysis of patients' brains demonstrated the presence of axonal spheroids, Lewy bodies, axonal swellings, and hyperphosphorylated tau in the cortex, GP, caudate/putamen, SN, and brain stem.

In silico evaluation of the *C19orf12* protein indicated that it contains a transmembrane glycine zipper. The protein has two isoforms deriving from different start codons: the longer isoform has multiple sub-cellular localization: it is present into mitochondria, endoplasmic reticulum [88], and a small fraction in the MAM, contact regions between mitochondria and ER functionally-relevant for phospholipids metabolism [88]. The protein has high levels of expression in the brain, blood cells and adipocytes, while transcriptome analysis indicated aco-regulation with genes involved in lipid metabolism with a possible connection with CoA synthesis [80]. The function of the protein is still not clarified and the simultaneous knock-down of two *C19orf12* orthologs CG3740 and CG11671 in *Drosophila*, demonstrated a defect in climbing activity, bang sensitivity, and the presence of vacuoles in the brain and optical lobe but the absence of iron accumulation [89].

1.3.4. SCP2 (OMIM*184755) and CRAT (OMIM*600184)

Mutations in the sterol carrier protein-2 (SCP2) gene (OMIM #613724) cause a complex phenotype characterized by leukoencephalopathy, dystonia, torticollis, azoospermia, cerebellar ataxia, and gait impairment [90,91]. Brain MRI analysis showed bilateral hyperintense signals in the thalamus, indicating iron overload, butterfly-like lesions in the pons, and lesions in the occipital region. The *SCP2* gene is located on chromosome 1p32.3 and exons 1–16 encode for the sterol carrier protein X (SCPx), while exons 12–16 encode the sterol carrier protein 2 (SCP2) [92]. Both enzymes have thiolase activity required for the breakdown of branched chain fatty acids and are located into peroxisomes. The pathogenic effects are probably due to elevated levels of branched chain fatty acids in patients with SCP2 mutations [93]. Abnormal fatty-acid acyl-CoA metabolism is again suggested as the culprit for a disease belonging to the big group of NBIA, highlighting the role of lipids as a common pathogenic mechanism for this category of disorders.

Mutations in *CRAT*, coding for a protein involved in the transfer of acyl groups from carnitine to coenzyme A and in the transport of fatty acids for beta-oxidation have been recently identified in one subject with an NBIA phenotype [94]. Intensive investigations of fibroblasts derived from patients with *CRAT* and *REPS1* [92] mutations as well as fibroblasts derived from patients with *PANK2*, *PLA2G6*, *C19orf12*, and *FA2H* mutations, led to the identification of a common pathogenic mechanism shared by the different diseases. Iron overload was found to be due to abnormalities in transferrin receptor (TFR1) recycling and reduction of its palmitoylation [94]. This is the first time that a common pathogenic mechanism has been, not only hypothesized, but also experimentally demonstrated. Interestingly enough, it was also showed that the antimalarian drug artesunate, was able to increase Tfr1 palmitoylation and decrease ferritin levels in fibroblasts of different NBIA subgroups [94].

This potential therapeutic treatment of NBIA should be seriously taken into consideration and could also be relevant for other more common neurodegenerative disorders, such as Parkinson's or Alzheimer's, in which iron overload is a common finding.

1.4. Iron Homeostasis and Autophagosome/Lysosome Regulation

Three genes, whose mutations cause neurodegeneration with brain iron accumulation related to malfunctioning of the autophagosome and/or lysosome activity, will be described in this chapter and include: *WDR45*, *ATP13A2*, and possibly *AP4M1*.

1.4.1. WDR45 (OMIM*300526)

Mutations in the WD repeat domain 45 (*WDR45*) gene are responsible for β -propeller-associated neurodegeneration (BPAN) (OMIM #300894), a very peculiar disorder mapping on X chromosome,

in which the deleterious mutations are invariable de novo. Patients of both gender display similar symptoms characterized since male patients were shown to have somatic or germline mosaicism [95]. Onset of the disease is in childhood but remains somehow silent until adulthood when neurological deterioration manifests as dystonia, parkinsonism, cognitive decline, and seizures. Iron overload in GP and SN was revealed by MRI that also showed, on T1 sequence, a dark central band surrounded by a halo of hyperintense signal in SN and cerebral peduncles.

WDR45 belongs to the WD40 scaffold-protein family involved in protein-protein interactions. It is the human ortholog of the yeast *atg18*, which has been demonstrated to be crucial for the formation of the autophagosome. In fact, the phosphatidylinositol-3-phosphate binding motif present in *atg18* determines its binding to the ER membrane, allowing the formation of protein complex [96]. A possible link between WDR45 autophagosome and mitochondria has been suggested by the evidence that proteins coded by *atg* genes are found transiently associated to the outer membranes of mitochondria [97].

Recently, it was reported that a brain-specific *Wdr45* knock-out mouse model showed learning and memory defect, axonal swelling, and impaired autophagic flux. The hippocampus and caudate nucleus revealed the presence of aggregates, which stained positive for ubiquitin, but the presence of high levels of iron was not observed [98]. Finally, Ingrassia et al. [99] demonstrated in fibroblasts of two BPAN individuals under conditions of nutrient deprivation, up-regulation of the divalent metal transporter 1 (DMT1), which is essential for uptake of iron in the cells, and the simultaneous down-regulation of the Transferrin receptor (TfR1) causing accumulation of ferrous iron within the cells. TfR1 down-regulation is in line with what observed in other NBIA derived fibroblasts [94] and could again represent a common pathogenic mechanism unifying several different forms of the disease. It is possible that the presence of altered autophagy process, as observed in fibroblasts carrying WDR45 mutations, could affect the removal of proteins such as DMT1, determining iron overload [99].

1.4.2. *ATP13A2* (OMIM*610513)

Kufor-Rakeb syndrome (KRS), an extremely rare autosomal recessive disorder characterized by early onset parkinsonism, pyramidal signs, dementia, and eye movements alteration, belongs to the NBIA group since some patients show iron accumulation in basal ganglia at MRI analysis [100]. Mutations in *ATP13A2*, located on chromosome 1p36.13 and encoding for a P-type ATPase, are causative of this disorders but have also been reported in different neurodegenerative diseases, such as juvenile onset parkinsonism and dementia (PARK9), and Ceroid-Lipofuscinosis [101]. This protein works mainly in the lysosome membranes as a cation pump, although modulation of its expression results in a variety of effects ranging from reduced intracellular calcium levels in cortical neurons to mitochondrial fragmentation [102]. Patients' fibroblasts showed increased fragmentation of mitochondrial network, disrupted mitochondrial DNA integrity, increased ROS production, reduced ATP production [103]. *ATP13A2* deficiency leads to lysosomal dysfunctions, which could ultimately cause impaired degradation of substrates and damage of autophagosome clearance [104]. Alteration of autophagy, cell death, and accumulation of α -synuclein occur after down-regulation of *ATP13A2* in dopaminergic neurons and primary cortical neurons derived from mice [105].

Ablation of *Atp13a2* gene in mice causes lipofuscin and α -synuclein accumulation, gliosis, increased level of ubiquitinated proteins, endolysosomal abnormalities, and deficiency of sensory and motor coordination probably due to dopaminergic pathology [106,107].

The only experimental evidence, demonstrating a direct role of this P-type ATPase in iron intracellular handling, was reported by Rajagopalan et al. [108]. The authors identified *ATP13A2* as a gene target of HIF1 and, therefore, it is subjected to the iron-dependent regulation of the PHD2-HIF1 signaling pathway [108]. This study suggests that the perturbation of lysosomes and acidic endosomes functions, which are important for iron homeostasis, may promote iron accumulation observed in the brains of KRS patients.

1.4.3. *AP4M1* (OMIM*602296) and *REPS1* (OMIM*614825)

Iron accumulation in the globus pallidus has been recently reported in three patients from a large consanguineous Moroccan family affected by early-onset developmental delay and deterioration of motor function, tetraparesis and intellectual disability. WES analysis of the affected members revealed mutations in a gene denominated *AP4M1* [109]. Mutations in this gene have been previously described in cases with severe congenital microcephaly [110], and in an autosomal-recessive type of tetraplegic cerebral palsy with mental retardation, reduction of cerebral white matter, and atrophy of the cerebellum [111]. The clinical and neuroradiological features of the Moroccan family are not only very different from the previously reported cases, but represent the first time in which iron accumulation is documented in association with *AP4M1* mutations.

AP4M1 encodes for the mu subunit of the heterotetrameric adaptor protein complex-4 (AP-4), which is ubiquitously expressed in the CNS and involved in vesicle formation, post-Golgi protein trafficking, and sorting processes. AP-4 dysfunction might impair endosomal formation and affect autophagic process, which has already been demonstrated to play a pathogenic role in other NBIA forms related to *ATP13A2* and *WDR45* mutations.

Mutations in *REPS1* were identified by exome sequencing in two sisters, of unrelated healthy parents, presenting with typical clinical features of NBIA and iron overload in the globus pallidi and peduncles revealed by T2* evidence. MRI also showed progressive cerebellar and cerebral atrophy. *REPS1* codes for a protein involved in endocytosis and vesicle transport [94]. As previously discussed for the *CRAT* gene [92], a defective TfR1 palmitoylation is present in fibroblasts derived from *REPS1* mutant patients, and artesunate treatment is able to increase TfR1 palmitoylation and decrease steady-state ferritin levels, which could be considered as indirect evidence of total iron content reduction [92].

1.5. Iron Homeostasis in Disease Genes with Unknown Functions

1.5.1. *DCAF17* (OMIM*612515)

Woodhouse-Sakati syndrome (WSS) (OMIM #241080) is a rare disease transmitted as autosomal recessive trait and characterized by a combination of movement and metabolic impairment. Iron overload is present in the *GP* and *SN* in a fraction of affected patients [112]. The disorder is caused by mutations in *DCAF17* (*C2orf37*) gene, located on chromosome 2q31.1 and encoding for a protein located in the nucleolus. *DCAF17* is the acronym for DDB1 and CUL4-associated factor 17 and the gene belongs to the *DCAF* gene family, which encode receptor proteins for specific ubiquitin ligases involved in DNA damage and cell cycle control [113], but the exact role of the *DCAF17* protein remains to be defined.

Dcaf17 constitutive knockout mice showed male infertility due to abnormal sperm development [114]. An additional mouse model generated by CRISPR/Cas9 approach determining loss of function mutation in exon 2 of *Dcaf17*, showed also female subfertility in addition to male infertility [115]. This phenotype recapitulates hypogonadism and infertility, which are consistent findings in patients carrying *DCAF17* mutations [112] and indicates that this gene plays a role in mammalian gonadal development. It is however, not clear why loss of function mutations of *DCAF17* lead to the complex clinical presentation of affected patients and why knock-out mice only develop infertility and no other signs typical of the human disorder.

1.5.2. *GTPBP2* (OMIM*607434)

Mutations in this gene have been reported for the first time in four subjects presenting with mental retardation, ataxia and dystonic features. Cerebellar atrophy and hypointensity in the *GP* and *SN* suggestive of iron overload were detected by MRI [116]. More recently, biallelic inactivating variants in the same gene have been reported in three unrelated families with a neurological phenotype different from the previously published family and displaying agenesis of the corpus callosum without signs

of iron accumulation [117]. The *GTPBP2* is located on human chromosome 6,p21.1 and encodes for a GTP-binding protein, which could play a role as regulator and adaptor of the exosome-mediated mRNA turnover pathway [118].

In mice, a homozygous *GTPBP2* mutation interfering with the normal splicing process, determined a neurodegenerative phenotype characterized by apoptosis of neurons accompanied by locomotor dysfunctions, and degeneration of the retinal neurons [119]. These features are evident only in a mouse strain harboring an additional mutation in a gene, expressed in the brain and encoding tRNA^{Arg}_{UCU}, indicating that GTPBP-2 may play a role in mRNA surveillance and ribosome-associated quality control. A recent study aimed at understanding the role of GTPBP1 and GTPBP2, demonstrates that although GTPBP2's function involves interaction with aa-tRNA, it does not stimulate exosomal degradation and have a different function as compared to GTPBP1 [120].

2. Conclusions

Iron overload in the brain is a distinct finding in rare neurodegenerative disorders classified as NBIA, but is also observed in common neurodegenerative disorders, including Parkinson's, Alzheimer's, and ALS. Two NBIA gene products play a direct role in iron homeostasis, but whether other NBIA-related gene products also control iron metabolism in the human brain, and whether high levels of iron contribute to the progression of neurodegeneration is still a matter of investigation. An interesting study based on a systems biology approach aimed at clarifying the pathogenic mechanisms of these disorders and identifying the network connecting known NBIA transcripts with specific partners. This analysis indicated that multiple cell types contribute to the clinically heterogeneous group of NBIA disorders, revealed strong links with iron metabolism and demonstrated the presence of common pathways shared by NBIA and overlapping neurodegenerative disorders [121].

More recently, interesting unifying mechanisms relating altered iron metabolism to the different NBIA sub-types start to be experimentally proved. The impairment of transferrin receptor palmitoylation and recycling was demonstrated to be common to fibroblasts derived from patients carrying mutations in *PANK2*, *PLA2G6*, *C19orf12*, *FA2H*, and in two recently identified disease genes *CRAT* and *REPS1* [94]. An altered recycling of transferrin receptor was also independently demonstrated in *WDR45* mutant fibroblasts [99]. Additional investigations revealed that iron accumulation might be also related to dysfunctions of neural cells caused by alterations of mitochondrial activities, lipid metabolism, membrane remodeling, and autophagy [122]. Alteration of the structure of mitochondrial cristae is a common feature in NBIA sub-types [60,71] and can cause energy deficiency determined by defective assembly of respiratory chain super complexes [53,64].

Mitochondria and their interaction with the endoplasmic reticulum play a crucial role in lipid metabolism and in the formation of autophagosomes. Finally, mitochondria are crucial organelles for the regulation of iron metabolism in other neurodegenerative disorders, such as Parkinson's and Alzheimer's, but also Friedreich ataxia and X-linked sideroblastic anaemia and ataxia [72].

During the last years the research in this field have massively contributed to the clarification of the pathogenic mechanisms, which are now emerging specially for the most frequent causes of NBIA. Nevertheless, the intricate connection between all the different players acting in NBIA remains to be clarified, paving the way to the identification of tailored approaches to therapy.

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Review

Mitochondrial Targeting in Neurodegeneration: A Heme Perspective

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Abstract: Mitochondrial dysfunction has achieved an increasing interest in the field of neurodegeneration as a pathological hallmark for different disorders. The impact of mitochondria is related to a variety of mechanisms and several of them can co-exist in the same disease. The central role of mitochondria in neurodegenerative disorders has stimulated studies intended to implement therapeutic protocols based on the targeting of the distinct mitochondrial processes. The review summarizes the most relevant mechanisms by which mitochondria contribute to neurodegeneration, encompassing therapeutic approaches. Moreover, a new perspective is proposed based on the heme impact on neurodegeneration. The heme metabolism plays a central role in mitochondrial functions, and several evidences indicate that alterations of the heme metabolism are associated with neurodegenerative disorders. By reporting the body of knowledge on this topic, the review intends to stimulate future studies on the role of heme metabolism in neurodegeneration, envisioning innovative strategies in the struggle against neurodegenerative diseases.

Keywords: neurodegeneration; mitochondria; therapy; heme; haem

1. Implication of Heme in Neurodegeneration

Heme is a molecule composed by protoporphyrin IX and iron produced by all the cells in the organism, including neurons. Heme mediates a series of functions that encompass oxygen transport, the regulation of gene expression and the modulation of enzyme activity, just to cite the most relevant ones. Moreover, heme can also promote oxidative stress, thus performing as a double-face molecule with both positive and negative properties [1]. This concept is also true for neuronal cells. Indeed, on one hand heme is required for the survival and differentiation of neuronal cells, as demonstrated by the observation that heme deficiency interferes with neurite outgrowth in nerve growth factor (NGF)-induced PC12 cells [2,3] and results in apoptosis in PC12 pheochromocytoma cells, SHSY5Y neuroblastoma cells and U373 astrocytoma cells, as well as in rat primary hippocampal neurons [2–4]. However, on the other hand, an excess of free-heme is associated with neurodegeneration. The large amount of hemoglobin and heme released in the brain during intracerebral or subarachnoid hemorrhages promotes oxidative stress, lipid peroxidation, inflammatory response and finally, neuronal cell death [5–7]. Moreover, loss of the heme scavenger hemopexin (Hx) causes defective myelination in mice [8–10]. Furthermore, impairment of cellular heme export reduces SHSY5Y cells survival [11]. Together, these data indicate that both heme deficiency and excess are deleterious for the survival of neuronal cells (Figure 1), thus suggesting that heme levels must be finely controlled both at the systemic and cellular level. At the systemic level, circulating free-heme is scavenged by the plasma proteins haptoglobin and hemopexin [6–8,10,12–14]. However, at cellular level, the amount of intracellular free-heme (*labile heme* or *heme regulatory pool*) is regulated at multiple steps: heme synthesis, incorporation into hemoproteins, catabolism, import and export [1,15].

Although this tight regulation has been extensively studied in non-neuronal cells, similar mechanisms likely occur in the nervous system. Indeed, the main proteins involved in the control of labile heme are also expressed in the nervous system [16].

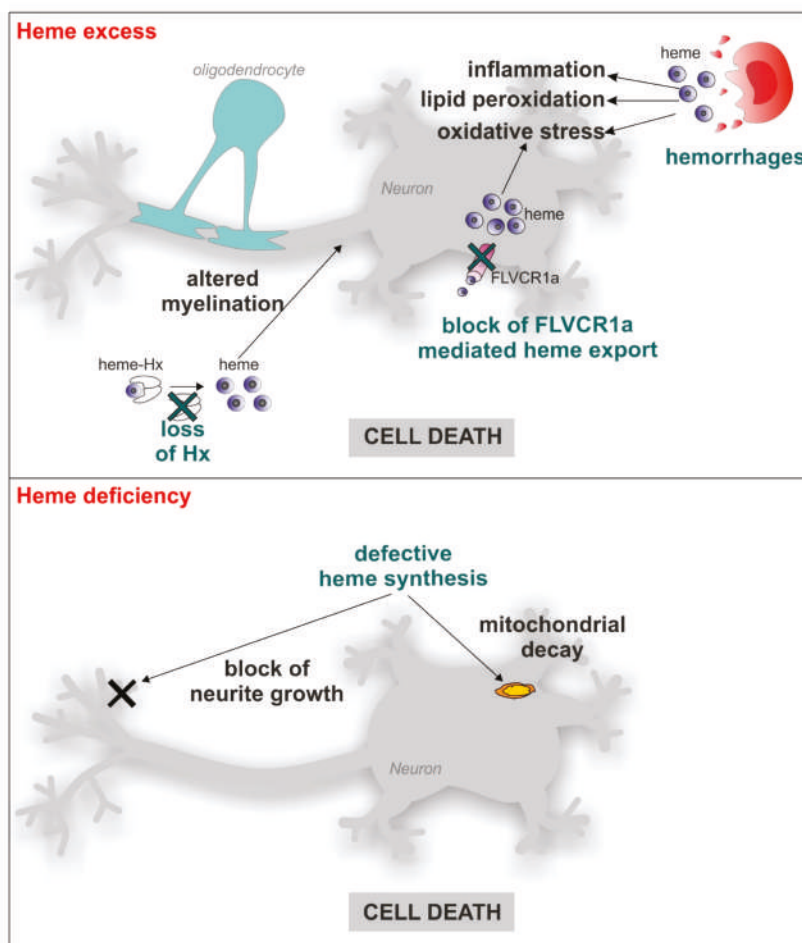


Figure 1. Implication of heme in neurodegeneration. Both heme excess and heme deficiency contribute to neurodegeneration. Heme released during hemorrhages leads to inflammation, lipid peroxidation and oxidative stress; the loss of the heme scavenger Hx causes defective myelination of axons; the impairment of intracellular heme export by FLVCR1a is associated with increased oxidative stress. On the other hand, heme deficiency, due to defective synthesis, leads to mitochondrial decay and the blocking of neurite growth. These events all result in neuronal cell death. In the figure, neurons are represented as the main target for heme-mediated effects; however, other cell types of the nervous system could be affected by the same phenomena.

Neurodegenerative disorders are a common and growing cause of mortality and morbidity worldwide [17]. Recently, a series of rare neurodegenerative disorders have been directly linked to alterations of heme metabolism (see Table 1). Defective heme synthesis causes porphyrias, some of which are associated with a wide array of neurological disturbances involving both the central and peripheral nervous systems (neuropathic porphyria). Neuropathic porphyria includes acute

intermittent porphyria (AIP), hereditary coproporphyria (HCP), variegate porphyria (VP) and 5-aminolevulinatase dehydratase deficiency (ALAD deficiency) [18–20]. Furthermore, reduced heme synthesis has been observed in Friederich Ataxia (FRDA), an autosomal recessive disorder caused by mutations in Frataxin (FXN), a mitochondrial iron chaperone involved in iron-sulfur (Fe-S) clusters and heme biosynthesis [21–24]. Finally, reduced heme synthesis has been observed during aging [4,25].

Table 1. Rare neurodegenerative disorders linked to defective heme metabolism.

<i>Disease</i>	<i>Gene</i>	<i>Inheritance</i>	<i>Clinical Features</i>	<i>OMIM</i>
ALAD deficiency	5-aminolevulinatase dehydratase (ALAD)	autosomal recessive		612740
Acute intermittent porphyria (AIP)	Hydroxymethylbilane synthase (<i>HMB</i> S)	autosomal dominant	Neuropathic Porphyria: acute neurovisceral attacks involving severe abdominal pain, peripheral neuropathies and psychiatric disturbances	176000
Hereditary coproporphyria (HCP)	Coproporphyrinogen oxidase (<i>CPO</i> X)	autosomal dominant		121300
Variegate porphyria (VP)	Protoporphyrinogen oxidase (PPOX)	autosomal dominant		176200
Friederich Ataxia (FRDA)	Frataxin (FXN)	autosomal recessive	Progressive gait and limb ataxia associated with cardiomyopathy and diabetes	229300
Posterior Column Ataxia and Retinitis Pigmentosa (PCARP)	Feline Leukemia Virus Subgroup C Receptor 1 (FLVCR1)	autosomal recessive	Sensory ataxia and retinitis pigmentosa	609033
Non syndromic Retinitis pigmentosa (RP)	Feline Leukemia Virus Subgroup C Receptor 1 (FLVCR1)	autosomal recessive	Retinitis pigmentosa	268000
Hereditary Sensory and Autonomic Neuropathy (HSAN)	Feline Leukemia Virus Subgroup C Receptor 1 (FLVCR1)	autosomal recessive	Loss of pain perception	201300
Fowler syndrome (PVHH)	Feline Leukemia Virus Subgroup C Receptor 2 (FLVCR2)	autosomal recessive	Proliferative glomerular vasculopathy in the central nervous system associated with severe hydrocephaly, ventriculomegaly, cortical thinning and hypoplastic cerebellum.	225790

In addition, other rare neurodegenerative disorders have been associated with defective heme transport across membranes. Several proteins are involved in this process [1,15]. Among them, Feline Leukemia Virus Subgroup C Receptor 1 (FLVCR1) and 2 (FLVCR2) are implicated in heme export and import, respectively [11,26–31]. Mutations in the heme exporter FLVCR1 are associated with three distinct disorders affecting the sensory nervous system: posterior column ataxia and retinitis pigmentosa (PCARP) [32–34], non-syndromic retinitis pigmentosa (RP) [35,36] and hereditary sensory and autonomic neuropathy (HSAN) [11,37]. Mutations in the heme importer FLVCR2 are responsible for the Fowler syndrome, a proliferative glomerular vasculopathy [30,38,39].

Furthermore, several lines of evidence suggest that heme may also contribute to the pathogenesis of common neurodegenerative disorders. The deregulation of enzymes critically involved in heme synthesis has been reported in both Alzheimer’s disease (AD) and Parkinson’s disease (PD). Reduced 5-aminolevulinatase synthase 1 (ALAS1) and porphobilinogen deaminase (PBGD) mRNA were observed in AD brains [40], suggesting decreased heme synthesis rates in AD. Moreover, heme deficiency has been reported in the brain of patients with AD [41]. It has been proposed that heme deficiency may arise from either decreased heme synthesis rates or heme depletion as a consequence of heme binding to amyloid- β [41–43]. However, increased Ferrochelatase (FECH) levels were reported in another study [41].

Heme binding to α -Synuclein has also been reported [44], suggesting that heme depletion may also occur in PD. In addition, blood transcriptomic meta-analysis showed downregulation of 5-aminolevulinatase synthase 2 (ALAS2) and FECH in PD [45]. However, within PD erythroid cells, α -synuclein gene (SNCA) was co-expressed with crucial enzymes involved in heme metabolism, including ALAS2, FECH and biliverdin reductase B (BLVRB) [46]. Moreover, increased striatal 5-aminolevulinatase dehydratase (ALAD) activity was observed in the MPTP-induced mouse model of PD, indicative of increased heme synthesis rates [47]. It is difficult to conclude from these studies

whether heme synthesis is increased or reduced in AD and PD. Data are still controversial due to the low amount of human samples analyzed and the different experimental approaches adopted. Further studies are required to definitively determine heme synthesis rates in these pathological conditions. Mouse models of AD and PD will be extremely useful to analyze in detail the role of heme metabolism in these disorders.

A role for heme derived from extracellular sources in the pathogenesis of neurodegenerative disorders has also been proposed. The heme scavenger Hx has been found strongly increased in the cerebrospinal fluid of AD patients [48–50]. Similarly, altered expression of the hemoglobin scavenger haptoglobin was shown in AD [51,52], PD [53,54] and Huntington’s disease (HD) [55]. The induction of heme oxygenase 1 (HO1), the enzyme responsible for heme degradation, is common in patients affected by neurodegenerative conditions. Although HO1 is induced by a plethora of stimuli, it has been proposed that HO1 overexpression in AD and PD may be a consequence of increased brain-blood barrier permeability and hemoglobin-derived heme levels in AD and PD [16]. Moreover, increased expression of HO1 was reported in a mouse model of amyotrophic lateral sclerosis (ALS) [56] and ALS patients [57].

All the reported examples highlight the importance of the maintenance of heme homeostasis in the context of neurodegeneration. However, the molecular mechanisms underlying these disorders and the precise mechanism through which heme participates to them remains elusive and requires further investigations.

2. Role of Mitochondria in Neurodegenerative Diseases

Neurodegeneration can be elicited by several systems. Among them, mitochondrial-dependent processes have become increasingly relevant [58–63]. Several mechanisms account for mitochondria-dependent neurodegeneration (see Figure 2 for a graphic list of them) and below we attempt to summarize the most important ones.

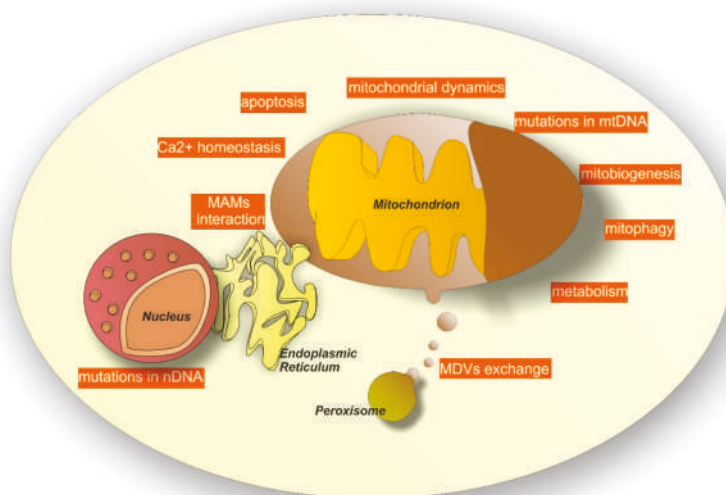


Figure 2. Mitochondrial dependent mechanisms in neurodegeneration. Mitochondria contribute to neurodegeneration by several mechanisms, including alterations in calcium homeostasis, mitochondrial

biogenesis (mitobiogenesis), mitochondrial dynamics, metabolism and mitophagy. Moreover, mutations in mitochondrial DNA (mtDNA) and inappropriate activation of apoptosis can be alternative mechanisms. Finally, additional systems include mutations in nuclear DNA (nDNA) at the level of genes encoding for mitochondrial proteins, the compromised exchange of mitochondria-derived vesicles (MDVs) among mitochondria and peroxisomes and the inefficient interaction among mitochondria and the endoplasmic reticulum at the level of mitochondrial associated membranes (MAMs).

2.1. Mutations on Mitochondrial DNA (mtDNA) Genes

Mitochondria contain their own genome, which is made of multiple copies of a circular double stranded molecule, which is 16.6 kb long in humans. It comprises 37 genes, 13 encoding for proteins involved in adenosine triphosphate (ATP) production and the other 24 encoding for two rRNAs and 22 tRNAs. Cells contain thousands of molecules of mtDNA and the majority of them have the same sequence, a condition known as homoplasmy. Inefficient mtDNA repair, localized oxidative environment and increased replication, however, can promote mtDNA mutations that, due to the polyploidy nature of mtDNA, often co-exist with their wildtype counterpart in various proportions (a condition termed heteroplasmy). mtDNA mutations are usually responsible for defects in the respiratory chain functions, but only if they are present above a certain threshold level.

The replication of mtDNA occurs independently on cell cycle, and a particular mtDNA molecule may be strongly replicated (or not at all) during cell division. Moreover, replication occurs also in postmitotic cells. These phenomena account for the clonal expansion of mutated mtDNA molecules and in association with heteroplasmy, result in mosaicism, with the levels of mutated mtDNA varying dramatically between tissues in the same organism and in different regions of the same tissue.

Somatic mtDNA mutations accumulate during a person's lifetime and undergo clonal expansion, so aging is typically associated with mosaic occurrence of respiratory chain-deficient cells in tissues [64].

Mitochondrial reactive oxygen species (ROS) production is the major cause for the higher mtDNA nucleotide instability when compared with nDNA.

Mitochondrial DNA mutations potentially affect tissue that require a large amount of ATP to function, such as heart and brain. Some haplogroups [65] (evolutionary selected population subgroups carrying neutral single-base pair variants of mtDNA) have been associated with susceptibility to a variety of human diseases, including age-related neurodegenerative disorders such as PD and AD. Moreover, inherited point mutations and sporadic rearrangements on mtDNA have been described in association with neurodegeneration [65–69].

Although the presence of mtDNA deletions below a certain threshold is not sufficient to induce PD, small changes inside the genome of mitochondria could represent a risk factor for this pathology [70–74]. In addition, the accumulation of mutations in mtDNA over the course of PD has been observed to correlate with severity and burden of the disease [75]. Accumulation of mtDNA damage is also considered a possible mechanism of neurodegeneration over the course of HD [76]. Moreover, mtDNA mutations have been associated with AD [77], although the degree of mtDNA damage does not seem to correlate with the severity of AD symptoms [78].

As stated before, mtDNA alterations often result in defects on a particular component of the electron transport chain (ETC). The three main mechanisms through which mtDNA damage can contribute to neurodegeneration are therefore consequences of ETC alteration and include the decrease of ATP synthesis, the increase of ROS production and the enhanced sensitivity to neurotoxins associated to ETC disruption.

2.2. Mutations on Nuclear DNA Genes Encoding Proteins Crucial for Mitochondrial Functionality

Besides mutations on mitochondrial DNA, mutations in nuclear DNA (nDNA) at the level of genes encoding for mitochondrial proteins have also been associated with neurodegenerative disorders. For example, many of the identified ALS genes have a role in mitochondrial-associated functions (see [79] for a comprehensive description on this topic). In addition, mitochondrial dysfunction

and oxidative stress in PD have been linked to mutations in genes encoding for parkin RBR E3 ubiquitin protein ligase (PRKN, commonly referred to as parkin), PTEN induced putative kinase 1 (PINK1, a protein that acts in the same pathway of parkin) and parkinsonism associated deglycase (DJ-1) [80], just to cite some of them. Parkin promotes autophagy of damaged mitochondria [81] and its deficiency is associated with defects in mitochondria morphology [82] and low levels of proteins involved in mitochondrial functions, thus resulting in decreased mitochondrial respiration [83]. Similarly, mutations in *PINK1* gene lead to decreased mitochondrial respiration [84] and alterations in mitochondria functions [85–88]. Regarding DJ-1, this protein localizes into mitochondria [89] and exerts crucial antioxidant functions [80,90–92]. Mutations in DJ-1 impair mitochondrial respiration, reduce mitochondrial membrane potential, increase ROS within the mitochondria and alter mitochondrial morphology [93]. Also, nDNA encoded proteins implicated in AD, like presenilin-1 and presenilin 2 (PSEN1 and 2) are related to mitochondria [94,95].

2.3. Alterations of Mitochondrial Dynamics (Fusion, Fission, Motility)

Mitochondria are not rigidly structured. They form a complex reticulum that undergoes regulated processes of fusion (the combination of two smaller mitochondria into a single organelle) and fission (the division of one large mitochondrion into two smaller fragments). Fusion allows mitochondria to mix their contents, enabling protein complementation, mtDNA repair and equal distribution of metabolites. Fission facilitates equal segregation of mitochondria into daughter cells during cellular division, enhances the distribution of mitochondria along cytoskeletal tracts and participates in the targeting of damaged segments of mitochondria to the autophagic process. Fusion and fission also contribute to the movement of mitochondria necessary for mitochondria distribution along neuronal axons and dendrites. Mitochondrial dynamics are crucial for neurotransmission, synaptic maintenance and neuronal survival. Proper mitochondrial trafficking is particularly important in neurons compared to other cell types, due to their exceptional cellular morphology. Indeed, neurons extend their axons and dendrites for very long distances that, in the case of human peripheral nerves or corticospinal tracts, extend up to a meter. Thus, the neuron represents an extreme case of mitochondrial distribution: dysfunctions in mitochondrial distribution that are not dangerous for other cells could be fatal for neuronal survival [96].

Alterations in mitochondria motility have been reported in several neurodegenerative disorders and neuropathies [97,98]. Aberrant activity of the fission-fusion machinery contributes to the pathogenesis of PD [99–102]. Moreover, alterations of mitochondrial dynamics have been observed in AD [61,102] and HD [100,103]. Particularly in HD, mitochondrial fission is promoted and mitochondrial fusion proteins are downregulated as the severity of the pathology increases [104]. Finally, defects in mitochondrial dynamics and disruption of the axonal transport of mitochondria have been reported in ALS [60,105–107].

2.4. Inappropriate Activation of Cell Apoptosis by Mitochondria

Mitochondria are pivotal organelles for the execution of apoptosis. The inappropriate activation of apoptosis leads to the disruption of the cellular proliferation-death balance. Neurodegenerative disorders are believed to partly depend on alterations of this equilibrium. *PINK1* loss-of-function mutations lead to early signals for apoptosis, promoting neurodegeneration in the context of PD [108]. Moreover, the low levels of *PTPA* (phosphotyrosyl phosphatase activator) observed in AD affected-people contribute to induce cell apoptosis in the brain of these patients [109]. Furthermore, caspase-6, an effector of the caspase-dependent apoptotic pathway, is known to be involved in the cleavage of mutant huntingtin resulting in neurodegeneration in HD patients [110]. Finally, in ALS the mutant *SOD1* can trigger cytochrome c release from mitochondria to operate apoptosis [111].

2.5. Alteration of Mitochondria-Dependent Ca^{2+} Homeostasis

An additional neurodegenerative mechanism related to dysfunctions of mitochondria concerns the modulation of calcium. Mitochondria are involved in Ca^{2+} homeostasis as they are able to both accumulate and release Ca^{2+} . Mitochondrial Ca^{2+} concentration is fundamental for the regulation of specific mitochondrial key functions, such as the apoptotic process and the activity of several mitochondrial enzymes. The deregulation of Ca^{2+} homeostasis is a hallmark of different neurodegenerative diseases including PD, AD, HD and ALS [112,113]. Moreover, alterations of calcium levels have been observed in neuropathies. Neuropathic pain phenotypes include chemotherapy induced neuropathy, diabetic neuropathy, human immunodeficiency virus (HIV)-associated neuropathy and Charcot-Marie-Tooth neuropathy. Neuropathies have been associated with mitochondrial dysfunctions [63], and particularly in diabetic neuropathy, impaired cellular calcium homeostasis, including alterations in mitochondrial Ca^{2+} concentration, has been reported. Indeed, in the context of diabetes, sensory neurons, above all the lumbar dorsal root ganglia neurons (which have the longest axons), show an increased intracellular Ca^{2+} concentration that triggers elevated mitochondrial Ca^{2+} levels. This condition induces mitochondrial membrane depolarization and can favor the generation of reactive oxygen species (ROS) and oxidative stress, as well as alterations in mitochondrial functionality that can ultimately lead to neuronal damage [114].

2.6. Additional Alterations of Mitochondrial-Related Processes: Biogenesis, Mitophagy, MDVs Exchange, Interaction with Mams, Control of Cellular Metabolism

Besides the mechanisms reported above, a series of additional mechanisms involving mitochondria have been described. Among them, neurodegeneration has been associated with the impairment of mitochondrial biogenesis. Particularly, the deficit of peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α), a key regulator of mitochondrial biogenesis, has been associated with HD, PD and AD [115–120].

Mitophagy is also linked to neurodegenerative disorders [121–123]. Mitophagy is the selective autophagic process responsible for the elimination of damaged or excess mitochondria. In this process, a peculiar role is played by PINK1, that recruits parkin to dysfunctional mitochondria, where it induces their degradation by mitophagy [124]. Thus, it is not surprising that in addition to other neurodegenerative disorders, defective mitophagy is highly implicated in PD and is considered one of the major pathological mechanisms of mitochondrial dysfunction in autosomal recessive forms of PD [125].

Mitophagy is a cellular process that eliminates whole mitochondria, but other mechanisms exist to partially eliminate portions or components of mitochondria [122]. Mitochondria-derived vesicles (MDVs) exchange between mitochondria and peroxisomes represent one of these possible mechanisms. MDVs are crucial for the transport of cargo from mitochondria to peroxisomes. This process is regulated by the retromer complex. Studies on vacuolar protein sorting 35 (VPS35), a component of the retromer complex, indicate that mutations or alterations in VPS35 expression are associated to PD [126,127]. Moreover, it has been demonstrated that Parkin and PINK1, two genes highly implicated in PD, play crucial roles in the control of this process [128,129].

Other than with peroxisomes, mitochondria also physically interact with other subcellular organelles to ensure efficient and rapid metabolism and signaling. For example, the interaction between mitochondria and the endoplasmic reticulum (ER) occurs at the level of MAMs (mitochondrial associated membranes), a subdomain of the ER. The proteins involved in neurodegenerative diseases such as DRP1 (dynamin related protein 1) and MFN2 (mitofusin 2) are enriched in MAMs [130,131] and the perturbation of mitochondria-ER contacts has been described in neurodegenerative disorders, including PD, AD and ALS [132].

A further mitochondrial-dependent mechanism has been highlighted in neurodegeneration. This is the impairment of cell metabolism [133]. Mitochondria are the main energy-producing organelles of the cell, thus any process impairing mitochondrial functionality may lead to metabolic switching

aimed to compensate for their decreased ATP production. Among the different neurodegenerative disorders, many lines of evidence suggest that mitochondria-dependent mechanisms are responsible for the metabolic changes observed in dopaminergic neurons in the context of PD. Mitochondrial ROS are particularly abundant in dopaminergic neurons due to dopamine oxidative metabolism, enhanced Fenton's reaction initiated by the high iron content of these cells, and the high rate of ATP production required to sustain the activity of a particular L-type voltage dependent Ca^{2+} channel expressed by these neurons. An excess of mitochondrial ROS can induce a series of cellular modifications, including hypoxia inducible factor 1α (HIF1 α)-dependent up-regulation of glucose transporters [134], favoring the switch of energy metabolism towards glycolysis. When sustained for a long period, this metabolic change can be deleterious for dopaminergic neurons. Indeed, neurons need to deliver glucose in the pentose phosphate pathway (PPP), a process that produces NADPH (nicotinamide adenine dinucleotide phosphate), crucial for the recycling of the antioxidant glutathione. PPP is especially important in neurons as these cells show less robust antioxidant systems and are more vulnerable to oxidative stress than other cell types. Switching from PPP to glycolysis promotes oxidative stress and, consequently, neurodegeneration [135].

3. Heme and Mitochondrial Dysfunction Related to Neurodegenerative Diseases

The examples reported above sustain the notion that mitochondrial dysfunctions play a critical role in neurodegenerative disorders. The mitochondrion is a critical organelle for cells, representing a crossroad for a plethora of reactions contributing to a variety of metabolic processes, including heme metabolism. Considering the impact of impaired heme homeostasis in several neurodegenerative disorders and the interesting potential that heme takes on for the research in the context of neurodegeneration, it is curious to notice that heme is an under-investigated molecule in the field. This discrepancy could be due to the lack of knowledge on the possible systems through which heme can influence pivotal processes implicated in neurodegeneration. Among the possible ways, it is tempting to speculate that heme could directly or indirectly affect many of the mitochondria-dependent mechanisms of neurodegeneration described in the previous paragraphs. Indeed, the relationship between heme and mitochondria is based on several elements (Figure 3): heme is produced through a series of reactions that occur partly in the mitochondria and partly in the cytosol [136]; heme acts as a cofactor for cytochromes c and cytochromes in complexes II-III-IV of the mitochondrial ETC [137]; heme has been reported to directly or indirectly influence ATP translocation between mitochondria and cytosol [138–140] mediated by adenine nucleotide translocases (ANTs); finally, heme biosynthesis is considered a cataplerotic pathway for the Krebs's cycle due to the fact that the first step of heme production consumes succinyl-CoA [141,142]. Thus, modulation of heme homeostasis can affect mitochondrial functions.

Considering the mechanisms by which mitochondria contribute to neurodegeneration, it is interesting to note that heme levels can influence iron homeostasis, and both iron deficiency and iron excess are reported to cause damage on mitochondria [143,144] and on mitochondrial DNA [145,146]. Moreover, a decrease in heme itself leads to mitochondrial decay [4,147].

Furthermore, a connection exists between heme and nDNA genes encoding mitochondrial proteins typically implicated in neurodegenerative disorders. Indeed, it has been demonstrated that amyloid precursor protein, particularly when mutated, interacts and negatively regulates the heme-degrading enzyme HO1 [148]. Also PINK1 mutation is related to alterations in HO1 expression [149]. Moreover, DJ1 regulates nuclear factor-E2-related factor 2 (NRF2) [150], a key transcription factor for the induction of HO1 expression [151].

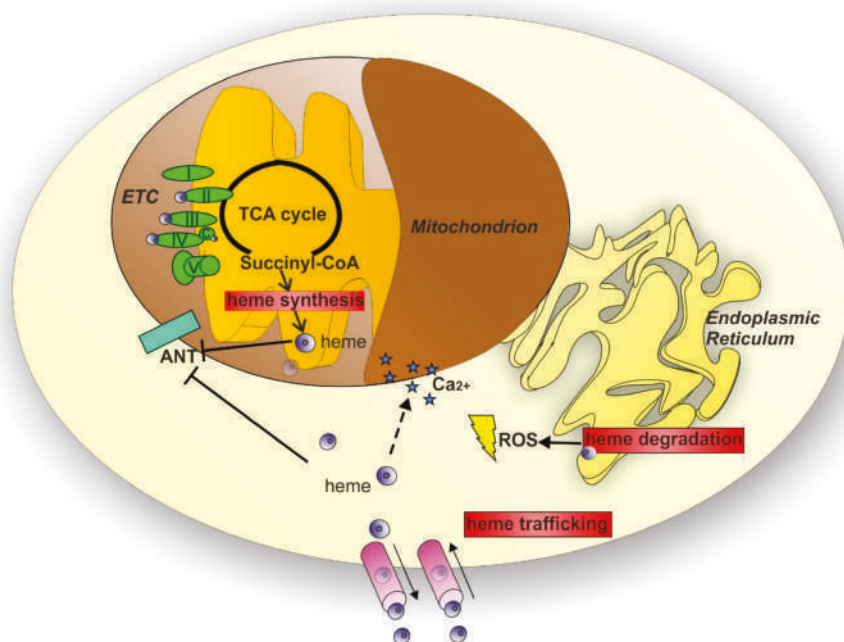


Figure 3. The “heme-mitochondria” relationship and the putative heme-related targets for the therapy of neurodegenerative disorders. Heme and mitochondria share a strong relationship based on several elements: heme synthesis occurs partly in the mitochondrion and acts as a cataplerotic pathway for the Krebs’s cycle; heme is a cofactor for cytochromes c and cytochromes in complexes II-III-IV of the mitochondrial ETC [137]; heme influences the ATP translocation between mitochondrion and cytosol mediated by adenine nucleotide translocases (ANTs); heme export influences calcium (Ca^{2+}) flux in mitochondria. Therefore, modulation of heme metabolism can lead to modification of mitochondrial functions. The control of intracellular heme levels is achieved by a balance among synthesis, catabolism and proper trafficking of heme. Thus, all these processes (highlighted with red boxes in the figure) represent putative good targets for the therapy of neurodegenerative disorder.

In addition, heme and the heme-degrading enzyme HO1 are implicated in the regulation of mitophagy, mitochondrial biogenesis and morphology [152–154].

In endothelial cells, it has been demonstrated that alterations in heme metabolism, in addition to promoting lipid peroxidation and activation of autophagy, induce mitophagy and apoptosis, indicating mitochondrial dysfunction [155]. Similarly, FLVCR1 loss is associated with alterations in mitochondrial morphology in human microvascular endothelial cells [26].

Furthermore, FLVCR1-deficient HeLa cells show impaired mitochondrial calcium uptake [156].

These data have been obtained in non-neuronal cells. However, similar mechanisms could also occur in neuronal cells.

Finally, compromised ETC complexes activity has been observed in the brain of a mouse model for acute intermittent porphyria, a type of porphyric neuropathy caused by alterations of heme biosynthesis [157]. Moreover, in three cases of Fowler syndrome it was suggested to be the presence of a defect in complex III and IV of the ETC [158,159].

These examples directly suggest that a connection between heme-mitochondria-neurodegeneration exists and open the possibility that future studies on this topic will further strengthen this notion.

4. Current Therapies and Potential Future Approaches to Face Mitochondrial Dysfunction in Neurodegenerative Diseases

Currently, there is no cure for reversing neurodegeneration and the treatment of neurodegenerative disorders is mainly symptomatic [160]. In order to face neurodegeneration, several pathways could be targeted to improve and/or restore mitochondrial functions, including mitochondrial biogenesis and metabolic flexibility, mitochondrial dynamics and mitophagy [161].

There are several pharmacological approaches to induce mitochondrial biogenesis and metabolic flexibility, that is the ability to switch from one fuel source to another. Several compounds have been generated to target the upstream sensors of energy production, including AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR) and sirtuins, or downstream transcriptional factors and co-factors, such as nuclear receptors, nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM) [161]. The therapeutic potential of these drugs has been evaluated mostly in the context of metabolic diseases, but also seems promising for neurodegeneration. For example, resveratrol is a natural compound that mimics caloric restriction and activates the sirtuin family of histone deacetylases. In humans, resveratrol improves mitochondrial function in obese patients and type 2 diabetes [162]. Furthermore, resveratrol counteracts neurodegeneration in worms and mice [163].

As described above, the disruption of the balance between mitochondrial fusion and fission contributes to neurodegeneration. Therefore, targeting mitochondrial dynamics represents another important strategy to improve mitochondrial function in neurodegenerative diseases. Strategies aimed at increasing mitochondrial fusion or inhibiting mitochondrial fission might improve mitochondrial function and are therapeutically interesting. The promotion of mitochondrial fusion by the overexpression of key components of the fusion machinery, like MFN2 or OPA1, rescues ATP production and mitochondrial morphology in a cellular model of PD [164]. Similar results were obtained with the inhibition of fission through the genetic deletion of DRP1 [164,165]. Although the understanding of the regulation of mitochondrial dynamics is still in its infancy, novel compounds have been identified to promote fusion or inhibit fission [166]. M1 hydrazone [167] and S3-derivative [168] promote mitochondrial fusion in cells deficient for mitofusin 1 (MFN1) and MFN2. Mdivi-1 (mitochondrial division inhibitor) attenuates fission in yeast and mammalian cells by inhibiting DRP1. In vitro, Mdivi-1 delays apoptosis by inhibiting mitochondrial outer membrane permeabilization and blocking cytochrome c release from mitochondria [169]. The therapeutic potential of Mdivi-1 seems promising for neurodegenerative disorders. Indeed, the administration of Mdivi-1 in mouse and cellular models of PD attenuates disease-associated phenotypes [165,170]. Although initially reported as an inhibitor of fission, Mdivi-1 was recently reported to reversibly inhibit complex I in a DRP1-independent manner [171]. The complete inhibition of complex I in vivo would be expected to cause neurodegeneration. Indeed, rotenone completely inhibits complex I, induces ROS levels and causes parkinsonian neurodegeneration in mice [172]. In contrast, Mdivi-1 lacks neuronal toxicity in vivo and is neuroprotective. This is likely due to the ability of Mdivi-1 to attenuate complex I-dependent reverse electron transfer (RET)-mediated ROS production. Indeed, Mdivi-1 fails to increase ROS levels in intact neurons and in isolated brain mitochondria [171].

The accumulation of dysfunctional mitochondria is another key event in several neurodegenerative conditions [173,174]. In this context, mitophagy is essential for the maintenance of mitochondrial integrity. As stated before in this review, the impairment of autophagy/mitophagy is common in neurodegenerative disorders. Therefore, mitophagy may be an additional pathway amenable for therapeutic intervention to ameliorate mitochondria function and counteract neurodegeneration [161,175]. Interestingly, both genetic and pharmacological induction of the mitochondrial autophagy receptor Nip3-like protein X (NIX) restores mitophagy in patient-derived fibroblasts [176].

Considering the crucial role of heme in maintaining mitochondrial function, it is tempting to speculate that targeting heme metabolism might be a promising strategy for the treatment of

neurodegenerative diseases. Multiple approaches can be used to target heme metabolism (Figure 3); theoretically, targeting any of the enzymes involved in the heme biosynthetic pathway or proteins involved in the control of the intracellular heme pool may be a good strategy. Among these methods, HO1 represents a potentially interesting target. Due to its anti-oxidant and anti-inflammatory properties, HO1 plays a well-established neuroprotective role. The improvement of HO1 expression has been initially proposed for neurodegenerative conditions [177]. However, it has been reported that the overexpression of HO1 induces oxidative mitochondrial damage [178,179] and macroautophagy [179] in cultured astroglia. More importantly, HO1 induction has been associated with the later phases of neurodegeneration [180] and the deletion of HO1 has been proposed as a therapeutic option [181]. HO1 activity can be suppressed by synthetic metalloporphyrin compounds that unfortunately present important limitations [182]. However, novel HO1 inhibitors have been synthesized to overcome these side effects. Interestingly, these inhibitors confer neuroprotection in a mouse model of AD [182]. Considering the complex role of HO1 in neurodegeneration and the still controversial data reported in literature [180], further work is needed to fully elucidate the therapeutic potential of HO1 targeting. Recently, long-term 5-aminolevulinic acid (ALA) treatment has been exploited as a therapeutic approach in a mouse model of AD. Omori C. et al. reported that the oral administration of ALA increased cytochrome *c* oxidase (COX) activity and protein expression as well as mitochondrial membrane potential in the brain of treated mice [183]. Additional studies are required to understand more in detail the functional consequences of ALA administration in AD pathogenesis and the translation of this therapeutic approach to other neurodegenerative disorders. Considering that ALA formulations are already used for photodynamic therapy in a variety of cancer types [184], results obtained by Omori C. et al. are extremely encouraging for therapeutic purposes and further research in this direction is desirable.

5. Conclusions

The information reported over the course of the present review showed that mitochondria participate to neurodegenerative disorders by different mechanisms, encompassing DNA mutations, mitobiogenesis, mitophagy, mitochondrial dynamics, metabolism and mitochondrial interactions with other organelles. The literary contributions on the role of mitochondria in neurodegeneration are constantly growing and the present review attempted to make an excursus on the most important mechanisms by which these crucial organelles contribute to neurodegenerative diseases, with the awareness that not all the wide literature on this topic has been covered. Some of these mechanisms are currently considered as strategic targets for pharmacological interventions to counteract neurodegeneration. However, the investigation on additional elements contributing to the control of mitochondrial functions in neuronal cells will offer a wider window of intervention. In this perspective, heme metabolism provides an interesting opportunity. Indeed, as reported above in the review, heme participates to crucial processes occurring in mitochondria, influencing their functions and properties. The tight relationship between heme and the mitochondrion has been curiously underestimated and poorly investigated in the context of neurodegeneration. However, the implication of heme in crucial mitochondrial functions and the involvement of heme in a subset of neurodegenerative diseases strongly suggest an implication of heme in these disorders.

The comprehension of these mechanisms will allow the consideration of possible therapies based on the targeting of heme metabolism as an additional option to promote mitochondrial function. Alternatively, targeting heme metabolism may improve the efficacy of other drugs targeting mitochondria. The hope is that the understanding of the role of heme in mitochondria and its implication in neurodegeneration will open new perspectives in the struggle against neurodegenerative diseases.

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Review

Reductive Mobilization of Iron from Intact Ferritin: Mechanisms and Physiological Implication

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Abstract: Ferritins are highly conserved supramolecular protein nanostructures composed of two different subunit types, H (heavy) and L (light). The two subunits co-assemble into a 24-subunit heteropolymer, with tissue specific distributions, to form shell-like protein structures within which thousands of iron atoms are stored as a soluble inorganic ferric iron core. In-vitro (or in cell free systems), the mechanisms of iron(II) oxidation and formation of the mineral core have been extensively investigated, although it is still unclear how iron is loaded into the protein in-vivo. In contrast, there is a wide spread belief that the major pathway of iron mobilization from ferritin involves a lysosomal proteolytic degradation of ferritin, and the dissolution of the iron mineral core. However, it is still unclear whether other auxiliary iron mobilization mechanisms, involving physiological reducing agents and/or cellular reductases, contribute to the release of iron from ferritin. In vitro iron mobilization from ferritin can be achieved using different reducing agents, capable of easily reducing the ferritin iron core, to produce soluble ferrous ions that are subsequently chelated by strong iron(II)-chelating agents. Here, we review our current understanding of iron mobilization from ferritin by various reducing agents, and report on recent results from our laboratory, in support of a mechanism that involves a one-electron transfer through the protein shell to the iron mineral core. The physiological significance of the iron reductive mobilization from ferritin by the non-enzymatic FMN/NAD(P)H system is also discussed.

Keywords: ferritin; iron mobilization; chaotropes; flavin nucleotide; electron transfer; kinetics

1. Introduction

Iron is an essential element required for virtually all living organisms and is involved in numerous biological reactions including respiration, oxygen transport, electron transfer, oxidative metabolism, deoxyribonucleotide synthesis, and gene regulation [1,2]. Due to the wide range of redox potentials of $\text{Fe}^{3+}/\text{Fe}^{2+}$ metal complexes (from approximately -500 to $+600$ mV), iron cations are indispensable cofactors for numerous enzymes catalyzing oxidation–reduction reactions [3–5]. Almost all iron cations in living cells are tightly bound to metalloproteins, leaving less than one percent as “labile iron pool” in the cytosol. The pool contains “free” or loosely bound iron cations predominantly in the reduced Fe^{2+} form. The labile iron pool serves as the main source of iron for the synthesis of iron proteins. However, the presence of labile ferrous ions constitutes a considerable problem for all aerobic organisms, due to their involvement in Fenton reaction in the presence of hydrogen peroxide, an inevitable byproduct of cellular respiration. The resultant hydroxyl radicals react rapidly with cellular biopolymers, including proteins, lipids, and nucleic acids, resulting in their oxidation and loss of functionality. In living cells iron(III) cations produced via Fenton reaction can be rapidly reduced back to iron(II) cations,

leading to a continuous cycle of hydroxyl radicals (HO^\bullet) production. To avoid this dangerous cycle of hydroxyl radicals formation, the concentration of labile iron is regulated by a strict control of the rates of iron uptake and mobilization in iron transport and storage proteins. While cellular iron trafficking is suggested to occur via iron chaperones [6], the reported affinity of such chaperones to iron cations is relatively low, suggesting an easy iron exchange mechanism with the labile iron pool.

The most efficient way to protect cells from the toxic effect of elevated levels of labile iron cations is ferritin, a ubiquitous and well-characterized iron storage and detoxification protein. A single ferritin molecule is capable of sequestering up to thousands of iron atoms, in the form of insoluble inorganic ferrihydrite core [7–10]. In bacteria, plants, fish, and amphibians, ferritins are generally homopolymers composed of H-type subunits, while in animals, ferritin is a heteropolymer, typically consisting of 24 subunits of two types, H and L (Figure 1) [10,11]. The resultant protein nanocage, separating the inner cavity of ferritin from the outside environment is remarkably stable in the pH range of 3–9, and temperatures up to 80 °C. Small molecules and various cations have been shown to enter the ferritin interior through eight narrow hydrophilic three-fold channels, and possibly through six hydrophobic four-fold channels as well [10]. The natural Fe^{2+} cations rapidly diffuse through the ferritin shell, where it is oxidized to insoluble iron(III) cations to form the inorganic ferrihydrite core. In mammals, the H-subunit is responsible for the rapid oxidation of Fe(II) to Fe(III) by molecular oxygen (or by hydrogen peroxide) at a dinuclear center, while the L-subunit helps iron clearance from the center in support of iron nucleation and mineralization [12–15]. Ferritin composed exclusively of L-subunits is still capable of oxidizing iron(II) cations, although at a much lower rate.

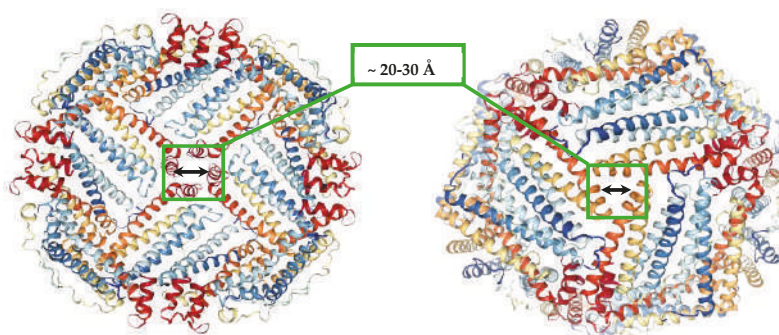


Figure 1. Ferritin molecule (PDB 1R03) with highlighted hydrophobic four-fold (left), and hydrophilic three-fold channels (right), that allow the transport of small molecules and ions to the inner cavity.

Bacteria can express at least four types of ferritin-like proteins with iron storage capability, two of which (FtnA and FtnB) are structurally similar to conventional ferritins, in term of subunit arrangement and the formation of nanocages (i.e., composed of 24 identical H-like subunits) [16]. The third type, bacterioferritin (Bfr), contains up to 12 protoporphyrin IX heme groups sandwiched between two-fold symmetry-related subunits, where they are bound covalently to methionine groups. The fourth ferritin type is called Dps (or DNA-binding protein from starved cells), contains 12 identical subunits, and thus has half the size of typical ferritins [17,18]. All ferritin types (except FtnB) possess rapid ferroxidase activity, and form ferrihydrite iron cores similar to conventional ferritins [19,20]. In bacteria, iron release from ferritin occurs through reduction of the inorganic iron core by bacterioferritin associated ferredoxin (Bfd) [19,20]. Transfer of electron from Bfd to the iron hydroxide core is mediated by bacterioferritin-bound heme molecules, most likely through electron transfer mechanisms [21]. However, very little is known about how the mechanisms of oxidative storage and reductive mobilization of iron are regulated in bacteria.

2. Brief Overview of Iron Reductive Mobilization from Intact Ferritin

While iron uptake and oxidation in conventional ferritin is relatively well understood, the mechanism of iron mobilization is rather controversial [22–26]. It is well established that the major pathway of iron mobilization from ferritin is based on proteolytic degradation, which involves transport of ferritin molecules into the lysosomes, followed by dissolution of the exposed iron hydroxide core [22,27,28]. The half-life of ferritin circulation in eukaryotic cells is about 19–24 h, under conditions of iron deficiency, but is much longer in iron-abundant environments [29], suggesting a tightly regulated cellular iron mobilization, although the exact mechanism remains unclear. We still do not know if proteolytic degradation of ferritin is the only mechanism by which iron is released from the protein, and whether other auxiliary iron mobilization mechanisms for the immediate access of cells to iron exist. Multiple reports on the reductive mobilization of iron cations from intact ferritin appeared in the literature, demonstrating the easy reduction of the iron core by a variety of one electron reducing agents, including flavin mononucleotide [30,31], ascorbate [32,33], sodium dithionite [34] and superoxide [35,36]. The rate of reduction is strongly dependent on the reduction potential of the electron donor [30]. In contrast, while two electron reductants, such as NADH [23] and glutathione [31], possess strongly negative reduction potentials, they generally reduce ferritin very slowly. One notable exception is thioglycolic acid [37], which reacts much more rapidly with the ferritin iron core, and is commonly used in the preparation of iron-free apoferritin. It has been shown that FtnA ferritin, from *Pseudomonas aeruginosa*, undergoes reductive mobilization of iron(III) by treatment with NADPH in the presence of ferredoxin-NADP⁺ reductase and 2,2'-bipyridine; however, the physiological relevance of this process is not clear, even for anaerobic conditions [19,38].

The reduction of ferritin iron by these reducing agents, produces soluble Fe(II) cations that exit the ferritin internal cavity, through the eight hydrophilic three-fold channels, and join the cellular labile iron pool (Figure 2). The newly formed Fe(II) cations can be easily re-oxidized to Fe(III), through the catalytic activity of the ferritin's ferroxidase centers. The rate of ferrous ions re-oxidation is very high [39,40], and their concentration is likely very low, and difficult to observe. This difficulty in detecting reduced iron(II) cations, can be resolved by the addition of iron(II) specific chelate ligands, such as 1,1'-bipyridine, phenanthroline, or ferrozine. The presence of excess chelators prevents the re-oxidation of iron(II) cations, and provides straightforward spectrophotometric detection of the resultant Fe(II)-chelate complexes, which typically have high ($\sim 10^3$ – 10^4 M⁻¹cm⁻¹) absorbance metal-to-ligand-charge-transfer (MLCT) bands [41].

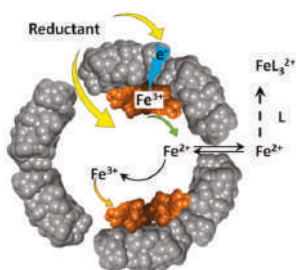


Figure 2. Schematic depiction of the oxidative deposition and reductive mobilization of iron in ferritin.

Amongst the most potent ferritin reducing agents in cell free systems are reduced flavins, including FMNH₂ and FADH₂ [31]. Reduced flavins can be prepared by chemical reduction of flavins, under oxygen-free conditions [31], or by in-situ reduction of NADH or NADPH. The latter reaction can be catalyzed by NAD(P)H:flavin oxidoreductase [30], or can proceed without enzymatic catalysis at higher concentrations of flavins and NADH [42]. Because a significant concentration (several mM) of flavins is commonly found in the cytosol of living cells, reduced flavins are possible candidates for the hypothetical reductive mobilization of iron cations from intact ferritins [30,31],

although the physiological relevance of this mechanism is far from being obvious. Free reduced flavins, such as FMNH₂ and FADH₂, that are not protein-bound, are rapidly oxidized by molecular oxygen [43]. While FMN can be reduced back to FMNH₂ by cellular NAD(P)H:flavin oxidoreductases [30], the rate constants for the re-oxidation of reduced flavins by molecular oxygen are very high [43], suggesting that the stationary concentrations of reduced flavins is extremely low. Unfortunately, there have been multiple studies on the reduction of ferritin iron by NADH without any consideration of the amount of dissolved oxygen present in solutions.

3. Iron Mobilization by the FMN-NADH System

To clarify the influence of each player on the reductive mobilization of iron from intact ferritins, we studied the kinetics of iron reduction by the FMN-NADH system, using horse spleen ferritin (HosF), and recombinant human heteropolymer apoferritin (~21H- and 3L-subunits, referred to as HuHLF), under a tight control of O₂ concentration [23]. In both cases (Figure 3), the iron release kinetics showed a biphasic behavior. The initial lag phase was followed by a second phase featuring a rapid iron release kinetic. The initial lag phase exhibited a small increase in absorbance at 530 nm, indicating a slow iron release process. The second phase is characterized by a dramatic increase in the iron release rates, which depend on the concentrations of FMN and NADH, but not on the concentration of ferritin. The linear increase in the rates of iron mobilization suggests that the generation of FMNH₂ by the FMN-NADH system, is the rate-limiting step under these conditions. Notably, the gradual decrease in the rates of iron mobilization from ferritin (as observed towards the end of the reactions in Figure 3 (right), is due to the depletion of the iron(III) hydroxide core, such as the reduction of the ferritin iron core becomes the rate limiting step [23].

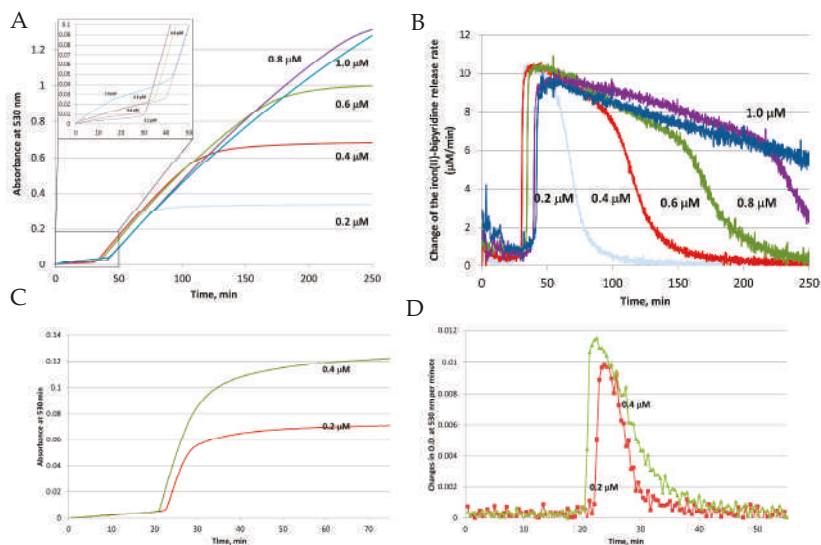


Figure 3. Reductive mobilization of iron from HosF containing 2250 Fe/shell. Conditions: 0.2–1 μM ferritin, 2 mM NADH, 200 μM FMN, 2 mM 2,2'-bipyridine, 2650 U/mL catalase, at pH 7.0 and 22 °C. (A) Absorbance change of [Fe(bipy)₃]²⁺ as a function of time. (B) Change of the iron(II)–bipyridine release rate versus time for different concentrations of HosF. (C,D) Reductive mobilization of iron from human recombinant heteropolymer ferritin (0.2 and 0.4 μM) loaded with 500 Fe/protein, in the presence of 2 mM NADH, 200 μM FMN, 2 mM 2,2'-bipyridine, 2650 U/mL catalase, at pH 7.0 and 28 °C. (C) Absorbance change of [Fe(bipy)₃]²⁺ as a function of time. (D) Change of the iron(II)–bipyridine release rate versus time, for different concentrations of heteropolymer ferritin. Reprinted with permission from Ref. [23].

The significant release of iron from ferritin does not occur until essentially all the dissolved oxygen has been depleted, as observed in Figure 3 (left). The duration of the lag phase depends on the concentration of dissolved oxygen, as well as the concentrations of NADH and FMN, but not on the concentration of ferritin. This indicates that most of the FMNH₂, produced from the reaction between FMN and NADH, is re-oxidized back to FMN by dissolved molecular oxygen, without an appreciable release of iron. This re-oxidation reaction is very fast, even at a low concentration of dissolved oxygen, which can be expected, since the rate of FMNH₂ oxidation by oxygen is much faster than that of iron reduction [43].

To further determine the effect of oxygen on the amount and rate of iron release from ferritin, the experiments were repeated, while simultaneously monitoring the concentration of molecular oxygen by a MI 730 Clark oxygen microelectrode [23]. As shown in Figure 4, the concentration of dissolved oxygen decreases sharply during the induction period (lag period), without any noticeable iron release. Only when oxygen concentration reaches a level of ~5 μM, that iron reduction and chelation by bipyridine is observed [23]. Because oxygen concentration in this experiment is well below typical normoxic conditions, the results presented in Figure 4 suggest that free reduced flavins are unlikely to serve as ferritin-reducing agents, in normally oxygenated cells. Significantly, under these conditions, the rates of iron mobilization from ferritin would be too low, even in the absence of inevitable re-oxidation of newly formed iron(II) cations. It is also highly improbable that the rates of iron mobilization are strongly dependent on cells oxygenation level, which is known to vary widely under different circumstances.

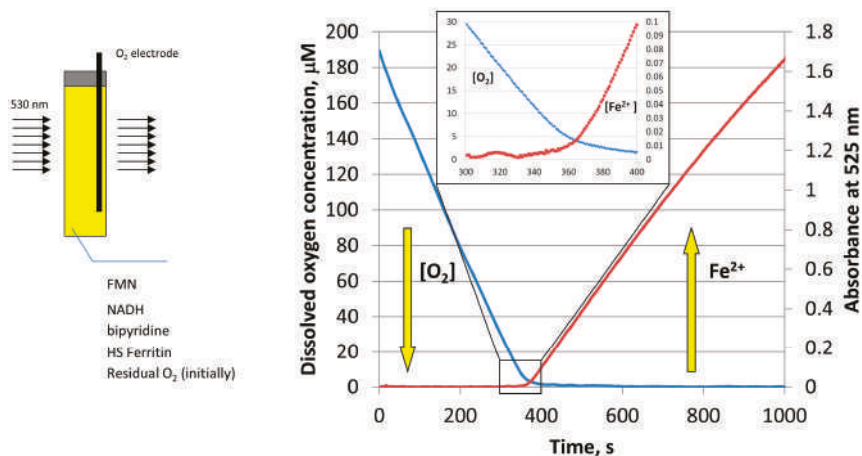


Figure 4. Schematic of the simultaneous monitoring of the dissolved oxygen concentration (blue curve), and light absorption of Fe(II)–bipyridine complex at 530 nm (red curve), during the reductive release of iron from HosF (0.6 μM), in the presence of 2 mM NADH, 2 mM FMN, and 2 mM 2,2'-bipyridine. Reprinted with permission from Ref. [23].

4. Iron Mobilization by Reduced Flavins

Reduction of ferritin iron cores by reduced flavins (and other reductants) has been the subject of intense investigation [23,25,26,30,31,44–46]. Iron reduction can proceed through two possible mechanisms that involve either a passive diffusion of reducing agents across the ferritin's shell [30], or the transfer of electrons through specific pathways along the protein shell [46] (Figure 5). Literature evidence in support of the diffusion mechanism is based on two experiments, the first of which employed agarose-bound FMNH₂, produced by a reaction of agarose-bound FMN with NADH [30]. The inability of agarose-immobilized FMNH₂ to induce reductive mobilization of iron cations from horse spleen ferritin [31], was considered as proof of the inability of FMNH₂ to diffuse to the protein's

interior, and that ferritin iron reduction reactions can only proceed through FMNH₂ diffusion into ferritin. The problem with this experiment is that it failed to account for possible changes in the reactivity of reduced flavins caused by agarose binding. Alternatively, derivatization of agarose by FMN may occur at the surface of the agarose grains, but also inside the agarose beads, which may not be available for ferritin diffusion and subsequent iron reduction [31].

The second piece of evidence is the observation of increased rates of iron reductive mobilization in the presence of chaotropes, or specific peptide molecules in solution [42,47,48]. It was shown that the presence of 1 M urea resulted in a dramatic (more than five-fold) increase in the rates of iron reduction in ferritin, by the FMN-NADH system, in the presence of 2,2'-bipyridine [42]. The data were interpreted as a weakening of the ferritin inter-subunits interactions, which resulted in an enlarged diameter of the three-fold or four-fold axes, thus facilitating the diffusion of FMNH₂ molecules into the ferritin interior. Furthermore, certain short peptide sequences (i.e., HSNTYYFPKGG) were shown to exhibit more than three-fold increased iron release rates from ferritin [48]. These later results, however, cannot be easily interpreted, given that the rates of iron reduction in ferritin by the non-enzymatic FMN-NADH system are limited by the rate of FMNH₂ production [23], and not by the rate of iron reduction. Additionally, these experiments did not disclose the initial amount of dissolved oxygen in solution, or the possibility of oxygen diffusion inside the reaction cell, which, as reported above, dramatically alters the amount, and rates of iron release from ferritin.

Evidence for electron transfer reactions across the protein shell are based on the rapid oxidation of iron(II) cations inside the ferritin cavity, under anaerobic conditions, by large electron acceptors, such as plastocyanin, cytochrome c, or stellavyanin [26,46,49]. Because of the large size of these proteins, and their inability to diffuse across the protein shell, iron oxidation reactions can only be explained by electron transfer, from iron(II) cations to these electron acceptors. In further support of this mechanism, iron oxidation and deposition reactions were also observed in L-subunit ferritins, which lack ferroxidase centers, and therefore ferroxidase activity [49]. Such a long range electron transport across the 2 nm protein shell, can be facilitated by the redox activity of the protein shell [46].

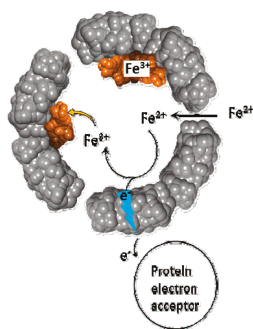


Figure 5. Schematic of Fe²⁺ cations diffusion and electron transfer across the ferritin shell, followed by Fe²⁺ oxidation and deposition.

To further understand the iron reductive mobilization from ferritin, and differentiate between the two mechanisms (diffusion vs. electron transfer), more data is needed, using different types of ferritin (i.e., heteropolymer horse spleen ferritin (HosF), homopolymer human H-chain or L-chain ferritin (HuHF or HuLF), and heteropolymer human H/L ferritin (HuHLF)). Recent experiments from our laboratory [25], using the FMN-NADH system (or reduced flavin FMNH₂), were performed with HosF and HuHF, under controlled concentration of oxygen. In contrast to literature reports, our data showed no (or insignificant) difference in the reaction rates, in the presence of 0–2 M urea, guanidine chloride, or Triton X100 (Figure 6), suggesting that the kinetics of iron release from ferritin by flavins are independent on the type, or amount of chaotropes present. Additional experiments performed

under strict anaerobic conditions, using solutions of FMNH₂ prepared by catalytic hydrogenation with Adams catalyst, revealed much faster reduction reactions, with no effect from chaotropes on the rates of the iron mobilization reactions [25].

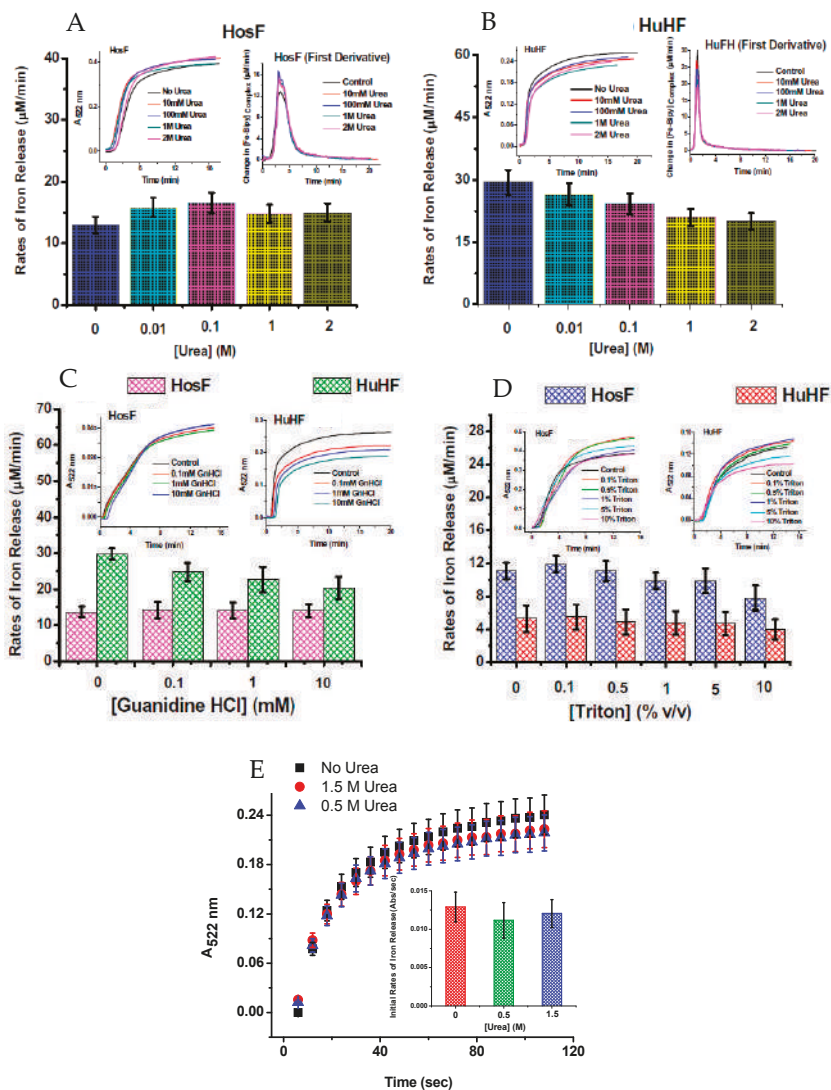


Figure 6. Rates of iron release from HosF and HuHF by the FMN-NADH system (A–D), and by FMNH₂ (E), in the absence or presence of different concentrations of chaotropes. In both experiments, the presence of urea does not affect the rates of iron mobilization. Reprinted with permission from Ref. [25].

The above results suggest that the earlier studies may have been inaccurately interpreted, and that the diffusion mechanism should be revisited. If FMNH₂ is able to diffuse into the ferritin interior and reduce the iron(III) hydroxide core, then the produced FMN should be able to diffuse out of ferritin. Notably, the diffusion of various molecules of similar size to FMN, including cisplatin [50],

curcumin [51], or anthocyanin [52] is known to be extremely slow, suggesting that diffusion of FMNH₂ is unlikely to be responsible for the observed fast rates of iron reduction. To further explore the origin of these discrepancies, we carried out an experiment to unambiguously demonstrate that, neither FMN, nor FMNH₂, both of which possess virtually identical steric size, can diffuse through the ferritin shell [25]. All of the aforementioned molecules, including FMN, can be trapped inside the inner cavity of ferritin, by first denaturing the protein down to its individual subunits at pH 2, followed by their re-assembly at neutral pH, and dialysis [50–53]. The resulting ferritin-FMN entrapped molecules, were found to be stable for days, without any sign of diffusing out. These results clearly indicate that the ferritin channels are too small for FMN (or FMNH₂) diffusion, implying that electron transfer is most likely the pathway by which ferritin iron core is reduced by flavins. Given the thickness of the protein shell (~2 nm), it is reasonable to assume that these electron transfer processes are not arbitrary, but rather a conserved evolutionary feature of ferritin, accompanied by specific protein structural arrangements. Interestingly, recent reports demonstrated the importance of electron transfer reactions, carried by three aromatic residues surrounding the diiron center of *E. coli* bacterial ferritin in the formation of the iron mineral core [54,55].

We propose that these electron transfer pathways might be physiologically important processes for iron reductive mobilization from ferritin. However, as discussed above, FMNH₂ is an unlikely electron donor candidate in cells. Protein-bound reduced flavins are more stable complexes in the presence of oxygen, and could serve as potential reducing agents, similarly to the ferredoxin-bacterioferritin complex [19,21], but this is purely speculative at the moment and requires further investigation. Some exogenous one-electron reducing agents, with less negative reduction potential than FMNH₂, were reported to reduce ferritin iron cores in the presence of oxygen [56]; however, these measurements were conducted without the continuous monitoring of oxygen concentration in solution, and require further clarification.

5. Iron Mobilization by Other Reducing Agents

It has been reported that ascorbate and glutathione are capable of mobilizing iron(II) cations from ferritin [31]. While the rates of iron reduction were substantially lower than with reduced flavins, the high intracellular concentration (several mM) of the reduced forms of ascorbate and glutathione, could be sufficient for significant mobilization of iron from cellular ferritin, under physiological conditions. Surprisingly, under anaerobic conditions, iron core reduction by ascorbate and glutathione in horse spleen ferritin comes to a complete stop only after ~17–18% of the total iron has been reduced, in direct contrast with reduced flavins and dithionite, where complete iron mobilization was observed. The sudden halt of iron mobilization cannot be the result of a thermodynamic equilibrium, since the reduction potential of both ascorbate and glutathione is much more negative than that of iron(III). Damaged horse spleen ferritin molecules that cannot protect the inorganic iron core from reducing agents, is one possible explanation for this partial iron mobilization. If this were true, then iron(III) cations in undamaged ferritin can NOT be reduced by ascorbate and glutathione under anaerobic conditions, at least in the absence of other agents. In that regard, it is interesting to compare the iron reducing ability of glutathione, with that of thiolactic acid, which is known to induce complete iron mobilization from ferritin. This clear difference in outcome can be rationalized by the small size of thiolactate molecules, and their ability to easily diffuse through ferritin channels, in contrast to glutathione. Under anaerobic conditions, iron mobilization from ferritin by ascorbate is extremely slow, but the presence of oxygen, iron, or copper cations causes a dramatic increase in the rates of iron reduction [57,58]. This effect can be ascribed to the formation of different redox species, most likely free radical species [59].

Superoxide anion radicals were also reported to induce reductive mobilization of iron from ferritin [35,36]. Because superoxide anions are continuously produced by mitochondria, as a byproduct of oxidative phosphorylation, its tiny size would allow its fast diffusion into the ferritin interior, and the subsequent reduction of the inorganic iron core. However, the physiological importance of this

hydroxides, embedded within the iron(III) hydroxide core forming a magnetite phase [67]. Reduction of magnetite by one superoxide anion can produce two iron(II) cations, instead of one iron(II) cation in the case of a ferrihydrite phase (composed of only iron(III) hydroxides), thus continuously “breeding” superoxide anions.

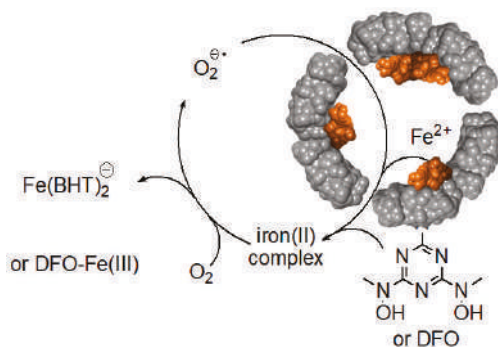


Figure 8. Superoxide mediated mobilization of iron(III) cations from the ferritin iron core by BHT (or DFO) chelators.

This unusual iron reduction mechanism is highly specific for exogenous chelators, possessing very high affinity to iron(III) cations, and is unlikely to be physiologically relevant for cells not exposed to these ligands. Endogenous iron(III) specific ligands with strong affinity for iron(III) (i.e., at least equal to that of pyrophosphate with a $\log \beta = 22.2$), including nucleic acids [68] and nucleotide triphosphates, should be considered, although their ability to reach the inner cavity of ferritin is unknown. Smaller ions having lower affinity to iron(III) cations, such as phosphate, lactate, glutamate, or citrate may represent a better option that could assist with the dissolution of the inorganic iron core, similarly to that observed with acetohydroxamate [66]. In this latter case, iron(III) cations, complexed with these small chelates, are released from the interior cavity of ferritin, which upon exiting the ferritin shell encounter the stronger chelate agents (i.e., nucleotide triphosphate), followed by complexation and reduction to iron(II). Such process could constitute a futile cycle for the release of small amounts of iron cations, to help maintain the labile iron pool, under conditions that do not require large spikes in iron protein synthesis.

7. Conclusions and Perspective

Under anaerobic conditions, the reductive mobilization of iron from ferritin, in cell free systems, proceeds via electron transfer reactions across the protein shell. This process is likely to be physiologically relevant in anaerobic bacteria, where ferredoxin serves as the electron source, but its relevance under aerobic conditions is unknown. Reduced flavins are one-electron reducing agents that react much more rapidly with molecular oxygen than with the ferritin iron core, and are unlikely relevant iron reducing agents in oxygenated cells. Although some phenazine derivatives have been described as more efficient electron transfer mediators that facilitate the mobilization of iron from ferritin, even in the presence of dissolved oxygen [68], the exact mechanism of this electron mediation process is unclear. Additionally, the efficiency of these mediators in the presence of different concentration of molecular oxygen, and the relevance of this mechanism *in vivo*, remains to be determined. Nonetheless, this potential chemoselectivity for ferritin iron core reduction vs. oxygen reduction, is an attractive possibility that could support an auxiliary iron mobilization mechanism, in addition to ferritin proteolytic degradation. Other endogenous compounds including oxidoreductases are also worth exploring as potential ferritin-reducing agents.

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Review

Links Between Iron and Lipids: Implications in Some Major Human Diseases

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Abstract: Maintenance of iron homeostasis is critical to cellular health as both its excess and insufficiency are detrimental. Likewise, lipids, which are essential components of cellular membranes and signaling mediators, must also be tightly regulated to hinder disease progression. Recent research, using a myriad of model organisms, as well as data from clinical studies, has revealed links between these two metabolic pathways, but the mechanisms behind these interactions and the role these have in the progression of human diseases remains unclear. In this review, we summarize literature describing cross-talk between iron and lipid pathways, including alterations in cholesterol, sphingolipid, and lipid droplet metabolism in response to changes in iron levels. We discuss human diseases correlating with both iron and lipid alterations, including neurodegenerative disorders, and the available evidence regarding the potential mechanisms underlying how iron may promote disease pathogenesis. Finally, we review research regarding iron reduction techniques and their therapeutic potential in treating patients with these debilitating conditions. We propose that iron-mediated alterations in lipid metabolic pathways are involved in the progression of these diseases, but further research is direly needed to elucidate the mechanisms involved.

Keywords: iron; lipid; obesity; cancer; neurodegeneration; iron chelation; phlebotomy

1. Introduction

Iron, one of the most essential elements in the human body and indispensable for life, exists in complex forms, including (a) the iron storage complex in which iron is trapped (i.e., hemosiderin), (b) heme containing proteins (i.e., hemoglobin), (c) heme-containing enzymes, (d) transferrin (i.e., holo-transferrin), and (e) the ferritin complex (comprised of 4500 Fe(III) molecules in a complex with ferritin heavy and light chains) [1]. This metal is essential for cellular processes, including metabolic reactions, oxygen transport via hemoglobin, and DNA synthesis [2]. An average adult has 3–5 g of iron in their body [3], while only 1–2 mg of iron is normally absorbed in the intestinal tract, which would then be available for body-wide circulation [4]. Free iron also exists intracellularly in the labile iron pool (LIP) and leads to the production of reactive oxygen species (ROS) via the reaction of hydrogen peroxide (H₂O₂) with Fe(II), a process known as the Fenton reaction [5,6]:



Since the body has no mechanism to eliminate excess iron (other than conditions like pregnancy, menstruation, and blood-letting [3]), iron levels must be appropriately maintained to hinder the potentially toxic effects if present in excess [7]. On the other hand, insufficient quantities of iron also leads to detrimental cellular processes [7]. Normal body serum iron levels range from 9–27 μM;

however, acute toxicity will be observed in excess of 45 μM . This can result in death if $>160 \mu\text{M}$, which can be induced by taking iron supplements [3]. On the other hand, chronic iron overload will arise in response to sublethal doses over extended periods of time (i.e., blood transfusions) leading to development of diseases, including cancer [3]. Indeed, the carcinogenic effects of excessive iron have been well established [8]. In this regard, it is interesting that medical conditions, such as hereditary hemochromatosis and β -thalassemia, are associated with an increased risk of developing cancer [3].

Specific mechanisms, such as iron absorption, iron recycling, and iron mobilization, are in place to regulate iron content at both a cellular and systemic level [1]; for comprehensive reviews, see [2,9]. Briefly, uptake of iron, either as transferrin-bound iron (TBI, holo-transferrin bound Fe(III)) or as non-transferrin bound iron (NTBI, Fe(II)), is mediated, respectively, via the transferrin receptor (CD71) and by solute carrier family 39 member 8 (SLC39A8/ZIP8) or solute carrier family 39 member 14 (SLC39A14/ZIP14) [10–12]. For uptake of NTBI in liver enterocytes, Fe(III) is oxidized to Fe(II) by duodenal cytochrome b (DCYTB) before being imported into the cell by the divalent metal transporter 1 (DMT1) [13]. After TBI is endocytosed, Fe(III) is released from transferrin and then reduced to Fe(II) by the ferrireductase STEAP3 (six-transmembrane epithelial antigen of prostate 3) [14] prior to its release from the endosome via the DMT1 channel. Cytosolic iron may then (a) remain available for use in the LIP, (b) be transported to mitochondria to generate iron-sulfur (Fe-S) clusters, or (c) be stored within the ferritin complex (a process mediated by poly(RC) binding protein 1 (PCBP1)) [9,15]. Iron is released from the ferritin complex via the action of nuclear receptor coactivator 4 (NCOA4), which is involved in autophagy-mediated degradation of these iron complexes [16,17]. With regards to iron export, ferroportin (FPN1), the only known iron exporter [12], is tightly regulated by hepcidin (HAMP), a protein hormone that is secreted in a controlled manner from the hepatic tissue [12]. The exported iron (in the Fe(II) form) is then oxidized via hephaestin (HEPH) to Fe(III) [18].

Increased circulating transferrin saturation leads to elevated NTBI, which is deposited primarily into the heart, pancreas, liver, and brain [19–23]. Such iron deposits can be observed via transmission electron microscopy (TEM) and are present prior to the development of iron overload symptoms [3]. Under these conditions, ROS accumulates—which can then mediate damage to proteins, lipids, nucleic acids, and other cellular components [3,24,25]. In addition, elevated ROS can induce a ferroptotic response, which is characterized by accumulation of lipid peroxides [15,26]. Activation of ferroptosis promotes cell death in various pathological conditions, such as diffuse large B-cell lymphoma, acute kidney failure, chromophobe kidney cancer, and periventricular leukomalacia [27]. As described later in Section 3, dysregulated iron levels have also been implicated in the development of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and Amyotrophic lateral sclerosis [28], as well as in cancer [8].

In addition to systemic iron overload, localized increases in this metal can give rise to conditions, such as endometriosis, a benign gynecological disorder characterized by the presence of endometriotic cysts, which contain old blood components (including heme and its breakdown products) [29]. This source of redox active iron present within these cysts or that arising from follicular fluid, retrograde menstrual effluent, and the process of ovulation have been proposed to contribute to ovarian cancer risk [30–32]. Consumption of red meat as a source of dietary iron may also contribute to development of other cancers, namely colorectal cancers [33].

Similar to iron, lipid levels must be regulated in an appropriate manner to ensure cellular homeostasis. Lipids are a critical source of cellular energy and also have roles as signaling metabolites [34]. When these are in excess, they are stored within lipid droplets to hinder the detrimental effects of lipotoxicity [35]. On the other hand, when lipids are depleted, cellular biosynthetic pathways are activated to generate these macromolecules [35]. Within the body, lipids are primarily stored in adipose tissue, an organ that is also involved in endocrine signaling to regulate energy balance and insulin resistance [36]. Deregulated lipid biosynthetic and catabolic pathways may therefore interfere with crucial biological processes, ultimately producing deleterious effects and potentially causing serious medical issues. Herein, we present a review of the literature pertaining

import and lipid droplet formation. Synthesis of triacylglycerides, as well as phospholipids (and their modification), are presented in pink, whereas the sphingolipid metabolic pathway is displayed in dark blue. Iron can promote the production of ceramide (indicated with a brown triangle), and in turn induces HAMP expression, which negatively regulates FPN1 (refer to Section 2.2). Furthermore, our own unpublished work suggests iron treatment in human fallopian tube secretory epithelial cells (FTSECs) increases mRNA expression of autotaxin (ATX), which is involved in generating lysophosphatidic acid (see Section 2.5). **(Bottom)** TBI can be imported via endocytosis by binding to CD71; Fe(III) is then converted to Fe(II) by STEAP3 prior to being transported from the endosomal compartment to the cytosolic LIP by DMT1. Alternatively, NTBI can be imported into cells by DMT1 (following conversion of Fe(III) to Fe(II) by DCYTB), ZIP8, or ZIP14 [37]. From the LIP, iron may be (a) transported to the mitochondria for use in Fe-S cluster generation, (b) loaded to ferritin by PCBP1, or (c) used by the cell for other cellular processes. Iron can also be released from the ferritin complex via NCOA4-mediated ferritinophagy. Increased iron levels in the cell can promote the formation of lipid peroxides, a process critical for ferroptosis (shown in grey). Please see [2,38–45] for comprehensive reviews of these pathways and the contributing enzymes.

2. Interactions between Iron and Lipids in Model Systems

2.1. Iron and Cholesterol

Cell membranes are comprised of not only protein, but also lipids and cholesterol, which play important roles in cell signaling and maintenance of cell structure [46]. Similar to iron, excess levels of cholesterol can also elicit a toxic effect by elevating oxidative stress responses [47]. Specifically, hepatic iron levels were correlated with increased cholesterol content, which was associated with elevated mRNA levels of seven key enzymes involved in the cholesterol biosynthetic pathway: 3-hydroxy-3-methylglutarate-CoA reductase (HMGCR), lanosterol-14 α demethylase (CYP51), Δ 14-sterol reductase (TM7SF2), sterol-4 α -carboxylate-3-dehydrogenase (NSDHL), cholesterol- Δ -isomerase (EBP), phosphomevalonate kinase (PMVK), and lathosterol oxidase (SC5D) [47]. The authors of this work propose that these changes could contribute to the development of fatty liver disease [47].

Cholesterol is also found in lipoprotein particles along with apolipoproteins, such as apolipoprotein E (ApoE), a major brain Apo, which is critical for learning, memory, and brain repair [48]. In Alzheimer's disease, current evidence implicates impaired levels of ApoE4 (which correlates with neurodegeneration while also being able to bind to metals, such as iron) in the sequestration of iron to amyloid- β deposits [48]. Although elevated levels of iron contribute to increased ApoE mRNA and protein expression, the secretion of this apolipoprotein was reduced [49]. In addition to altered ApoE levels, patients with Alzheimer's disease have increased ferritin levels in their cerebrospinal fluid (CSF), a marker of brain iron content [50–52]. Interestingly, patients with elevated ApoE4 (specifically, the ϵ 4 variant) have >20% increase in CSF ferritin correlating with increased kinetics of cellular degeneration in the hippocampus, as well as with cognitive decline [50–52].

The effects of ApoE extend beyond its role in neurodegenerative diseases. For example, ApoE is proposed to protect against NASH (non-alcoholic steatohepatitis) as ApoE knockout mice were characterized by hepatosteatosis [53]. A link to iron was identified in a recent SILAC proteomic study in which adipocytes were treated with ferric ammonium citrate (FAC, a source of NTBI) resulting in an 11-fold increase in ApoE (amongst two other markers), although ApoE secretion was reduced by >55% [53]. Further studies are needed to elucidate the mechanism underlying increased ApoE expression in spite of its reduced secretion.

2.2. Iron and Sphingolipids

Like cholesterol, sphingolipids (i.e., sphingomyelin, ceramide, and sphingosine amongst others) are essential membrane and signaling components [54]. The initial link between iron and sphingolipid regulation in eukaryotic systems was derived from *S. cerevisiae* in which iron-induced toxicity was

correlated with increased synthesis of sphingolipids [55]. More recently, this finding has been extended to *D. melanogaster* and *M. musculus*, as well as in mammalian cell lines [56,57] in which iron-induced toxicity was mediated by a deficiency in frataxin, a key modulator of iron-sulfur cluster biogenesis, also lacking in patients with Friedreich's ataxia [58].

Intriguingly, iron uptake via CD71 is increased following cellular treatment with C2-ceramide, a sphingolipid involved in lipid signaling in bovine aortic endothelial cells (BAECs), and could be reversed with an iron chelator (deferrioxamine (DFO)) or with an antibody targeting endocytosis of CD71 [59]. C2-ceramide treatment in human hepatocellular carcinoma (HepG2) cells was found to transcriptionally upregulate HAMP mRNA via the JAK/STAT3 signaling cascade [60]. In another report, loss of sphingomyelin in murine lymphoma cells (WR19L) hindered clathrin-mediated endocytosis of CD71 whereas overexpression of sphingomyelin synthase, as well as presentation of exogenous sphingomyelin increased transferrin uptake [61]. Whether alterations in other elements of iron signaling are induced in response to ceramide and other sphingolipids has yet to be determined.

2.3. Iron-Sulfur Cluster and Lipids

Iron-sulfur cluster containing proteins play key roles in the Krebs (TCA) cycle (i.e., aconitase) [62] and the electron transport chain (i.e., complex I) [63], which contribute to ATP production. Iron-sulfur clusters are also needed for regulation of enzymes involved in key cellular processes (i.e., DNA polymerase, base excision repair) in addition to iron-sensing molecules (i.e., iron response proteins IRPs) [62]. In human embryonic kidney cells (HEK293) overexpressing a dominant negative form of ISCU (an iron-sulfur cluster assembly enzyme), a 10-fold increase in citrate levels (as a result of a deficiency in aconitase activity) was noted; this citrate was redirected for its use in fatty acid biosynthesis, which increased lipid droplet formation [64,65]. The mitochondrial phospholipid, cardiolipin, is also involved in multiple processes for generating cellular energy by regulating activities of protein complexes involved in the electron transport chain and mitochondrial membrane dynamics [66]. Using the yeast model, researchers identified that deficiency in cardiolipin synthase (*crdΔ*) increased expression of the iron regulon (iron uptake genes for mitochondria) and biogenesis of iron-sulfur clusters in the mitochondria and their export to the cytosol [67]. Additional investigations into cardiolipin regulation needs to be addressed to have an improved understanding of iron-sulfur cluster biogenesis.

2.4. Iron, Lipid Droplets, and Leptin

According to the World Health Organization (WHO), obesity (defined as a body-mass index (BMI) ≥ 30) has increased 3-fold over the past 40-decades throughout the world [68]. Additionally, patients who are obese (characterized by a gain in adipose tissues (including visceral and subcutaneous) [69]) are at an increased risk of developing co-morbidities, including cancer [70]. Adipocytes, the major component of these tissues, contain $\sim 100 \mu\text{m}$ large-sized lipid droplets, composed of triacylglycerides and cholesterol esters [71]. In *C. elegans*, iron supplementation significantly increased the abundance and size of lipid droplets [72]. Specifically, iron treatment in this model organism increased the expression of *sgk-1* (an ortholog for mammalian glucocorticoid-induced kinase), which was found to increase expression of *acs20* (mammalian homolog, FATP1/4) involved in fatty acid import and thus transport to lipid droplets while simultaneously promoting iron storage in ferritin [72].

Leptin is an adipokine that is produced by adipocytes to regulate hunger [73]. In HuH7, a human hepatoma cell line, leptin treatment resulted in increased HAMP mRNA, which was regulated by the JAK2/STAT3 signaling cascade [74]. Additionally, using mice deficient in leptin (*ob/ob*), leptin treatment increased both plasma HAMP levels and liver HAMP mRNA, which were associated with an increase in liver iron levels [75]. Interestingly, in mice lacking mediators important for iron efflux (hephaestin and ceruloplasmin), leptin levels were reduced [76]. Furthermore, C57BL/6 mice, fed a high-fat diet, showed increased leptin levels and increased liver HAMP mRNA associated with increased liver iron [77]. Whether leptin alters other elements of iron signaling has yet to be determined.

2.5. Iron, LPP1, and Other Enzymes Involved in Lysophospholipid Metabolism

Recent work has identified that overexpression of LIPIN1, an enzyme involved in the conversion of phosphatidic acid (PA) to diacylglycerol (DAG), can reduce iron levels in human hepatic cancer cells (BEL7402) [78]; this phenomenon appeared to be mediated by FPN1, which was increased upon LIPIN1 expression [78]. On the other hand, from our own work (*unpublished results, Rockfield and Nanjundan*), we have identified that addition of exogenous NTBI iron (presented as FAC) to transformed gynecological cell lines induced autotaxin (ATX) mRNA. ATX, an adipokine, catalyzes the conversion from lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) and is noted to be altered in multiple cancer types, including non-small cell lung cancer, glioblastoma multiforme, melanoma, thyroid cancer, follicular lymphoma, ovarian cancer, hepatocellular carcinoma, breast cancer, and colon cancer [79,80]. To our knowledge, the only other reported link between ATX/LPA and iron is in the H9c2 cardiomyoblast cells; ATX overexpression protected these cells from ferroptotic cell death (an iron-dependent cellular response) by reducing the levels of intracellular ROS [81]. Further work must be performed to improve our understanding of these initial findings.

2.6. Iron and Fatty Acid Metabolism

Cancer cells are described to be “addicted” to iron [8]; indeed, their increased proliferative capacity is negatively regulated upon cellular treatment with iron chelators [82]. Links between iron and lipid pathways in cancer are only beginning to come to the forefront. Recent work using a systems biological approach (using existing microarray datasets followed by data mining approaches) implicates associations between the iron pathway and fatty acid synthesis and regulation in high-grade serous epithelial ovarian carcinomas [83]. Specifically, peroxisome proliferator-activated receptor gamma (PPARG), sterol regulatory element binding transcription factor 1 (SREBF1), ATP citrate lyase (ACLY), fatty acid synthase (FAS), acyl-CoA synthetase long-chain (ACSLx), fatty acid desaturation (fatty acid desaturase 2 (FADS2), stearoyl-CoA desaturase (SCD), elongation of very long chain fatty acid elongase 2 (ELOVL2), elongation of very long chain fatty elongase 5 (ELOVL5)), and glycerolipid metabolic pathways (1-acylglycerol-3-phosphate O-acyltransferase (AGPATx), DGAT1, LIPIN1, LIPIN2, glycerol kinase (GK), glycerol-3-phosphate-acyltransferase (GPAM)) were perturbed along with iron-related genes (iron-ion binding, as well as intracellular iron regulation) [83]. The functional outcomes of these initial associations must be further investigated.

2.7. Ferroptosis and Lipids

Lipid peroxides are a form of ROS, which serve as signaling molecules that can alter the properties of cell membranes, lipid interactions, and protein functions. In addition, these ROS promote cellular apoptosis, including iron-dependent ferroptotic cell death [84]. In ferroptosis, recent research has identified alterations in lipid metabolic pathways in addition to the well-established lipid peroxidation [85]. Using retrovirus-generated insertional mutagenesis in KBM7 (haploid chronic myeloid leukemia cells), 9 genes were identified as increased upon ferroptosis induction with multiple small molecule ferroptosis inducers, including ACSL4 (acyl-coA synthetase long-chain family member 4, which produces the arachadonic acid metabolite 5-HETE (5-hydroxyeicosatetraenoic acid)) and LPCAT3 (lysophosphatidylcholine acyl-transferase 3) [86]. Similarly, in a genome wide CRISPR-mediated genetic screen and microarray screen involving ferroptosis resistant cells, ACSL4 was also identified as a regulator of this pathway [87]. Furthermore, ACSL4 mRNA and protein were reduced in ferroptosis-resistant (LnCaP and K562) cells relative to sensitive (HL60 and HepG2) cancer cells [85]. Likewise, in breast cancer cell lines, ACSL4 expression corresponded with ferroptosis sensitivity [87]. When ACSL4 is reduced (via shRNA-mediated knockdown) in HL60 and HepG2 cells, ferroptosis is inhibited; in contrast, when it is overexpressed in LnCaP and K562 cells, ACSL4 promotes ferroptosis [85].

In a ferroptosis mouse model for which glutathione peroxidase 4 (GPX4) is deficient, inhibition of ACSL4 was found to improve tissue health [87]. Furthermore, a novel ferroptosis-inducing compound (CIL56) was identified to be dependent on the activity of acetyl-coA carboxylase 1 (ACC1, the rate-limiting enzyme involved in fatty acid biosynthesis) [86]. Upon knockout of ACC1 via CRISPR-Cas9, a 5-fold increased resistance to ferroptosis was noted in response to CIL56 [86]. In another study, researchers identified LSH (lymphoid specific helicase, a DNA methylase modified, which is part of the SNF2 chromatin remodeling ATPase family) as hindering ferroptosis through its interaction with WD repeat domain 76 (WDR76, via direct promoter binding activity). This in turn corresponded with increased expression of fatty acid desaturases (i.e., FADS2 and FADS5) and was dependent on both iron and lipid peroxidation [88]. In HepG2 and Hep3B liver cancer cells, knocking out the expression of iron sulfur domain 1 (CISD1, localized to the outer mitochondrial membrane) also was found to promote lipid peroxidation and ferroptosis [89]. The clinical utility of such ferroptosis inhibitors could be tested in future work.

3. Iron and Lipids: Neurodegenerative Diseases

3.1. Brain Iron Localization

Neurodegenerative diseases (i.e., Alzheimer's, Parkinson's, and Huntington's, among others) are considered age-related diseases, in part due to accumulation of iron, and its physiological consequences [90]. Notably, this metal causes inflammation of the brain and thus, its degeneration [90]. Iron response proteins (IRP1 and IRP2) increase amyloid precursor protein (APP) expression, which is the precursor to amyloid- β in Alzheimer's disease, as well as the expression of α -synuclein, which is a critical component of the Lewy bodies in Parkinson's disease [90]. The mTOR pathway can regulate expression of CD71, which is responsible for cellular iron uptake [90]; indeed, it has been recently proposed that inhibition of mTOR could reduce iron accumulation and thus, lessen the neurodegenerative effects induced by this metal [90]. Specific brain regions that accumulate iron include the hippocampus [91], the globus pallidus, red nucleus, substantia nigra, dentate nucleus, and caudate-putamen [92], whereas increased iron content in the basal ganglia is a unique feature of a rare brain disease called neurodegeneration with brain iron accumulation (NBIA) [93].

3.2. Iron-Mediated Lipid Peroxidation and Ferroptosis

As mentioned earlier, iron participates in the Fenton reaction to generate ROS, which damage lipids via peroxidation [6], a phenomenon observed in neurodegenerative diseases [94]. Indeed, increased redox active iron in certain regions of the brain contributes to the development of neurological diseases, which is associated with programmed cell death [95]. The exact mechanism contributing to this cell death process has been unclear until recently. It is now recognized that iron-dependent cell death pathway, namely ferroptosis, may be involved in the development of such neurodegenerative diseases [95,96].

Lipoxygenases (iron-dependent enzymes) can also promote oxidation of polyunsaturated fatty acids and are localized to the hippocampal region of the brain [97]. One oxidative stress stimulator, namely tert-butylhydroperoxide (t-BHP), promotes cell death via ferroptosis in PC12 cells (a model cell line for neurobiology) by reducing GPX4 protein and glutathione (GSH) levels leading to increased lipid peroxidation [98]. Mitochondrial alterations, including (a) reduced mitochondrial membrane potential, (b) reduced ATP levels, and (c) increased ROS in the mitochondria, were also noted [98]. They further identified that these effects could be reversed upon treatment with ferrostatin or iron chelation with DFO [98]. Neurons in the forebrain (cerebral cortex and hippocampus) are susceptible to ferroptosis [99]. Interestingly, an inducible tissue-specific GPX4 knockout mouse (specifically in forebrain neurons) resulted in massive deficits in cognitive and memory functions concurrently with increased lipid peroxidation, increased MAPK pathway activation, and increased oxidative damage [99]. Furthermore, maintaining these mice on a vitamin E (an antioxidant) deficient diet

accelerated the neurodegenerative processes in these mice, whereas treatment with a ferroptosis inhibitor (liproxstatin-1) ameliorated brain functions [99]. In a model of Huntington's disease using brain slices, ferrostatin-1 was also found to reduce cell death [100]. The clinical application of these ferroptotic inhibitors could be tested in the future.

3.3. Iron and the Sphingolipid Pathway

In addition to iron-induced lipid peroxidation, iron can also promote sphingomyelin breakdown via activation of sphingomyelinases, generating the product ceramide (which is involved in mediating the regulated cell death response) [101] that then contributes to neuronal apoptosis, a feature of neurodegenerative diseases. In support, increased levels of sphingomyelin coinciding with reduced ceramide content is associated with neuronal protection and may thus be a targetable pathway (using iron chelators) for treatment [102]. In *D. melanogaster* and *M. musculus*, targeting of frataxin (via mutations or knockout strategies) led to iron-induced toxicity, which was mediated through the sphingolipid/PDK1/MEF2 signaling cascade [56,57]; this was detrimental to the health of these model organisms and recapitulated the neurodegenerative disease, Friedreich's ataxia [56,57]. Links between iron and the sphingolipid metabolic cascade have been identified in other neurodegenerative diseases, including NBIA; in this disease, sphingolipids were enriched in the compartment with the highest iron levels (i.e., basal ganglia), identified via gene network analyses [93]. The functional contribution of this observation needs to be investigated further in NBIA and in other neurodegenerative diseases.

4. Treatments and Concluding Perspectives

Targeting iron and its downstream effectors (i.e., alterations in lipid peroxidation and/or lipid metabolism) would be of high benefit to patients afflicted by detrimental effects of iron accumulation. Methods of iron reduction thus far utilized include iron chelators and the process of bloodletting.

In the case of neurodegenerative diseases, iron treatment with DFO reduced symptoms of Alzheimer's disease in an amyloid precursor protein (APP) overexpressing transgenic mouse model; specifically, amyloid- β deposits were reduced coinciding with improved cognitive functions [103]. However, in patient studies for a variety of neurological disorders (such as pantothenae kinase-associated neurodegeneration (PKAN), aceruloplasminemia, NBIA, Friedreich's ataxia, superficial siderosis, Parkinson's disease, Alzheimer's disease, and multiple sclerosis), only low to moderate improvement of clinical symptoms was noted in a small proportion of conducted studies, with most observing no improvement [104]. Similarly in cancer, the use of iron chelators, such as DFO, has shown some efficacy in both animal models and clinical studies [105]; for additional iron chelators used in cancer studies, please see citation [105] for more details.

To the best of our knowledge, improvements in health following iron reduction via the process of phlebotomy have been assessed in six independent studies. Patients afflicted with nonalcoholic fatty liver disease (NAFLD) that underwent bloodletting had reduced blood ferritin levels [106]. Administration of phlebotomy in metabolic syndrome (METS) patients reduced blood pressure and heightened insulin sensitivity [107]. Similarly, phlebotomy administration in type II diabetics, characterized by high blood ferritin, had a marked reduction in not only ferritin (at 4-month follow-up), but also in insulin resistance [108]. With respect to cancer patients, there has been a variation in cancer incidence following such bloodletting procedures. In one study, phlebotomy reduced risk of cancer development in 36% of patients [109] while another reported only 4% [110]. Yet another showed a lack of association between iron reduction and overall risk of cancer [111]. Further investigations into implementation of iron reduction therapies can be pursued in future studies.

Although ferroptosis inhibitors have been utilized in *in vivo* animal studies, as well as *in vitro* studies, novel inhibitors could be designed that could be utilized to treat patients with neurological diseases described herein.

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Review

Multilevel Impacts of Iron in the Brain: The Cross Talk between Neurophysiological Mechanisms, Cognition, and Social Behavior

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Abstract: Iron is a critical element for most organisms, which plays a fundamental role in the great majority of physiological processes. So much so, that disruption of iron homeostasis has severe multi-organ impacts with the brain being particularly sensitive to such modifications. More specifically, disruption of iron homeostasis in the brain can affect neurophysiological mechanisms, cognition, and social behavior, which eventually contributes to the development of a diverse set of neuro-pathologies. This article starts by exploring the mechanisms of iron action in the brain and follows with a discussion on cognitive and behavioral implications of iron deficiency and overload and how these are framed by the social context. Subsequently, we scrutinize the implications of the disruption of iron homeostasis for the onset and progression of psychosocial disorders. Lastly, we discuss the links between biological, psychological, and social dimensions and outline potential avenues of research. The study of these interactions could ultimately contribute to a broader understanding of how individuals think and act under physiological and pathophysiological conditions.

Keywords: iron; brain; neurophysiology; cognition; social behavior

1. Introduction

Iron is a vital element for most organisms. Iron actively participates in many physiological processes, such as oxygen transportation, cellular respiration, energy production, cell growth and differentiation, DNA synthesis, and more. Its ability to transfer electrons among different substrates is a property that renders this metal essential for life. However, it also implies the existence of fine-tuned regulatory mechanisms strictly controlling iron homeostasis, since minimum disruption of iron balance significantly alters organs' functionality [1]. Many mechanisms contribute to maintain iron homeostasis, with a vast number of genes being involved in iron uptake, storage, and recycling. So far, there is no description of physiological mechanisms of iron excretion.

In the specific case of the brain, which is a metabolically active organ and particularly sensitive to changes in iron homeostasis [2], there are still many uncertainties. This is the case since the distribution and roles of genes devoted to iron homeostasis are still under discussion. Current knowledge points at iron crossing the blood brain barrier (BBB) as iron-loaded transferrin (holotransferrin, HTf) complexes. In addition, binding of iron to transferrin receptors on capillary endothelium results in their subsequent internalization by forming endocytic vesicles. Iron is then pumped out via the expression of divalent metal transporter 1 (DMT1) in its ferrous form. In the cytosol, ceruloplasmin is the oxidase responsible for converting this metal into its ferric form, which can be exported in the extracellular

space by ferroportin [3]. In the brain, disruption of iron homeostasis significantly impairs oxidative metabolism of neural cells, with dramatic consequences for synaptic plasticity, myelination, and synthesis of neurotransmitters [4,5]. This means that both iron deficiency and overload are associated with disruption of neurophysiological mechanisms, which were previously associated with impaired cognition and altered social behavior, as described in the following sections of this paper.

Regarding iron deficiency, which is the most common nutrient deficiency worldwide [6,7], the absence of mobilizable iron stores and compromised supply of iron to tissues, including erythrocytes, can identify it. The most severe form of iron deficiency, i.e., iron deficiency anemia, is characterized by the levels of iron associated with hemoglobin lower than two standard deviations of the distribution of mean hemoglobin in a population of the same age, sex, living at the same altitude, and without any other pathologies [6]. The causes underlying iron deficiency are diverse and include: inadequate oral iron intake, resulting from poor diets, excessive milk intake or vegetarian diets, inadequate iron absorption, as a result of Celiac disease and others, or excessive iron loss, mainly occurring via blood loss or as a result of parasitic infection (Figure 1) [6,8]. Although individuals can suffer from iron deficiency at any stage in life, the most severe consequences of iron deficiency are present in early childhood, which is a period of rapid growth and high needs for iron intake. These range from the disruption of neurophysiological mechanisms, to impaired cognitive and altered social behavior. Most importantly, iron deficiency has been reported in low-income and high-income countries, with increased prevalence of iron deficiency in lower socio-economic groups. These have been concomitantly characterized by worse eating habits, lower levels of education, and acknowledgement of the impacts of nutritional patterns in physical and cognitive development and health [9–15]. In this population, dietary iron intake was shown not only to be influenced by personal taste, attitude, and knowledge, but also by food access and cost, which render low-income groups at a multiple disadvantageous position from the go ahead [9,16]. Altogether, this could explain the higher risk for iron deficiency in lower socio-economic groups.

Currently, the World Health Organization (WHO) recommends iron replacement therapies as an initial intervention to address iron deficiency [17]. These supplementations have been successful in the control of anemia and iron deficiency in different age groups. However, the long-term deficits in motor and cognitive functions, as well as the modifications in social behavior, were mostly shown to persist and deserve great attention and debate [8,18–20]. Several reasons can account for the absence of (or very small, in a few cases) motor, cognitive, and behavioral effects. On the one hand, methodological insufficiencies (i.e., study design limitations, indirectness of presented evidence, imprecisions, or reporting bias) and heterogeneity of intervention protocols (precluding a straight comparison among studies) can partly account for the reported inconsistencies. On the other hand, these studies mostly assume that correction of the nutrient deficiency per se, would suffice to address long-term motor and cognitive impairments, as well as modifications in social behavior. However, as we will discuss in the following sections of this paper, one can argue that there seems to be an overlap between the biological effects of a nutrient insufficient diet and its effects on neurophysiological mechanisms and cognition, as well as a potential absence of social stimuli to promote adequate physical and cognitive development. Most studies do not take into account, or analyze superficially at most, the interactions between biological, psychological, and social dimensions of a nutrient deficiency, subsequent multi-organ disruption of iron homeostasis, and its impacts on motor deficits, cognitive impairments, and modifications of social behavior. Future research should address these interactions much more profoundly, by integrating the immense knowledge patrimony of diverse scientific disciplines, and characterizing the role of each dimension, their interactions, and potential contributions for the multiple reported outcomes. This has not been previously done.

On the opposite pole of iron deficiency, we have iron overload, which is present, for instance, in the aging population and in protein misfolding neurodegenerative diseases, such as Alzheimer's or Parkinson's disease (AD and PD, respectively) (Figure 1) [21]. In the specific case of AD and PD patients, mental decline, as evaluated by the Dementia Rating Scale and the total Frontal Systems

Behavior Scale, was shown to be present [22]. More specifically, AD patients have difficulties in storing information and learning new data. PD patients are unable to prevent memory retrieval and mind forgetfulness, which are characteristics that can alter the cognitive and behavioral profiles of these subjects [23]. Lastly, AD and PD progression are associated with depression, anxiety, psychotic symptoms, and sleep disturbances [24,25] and likely impacted by a dysregulation of neural circuitries. This dysregulation can be triggered by the brain iron accumulation present in these patients.

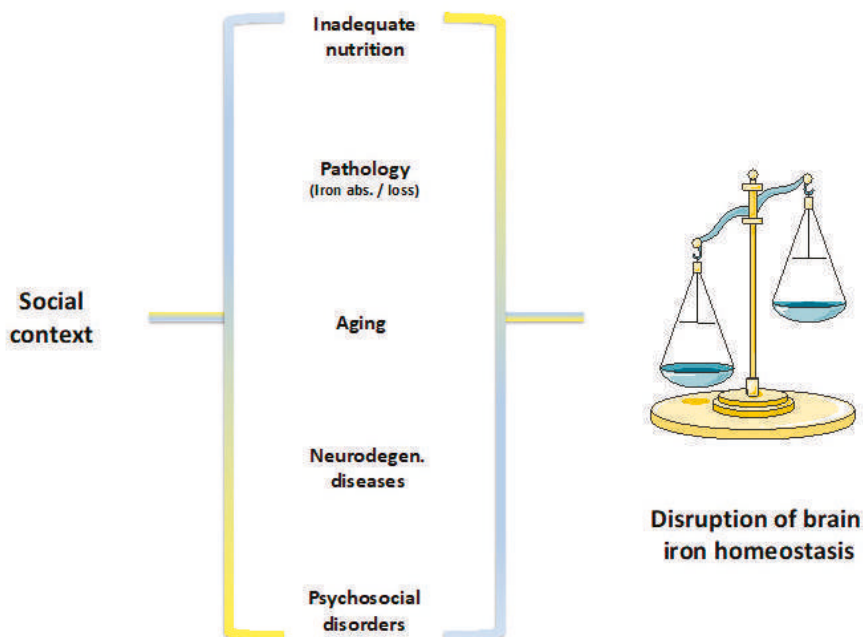


Figure 1. Processes underlying disruption of brain iron homeostasis. Social context, integrating structural (e.g., socio-economic context, life trajectories, socio-economic status, education, knowledge and skills, norms and values), social interactions (e.g., the specific context of interactions; who is present) and individual dimensions (e.g., appraisal, perceptions, motivations and expectations, and reflexivity) frames the pathophysiological mechanisms of an inadequate nutrition (e.g., poor diets, excessive milk intake, vegetarian diets), pathologies associated with inadequate iron absorption (e.g., Celiac disease) or excessive iron loss (e.g., blood loss, parasitic infections), aging, neurodegenerative diseases (e.g., Alzheimer’s and Parkinson’s disease) or psychosocial disorders (e.g., chronic social defeat stress, anxiety, depression). All these conditions are associated with a disruption of brain iron homeostasis.

In psychosocial disorders like chronic social defeat stress, anxiety, and depression, iron’s ability to modulate the levels of neurotransmitters released in the synaptic clefts was shown to contribute to commonly observed cognitive impairments and behavioral modifications [26]. These conditions are also characterized by changes in both structure and function of the prefrontal cortex, where the expression of genes regulating iron metabolism appears to be significantly altered. This means that brain iron accumulation is likely involved in the outcome of stress-induced depression, and pathophysiological changes typical of mood and anxiety disorders (Figure 1) [27]. In the body, 80% of iron is incorporated within the protoporphyrin ring of heme molecules, which is the prosthetic group of heme proteins. Among heme proteins, hemoglobin is the most abundant [2,28]. Interestingly, studies in animal models revealed an increase in hemoglobin gene expression in the brain of animals that were exposed to certain levels of stress. Even though the function of hemoglobin in the brain is still unknown, it has been proposed to control the vascular tone. If that is the case, its increase might

protect neurons against stressful social encounters that could, otherwise, compromise brain functions by damaging its vascular system [29]. In mice, the expression of genes that directly regulate iron metabolism was also found to be changed under stressful conditions. A reduction in the iron importer Transferrin Receptor 1 was observed in response to stress. This reduction could possibly prevent further intracellular iron entry, which would subsequently harm neurons in the pre-frontal cortex, i.e., an area that is mainly involved in conscious decision-making and social action [27]. Conversely, an increased expression of lipocalin 2, which is an iron transporting protein that regulates the morphology and excitability of neurons in the hippocampus and amygdala when mice are exposed to stress [27,30], was associated with a number of modifications in behavioral responses, depression, neuronal excitability, and anxiety [31]. The reasoning for this is that, in the absence of a neutralizing system, an increased brain iron accumulation is capable of promoting oxidative stress and neuronal death, processes that could be undermining cognition, which potentially frames social behavior and eventually leads to pathological development [32].

Given the increasing life expectancy of the world's population, as well as the increasing prevalence of both mentioned neurodegenerative and psychosocial disorders, the study of iron overload and of its etiology is of the utmost relevance. Since the role of iron in neurodegenerative pathologies was the focus of several reviews [3,33–36], for the purpose of this article, we will only address its impacts in psychosocial disorders. As for iron deficiency, we will review previous studies, and discuss the pressing need for research integrating the causes underlying iron overload and its impacts on neurophysiological mechanisms, as well as on concomitant modifications of motor, psychological, and social processes of populations affected by excessive iron.

This paper argues that brain iron dysregulation has profound neurophysiological impacts and is associated with cognitive and behavioral modifications that could be further exacerbated by social contexts that do not promote regular cognitive function and normative social behavior (Figure 2). Hence, this review starts by exploring the mechanisms of iron action in the brain and presents an overview of iron metabolism and of its cytotoxic mechanisms of action. A discussion of cognitive, behavioral, and pathological implications of iron deficiency and overload, and how these can be framed by the social context, will follow. The links between biological, psychological, and social dimensions will be explored and potential avenues of research will be pointed out.

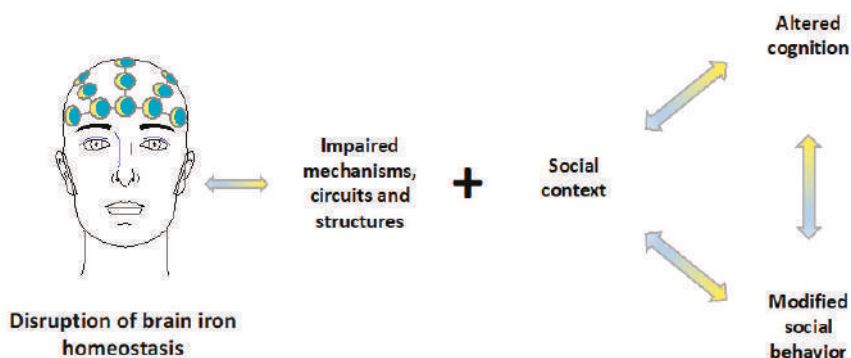


Figure 2. Impacts of disruption of brain iron homeostasis. Dysregulation of brain iron metabolism profoundly affects neurophysiological mechanisms (e.g., neurotransmitter synthesis and metabolism, axon myelination, neural transmission), neural circuits (e.g., mesocortical, mesolimbic, nigrostriatal, and tuberohypophyseal dopaminergic pathways) and brain structures. In the presence of a social context that does not provide stimuli to promote regular cognition and normative social behavior, disruption of brain iron homeostasis is associated with altered cognition (e.g., executive function, attention, memory) and modified social behavior (e.g., functional isolation, wariness, and hesitance behaviors).

2. Iron in the Brain

It has been previously shown that the brain is particularly sensitive to changes in iron homeostasis [2,4,5]. This is the case since iron is critical for maintenance of the high metabolic and energetic requirements of neuronal tissues, neurogenesis, axon myelination, synaptic development, neurotransmitter synthesis, and metabolism [37]. As such, the brain requires considerable amounts of iron, particularly in periods of rapid growth, such as early childhood. These levels are mainly up-taken from the blood, which is the source of brain iron through adult life, when its up-take is considerably lower. In addition, presenting a considerable variation is the distribution of iron in the brain. More specifically, the highest concentrations of iron are found in the basal ganglia, particularly in the substantia nigra, globus pallidus, nucleus caudate, red nucleus, and putamen [38]. Since brain iron is mainly up-taken from the blood, the brain iron status is directly affected by peripheral iron levels and nutrition. In fact, iron deficiency, which is the most prevalent nutrient deficiency worldwide, has been proposed to impact motor function, cognition, and social behavior [26]. In contrast, accumulation of iron in the brain has been associated with aging, neurodegenerative diseases, and psychosocial disorders [26,32,39]. This raises the question about potential adverse effects induced by iron food biofortification, which, in the long term, might contribute to tissue iron accumulation and, ultimately, mediated damage. Nevertheless, iron overload was mostly studied in animal models and the results might not be fully translatable into human subjects, especially in studies exposing mice to non-physiological levels of dietary iron. It is not surprising that behavioral dysfunctions and neurological effects appear when animals are fed with exacerbated concentrations of this nutrient [26,40]. Similar manifestations may also occur in response to other micronutrients excess, as observed upon acute overexposure to the metalloid selenium [41]. As such, a tight regulation of brain iron homeostasis is of the utmost importance.

One of the processes for which the maintenance of iron homeostasis is vital is the regulation of axon myelination and, subsequently, neural transmission. In fact, a great number of studies present iron deficiency in early development as capable of disrupting synaptic function, with potential long-lasting effects that were shown to be associated with impaired cognitive and/or altered social behavior [26,42–44]. However, the importance of iron in the brain further relies on iron being part of the structure of many enzymes involved in energy metabolism and neurodevelopmental mechanisms [42,45]. In many of those, iron acts as a co-factor, which ensures proper functioning. This is the case for cytochrome c oxidase, which is a central enzyme for energy metabolism, tyrosine hydroxylase, which is responsible for dopamine synthesis, and for tryptophan hydroxylase, which is responsible for serotonin synthesis. Iron deficiency anemia has also been associated with decreased monoamine oxidase activity in humans, which promotes a disruption of the metabolism of monoamines, including dopamine and serotonin. In addition to iron's role in the monoaminergic system, it further regulates the balance between inhibitory and excitatory neurotransmitters, γ -aminobutyric acid (GABA) and glutamate, respectively. Moreover, iron was shown to interact with other metals such as zinc, and to be involved in processes such as dendritogenesis, neuro-metabolism, gene profiles, and protein profiles [26,42,46].

Among all age populations, infants and young children are the most sensitive to variations in iron levels [20,47]. Presumably, this is associated with their rapid growth, which requires iron to prevent a decline in functions that are mainly exerted by the basal ganglia and that could lead to an impaired psychomotor development, as well as disrupted cognition and behavior [43]. Contrary to expectations, the effects of iron supplementation to children affected by iron deficiency are still controversial. These interventions were able to correct for iron deficiency and anemia, but their impacts on motor, cognitive, and behavioral outputs are ambiguous. This has been reasoned to result from methodological insufficiencies and heterogeneity of the study design [8,18,20]. These data will be thoroughly discussed under the section "Linking iron deficiency to cognition and social behavior in human subjects."

While there is widespread acknowledgment of the highly deleterious effects of an increased iron status, particularly in the brain, the use of iron-enriched foods and beverages, used to overcome the negative impacts of iron deficiency, is widespread. However, the potential negative impacts of very

high iron intake have not been established [48]. As such, several studies have started to address the effects of high iron intake or high peripheral iron status on neurophysiological mechanisms, cognition, and pathological development. While, in mice, there seems to be very strong indications that high iron intake in early post-natal life has adverse effects on cognition and neuropathological outcomes. The results of studies with human subjects are controversial. This is presumably due to methodological variations. Moreover, the effects of iron supplementation on cognition and pathological progression were only addressed in adults. While the findings are somewhat supportive, high iron intake in adults contributes to the degradation of neuronal function and pathophysiological development. No studies have addressed whether and how iron supplementation in early childhood impacts on adult cognition and behavior, as well as on brain aging and health [32]. Data addressing potential links between iron accumulation, cognition, and social behavior are either inconsistent or absent. Before discussing this topic in depth, we will focus on the details of iron metabolism and cytotoxicity, since these mechanisms are critical for understanding the processes involved in disruption of iron homeostasis and its potential impacts.

3. Iron Metabolism

Since the disruption of iron homeostasis has such severe consequences, the levels of systemic and intracellular iron need to be tightly regulated. In fact, many mechanisms contribute to maintain iron homeostasis, with a vast number of genes being responsible for the control of iron uptake, storage, and recycling. As for iron excretion, no physiological mechanisms have been described so far, with iron losses being presently described through menstruation and skin desquamation [49].

In the specific case of the brain, iron crosses the blood brain barrier (BBB), as iron-loaded transferrin (holotransferrin, HTf). Internalization of the complex iron-Tf:Tf receptors (TfR), expressed on the luminal side of brain capillary endothelial cells, allows its endocytosis. Subsequently, iron is released in acidic endosomes from Tf, and then released from the Tf-TfR complex. Once reduced to ferrous iron, it is translocated into the cytosol by the action of DMT-1. This iron contributes to the labile ferrous iron pool. However, poor expression of DMT-1 on brain capillary endothelial cells raised concerns about the requirement of this transporter for iron entry to the brain. In fact, alternative mechanisms indicated that iron could also be released from endosomal compartments via the expression of transient receptor potential mucolipin-1 (TRPML1) channels. Disrupted iron homeostasis observed in the brain of TRPML1-deficient mice further supports this notion. In addition, dysregulated iron metabolism has been observed in patients suffering from mucopolidosis type IV (ML4), which is a neurodegenerative disease in which a TRPML1 mutation is responsible for reduced brain ferric iron. This could lead to impaired myelination and neuronal damage [50,51]. Whether other iron transporters, like TRPML2 or ZIP8, can also play a role in regulating iron metabolism in brain capillary endothelial cells has not been fully elucidated.

Similarly to DMT-1, TRPML1 transports ferrous but not ferric iron. This suggests that a ferric reductase is required for TRPML1 to release iron from endosomal compartments. Whether this is ensured by one of the Steap family members remains to be established, but the ubiquitous brain expression of Steap 1 and high levels of Steap 2 render these proteins to be strong candidates for this function. Thus, the enzymatic activity of these proteins might increase iron uptake into the cells [52]. Additionally, stromal cell-derived receptor-2 (SDR2), which is a homologue of duodenal ferrireductase DcytB, is expressed in the brain, although its specific localization and function remain to be fully established [53].

Still the object of the discussion is the possibility of iron entering the brain via a transcytosis mechanism. This implies the exocytosis of the recycling endosome, which contains iron-Tf complexes. Their dissociation from TfR at the abluminal membrane results in the detachment of iron from Tf and subsequent release of Tf into the brain interstitium [54]. This potential mechanism of iron entry into the brain is supported by the reported normal amounts of iron in the brain of hypo-transferrinemic mice, i.e., animals presenting less than 1% of circulating Tf [55]. Regardless of the mechanisms, once inside the cells, iron is either directed to mitochondria, to participate in processes ensuring cellular function, or is stored in the cytosol, within the multimeric ferritin L and H chain subunits [33,56].

Excess iron is then exported to the extracellular space by ferroportin, which is the only iron exporter that has been identified so far [57]. The expression of ferroportin on the abluminal membrane of brain capillary endothelial cells is coupled with ceruloplasmin, which presents a ferroxidase activity capable of re-oxidizing ferrous iron into ferric iron and allowing it to enter the Tf distribution cycle. This has led the scientific community to hypothesize that endothelial cells regulate iron entry into the brain by acting as gate-keepers for iron and releasing it when required.

Most importantly, levels of circulating Tf in the brain interstitium are quite low, which implies Tf to become saturated with small amounts of iron. This justifies non-Tf bound iron (NTBI) to be the main source of iron delivery to neural cells. NTBI might have entered neural cells by binding to DMT-1 and should be, subsequently, redistributed to the extracellular space in the brain parenchyma. High levels of ferrous iron can also be found in the cerebrospinal fluid [58], and possibly represent the biological underpinning of the existence of multiple mechanisms guaranteeing iron homeostasis. NTBI can also be up-taken by neurons and enter these cells via DMT-1, Zip-14, or other molecules ensuring non-vesicular import mechanisms [59]. Ferritin can also bind NTBI and cross the BBB via a transcellular transport through the T-cell immunoglobulin and mucin domain-containing 1 receptor 1 (TIM-1) [60], which is the human homologous of the rodent gene TIM-2. This mechanism ensures endocytic iron reutilization in oligodendrocytes [61]. Moreover, the relevance of ferritin as a possible route for iron delivery to the brain was also shown in microglia. These cells can act as an exogenous iron source considering their ability to increase ferritin expression when attempting to limit the amount of extracellular-free iron in the brain. This means that the upregulation of ferritin in microglia is a direct consequent of NTBI uptake [62].

Fenestrated capillaries of the choroid plexus are also playing a dominant role in regulating iron entry into the brain. Their peculiar permeability facilitates not only the diffusion of molecules of different sizes to which iron can bind, but also their regulated entry into the brain through the ferroportin-hepcidin axis present in these cells [63]. In the brain, iron can be transported as described or bound to low-molecular-weight agents such as citrate, ascorbate, or ATP [4]. While ATP and other nucleotides may contribute to the release of iron via the exocytosis-dependent abluminal membrane transport [64], the higher concentrations of citrate and ascorbate when compared to their concentrations in circulation could be justified by the need to prevent NTBI from dysregulating iron homeostasis. Although the affinity of these molecules for iron is lower than that of Tf, their abundance allows to bind significant amounts of NTBI.

Another major player in iron metabolism is hepcidin, which is the iron regulatory hormone. Contrarily to hepcidin role in the peripheral compartment, its role in the brain is still under debate. Currently, it is known that hepcidin targets ferroportin in the brain. However, growing evidence supports the ability of systemic hepcidin to cross the BBB and enter the brain. On the other hand, the existence of an autonomous mechanism justifies the absence of brain iron accumulation in mice or patients presenting hepcidin mutations, such as in hemochromatosis. Additional mechanisms can then be responsible for the maintenance of brain iron homeostasis. These mechanisms should involve microglia and astrocytes, which are the brain cells producing higher levels of hepcidin [65].

Despite the numerous mechanisms regulating iron homeostasis in the brain, this tight regulation can still be disrupted. The following section addresses these mechanisms and the impacts of their disruption.

4. The Cytotoxicity of Iron in the Brain

The main causes underlying brain iron accumulation still need to be studied in more detail. Nonetheless, and in addition to other types of brain iron deposits and superficial siderosis, the literature points at cerebral microbleeds as one of the major factors involved in brain iron overload [66]. These microbleeds are brain microhemorrhages that can occur physiologically in the elderly and affect both brain tissue and ventricles. Microbleeds are often asymptomatic and a recurrent headache is the major complaint. However, they are not always silent. This is the case upon release of hemoglobin into

intracranial cavities, which can lead to clinical manifestations such as weakness, vomiting, seizures, decreased level of consciousness, neck stiffness, and others [67].

When unlocked from cells, hemoglobin is oxidized and rapidly broken-down to its by-products, heme, and iron [2]. Therefore, depending on the extent of cerebral microbleed, brain iron accumulation can compromise synaptic transmission and neuronal functions [68]. This suggests that individuals that are more susceptible to vasculature impairments, such as patients affected by genetic blood disorders like sickle cell or thalassemia, could present higher risk of cerebral microbleeds. Those microhemorrhages could contribute to neurological conditions that these subjects might suffer from [69]. In fact, this is supported by a study with children affected by hemoglobinopathies [70], and developing a reversible neurological complication, known as leukoencephalopathy syndrome, upon blood transfusion [71]. This means that blood treatments might cause long-term effects to organs such as the brain, which might result from potential iron accumulation over time. Still, iron toxicity is prevented by storing excess iron in ferritin and intracellular vacuolar-like structures, like lysosomes. Therefore, this does not come as a surprise regarding the importance attributed to lysosomal impairment during aging [72]. The role of lysosomes in ferritin degradation [73] allows us to understand their strict relationship with brain pathologies [74] like neurodegenerative diseases such as Alzheimer's disease [75,76]. These disorders have been defined as iron storage diseases, pathologies in which iron accumulates as encapsulated iron, i.e., iron contained within vesicles [77,78]. Currently, it remains to be elucidated whether this iron is toxic or accumulates in response to chronic stress, acting as a stress responsive intracellular second messenger, as recently proposed [79]. It is, therefore, possible that iron becomes toxic only after certain thresholds. All these questions will remain opened until final results of iron chelation therapy on neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, are fully disclosed. Nevertheless, the reduction of iron accumulation in patients with Parkinson's disease and treated with deferiprone was considered satisfactory [80,81]. New compounds, like the chiral 3-hydroxypyrid-4-one derivative, which present the same properties but prevent the side effects of neutropenia and agranulocytosis after prolonged exposure to iron chelation treatment, are currently being tested in different models of Parkinson's disease models.

Despite the numerous mechanisms to prevent iron cytotoxicity, the ability of iron to transfer electrons allows its participation in the Fenton chemistry and the generation of highly reactive and harmful hydroxyl radicals (HO•), from the conversion of diffusible H₂O₂ [82]. Once produced, reactive oxygen species (ROS) can damage lipids, proteins, and nucleic acids, which leads, ultimately, to cell death. In the specific case of the brain, the high abundance of lipids renders it very susceptible to oxidative stress. Since these lipids are ensuring neuronal communication by mediating impulse transmission, brain oxidative stress is considered among the etiology of most brain disorders [34,83]. In addition, the irreversible damage caused by iron fueling ROS production in the brain is also due to the lower tolerability and repair capacity of this organ when compared to other tissues [84].

Therapeutic interventions based on antioxidant administration have not been successful to prevent the neural toxicity of iron-driven oxidative stress. The inefficiency of drugs to cross the BBB could justify the lack of positive outcomes. However, this could also be influenced by the need of a small amount of ROS production for cell metabolism, which these drugs are suppressing. For example, microglia produce ROS to activate phagocytosis and eliminate possible harmful stimuli in the brain parenchyma. ROS further regulate the activity of both kinases and phosphatases, and modulate signaling pathways activated by transcription factors. In neurons, most ROS are derived by oxygen consumption, as by-products of mitochondria energy production [85]. The abundance and intense activity of these organelles in the brain, as they provide the energy for daily activities, renders the brain highly susceptible to oxidative stress. For example, meta-analytic studies have shown that increases in oxidative stress are associated with depressive disorders, including major depressive disorder and bipolar disorder [86]. This notion is also supported by the fact that mitochondria are the main source of intracellular iron [87]. A link between chronic exposure to stressors, maladaptive mitochondrial changes, and neurodegenerative disorders like dementia and AD, has also been suggested. Both

heme synthesis and assembly of iron-sulphur clusters occur in the mitochondria, where density and activity vary, according to brain regions. This explains the existence of areas that are more sensitive to iron-driven oxidative damage than others. As an example, the substantia nigra and the striatum are areas where iron mostly accumulates and reaches higher concentrations when compared to the hippocampus, cortex, pons, and medulla. These latter regions present the lowest amount of iron [33]. The oxidation of dopamine to neurotoxic metabolites, which occurs in the substantia nigra and is coupled to increased iron levels, further boosts oxidative damage and justifies the sensitivity of this region to neuronal death [88]. As mentioned above, the main target of ROS production in the brain is neuronal membranes rich in polyunsaturated fatty acids (PUFA). Lipid peroxidation is a key feature defining iron-mediated cell death, also known as ferroptosis [34,89]. Still, iron's role in impairing brain activity, cognitive functions, neuroplasticity synaptogenesis, and synaptic transmission remains to be elucidated. Nevertheless, epidemiological studies with PUFAs supplementation were shown to reduce the risk of neurological and psychosocial disorders [90]. Thus, a better understanding of causes leading to iron-driven oxidative stress is important for the development of effective therapies that prevent neuronal damage and functional impairment. The following section will precisely start to address the potential links between the neural circuits involved in iron regulation, cognition, and social behavior.

5. Neural Circuits and Social Behavior

Social behavior is one of the main areas of current research in fields as diverse as anthropology, sociology, psychology, or neuroscience. Simply put, social behavior is how we do things in social contexts. If one focuses on human social behavior, we are talking, for instance, about how we perform our jobs or how we spend our holidays, how we discuss things with others, or how we run away from them. Any of the above-mentioned behaviors are framed by knowledge and dispositions that we have embodied through our life trajectories, perceptions of the situation that we are being confronted with, and its broader social-economic contexts, our motivations, and expectations, as well as biological factors, mechanisms, and the more general physiological status. Changes in these processes characterize many neuropsychiatric disorders and psychosocial dysfunctions. This justifies an increasing interest in understanding the molecular mechanisms regulating neural circuits involved in the development of social behaviors. Since most genes are conserved across species, once again, research in rodents was fundamental to dissect specific brain networks involved in the development of social behavior. In human subjects, the brain structures involved in cognitive processes were originally identified through magnetic resonance imaging (MRI) studies, which revealed activation of specific regions upon the development of specific social tasks [91]. The somatosensory and temporal cortex are involved in perception and recognition of facial emotions [92]. The amygdala is involved in fear processing and threat detection [93] and the prefrontal cortex is responsible for decision-making and behavior, as well as performing executive functions [94]. Changes in the amygdala response can result in social anxiety disorders, and damages to the frontal cortex can underlie psychopathological and antisocial behavior [95]. Still, the existence of neural circuits interlinking specialized cortical and sub-cortical structures ensure that these areas work together to ensure that a certain behavior takes place [91].

From a molecular point of view, a series of neurotransmitters and neuromodulators regulate the activity and maturation of these brain areas. Although glutamate, GABA, serotonin, and noradrenaline contribute to modulate pre-frontal-striatal circuits, among neurotransmitters, dopamine stands out. The involvement of dopaminergic projections is responsible to exert functions resulting from the cooperation of all previously described areas for sustained attention, memory storage and retrieval, emotion regulation, and motivation. Since iron impairs dopamine synthesis, by influencing the activity of the enzyme that produces it, i.e., tyrosine hydroxylase, and fosters the production of ROS through dopamine catabolism [96], others have suggested that the outcomes of neurocognitive tasks are highly influenced by any disturbance in iron homeostasis [47]. It is precisely the link between brain iron homeostasis, cognition, and social behavior that will be addressed in the following sections of this article. Special attention will be devoted to studies with human subjects.

6. Linking Iron Deficiency to Cognition and Social Behavior in Human Subjects

Many studies have shown that iron deficiency with or without anemia is associated with short-term and long-term impacts on psychomotor development, cognition, and social behavior in human subjects (Figure 3). While iron deficiency is more prevalent and studied in early childhood, which is a period of rapid growth and high needs for iron, an elevated risk for iron deficiency is also present in women of reproductive age, as a result of regular blood loss during menstrual cycles, and during pregnancy, which is characterized by increased iron requirements [8]. In fact, some of the previously mentioned neurophysiological, cognitive, and behavioral features of early iron deficiency were also reported to be present in iron deficient women of reproductive age [97–99]. During pregnancy, iron deficiency anemia has been associated with prematurity, low birthweight neonates, maternal morbidity, and impaired mental and psychomotor development [4,8,100]. Even though the number of studies is presently limited, future studies should address whether these modifications are the strict result of iron deficiency or, rather, of its interactions with other factors.

In what concerns the mechanisms connecting a disruption of iron status to neurophysiological and cognitive impairments and alterations in social behaviors, it has been previously suggested that an iron-induced disruption of major dopamine pathways (i.e., mesocortical, mesolimbic, nigrostriatal, and tuberohypophyseal), would possibly impair its ability to propagate neuronal impulses. This disruption could potentially lead to motor and cognitive impairments, altered social behavior, and/or pathological development [47]. A different line of research has suggested that iron deficiency can be deleterious to axon myelination. In fact, it has been documented that early iron deficiency is associated with short-term and long-term latency delays in visual and auditory evoked potential studies. The latest study results that are consistent with impaired axon myelination have lower amplitude in the variability of heart rate in the sleep-wake cycle [101–103]. This could be due to the documented alterations in iron status, but also due to its impacts on different neurotransmitters such as serotonin, norepinephrine, or others. It is also possible to find some studies in which the evoked potential analysis was inconclusive and the results were attributed to problems in the study design [104].

Specifically, regarding the dopamine-dependent pathways, studies have shown that children and young adults with chronic, severe iron deficiency with or without anemia in infancy, present altered dopamine frontal-striatal circuits, known to be involved in the control of executive functions, sustained attention, memory, emotion regulation, and motivation. In particular, the authors show that early severe iron deficiency was most likely contributing to impairments in neurocognitive and motor functioning and modifications in social behavioral in infancy, at 5 years of age and at 11–14 years of age. At the age of 19, cognitive impairment, as revealed by poor inhibitory control and executive functioning, was shown to be present [47,103].

The negative impacts of iron unbalance on dopamine action can also affect the mesolimbic pathway, which connects the ventral tegmental area in the midbrain to the ventral striatum of the basal ganglia in the forebrain. Alterations in this region, where dopamine plays a central role in behavioral activation, inhibition, positive affect, and reward [105,106], were proposed to be involved in increased prevalence of wariness and hesitance behaviors, lack of positive affect, diminished response to novelty, and social engagement. These modifications, present in infants with iron deficiency, with or without anemia, seem to show a dose response to the severity of the iron deficiency [43,107–114].

The dopamine nigrostriatal pathway is involved in movement control and regulation. In fact, infants with iron deficiency with or without anemia were shown to have impaired motor sequencing and bi-manual coordination, which is a symptom that was more severe in anemic infants [115,116]. In a different study, iron deficient infants, with or without anemia, presented a lower rate of spontaneous eye blink, which is a widely accepted measure of the nigrostriatal pathway (anemic infants had the lowest levels). The study further showed that a spontaneous eye blink could be partly corrected with iron supplementation in anemic infants [117]. Children with iron deficiency also present a disrupted learning process, which results in impaired cognition and altered social behavior.

Lastly, the involvement of the dopaminergic tuberohypophyseal pathway has been studied fewer times. Nevertheless, dopamine production by the hypothalamus inhibits prolactin release by the anterior pituitary. As such, decreased dopamine function as present in iron deficiency should be associated with increased prolactin levels. This was shown to be the case in iron deficiency anemia in infancy [118]. Even though these results are indicative of an impaired tuberohypophyseal pathway and long-term dysregulation of prolactin, one should note that the regulation of prolactin is complex and involves other neurotransmitters such as serotonin.

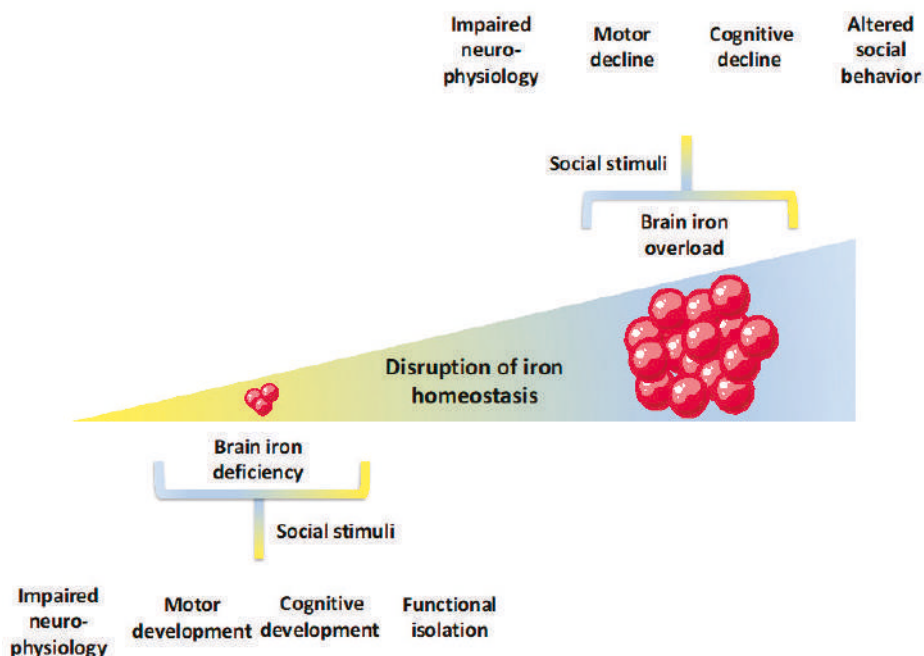


Figure 3. Multilevel repercussions of iron deficiency and iron overload in the brain. Brain iron deficiency is associated with disruption of neurophysiological mechanisms that, within a social context that does not provide regular stimuli, compromises motor and cognitive development (e.g., impaired motor sequencing and bi-manual coordination, poor executive function, attention, and memory). Subsequently, these can impact social behavior, which leads to, for instance, functional isolation. As for the impaired neurophysiological mechanisms associated with brain iron overload (e.g., exacerbated brain oxidative stress, neuronal cell death), these are associated with motor and cognitive declines (e.g., motor slowing, insufficient adjustment and altered feedback processing and sensitivity, memory loss, and impaired decision-making). Within a social context that does not provide suitable stimuli, these might impact social behavior leading to, for instance, hesitant behavior and unwillingness to interact with others.

At this point, there are several important arguments to put forward. First, the brain works as an integrated system and, as such, responses are unlikely the result of impairments in the action of specific neurotransmitters, regions, circuits or processes, but rather result from the concerted action of interconnected and interdependent neurotransmitters, processes, circuits, and regions. Second, other dimensions could have a role in mediating or potentiating the biological and neurophysiological impacts of iron deficiency. In this line of reasoning, a very important dimension to consider when analyzing the impacts of iron deficiency in cognition and social behavior in human subjects was not yet addressed in this section. What is the role, if any, of the social dimension? Social psychology and

sociology have shown that the social context in which individuals are embedded influences individual cognition and action. However, there is little, if any, evidence of how it acts concertedly with the neurophysiological impacts of iron deficiency.

In fact, some studies have started to give innovative inputs into this discussion. In 2013, Algarín and colleagues developed a study with 10-year-old children who had early iron deficiency anemia and were given iron supplementation for at least six months [103]. At 10, these children presented hemoglobin levels within a normal range but still had impaired executive functions, as assessed by slower reaction times, less accuracy, and poorer inhibitory control as compared with children that did not present early iron deficiency anemia. Since the iron deficiency anemia and the control groups were matched for a socio-economic status, Algarín and colleagues discarded the potential effects of the social context. In line with the “critical period hypothesis,” stating that an absence of adequate nutrients in critical developmental periods results in permanent structural deficits that cannot be corrected with nutrient supplementation after that period [119], the authors argue that these results are consistent with long-term effects of iron deficiency anemia on axon myelination and dopamine-dependent prefrontal-striatal circuits. These results could also be consistent with the “Altered-regulation hypothesis,” which states that early nutrient deficiency modifies the brain epigenetic landscape via CpG methylation, histone modification, or hydroxymethylation [120].

In a different study, Carter and colleagues have shown that nine-month old infants with iron deficiency anemia presented poorer attention and recognition memory than infants without anemia (both with and without iron deficiency) [121]. These effects were at least partially mediated by a decreased ability to engage affectively with the environment and by other socio-emotional modifications. The authors further show that there was no relation between the impairment of cognitive function and maternal socio-demographics. These data raise questions about the role of the social dimension in the cognitive and behavioral factors that characterized these children, which shows that it probably involves other factors beyond socio-demographic characteristics.

However, one could easily argue that cognition and behavior are framed by a multitude of psychological and social dimensions (including norms, beliefs, knowledge and dispositions, interaction context, actors’ perceptions, appraisals, expectations and motivations, among others) [122,123]. To give a specific example, one of these processes is known as personal reflexivity, which is the capacity that individuals have to think about themselves and the world, their decisions and interactions, as well as to project future behavior [124–126]. The authors have found diverse profiles of personal reflexivity, framed, among others, by the individuals’ social origins. The social-economic contexts in which they live, their life trajectories and interactions, as well as the available resources. Most importantly, these profiles of individual reflexivity impacted not only how individuals think about themselves and the world, but also how they planned for their future and acted in social contexts. As such, cognition and behavior cannot be subsumed to a one-to-one linear relation either with the social-economic status or with the maternal socio-demographics. In fact, an immense number of studies have shown that the development of executive functions and subsequent social behavior, are also highly dependent on the stimulation cues that one receives from the social environment throughout their lives. These cues characterize social interactions in general (i.e., who you interact with, what type of interactions you have, how frequently you have them and others), as well as interactions during formal learning processes [127]. More specifically, it has been consistently shown that environmental interventions can potentiate cognitive functions, such as working memory [128–130]. If this is the case, an analysis of the social-economic status, or of the maternal socio-demographics, in spite of providing most relevant inputs, is not able to capture the complexity of the social environment and the individuals’ appraisal of that same environment. Given the above, even though these studies do present clear associations between early iron deficiency anemia and cognition, they definitely do not allow us to rule out the role of the social dimension in cognition.

In fact, a couple of studies already shed some light into this discussion. One study shows that five-year-old children with early iron deficiency anemia are less active, more inhibited, and shy in

a mother-child interaction [131]. These behavioral patterns are similar to the ones presented by other malnourished children [132,133]. Concomitantly, the same study reports that the mothers of children with normal iron status are more responsive and have richer interactions with their children. As such, the modifications that children present in social behavior could result from the combined effect of early iron deficiency anemia and decreased social interaction patterns that these children were subjected to. Adding on to this line of reasoning, more recent studies have confirmed children with iron deficiency to be less active, emotionally dull, and disengaged [112]. In addition, iron-deficient children seek and receive less stimulation from caregivers, which is a process described as “functional isolation” [134–136]. The authors argue that, over time, these behaviors result in smaller motor, cognitive, and social inputs from the environment and could interfere with social learning processes and development. To address this issue, it has been proposed that interventions for iron-deficient children should target social interactions [135]. In fact, this strategy was already mobilized on a study combining iron supplementation with a home intervention, designed to foster child development by providing support to the mother-infant relationship and has shown some positive effects on cognition and behavior [137]. Other authors have suggested that iron supplementation should be accompanied with cognitive training [138], such as working memory training, shown to present positive outcomes in cognitive functions [127].

Altogether, this clearly points to a role of iron deficiency and psychological and social processes in the development of cognition and social behaviors. The point being made here is that, despite the relevance of iron and of its neurophysiological action, these mechanisms are most likely interdependent with psychological and social dimensions, as individuals live in social contexts, grow in social contexts, learn in social contexts, and construct their memories in social contexts. Future studies should provide an in-depth characterization of all these dimensions, as well as of the specific interconnections among them.

7. Increased Iron Levels, Cognition, and Social Behavior in Human Subjects

The link between cognitive impairment, modifications of social behavior, and brain iron accumulation in human subjects is not well-understood. The few studies focusing on the impacts of elevated iron levels in human subjects are characterized by wide differences in research design and socio-demographic characteristics of the analyzed population. Furthermore, wide variations on population size and outcome measures render available data to present inconsistencies that are difficult to reconcile [32]. Additionally, unavailability is a characterization of social behaviors and social contexts of the studied population, which is a characterization that, as previously pointed out, is of major relevance when studying cognitive processes. In addition, while it would be of major relevance to understand whether high iron intake in the early childhood period, a common supplementation for neonate/infant iron deficiency, impacts brain mechanisms and cognition in adult life, no studies have addressed this issue. These studies are necessarily timely and costly, but, considering the increasing life expectancy, prevalence of neurodegenerative diseases as well as the relatively widespread intake of iron-enriched food and beverages to prevent or overcome early iron deficiency, these studies are of the utmost relevance [32].

Nevertheless, previous scientific studies still present some hints that should be taken with careful consideration when discussing potential impacts of iron overload in cognition and social behavior (Figure 3). Studies with human subjects have mostly focused on whether high iron intake or high peripheral iron in adults were associated with poorer cognitive outcomes (we will address the impacts of brain iron accumulation in psychosocial disorders in the following sections of this paper). In this line of research, some studies have shown no associations between plasma or serum iron levels and cognitive outcomes [139–141] (one cross sectional study with 2000 subjects with more than 65 years old and two cohort studies with approximately 800 subjects with ages above 50 years). Still, other studies present the opposite outcome, which reveals an association between diverse measures of iron status and cognition. More specifically, a cross sectional study involving 1451 subjects (mean age of 75 years

old) revealed an association between plasma iron levels and cognition [142]. On their hand, Umer and colleagues have shown that mild cognitive impairment is present upon high serum iron (98%), ferritin (56%), and transferrin saturation (107%). This was a cross sectional study, developed on 87 nursing home residents with more than 65 years old and who presented no neurologic diagnosis [143]. In 2012, Mueller and colleagues have shown, in a relatively small study, that the progression from mild cognitive impairment to Alzheimer's disease was associated with serum copper to non-heme iron ratio (19 control subjects, 11 cases of stable mild cognitive impairment, 7 cases of progressive cognitive impairment, and 19 cases of early dementia, mean age of 78 years old). Lastly, pre-menopausal and peri-menopausal women presented an association between improved cognitive outcomes and lower serum ferritin levels (n = 4959 men and women, 35–60 years old) [144]. Taken together, the data gathered with human subjects with results obtained with animal models, which show a clear association between increased iron and cognitive impairment [32], there seems to be some indications for a link between systemic iron status and cognition that needs to be studied in more detail.

A diverse approach further addressed iron's impacts on cognitive decline in older populations. These studies focused on the association between cognitive function and brain iron deposits, which are shown to be present in several brain regions. The presence of these deposits has been mainly attributed to impairments in the regulation of brain iron [145], brain microbleeds, focal micro-hemorrhages, with iron deposits in lobar white matter, basal ganglia, and internal capsule [67], and superficial siderosis, which are accumulations of hemosiderin in subapial layers of the brain [146]. These studies have reported that increased iron deposit volume was associated with decreased cognitive ability [147–153]. However, the association of aging, iron deposits, and impaired cognition remains to be clarified. While one extant cross-sectional study presented data consistent with such a notion [150], the only longitudinal approach to the role of iron aging-associated cognitive decline failed to confirm the previous results [151]. Despite the divergences in the scientific literature, since the reported patterns of iron deposition in the brain of healthy human subjects were similar to the ones present in neurodegenerative diseases, it has been proposed that iron deposits could be used as an indicator of early prediction and diagnosis of neuropathological progression [152].

There is yet a different line of studies focusing on the relation between increased iron levels, neuronal mechanisms, cognition, and social behavior, which could reveal some interesting cues. Studies focusing on iron-induced oxidative stress [86] suggest that iron might play a key role in reactions to psychosocial stress, which impact neuronal mechanisms and cognition. In fact, it has been shown that psychosocial stress, which was previously associated with oxidative stress in animal models and human subjects [154,155], can lead to iron accumulation in the brain, which exacerbates brain oxidative stress and leads to a neuronal cell in rats [156]. This mechanism is aggravated by iron overload [157], which is a condition with increasing prevalence in healthy men, postmenopausal women, and the elderly population and has been attributed to changes in dietary habits [158,159]. These data suggest a reinforcement mechanism. Not only stress can disrupt brain iron homeostasis, contributing to iron accumulation in the brain, but also increased iron uptake further impairs brain oxidative balance and, subsequently, brain functioning. Taking in account the increasing age of the world's population and changes in diet, it would be particularly relevant to have a more complete understanding of the mechanisms mediating stress-induced iron accumulation, iron overload, and brain functioning.

In order to have a full sketch of this system, one should look further at the relation between psychosocial stress and cognition. In fact, it is known that decision-making, the process of choosing between alternative courses of action, can become impaired under stressful conditions [160]. Decision-making processes develop via neurophysiological mechanisms and are framed by broad social-cultural contexts, perceptions, and appraisal of the situation, available knowledge, decision content, motivations, and expectations [161–163]. Furthermore, it was also shown that psychosocial stress activates internal biological processes favoring the occurrence of an inflammatory response [164–166]. This strict correlation between stress and inflammation is detectable even in healthy individuals but overwhelmed with time constraints and increased task demand [167,168]. Upon

prolonged stressful conditions, neuronal activity, motivation, and goal-directed motor behavior start to decrease, which is an effect that is dependent on increased levels of pro-inflammatory cytokines [169]. Lastly, these changes in brain function result in anhedonia, fatigue, motor slowing, psychomotor retardation, and isolation in mice [170]. In human subjects, stress promotes the mobilization of dysfunctional strategies such as looking for premature closure and non-systematic scanning, insufficient adjustment, and altered feedback processing and sensitivity [160]. Additionally, the association between stress and decision-making was shown to be shaped by several social moderators, such as the nature of the stressor, the individual stress reaction, or individual characteristics [171]. Research also shows that the appraisal of the stressor itself, either as a challenge (i.e., the situation is seen as an opportunity for mastery or personal growth) or as a threat (i.e., when demands are seen as exceeding the available resources), should be taken into account, since it influences performance [172]. As such, we know that brain iron accumulation is present under conditions of psychosocial stress. We also know that psychosocial stress impacts on cognitive processes such as decision-making as well as psychological and social dimensions are critical for decision-making processes in the presence or absence of stressful conditions. It remains to be ascertained whether and how iron accumulation plays a role in these processes, directly or indirectly contributing to compromising cognitive function, including impaired appraisal of the environment and decision-making processes. Some hints can be given by looking closely at studies focusing on chronic social defeat stress (CSDS), which is a neuropathology that we will focus on in the following section.

Lastly, as for iron deficiency, it is our understanding that future studies should definitely consider an integrated analysis of iron neurophysiological mechanisms, cognitive processes, and thoroughly characterize social behaviors and its contexts. This is the case since it is known that cognition and behavior are also dependent on the stimulation cues one is subjected to from the social context in which we live in [128–130] as well as by broader social-cultural structures, interaction contexts, actors' perceptions, dispositions to act, motivations, and expectations [162,163]. These social cues, which are expected to act concertedly with the biological mechanisms, could interfere with the biological footprints of an iron dysregulation on cognition and behavior. This renders it difficult to understand the mechanisms at stake.

8. Linking Iron with Chronic Stress, Anxiety, and Depression

Social behavior and stress are deeply connected, and this interrelation was firstly revealed by experiments conducted in animal models describing how social interactions in rodents can cause stress and how social behavior can change in response to environmental stressors [173]. In human subjects, stress caused by social interactions was shown to profoundly impact individuals' health and well-being [174]. In fact, both biological and neurobiological mechanisms underlying social behavior are influenced by molecular changes characterizing stress, which are understood as the physiological or psychological response to a prolonged internal or external stressful event (i.e., a stressor), even when it is no longer physically present but is recollected by the individual [175]. When stress is prolonged, the constant state of alertness that individuals are subjected to results in the development of chronic stress, which is associated with chronic fatigue and characterized by low levels of vitality, vigor, and energy [176]. Chronic stress leads to worse cognitive performance and impairs decision-making, including information processing, learning, and risk-taking [177].

Chronic stress has a major impact on the human brain, which alters the structure, connectivity, and neurochemistry of affected areas. Profound changes are mainly observed in hippocampal morphology, function, and neurogenesis [178], which are then associated with a neuroinflammatory profile and impaired neuronal plasticity. Dysfunction in neuronal connections of the basolateral amygdala can be triggered by stress, and compromise the structure, connectivity, and density of excitatory and inhibitory neurons of this region [179]. The release of molecules known as stress mediators and their binding to correspondent receptors activates biological mechanisms, which allows a subsequent adaptation to the challenging situation and shifts the physiological balance toward a new homeostasis. In this phase,

oxidative damage and cognitive decline were pointed out as capable of modulating the response to chronic stress and the brain's ability to fight against it [180].

From a molecular point of view, exposure to chronic stress activates a neuroendocrine response, which results in the production of the stress hormone, cortisol, and in its subsequent release into circulation. Although there is little research on the ability of iron to modulate the neuroendocrine system in human subjects, lower cortisol levels were shown to be produced in conditions of serum iron deficiency [181]. The correlation between cortisol and iron could be justified by the notion that the enzyme necessary for the synthesis of this stress hormone is an iron-containing protein. Hence, cortisol synthesis strictly depends on iron levels [182].

The autonomic nervous system and endocrine signals involved in cortisol modulation of neural and behavioral responses are influenced by the circadian rhythm, which, in turn, is highly dependent on the levels of circulating iron [183]. More specifically, while cortisol levels are low around midnight, rise approximately three hours after and reach their peak in the morning [184], the opposite occurs for serum iron. Assessed as transferrin saturation, iron is higher at night and starts to gradually decrease during the morning. No circadian variations have been detected for transferrin receptors or ferritin expression [185]. The impact of iron on the circadian regulation of cortisol is due to most clock-related genes being iron-containing molecules [186], so one can extrapolate the effects of iron deficiency or iron accumulation for the production of this hormone. Since iron and stress were shown to have strong impacts on circadian rhythms, one can speculate that variations in this mechanism could be the basis of modifications in social behavior, and, eventually, a decline in health and well-being.

Disruption of the circadian clock is also associated with anxiety, which can be conceptualized as the response to stress and dangerous situations, and is present in more than 12% of the entire population. Anxiety is described by the current Diagnostic and Statistical Manual (DSM-5), published by the American Psychiatric Association (APA), as "characterized by feelings of tension, worried thoughts, and physical changes like increased blood pressure." Thus, under the definition of anxiety disorders, the APA refers to those that share "features of excessive fear and anxiety and related behavioral disturbances" [187]. Anxiety is characterized by a high arousal negative state [188]. In the later decades of the twentieth century, its prevalence and consistent increase led researchers to claim that we live in "the age of anxiety" [189]. A number of different factors can trigger anxiety, and these were shown to be modulated by iron levels. As the first example, the more intuitive, anxious feelings are associated with iron deficiency, since low iron levels translate into a lack of oxygen to tissues. This means that this anxiety state can be reverted as soon as normal iron levels are restored [190]. The negative influence of iron on dopamine synthesis, as previously described, also contributes to develop a state of anxiety, given the importance of this neurotransmitter in maintaining attention and focus. While conditions of iron deficiency compromise the activity of tyrosine hydroxylase and results in lower dopamine production, the same occurs with excess iron, since this metal causes dopaminergic neurodegeneration by exacerbating the oxidative stress produced by dopamine degradation. Consequently, individuals suffer from cognitive changes, altered performance, and memory loss. In mice, the development of anxiety is likely accompanied with the accumulation of iron in the brain, which occurs upon a reduced expression of antioxidant enzymes and oxidative-mediated damage to catecholaminergic innervated regions of the anterior forebrain [191]. In rodents, anxious behavior can be improved by restoring the redox balance in these areas through the uptake of dietary or pharmacological antioxidants. Whether this occurs in human subjects needs to be fully elucidated. However, the consumption of fish-derived omega-3 poly-unsaturated fatty acids as well as docosahexaenoic and eicosapentaenoic acid aids preserve cognitive capacities via a mechanism that can possibly reduce iron-driven oxidative damage and restore the expression of antioxidant enzymes [90]. This further points at the need to monitor iron levels in psychosocial disorders, since the development of new interventions maintaining iron homeostasis could contribute to better outcomes.

The effects of iron deficiency on psychosocial disorders were further reported in depression. Several studies indicate anemia as a pre-disposing factor [192,193] for a condition that, unlike anxiety,

is characterized by a low arousal negative state accompanied by pervasive negative thoughts and emotions about oneself and the future [188]. Individuals with major depressive disorder have difficulties engaging in cognitive reappraisal (i.e., an effort to change the subjective evaluation of a situation in order to modify its emotional impact), which is a key emotional regulation strategy to deal with stressful or uncontrollable events [194]. Early adversity, conflict occurrences, maternal depression, socioeconomic status, and others, can be considered as major causes for depression, and have been associated with low-grade inflammation. This is corroborated by prospective, longitudinal, and meta-analyses research. These studies show depression to be correlated to an increased immune response. Inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-6, C-reactive protein, the hemoglobin scavenger protein, and haptoglobin, have been found in the peripheral blood of patients suffering from severe depressive symptoms [195,196]. Thus, given the cross-talk between iron metabolism and inflammation [197], one can understand the involvement of genes controlling iron hemostasis in the pathogenesis of this disorder.

Studies in mice show that stress-induced depression causes brain iron overload and affects specific regions of this organ, such as the hippocampus. Administration of iron chelation therapy to these animals prevents neuronal loss [198], which is an effect that is also observed in human subjects with depression when subjected to a similar treatment. Widespread iron deposition was found in the brain of these individuals and was recognized as capable of predicting both depression and memory impairment [199].

Thus, although the biological basis of chronic stress, anxiety, and depression remains very elusive [26,31,191,200,201], overall, iron is considered to be a key biological marker for psychosocial disorders associated with impaired cognitive functions and altered social behavior. As previously discussed, the potential effectiveness of iron-dependent treatment strategies should take into consideration that, even though cognition and behavior do develop through iron-dependent physiological and neurophysiological mechanisms, they are unequivocally framed by psychological and social processes that should also be addressed in future research.

9. Discussion

Dysregulation of iron levels in human subjects is a relatively common phenomenon. On the one hand, we have iron deficiency, which is the most prevalent nutrient deficiency worldwide, and, on the other hand, iron overload, which is common in the increasing aging population as well as in pathologies such as neurodegenerative diseases and psychosocial disorders. For both cases, the impacts of iron dysregulation in neurophysiological structures and mechanisms have been studied, with consistent data published in both animal models and human subjects. The association between iron dysfunction and motor and cognitive impairment, as well as modified social behavior, is also very consistent. However, while most studies show that iron interventions could correct for altered iron levels, this type of intervention was mostly unable to revert cognitive or behavioral modifications. Within the iron community, many authors either underline the methodological insufficiencies of intervention studies or argue that iron dysregulation during critical periods, cannot be reverted. Other authors argue that correction of iron levels necessarily needs to be complemented with cognitive stimuli and other types of social interventions, which is a proposal that is completely in line with the findings of social psychology and sociology.

It is our understanding that, while biological impacts or iron dysregulation are undeniable and deserve to be studied and discussed in great detail, its associated cognitive and social blueprints could be further exacerbated by psychosocial contexts that do not promote regular cognitive function and normative social behavior. The study of these interactions is of the utmost relevance for the design of future research aiming to understand how individuals think and act under physiological and pathophysiological conditions.

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Review

Potential Treatment of Retinal Diseases with Iron Chelators

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Abstract: Iron is essential for life, while excess iron can be toxic. Iron generates hydroxyl radical, which is the most reactive free radical, causing oxidative stress. Since iron is absorbed through the diet but not excreted from the body, it accumulates with age in tissues, including the retina, consequently leading to age-related toxicity. This accumulation is further promoted by inflammation. Hereditary diseases such as aceruloplasminemia, Friedreich's ataxia, pantothenate kinase-associated neurodegeneration, and posterior column ataxia with retinitis pigmentosa involve retinal degeneration associated with iron dysregulation. In addition to hereditary causes, dietary or parenteral iron supplementation has been recently reported to elevate iron levels in the retinal pigment epithelium (RPE) and promote retinal degeneration. Ocular siderosis from intraocular foreign bodies or subretinal hemorrhage can also lead to retinopathy. Evidence from mice and humans suggests that iron toxicity may contribute to age-related macular degeneration pathogenesis. Iron chelators can protect photoreceptors and RPE in various mouse models. The therapeutic potential for iron chelators is under investigation.

Keywords: chelation; iron; retina; age-related macular degeneration (AMD)

1. Introduction

Iron is essential for life. In the retina, iron is particularly significant for phototransduction. Each day, photoreceptors shed and regenerate disc membranes, using the iron-containing enzyme fatty acid desaturase to synthesize lipids used in disc membrane generation [1]. RPE65 is an iron-dependent enzyme used by the retinal pigment epithelium (RPE) to catalyze the conversion of all-trans-retinyl ester to 11-cis-retinol, a critical step in the visual cycle [2,3].

While iron is essential for retinal metabolism and the visual cycle, excess iron can be toxic. Ferrous iron can catalyze the conversion of hydrogen peroxide to the highly reactive oxygen species (ROS), hydroxyl radical. Hydroxyl radicals cause oxidative damage to proteins, DNA, and lipids [4]. Hydroxyl radicals have also been implicated in the pathogenesis of the neurodegenerative diseases Alzheimer's [5] and Parkinson's [6], and iron chelation is currently in clinical trials for these diseases [7–9]. The retina is prone to oxidative stress due to the combination of photo-oxidation and high oxygen tension due to high perfusion. Photoreceptor outer segments, which are phagocytosed by RPE cells daily, are rich in easily oxidized lipids. Hence, iron must be tightly regulated to provide needed iron while shielding retinal cells from iron-induced oxidative damage.

This regulation is provided in the retina by a series of iron handling proteins. The transferrin receptor (TfR) takes transferrin bound iron into retinal cells. Iron can then be transported to

mitochondria to initiate its incorporation into numerous metabolic proteins, or can be stored safely within cytosolic ferritin. The iron can be exported from cells by the iron exporter ferroportin (Fpn), working in conjunction with the ferroxidases ceruloplasmin (Cp) or hephaestin (Hp). The localization of Fpn on the abluminal surface of the vascular endothelial cells and basolateral surface of the RPE suggests that iron is trafficked from the retinal vasculature, through the neural retina, and eventually out of the RPE into the choriocapillaris (Figure 1).

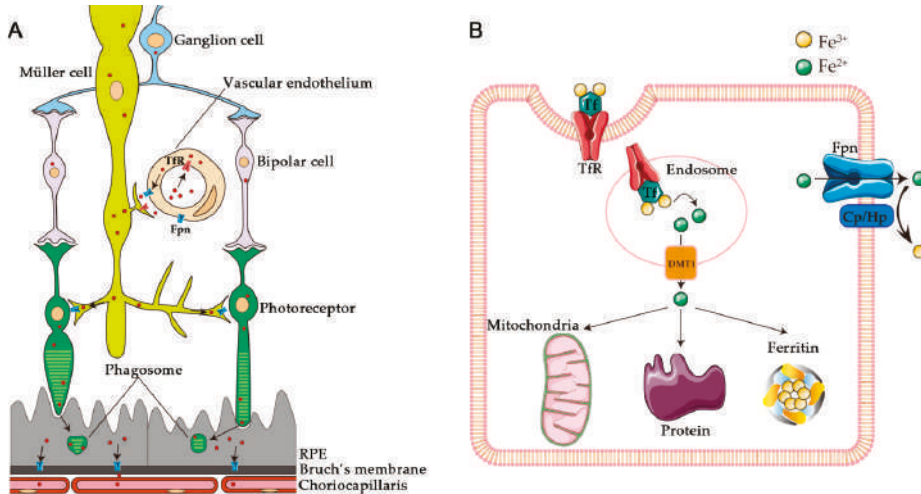


Figure 1. Schematic illustration of retinal iron transport. (A) Iron (red dots) moves through the retina as shown by arrows. Based on immunolocalization of TfR and Fpn, iron can enter retinal vascular endothelial cells by TfR-mediated endocytosis. It exits the vascular endothelial cells through Fpn (transmembrane protein indicated in blue). It is distributed through the neural retina by Müller glial cells. Iron is transferred from photoreceptors to the RPE through daily phagocytosis of the photoreceptor outer segments by the RPE. It is then exported by Fpn on the basolateral RPE into the choriocapillaris. (B) Iron transport at the cellular level. Iron can be taken into the cell by TfR, exported from the endosome by Dmt1, imported into mitochondria, stored in ferritin, or exported from the cell by Fpn in cooperation with ferroxidases Cp or Hp. Cp, ceruloplasmin; Dmt1, divalent metal transporter-1; Fpn, ferroportin; Hp, hephaestin; Tf, transferrin; TfR, transferrin receptor. “Mitochondria” and “Protein” by Servier Medical Art by Servier are licensed under CC BY 3.0.

2. Retinal Degeneration Resulting from Iron Dysregulation

Abnormal iron homeostasis in hereditary diseases can lead to retinal iron overload and degeneration. In addition to hereditary iron overload, ocular siderosis from an iron-containing intraocular foreign body or from iron released from red blood cells after intraocular hemorrhage can promote retinopathy. Recent evidence suggests that iron toxicity can contribute to the pathogenesis of age-related macular degeneration. Dietary or injected iron contributes to systemic iron overload and may promote retinal degeneration. Antioxidants and iron chelators could be beneficial in the prevention or treatment of these retinal disorders.

2.1. Hereditary Iron Overload

Several forms of hereditary iron overload have been associated with retinal disorders: aceruloplasminemia, hereditary hemochromatosis, pantothenate kinase-associated neurodegeneration, and Friedreich’s ataxia.

2.1.1. Aceruloplasminemia

Aceruloplasminemia is a rare autosomal recessive disease resulting from mutations in the gene encoding ceruloplasmin [10]. Ceruloplasmin promotes iron export by converting ferrous to ferric iron, the only form of iron that can bind to transferrin. There is iron export impairment-induced iron overload in the brain, retinas, and pancreas of patients with aceruloplasminemia. Clinical manifestations of aceruloplasminemia include retinal degeneration, diabetes mellitus, dementia, and cerebellar ataxia [11]. Cases of aceruloplasminemia-related retinopathy have been reported. In the case of a 56-year-old Japanese woman, fundus photography showed generalized yellow discoloration of the retinas in both eyes [12]. Fluorescein angiography (FA) revealed RPE atrophy, visible as window defects in the maculae and mid-periphery. In comparison, in the case of a 47-year-old Caucasian patient, subretinal lesions were found in the macula, similar to drusen in AMD patients [13]. The drusen, as well as RPE iron accumulation, hypertrophy, and RPE cells with increased or decreased melanin were all revealed through the histopathology of this case [14].

Mice that model human aceruloplasminemia through knockout of ceruloplasmin (*Cp*) and hephaestin (*Hp*), its homolog, have been studied, showing progressive retinal degeneration [15]. In the *Cp/Hp* double-knockout mice aged six to nine months, as in the aceruloplasminemia case, RPE cells were iron overloaded, and hypertrophic. Electron microscopy showed increased numbers of lipofuscin-containing vesicles in the RPE, and wide-spaced collagen in sub-RPE deposits. Focal areas of RPE hyperplasia, photoreceptor degeneration, and subretinal neovascularization were also observed.

2.1.2. Hereditary Hemochromatosis

Hereditary hemochromatosis is a common condition caused by enhanced absorption of dietary iron. This disease can be caused by the mutation of any of several genes. Most commonly, it is caused by the mutation of the histocompatibility leukocyte antigen class I-like protein involved in iron homeostasis (*HFE*) gene [16]. Normally, *HFE* regulates iron uptake into the cell through transferrin receptor, and also regulates levels of the iron regulatory hormone hepcidin [17]. Patients with the *HFE* gene mutation form fewer transferrin receptor complexes, leaving more transferrin receptor available to bind transferrin, resulting in more iron intake into the tissues. Mutations in the genes transferrin receptor 2 (*TfR2*), ferroportin, and hemojuvelin (*HJV*) can also cause hemochromatosis [18]. In most forms of hemochromatosis, deficiency of the iron regulatory hormone hepcidin appears to be the ultimate cause of excess iron [19]. Hepcidin deficiency leads to iron overload because hepcidin normally triggers degradation of ferroportin on the membranes of gut enterocytes, preventing iron export from the enterocytes into the bloodstream. High serum iron levels lead to iron accumulation in heart, liver and pancreas [20]. The reported ocular studies on patients with hereditary hemochromatosis show peri-papillary RPE pigment changes, with mild iron accumulation in this region [21]. These minimal findings in patients with presumed *HFE* point mutations (by far the most common cause of hereditary hemochromatosis) suggest that the degree of blood iron elevation in these patients may not lead to retinal degeneration. The genetic penetrance of hemochromatosis in patients with *HFE* mutations is highly variable, so it would be worthwhile to screen for retinal abnormalities in those with the highest blood ferritin and iron levels. In animal studies, *HFE* and *HJV* were found to be expressed in mouse retina and RPE cells, suggesting that they can play roles in retinal iron homeostasis [22,23]. Complete knockout of each gene led to retinal iron accumulation as well as degeneration [24,25]. Knockout of hepcidin also causes severe retinal iron overload and degeneration [26].

2.1.3. Friedreich's Ataxia (FRDA)

Friedreich's ataxia (FRDA) is an autosomal recessive hereditary disease involving progressive neurodegeneration. The *FXN* gene encodes the iron-binding mitochondrial protein frataxin. *FXN* trinucleotide repeat expansion causes reduction of frataxin messenger RNA and protein levels in diverse tissues. A result of frataxin deficiency is mitochondrial iron overload, which is

potentially damaging. Symptoms of Friedreich's ataxia include progressive ataxia, dysarthria, and cardiomyopathy [27]. Ocular manifestations include a progressive optic neuropathy, which can result in severe vision loss. A case reported by Porter showed a 59-year-old woman who had optic neuropathy with rapid-onset severe vision loss [28]. She was a compound heterozygote for the GAA expansion and a Gly130Val missense mutation. Color fundus photos revealed a pale optic nerve and multiple yellowish flecks in both maculas and nasal to the optic discs on her first visit. These deposits were auto-fluorescent, suggesting RPE lipofuscin accumulation. In 12 months, her visual acuities dropped from 6/6 OD and 6/24 OS to bilateral light perception only.

2.1.4. Pantothenate Kinase Associated Neurodegeneration (PKAN)

Pantothenate kinase-associated neurodegeneration (PKAN) is an autosomal recessive neurodegenerative disease with iron accumulation [29]. Patients harbor mutations in the pantothenate kinase 2 gene (*PANK2*) [30]. Pantothenate kinases produce phosphopantothenate, which condenses with cysteine during coenzyme A synthesis. In patients with PKAN, *PANK2* mutation causes reduced levels of phosphopantothenate and increased levels of cysteine, which can bind iron, potentially explaining the iron accumulation in the brain. The regions in the brain that have the most iron in normal subjects are those that are most affected by PKAN, including the medial globus pallidus and substantia nigra pars reticulata. In the photoreceptors, where coenzyme A is in high demand for disc membrane regeneration, coenzyme A deficiency is likely. Since rod photoreceptor cells shed outer segment tips daily and generate membrane discs, this may contribute to the retinopathy resulting from PKAN. Patients with PKAN also have childhood-onset movement disorder and dementia [29]. Vision loss is associated with pigmentary retinopathy. A pair of dizygotic twins with PKAN showed retinopathy at the age of seven, including hyperpigmented foveas and depigmented posterior poles [31]. In a histopathological report, a 10-year-old girl had retinal abnormalities visible upon ophthalmoscopy, including retinal flecks, bone spicules, and bull's eye maculopathy [32]. The flecks and bull's eye corresponded histologically to macrophages containing melanolipofuscin and hypertrophic RPE cells containing melanolipofuscin aggregates. The photoreceptor inner and outer segments were absent through the retina. A mouse with *PANK2* knockout also exhibited progressive photoreceptor degeneration, providing a PKAN retinopathy model [33]. Whether iron toxicity contributes to the retinopathy is a topic requiring further study.

2.2. Age-Related Macular Degeneration (AMD)

Age-related macular degeneration is a major cause of irreversible vision loss worldwide [34]. AMD is divided into several stages based on the histopathological features. Drusen, extracellular deposits that are rich in proteins and lipids sitting between RPE and Bruch's membrane, are features of early and intermediate AMD. Advanced AMD includes geographic atrophy (GA) and neovascular AMD [35]. Although the pathogenesis of AMD is still incompletely understood, oxidative stress and free radical damage most likely play a role [36]. The Age-Related Eye Disease Study (AREDS) reported in 2001 that antioxidant vitamins plus zinc were effective in reducing the risk of AMD progression [37]. During 10 years of follow-up, the protection against neovascular AMD persisted [38].

Iron is likely to be an important cause of oxidative stress in AMD. Compared to healthy age-matched controls, AMD-affected maculas have increased iron detected by enhanced Perls' Prussian blue stain, especially in the RPE and Bruch's membrane of early AMD, GA and neovascular AMD retinas [39]. The iron chelator deferoxamine was used to treat sections, showing that a portion of the iron in these tissue sections was chelatable. In another lab, sections from six post-mortem eyes of AMD donors and seven age-matched healthy donors were investigated using Analytical Electron Microscopy, showing that more iron accumulated in RPE melanosomes and within calcified Bruch's membrane of donors with AMD compared to age-matched controls [40]. Elevated iron levels have also been found in the photoreceptor layer of the *post mortem* macula of a patient with GA [41]. Perls' stain showed iron in the photoreceptors of this donor. In contrast, the stain was not sensitive enough to

detect any signal in the photoreceptors of nine control elderly donors with normal retinas. The maculas from the GA patient also had higher levels of ferroportin and ferritin, proteins whose abundance is increased by iron, in the photoreceptors than the normal donor retinas. In addition, when iron levels in aqueous humor were measured in patients having cataract surgery, they were found to be increased by more than two-fold in patients with non-exudative AMD [42].

Though iron accumulation was discovered in AMD retinas, its role in the pathogenesis remains unproven, but several lines of evidence suggest a causal link. First, retinal iron levels increase with age [43]. Eyes from donors younger than 35 and older than 65 were dissected. Atomic absorption spectrophotometry (AAS) revealed significantly increased iron levels in the retinas of older versus younger eyes, and no increase detected in RPE/choroid. Since photoreceptors are the cell type containing the highest iron levels in the rat retina, and they are the most abundant retinal cell type, elevated photoreceptor iron levels most likely contributed to the increased AAS reading. [44]. Second, as mentioned above, a patient with RPE iron overload caused by aceruloplasminemia had drusen-like deposits in the retina at an uncharacteristically young age [13]. Third, *Cp/Hp* double knockout mice with iron accumulation in the neural retina and RPE have retinal degeneration sharing features of AMD [15]. Among these features is sub-RPE wide-spaced collagen, which is associated with drusen in humans; RPE hypertrophy with lipofuscin-containing vesicles; and RPE death. In addition, they have focal photoreceptor degeneration and subretinal neovascularization. Fourth, iron is a potent catalyst of radicals and causes photoreceptor/RPE toxicity when it accumulates locally in conditions like ocular siderosis. Even though siderosis leads to pan-retinal photoreceptor degeneration, but not drusen, GA, or choroidal neovascularization, this difference in retinal manifestations could be explained by the different iron delivery route, as well as the spatial and temporal differences in iron accumulation. Last but not the least, the extracellular iron binding protein transferrin is upregulated in AMD retinas [45]. Transferrin mRNA levels are increased in both dry and wet AMD. Consistent with this, Western analysis revealed an increase in transferrin protein levels in AMD retinas compared to normal controls and immunohistochemistry showed increased transferrin labeling in AMD retinas, which were localized to drusen, photoreceptors, and Müller glia.

The mechanisms of iron accumulation in AMD retinas are an area of active investigation. Plausible pathways include oxidative stress, inflammation, and hypoxia, all of which are involved in AMD pathogenesis. Inflammation has been shown to induce cellular iron sequestration through IL-6-mediated upregulation of hepcidin [46]. Hypoxia leads to elevated iron uptake through HIF-mediated increases in *DMT1* and *TfR1*, both of which are cellular iron importer genes [47]. Oxidative stress can also upregulate *TfR1* [48]. Further, subretinal hemorrhage in a rabbit model led to iron accumulation in photoreceptors and RPE cells following its release from lysing red blood cells [49].

2.3. Iron Overload from Supplementation

In addition to an inherited abnormality in a specific iron metabolic protein or pathway, systemic or focal iron accumulation and the resulting toxicity can result from dietary or parenteral iron supplementation. It has been reported that anemia occurs in one-quarter of the world's population [50], primarily in preschool-aged children and women, making it a global public health problem [51]. Iron deficiency anemia (IDA) is the leading cause of anemia [52]. In developing countries, IDA typically results from inadequate dietary iron intake, blood loss due to intestinal worm infection, or both. In developed countries, low-iron diets (e.g., a vegetarian diet or no red meat intake), iron malabsorption, or chronic blood loss from menorrhagia or intestinal bleeding are the most common reasons. In addition to identification of the source of iron loss, dietary iron supplementation is a common treatment for IDA patients. Iron deficiency makes the body increase its ability to take up any available dietary iron, primarily by downregulating hepcidin production [53]. Standard therapy for IDA in adults is oral administration of a 300-mg tablet of ferrous sulfate three or four times daily, but excessive iron supplement could theoretically be harmful to the retina. Rats placed on a high iron diet had increased

retinal iron levels, oxidative stress, and photoreceptor death [54]. Mice placed on a high iron diet for 10 months had RPE iron accumulation, but not enough to cause degeneration [55].

Heme iron is found at much higher levels in red meat than white meat. Heme, the iron-containing porphyrin pigment of meat, is not only a prooxidant in itself, but also responsible for the endogenous intestinal N-nitroso compounds in humans arising from red meat [39,56]. A cohort study of 6734 people aged 58–69 years was conducted in Australia to evaluate the association between red meat or chicken intake and AMD [57]. Higher red meat intake was associated with early AMD, with an odds ratio for red meat consumption more than 10 times per week versus fewer than five times per week of 1.47. In contrast, consumption of chicken more than 3.5 times per week versus fewer than 1.5 times per week was inversely associated with late AMD (Odds ratio: 0.43).

Parenteral iron therapy for iron deficiency increases hemoglobin levels more rapidly than oral iron because it circumvents the limitations of intestinal iron absorption [58,59]. The established indications for parenteral iron therapy include failure of oral iron to increase the hematocrit, oral iron intolerance, most often due to constipation, or the need for quick recovery. However, in intravenous iron-injected mice, serum iron levels increased and caused elevated iron levels in the RPE and neurosensory retina despite an intact blood-retinal barrier [60]. In a similar study, intravenous iron not only elevated mouse serum and RPE iron levels, but also led to AMD-like histological lesions, including Bruch's membrane thickening showing complement C3 deposition, as well as hypertrophy and vacuolization of the RPE [61]. A 43-year-old patient with IDA who received IV iron therapy developed numerous retinal drusen within 11 months of receiving the iron, suggesting that intravenous iron therapy may have caused retinal iron accumulation that promoted early AMD [61]. We are currently conducting clinical studies to determine whether intravenous iron can promote drusen formation in susceptible individuals.

Acquired hemochromatosis can also result from multiple blood transfusions, since red blood cells contain abundant iron. Patients with sickle cell anemia or thalassemia often receive numerous transfusions. These diseases have been associated with retinal abnormalities, including RPE atrophy and angioid streaks. Since these patients have iron overload, chelation therapy and intraocular hemorrhage, it is difficult to separate the effects of each of these on the retina. Nevertheless, several patients with thalassemia exhibited retinopathy in the absence of any iron chelation [62].

2.4. Siderosis

Ocular siderosis results from intraocular iron deposition following ocular penetration of a metallic foreign body. In this condition, ferrous iron generates radicals and causes oxidative stress. [63]. The clinical features include corneal iron deposition, iris heterochromia, pupillary mydriasis, accommodation failure, anterior subcapsular cataract, lens discoloration, retinal arteriolar narrowing, retinal detachment, retinal pigment epithelium clumping and RPE atrophy. Glaucoma may occur if the trabecular meshwork and Schlemm's canal are involved [63–65]. Electroretinography (ERG) results vary in different stages of the disease [66]. ERG a- and b-wave amplitudes may increase initially, and gradually decrease as the siderosis progresses and photoreceptors degenerate.

Animal models of the disease have illustrated the histopathologic and functional effects on the retina. Retinal changes in squirrel monkeys after they received intravitreal injection of iron-containing solutions were studied, finding geographic patches of retinal whitening and RPE disruption by ophthalmoscopy [67]. ERG changes occurred as soon as the first few hours, with reduced amplitudes or absent signals with higher doses. In another animal model, solid iron foreign bodies were placed into the rabbit vitreous, and degeneration of the outer nuclear layer and RPE was observed 10 days later [68]. ERG results were consistent with morphological changes, showing decreased a- and b- wave amplitudes under both scotopic and photopic conditions.

2.5. Subretinal Hemorrhage

Subretinal hemorrhage, which may lead to vision loss, occurs in several conditions, most commonly associated with neovascular AMD, but also occurring in presumed ocular histoplasmosis, angioid streaks, myopic degeneration, and trauma. A clinical study on the patients with intra and subretinal hemorrhage in 1983 showed that the visual acuity loss significantly depended on hemorrhage size and the ability of the tissue to clear the blood [69]. Possible mechanisms of vision loss followed by subretinal hemorrhage include iron toxicity to the photoreceptors and RPE, mechanical damage, development of a fibrovascular membrane, or separation of photoreceptors from the RPE [69]. Fresh autologous blood injection into the rabbit subretinal space caused progressive photoreceptor degeneration with edematous change, as well as iron accumulation in the photoreceptor outer segments and RPE [70]. An iron chelator, deferoxamine, was shown to protect these rabbits as measured by ERG [71]. In a similar rabbit model, iron was detected by Perls' stain in photoreceptors and RPE, and triamcinolone acetonide intravitreal injection reduced apoptosis of photoreceptors [49]. In vitro, oxyhemoglobin causes lipid peroxidation in retinal tissue, and oxyhemoglobin in subretinal blood may similarly cause damage to the retina after it is released from red blood cells [72]. Hemopexin, the hemoglobin-binding protein, may protect retina from heme-mediated toxicity. In addition, RPE cells can bind and internalize the heme-hemopexin complex, facilitating clearance of heme from the retina [73].

3. The Potential for Retinal Protection by Iron Chelators

Since iron overload-induced oxidative damage may be involved in the pathogenesis of AMD and other retinal diseases, iron chelators may help reduce the occurrence and progression of AMD. Several reports suggested that iron chelators could be helpful in the treatment of neurological diseases, such as Alzheimer's, Parkinson's, Huntington's, and FRDA [74,75]. Iron chelators may also be beneficial in the treatment of retinal diseases associated with iron overload. Studies in mouse models support this assertion (see below). The iron chelators that have been approved by the U.S. Food and Drug Administration (FDA) approved for treatment of patients with systemic iron overload include deferoxamine, deferasirox, and deferiprone. Another iron chelator, salicylaldehyde isonicotinoyl hydrazine is effective in cell culture but has not yet been tested extensively in vivo. In this review, the chelators' potential protective role in the retina is discussed.

3.1. Deferoxamine

Deferoxamine (DFO) was the first iron chelator approved by the FDA, in 1968, for the treatment of acute iron intoxication and for chronic iron overload in patients with transfusion-dependent anemias. Synthesized by a bacterium to steal iron from the host, this hexadentate molecule binds iron in a 1:1 ratio. Despite the remarkable effect on reduction of serum ferritin level, DFO has many limitations. As a hydrophilic iron chelator, DFO must be administered as a slow subcutaneous infusion, intramuscularly or intravenously [76]. It has a short plasma half-life, so continuous injection is often required for patients with iron overload. For patients who need chronic treatment, DFO is costly and inefficient. The most common side effects of DFO are local skin reactions and infections. Visual neurotoxicity of DFO has been reported, including defects in the visual field, diminished visual acuity, color vision defects, and decreased ERG and electrooculogram responses [77–79], making DFO a poor candidate to treat AMD or other iron-related ocular diseases. The retinal toxicity of DFO may result from its high affinity for iron, causing retinal iron deficiency.

Modification of DFO, in a DFO-zinc complex, however, attenuates retinal degeneration in the rd10 mouse model of retinitis pigmentosa [80]. DFO-zinc delivery to rd10 mice resulted in protected ERG responses and more photoreceptor survival, with retinal ferritin and lipid peroxidation levels reduced. Intraperitoneal DFO also protects albino rats from light damage. In rats treated with DFO,

there was partial preservation of photoreceptor nuclei and fewer subretinal macrophages than in the control rats [81].

3.2. Deferasirox

Deferasirox (DFX), a tridentate molecule, which binds iron in a 2:1 ratio, was approved by the FDA in 2005. DFX is administered orally, and its long half-life allows for once-daily dosing [82]. Because of its relatively small molecular weight, DFX is better absorbed, showing over twofold increase in potency compared to DFO for iron mobilization from tissue [83]. DFX has a well-characterized safety profile that requires regular monitoring both adult and pediatric patients [84–86]. The most common side effects include abdominal pain, gastrointestinal upset, vomiting, and transient skin rashes [87]. Higher doses may cause hepatic, renal and ocular toxicities. Several cases of retinopathy or lens opacities have been reported among patients with thalassemia major treated with DFX [88,89]. The pathogenesis of the retinopathy and cataract development was unclear but age-related. Given that DFO, a structurally unrelated molecule, can also cause retinopathy, the most likely mechanism is RPE iron deficiency [90,91].

3.3. Deferiprone

Deferiprone (DFP), a bidentate iron chelator that binds iron in a 3:1 ratio, was approved by the FDA in 2011. DFP has a shorter half-life than DFX, requiring a three-times-daily oral administration. Side effects can include increased liver enzymes, gastrointestinal disorders or arthralgia. The most serious adverse effects are agranulocytosis and neutropenia with an incidence of 0.2 and 2.8 per 100 patients over one year. These are reversible by discontinuing the medication [92]. Importantly, DFP, which has a lower affinity for iron than DFO or DFX, has not been reported to cause retinal toxicity in human patients or mice. In animal studies, our team reported that DFP could diminish retinal iron levels and oxidative stress, and thereby protecting *Cp/Hp* double-knockout mice and hepcidin knockout mice against iron overload-induced retinal degeneration [93,94]. While it is difficult to measure mouse retinal iron levels directly, it was possible to show that DFP caused an increase in neural retina transferrin receptor mRNA levels, indicating that DFP diminished levels of potentially toxic labile (free) iron. It may have done this by removing iron from the retina, or by redistributing labile iron to proteins that can bind it tightly and safely, such as transferrin. DFP also protected mice against retinal degeneration initiated by *rd6* mutation, sodium iodate (NaIO_3) treatment or light exposure, indicating that iron chelation can be retina-protective even when iron toxicity is not the primary cause [95,96]. Presumably, iron potentiates photoreceptor cell death even when iron overload isn't the initial insult. Therefore, retinal diseases in which iron levels are not elevated but free iron still potentiates oxidative stress might be protected by chelation of labile iron. It may do so by increasing oxidative stress or through a recently characterized form of iron-dependent cell death called ferroptosis [97].

3.4. Salicylaldehyde Isonicotinoyl Hydrazine

Salicylaldehyde isonicotinoyl hydrazine (SIH) is a tridentate iron chelator that binds iron in a 2:1 ratio. Due to its lipophilicity, SIH can be administered orally, easily penetrating cell membranes and firmly chelating intracellular Fe^{3+} [98]. SIH showed cardioprotective potential *in vivo* [99,100]. It is also highly effective in the protection of cultured RPE cells against oxidative damage induced by diverse stimuli [101]. SIH provided better protection for ARPE-19 against H_2O_2 -induced cell death than the antioxidant N-acetyl cysteine (NAC) and other iron chelators, including DFP and the extracellular iron chelator diethylenetriaminepentaacetic acid (DTPA). It is notable that SIH can also play a protective role by activating Nrf2 transcription factor, which regulates the expression of antioxidant genes [102]. Despite all the positive outcomes, multiple studies revealed a short biological half-life of SIH due to the rapid hydrolysis of its hydrazine bond in plasma [103]. Hopefully a stabilized, optimized form of SIH can become another potent agent for iron chelation therapy.

4. Future Directions

The association of iron with several retinal diseases and the development of mouse models of iron-induced retinopathy have paved the way for studies on the therapeutic potential of retinal iron chelation. The oral iron chelator DFP reduces retinal iron levels and protects the retina against a broad range of insults in animal models, without causing retinal toxicity. The future testing of iron chelators for retinal disease will need to take into account the potential for systemic toxicities, including iron deficiency. Careful dosing of relatively lower affinity iron chelators, such as DFP, has the potential to chelate loosely-bound, toxic iron, while avoiding cellular or systemic iron deficiency. Taken together, new iron chelators in development provide promise for the treatment of retinal diseases.

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Review

Iron as a Therapeutic Target in *HFE*-Related Hemochromatosis: Usual and Novel Aspects

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Abstract: Genetic hemochromatosis is an iron overload disease that is mainly related to the *C282Y* mutation in the *HFE* gene. This gene controls the expression of hepcidin, a peptide secreted in plasma by the liver and regulates systemic iron distribution. Homozygous *C282Y* mutation induces hepcidin deficiency, leading to increased circulating transferrin saturation, and ultimately, iron accumulation in organs such as the liver, pancreas, heart, and bone. Iron in excess may induce or favor the development of complications such as cirrhosis, liver cancer, diabetes, heart failure, hypogonadism, but also complaints such as asthenia and disabling arthritis. Iron depletive treatment mainly consists of venesections that permit the removal of iron contained in red blood cells and the subsequent mobilization of stored iron in order to synthesize hemoglobin for new erythrocytes. It is highly efficient in removing excess iron and preventing most of the complications associated with excess iron in the body. However, this treatment does not target the biological mechanisms involved in the iron metabolism disturbance. New treatments based on the increase of hepcidin levels, by using hepcidin mimetics or inducers, or inhibitors of the iron export activity of ferroportin protein that is the target of hepcidin, if devoid of significant secondary effects, should be useful to better control iron parameters and symptoms, such as arthritis.

Keywords: iron; genetic hemochromatosis; non transferrin bound iron; hepcidin; ferroportin; venesections

Iron metabolism must be tightly controlled in order to avoid deleterious situations, including iron overload diseases, especially *HFE*-related hemochromatosis. In such conditions an iron depletive treatment is engaged in order to avoid the development of complications. However, iron depletive therapy targets only iron excess and alternative, possibly complementary, novel approaches are needed.

1. Normal Iron Metabolism

1.1. The Ferroportin/Hepcidin Duo Controls Systemic Iron Metabolism

Systemic iron metabolism is characterized by a continuous distribution of iron from plasma toward cells which require its presence for participating in biological functions, including oxygen transport and enzymatic processes [1]. Plasma iron must be continuously renewed. Plasma iron originates predominantly from macrophages that recycle iron from senescent erythrocytes during the erythrophagocytosis process. The erythropoietic cells contains 70% of total body iron. The macrophages provide schematically 19 mg out of the 20 mg of iron required from the plasma by cells every day. The second source of iron is digestive absorption. Iron is absorbed in two steps from the nutrients. The first step takes place at the apical pole of the enterocytes, non-heme and heme iron

being transferred from nutrients to the enterocyte cytoplasm. The transfer of non-heme iron implicates DMT1 (divalent metal transporter 1) that takes up ferrous iron [2] after reduction by the ferrireductase dCytb [3]. Heme iron is taken up through a receptor that could be HCP1 (heme carrier protein1) (controversial) [4–6]. The second step of iron absorption is the transfer of iron from the cytoplasm toward the plasma through the basal membrane of the enterocyte. This process limits the iron entry into the body, and only 1–2 mg are transferred each day toward the plasma, whereas the remaining iron is transiently stored within the cytoplasm and will be lost during enterocyte desquamation. This selective process is the classical mucosal block [7]. Ferroportin is the iron export protein that allows iron egress from enterocytes (concerning both the non-heme and heme iron absorbed at the apical side) and macrophages toward the plasma [8–10]. Once transferred into the plasma, and after its oxidation by ferroxidase enzymes—ceruloplasmin [11] and/or hephaestin [12]—ferric iron is associated to transferrin, the protein that can link up to two iron atoms and delivers iron to cells. Cellular uptake of transferrin iron is mediated by the transferrin receptor 1 (TFR1) [13]. Thereafter, endocytosis of the complex permits the transport of iron into endocytic vesicles and subsequently, its transfer to the cytosol by DMT1, that is also expressed in endocytic vesicles, after oxidation of iron by STEAP3 (*Six-Transmembrane Epithelial Antigen of the Prostate 3*) [14]. Another transferrin receptor, transferrin receptor 2 (TFR2) is expressed by cells, especially hepatocytes, but its affinity coefficient for transferrin is very low [15] so that its function is related to iron sensing rather than iron transport [16].

Hepcidin is a small cysteine rich peptide [17–19], mainly expressed by hepatocytes and secreted in the plasma, that interacts with ferroportin [20], the only known protein involved in cellular iron export. An increase of plasma hepcidin level leads to a decrease of both digestive iron absorption and iron leakage by macrophages [21]. Hepcidin decreases plasma iron concentration and the saturation of transferrin by iron. Conversely, a decrease of hepcidin expression favors cell membrane expression of ferroportin, and, in turn, the iron release from cells into the plasma, thus, increasing the transferrin saturation by iron. The regulation of hepcidin expression plays a major role in the maintenance of iron homeostasis.

1.2. Regulation of Hepcidin Expression

Hepcidin expression is regulated by many factors, including iron status [19] and inflammation [19,22] that induce an upregulation of hepcidin expression, and hypoxia/erythropoiesis activity [23–25] that decrease hepcidin expression. Mechanisms related to the induction of hepcidin expression by iron status are mainly transcriptional.

The first iron-related mechanism that regulates hepcidin expression is reported to be linked to transferrin saturation level in plasma [26,27]. This pathway involves the *HFE* gene, located on the chromosome 6, that encodes a HLA like class I protein that is expressed on cell membrane in association with the β 2-microglobulin [28]. It has been reported that HFE protein may interact either with TFR1 [29,30] or TFR2. The mechanism potentially involved in such regulation is a decrease of the physical interaction between TFR1 and HFE proteins when transferrin saturation increases [31]. This could lead to the stimulation of a MAP (mitogen-activated protein kinase) signaling pathway that promotes the hepcidin transcription level [27]. However, while the HFE/TFR2 interaction has been documented in *in vitro* experiments, the *in vivo* relevance of these findings is questionable [32]. The increase of hepcidin expression promotes ferroportin degradation, and thus, reduces plasma iron concentration and transferrin saturation by iron (Figure 1).

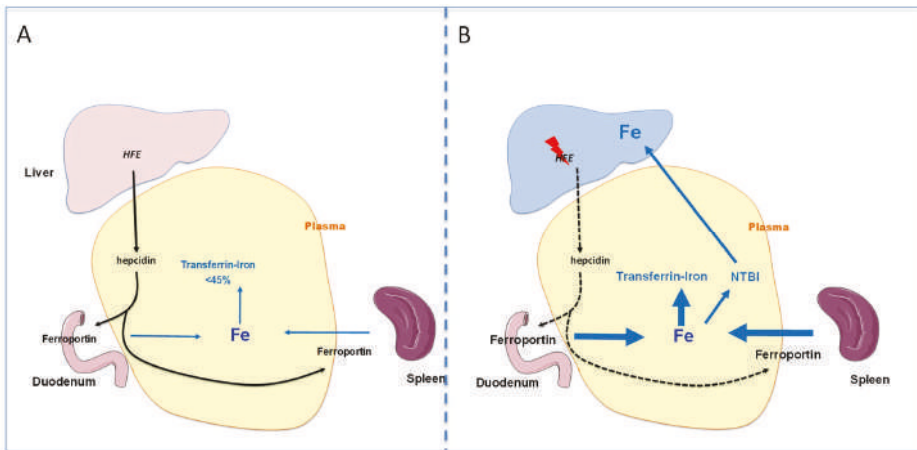


Figure 1. Schematic representation of the pathophysiological mechanisms leading to the development of iron overload during *HFE*-related hemochromatosis. (A): Normal situation with adequate *HFE* signaling allowing control of transferrin saturation level (<45%). (B): Genetic hemochromatosis with low activity of *HFE*-related signaling that limits hepcidin expression and in turn favors iron release in plasma, despite the presence of a sufficient amount of iron. Thus, the transferrin saturation increases and non-transferrin bound iron appears and targets organs such as liver, leading to abnormal iron accumulation.

The second regulatory mechanism implicates the bone morphogenetic protein/hemojuvelin/son of mothers against decapentaplegic homolog (BMP/HJV/SMAD) pathway [33–36]. When cell iron concentration increases, BMP6 and BMP2 proteins are produced and secreted by hepatocytes and more likely sinusoidal cells [37–39]. BMPs interact with BMP receptor proteins that are associated to HJV, acting as BMPs co-receptor. The interaction induces a phosphorylation of SMADs (Son of Mothers Against Decapentaplegic) 1, 5, 8 that are translocated toward the nucleus after association with SMAD4 in the nucleus [40,41]. Then, they interact with BMP-responsive element sequence within the hepcidin gene promoter and promote hepcidin transcription [35,36,42].

It is noteworthy that the hemochromatosis proteins HFE and HJV form a membrane-associated protein complex for hepcidin regulation. This suggests that the two pathways are not fully independent [43,44], and further work should help to better specify the respective roles of the different actors.

2. Pathophysiology of *HFE* Hemochromatosis

2.1. *HFE* Hemochromatosis

HFE hemochromatosis is a disease mainly related to homozygosity of the C282Y (*p.Cys282Tyr*) mutation in the *HFE* gene [45]. The *p.Cys282Tyr* mutation alters the structure of the *HFE* protein due to the substitution of a cysteine that is engaged in intra-molecular disulfide bonds, that play a role in the protein shape, by a threonine. Thus, the expression of HFE protein on cell membrane, as well as its interaction with the Beta2 microglobulin are altered [29]. Some exceptional private mutations in the *HFE* gene can also lead to hemochromatosis, when present either at the homozygous state or in association with the C282Y mutation [46]. Homozygosity for H63D (*p.His63Asp*) that is present at homozygous state in approximately 2% of the Caucasian population cannot by itself generate clinically significant iron excess, so that, when associated to iron overload, one must search for associated genetic or acquired associated factors that promote iron excess [47]. It must be pointed out that the penetrance of *HFE*-related hemochromatosis is very incomplete. In terms of clinical penetrance, some studies

have estimated a prevalence of 25–60%, while a single study reported a prevalence of 28% in males and 1% in females [48]. However, in the same study the biochemical penetrance, assessed by increased ferritinemia, was much higher (82% and 55%, respectively) [48].

2.2. Pathophysiology of Iron Overload during HFE Hemochromatosis

In hemochromatosis, iron overload is a two-hit phenomenon.

The first hit (Figure 1), which is related to the structural change of the protein is a deficiency in hepcidin expression and secretion—meaning hepcidin deficiency—compared to the iron stores [22,26]. In other words, hepcidin expression is lower than expected when considering plasma transferrin saturation and body iron stores [49]. This hepcidin deficiency results from the mutation in the *HFE* gene that alters the efficacy of the transduction pathway regulating hepcidin expression. Consecutively, despite iron excess, ferroportin expression on cell membranes of enterocytes and macrophages remains elevated and favors an increase of both plasma iron concentration and transferrin iron saturation [20]. As previously mentioned, transferrin iron ingress into cells is modulated by the expression level of its receptor TFR1. In physiological situation, TFR1 expression on cell membranes is downregulated when cellular iron is in excess [50], in order to avoid cellular iron accumulation with subsequent toxicity, especially through the production of reactive oxygen species (ROS) [51,52]. The iron responsive element/iron regulatory protein system (IRE/IRP) regulates TFR1 and ferritin expression, adapting iron entry into the cell (TFR1) and the capacity of iron storage in cells (ferritin), to the variations of cellular iron content [50,53].

The second hit (Figure 1) involved in the development of iron overload in hemochromatosis results from the appearance of the non-transferrin-bound form of iron (NTBI) [54]. Indeed, transferrin saturation increase favors the presence of NTBI in plasma [55]. The NTBI is constituted of low molecular forms of iron linked to citrate or acetate [56]. The NTBI, in contrast to transferrin iron, constantly enters the cells, especially through the Zip14 transporter [57], even when they are already overloaded [58,59], whereas transferrin iron ingress is physiologically reduced due to the decrease of TFR1 on cell membrane [60,61]. The transporters involved in the uptake of NTBI are mainly expressed in the liver, the pancreas [57,62], and the heart, explaining that these organs are the primary targets of iron excess.

It is important to note that rare or very rare non-*HFE* mutations may also favor hepcidin deficiency. Homozygous and compound heterozygous mutations in the *HAMP* [63] or *HJV* [64] genes induce an early and severe iron overload disease (juvenile hemochromatosis) that is related to severe hepcidin deficiency with major complications that quickly impact well-being and life expectancy. In addition, mutations in the *TFR2* gene induce an hepcidin deficiency that provokes a clinical iron overload phenotype which is in between juvenile hemochromatosis and the classical *HFE*-related hemochromatosis form [65].

2.3. Pathophysiology of Organ Damage in Hemochromatosis

Complications of *HFE*-related genetic hemochromatosis, and more globally of hemochromatosis related to hepcidin deficiency, include hepatic damage with the development of liver fibrosis, with the risks of cirrhosis and hepatocellular carcinoma, diabetes, and at a lesser degree, heart dysfunction, which are sources of morbidity and mortality [66,67]. The risk of hepatic fibrosis increases with the severity of iron overload [68], and it is recommended to perform a liver biopsy in patients exhibiting very high levels of ferritinemia [69]. This risk is associated with the presence of sideronecrotic lesions of hepatocytes [70], that likely corresponds to the recently identified ferroptotic cell death process [71,72], a new cell death pathway in cells containing high iron content. Whereas hepatocyte iron loading is the pathophysiological basic feature of iron overload in hemochromatosis, it is noteworthy that Kupffer cell iron load finally occurs in advanced iron hemochromatosis and also represents a risk for the development of hepatic damage [70]. The role of an iron-related induction of TGF (transforming growth factor)-beta in the development of fibrosis has been reported [73,74]. It should be underlined

that hepatocellular carcinoma, that mostly develops in patients with a cirrhotic liver, can also be rarely found in non-cirrhotic patients which suggests the role of iron itself [75] and/or additional cofactors in the development of hepatocellular carcinoma (HCC) (see below). Finally, patients exhibiting cirrhosis before iron depletive treatment still present a risk for developing HCC even despite completion of iron depletive treatment [67].

Other complications may include hypogonadism—mostly in juvenile hemochromatosis—and much more frequently osteoporosis and arthritis that have a strong impact on the quality of life. Arthritis is characterized by absence of systemic inflammation and sometimes presence of calcium pyrophosphate crystals in the synovial fluids and visible on X-rays (review in [76]).

The ability of excessive iron to generate oxygen reactive species (ROS) (Figure 2) through the Haber–Weiss and Fenton reactions is strongly involved in the development of tissue lesions [77]. Indeed, ROS induces peroxidation that alters lipids, proteins, and DNA, generating dysfunctions of organelles, including mitochondria and cells, thus leading to tissue and organ damage [78–80]. One of the abnormal forms of NTBI, called Labile Plasma iron-LPI (or reactive plasma iron), is found in plasma when transferrin saturation reaches 80% [81]. This iron species is highly reactive and participates strongly in oxidative stress. Labile plasma iron is considered as a major determinant in the development of organ damage during hemochromatosis [82].

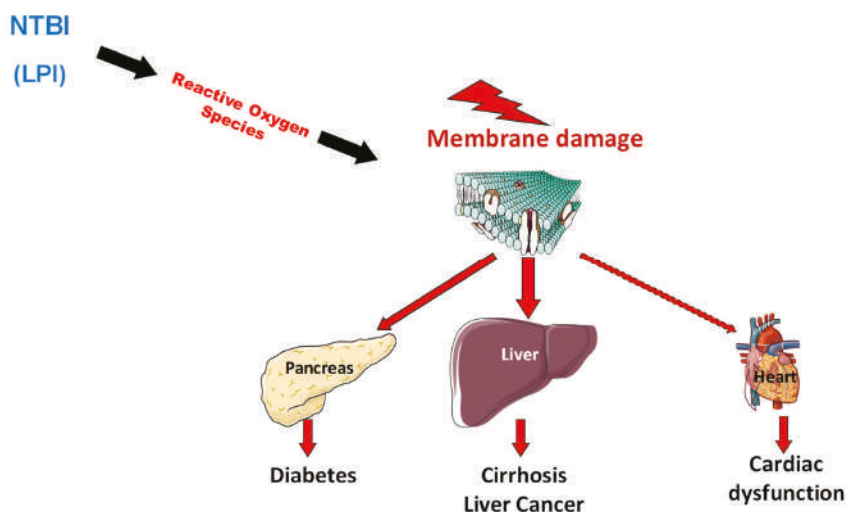


Figure 2. The appearance of non-transferrin bound forms of iron in plasma favors organ iron deposition but also the occurrence of oxidative stress that alters organelles in cells especially in iron overloaded organs.

In addition to the typical disease causing mutations associated with hemochromatosis, additional genetic modifiers have recently been described to elucidate in part, the large disparity in disease manifestations in patients. In this context, the importance of gene polymorphisms, e.g., GNPAT (glyceronephosphate O-acyltransferase) remains controversial and awaits additional confirmatory studies [83–88]. Alcohol consumption [68,89], non-alcoholic fatty liver disease [90], and viral hepatitis [91,92] may also be involved in disease penetrance, as well as a polymorphism in the PCSK7 [93] or PNPLA3 [94] genes.

3. Iron Is Presently the Main Therapeutic Target in HFE-Related Hemochromatosis

It is well known that iron depletion is a treatment of choice for hemochromatosis patients [95]. Indeed, when performed in patients prior to severe complications, the iron depletive treatment

restores normal life expectancy, meaning that hepatic, pancreatic, and cardiac dysfunctions can be fully prevented [67]. In addition, partial regression of liver fibrosis after completion of iron removal has been reported [96].

3.1. Venesections Are the Mainstay Treatment for Iron Removal

The principle of repeated venesections [47,95] (Figure 3) is to remove red blood cells that are known to be very rich in iron, as part of hemoglobin. Erythropoiesis is stimulated to compensate hematocrit loss, and iron is taken up from the plasma by erythroblasts to produce new erythrocytes. In order to maintain enough plasma iron for the stimulated erythropoiesis, iron is released from macrophages and enterocytes but also from iron storage cells, including parenchymal cells such as hepatocytes. Removing iron at a faster rate than that excessively reaccumulated by intestinal absorption is critical to negative iron balance, but determining this rate prior to starting depletive treatment is difficult due to individual differences between hemochromatosis patients. It should also be recalled that hepcidin synthesis can be further suppressed by increasing erythropoiesis as a consequence of erythroferrone production. In practice, the depletive treatment includes two successive phases. The first one, called induction phase, aims at totally removing the iron excess present at the time of diagnosis. It usually consists of weekly venesections (≤ 7.5 mL/kg body weight per venesection). Once excess iron has been removed, the second phase, called maintenance therapy, aims to avoid recurrent iron overload using lifelong venesections, performed every 1–4 months.

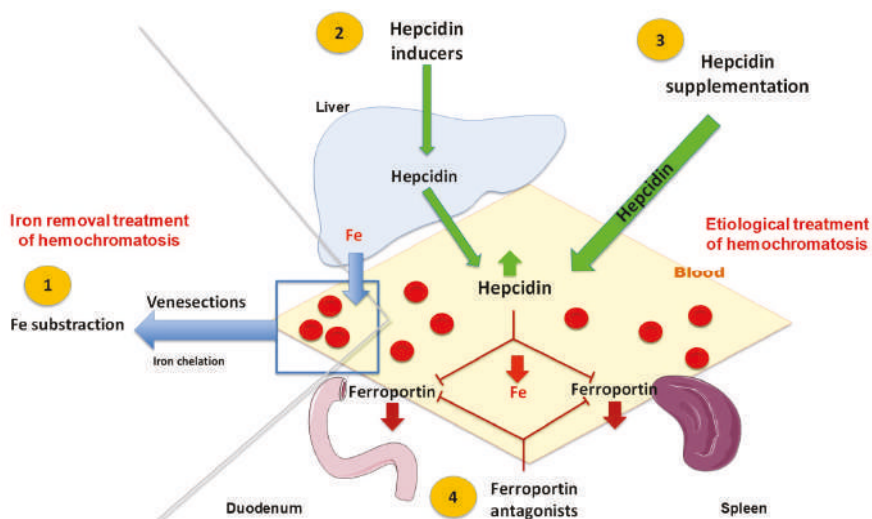


Figure 3. Schematic representation of currently used iron removal treatments during hemochromatosis and potential pathophysiological actions. (1) Iron depletive treatment is classically operated through repeated venesections that remove red blood cells. In some rare cases, iron chelators can be also used as complementary or suppletive treatment. (2) Hepcidin supplementation by endogenous or exogenous hepcidin. (3) Alternatively (4) the use of ferroportin antagonists could be also a way of treatment.

3.2. Use of Chelation Therapy

Another way to remove iron is the use of iron chelators that promote iron mobilization and excretion. However, in contrast to venesections, these drugs, including mainly today new oral iron-chelators, may have potential side effects [97]. Therefore during genetic hemochromatosis, oral chelation is only used, and as an off-label drug, in rare situations including cardiac failure,

recent cerebral ischemic stroke, venous access problems limiting the possibility of venesections, or psychological intolerance to venesections.

3.3. Biochemical Follow-Up of Venesection Therapy

Three main parameters are classically followed: plasma ferritin and transferrin saturation levels that reflect the efficacy of iron depletive treatment, whereas hemoglobin levels reflect the tolerance of venesections. During the induction phase ferritinemia—that reliably reflects the amount of iron excess in hemochromatosis (provided other frequent acquired causes of hyperferritinemia have been excluded, such as inflammation, alcoholism, cytolysis or metabolic syndrome; genetic hyperferritinemia, as seen in the ferritin-cataract syndrome, is much rarer)—is the first parameter that decreases. This decrease is only slowly progressive, especially in patients exhibiting a severe iron load phenotype. Conversely, the decrease of transferrin saturation levels, that reflect plasma iron bioavailability, is a very late event. Normalization of transferrin saturation occurs only when the induction treatment is in its final phase. It is noteworthy that during hemochromatosis, despite the impressive frequency of venesections, hemoglobin levels remain stable due to the high capacity of cellular iron release by ferroportin hyperactivity, related to hepcidin deficiency. However, transient hypoxia and relative iron deficiency occurring after venesection, together with increased erythropoiesis, tend to decrease hepcidin plasma levels, thus contributing to further enhance iron entry into the plasma [98,99]. When considering NTBI during the induction therapy, its plasma concentration decreases parallel to transferrin saturation levels [55]. Altogether, these data suggest that induction therapy must be fully completed to remove iron excess, but also to prevent the appearance of toxic forms of iron, including plasma NTBI.

During the maintenance phase, it is essential to pursue the patient follow-up in order to ideally maintain both transferrin saturation and ferritin levels in the normal range [47,95].

4. Iron Removal Is not the Unique Therapeutic Target in HFE-Related Hemochromatosis

4.1. Other Preventive Actions to Avoid Iron Overload Complications

Besides iron removal, it is important to prevent associated causes of organ damage, as mentioned previously. This holds especially true for hepatic lesions knowing the impact of excessive alcohol intake and viral hepatitis as cofactors of liver fibrosis, cirrhosis, and hepatocellular carcinoma. Moreover, NAFLD (Non Alcoholic Fatty Liver Disease) with the risk of NASH (Non-Alcoholic Steato-Hepatitis) represents an additional risk for hemochromatosis patients. The practitioner must complete the iron removal approach by nutritional recommendations on alcohol and glucido-lipidic regimens [47,95]. Moreover, avoiding the occurrence of viral B hepatitis through vaccination is critical. In addition, tea [100] has been demonstrated to limit iron absorption, and therefore, could be used as adjuvant to avoid recurrence of excessive iron stores after completion of iron removal. In the same way, the use of therapeutic oral calcium channel blockers has also been proposed due to its positive impact on urinary iron excretion related to DMT1 activity increase in the kidney [101]. The use of proton-pump inhibitors could also represent an interesting adjunct [102].

4.2. The Development of a Pathophysiological Treatment Is a Major Goal

Iron removal is a treatment that only cures iron excess, but clearly does not correct the hepcidin deficiency that generates iron metabolism disturbances, and some clinical observations do suggest that the removal of iron excess is not sufficient to treat hemochromatosis. Considering arthropathy, it is noteworthy that, despite a well-conducted iron depletive treatment, the symptomatology may persist and even become more severe [76]. This suggests that: (i) synovial iron deposition [103] may represent an inaccessible compartment for iron depletion by venesections because synovial fluid is not contiguous with serum; (ii) synovitis, that has been documented in hemochromatosis [104,105] is irreversible despite effective iron depletion; and/or (iii) hepcidin deficiency leading to iron excess

could be involved directly in symptomatic arthropathy and/or (iv) the mutations of the *HFE* gene, that encodes an HLA-like class I protein, could be involved directly in disease expression [106].

Considering the biochemical follow-up of patients, it is noteworthy that transferrin saturation levels may be found to be frequently increased during the maintenance therapeutic period, despite the absence of increased body iron stores. This could contribute to the appearance of clinical manifestations such as fatigue and arthropathy. It should be emphasized that, during the maintenance period, serum hepcidin levels are even lower than in iron overloaded patients [98,107]. Such hypohepcidinemia is expected to favor transferrin saturation increase and the appearance of NTBI that could participate in the arthritis of hemochromatosis patients.

Targeted treatment in the setting of hepcidin deficiency itself could represent an efficient way, as suggested by clinical observations obtained in hemochromatosis patients that have been transplanted for hepatocellular carcinoma [108]. The development of substitutive hepcidin treatment and/or of drugs stimulating hepcidin expression is under development and could be useful to complete the therapeutic arsenal, as suggested by results obtained with mini-hepcidins [109] and BMP known to induce hepcidin expression [110]. Clinical trials with hepcidin supplementation are ongoing (Clinical.trial.gov). Using antisense oligonucleotides for increasing hepcidin synthesis [111] or ferroportin antagonists [112] represents also further interesting innovative approaches. These different treatments could be especially indicated as an adjunct to venesections during the induction phase, and possibly as replacing venesections during maintenance therapy. Considering the overall safety and efficacy of the venesections, every putative treatment of hemochromatosis targeting iron metabolism must be without side-effects and easy to follow, suggesting especially that oral treatments providing exogenous hepcidin or inducing endogenous hepcidin synthesis might be preferable.

5. Conclusions

Iron remains the major therapeutic target during *HFE*-related hemochromatosis. However, targeting directly the iron stores by iron depletive treatment does not correct the pathophysiological defect of the disease. The development of treatments aimed at restoring iron homeostasis, especially by correcting hepcidin deficiency, could be useful to optimize the treatment, especially during the maintenance phase by controlling transferrin saturation with hopefully a favorable impact on the arthritis of hemochromatosis patients.

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Article

HFE Related Hemochromatosis: Uncovering the Inextricable Link between Iron Homeostasis and the Immunological System

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Abstract: The *HFE* gene (OMIM 235200), most commonly associated with the genetic iron overload disorder Hemochromatosis, was identified by Feder et al. in 1996, as a major histocompatibility complex (MHC) class I like gene, first designated human leukocyte antigen-H (HLA-H). This discovery was thus accomplished 20 years after the realization of the first link between the then “idiopathic” hemochromatosis and the human leukocyte antigens (HLA). The availability of a good genetic marker in subjects homozygous for the C282Y variant in *HFE* (hereditary Fe), the reliability in serum markers such as transferrin saturation and serum ferritin, plus the establishment of noninvasive methods for the estimation of hepatic iron overload, all transformed hemochromatosis into a unique age related disease where prevention became the major goal. We were challenged by the finding of iron overload in a 9-year-old boy homozygous for the C282Y *HFE* variant, with two brothers aged 11 and 5 also homozygous for the mutation. We report a 20 year follow-up during which the three boys were seen yearly with serial determinations of iron parameters and lymphocyte counts. This paper is divided in three sections: Learning, applying, and questioning. The result is the illustration of hemochromatosis as an age related disease in the transition from childhood to adult life and the confirmation of the inextricable link between iron overload and the cells of the immune system.

Keywords: hemochromatosis; HFE; natural history; T lymphocytes; MHC; CD8+ T cells; prevention

1. Introduction

The practice of vaccination and the use of antibiotics provoked a significant decrease of infectious disease in the spectrum of modern clinical practice. That decrease uncovered simultaneously the appearance of other diseases whose presentation relates mainly to age. HFE-related hemochromatosis is a genetic disorder of iron overload. The combination of some luck, progress in the immunology of transplantation, progress of the understanding of the regulation of iron metabolism, progress in genomics, progress in the development of transgenic mice and in the characterization of adaptive immunity cell populations resulted in a sequence of events responsible for permitting today the early diagnosis and prevention of the iron overload induced clinical manifestations of the disease. Human HFE-related hemochromatosis has been the subject of several reviews by prominent researchers in the field [1,2]. In general, emphasis in the pathophysiology of the disease has been focused on the role of hepcidin in iron homeostasis [3,4]. Less attention has been directed to the role of HFE in immunology [5–7] and to the potential contribution of lymphoid cell numbers to that same pathophysiology [8–10].

In the present paper, under “learning”, we briefly review the evidence sustaining the importance of immunology in HFE-related hemochromatosis, starting by the improbable discovery of its link with HLA to the discovery of the *HFE* gene as a MHC class I-like gene and its later association with selected immune defects. Under “applying”, we report a 20 year follow up of iron parameters and lymphocyte counts in three children homozygous for the C282Y HFE variant who started to express signs of iron overload in their transition from childhood to adult life. Under “questioning”, we explore the issue of the still unexplained immunological functions of HFE. The paper concludes with a brief overview of its implications for the clinical practice and for the immunological theory.

Learning: A Brief Historical Sequence Where the Improbable Led to Discovery

In 1975 it was improbable to think that HLA could be of clinical importance beyond histocompatibility or diseases with an immunological background. However, Simon thought the improbable and encountered in Fauchet an immunogeneticist open to doing the HLA typing of 20 “idiopathic” hemochromatosis” patients to find 17 with HLA A3 [11]. The result was the discovery, first published in 1975 in French [11] and in 1976 in the Lancet paper with the title, “HLA and “non-immunological” disease: idiopathic haemochromatosis” [12].

The publications of Simon, Fauchet and coworkers were to provide some background to a postulate published in 1978 saying that the immunological system could have a role in the surveillance of iron toxicity [13]. That postulate was followed by a series of studies of immunological cell populations in patients with “idiopathic” hemochromatosis [8,9,14,15], preceding the finding by Feder et al. in 1996 of the hemochromatosis gene as a novel MHC class I-like gene [16], closing a circle of 21 years from the improbable to the discovery.

Radical changes followed the discovery of the hemochromatosis gene and its association with the C282Y HFE variant. The introduction of genetic testing, combined with Magnetic Resonance Imaging (MRI) as a non-invasive measure of liver iron concentration, the systematic family screening, approaches to disease penetrance from large population studies, all led to a radical change in the clinical presentation and timing of diagnosis well demonstrated by the decreasing frequency of severe liver disease [17].

HFE-related hemochromatosis appears thus as a “dream like” age related disease, with a strong genetic marker, reliable and reproducible serum biochemical markers, namely transferrin saturation and serum ferritin, and confirmatory non-invasive tools all enabling diagnosis much before the clinical presentation of the disease.

The surprise, however, arises from the fact that a disease thought of as non-immunological, can be seen inevitably as an immunological disease. Firstly, the gene is an MHC class I like gene in strong linkage with other genes within the MHC cluster [18,19]. Secondly, the very early studies of lymphoid cell populations in patients with (at the time) idiopathic hemochromatosis demonstrated abnormally high CD4/CD8 ratios in those with a more severe iron overload [14]. At that time, it was also demonstrated that after complete iron depletion by repeated phlebotomies, entry of iron measured by changes in transferrin saturation was faster in patients with the highest CD4/CD8 ratios [14]. Later, a greater importance was attributed to the finding of low numbers of CD8+ T cells associated with the severity of iron overload [9,20] and the demonstration that the low CD8+ T lymphocyte numbers in hemochromatosis are due to defects in the most mature effector memory cells [21]. When those defects were described, they generated some surprise and confusion amongst researchers and clinicians because of the previously existing evidence of iron induced expansions of T lymphocyte populations, namely, relative expansions of CD8+ T lymphocytes, both in experimental models of Fe-citrate injection [22,23] and in clinical models of transfusional iron overload [24,25]. The reciprocal effect, i.e., that primary immune defects could, in turn, contribute to iron overload, was next confirmed with a number of experimental studies examining and confirming the presence of iron overload in mice deficient in selected [26–28] or total lymphocytes [29] and a more severe phenotype in mice lacking

both HFE and β 2-microglobulin [30]. Surprisingly, mice lacking only classical MHC class I molecules also developed iron overload [31].

The mechanisms underlying the lymphocyte defects in hemochromatosis are still poorly understood. The most recent evidence points to the possibility of a continuous effect where iron may sustain a constant activation, self-renewal and proliferation of CD8+ cells, and this may eventually lead to exhaustion of the effector memory T cells [32].

Regarding the mechanism “how” could lymphocyte defects contribute to iron overload, it is not until 2014 that Pinto and co-workers, in an extensive analysis of the interaction of lymphocytes with non-transferrin bound iron (NTBI), demonstrated that lymphocytes can take in NTBI in vitro [33] and that in vivo lymphocyte transfer can correct the iron overload of immune-deficient mice [10], closing thus another circle: From firm but unexplained observations to the demonstration of how lymphoid cell numbers could have a role in the control of iron overload, i.e., by acting as a circulating pool capable of “buffering” NTBI.

Evidence of the inextricable connection between iron homeostasis and the adaptive immune system recently gained a novel impulse with the demonstration that patients with a homozygous p.Tyr20His mutation in the transferrin receptor 1 (TfR1) have a combined immunodeficiency characterized by normal numbers but impaired function of T and B cells [34]. Besides TfR1, other iron regulatory genes had also been previously found to be critical for lymphocyte activation and function, namely H-ferritin, whose conditional deletion in mice was shown to reduce B and T lymphocyte populations [35] or hepcidin, whose expression is increased during lymphocyte activation and shown to be necessary for proper lymphocyte proliferation [36].

With regard to the cells of the mononuclear/phagocytic system, their role in the recycling of senescent red blood cells provides perhaps the most significant illustration of the close interactions between iron metabolism and the immunological system [24]. In the case of secondary iron overload, as a result of dyserythropoiesis, hemolysis, or transfusions, macrophages are heavily loaded with iron which is released in the form of low-molecular weight (LMW) iron. This leads not only to increased transferrin saturation but also iron circulating as NTBI that will inappropriately enter tissues and cells [37]. In HFE-related hemochromatosis, the scenario is somehow different. In spite of the high transferrin saturation and circulating NTBI, as a result of increased iron absorption, little iron is seen in the Kupffer cells and other macrophages, while hepatocytes already show iron overload [2]. In a previous study of iron release by monocytes after erythrophagocytosis, Moura and co-workers demonstrated that monocytes from hemochromatosis patients released twice as much iron in a LMW form as control cells [38]. Based on those observations, they proposed for the first time the existence of a basic abnormality in the retention of iron in macrophages and probably from intestinal mucosal cells [38], a mechanism that is presently well established with the demonstration that hepcidin, which is functionally defective in hemochromatosis, regulates cellular iron efflux by binding to ferroportin and inducing its internalization [3]. Finally, one may question how the handling of iron in other compartments of the mononuclear/phagocytic system may also impact in their response to other types of toxicity. As an example, increasing evidence suggests that the accumulation of iron in the brain and the consequent microglia activation are hallmarks of neurodegenerative disorders, including Alzheimer’s disease [39]. However, substantial efforts are still needed to fully understand this and many other aspects of the complex interactions of iron with inflammation and immunity. In our view, HFE hemochromatosis continues to offer a particularly good model to approach new questions.

2. Results

2.1. Applying: A Case and Family Report

Armed with all the tools available in 1999 and the possibility to perform at our center systematic individual longitudinal studies, we were in a position to follow the real natural course of a disease, not just the course deduced from cross sectional data at different ages. Every clinician following a

patient with an age related disease hopes to be able to have tools for an early detection of the disease, and fortunately, this is the case for hemochromatosis. We were challenged by the finding, by an attentive pediatrician, of a 9-year-old boy with an abnormally high transferrin saturation. This case, the subsequent family study with detection of two additional cases, and their clinical progression are described below. Highly motivated by the search of the reciprocal interactions between iron and the immunological system, we included in our clinical follow-up not only the iron related parameters but also the lymphocyte counts.

The proband was thus a 9-year-old boy, referred to our center for investigation of an abnormally high transferrin saturation (TS) value (65%) accidentally found on a routine examination and confirmed on a second determination (70%). He had a normal healthy growth and no signs or symptoms of any disease; the physical examination was normal and had normal blood counts for age. Serum ferritin (SF) was normal (79 ng/mL). *HFE* genotyping was performed with the parents' informed consent and revealed homozygosity for the C282Y variant. Genetic counseling was offered to the family and consent obtained to perform *HFE* genotyping in the parents and in the two only brothers of 5 and 11 years of age, all apparently healthy. Results of the family screening are illustrated in Figure 1, showing that both mother and father were heterozygous for the C282Y variant, with normal iron parameters.

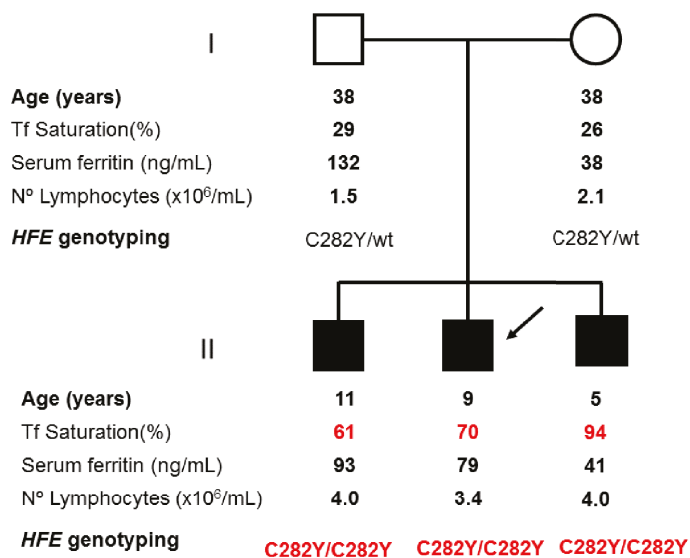


Figure 1. Family Pedigree of the case study. Age, serum markers of iron metabolism, total lymphocyte counts, and *HFE* (OMIM 235200) genotype are shown for each subject. The C282Y variant in the *HFE* gene is characteristically found in the homozygous form in patients with hemochromatosis. Homozygous subjects (C282Y/C282Y) are here represented by solid symbols and heterozygous (C282Y/wt) for half solid symbols. The proband is indicated by an arrow. Values in red are outside normal range.

Surprisingly, the two brothers were C282Y homozygous, with abnormally high TS (respectively 94% and 61%) with normal SF values of 41 ng/mL and 93 ng/mL, respectively. Of note, all 3 siblings had high lymphocyte counts as appropriate for their age. All procedures involving the collection of human samples and data were carried out following the rules of the Declaration of Helsinki of 1975, revised in 2013 [40], with the approval of the Ethical Committee of Centro Hospitalar Universitário do Porto.

The 3 siblings were regularly followed at our center with longitudinal determinations of iron parameters and lymphocyte counts for the last 20 years. They were recommended to become volunteer

blood donors at the age of 18. Results of the follow-up are shown in the graphs on Figure 2 with the illustration of a representative case.

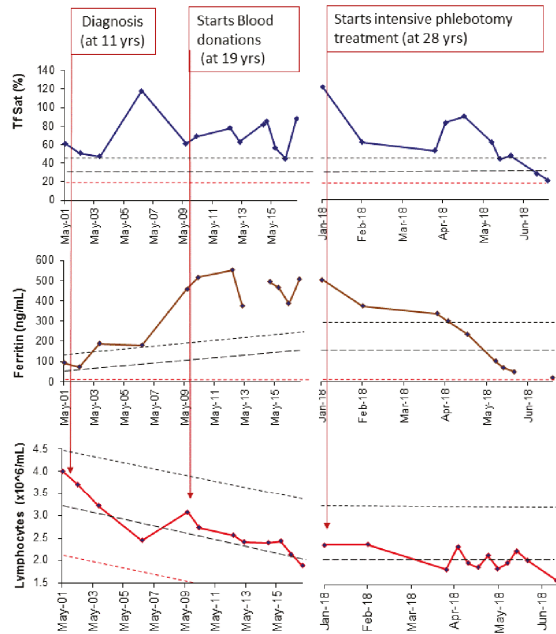


Figure 2. Follow-up of transferrin saturation (TfSat), serum ferritin, and total lymphocyte counts during a 17-year period. A representative case is shown (case II-1). Time at diagnosis, start of blood donation, and beginning of intensive phlebotomy treatment are marked in graphs by arrows. The large dashed lines represent the expected average values depending on age, derived from the simple regression equations in a population of sex and age matched normal controls recruited in the context of family studies. The small dashed lines represent the upper (in blue) and lower (in red) 95% confidence limits for each regression.

The profiles of SF progression together with the change of lymphocyte counts offer a remarkable illustration of how early does iron start to accumulate in C282Y homozygous subjects, how it progresses during the transition from childhood to adult life and how the total numbers of lymphocytes may influence that progression. In all cases, SF started to be elevated before adult age (< 18 years) and continued to rise inversely to the fall of lymphocyte counts that continued to decline until reaching the adult levels at around 25 years (only case II-3 did not reach yet 25 years of age). The graphs also illustrate that intervention with intensive treatment was necessary to normalize SF in spite of regular blood donation. A better illustration of the inverse correlation between SF values and lymphocyte counts, before the impact of intensive treatment, is given in Figure 3 where the individual values in all 3 cases are plotted against age.

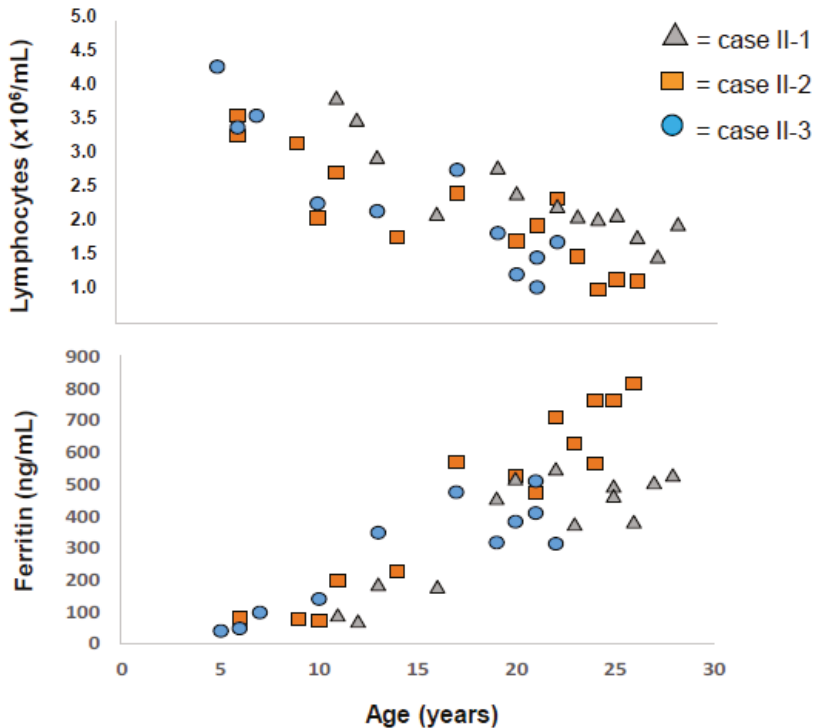


Figure 3. Inverse correlations between lymphocyte counts (A) and serum ferritin (B) before intensive treatment. Individual values from all three cases (II-1, II-2 and II-3) are plotted against age.

Notably, case II-2, whose lymphocytes declined to values $<1.5 \times 10^6/\text{mL}$ (considered lymphopenia) at the age of 26, reached SF levels much higher ($>700 \text{ ng/mL}$) than his brother, case II-1, whom, at the same age, had lymphocytes stabilized at around $2.0 \times 10^6/\text{mL}$, and a lower SF value (500 ng/mL).

In order to determine which sub-populations contributed the most for the setting of lymphocyte total numbers, we also determined, by flow cytometry analysis, the numbers of CD4+ T cells, CD8+ T cells and non-T subpopulations along the follow-up time. The results are shown in Figure 4 where it is clear that the change in lymphocyte counts is due to the continuous decline in T cell numbers and not related to the numbers of non-T cell populations (a combination of B and natural killer cells), which are more variable and probably related to immune responses against foreign antigens.

Naturally, the question could be raised if it is the lymphocyte depletion that is contributing to the progressive iron accumulation or, on the contrary, is the lymphocyte depletion a consequence of iron overload? In order to answer that question, we retrospectively reviewed the iron parameters and lymphocyte populations determined in 190 apparently healthy, non-C282Y homozygous family members of hemochromatosis patients, aged 5–20 years, who were studied in the context of a systematic family screening program at our center. The results, illustrated in Figure 5, confirm that the decline in lymphocyte counts is a normal physiological process and that, although SF slightly increases with age (particularly in males), it never reaches abnormal levels above 200 ng/mL in females or 300 ng/mL in males. These findings come to support the notion that, in order to start accumulating iron in hemochromatosis, it is necessary to combine two conditions: The genetic predisposition given by C282Y homozygosity to increase the iron in circulation and the lack of a sufficiently high number of T lymphocytes to buffer the excessive serum iron.

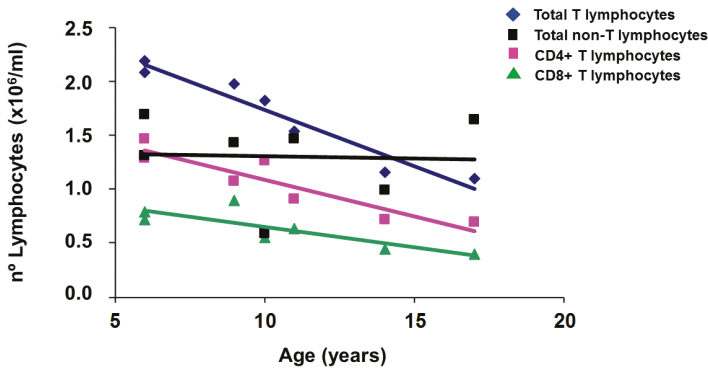


Figure 4. Follow-up of lymphocyte subpopulations. Total T-lymphocytes, CD4 and CD8 T lymphocytes, and non-T lymphocyte counts from a representative case (II-2) are plotted against age. Total T lymphocytes were calculated by the sum of CD4+ and CD8+ T cells. Non-T lymphocytes were calculated from the difference between the absolute lymphocyte numbers and the total T lymphocytes.

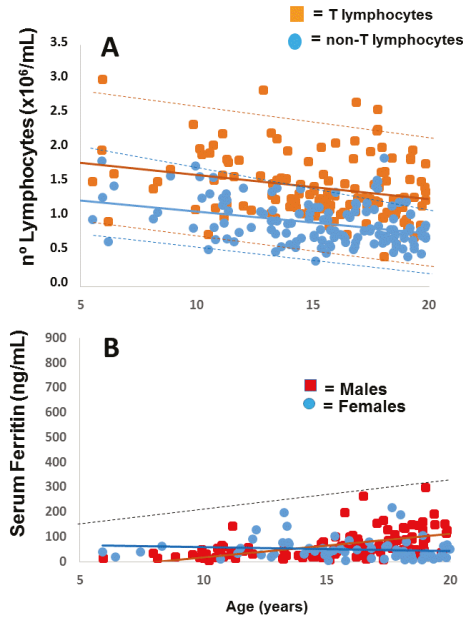


Figure 5. (A) Change in total T and non-T lymphocytes with age in non-homozygous apparently healthy family members screened before 20 years of age ($n = 190$). (B) Variation in serum ferritin with age in the same subjects, grouped as males or females. Full lines represent the regression lines for each correlation. Dashed lines in panel A represent the upper and lower 95% confidence limits for T (in brown) and non-T (in blue) lymphocytes. The dashed line in panel B represents the upper 95% limits.

2.2. Questioning: A Dual Function for HFE?

In spite of all advances supporting the cross-relationships between iron and adaptive immunity, many questions still remain unexplored and may keep immunologists and iron biologists busy for some time. Among them, perhaps the most critical question relates to the HFE function and how does it impact simultaneously on iron metabolism and on the adaptive immune functions. Interestingly, 20

years before the discovery of HFE, Svejgaard and Ryder postulated that HLA alloantigens could interfere with ligand/receptor interactions not directly involved in immune reactions, and those interactions could under certain conditions explain some associations between HLA and non-immunological diseases giving as an example hemochromatosis [41]. This view came to be vindicated with the demonstration that HFE, a non-classical HLA molecule, interacts with both the Tfr1 to regulate transferrin-mediated iron uptake [42] and with transferrin receptor 2 (Tfr2) as an iron sensor for hepcidin signaling [43]. The role of HFE, however, is not limited to iron metabolism; it is also implicated in adaptive immune functions. Sometime ago, it was suggested that HFE could be immunogenic [44], and more recently Reuben and co-workers proposed that HFE could have a role in antigen processing and presentation leading to an inhibition of CD8+ T-lymphocyte activation [7]. These were *in vitro* studies based on several T-lymphocyte activation read-outs in cells transfected with wild type and mutated HFE molecules. The first demonstration *in vivo* that HFE acts as a negative regulator of CD8+ T-lymphocyte activation and differentiation was provided with the study by Costa et al. [32] of lymphocyte gene expression signatures from HFE-related hemochromatosis patients and mouse models, where it was shown that the lack of HFE impacts on several activation markers in CD8+ T lymphocytes [32]. Whatever the mechanism how this interaction may happen, it may imply the cross-talk between HFE and the MHC class I antigen presentation pathway, as suggested by Almeida et al in their study of peripheral blood mononuclear cells from patients homozygous for the p.C282Y variant in HFE [5]. In that study they found a reduced cell-surface expression of MHC class I due to an enhanced endocytosis rate of MHC class I molecules caused by premature peptide and β 2-microglobulin dissociation [5]. Whether this could happen through a direct effect or through the indirect influence of an unfolded protein response [45], this remains a pending question.

In summary, the mutated HFE can be now appropriately defined with a Janus like nature, implicated in two relevant pathways: Regulation of systemic iron homeostasis, through its interaction with the transferrin receptors and hepcidin signaling [42,43]; and the core of the immune system, by influencing MHC assembly and surface expression [5], and CD8+ lymphocyte expansion [7,32]. Failure of this dual function may explain the phenotypes observed in hemochromatosis of both iron overload and defective numbers of CD8+ T lymphocytes. To better illustrate this view, we present in Figure 6a diagram summarizing how iron is essential for lymphocyte functions and, in turn, how lymphocytes, equipped with the capacity to uptake, hold and mobilize iron, contributing to the control of systemic iron homeostasis and the protection against iron toxicity.

At the systemic level, HFE is one of the players of the complex regulatory machinery of iron sensing and hepcidin signaling [43], thus contributing to the prevention of inappropriate iron accumulation. In HFE-related hemochromatosis, this regulation fails and the consequence is systemic iron overload. One of the multiple physiological implications of a well-controlled systemic iron homeostasis is the maintenance of appropriate adaptive immune functions. Lymphocyte responses depend on the interplay between the MHC dependent activation pathway, and their capacity to proliferate and differentiate, for which iron availability is essential through Tfr1 mediated endocytosis [34]. The control of ferroportin mediated iron export through autocrine hepcidin signaling also impacts on the lymphocyte proliferation capacity of relevance [36], the hemochromatosis protein HFE was shown to have an additional role in the assembly and expression of the classical MHC class I for antigen presentation [5]. In the event of acquired systemic iron overload, such as occurs in transfusion dependent thalassemia patients or in experimental models with iron injection, lymphocytes (particularly CD8+ T lymphocytes) will be activated and expand in response to iron [22]. The recent demonstration that lymphocytes have the capacity to uptake the non-transferrin bound iron (NTBI) [33] and by doing so, to act as “buffers” of the systemic iron overload and protect from the iron accumulation in other tissues [10], came to vindicate the postulated reciprocal role of lymphocytes in the protection against iron toxicity [13]. Of relevance, the hemochromatosis protein HFE also has a role in this setting, by influencing the CD8+ T lymphocyte gene expression of molecules involved in activation and differentiation that finally have an impact on the number of circulating CD8+ T cells and consequently [32], on its NTBI

“buffering” capacity. In summary, iron is essential for lymphocyte functions and, in turn, lymphocytes equipped with the capacity to uptake, hold, and mobilize iron contribute to the control of systemic iron homeostasis and the protection against iron toxicity. Pharmaceutical and/or therapeutic targets in the context of this new awareness are not completely obvious but evidence consistently points to TFR1 [34] and HFE as key molecules in the regulation of the crosstalk between the two systems. The molecule responsible for NTBI uptake by lymphocytes remains to be discovered. In summary, as usual, answers can only be found when the importance of the questions is acknowledged, and that takes time. The authors hope that the openness of Pharmaceuticals to the views presented here is a sign that the distance between questions and answers is being shortened.

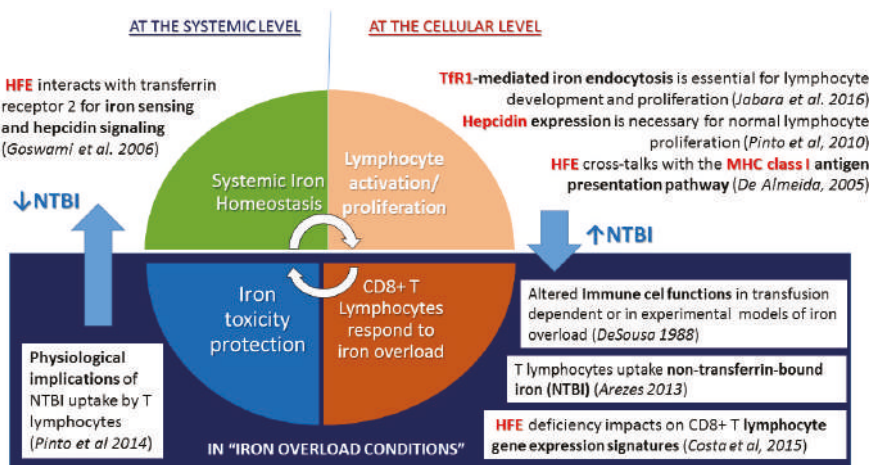


Figure 6. Diagram representation of the dual role of the human hemochromatosis protein HFE as a critical molecule in the reciprocal interactions between iron homeostasis, the major histocompatibility complex (MHC) and lymphocyte functions.

3. Discussion

3.1. Implications for Disease Prevention in HFE-Related Hemochromatosis

The prevailing view regarding the onset of HFE-related hemochromatosis is that patients develop symptoms only at around 30–40 years of age [2]; it is well established, however, that they may have biochemical evidence of iron overload some time before [46]. Although early diagnosis is well accepted as the best certainty of therapy in this age related disease, no available guidelines specifically address the question of when should biochemical screening be performed on a population basis. Two large population-based prospective studies with long-term follow-up of untreated p.C282Y homozygous subjects described the progression of iron overload with age but including only subjects older than 35 years in one study [47] and older than 40 years in another [48]. In another HH cohort study with a long-term (24 years) follow-up of subjects screened by family or primary medical practice screening, it was estimated that the mean age of homozygotes who could develop a severe liver iron overload would be approximately 21 years after the hepatic iron stores begin to increase in both men and women [49]. In spite of this knowledge, the average age of diagnosis of HH patients continues to be at around 40 years [17], and there is still no evidence-based recommendation for screening earlier. The results described above challenge this view by showing that markers of the disease may be found much before adulthood and that, at least in some cases, at the age of 28 years there may be already a significant amount of iron accumulated. Knowing the silent toxicity of iron, the question of when should screening be ideally performed deserves a wider discussion.

3.2. Implications for Immunological Theory

The consistent associations found between iron overload and lymphocyte numbers or function vindicate the postulated notion that the immunological system has a surveillance role of the toxicity of iron [13] much before iron becomes a threat posed by its use by microbes [50]. How exactly that role is exercised is still not fully understood. Searching first in the β 2-microglobulin (β 2m) knock-out mice, spontaneous iron overload was found in organs such as liver and pancreas [26]. The subsequent finding of spontaneous hepatic iron overload in mice lacking classical MHC Class I molecules was surprising and signals the involvement of MHC class I itself in the exercise of control of iron overload [31]. A similar explanation may apply to the finding in the two separate studies by Rodrigues et al. [51] and Levy et al. [30] of higher hepatic iron overload in older β 2m deficient mice than in HFE deficient mice. No matter how results affirm themselves, MHC class I appears always as a “culprit” or a motive for tissue iron accumulation: Either by its absence, such as in β 2m knock out mice, or because its assembly and expression are affected by the C282Y mutated HFE.

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Review

Twenty Years of Ferroportin Disease: A Review or An Update of Published Clinical, Biochemical, Molecular, and Functional Features

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Abstract: Iron overloading disorders linked to mutations in ferroportin have diverse phenotypes *in vivo*, and the effects of mutations on ferroportin *in vitro* range from loss of function (LOF) to gain of function (GOF) with hepcidin resistance. We reviewed 359 patients with 60 ferroportin variants. Overall, macrophage iron overload and low/normal transferrin saturation (TSAT) segregated with mutations that caused LOF, while GOF mutations were linked to high TSAT and parenchymal iron accumulation. However, the pathogenicity of individual variants is difficult to establish due to the lack of sufficiently reported data, large inter-assay variability of functional studies, and the uncertainty associated with the performance of available *in silico* prediction models. Since the phenotypes of hepcidin-resistant GOF variants are indistinguishable from the other types of hereditary hemochromatosis (HH), these variants may be categorized as ferroportin-associated HH, while the entity ferroportin disease may be confined to patients with LOF variants. To further improve the management of ferroportin disease, we advocate for a global registry, with standardized clinical analysis and validation of the functional tests preferably performed in human-derived enterocytic and macrophagic cell lines. Moreover, studies are warranted to unravel the definite structure of ferroportin and the indispensable residues that are essential for functionality.

Keywords: SLC40A1; ferroportin; iron overload; non-HFE; ferritin; hemochromatosis

1. Introduction

Hereditary hemochromatosis (HH) type 4 or ferroportin disease (OMIM-code: 606069 Orphanet-code: 139491) is associated with variants in *SLC40A1* and inherited in an autosomal-dominant manner. Since 1999, ferroportin disease was classified into two entities [1–3], based on experimental *in vitro* models of genetic variants found in patients [4–10]. Classical ferroportin disease (Type 4A) is associated with loss-of-function (LOF) variants with diminished cell surface expression of ferroportin and lower iron export capacity, and is characterized by macrophage iron retention and iron restriction for erythropoiesis. It is clinically recognized by the presence of high serum ferritin concentrations with

low to normal transferrin saturation (TSAT) and poor tolerance to phlebotomy. Non-classical (atypical, type 4B) ferroportin disease is associated with gain-of-function (GOF) variants that render ferroportin protein resistant to hepcidin, resulting in continued iron export, and leading to a phenotype that mimics classical HFE HH characterized by parenchymal (hepatocellular) iron overload with elevated serum ferritin and TSAT.

Systematic and narrative reviews on ferroportin disease revealed the great variety in iron parameters among patients with ferroportin variants and emphasize the poor correlation between iron export capacity in functional studies and clinical characteristics (phenotype) [7,9–11] and question the pathogenicity of various variants [8,12].

To better guide the management of this disorder, a better understanding of the clinical and biochemical features of ferroportin disease and its underlying pathological mechanisms is required. In this report, we evaluated and describe clinical and laboratory data of patients reported between 1999 and June 2019, in whom the identified ferroportin variant was assumed to be associated with the defined state of iron overload. The relation between patient's clinical features and the functional in vitro features of underlying variants is assessed in context of current knowledge on the function and structure of ferroportin and its regulatory mechanisms. We comment on the shortcomings of describing the phenotype based on case and family reports, and review the limited applicability of functional studies and available in silico prediction models to determine pathogenicity. We recommend the institution of a global registry with a (1) standardized diagnostic protocol, (2) validated functional tests, and (3) studies to establish the definite secondary and tertiary structure of ferroportin and residues involved in its binding with hepcidin for ubiquitination and to ferroportin for iron transport.

2. Methods

2.1. Data Collection and Selection

Identification, Demographic Characteristics, and Iron Parameters

A literature search was done using Medline and Embase (searching strategy: #1 (SLC40A1 or ferroportin or IREG or IREG-1 or FPN or FPN1 or non-HFE hemochromatosis), #2 (mutation or variant) from 1999 to June 2019. Papers with only an abstract in English were also selected. We included all the cases (including family studies) of ferroportin variants that are associated with unexplained elevated serum ferritin concentration. Since the aim of this analysis was to evaluate the relation between the clinical features and the functional in vitro characteristics in experimental cell lines of the various potentially pathogenic variants, we excluded variants for which these functional studies have not been performed, e.g., variants in promoter and untranslated regions, splicing variants as well as variants leading to synonymous amino acids such as Iso109Iso, Leu129Leu, and Val221Val [13–17]. In addition, we excluded patients with previously established neutral or (possibly) iron-modifying polymorphisms with a variant population frequency > 1%, such as the African Gln248His variant and variants in residues Leu348 and Leu384 [7,18–21].

Only patients with reported numerical value of at least transferrin saturation (TSAT) or ferritin concentration were selected. Demographic features, presenting symptoms, blood test results (hemoglobin (Hb), mean corpuscular volume (MCV), serum iron, TSAT, ferritin, and alanine aminotransferase (ALT)), hepatic iron content and hepatic histology, data on tolerability of phlebotomy (including the amount of iron removed), and the presence of p.Cys282Tyr and p.His63Asp HFE variants and other potential confounders, such as alcohol consumption and signs of metabolic syndrome, were collected. Organ iron accumulation as assessed by MRI and histologic examination was evaluated. Hepatic damage was histologically scored using the Meta-analysis of Histological Data in Viral Hepatitis (METAVIR) score (0 = no fibrosis, 1 = mild fibrosis (portal fibrosis without septa), 2 = moderate fibrosis (portal fibrosis and few septa), 3 = severe fibrosis (numerous septa without cirrhosis), and 4 = cirrhosis). Differences between categorical variables were compared with the Chi-square test with Yates correction while the comparison of continuous variables was done with the Mann-Whitney U-test

and Student's *t*-test of independent means. A two-sided *p*-value < 0.05 was considered significant. The correlation between variables was determined by the Pearson correlation coefficient.

2.2. Variant Classification Based on Functional In Vitro Studies

Functional studies on iron export and/or ferroportin expression were performed in cell lines transfected with the ferroportin protein variant. We reviewed the reported functional studies on iron export capacity and ferroportin expression that were performed in cell lines transfected with the wild-type and the variant ferroportin protein. We assigned a variant as an LOF variant when the iron export capacity was significantly reduced, in comparison to the wild-type (WT) protein. The variant was assigned as a GOF variant when the iron transport capacity was preserved. When the addition of hepcidin significantly inhibited the iron export capacity and/or ferroportin expression, the variant was defined as hepcidin-sensitive, and the variant was hepcidin-resistant in the absence of a modulating effect of hepcidin. A variant was scored as "non-classified" when no relevant functional data were available, and as "conflicting" when data on the hepcidin effect were inconsistent or inconclusive.

2.3. Bioinformatics Prediction Software Analysis

To assess co-segregation in multiple affected family members, the simplified method of segregation analysis (SISA) was applied [22,23] and classified according to recent recommendations to define co-segregation to fit the guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP) [22,24]. Briefly, to determine the co-segregation we used the classification suggested by Jarvik et al. and the method to calculate the probability that the phenotype of a variant carrier is caused by chance rather than genotype, as suggested by Moller et al. [22,23]. This probability is described as $(1/2)^n$, wherein *n* is the number of informative meioses and will be the number of the carriers with the affected phenotype of interest and all the carriers in between them, minus one. The genome aggregation data base (gnomAD), was applied to determine of the allele frequency of a variant. In order to further determine the potential pathogenicity of the various variants three commonly used algorithms were applied [25]. Polyphen2 predicts the impact of amino acid substitution on the structure and function of human protein using physical and comparative considerations [26]. The method "Sorts intolerant from Tolerant" (SIFT) (<http://www.blocks.fhcr.org/sift/SIFT.html>) classifies non-synonymous single nucleotide polymorphisms on the basis of the evolutionary conservation of amino acids within protein families [27]. Align-GVGD predicts variants in the query sequence based on a combination of Grantham Variation (GV) which measures the evolutionary variation at a particular position in the alignment and Grantham Deviation (GD), measuring the biochemical difference between the reference and amino acid encoded by the variant [28].

3. Evaluation and Analysis

3.1. Description of the Phenotype of Patients (Including Related Family Members) in Relation Findings in Functional Studies

3.1.1. Patients Characteristics

Ferroportin Disease Occurs in Different Parts of the World and Has no Specific Symptoms

We retrieved 359 individuals with 60 variants (59 missense variants and one deletion) in the ferroportin protein (Table 1). The reported patients, were predominantly male and of European descent. When reported, fatigue and arthralgia were the major presenting symptoms. Overall, patients had normal Hb and MCV with a serum iron level and TSAT at the upper range of normal and moderately elevated ferritin levels.

Table 1. Patient Characteristics. MCV: mean corpuscular volume, TSAT: transferrin saturation.

Characteristics	<i>n</i>	Value (Median)	Range
Patients	359		
Probands		68	
Relatives from probands		191	
Individual patients		88	
Gender	342		
Female		131	
Male		211	
Age (years)	322	41	2–87
Continent	352		
Europe		258	
North–South America		37	
Asia		44	
Australia/Oceania		13	
Presenting clinical symptoms	71		
Fatigue		34	
Elevated liver enzymes / hepatomegaly		30	
Joint complaints/arthralgia		36	
Miscellaneous		47	
Referral because of high ferritin level	131		
Hematological Parameters			
Hemoglobin (g/dL) [#]			
Female	60	13.1	7.5–16.3
Male	92	15.0	6.5–18.4
MCV (fl)			
Female	38	91	77–107
Male	46	91	70–108
Serum iron (μmol/L)			
Female	34	20.9	6.7–94.0
Male	56	26.3	6.2–86.0
TSAT (%)			
Female	116	31.5	2.0–100.0
Male	180	38.0	7.0–104.0
Ferritin (μg/L)			
Female	122	1026	4–8943
Male	201	1514	12–18695

Iron Parameters are Determined by Gender and Age. [#]Significant difference between females and males ($p < 0.05$, also after correcting for multiple testing according to Bonferroni (<0.01)).

Women had significantly lower Hb, TSAT, and serum ferritin concentration (Table 1). There was a weak correlation between age and TSAT ($r = 0.1796$) and serum ferritin ($r = 0.3461$) (Figure S1a–c). Probands were significantly older than affected family members (45.5 versus 34 years) with significantly higher (1896 versus 948 μg/L) serum ferritin levels (Table S1). Ten percent (20/191) of affected family's members (6/14 males/females, median age 42 years) had no biochemical signs of iron overload (ferritin level ≤ 200 μg/L and normal TSAT).

Modification by Additional Hereditary and Acquired Conditions

We found no effect of the presence of p.Cys282Tyr (13 heterozygotes) and p.His63Asp (35 heterozygotes, five homozygotes) HFE variants on iron parameters (Table S2). In only 84 patients, the current alcohol consumption was reported without demonstrable effect (Table S3). The reported clinical data were insufficient for the proper determination of the presence of metabolic syndrome to explore its modifying effect.

3.2. Relation between In Vitro Functional Studies of Ferroportin Variants on Clinical Features

We identified 27 different GOF variants (110 patients), 21 LOF variants (205 patients), and 12 unclassified variants (33 patients) (Table 2). A total of 355 cases involved heterozygosity for ferroportin variants. One homozygous Gly204Ser GOF variant was reported in a 52-year-old female with ferritin concentration of 5236 µg/L, TSAT of 100%, and symptoms of hepatic fibrosis revealed by radiology [15]. A patient with homozygosity for the GOF variant Arg561Gly had biochemical signs of severe iron overload reflected by a serum ferritin of 2750 µg/L and TSAT of 84% [29].

Table 2. Functional and phenotypical characteristics of 359 patients with 60 ferroportin variants. GOF: gain of function, LOF: loss of function.

Protein Variant	Iron Export			Hepcidin Effect on			TSAT			Reference %
	Expression	Capacity	Export Capacity	Expression	Export Capacity	Fate of Hepcidin	Patients (n)	Normal	High	
Tyr64Asn	PM	=	no	no	no	↓ubiquitination/uptake	8	-	8	[11,30–36]
Cys326Ser	PM	=	no	no	no	↓binding/no uptake	7	-	6	[30,32,34,37,38]
Cys326Tyr	PM	=	no	no	no	↓no uptake	4	-	4	[8,31,34,35,39–44]
Tyr333His	PM	=	no	no	no	↓no uptake	5	-	5	[45]
Tyr501Cys	PM	=	no	no	no	↓binding	7	2	5	[30,33,46]
Asp504Asn	PM	=	no	no	no	↓binding/uptake	2	-	2	[8,30]
His507Arg	PM	=	no	no	no	↓ubiquitination	4	-	4	[30,41,47]
Sum: 7	GOF						37	2	34	
Ile180Thr	PM	=	yes	-	-	normal uptake	3	2	1	[8,48,49]
Thr230Asn	PM	=	yes	-	-	normal uptake	1	1	-	[8]
Met266Thr	PM	=	yes	-	-	normal uptake	1	1	-	[8]
Leu345Phe	PM	=	yes	-	-	normal uptake	1	1	-	[8]
Ile351Val	PM	=	yes	-	-	↑uptake	1	1	-	[8]
Pro443Leu	PM	=	yes	-	-	↑uptake	1	-	-	[8]
Arg561Gly	PM	=	yes	-	-	normal uptake	4	1	2	[8,29,50]
Sum: 7	GOF						12	7	3	
Ala69Thr	PM	=	-	-	-	-	4	2	2	[42,51,52]
Val72Phe	PM	=	yes	↓!	↓!	-	5	2	3	[30,53]
Asn144Asp	PM	=#	conflicting	no#	no#	↓binding/uptake	2	-	1	[30–32,35,54]
Asn144His	PM	=	conflicting	yes	yes	↓uptake	20	13	7	[3,6,31,34,35,39,40,42,55–59]
Asn144Thr	PM	=	conflicting	yes#	yes#	↓uptake	1	-	1	[6,31,32,34,60]
Gln182His	PM	=	conflicting	yes	yes	normal uptake	2	1	-	[55,56,61]
Gly204Arg~	PM	=	no\$	no\$	no\$	-	1	-	1	[51]
Gly204Ser	PM	=	conflicting	no#	no#	↓ubiquitination	14	4	10	[9,11,15,30]
Ser209Leu	PM	=	no\$	no\$	no\$	-	7	7	-	[62,63]
Asp270Val	PM	=#	yes	↓!	↓!	↑ubiquitination	2	1	1	[14,30,64]
Arg296Gln~	PM	=	no\$	↑	↑	-	1	1	-	[51]
Ser338Arg	PM	=	yes	↓!	↓!	↑ubiquitination	1	-	1	[6,30,65]
Arg371Gln	IC/PM	=	no	yes	yes	-	1	1	-	[11]

Table 2. *Cont.*

Protein	Iron Export			Hepcidin Effect on			TSAT			Reference %
	Expression	Capacity	Export Capacity	Expression	Export Capacity	Fate of Hepcidin	Patients (n)	Normal	High	
Sum: 13	GOF		Uncertain/Conflicting/Unknown				61	32	27	
Asp84Glu	-	↓	no	no	-	-	1	-	-	[43]
Val1162del	IC/JPM	↓	no	no	no	↓↓uptake	50	39	2	[6,8,35,43,51,55,56,66-79]
Asp181Asn	PM	↓	no	no	no	-	6	6	-	[51]
Asp181Val	JPM	↓	no	no	no	-	10	10	-	[8,42,46,80]
Gly490Asp	IC/JPM	↓	no	no	no	no uptake	12	9	3	[8,34,55,81]
Sum: 5	LOF		Hepcidin Resistant				79	64	5	
Arg88Gly	IC/PM	↓	yes	-	-	-	7	3	3	[8,11,82]
Leu129Pro	PM	↓	yes	↑yes	-	-	5	3	2	[83]
Arg178Gln	PM	↓	yes	-	-	-	26	17	-	[75,82,84,85]
Asn185Asp	PM	↓	yes	-	-	-	19	4	6	[9,11,86]
Sum: 4	LOF		Hepcidin Sensitive				57	27	11	
Ala77Asp	IC/PM	↓	conflicting	not reliable@	no uptake	no uptake	26	15	11	[1,2,8,11,34,35,39,41,42,56,57,66,71,73,87-90]
Gly8Ser	JPM	↓&	not reliable@	not reliable@	normal uptake&		24	15	3	[8,57,73,87,88,91,92]
Ile152Phe	PM	↓	↓	↓	-	-	2	-	-	[39,93]
Asp157Gly	IC/PM	↓	conflicting	no&	-	-	4	3	1	[8,34,55,56,61]
Asp157Tyr	JPM	↓	-	-	-	-	2	1	1	[8,94]
Trp158Cys	IC	↓	not reliable@	not reliable@	-	-	4	4	-	[41,62]
Trp158Leu	IC	↓	not reliable@	not reliable@	-	-	2	2	-	[11]
Asn174Ile	IC/PM	↓	conflicting	not reliable@	↓uptake		3	1	2	[34,39,46,87,88]
Leu233Pro	IC/JPM	↓	not reliable@	not reliable@	-	-	3	1	-	[8,55,93]
Gly323Val	IC/PM	↓	conflicting	not reliable@	↓uptake		1	-	-	[34,55,56,61]
Arg489Lys	IC	↓	-	-	-	-	6	4	-	[40]
Gly490Ser	JPM	↓	-	-	-	-	3	2	1	[8,35,82]

Table 2. *Cont.*

Protein Variant	Iron Export		Hepcidin Effect on			TSAT			Reference%
	Expression	Capacity	Expression	Export Capacity	Fate of Hepcidin	Patients (n)	Normal	High	
Sum: 12	LOF		Uncertain/Conflicting/Unknown			80	48	21	
Tyr64His						1	-	1	[95]
Gly80Val						2	2	-	[80]
Arg88Thr						7	3	4	[48]
Asp157Ala						5	2	3	[96–98]
Asp157Asn						5	5	-	[45,53]
Ala232Asp						2	2	-	[99]
Lys240Glu						1	-	1	[100]
Gly267Asp						1	1	-	[80]
Cys326Phe						1	-	1	[101]
Gly468Ser						3	3	-	[102]
Arg489Ser						4	2	2	[94,103]
Val511Ile						1	-	1	[45]
Sum: 12	Non-Classified					33	20	13	
Sum: 60						359	200	114	

%reference before the comma refers to reports on functional data; reference after the comma points to reports on clinical data; PM plasma membrane; IC intracellular; = comparable; - no data available; ~ discrepancy in the classification with respect to the original report [51], † dependent on the dose of hepcidin and time of exposure; # data not fully consistent; \$ not strongly established; * In one study [104], this variant had diminished iron efflux; ‡‡ severe; †† moderate/mild; ‡ borderline impaired; @ interpretation is hampered since the variant is mainly localized intracellularly in the experiments; & data of Domenico et al. [55,87] were disregarded in view of an unexpected discrepancy with other reports.

3.2.1. Clear Distinct Phenotypical Features in Patients with GOF and LOF Variants

Patients with GOF variants had significantly lower ferritin (755 µg/L versus 1595 µg/L) concentration (Table 3) and higher serum iron (36.0 µmol/L versus 17.5 µmol/L) concentration and TSAT (62% versus 32%) than patients with LOF variants. Inter-individual variation in TSAT levels was high in both LOF and GOF patients (Figure 1). In contrast to previous reports [7,105] and the prevailing opinion [10], patients with LOF and GOF variants were found to have a comparable Hb and prevalence of anemia (according to the World Health Organization criteria). While hepatic iron content was similar in patients with LOF and GOF variants, patients with LOF variants display iron deposition predominantly in macrophages (Kupffer cells), while the iron in GOF patients is predominantly present in hepatocytes, and is associated with more hepatic damage as reflected by the significantly higher serum ALT and amount of fibrosis (scored according to the METAVIR scale).

Table 3. Phenotypic features by ferroportin functional gene variant.

Phenotypic Features	Gain-of-Function (n = 110)	Loss-of-Function (n = 216)	p
Age (years) median (range)	n = 93 46 (2–80)	n = 203 36 (6–87)	0.032 ^{&} 0.055 ^{&&}
Gender (n)			
Female	35	82	
Male	66	126	ns
Hb (g/dL) median (range)	n = 39 14.4 (9.5–16.5)	n = 104 14.4 (10.1–18.4)	ns
Anemia			
Yes	4	14	
No	35	90	ns
MCV (fl) median (range)	n = 29 93 (70–108)	n = 50 91 (73–98)	ns
Serum iron (µmol/L) median (range)	n = 45 36.0 (8.0–74.0)	n = 51 15.7 (6.2–94.0)	0.0002 ^{&} 0.00017 ^{&&}
TSAT (%) median (range)	n = 105 62 (2–104)	n = 174 32 (5–99)	< 0.0001 ^{&} < 0.0001 ^{&&}
Ferritin (µg/L) median (range)	n = 100 755 (4–15000)	n = 208 1595 (24–21665)	< 0.0001 ^{&} 0.0013 ^{&&}
Iron Removed (g) median (range)	n = 10 10.2 (2–24.4)	n = 31 8.0 (1.6–80.0)	ns
Tolerance to Phlebotomy (n)			
Good	16	38	
Poor	2	13	ns

Table 3. Cont.

Phenotypic Features	Gain-of-Function (n = 110)	Loss-of-Function (n = 216)	p
HIC($\mu\text{g/g}$) median (range)	n = 19 11718 (925–38665)	n = 53 10052 (307–58590)	ns
Grade of fibrosis			
0–2	14	20	
3–4	10	2	0.012
Hepatic Iron distribution			
Hepatic (predominant)	18	7	< 0.00001 [@]
Macrophagic (predominant)	0	28	
Mixed	10	16	< 0.00001 ^{@@}
ALT (IU/L) median (range)	n = 31 52.0 (14.0–304.0)	n = 48 29.5 (6.0–98.0)	0.0026 ^{&} 0.004 ^{&&}

HIC, hepatic iron content; ALT, alanine aminotransferase. [&]Mann–Whitney U-test, ^{&&}Student's t-test of independent means, p is significant < 0.0033 when corrected for multiple testing according to Bonferroni. [@] difference between hepatic and macrophagic, ^{@@} difference between hepatic, macrophagic, and mixed.

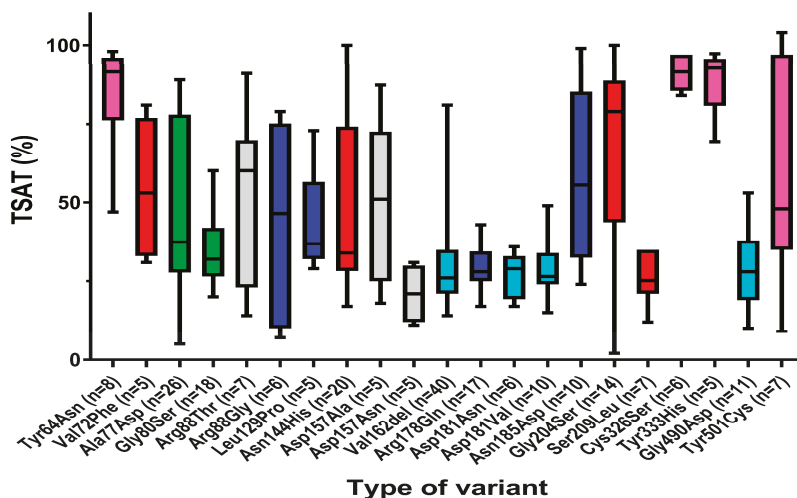


Figure 1. Box plots (Whiskers: min to max) of transferrin saturation (TSAT) in individuals with ferroportin variants. Only the variants with at least five reported TSAT levels in patients are included. Red: GOF without or conflicting data about hepcidin sensitivity; Pink: GOF hepcidin-resistant variants; Green: LOF without or conflicting data about hepcidin sensitivity; Light blue: LOF hepcidin-resistant variants; Blue: LOF hepcidin-sensitive variants; Gray: No data available.

Long-term follow-up information and data to determine a difference in clinical course between GOF and LOF variant patients are limited [12,106]. We found three documented deaths related to ferroportin disease: fatal hepatocellular carcinoma in one GOF and one LOF variant patient [48,82]. One patient with a GOF mutation died of multiorgan failure due to widespread iron deposition [50].

3.2.2. Strong Association of Hepcidin Sensitivity of Ferroportin Functional Gene Variants with Serum Iron Parameters

In vitro hepcidin sensitivity studies were performed in 45 variants. We identified seven hepcidin-resistant and seven hepcidin-sensitive GOF variants, and five hepcidin-resistant and four

hepcidin-sensitive LOF variants (Table 2). Twelve LOF and 13 GOF variants were designated as uncertain/conflicting/unknown, including those variants with partial resistance or sensitivity.

Patients with hepcidin-resistant LOF variants had significantly lower TSAT (27% versus 35%) and serum iron (14.1 $\mu\text{mol/L}$ versus 28.7 $\mu\text{mol/L}$) but higher ferritin (1810 $\mu\text{g/L}$ versus 1066 $\mu\text{g/L}$) than patients with a hepcidin-sensitive LOF variant (Table S4, Figure 2).

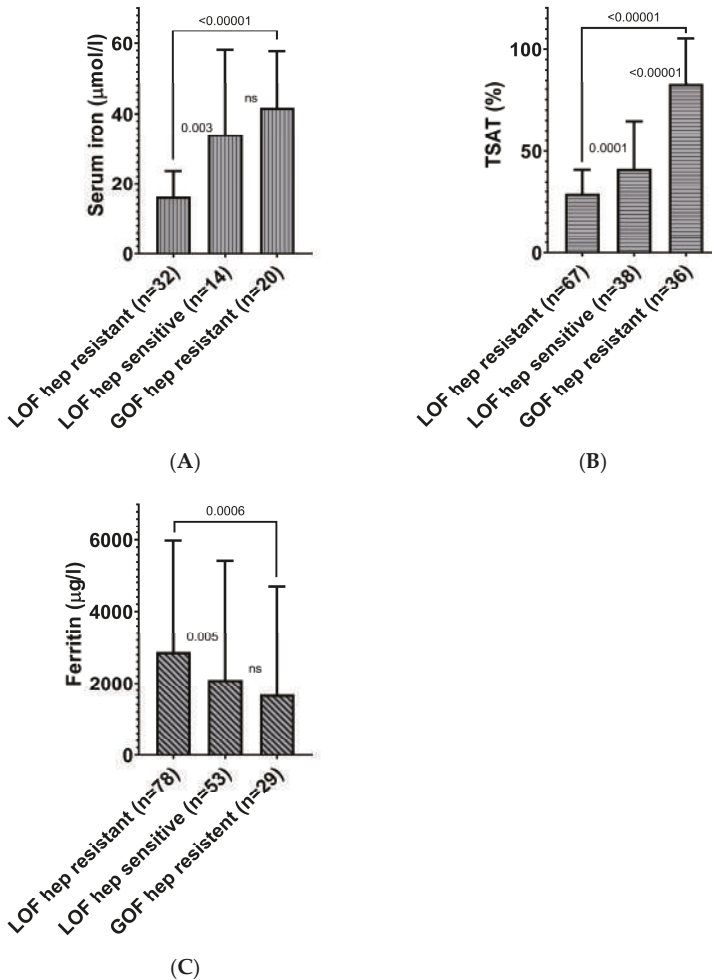


Figure 2. Iron parameters for the different ferroportin variants. Mean (+ SD) of (A) serum iron, (B) transferrin saturation, and (C) ferritin as measured in patient samples for the function of variant (LOF = loss-of-function, GOF = gain-of-function) and the effect of hepcidin (hep resistant = hepcidin-resistant, hep sensitive = hepcidin-sensitive) on the iron transport capacity, as assessed by in vitro functional tests. TSAT = transferrin saturation. The Student’s t-test was applied to determine the difference between serum iron and TSAT, while the Mann–Whitney U-test was applied for ferritin. Displayed p values, serum iron, and TSAT are according to the Student’s t-test. Displayed p values ferritin according to the Mann–Whitney U-test.

However, patients with hepcidin-resistant GOF variants had highly significant higher serum iron (44.0 $\mu\text{mol/L}$ versus 14.0 $\mu\text{mol/L}$) and TSAT (92% versus 28%) and lower ferritin (642 $\mu\text{g/L}$ versus

1810 µg/L) concentration than patients with hepcidin-resistant LOF variants (Table S5, Figure 2). All seven patients with a hepatocyte predominant iron deposition carried a hepcidin-resistant GOF variant, while all nine patients with a macrophage predominant iron deposition had a hepcidin-resistant LOF variant.

3.3. Therapeutic Considerations

In patients with primary iron overload, phlebotomy is the cornerstone of treatment. In 93 of 102 patients who were reported to be phlebotomized, the tolerance of the phlebotomy was reported. A total of 73 patients tolerated phlebotomy without side effects. In 17 of 20 patients with poor tolerance to phlebotomy, the cause of the intolerance could be analyzed. In 12 of the 14 patients with intolerance to phlebotomy due to the development of early anemia, phlebotomy could be continued by the application of a less intensive phlebotomy schedule. In one patient, anemia subsided despite the continuation of the same regimen. In three additional patients, the administration of erythropoietin resulted in an increase of Hb and continuation of the phlebotomy regimen [59,107]. In two patients, phlebotomy was discontinued because of the occurrence of anemia. Treatment with deferasirox resulted in a dramatic decrease in serum ferritin concentration in two patients, but was not successful in another [52,76,96].

In contrast to the current consensus [1,2,4,6,8,40,71,83,92,105], patients with LOF variants were not less tolerant to phlebotomy than patients with GOF variants (Table 3). Nevertheless, in five of 22 (23%) patients with hepcidin-resistant LOF variants the reported phlebotomy was poorly tolerated (Table S5) due to early anemia.

4. Critical Annotations on Pathogenicity of Variants

In Table 4, we depicted the potential pathogenicity of the various variants derived from available clinical, epidemiological, and genetic data from the original reports. We scored the likelihood of the pathogenicity of a variant based on the available data from the genome aggregation data base (gnomAD), data derived from the functional tests, at the molecular level in context of the current knowledge of the function and structure of the ferroportin molecule, and based on three in silico predicting models.

Table 4. Likelihood of pathogenicity derived from the reported data, and current databases and prediction models.

Variant	Pheno-Type	Co-Segregation	VO/Controls	gnomAD	Functional	Molecular	In silico Prediction		
							Poly-phen2	SIFT	Align-GVGD
Tyr64Asn	+++	+++	0/100		++++	++	++	0	0
Tyr64His	+++ ^κ					++	++	0	0
Ala69Thr	+++ ^κ	0			++	0	++	+	0
Val72Phe	+	+			+++	0	++	0	0
Ala77Asp	+++	++, +++#	0/100		+++	0	++	+	0
Gly80Ser	++		0/734		+++	+	++	+	0
Gly80Val	++		0/100		++	+	++	+	0
Asp84Glu	+++ ^κ				++	+	++	+	0
Arg88Gly	+++		0/734		++	+	++	+	0
Arg88Thr	++	+++	0/60		++	+	++	+	0
Leu129Pro	0	+			++++	0	++	+	+++
Asn144Asp	+++				+++	+	++	0	0
Asn144His	0	+++	0/200		+++	+	+	0	0
Asn144Thr	0 ^κ		0/100		+++	+	++	0	0
Ile152Phe	+++	+			+++	+	++	0	0
Asp157Ala	++	++			++	+	++	+	0
Asp157Asn	++	0			++	+	++	0	0
Asp157Gly	+++		0/80, 0/734		+++	+	+	0	0
Asp157Tyr	+++		0/734		+	+	++	+	+
Trp158Cys	+++	0,+	0/50		+++	0	++	+	++
Trp158Leu	+++				+++	0	+	0	0
Val162del	++	+, +++	0/100, 0/103, 0/734		++++	0	@	@	@

Table 4. Cont.

Variant	Pheno-Type	Co-Segregation	VO/Controls	gnomAD	Functional	Molecular	In silico Prediction		
							Poly-phen2	SIFT	Align-GVGD
Asn174Ile	+++				+++	+	++	+	+++
Arg178Gln	+++	0-+++##			++	+++	++	0	0
Ile180Thr	0	0	0/100	7.076 ⁻⁵	++	0	++	+	+++
Asp181Asn	++				++	+	++	+	+
Asp181Val	++	+,++	0/100, 0/734		++	+	++	+	+++
Gln182His	+++		0/80		+++	0	++	+	+
Asn185Asp	+++	+,+++	0/50		+	0	++	+	+
Gly204Arg	+++&				++	0	++	+	+++
Gly204Ser	+	+++	2/100	7.955 ⁻⁶	+++	0	++	0	0
Ser209Leu	0	++		1.485 ⁻⁴	+++	0	0	0	+
Thr230Asn	0&		0/734	1.096 ⁻⁴	++	0	+	0	0
Ala232Asp	0			4.95 ⁻⁵		0	+	0	+
Leu233Pro	+++	0	0/734		++	0	++	+	+++
Lys240Glu	+++&		0/50			0	0	+	0
Met266Thr	0&		0/734		++	0	0	+	0
Gly267Asp	+++&		0/100	4.382 ⁻⁵		0	+	0	0
Asp270Val	+		4/100, 1/516	7.09 ⁻⁵	+++	0	0	0	0
Arg296Gln	0&				++	0	+	0	++
Gly323Val	+++&		0/80		+++	0	++	+	+++
Cys326Phe	+++&	0				++	++	0	+
Cys326Ser	+++	+++			++++	+++	+	0	0
Cys326Tyr	+++		0/800		++++	++	+	0	0
Tyr333His	+++		0/40		++	+	++	+	+++
Ser338Arg	+++&				+++	0	0	0	0
Leu345Phe	0&		0/734		++	0	++	0	0
Ile351Val	0&		0/734	2.125 ⁻⁵	++	0	0	0	0
Arg371Gln	0&			2.388 ⁻⁵	+++	0	0	0	0
Pro443Leu	0&		0/734	5.87 ⁻⁴	++	0	0	0	0
Gly468Ser	++	0		4.005 ⁻⁶		0	++	0	+
Arg489Lys	+++	+++	0/50		+	+	++	+	+++
Arg489Ser	+		0/734		+	+	++	+	+++
Gly490Asp	+++	0	0/734		++++	0	++	0	0
Gly490Ser	+++		0/734		+	0	++	0	0
Tyr501Cys	+		0/200	3.184 ⁻⁵	++++	+++	+	0	+
Asp504Asn	+++		0/734		++++	+++	++	0	0
His507Arg	+++	+	0/50		++++	++	++	0	0
Val511Ile	+++&					0	++	+	++
Arg561Gly	+		0/734	1.638 ⁻³	++	0	0	0	0

Open space: no data available. **Phenotype**: bold: at least five patients; 0 = < 50%, + = 51–80%, ++ = 81–99%, +++ = 100% presence of an elevated TSAT in GOF patients and elevated ferritin in LOF patients. Variants in italic are unclassified; + = elevated TSAT and/or ferritin (but not in all patients), ++ = elevated TSAT or ferritin in all patients, +++ = elevated TSAT and ferritin in all patients. & only one reported patient. # enumeration of a limited number of studies; ## range in multiple studies. **Co-segregation** from pedigrees in the original reports using the simplified method of segregation analysis (SISA) according to the following scoring system: single family: 0 = no evidence, + = ≤ 1/8 (supporting), ++ = ≤ 1/16 (moderate), +++ = ≤ 1/32 (strong); bold: multiple family studies: 0 = no evidence, + = ≤ 1/4 (supporting), ++ = ≤ 1/8 (moderate), +++ = ≤ 1/16 (strong); all according to the recommendations of Jarvik et al. [22] to define co-segregation as criteria to fit the ACMG-AMP guidelines [24]. **VO/Controls**: Variant Occurrence in controls reported in the original reports. **GnomAD**: allele frequency derived from GnomAD data base [25]; the variants for which no allele frequency are given are absent in the database and thus extremely rare. **Functional**: + = effect of hepcidin not performed, ++ = effect of hepcidin established only on membrane expression or iron transport, +++ = conflicting or unreliable results on the effect of hepcidin, with reported fate of hepcidin, ++++ = established effect of hepcidin on membrane expression and iron export with/without reported fate of hepcidin; Bold = ≥ 3 independent studies. **Molecular**: 0 = variant present on a site without established role in ferroportin molecule, + = variant present at a site potentially involved in the formation of the intracellular and extracellular gate, internalization and degradation of the ferroportin molecule or at a site potentially involved in iron binding or egress, ++ = variant present at a site with established function in hepcidin binding, +++ = variant present at a site leading to established conformational changes in the structure of the ferroportin molecule as predicted in the available three-dimensional (3D) models. **In silico prediction**: Polyphen 2: 0 = benign, + = possibly damaging, ++ = probably damaging; “Sorts intolerant from Intolerant” (SIFT): 0 = tolerated, + = deleterious; Align-GVGD: 0 = class 0, + = Class 15, ++ = Class 25 and 35, +++ = Class 55 and 65; @ = It is not possible to test deletions in Polyphen2, SIFT, and Align-GVGD.

4.1. Shortcomings in Description of Clinical Phenotypes

The collected data to describe the clinical phenotype were derived from extensively documented family studies to isolated patients with minimal reported clinical data as part of national surveys of patients with iron overload. So, the available clinical, biochemical, radiological, and histological data

were the most heterogenous. In addition, in family studies, relatives were more than 10 years younger with a milder phenotype, including 11% with normal iron parameters. In many patients, a genetic diagnosis was made after referral, and reported iron parameters may potentially have been affected by previous phlebotomies. The demonstrated lack of effect on the presence of additional acquired iron-modulating conditions must be interpreted with caution in view of insufficient reported data. The presence of polymorphisms in ferroportin may affect the degree of iron overload in homozygotic patients for p.Cys282Tyr variants in the HFE molecule [13]. The lack of effect of presence of HFE variants on the iron parameters in our analysis is likely to be explained by the absence of patients with homozygotic p.Cys282Tyr HFE variants.

The phenotype of a number of reported variants raised doubt on the pathogenicity of these variants. An elevated TSAT is considered to be the hallmark of GOF variants. In 41 of 116 GOF patients, TSAT was normal. Since 13 of them also had a ferritin level ≤ 200 $\mu\text{g/L}$, 11% of GOF patients had normal serum iron parameters, especially patients with Asn144His (8/20 pts) and Val72Phe (2/5 pts) variants. Also, 7/9 reported patients with the seven hepcidin-sensitive GOF variants had a normal TSAT. The Gly204Ser GOF variant displayed evident pathologic iron parameters in a homozygous patient, with normal iron parameters in the two heterozygous relatives, and four of 14 additional reported patients had normal TSAT. Six of 197 LOF patients had normal reported ferritin level including 3/5 Leu129Pro variant patients. In a 10-year follow-up study of a proband and his father with a Arg489Ser variant, the serum iron spontaneously decreased with a slight reduction in the MRI estimated HIC [12].

4.2. Shortcomings in Establishment of Pathogenicity

4.2.1. Co-Segregation with Disease in the Family

SISA was applicable for 26 variants and hampered by the small pedigrees in most reports. In 13 variants, there was no or only supporting evidence for pathogenicity; in nine variants, evidence was moderate or strong, and for four variants, the score for pathogenicity was inconclusive (Table 4).

4.2.2. Variant Occurrence and Allele Frequency

In the original reports, variant occurrence in healthy controls was determined for 37 variants in relatively small cohorts (range 40 to 734 persons) with an established occurrence of $< 1/100$ for 26 variants. In the genome aggregation data base (gnomAD, [25]), 213 missense variants were observed with subsequent determination of the allele frequency. Of these, only 13 variants displayed clinical symptoms to be reported and analyzed in the current review. Notably, 47/60 variants in our analysis are so rare that the allele frequency is not depicted in gnomAD.

4.2.3. Limitations of Functional In Vitro Tests

In most of the transfection studies HEK293T, a human embryonic kidney cell line, was used [6,8,11,31–33,35,39–42,46,55–57,83,87,93] and occasionally *Xenopus* oocytes, colon carcinoma CaCo-2, or hepatoma HuH7 cell lines [6,57,66]. Protocols are not standardized. Iron loading was done with 1 to 2 mg/mL of holotransferrin [8,41], 1 to 4 mg/mL of ^{55}Fe [8,31,33,35,39,46], 20 to 40 $\mu\text{g/mL}$ of ^{59}Fe bound to transferrin [31,40,42], nitrilotriacetate [46] or ascorbate [66], or 10 to 100 μM of ferric ammonium citrate [6,32,55,57,83,93] for 24 h before to 48 h [8,31–33,39,40,42,46,55,66,83,87] after transfection. Cells were lysed 4 to 72 h after iron loading [8,31,33,46,56]. Iron export capacity was determined either by the measurement of radioactive Fe [8,31,33,35,39,40,42,46,56,66] or ferritin concentration [6,8,11,32,33,35,41,42,55,57,83,87,93] in lysed cells [6,32,33,35,40,42,57,93] or in the supernatant [8,11,31,33,35,39,46,56,83]. Results obtained in cells transfected with the variant ferroportin were compared with results in untransfected cells and in cells transfected with wild-type ferroportin. The effect of hepcidin on iron export capacity and the expression of the variant ferroportin protein were analyzed. Experimental conditions are also not standardized; specific differences include: (i) applied hepcidin concentration, which varied between 0.01 and 10 μM , and (ii) incubation times,

which varied between 3 and 56 h [11,31–34,39,41,42,55,56,83,87,93]. The membrane expression of variant ferroportin proteins was determined by surface biotinylation and Western blotting [8,39], immunofluorescence [6,31–33,40,42,46,55–57,65,87] and confocal microscopy [11,34,41,83], or flow cytometry [31,35,41,42,56]. These differences may be responsible for the variable results reported on the effect of hepcidin on the membrane expression and iron export capacity of cells transfected with WT ferroportin.

In a significant number of the tested variants, the effect of hepcidin was determined by means of ferroportin expression and not by changes in iron export. Furthermore, in the vast majority of the transfected LOF variants, the membrane expression of the protein was strongly reduced or even absent, and therefore the effect of hepcidin is difficult to determine reliably. In a number of variants, the inhibitory effect of hepcidin was tested at various concentrations of hepcidin and/or exposure times, which resulted in a time and/or concentration dependent effect, ranging from hepcidin resistant to more or less hepcidin sensitive [6,30,42,65]. Therefore it is not unthinkable that the same variant is considered hepcidin resistant by one investigator and hepcidin sensitive by the other, depending on the design of the applied assay.

So, large inter-assay variation may not only lead to conflicting results in ferroportin variants tested in multiple studies, but also to reluctance to draw firm conclusions for data obtained in a single study. In addition, our definition of LOF (i.e. a significant reduced iron transport in functional tests) and hepcidin sensitivity (i.e. a significant reduction in iron transport or ferroportin expression in functional assays upon the addition of hepcidin) may lead to discrepancies in the interpretation of the functional characterization of some individual variants such as Gly204Arg and Arg296Gln, between us and the authors of the original publication [51].

4.2.4. Lack of Concordance between Various In Silico Prediction Models

As illustrated in Table 4, the concordance between the three methods is poor, with a weak albeit significant correlation between SIFT and Align-GVGD ($r = 0.5441$), polyphen2 and SIFT ($r = 0.3832$), and polyphen2 and Align-GVGD ($r = 0.3066$).

4.3. Assessment of Pathogenicity of Ferroportin Variants on Molecular Level in Context of Current Knowledge on the Function and Structure of Ferroportin

4.3.1. Ferroportin, the Protein, Its Variants, and Function: Current Knowledge

Ferroportin is a 571 amino acid cation transporter of the major facilitator superfamily (MFS) encoded by the *SLC40A1* gene [46,108–111]. It is the only known cellular iron exporter, and primarily expressed in the basolateral membrane of duodenal enterocytes, macrophages, and hepatocytes [108,110–112]. In the most widely accepted secondary structure, ferroportin comprises 12 helices located in 12 transmembrane (TM) domains bound via six extracellular (ES) and five intracellular (IS) segments with a large intracellular loop between the sixth and seventh transmembrane helix and intracellularly located N and C terminus [6,8,34,46,56,113]. Although the amount of polymerization is still a matter of debate, most investigators assume that ferroportin is expressed on the membrane as a dimer [6,34,35,55,114]. Available three-dimensional models are based on comparison with ferroportin from other species with only 10–24% sequence homology and 40% similarity [8,39,46,115]. Recent studies reveal an open-inward and an open-outward structure with an intracellular and extracellular gate between the sixth and seventh transmembrane domain. Extrapolating the experimental model to the human protein reveals that residues Asp84, Arg88, Asp157, Asn174, Gln481, Glu486, and Arg489, which are located at IS1, IS2 and IS5, are important in intracellular gate interaction, while the residues Phe44, Val48, Val 51, Leu58, Asp325, Thr329, Leu342, and Phe520, which are located at TM1, TM12, ES1 and ES4, are involved in extracellular gate interaction (Figure 3) [46,115]. It is postulated that intracellular iron is transported outside the cell, and that

extracellular hepcidin is transported inside the cell through this gate via a so called “rocker-switch” mechanism [46,115,116].

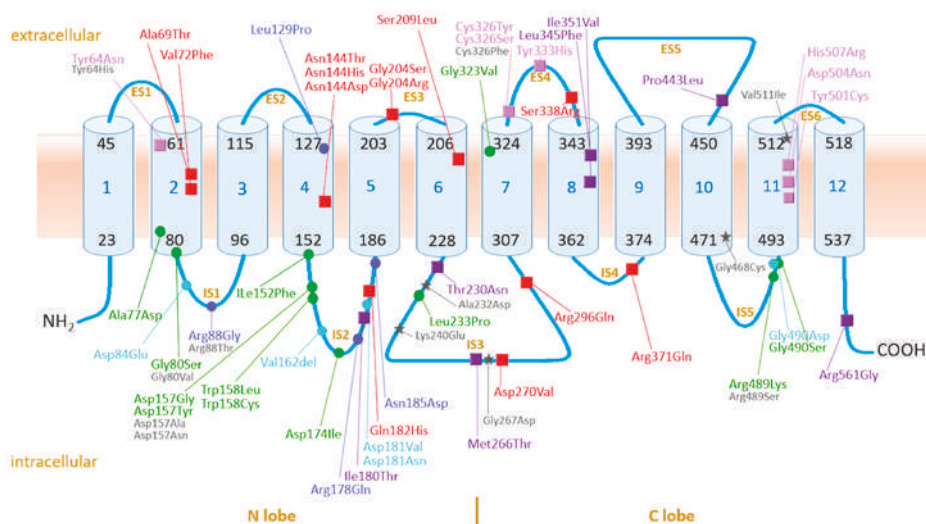


Figure 3. The two-dimensional (2D) structure of ferroportin protein adapted from Liu and Wallace [6,56], revealing 12 transmembrane helices and six extracellular and five intracellular segments. All the variants described in this review are shown as hepcidin-resistant (pink), hepcidin-sensitive or neutral (purple) and hepcidin conflicting/uncertain/unknown (red) GOF variants (squares). and hepcidin resistant (light blue), hepcidin sensitive (dark blue), and hepcidin conflicting-uncertain-unknown (green) LOF variants (dots), or non-classified variants (grey asterisk).

Since sites containing an Asp, Glu, His, or Tyr residue are reported to be involved in iron binding, Asp39, Glu52, Tyr54, Asp181, Tyr318, Asp325, Tyr331, Tyr501, and Glu518 are potential iron binding sites and Arg88, Asn157, Asn174, and Arg489 are involved in the intracellular gate interaction [46,115]. Functional studies revealed cation binding at Ser35, Asp39, Asn212, and Ser215 and site mutagenesis studies suggest that Asp39 and Asp181 are essential iron binding sites [46,115]. The residues Arg88, Ile152, and Asn174 are thought to be directly involved in iron egress [39].

Ferroportin-mediated cellular iron export is systemically regulated by the hepatocyte-derived peptide hormone hepcidin. The way hepcidin binding inhibits iron efflux is not fully elucidated [117–119]. Functional studies reveal the internalization of ferroportin upon hepcidin binding with subsequent endosomal and lysosomal degradation resulting in the diminution of cellular iron export, while three-dimensional (3D) structural studies suggest that hepcidin binding disrupts the conformational transition into the intracellular gate with the subsequent inhibition of the access of cytoplasmic iron to the substrate binding-sites [115,120,121]. Site mutagenesis studies and the exploration of various three dimensional models suggests that the Phe324, Cys326, Tyr333, Asp504, and His507 residues are important binding and docking sites for hepcidin [32,39,46,115]. Recent structural models indicate that these hepcidin binding residues are clustered in the extracellular gate in the open-outward structure. Residues found to be involved in internalization include Tyr64, Gly80, and Asn144, and the residues Lys229, Lys253, and Lys269 are present in the large intracellular loop, and seem to be involved in the degradation of ferroportin [39,114,122].

In an effort to unravel the pathogenicity of ferroportin variants, we depicted the various variants in the structure of ferroportin molecule. For the two-dimensional (2D) model (Figure 3), we adapted the structure constructed by Liu and Wallace [6,56] and for three-dimensional (3D) model (Figure 4), we used

the recently developed model (Bacterial ferroportin, PDB file 5AYN) and homology modeling in the Yet Another Scientific Artificial Reality Application (YASARA) [123] and WHAT IF Twinset [124] programs.

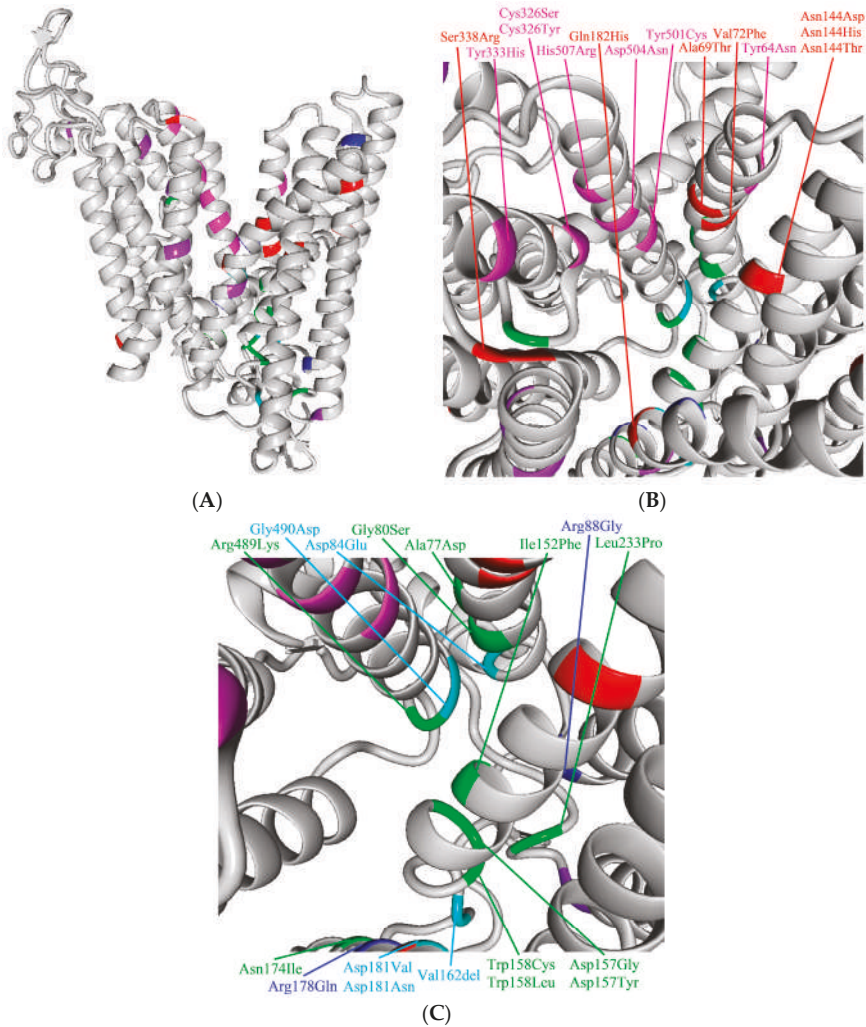


Figure 4. 3D model of ferroportin. (A). Overview. Ferroportin facing outward position. Top: extracellular; Bottom: intracellular. (B). Localization of hepcidin-resistant GOF variants. View inside the "channel" from extracellular to intracellular. Six (Cys326Tyr, Cys326Ser, Tyr333His, Tyr501Cys, Asp504Asn, and His507Arg) of the seven residues of GOF hepcidin-resistant variants are located inside the channel. The Ala69, Val72, Asn144, Gln182, and Ser338 residues are also located inside the channel. However, these variants have conflicting or no data on hepcidin sensitivity. (C). Clustering of LOF variants at the intracellular side of ferroportin. View inside the channel from extracellular to intracellular. Red: GOF without or conflicting data about hepcidin sensitivity; Pink: GOF hepcidin-resistant variants; Purple: GOF hepcidin-sensitive or neutral variants; Green: LOF without or conflicting data about hepcidin sensitivity; Light blue: LOF hepcidin-resistant variants; Blue: LOF hepcidin-sensitive variants. We used bacterial ferroportin for the 3D model (PDB file 5AYN).

4.3.2. GOF Variants

We identified Tyr64Asn, Cys326Tyr, Cys326Ser, Tyr501Cys, Asp504Asn, and His507Arg as definitely hepcidin-resistant GOF variants, and a co-culture of hepcidin with cells transfected with these variants does not lead to the internalization of the ferroportin variant with maintained iron export capacity and variant membrane expression [8,11,31–35,39–42,46]. Consequently, there is increased iron export from enterocytes and macrophages leading to hepatocellular iron overload. Observations in mice with the Cys326Ser variant reveal that increased dietary iron absorption is maintained by the upregulation of the Duodenal cytochrome b (Dcytb) and Divalent Metal Transporter 1 (DMT1) at the apical site of the enterocyte, which is likely to compensate for the pending intraenterocytic iron depletion [125]. For Cys326Tyr, Cys326Ser, Tyr501Cys and Asp504Asn, the blocked internalization of ferroportin on hepcidin exposure has been attributed to the impaired binding of hepcidin to ferroportin in the extracellular gate, while for Tyr64Asn and His507Arg—of which the location in relation to this gate varies between the available models—the impaired ubiquitination of hepcidin, which was most likely due to interference with conformation, is the most likely mechanism (Figure 4B) [30]. The Tyr333Ala, Tyr333His (both hepcidin-resistant variants in a functional test) and Tyr333Phe (hepcidin-sensitive) GOF variants display both membrane localization and intact iron export, but impaired ubiquitination after normal hepcidin binding; however, there are no data on configurational changes due to presence of these variants [30,45].

The Ile180Thr, Thr230Asn, Met266Thr, Leu345Phe, Ile351Val, Pro443Leu, and Arg561Gly were designated as hepcidin-sensitive GOF variants. In functional studies, the iron export capacity, hepcidin uptake, and hepcidin-induced reduction of the cell surface expression of these variants were comparable with WT ferroportin [8,11,31,35]. None of these variants are located at residues that are known to be involved in hepcidin binding, ferroportin ubiquitination, or iron binding/egress. Notwithstanding that these seven variants behaved as WT ferroportin in functional studies and are located at residues without an established functional role, the reported 16 patients had otherwise unexplained mild iron overload, which was reflected by a median TSAT of 42.5% and serum ferritin concentration of 980 µg/l. Nevertheless, the pathogenicity of the variants may be questioned, and therefore, the variants may as well be classified as neutral [8].

4.3.3. LOF Variants

Except for Leu129 and Gly323, the residues of the alterations that are associated with LOF variants are located at the intracellular site of the molecule, especially in the IS1, IS2, and IS5 (Figure 3) structures, which are involved in the formation of the intracellular gate responsible for iron transport (Figure 4C) [115]. In transfected cells, the majority of LOF variants are reported to be located intracellularly or have reduced membrane expression, although the data are not consistent for some variants (Table 2). Alteration in these residues may lead to conformational changes due to improper folding with mislocalization and disrupted intracellular gate formation. The substitution of Arg by Gly at residue 88 may lead to direct impaired iron handling with disrupted iron egress. That four hepcidin-sensitive variants display more or less intact membrane expression suggests intact extracellular gate formation enabling hepcidin binding. It was recently demonstrated that the hepcidin-sensitive LOF variant Arg178Gln, which is properly localized on the cellular membrane with disappearance after hepcidin exposure, is likely to form a non-covalent interaction between Arg178 and Asp473, which are located on the N and C lobe, respectively. This interaction may lead to a loss of stabilization of the open-outward conformation that is needed to preserve iron egress [84]. Residues of the four hepcidin-resistant LOF variants are not involved in hepcidin binding, so hepcidin resistance is likely to result from conformational changes in the extracellular gate, even though these variants have demonstrable but reduced membrane expression.

Overall, we conclude from that for most variants, clinical, functional, molecular, and in silico assessments of pathogenicity are not fully concordant, complicating the assessment of their likelihood of being the disease-causing variant (Table 4). For instance, the hepcidin-resistant GOF variants

Tyr64Asn Cys326Ser, Cys326Tyr, Asp504Asn, and His507Arg are benign in two of the predicting in silico models, while clearly damaging variants in the three applied prediction models such as Leu129Pro and Ile180Thr were clinically likely to be benign. On the other hand, six out of the seven hepcidin-sensitive GOF variants had a low likelihood for pathogenicity both on clinical and molecular ground as well as in the predicting models.

4.4. Is the Mode of Iron Overload in LOF Variants Related to a Difference in Ferroportin Expression and Activation between Enterocytes and Macrophages?

Despite clear differences in iron distribution between patients with GOF and LOF variants, hepatic iron content and iron that is needed to be removed by phlebotomy to obtain a normalized iron phenotype are not found to differ, which might suggest that the total amount of absorbed iron in GOF and LOF patients is similar. Therefore, we hypothesize that in enterocytes, all the pathogenic ferroportin variants are associated with increased iron transport, irrespective of the (in vitro) function of the variant, while iron transport in macrophages and thereby the mode of systemic iron distribution is dependent on variant function. For patients with LOF variants, this hypothesis includes iron export being relatively decreased in macrophages, and being increased in enterocytes.

Ferroportin knock-out mice are not viable [110]. Mice with mono-allelic expression of wild-type (WT) ferroportin [111] and *TMPRSS6* knock-out mice with subsequent reduced ferroportin expression on enterocytic membrane, develop iron deficiency anemia with intra-enterocytic iron retention [126] and patients with *TMPRSS6* variants develop Iron Refractory Iron Deficiency Anemia (IRIDA) due to impaired intestinal iron absorption [127–129]. In contrast, patients heterozygous for LOF variants, which fail to export iron in functional tests, do not develop iron deficiency anemia; instead, they develop systemic, predominantly reticuloendothelial iron overload with limited but consistent proof for iron depletion in the enterocytes [79,88].

It has been recently suggested that differences in amount of intracellular iron turnover between enterocytes and macrophages may influence iron export capacity of these cells in patients with ferroportin disease due to LOF variants [130]. The monoallelic-expressed WT ferroportin protein would be sufficient to preserve iron export in cells with low iron turnover, such as enterocytes, but insufficient to maintain iron export capacity in cells with high iron turnover, such as splenic and hepatic macrophages, with subsequent intracellular iron retention in these cells. However, this hypothesis is insufficient to explain the state of iron overload. Functional consequences of a variant leading to the designation LOF is foremost established in HEK293T, which is a human embryonic kidney cell line, and are in accordance with events as observed in macrophages but fail to elucidate events that must be present in the enterocytes. So, HEK293T cells might not be the most appropriate experimental cell line to predict the functional consequences of at least LOF variants.

Experimental data provide evidence for differences in ferroportin expression and the activation between enterocytes and macrophages. Ferroportin is expressed on the enterocytic basolateral membrane and predominantly in intramacrophagic vesicles [108,131], and there are indications that the mode of hepcidin-induced internalization differs in macrophages [120,132].

Intracellular transcription and the translation of *SLC40A1* is primarily regulated by iron and oxygen in enterocytes and heme and iron in macrophages, [113,131,133]; also, the modulatory capacity by the iron-regulatory protein-iron-responsive element (IRP-IRE) system may differ between both cell types [134–138]. After iron egress from enterocytes, ferrous iron is oxidized by membrane-bound hephaestin, co-located with ferroportin, while ferrous iron release from macrophages is oxidized by circulating ceruloplasmin [139,140].

Ferroportin expression differs between enterocytes and macrophages in response to systemic regulatory stimuli such as erythropoietin and hepcidin, changes in iron state, and inflammation (hepcidin independent) in experimental animals, as well as in isolated cell cultures [132,141–152].

Taken together, these experimental data suggest a regulation difference of ferroportin production and activation, between enterocytes and macrophages [153]. However, the extrapolation of results obtained from experimental models to physiology must be done with caution.

5. Conclusions

We analyzed 60 low-frequency missense variants in the coding sequence of the ferroportin gene reported over the past 20 years, which has been assumed to be responsible for iron overload in individual patients reported in the English literature with or without subsequent evaluation of the relatives. The clinical and laboratory features of the patients (phenotype) with these ferroportin variants were correlated with data on variant properties in functional *in vitro* tests, which were available for 80% of the variants. However, the reported clinical features are often incomplete and functional tests are artificial, not standardized, or unvalidated, which impedes the interpretation of the correlation between the functional properties and phenotype. The establishment of variant pathogenicity is hampered by a lack of sufficiently reported data regarding the presence of iron-modifying confounders and because the outcomes of available *in silico* prediction models is limited in the absence of a fully elucidated structure and function. Therefore, it is likely that not all reported variants are indeed pathogenic, but from the available data, it is nearly impossible to accurately establish the pathogenicity of individual variants. Notwithstanding these limitations, we can conclude that ferroportin disease is a heterogeneous iron overload disorder. Clinical symptoms are non-specific, and mostly consist of fatigue and arthralgia. At presentation, patients are generally middle aged with strongly elevated ferritin concentrations and mildly elevated TSAT and iron concentrations. Iron parameters and the severity of iron overload show considerable inter-individual variation and are also related to gender, age, and type of variant. The mode of organ iron distribution is primarily determined by the effect of ferroportin variants on macrophage iron export, which is comparable with that observed in functional *in vitro* studies. LOF variants are associated with macrophage iron retention with a high serum ferritin and low to normal TSAT, in contrast to GOF variants, which are associated with high TSAT and hepatocellular iron deposition. These distinct phenotypes are typically present in patients with hepcidin-resistant variants. Patients with GOF variants had a higher prevalence and higher grade of hepatic fibrosis, indicating that parenchymal iron deposition is more toxic than macrophage iron overload. This corroborates the notion that high TSAT facilitates parenchymal iron deposition, which is considered to be more toxic than macrophage iron overload [154]. On the other hand, with regard to toxicity in macrophages, it has been stated that macrophage iron overload is resistant to iron withdrawal, and that iron-loaded Kupffer cells may contribute to the development of hepatic fibrosis and even carcinogenesis [106].

Since iron distribution patterns and clinical features of patients with hepcidin-resistant GOF variants are indistinguishable from patients with other types of HH, we support the proposition to categorize these variants as ferroportin-associated HH, and to confine the entity ferroportin disease to patients with LOF variants [116]. For both entities, repeated phlebotomies to normalize the iron parameters in order to prevent organ damage remain the mainstay of treatment. Although we found no increased intolerance to phlebotomy in patients with LOF variants, we advocate for the regular determination of Hb levels during the phlebotomy program, especially in patients with hepcidin-resistant LOF variants, to avoid the occurrence of early anemia.

The pathophysiology, especially of the LOF variants, is only partially understood. The iron handling within the various human cell types is not fully clarified, and the mechanisms by which ferroportin transports iron and is degraded are still not fully elucidated. To improve patient management, there is an unmet need for a better understanding of yet unresolved issues on the pathophysiology. A global registry, with a standardized diagnostic work-up and evaluation of the clinical, biochemical, radiological, and histological features of the liver and spleen, is needed, such as the registry sponsored by the European Association for the Study of the Liver (EASL) (<http://non-hfe.com/>) [155], a collaboration between various European Expert centers, or registries launched by the European Reference Network (ERN) of rare Hematological Disorders

(EUROBLOODNET). Also, the histologic and molecular examination of enterocytes in duodenal biopsies of these patients by assessing iron content, as well as the expression of iron storage and transport proteins, will add to a better understanding of the iron handling. The secondary and tertiary structure of ferroportin as well as the identification of residues that are involved in hepcidin binding, ferroportin ubiquitination, and iron transport need to be further elucidated. Finally, the standardization of validated functional tests, which are preferably performed in human-derived enterocytic and macrophagic cell lines, will enable exploring iron transport and the modulating effect of hepcidin of known and yet unclassified variants, and will improve our insights of the pathophysiology of ferroportin disease.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1424-8247/12/3/132/s1>, Figure 1S: Relation between age and serum iron/TSAT/ferritin, Table 1S: Characteristics and iron parameters of probands versus probands' relatives, Table 2S: Phenotypical features by the reported presence of hereditary HFE and non-HFE iron overload conditions, Table 3S: Phenotypical features by the reported current use of alcohol, Table 4S: Phenotypical features of patients with functional LOF ferroportin variant by hepcidin sensitivity, Table 5S: Phenotypical features by hepcidin resistant GOF and LOF ferroportin variants.

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Review

Hepcidin Therapeutics

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Abstract: Hepcidin is a key hormonal regulator of systemic iron homeostasis and its expression is induced by iron or inflammatory stimuli. Genetic defects in iron signaling to hepcidin lead to “hepcidinopathies” ranging from hereditary hemochromatosis to iron-refractory iron deficiency anemia, which are disorders caused by hepcidin deficiency or excess, respectively. Moreover, dysregulation of hepcidin is a pathogenic cofactor in iron-loading anemias with ineffective erythropoiesis and in anemia of inflammation. Experiments with preclinical animal models provided evidence that restoration of appropriate hepcidin levels can be used for the treatment of these conditions. This fueled the rapidly growing field of hepcidin therapeutics. Several hepcidin agonists and antagonists, as well as inducers and inhibitors of hepcidin expression have been identified to date. Some of them were further developed and are currently being evaluated in clinical trials. This review summarizes the state of the art.

Keywords: iron metabolism; hepcidin; ferroportin; hemochromatosis; anemia

1. Systemic Iron Homeostasis

Iron is an essential constituent of cells and organisms and participates in vital biochemical activities, such as DNA synthesis, oxygen transfer, and energy metabolism. The biological functions of iron are based on its capacity to interact with proteins and on its propensity to switch between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states. In spite of the high abundance of iron on the earth's crust, its bioavailability is limited by the fact that under aerobic conditions, ferrous iron is readily oxidized to insoluble ferric. Accumulation of excess iron in cells is toxic, because “free” ferrous iron catalyzes the generation of hydroxyl radicals via Fenton chemistry, which attack and inactivate cellular macromolecules [1]. The intricate chemistry of iron poses a major challenge for iron homeostasis: To satisfy metabolic needs and limit toxic side effects [2]. Mammals have developed mechanisms to efficiently acquire and retain dietary iron in the body and store, but not excrete the iron excess.

Approximately 70% of body iron (~3–5 g in adult humans) is bound to heme and used in hemoglobin of red blood cells [3]. Another 2–3% is present in muscles, where it is mostly utilized in the heme moiety of myoglobin. Excess of body iron accumulates in liver hepatocytes, where it is stored within ferritin and can be mobilized for erythropoiesis under iron deficiency. On a daily basis, erythropoiesis requires up to 30 mg of iron, while non-erythroid cell requirements are ~5 mg. Bone marrow erythroblasts and non-erythroid cells in other tissues acquire iron from plasma transferrin. This protein not only serves as an iron carrier, but also keeps circulating iron in a redox-inactive state. Under physiological conditions, only ~30% of transferrin molecules are saturated with Fe^{3+} and the iron-free apo-transferrin offers a redox buffering capacity. The iron content of transferrin (~3 mg) represents a small, but a highly dynamic fraction of body iron, which turns over more than 10 times per day to satisfy metabolic needs. It is mainly replenished by iron recycled from effete red blood cells via tissue macrophages during erythrophagocytosis. Dietary iron absorption (1–2 mg/day)

contributes to the buildup of iron stores during development; in adults it mainly serves to compensate for non-specific iron losses, for instance via bleeding or cell desquamation.

2. Hepcidin: The Key Iron Regulatory Hormone

Serum iron levels are under the control of hepcidin, a liver-derived peptide hormone and master regulator of iron metabolism [4]. It operates by binding to the iron exporter ferroportin in iron-releasing target cells, mainly tissue macrophages and duodenal enterocytes, but also other cell types (Figure 1). The binding of hepcidin occludes iron efflux [5] and triggers ubiquitination, internalization and lysosomal degradation of ferroportin [6]. This leads to intracellular iron retention and eventually to hypoferremia.

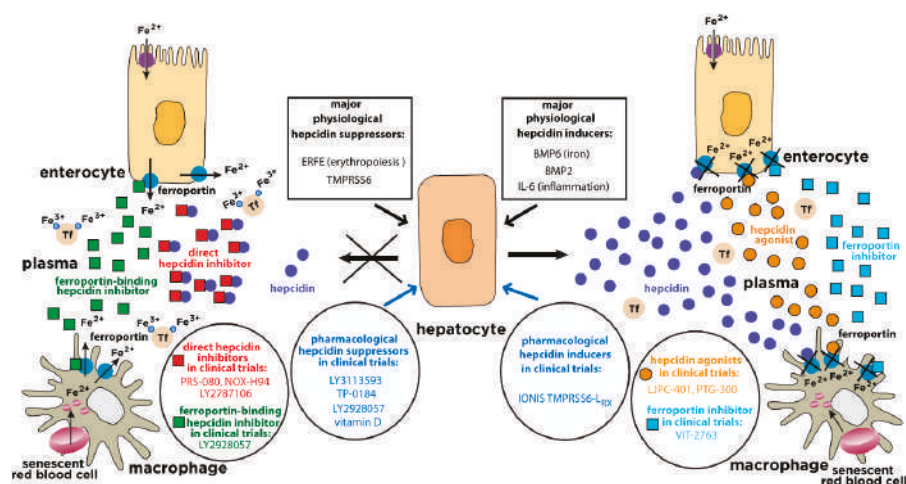


Figure 1. Physiological and pharmacological regulation of hepcidin activity. Hepcidin is synthesized in hepatocytes in response to iron or inflammatory stimuli. It operates by targeting the iron exporter ferroportin in tissue macrophages and duodenal enterocytes. The binding of hepcidin promotes ferroportin degradation, iron retention in target cells and hypoferremia. Suppression of hepcidin by erythropoietic stimuli allows iron efflux from cells into plasma via ferroportin. The expression of hepcidin can be manipulated pharmacologically with drugs that target hepatocytes (blue arrows). Hepcidin responses can be mimicked by hepcidin agonists (orange circles) or ferroportin inhibitors (light blue squares). Conversely, hepcidin responses can be antagonized by direct hepcidin inhibitors (red squares) or ferroportin-binding hepcidin inhibitors (green squares). The insets highlight major physiological hepcidin inducers or suppressors, as well as clinically relevant drugs that modulate the hepcidin-ferroportin axis and are currently being evaluated in randomized controlled trials for the treatment of hepcidin-related disorders (“hepcidinopathies”). Tf, transferrin.

The *HAMP* gene encodes pre-pro-hepcidin, an 84 amino acids long precursor, which is primarily expressed by hepatocytes in the liver, and at much lower levels by other cells in extrahepatic tissues. Pre-pro-hepcidin is processed to pro-hepcidin upon removal of its endoplasmic reticulum targeting sequence, consisting of 24 N-terminal amino acids. Further cleavage at the C-terminus yields mature, bioactive hepcidin, an evolutionary conserved, cysteine-rich peptide of 25-amino acids with antimicrobial properties. It folds to a distorted β -sheet with an unusual disulfide bridge between adjacent C13-C14 at the turn of a hairpin loop; according to this model, the structure is stabilized by further disulfide bonding between C7-C23, C10-C22 and C11-C19 [7] (Figure 2, left). An alternative structural model postulates disulfide bond connectivity between C7-C23, C10-C13, C11-C19 and C14-C22 [8] (Figure 2, right). Interestingly, the structural organization of hepcidin based on disulfide

bonding is not essential for iron-regulatory function, since the substitution of cysteines or the deletion of cysteine-containing segments do not impair hormonal activity [9,10].

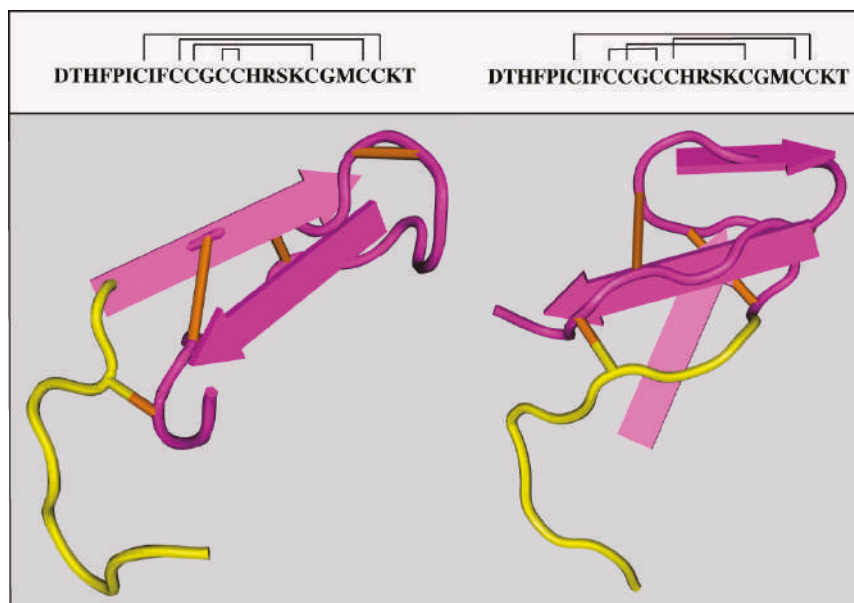


Figure 2. Proposed crystal structures of hepcidin. A structure with disulfide bonds between C7-C23, C10-C22, C13-C14 and C11-C19 (PDB ID: 1M4F) is shown on the left. An alternative structure with disulfide bonds between C7-C23, C10-C13, C11-C19 and C14-C22 (PDB ID: 2KEF) is shown on the right. The N-terminal amino acids which are essential for binding to ferroportin are highlighted in yellow.

Hepatocellular hepcidin expression responds to multiple stimuli, yet the major regulators are iron, inflammation and erythropoiesis [11]. Increases in serum or tissue iron trigger transcriptional induction of hepcidin via BMP/SMAD (Bone Morphogenetic Protein/Small Mothers Against Decapentaplegic) signaling (Figure 3). The mechanism involves secretion of *bone morphogenetic protein 6* (BMP6) from liver sinusoidal endothelial cells, which binds to type I (ALK2, ALK3, ALK6) and type II (ActRIIA, BMPRII) BMP receptors on hepatocytes and thereby activates the SMAD signaling cascade. Efficient iron signaling to hepcidin requires auxiliary factors, such as BMP2, the BMP co-receptor hemojuvelin (HJV), the hemochromatosis protein HFE, and the diferric transferrin sensor *transferrin receptor 2* (TfR2) [12]. The pathway is negatively controlled by the transmembrane serine protease matriptase-2 (also known as TMPRSS6), a hepcidin suppressor that appears to cleave HJV and other components of the hepcidin signaling pathway [13]. Iron-dependent upregulation of hepcidin serves to prevent excessive iron absorption when body iron stores are high.

Inflammatory cytokines promote hepcidin induction by several mechanisms. The most critical involves JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) signaling, which is activated in response to IL-6 (Figure 3). There is increasing evidence for a cross-talk between JAK/STAT and BMP6/SMAD signaling during inflammatory hepcidin induction [14–16]. Activin B, a JAK/STAT-induced ligand of BMP receptors can activate SMAD signaling to hepcidin [17], but its function is not essential [18]. On the other hand, BMP6 and HJV are critical components of the inflammatory hepcidin pathway [19,20], which is considered as an innate immune response to deprive invading bacteria of iron [21]. This is part of a broader iron withholding strategy within the context of “nutritional immunity” [22]. Under conditions of an increased erythropoietic drive, hepcidin

expression is suppressed by erythroferrone (ERFE), a hormone secreted by erythroblasts in response to erythropoietin [23] that neutralizes BMP6 [24]. This allows iron mobilization for erythropoiesis; the involvement of further erythropoietin-induced hepcidin suppressors in this response is also possible. Dysregulation of hepcidin expression leads to “hepcidinopathies”, which are iron-related disorders of hepcidin deficiency or excess (Table 1).

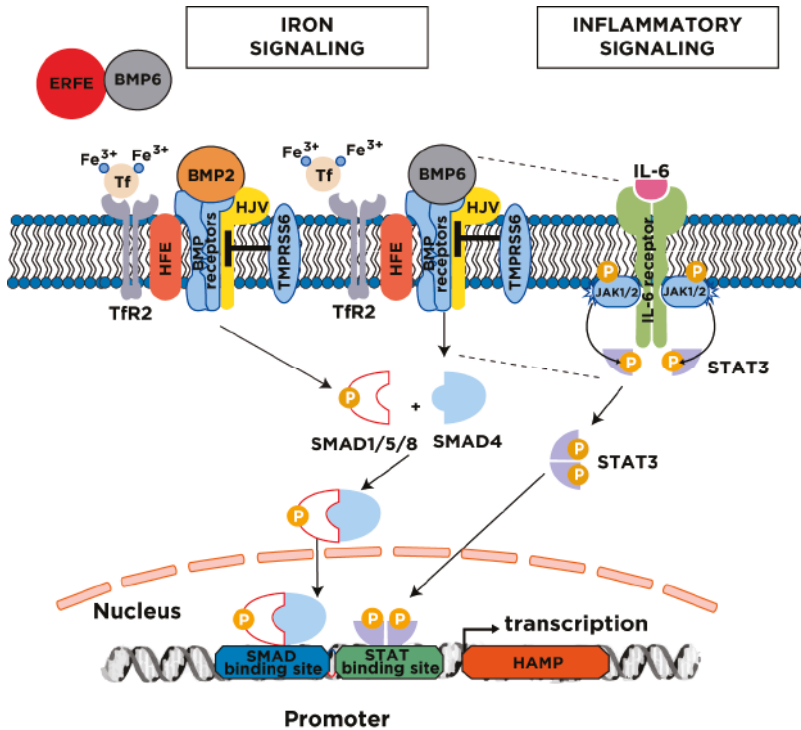


Figure 3. Major mechanisms for hepcidin regulation. Serum and tissue iron induce hepcidin transcription via the BMP/SMAD signaling pathway. The cascade is initiated following an increase in transferrin saturation and the secretion of BMP6 from liver sinusoidal endothelial cells; BMP2 is likewise secreted from liver sinusoidal endothelial cells but is less responsive to iron. Diferric transferrin binds to TfR2, while BMP6 and BMP2 bind to type I and II BMP receptors on hepatocytes. These events trigger phosphorylation of regulatory SMAD1/5/8, recruitment of SMAD4, and translocation of the SMAD complex to the nucleus for activating hepcidin transcription upon binding to BMP response elements in the *HAMP* promoter. Efficient iron signaling to hepcidin requires the BMP co-receptor HJV and the hemochromatosis protein HFE, and is negatively regulated by the transmembrane serine protease matriptase-2 (TMPRSS6). Under conditions of high iron demand for erythropoiesis, the erythropoietic regulator erythroferrone (ERFE) is released from bone marrow erythroblasts and suppresses hepcidin by sequestering BMP6. The inflammatory cytokine IL-6 induces hepcidin transcription via the JAK/STAT3 signaling pathway. The binding of IL-6 triggers dimerization of IL-6 receptors on hepatocytes, which leads to activation of associated JAK1/2 and subsequent phosphorylation of STAT3. Phospho-STAT3 dimerizes and translocates to the nucleus, where it activates hepcidin transcription upon binding to a STAT binding site in the *HAMP* promoter. Efficient hepcidin induction by the inflammatory pathway requires a threshold of BMP6/SMAD signaling (indicated by the dotted lines). BMP, Bone Morphogenetic Protein; SMAD, Small Mothers Against Decapentaplegic; HFE, high iron (Fe); HJV, hemojuvelin; TfR2, transferrin receptor 2; JAK, Janus kinase; STAT, Signal Transducer and Activator of Transcription.

Table 1. Iron-related disorders of hepcidin dysregulation. Deficiency or excess of hepcidin has a different physiological effect on intestinal iron absorption, iron release by macrophages, serum iron, and tissue iron. HH, Hereditary Hemochromatosis; CLD, Chronic Liver Disease; IRIDA, Iron-Refractory Iron Deficiency Anemia; AI, Anemia of Inflammation.

	Disorders								
	Hepcidin Deficiency			Hepcidin Resistance or Ferroportin Deficiency		Systemic Hepcidin Overexpression			Local Hepcidin Overexpression
	HH	Iron-Loading Anemias	CLD	Ferroportin Hemochromatosis	Ferroportin Disease	IRIDA	AI	Castleman Disease	Cancer
Hepcidin	↓	↓	↓	↑	↑	↑	↑	↑	↑ (in cancer cells)
Intestinal Fe absorption	↑	↑	↑	↑	↑	↓	↓	↓	Normal
Macrophage Fe release	↑	↑	↑	↑	↓	↓	↓	↓	Normal
Serum Fe	↑	↑	↑	↑	↓	↓	↓	↓	Normal
Tissue Fe	↑	↑	↑	↑	↑	Normal	Normal	Normal	Normal

3. Disorders with Hepcidin Deficiency

Hepcidin expression is suppressed in *hereditary hemochromatosis* (HH), in iron-loading anemias with ineffective erythropoiesis and in some chronic liver diseases.

3.1. Hereditary Hemochromatosis

Inactivating mutations in upstream regulators of hepcidin or direct disruption of hepcidin cause HH, an endocrine disorder of systemic iron overload [25]. As a result, hepcidin deficiency leads to increased dietary iron absorption (up to 8–10 mg/day) and uncontrolled release of iron to plasma, due to unrestricted expression of ferroportin in duodenal enterocytes and tissue macrophages. This promotes gradual saturation of plasma transferrin and buildup of unshielded *non-transferrin-bound iron* (NTBI), which accumulates within tissue parenchymal cells. Clinical complications of HH include liver cirrhosis, hepatocellular cancer, cardiomyopathy, diabetes mellitus, endocrinopathy, arthritis and osteoporosis. In addition, HH patients are susceptible to infection with siderophilic bacteria, but also various other pathogens [26,27].

HH is genetically heterogenous and its severity depends on the degree of hepcidin suppression relative to body iron stores [28]. The major form is linked to mutations in HFE (especially C282Y) and constitutes the most frequent genetic disorder in Caucasians. However, the clinical penetrance is variable and depends on further genetic and environmental factors. The disease phenotype is relatively mild, and symptoms typically develop after the fourth decade of life. Other forms of HH are rare and typically associated with more severe phenotypes. Thus, inactivation of either HJV or hepcidin cause *juvenile hemochromatosis* (JH) with early-onset iron overload in the late teens or early twenties. Inactivation of Tfr2 yields an intermediate clinical phenotype compared to HFE-related HH and JH.

3.2. Iron-Loading Anemias

Hereditary or acquired anemias, such as thalassemias, congenital dyserythropoietic anemias, sideroblastic anemias or myelodysplastic syndromes, are associated with bone marrow hyperplasia and ineffective erythropoiesis [29]. Patients with severe forms of these diseases are often treated with blood transfusions, causing secondary iron overload. Ineffective erythropoiesis stimulates expression of ERFE and other factors, such as GDF15, which suppress hepcidin expression. This in turn promotes a hemochromatosis-like phenotype of iron overload in non-transfused patients with milder forms of disease; in addition, it aggravates the already existing secondary iron overload in transfused patients. Importantly, repressive erythropoietic signals dominate over the stimulating iron signals creating a vicious cycle in iron homeostasis and hepcidin expression [3].

3.3. Chronic Liver Diseases

Patients with *alcoholic liver disease* and *chronic hepatitis C* often exhibit some degree of hepatocellular iron overload that aggravates liver disease progression to advanced stages (cirrhosis and hepatocellular carcinoma). This is linked to hepcidin suppression by oxidative stress and possibly additional mechanisms. Hepatic iron overload is also observed in patients with *non-alcoholic fatty liver disease* (NAFLD). The pattern of excessive iron distribution is variable with preference in either hepatocytes or macrophages, which may underlie differential hepcidin regulation by positive and negative stimuli [30,31]. In advanced liver disease of any etiology, the expression of hepcidin decreases dramatically due to severe injury of hepatocytes, and hepcidin levels offer a potential biomarker for liver disease progression [32].

Interestingly, experimental data provided evidence for a protective role of hepcidin against liver fibrosis. Thus, adenoviral expression of hepcidin attenuated the development of liver fibrosis in mice subjected to CCl₄ intoxication or to bile duct ligation [33]. The underlying mechanism involves a hepcidin-mediated increase in the iron content of hepatic stellate cells, which prevents their differentiation from collagen-secreting myofibroblasts. Another study using *Tmprss6*^{-/-} mice showed that genetic hepcidin overexpression protected these animals against high fat diet-induced obesity and liver steatosis by stimulating lipolytic pathways [34]. These data highlight a critical role of hepcidin in liver disease pathogenesis.

4. Disorders with Hepcidin Resistance or Ferroportin Deficiency

Ferroportin hemochromatosis is a distinct clinical entity that develops as a result of “gain-of-function” ferroportin mutations that prevent the binding of hepcidin [35]. Conversely, “loss-of-function” ferroportin mutations that inhibit intracellular trafficking of the protein are the hallmark of *ferroportin disease*, which is characterized by macrophage iron loading. Hepcidin levels are elevated rather than suppressed in these ferroportin-associated disorders of hepcidin resistance, or ferroportin deficiency, respectively. It should be noted that both ferroportin hemochromatosis and ferroportin disease are transmitted in an autosomal dominant fashion, contrary to all other forms of HH, which are autosomal recessive.

5. Disorders with Systemic Hepcidin Overexpression

Systemic overexpression of hepcidin occurs in anemias with iron-restricted erythropoiesis, such as *iron-refractory iron deficiency anemia* (IRIDA), *anemia of inflammation* (AI; also known as anemia of chronic disease) or Castleman disease. Local overexpression of hepcidin has been reported in tumors.

5.1. Iron-Refractory Iron Deficiency Anemia

IRIDA is an autosomal recessive disease caused by loss-of-function mutations in the hepcidin suppressor matriptase-2 (*TMPRSS6*), which lead to hepcidin overexpression [36]. It is characterized by hypochromic microcytic anemia, hyperhepcidinemia, hypoferremia, low transferrin saturation and unresponsiveness to oral iron therapy.

5.2. Anemia of Inflammation

AI is a heterogenous disorder caused by chronic inflammation due to infections, inflammatory bowel disease, inflammatory rheumatic disease, *chronic kidney disease* (CKD) or obesity [37]. It is also observed in cancer patients (see Section 6) and in frail, elderly persons. AI is the most common type of anemia among hospitalized patients in the developed world. Unresolved inflammatory induction of hepcidin is one of the contributors to AI, because it causes hypoferremia and iron sequestration in tissues, limiting its availability for erythropoiesis. Other contributing factors are reduced proliferation of erythroblasts, reduced expression of erythropoietin, impaired erythropoietin signaling and increased erythrophagocytosis. In CKD, hyperhepcidinemia is aggravated by ineffective renal clearance [38].

AI is normochromic and normocytic, but this phenotype can be affected by chronic blood loss or dietary iron malabsorption, which result in smaller and hypochromic red blood cells [39].

5.3. Castleman Disease

Castleman disease is a rare lymphoproliferative disorder characterized by generalized lymphadenopathy and multiple organ involvement that is linked to chronic overproduction of IL-6 [40]. Castleman disease patients present with iron-refractory hypochromic microcytic anemia, which develops in response to IL-6-mediated upregulation of hepcidin [41].

6. Disorders with Local Hepcidin Overexpression

Hepcidin is produced at lower levels in several extrahepatic tissues, including the heart or the brain, where it exerts local cell autonomous functions [42,43]. Interestingly, many tumor cells produce and utilize hepcidin for their own advantage. Thus, hepcidin targets ferroportin in tumor cells in an autocrine manner and thereby promotes retention of iron, which is essential for cell proliferation and tumor growth [44]. This mechanism has been documented in breast [45], prostate [46] and thyroid [47] cancers. It should also be noted that in advanced disease stages, the immune response against primary or metastatic tumor cells may trigger hepcidin induction in hepatocytes and thereby lead to systemic hepcidin overproduction. This may result in AI due to hepcidin-mediated iron sequestration in tissues and inhibition in iron absorption.

7. The Need for Hepcidin Therapeutics

Targeting the hepcidin-ferroportin axis could offer therapeutic benefits to patients with disorders related to aberrant hepcidin expression [48–50]. Hepcidin therapeutics could complement or even replace current treatment modalities, which have several limitations.

7.1. Narrowing the Management Gap in Iron Overload Disorders Linked to Hepcidin Deficiency

The standard of care for HH is therapeutic phlebotomy, which reduces circulating iron burden and also promotes iron mobilization from stores [51]. This approach is effective and essentially normalizes the life-span of HH patients without liver cirrhosis and diabetes [52]. However, there is no evidence base for optimal start time, frequency, duration and endpoint of phlebotomy. Moreover, this treatment cannot reverse liver cirrhosis, diabetes, arthritis, cardiomyopathy or hypogonadism, while a few patients may not tolerate, or exhibit low compliance, to life-long treatment. Another limitation is that phlebotomy stimulates dietary iron absorption by further suppressing hepcidin via erythropoietic regulators.

Iron overload in iron-loading anemias is managed by iron chelation therapy. Clinically approved iron chelating drugs include desferrioxamine (DFO), deferiprone (DFP) and deferasirox (DFX). DFO was first utilized for the treatment of transfused β -thalassemia patients in the 1970s. Due to the accumulated >40-year long experience and its low cost, it remains a first line therapeutic option in many guidelines [53]. However, DFO is poorly absorbed in the gastrointestinal tract and has a short (~20 min) plasma half-life. Thus, to reach effective pharmacological concentrations the drug is administered parenterally with the aid of a portable infusion pump at least 4–5 days per week for 8–10 h each time. The cumbersome procedure reduces compliance and significantly compromises the quality of patients' life. This prompted the development of the orally absorbed iron chelators DFP and DFX, which exhibit better pharmacokinetics, with plasma half-lives of 1–3 h and 8–16 h, respectively. Nevertheless, the use of iron chelating drugs is associated with the risk of side effects, such as neutropenia/agranulocytosis, skin rash, gastrointestinal disturbances, retinopathy or bone abnormalities. Another problem is that these drugs cannot prevent dietary iron absorption, which is highly induced in patients with iron-loading anemias, due to erythropoietic suppression of hepcidin.

Thus, hepcidin replacement therapy could provide an etiologic cure of HH, and correct aggravating dietary iron absorption in transfusional iron overload. Proof of principle is provided by

experiments with mouse models of HH (Hfe^{-/-}) and β -thalassemia (Hbb^{th3/+}). Thus, iron overload was corrected when these animals were crossed with mice overexpressing transgenic hepcidin [54,55], or with Tmprss6^{-/-} mice overexpressing endogenous hepcidin [56,57]. Moreover, the increased hepcidin expression by these manipulations led to improved erythropoiesis in Hbb^{th3/+} mice [55,57].

In light of the experimental evidence for the protective effects of hepcidin against liver fibrosis and obesity [33,34], hepcidin agonists, or inducers of hepcidin expression, could also be used for the treatment of these highly prevalent disorders. Finally, hepcidin agonists/inducers could enhance “nutritional immunity” during infection with extracellular pathogens.

7.2. Narrowing the Management Gap in Anemias and Other Disorders Linked to Hepcidin Overexpression

IRIDA patients are typically managed with intermittent parenteral iron therapy that only partially corrects anemia [36]. Improvement may need repeated dosing, which is associated with potential side effects and the risk for reticuloendothelial iron overload. Control of hepcidin overexpression by using hepcidin antagonists is expected to improve responses of IRIDA patients not only to parenteral, but also oral iron formulations.

AI can be corrected by treating the underlying cause of the primary disease, but this is not always feasible. Alternatively, AI patients can be treated with oral or parenteral iron administration, which increases the risk of bacteremia and allergic reactions [58]. Another option is the administration of *erythropoiesis stimulating agents* (ESA) based on recombinant human erythropoietin, often together with intravenous iron; however, prolonged use of ESAs may lead to cerebrocardiovascular complications [59]. When AI is combined with severe iron deficiency, blood transfusion is the preferred treatment; however, this is limited by the risk of infections, immune reactions or lung injury [60]. A major drawback that compromises efficiency of all these approaches is the inflammatory overexpression of hepcidin, which sequesters iron to tissues and reduces its availability for erythropoiesis. Thus, iron/ESA therapy could be complemented (and in some cases even replaced) by the use of hepcidin antagonists.

Proof of principle has been provided by pharmacological experiments showing improvement of anemia following hepcidin neutralization in a rat model of AI, based on injection with *group A streptococcal peptidoglycan-polysaccharide* (PG-APS) [15,61]. Moreover, inoculation of Hamp^{-/-} or IL6^{-/-} mice with heat-killed *Brucella abortus* yielded a milder AI phenotype [62,63].

8. Inducers of Hepcidin Expression

Various molecules exhibit pharmacological potential to induce hepcidin expression. These include recombinant BMP6, Tmprss6-silencing oligonucleotides, and a wide range of natural or synthetic small molecules. A detailed list is shown in Table 2. Clinically relevant hepcidin inducers currently undergoing evaluation in randomized controlled trials for disorders with hepcidin deficiency are highlighted in Figure 1.

8.1. Recombinant BMP6

Treatment of Hfe^{-/-} mice with the major hepcidin inducer BMP6 was shown to increase hepatic hepcidin expression, which in turn caused a decrease in serum iron levels, due to iron retention in the spleen and the duodenum [64]. However, this was associated with peritoneal calcifications, possibly because BMP6 was administered intraperitoneally. Based on these findings, the off-target effects of BMP6 and other BMPs in bone metabolism appear to limit their clinical applications in the context of iron overload.

8.2. Tmprss6-Silencing Oligonucleotides

Silencing of Tmprss6 mRNA using lipid nanoparticle formulated small interfering RNAs (siRNAs) or *antisense oligonucleotides* (ASOs) was shown to induce hepcidin and improve the phenotypes of hemochromatotic Hfe^{-/-} and thalassaemic Hbb^{th3/+} mice [65,66]. Of note, combined treatments of

Hbb^{th3/+} mice with Tmprss6 siRNAs or ASOs, together with the oral iron chelator deferiprone, resulted in better correction of erythropoiesis and prevention of secondary iron overload [67,68]. When conjugated with *triantennary N-acetyl galactosamine* (GalNAc) for targeted delivery to hepatocytes, the Tmprss6-ASOs exhibited improved efficacy [69]. GalNAc-conjugated Tmprss6 siRNAs demonstrated a therapeutic potential in splenectomised Hbb^{th3/+} mice [70], a finding of clinical interest for splenectomised thalassemic patients. Ionis Pharmaceuticals Inc. developed TMPRSS6-silencing molecules with the commercial name IONIS-TMPRSS6-L_{RX}, which are currently being tested in phase 1 clinical trials (ClinicalTrials.gov Identifier: NCT03165864).

8.3. Small Molecule Hecpudin Inducers

Several small molecules were shown to activate hepcidin pathways. A high-throughput chemical screen in zebrafish identified three steroid molecules i.e., progesterone, epitiostanol and mifepristone as potent hepcidin inducers. They operate via a pathway involving the *progesterone receptor membrane component-1* (PRMC-1) [71]. A small-scale chemical screen in zebrafish embryos uncovered the hepcidin-inducing capacity of genistein, a phytoestrogen that is abundant in soybean. Genistein was shown to upregulate hepcidin in HepG2 hepatoma cells and zebrafish embryos by promoting STAT3 phosphorylation [72].

A subsequent high-throughput screen of small molecules in HepG2 cells identified ipriflavone and vorinostat, which are synthetic drugs that inhibit bone resorption or histone deacetylase activity and are clinically applied for the treatment of osteoporosis or cutaneous T cell lymphoma, respectively. They both stimulate expression of hepcidin and other BMP- and STAT3-dependent genes without affecting SMAD1/5/8 or STAT3 phosphorylation, and exhibit 10-fold higher potency than genistein [73]. Ipriflavone was further tested in vivo and was shown to alleviate dietary iron overload in wild type mice; however, it failed to reduce iron overload in Hbb^{th3/+} mice [74]. The hepcidin-stimulating effects of vorinostat in Huh7 hepatoma cells were also validated in another study [75], and are consistent with earlier data showing that the histone deacetylase inhibitor trichostatin A induces hepcidin by inhibiting the binding of C/EBP α and STAT3 in the *HAMP* promoter [76]. Nevertheless, vorinostat failed to induce hepcidin in mice [75]. The non-steroidal anti-inflammatory drug diclofenac likewise stimulated hepcidin expression in vitro, but not in vivo [75]. In a screen of natural compounds, icariin, a plant flavonol glycoside, was documented to stimulate hepcidin expression in HepG2 cells by inducing SMAD1/5/8 or STAT3 phosphorylation. These findings were validated in mice, while similar results were obtained with the icariin analogue epimedin C [77]. Several polyphenolic small molecules or phytoestrogens that are found in fruits and vegetables likewise induced hepcidin in HepG2 cells and in rats. These include resveratrol, quercetin, kaempferol, naringenin, epi-gallo-catechin-3-gallate, and operate by activating Nrf2 for binding to an *antioxidant response element* (ARE) in the *HAMP* promoter [78].

A genetic siRNA screen revealed that Ras/Raf/MAPK and PI3K/Akt/mTOR signaling suppress hepcidin; consequently, pharmacological inhibitors of these pathways, such as sorafenib, wortmannin, rapamycin and metformin were shown to induce hepcidin in hepatoma cells and primary hepatocytes [79]. In another setting, dietary supplementation of Hfe^{-/-} mice with adenine was reported to induce hepcidin and attenuate iron overload by a mechanism requiring BMP/SMAD and *cAMP/protein kinase A* (PKA) signaling [80]. While some data obtained with small molecule hepcidin inducers are interesting, the potential for these drugs for clinical application in the context of hepcidin deficiency appears limited, due to lack of specificity.

Table 2. Inducers of hepcidin expression. Nrf2, nuclear factor (erythroid-derived 2)-like 2; PTGIS, prostaglandin I2 (prostaglyclin) synthase; MAPK, mitogen-activated protein kinase; PRMC-1, progesterone receptor membrane component-1; PKA, protein kinase A.

	Drug	Target	Evidence	Reference
In vitro studies	Genistein (small molecule)	STAT3	Hepatoma cells	Zhen et al. 2013 [72]
	Ipriflavone (small molecule)	Histone deacetylase, BMP-, STAT3-dependent genes	Hepatoma cells	Gaun et al. 2014 [73]
	Vorinostat (small molecule)	Histone deacetylase, BMP-, STAT3-dependent genes	Hepatoma cells	Gaun et al. 2014 [73] Mleczo-Sanecka et al. 2017 [75]
	Diclofenac (small molecule)	Not specified; independent of PTGIS and cyclooxygenases	Hepatoma cells	Mleczo-Sanecka et al. 2017 [75]
	Icariin (small molecule)	SMAD1/5/8, STAT3	Hepatoma cells	Zhang et al. 2016 [77]
	Resveratrol, quercetin, kaempferol, naringenin, epi-gallo-catechin-3-gallate (small molecules)	Nrf2	Hepatoma cells	Bayele et al. 2015 [78]
	Sorafenib, wortmannin, rapamycin, metformin (small molecules)	Ras/RAF/MAPK and mTOR signaling	Hepatoma cells, Primary hepatocytes	Mleczo-Sanecka et al. 2014 [79]
Preclinical studies	BMP6	BMP receptors	Mouse model of adult HH	Corradini et al. 2010 [64]
	siRNAs	Matriptase-2	Mouse model of adult HH Mouse model of β -thalassemia	Schmidt et al. 2013 [65]
	Antisense oligonucleotides (ASOs)	Matriptase-2	Mouse model of β -thalassemia	Guo et al. 2013 [66]
	GalNac-ASOs	Matriptase-2	Mouse model of β -thalassemia, splenectomised	Schmidt et al. 2018 [70]
	Progesterone, epitostanol, mifepristone	PRMC-1	Zebrafish	Li et al. 2016 [71]
	Ipriflavone (small molecule)	BMP-, STAT3-dependent genes	Wild type mice	Gaun et al. 2014 [73]
	Icariin (small molecule)	SMAD1/5/8, STAT3	Wild type mice	Zhang et al. 2016 [77]
	Epimedin C (small molecule)	SMAD1/5/8, STAT3	Wild type mice	Zhang et al. 2016 [77]
	Resveratrol, quercetin, kaempferol, naringenin, epi-gallo-catechin-3-gallate (small molecules)	Nrf2	Wild type rats	Bayele et al. 2015 [78]
Adenine (small molecule)	SMAD1/5/8 and cAMP/PKA	Mouse model of adult HH	Zhang et al. 2018 [80]	
Clinical trials	IONIS-TMPRSS6-Lrx (Antisense oligonucleotide) By: Ionis Pharmaceuticals Inc.	Matriptase-2	Healthy subjects—Phase 1 (Active)	ClinicalTrials.gov Identifier: NCT03165864

9. Hepcidin Agonists

Administration of synthetic hepcidin can effectively reduce plasma iron levels in wild type [81] or Hfe^{-/-} mice [82]. However, the clinical value of this approach is limited by the high cost of manufacturing correctly folded, bioactive hepcidin, and by the rapid clearance of native hepcidin peptide in the blood. This prompted the development of cheaper hepcidin derivatives with improved pharmacological and pharmacokinetic properties. A detailed list is shown in Table 3a. Clinically relevant hepcidin agonists currently undergoing evaluation in randomized controlled trials for disorders with hepcidin deficiency are highlighted in Figure 1.

9.1. Minihepcidins

Mutational studies revealed that the N-terminus of hepcidin is necessary and sufficient for binding to ferroportin, and the remaining sequence is permissive to mutations [9]. This paved the way for the development of stable, bioactive truncated hepcidin derivatives. The first generation minihepcidins consist of 7 to 9 N-terminal amino acids with a free sulfhydryl group at C7; among them Hep9 was the most potent in in vitro ferroportin internalization assays [83]. Minihepcidins were

further optimized by chemical modifications. To improve stability, peptides were circularized, but this resulted in decreased potency compared to Hep9. For protection against proteolysis, unusual amino acids, including N-substituted, beta-homo, and D-amino acids were introduced to the initial chemical structure resulting in retro-inverso (ri-) analogues (i.e., reversed sequence of parental L-peptides). To improve intestinal absorption, ri- analogues were conjugated to fatty acids (palmitoyl- groups) or chenodeoxycholic or ursodeoxycholic bile acids (cheno- and urso- groups, respectively). Modified minihepcidins were then tested in vivo. Injection of unmodified Hep9 into wild type mice failed to cause any effect on serum iron. By contrast, ri-hep9 and palmitoyl-ri-hep9 promoted hypoferrremia, while high doses of the latter had almost the same effect on serum iron levels to equivalent doses of full-length hepcidin [83].

Administration of PR65, another optimized minihepcidin, prevented iron loading and corrected iron distribution in previously iron-depleted Hamp^{-/-} mice. However, excessive amounts of the compound led to iron-restrictive anemia, underlying the need for dose optimization. The effects of PR65 in iron-loaded Hamp^{-/-} mice were less compelling, and limited to partial iron re-distribution from the liver to the spleen [84].

Because free sulfhydryl groups exhibit non-specific reactivity and may cause dermatological side effects, an S-protection strategy was used to develop the PR73 minihepcidin [85]. Daily administration of PR73 to Hamp^{-/-} mice for one week led to significant iron redistribution from pancreatic acinar cells to macrophages [86]. Attempts to further improve PR73 were made. Cyclization resulted in agonist mHS17, the most active analogue of PR73, but this was several times less active than PR73 in vitro and ineffective in vivo [87]. Thiol-protection with activated vinyl thioethers yielded the S-vinyl-PR73SH derivative, which exhibited almost the same potency with the parental compound in vitro and in vivo [85].

PR73 was also evaluated for possible protective effects in mouse models of bacterial infections. Treatment of Hamp^{-/-} mice with PR73, pre- or 3 h post-infection with siderophilic *Vibrio vulnificus*, caused hypoferrremia and eventually repressed bacterial growth and reduced mortality [88]. Similar results were obtained in Hamp^{-/-} or iron-loaded wild type mice infected with siderophilic *Yersinia enterocolitica* [89], or non-siderophilic *Klebsiella pneumoniae* or *Escherichia coli* [90,91]. These data uncover a therapeutic potential of minihepcidins, and probably other hepcidin agonists, against bacterial infections.

Merganser Biotech Inc., a start-up company, developed minihepcidins M004 and M009. The latter is a prodrug with an extra S-methyl group at C7, which is converted to M004 upon S-methyl removal in the blood. Daily injections of young Hbb^{th3/+} mice with M004, or biweekly injections of old Hbb^{th3/+} mice with M009, improved erythropoiesis and reduced splenomegaly and iron accumulation in the liver and kidneys within 6 weeks [92]. Further improvement was observed when M009 administration was combined with the iron chelator deferiprone. Interestingly, M009 was also shown to reduce erythrocytosis and splenomegaly in a mouse model of polycythemia vera [92].

9.2. Other Hepcidin Derivatives

La Jolla Pharmaceutical Company developed LJPC-401, a proprietary formulation of hepcidin. The molecule completed phase 1 clinical trials and has initiated phase 2 in patients with HH (ClinicalTrials.gov Identifier: NCT03395704) or transfusion-dependent β -thalassemia with myocardial iron overload (ClinicalTrials.gov Identifier: NCT03381833). Data from phase 1 show that LJPC-401 effectively reduces serum iron levels in a dose-dependent manner with a maximum at 8 h post injection, and in the absence of significant adverse effects [93].

Protagonist Therapeutics Inc. launched PTG-300, another proprietary hepcidin formulation. The compound reduces serum iron in cynomolgus monkeys and improves erythropoiesis in the Hbb^{th3/+} mouse model of β -thalassemia [94]. PTG-300 recently completed phase 1 clinical trial in healthy volunteers. The data show that the drug is well tolerated and reduces serum iron in a dose-dependent manner; notably this response is sustained for 6 days [95]. A phase 2 clinical trial

evaluating the effects of PTG-300 in β -thalassemia patients is expected to start in the fourth quarter of 2018 (<http://www.protagonist-inc.com/randd-pipeline.php#ptg300>).

Table 3a. Hepcidin agonists.

	Drug	Target	Evidence	Reference
Preclinical studies	Palmitoyl-ri-hep9 (minihepcidin)	Ferroportin	Mouse model of juvenile HH	Preza et al. 2011 [83]
	PR65 (minihepcidin)	Ferroportin	Wild type mice Mouse model of juvenile HH	Ramos et al. 2012 [84]
	PR73 (minihepcidin)	Ferroportin	Mouse model of adult HH Mouse model of adult HH, bacteria-infected	Arezes et al. 2015 [88] Lunova et al. 2017 [86] Stefanova et al. 2017 [89] Michels et al. 2017 [90] Stefanova et al. 2018 [91]
	M004, M009 (minihepcidins)	Ferroportin	Mouse model of β -thalassemia Mouse model of polycythemia vera	Casu et al. 2016 [92]
Clinical trials	LJPC-401 (hepcidin formulation) By: La Jolla Pharmaceutical Company	Ferroportin	HH, β -thalassemic patients—Phase 1 (Completed) Phase 2 (Recruiting)	Phase 1: Lal et al. 2018 [93] (congress presentation) Phase 2: ClinicalTrials.gov Identifiers NCT03395704, NCT03381833
	PTG-300 (hepcidin formulation) By: Protagonist Therapeutics Inc.	Ferroportin	Healthy subjects—Phase 1 (Completed) Phase 2 expected to start end of 2018	Bourne et al. 2018 [94] Nicholls et al. 2018 [95] (congress presentations)

Table 3b. Ferroportin inhibitors.

	Drug	Target	Evidence	Reference
Clinical trials	VIT-2763 (small molecule) By: Vifor Pharma	Ferroportin	Phase 1 planned in 2018	http://www.viforpharma.com/en/media/press-releases/201802/2167159

10. Inhibitors of Ferroportin Activity

Theoretically, the pharmacological effects of hepcidin replacement therapy could also be achieved by inhibiting the synthesis or the iron-exporting activity of ferroportin. Moreover, these approaches would be appropriate for the treatment of ferroportin hemochromatosis, which is caused by hepcidin resistance rather than deficiency. Currently, there is no reported attempt for therapeutic targeting of ferroportin synthesis, but efforts have been made to develop ferroportin inhibitors. Thus, Vifor Pharma has generated an orally administered small molecule that binds to ferroportin and inhibits iron efflux (Table 3b and Figure 1). The drug (VIT-2763) was expected to enter phase 1 clinical trial in early 2018 (<http://www.viforpharma.com/en/media/press-releases/201802/2167159>).

11. Inhibitors of Hepcidin Expression

Several inhibitors of hepcidin expression have been identified. These include macromolecular inhibitors of BMP6 or HJV, small molecule inhibitors of BMP/SMAD signaling, neutralizing antibodies against IL-6 receptor or IL6, small molecule inhibitors of JAK/STAT3 signaling, sex hormones, and vitamin D. A detailed list is shown in Table 4. Clinically relevant hepcidin inhibitors currently undergoing evaluation in randomized controlled trials for AI are highlighted in Figure 1.

11.1. Inhibitors of BMP6/HJV

BMP6 and HJV are essential for appropriate hepcidin induction during inflammation [19,20]. Thus, targeting these proteins is expected to be beneficial in disorders associated with hepcidin overexpression. Eli Lilly and Company developed LY3113593, a monoclonal humanized antibody

against BMP6, for the treatment of anemia in patients with CKD. The BMP6-neutralizing drug has been evaluated for safety and pharmacokinetics in two phase 1 clinical trials with healthy volunteers and CKD patients (ClinicalTrials.gov Identifiers: NCT02144285 and NCT02604160). The studies are completed, but the results are not yet publicly available.

Another BMP6-blocking strategy involves heparin, a glycosaminoglycan produced by mast cells, which is used in clinical settings as an anticoagulant. Biochemical experiments showed that heparin binds with high affinity to BMP6 and abolishes its hepcidin signaling function. Moreover, administration of heparin to mice downregulated hepcidin expression and increased serum iron, while a similar phenotype was documented in patients treated with heparin to prevent deep vein thrombosis [96]. Heparin was also shown to inhibit hepcidin expression in *Mycobacterium tuberculosis*-infected human macrophages [97]; however, the role of macrophage hepcidin in AI is not well understood. Modified glycol-split and oversulfated heparins lacking coagulant activity retain hepcidin suppressive properties. Thus, treatment with these drugs improved anemia in a heat-killed *Brucella abortus* mouse model of AI, and even further suppressed hepcidin in Bmp6^{-/-} mice [98,99]. Clearly, these promising results highlight the need for comprehensive evaluation of heparin derivatives for efficacy and safety in clinical trials.

Erythroferrone was recently identified as a potent competitive inhibitor of BMP6 [24]. This finding raises the interesting possibility for using this hormone to treat anemias with excessive hepcidin expression. The pharmacological potential of erythroferrone awaits experimental validation. Nevertheless, since erythroferrone moonlights as myokine (myonectin/CTRP15) with metabolism-modulating properties [23,100], potential pleiotropic effects should be considered.

A soluble HJV construct fused with the Fc domain of IgG (sHJV.Fc) specifically inhibits HJV-mediated induction of hepcidin in cultured hepatoma cells and in mice by competitive binding to BMP6 [101,102]. Moreover, sHJV.Fc was shown to correct hepcidin levels and anemia in the PG-APS rat model of AI [15]. FerruMax Pharmaceuticals, a start-up biotech company, aimed to investigate the pharmacological potential of sHJV.Fc for the treatment of AI in two phase 1 clinical trials with CKD patients (ClinicalTrials.gov Identifiers: NCT01873534 and NCT02228655). However, the trials were terminated early due to “inability to recruit patients meeting eligibility criteria”.

Abbvie developed humanized monoclonal antibodies against HJV. A single-dose of the HJV-neutralizing antibodies ABT-207 or H5F9-AM8 in rats and cynomolgus monkeys had long-lasting suppressing effects on hepcidin and promoted an increase in serum iron [103]. H5F9-AM8 was further evaluated in preclinical models of AI (chronic arthritis in rats, aseptic inflammation in mice) and IRIDA (Tmprss6^{-/-} mice). In all conditions tested, the antibody suppressed hepcidin and improved anemia [104]. H5F9-AM8 also reduced systemic and local tumor hepcidin in a mouse xenograft model; however, the suppression of hepcidin expression in cancer cells was not associated with inhibition of tumor growth [105].

11.2. Small Molecule Inhibitors of BMP/SMAD Signaling

Dorsomorphin was identified in a chemical screen as the first small molecule inhibitor of the BMP/SMAD signaling pathway [106], which is essential for inflammatory induction of hepcidin [14–16]. It operates by targeting type I BMP receptors, thereby blocking phosphorylation of SMAD1/5/8. The optimized dorsomorphin derivative LDN-193189 inhibited BMP/SMAD signaling to hepcidin in hepatocytes, which in turn stimulated erythropoiesis and attenuated anemia in the PG-APS rat model of AI [15,61]. Orally administered LDN-193189 was also shown to increase hemoglobinization in turpentine-challenged mice [107]. Surprisingly, hepcidin induction by activin B, which is mediated by ALK2 and the type II BMP receptor ActRIIA, is insensitive to LDN-193189 [108]. A major limitation of LDN-193189 as hepcidin inhibitor is its broad specificity that leads to off-target effects [109,110]. LDN-212854, another dorsomorphin derivative with increased selectivity to ALK2 versus ALK3, was shown to suppress IL-6-induced hepcidin expression in HepG2 cells, yet less effectively compared to LDN-193189 [111].

Further chemical screens identified additional compounds with inhibitory activity on BMP/SMAD signaling to hepcidin. The dietary flavonoid myricetin was shown to prevent lipopolysaccharide-induced hypoferrremia in mice [112]. An indazole molecule was derivatized to several indazole-based inhibitors [113], including DS28120313 [114] and orally active DS79182026, which could antagonize induction of hepcidin in mice injected with IL-6 [115]. Phenotypic screening by applying chemical proteomics and a radioactive compound-binding assay in HepG2 cells identified ALK2 and ALK3 as the primary targets of DS79182026 [116].

Tolero Pharmaceuticals, Inc., Lehi, UT, USA, launched TP-0184, a small molecule inhibitor of ALK2. The drug is currently undergoing a phase 1 clinical trial to determine its maximum tolerated dose and dose-limiting toxicities in patients with advanced solid tumors (ClinicalTrials.gov Identifier: NCT03429218). In preclinical mouse models of inflammation- and cancer-induced anemia, TP-0184 decreased hepatic hepcidin mRNA and improved hemoglobinization [117,118].

The value of ALK2 as a critical regulator of inflammatory hepcidin induction has been verified by independent studies using momelotinib, a JAK1/2 inhibitor that is under clinical evaluation for the treatment of myelofibrosis. A completed phase 1/2 clinical trial with myelofibrosis patients showed that this drug also improves anemia [119]. This finding was surprising because JAK1/2 signaling is required for erythropoiesis stimulation via erythropoietin [120]. Experiments with the PG-APS rat model of AI confirmed that momelotinib ameliorates anemia in response to hepcidin inhibition; moreover, they demonstrated that this response is caused by momelotinib-mediated targeting of ALK2 [121].

Evaluation of data from a genome-wide RNAi screen for hepcidin regulators, with a focus on targets of clinically applied drugs, identified spironolactone and imatinib as hepcidin suppressors [75]. Spironolactone is an aldosterone antagonist commonly used to treat hypertension and congestive heart failure, while imatinib is a tyrosine kinase inhibitor that is used in cancer therapy. Both drugs appear to reduce hepcidin expression in hepatoma cells, in primary hepatocytes and in mice. The mechanism is not well understood, but appears to require intact BMP/SMAD signaling.

11.3. Neutralizing Antibodies against IL-6 Receptor or IL-6

IL-6 is critical for inflammatory induction of hepcidin, and Il6^{-/-} (IL-6 knockout) mice exhibit an impaired hypoferrremic response to acute inflammation [122]. Thus, neutralizing IL-6 or its receptor offers another potential option for the management of AI. Tocilizumab, a humanized anti-IL-6 receptor antibody, has a good safety profile and is used as an immunosuppressive drug mainly for the treatment of rheumatoid arthritis [123]. Clinical studies showed that it can also significantly reduce serum hepcidin and improve anemia in rheumatoid arthritis patients [124–126]. In addition, tocilizumab [127], as well as the anti-IL-6 antibody siltuximab [128], lowered hepcidin and ameliorated anemia in multicentric Castleman disease patients. Tocilizumab or another IL-6 receptor antibody (MR16-1) had similar therapeutic effects in a cynomolgus monkey model of collagen-induced arthritis [129], and in a mouse model of cancer-related anemia [130], respectively. Nevertheless, the high cost of these drugs may restrict their applicability beyond the treatment of rheumatoid arthritis and Castleman disease.

11.4. Small Molecule Inhibitors of JAK/STAT3 Signaling

There are no appropriate small molecule inhibitors of hepcidin that specifically target the JAK/STAT3 signaling pathway downstream of IL-6 and the IL-6 receptor. Another caveat is that any specific inhibitors of JAK1/2 would also be incompatible with the requirement of this protein in erythropoietin signaling [120]. STAT3 inhibitors, such as curcumin, AG490 or PpYlKTK were reported to inhibit inflammatory induction of hepcidin in cultured cells [131], and in case of AG490 also in mice [132]. Moreover, a single oral dose of curcuma containing 2% curcumin decreased hepcidin in healthy volunteers; however, this had no effect on serum iron [133]. Acetylsalicylic acid (aspirin) was shown to downregulate hepcidin by decreasing JAK2 and STAT3 phosphorylation in murine BV-2 microglia [134] and rat PC12 pheochromocytoma cells [135], but the relevance of this finding to

hepatocellular hepcidin is unknown. Generally, STAT3 inhibitors exhibit low target specificity and selectivity, and therefore do not appear optimal candidates for the treatment of AI. Nevertheless, some promising data have been obtained in preclinical models.

For instance, maresin 1, a macrophage-derived ω -3 fatty acid metabolite, exhibited protective anti-inflammatory effects in Il10^{-/-} (IL-10 knockout) mice, which develop colitis with iron deficiency anemia [136]. Further experiments showed that maresin 1 inhibits hepcidin by reducing STAT3 phosphorylation, and thereby ameliorates anemia in this model [137]. A polysaccharide isolated from the roots of the medicinal herb *Angelica sinensis* was found to repress hepcidin expression in HepG2 cells and to relieve anemia in rat models of AI. The polysaccharide appears to operate by inhibiting both JAK/STAT3 and BMP/SMAD signaling pathways [138–140].

Hydrogen sulfide (H₂S) is a gasotransmitter with signaling properties [141]. Pharmacological administration of H₂S was shown to suppress hepcidin in mouse models of acute lipopolysaccharide-induced or chronic turpentine-induced inflammation; mechanistic experiments revealed that H₂S inhibits IL-6 production and STAT3 phosphorylation in response to IL-6 signaling [142,143]. Further studies showed that H₂S promotes JAK2 degradation by a pathway involving adenosine 5'-monophosphate-activated protein kinase (AMPK), a metabolic enzyme that controls intracellular AMP/ATP ratio. Moreover, activation of AMPK by metformin, a widely used antidiabetic drug, inhibited hepcidin expression and thereby relieved hypoferrremia and anemia in mouse models of inflammation. Notably, metformin intake was also associated with low circulating hepcidin and reduced anemia morbidity in diabetic patients [144]. On the other hand, activation of AMPK by metformin was reported to induce hepcidin in cultured Huh7 hepatoma cells [79]; these seemingly conflicting data highlight the need for more mechanistic studies.

An in-silico screen identified *guanosine 5'-diphosphate* (GDP) as a hepcidin-binding molecule. GDP was further shown to operate as a competitive inhibitor of hepcidin that prevents hepcidin-mediated internalization of ferroportin in cultured cells. GDP (combined with ferrous sulfate) also inhibited hepcidin and improved anemia in a mouse model of inflammation, which was partly due to reduced STAT3 phosphorylation [145].

11.5. Sex Hormones

Testosterone, the primary male sex hormone and anabolic steroid, inhibits hepcidin by enhancing *epidermal growth factor receptor* (EGFR) signaling [146] and by reducing BMP/SMAD signaling [147]. Along these lines, testosterone therapy is known to cause erythrocytosis [148]. In female heat-killed *Brucella abortus*-inoculated mice, administration of testosterone improved hemoglobinization by suppressing hepcidin and stimulating erythropoiesis [149]. Similar effects were observed in clinical trials with healthy or anemic men [150–152] and men with type 2 diabetes and concurrent hypogonadotropic hypogonadism [153]. Conversely, androgen deprivation therapy reduced erythropoiesis and increased hepcidin in men with prostate cancer [154]. These findings suggest that testosterone could be valuable for the treatment of AI; however, cardiovascular adverse effects associated with testosterone administration limit its clinical applications [155].

17 β -Estradiol, the major female sex hormone, was shown to suppress hepcidin transcription in Huh7 and HepG2 hepatoma cells via an estrogen responsive element half-site located in *HAMP* promoter. Nonetheless, while administration of 17 β -estradiol to mice reduced hepcidin, it did not affect serum iron levels [156]. These findings are in line with a study demonstrating a significant reduction in serum hepcidin without correlation with serum iron in female patients with elevated estrogens, due to fertility treatments [157]. On the other hand, in a study with premenopausal women during various phases of their monthly cycle, serum 17 β -estradiol was negatively correlated with hepcidin and positively correlated with iron [158]. Definitely, more work is required to clarify the mechanism and physiological relevance of hepcidin regulation by estrogens before considering any therapeutic applications.

11.6. Vitamin D

Vitamin D exerts various biological functions upon binding to *vitamin D receptor* (VDR); these include the stimulation of intestinal absorption of calcium, magnesium and phosphate, but also modulation of signaling pathways [159]. There is increasing evidence that vitamin D levels are inversely associated with anemia in chronic inflammatory diseases [160]. Experiments in cultured hepatoma cells, monocytic cells and peripheral blood monocytes demonstrated that vitamin D inhibits expression of inflammatory cytokines; and suppresses hepcidin by a mechanism involving VDR binding to the *HAMP* promoter [161,162]. Moreover, vitamin D supplementation to healthy volunteers decreased circulating hepcidin levels [161]. This finding was further validated in randomized controlled trials with healthy adults [163], stage 2–3 CKD patients [162] and mechanically ventilated critically ill patients [164]. However, in other randomized controlled trials, vitamin D supplementation failed to correct hepcidin in non-dialysis stage 2–5 pediatric CKD patients [165] and stage 3–4 adult CKD patients [166]. These controversial findings do not provide compelling evidence that vitamin D has a pharmacological value as a hepcidin-lowering agent in AI. Nevertheless, they may be confounded by differences in nutritional forms of vitamin D, dosage, and severity of disease. The interest in this area of research remains high, as illustrated by several clinical trials that are currently underway (ClinicalTrials.gov Identifiers: NCT03001687, NCT03145896, NCT02714361, NCT03166280, NCT01632761, NCT03718182, NCT03472833), which may provide more conclusive answers.

Table 4. Inhibitors of hepcidin expression. ALK, activin receptor-like kinase; IL-6, interleukin 6; EGFR, epidermal growth factor receptor; CKD, chronic kidney disease.

	Drug	Target	Evidence	Reference
In vitro studies	Genistein (small molecule)	BMP6	Macrophages	Abreu et al. 2018 [97]
	Erythroferone	BMP6	Hepatoma cells	Arezes et al. 2018 [24]
	sHJV.Fc (antibody-like fused protein)	BMP6	Hepatoma cells, Kidney cells	Babitt et al. 2007 [101] Andriopoulos et al. 2009 [102]
	Dorsomorphin (small molecule)	Type I BMP receptors (ALK2/3/6)	Hepatoma cells	Yu et al. 2008 [106]
	LDN-193189 (dorsomorphin derivative)	Type I BMP receptors (mainly ALK2)	Primary hepatocytes	Theurl et al. 2011 [15]
	LDN-212854 (dorsomorphin derivative)	Type I BMP receptors (mainly ALK2)	Hepatoma cells	Mohedas et al. 2013 [111]
	Spironolactone (aldosterone antagonist used to treat hypertension)	BMP/SMAD signaling?	Hepatoma cells Primary hepatocytes	Mleczko-Sanecka et al. 2017 [75]
	Imatinib (tyrosine kinase inhibitor used in cancer therapy)	BMP/SMAD signaling?	Hepatoma cells Primary hepatocytes	Mleczko-Sanecka et al. 2017 [75]
	AG490, PpYLKTK, curcumin (small molecules)	STAT3	Differentiated hepatocytes	Fatih et al. 2010 [131]
	Aspirin (cyclooxygenase inhibitor for pain treatment)	JAK2, STAT3	Microglia cells Pheochromocytoma cells	Li et al. 2016 [134] Huang et al. 2018 [135]
	<i>Angelica sinensis</i> polysaccharide (small molecule)	SMAD4, STAT3/5	Hepatoma cells	Wang et al. 2017 [139]
	GDP	STAT3	Hepatoma cells Colorectal adenocarcinoma cells	Angmo et al. 2017 [145]
	17 β -Estradiol	Estrogen responsive promoter	Hepatoma cells	Yang et al. 2012 [156]
Calcitriol	Vitamin D receptor	Hepatoma cells Leukemia cells	Bacchetta et al. 2014 [161] Zughaier et al. 2014 [162]	

Table 4. Cont.

	Drug	Target	Evidence	Reference
Preclinical studies	Heparin		Wild type mice	Poli et al. 2011 [96]
	Glycol-split heparin	BMP6	Mouse model of AI BMP6 knockout mice	Poli et al. 2014 [98]
	Oversulfated heparin	BMP6	Mouse model of AI	Poli et al. 2014 [99]
	sHJV.Fc (antibody-like fused protein)	BMP6	Mouse model of human SPTB Rat model of AI	Babitt et al. 2007 [101] Andriopoulos et al. 2009 [102] Theurl et al. 2011 [15]
	ABT-207 (monoclonal Ab)	HJV	Wild type rats Cynomolgus monkeys	Boser et al. 2015 [103]
	H5F9-AM8 (monoclonal Ab)	HJV	Mouse models of IRIDA and IA Rat model of AI, wild type rats Cynomolgus monkeys	Boser et al. 2015 [103] Kovac et al. 2016 [104]
	Dorsomorphin (small molecule)	Type I BMP receptors (ALK2/3/6)	Zebrafish embryos	Yu et al. 2008 [106]
	LDN-193189 (dorsomorphin derivative)	Type I BMP receptors (ALK2/3/6)	Rat model of AI Mouse model of AI	Theurl et al. 2011 [15] Theurl et al. 2014 [61] Mayeur et al. 2015 [107]
	Myricetin	SMAD 1/5/8	Wild type mice	Mu et al. 2016 [112]
	DS79182026 (small molecule)	ALK2	Mouse model of AI	Fukuda et al. 2017 [115] Sasaki et al. 2018 [116]
	TP-0184 (small molecule)	ALK2	Mouse model of AI	Peterson et al. 2015 [118] Peterson et al. 2016 [117]
	Momelotinib (JAK1/2 inhibitor for myelofibrosis treatment)	ALK2	Rat model of AI	Asshoff et al. 2017 [121]
	Spiroinolactone (aldosterone antagonist used to treat hypertension)	BMP/SMAD signaling?	Wild type mice	Mleczo-Sanecka et al. 2017 [75]
	Imatinib (tyrosine kinase inhibitor used in cancer therapy)	BMP/SMAD signaling?	Wild type mice	Mleczo-Sanecka et al. 2017 [75]
	Tocilizumab (monoclonal Ab for rheumatoid arthritis treatment)	IL-6	Cynomolgus monkey model of AI Mouse model of cancer anemia	Hashizume et al. 2010 [129] Noguchi-Sasaki et al. 2016 [130]
	MR16-1 (monoclonal Ab)	IL-6	Mouse model of cancer anemia	Noguchi-Sasaki et al. 2016 [130]
	AG490 (small molecule)	STAT3	Wild type mice	Zhang et al. 2011 [132]
	Maresin 1 (ω -3 fatty acid derivative)	STAT3	Mouse model of AI	Marcon et al. 2013 [136] Wang et al. 2016 [137]
	<i>Angelica sinensis</i> polysaccharide (small molecule)	SMAD4, STAT3/5	Rat model of IDA Rat model of AI	Liu et al. 2012 [138] Wang et al. 2017 [139] Wang et al. 2018 [140]
	H ₂ S (gasotransmitter)	JAK2/STAT3	Mouse model of AI	Xin et al. 2016 [142] Wang et al. 2017 [143]
GDP	STAT3	Mouse model of AI	Angmo et al. 2017 [145]	
Testosterone	SMAD1/4 or EGFR signaling	Liver-specific hepcidin-overexpressing mice Bmp6 ^{-/-} mice Mouse model of AI	Guo et al. 2013 [147] Latour et al. 2014 [146] Guo et al. 2016 [149]	
Clinical trials	LY3113593 (monoclonal Ab) By: Eli Lilly and Company	BMP6	Healthy subjects, CKD patients—Phase 1 (Completed)	ClinicalTrials.gov Identifiers: NCT02144285, NCT02604160
	sHJV.Fc (antibody-like fused protein) By: FerruMax Pharmaceuticals	BMP6	CKD patients—Phase 1 (Discontinued)	ClinicalTrials.gov Identifiers: NCT01873534, NCT02228655
	TP-0184 (small molecule) By: Tolero Pharmaceuticals Inc.	ALK2	Advanced solid tumor patients—Phase 1 (Active)	ClinicalTrials.gov Identifier: NCT03429218
	Momelotinib (JAK1/2 inhibitor used to treat myelofibrosis)	ALK2	Myelofibrosis patients—Phase 1/2 (Completed)	Pardanani et al. 2013 [119]
	Tocilizumab (monoclonal Ab for rheumatoid arthritis treatment)	IL-6	Rheumatoid arthritis patients Castleman disease patients	Song et al. 2010 [127] Song et al. 2013 [124] Isaacs et al. 2013 [125] Suzuki et al. 2017 [126]

Table 4. Cont.

	Drug	Target	Evidence	Reference
Clinical trials	Siltuximab (monoclonal Ab for neoplastic disease treatment)	IL-6	Castleman disease patients	Casper et al. 2015 [128]
	Curcumin (small molecule)	STAT3	Healthy subjects	Laine et al. 2017 [133]
	Testosterone	SMAD1/4 or EGFR signaling	Type 2 diabetes patients with hypogonadotropic hypogonadism	Dhindsa et al. 2016 [153]
	17 β -Estradiol	Estrogen responsive promoter	Patients with growth hormone deficiency/hyperthyroidism/hyperprolactinemia Premenopausal women	Lehtihet et al. 2016 [157] Bajbouj et al. 2018 [158]
	Vitamin D ₂	Vitamin D receptor	Healthy subjects	Bacchetta et al. 2014 [161]
	Vitamin D ₃	Vitamin D receptor	CKD patients Healthy subjects Critically ill patients	Zughaier et al. 2014 [162] Smith et al. 2017 [163] Smith et al. 2018 [164]

12. Hepcidin Antagonists

Hepcidin can be antagonized directly by hepcidin-binding inhibitors, which neutralize hepcidin and reduce its availability to interact with ferroportin. Alternatively, hepcidin can be antagonized indirectly by molecules that bind to ferroportin and competitively inhibit the binding of hepcidin without affecting iron export. A detailed list is shown in Table 5. Clinically relevant hepcidin antagonists currently undergoing evaluation in randomized controlled trials for AI are highlighted in Figure 1.

12.1. Direct Hepcidin Inhibitors

Administration of human hepcidin antibodies effectively neutralized hepcidin and triggered responses to hepcidin deficiency in human hepcidin knock-in mice [167,168] and in cynomolgus monkeys [168,169]. Furthermore, the therapeutic use of hepcidin antibodies improved hemoglobinization in the heat-killed *Brucella abortus* model of AI, established in hepcidin knock-in mice [167,168]. Eli Lilly and Company developed LY2787106, a human hepcidin monoclonal antibody. Its safety profile and efficacy to improve erythropoiesis were evaluated in a phase 1 randomized controlled clinical trial with patients having cancer-associated anemia. LY2787106 was well tolerated and promoted an increase in serum iron and transferrin saturation within 24–48 h. However, the changes were temporary, and values returned to baseline within a week after dosing. Moreover, there was no correction of anemia, even when the LY2787106 treatment was combined with iron supplementation [170].

Anticalins are engineered polypeptides that derive from human lipocalins and mimic antibody activity by binding to protein targets [171]. Like other therapeutic proteins, anticalins can be PEGylated (coupled with polyethylene glycol) to improve pharmacological properties, such as solubility, stability and plasma half-life [172]. Pieris Pharmaceuticals Inc launched PRS-080, a hepcidin-neutralizing PEGylated anticalin. The safety profile and efficacy of PRS-080 were verified in a phase 1 clinical trial with healthy subjects. Apart from mild adverse effects (mostly headache) in some patients, PRS-080 was well tolerated and decreased hepcidin one hour after infusion. This in turn led to increased serum iron, transferrin saturation and ferritin [173]. PRS-080 is currently being evaluated in phase 1b and 2a clinical trials with CDK patients undergoing hemodialysis (ClinicalTrials.gov Identifiers: NCT02754167 and NCT03325621, respectively).

Spiegelmers are synthetic aptamers made of L-oligoribonucleotides, which are engineered to bind with high affinity and selectivity to low-molecular weight protein or peptide targets [174]. NOXXON Pharma AG developed NOX-H94 (or Lexaptetid Pegol), a PEGylated Spiegelmer that binds to and inactivates hepcidin. In preliminary tests NOX-H94 effectively antagonized hepcidin-induced ferroportin internalization in cultured J774 macrophages, and reversed inflammation-related hypoferrinemia in cynomolgus monkeys [175]. In a phase 1 randomized control clinical trial with healthy volunteers, NOX-H94 inhibited hepcidin in a dose-dependent manner and promoted

increases in serum iron levels with only minor adverse effects (mild increase in transaminases at high doses) [176]. In another phase 1 trial with human volunteers who developed endotoxemia following lipopolysaccharide injections, NOX-H94 neutralized high levels of hepcidin and corrected endotoxin-induced hypoferremia [177]. In a phase 2b clinical trial with ESA-hyporesponsive CKD patients undergoing hemodialysis, NOX-H94 significantly elevated serum iron and improved hemoglobinization [178]. Similar effects were observed in a phase 2a clinical trial with patients having cancer-related anemia [179].

Table 5. Hepcidin antagonists.

	Drug	Target	Evidence	Reference
In vitro studies	Fursultiamine (small molecule)	Ferroportin	Kidney cells	Fung et al. 2013 [180]
	Quinoxaline (small molecule)	Ferroportin	Kidney cells, breast cells, leukemia cells	Ross et al. 2017 [181]
Preclinical studies	LY2928057 (monoclonal Ab)	Ferroportin	Cynomolgus monkeys	Witcher et al. 2013 [182]
Clinical trials	LY2787106 (monoclonal Ab) By: Eli Lilly and Company	Hepcidin	Patients with cancer-associated anemia—Phase 1 (Completed)	Vadhan-Raj et al. 2017 [170]
	PRS-080 (Pegylated anticalin) By: Pieris Pharmaceuticals GmbH	Hepcidin	Anemic CKD patients—Phase 1b/2a (Recruiting)	ClinicalTrials.gov Identifiers: NCT02754167, NCT03325621
	NOX-H94 (Pegylated spiegelmer)	Hepcidin	Healthy subjects—Phase 1 (Completed) Endotoxemia-induced in volunteers—Phase 1 (Completed) Patients with cancer-associated anemia—Phase 2a (Completed) ESA-hyporesponsive anemia in CKD patients—Phase 2b (Completed)	Boyce et al. 2016 [176] Van Eijk et al. 2014 [177] Macdougall et al. 2015 [178] Georgiev et al. 2014 [179]
	LY2928057 (monoclonal Ab) By: Eli Lilly and Company	Ferroportin	Healthy subjects and hemodialyzed patients—Phase 1 (Completed)	Barrington et al. 2016 [183]

12.2. Ferroportin-Binding Hepcidin Inhibitors

High-throughput screens have been performed to identify small molecule ferroportin-binding hepcidin inhibitors. In one of them the best hit was fursultiamine, a thiamine (vitamin B₁) derivative. This drug irreversibly reacts with the C326 thiol residue in ferroportin that is essential for binding to hepcidin. Consequently, fursultiamine efficiently prevented hepcidin-mediated internalization of ferroportin in an in vitro assay. However, it failed to elicit biologic responses in vivo, presumably due to its conversion to inactive metabolites in the bloodstream [180]. Another screen identified sulfonyl quinoxaline, a synthetic compound that likewise reacts with C326 in ferroportin and prevents its hepcidin-mediated internalization in vitro [181]; the in vivo efficacy of this drug is currently unknown. The screen also identified compounds that inhibit ferroportin internalization without interfering with the binding of hepcidin; however, these were shown to be non-specific [181].

Eli Lilly and Company developed LY2928057, a humanized monoclonal ferroportin antibody that recognizes the fifth extracellular loop of ferroportin and thereby precludes the binding of hepcidin. LY2928057 maintains iron efflux from ferroportin in the presence of hepcidin, and promotes a dose-dependent increase in serum iron and hepcidin levels in cynomolgus monkeys [182]. The antibody recently completed phase 1 clinical trials and was well tolerated in hemodialyzed CKD patients. As expected, it caused an increase in serum iron and transferrin saturation; however, the effects on hemoglobinization were modest [183]. Further monoclonal antibodies against human ferroportin that recognize epitopes in the fifth extracellular loop were developed by Amgen. They were shown to partially prevent hepcidin-mediated ferroportin internalization [181], but their therapeutic potential has not been evaluated yet.

13. Conclusions

Within a few years after its discovery in 2001, hepcidin emerged as a therapeutic target for the treatment of iron-related “hepcidinopathies”, and possibly also for metabolic and infectious disorders. The clinical relevance for pharmacological manipulation of hepcidin pathways was established by experiments in preclinical animal models of HH, iron-loading anemias, IRIDA and AI. This led to the development of hepcidin therapeutics. Drugs that enhance or reduce hepcidin action are currently being tested in randomized controlled trials and some of them are expected to reach the clinic.

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Review

Therapeutic Advances in Regulating the Hepcidin/Ferroportin Axis

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Abstract: The interaction between hepcidin and ferroportin is the key mechanism involved in regulation of systemic iron homeostasis. This axis can be affected by multiple stimuli including plasma iron levels, inflammation and erythropoietic demand. Genetic defects or prolonged inflammatory stimuli results in dysregulation of this axis, which can lead to several disorders including hereditary hemochromatosis and anaemia of chronic disease. An imbalance in iron homeostasis is increasingly being associated with worse disease outcomes in many clinical conditions including multiple cancers and neurological disorders. Currently, there are limited treatment options for regulating iron levels in patients and thus significant efforts are being made to uncover approaches to regulate hepcidin and ferroportin expression. These approaches either target these molecules directly or regulatory steps which mediate hepcidin or ferroportin expression. This review examines the current status of hepcidin and ferroportin agonists and antagonists, as well as inducers and inhibitors of these proteins and their regulatory pathways.

Keywords: iron metabolism; hepcidin; iron homeostasis; ferroportin

1. Introduction

Iron is an essential component of many cellular processes including oxygen transport, DNA replication and repair and as an enzyme cofactor [1,2]. While iron is abundant within the Earth's crust, ferrous (Fe^{2+}) iron spontaneously reacts with oxygen to form ferric (Fe^{3+}) iron, which is insoluble under physiological conditions [3]. This presents the first of many challenges that organisms face in meeting their needs for adequate iron absorption, as organisms require mechanisms to convert Fe^{3+} to Fe^{2+} . The second challenge organisms face is iron's ability to undergo redox reactions via Fenton chemistry, which result in reactive oxygen species (ROS). These destructive ROS have the potential to damage proteins, lipid membranes and nucleic acids and result in tissue damage [3,4]. As there is currently no known export mechanism for excess iron, its metabolism is tightly regulated through transcriptional, post-transcriptional, translational, and post-translational mechanisms to prevent toxic iron excess while maintaining the metabolic needs of the organism [5,6].

An adult human body contains 3–5 g of iron, much of which is supplied to developing erythroblasts for incorporation within the haem group of haemoglobin (70% of bodily iron) [3]. Other tissues which are major users of iron include the muscles (2–3%) where it is present in the haem group of myoglobin, tissue macrophages (5%) involved in red blood cell (RBC) recycling and liver hepatocytes (20%) where excess iron is stored within ferritin. Humans require around 20–25 mg of iron per day for the synthesis of new RBCs; however, only about 1–2 mg of iron is absorbed per day via the enterocytes of the duodenum. The majority of iron (90%) is supplied by the recycling of RBCs by macrophages [2,6].

2. Hecpidin Regulation

Hepcidin is an 84-amino-acid long prepropeptide produced in hepatocytes and excreted in urine as a 25-amino-acid mature form [7]. It was originally thought to function solely as an antimicrobial peptide as it is upregulated under inflammatory conditions and as such is considered to be a type two acute-phase reactant due to its regulation via interleukin 6 (IL-6) [7–9]. The 24-amino-acid N-terminal signal peptide of pre-prohepcidin is cleaved to produce prohepcidin, comprising 60 amino acids [10]. The mature 25-amino-acid form of hepcidin [7] is generated via cleavage of the pro-region by furin-like prohormone convertases [10]. The carboxy terminus of the mature hepcidin, comprising four highly conserved disulphide bonds, forms a β -hairpin; the N-terminus is unstructured and essential for ferroportin (FPN) interaction [11]. This interaction results in reduced membrane concentrations of FPN, thus lowering levels iron availability to tissues [12].

The interplay between the iron exporter FPN and the hepatic peptide hormone, hepcidin, is central to iron homeostasis. Hepcidin binds to membrane-bound FPN and induces its internalisation and eventual degradation. The expression of these two proteins is highly regulated. The main regulatory pathway for liver hepcidin expression is the bone morphogenetic protein/Sma mothers against decapentaplegic (BMP/SMAD) pathway (Figure 1). The ligand BMP6, believed to be produced by liver sinusoidal endothelial cells [13], has been found to be the primary BMP associated with hepcidin transcription [14,15]. However, BMPs 2, 4 and 9 have also been shown to increase hepcidin expression [16,17]. Increased body iron stores induce BMP6 expression in the liver; BMP6 then binds to membrane-bound BMP receptors (BMPRs) with hemojuvelin (HJV) acting as a co-receptor [18]. BMPRs type 1 (activin-like kinase (ALK) 2 and ALK3) and type 2 (BMPR2 and Actr2A) are critical for iron balance [19,20]. BMP6 binding to BMPRs results in the phosphorylation of intracellular SMAD1/5/8 [21]. Phosphorylated SMAD1/5/8 then complexes with SMAD4, which translocates to the nucleus [22], where it binds to BMP-responsive elements (BMP-RE) on the hepcidin gene promoter inducing hepcidin expression [23].

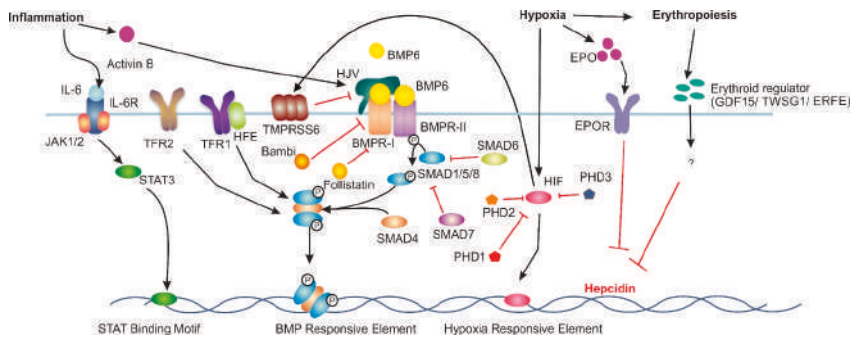


Figure 1. Major Signalling Pathways Involved in Hepcidin Regulation. Increased BMP6 levels induce hepcidin mRNA expression via the bone morphogenetic protein (BMP)/sma and mothers against decapentaplegic homologue (SMAD) pathway. Under inflammatory conditions, interleukin 6 (IL-6) induces hepcidin mRNA via the Jak/signal transducer and activator of transcription (STAT) pathway. Hypoxia induces hepcidin downregulation through increases in erythropoietin (EPO) expression. Hypoxia Inducible Factor (HIF) can also directly downregulate hepcidin. Additionally, HIF upregulates matriptase-2 expression, which results in decreased hepcidin expression. Lastly, erythropoiesis has been theorised to reduce hepcidin expression acting through growth differentiation factor 15 (GDF-15), twisted gastrulation factor 1 (TWSG1) or erythroferrone (ERFE), however the exact mechanism is not understood. Interleukin-6 Receptor (IL6-R), Transferrin Receptor 1 (TFR1), Hemojuvelin (HJV), Bone Morphogenetic Protein Receptor Type 1 (BMPR-I), Bone Morphogenetic Protein Receptor Type 2 (BMPR-II), Erythropoietin Receptor (EPOR) and prolyl hydroxylase domain containing enzymes (PHD) are involved in hepcidin regulation.

An et al. demonstrated that phosphorylation of SMAD1/5/8 is regulated by the inhibitory SMADs 6 and 7 [24]. Hepatocyte-specific *Smad7* knockout mice demonstrated a decrease in non-haem iron within the liver and spleen, in addition to decreases in L-ferritin and FPN levels [24]. Conversely, hepcidin and phosphorylated SMAD1/5/8 levels were increased in these mice [24]. SMAD6, BMP, activin membrane-bound inhibitor homolog (Bambi) and follistatin have been shown to be inhibitors of hepcidin expression in a *Smad7* knockout mouse model fed an iron-rich diet [24]. SMAD6 is known to inhibit the phosphorylation of other SMAD proteins while both Bambi and follistatin inhibit the BMP pathway through interacting with the BMPRs and BMPs respectively [24]. Interestingly, An et al. found that SMAD6 and Bambi were controlled by the BMP/SMAD pathway, while follistatin was unaffected [24]. This may indicate why SMAD6 and Bambi are unable to substitute for SMAD7 under normal iron conditions.

BMP6 and iron levels have also been shown to increase the expression of the transmembrane serine protease, matriptase-2 (TMPRSS6) [25]. TMPRSS6 acts as a negative regulator of hepcidin, having been shown to cleave HJV and thus reduce the available membrane-bound HJV [26]. In addition, Lin et al. found that soluble HJV (sHJV) competes with membrane-bound HJV for ligation with BMPs resulting in hepcidin suppression [27].

Hepcidin regulation under inflammatory conditions involves the IL6/signal transducer and activator of transcription (IL6/STAT) pathway [28]. IL6 released during inflammation binds to its receptors, which in turn induce Janus kinase 1 (JAK) to phosphorylate STAT3 [29]. STAT3 translocates to the nucleus where binding to the STAT binding motif on the *Hepcidin* gene promoter activates expression [28]. Interestingly, intact SMAD1/5/8 function is required for maximal induction of hepcidin via the IL6/STAT3 pathway [30]. It has been suggested that activin B may be responsible for the cross talk between the IL6/STAT3 and BMP/SMAD pathways. Activin B promotes hepcidin activation, acting as a surrogate ligand for SMAD1/5/8 in the BMP/SMAD pathway during infection. Activin B interacts with type 2 BMPR ActR2A and type 1 receptors ALK2 and ALK3 to stimulate *hepcidin* expression via SMAD1/5/8 phosphorylation as described above [30,31].

In addition to the BMP6/SMAD and IL6/STAT pathways, iron levels are also regulated by hypoxia. Hypoxia Inducible Factor (HIFs), members of the heterodimeric nuclear transcription factor family are the main protein complexes that result in changes in gene expression under hypoxic conditions [32].

HIF complexes regulate a large variety of genes, although the current review focuses on the genes involved with iron regulation. One of the most well studied iron pathway genes regulated by HIF is erythropoietin (EPO). Initially, it was believed that HIF1 α was the major HIF isoform involved with EPO regulation, however multiple knockout studies in mice have confirmed that HIF2 α is the primary regulator of hypoxia induced EPO expression [33,34]. This led to the discovery of EPO-dependent mechanisms of hepcidin downregulation. Lui et al. discovered HIF suppression of hepcidin required EPO-induced erythropoiesis in a *Vhl/Epo*^{-/-} mouse model [35]. EPO independent mechanisms for HIF regulation of hepcidin have also been discovered. Peyssonax et al. found murine and human hepcidin contains a Hypoxia-Response Element (HRE) within the hepcidin promoter that results in its down regulation [36]. The ability of HIF1 α to downregulate hepcidin was shown in vivo using a *Hif-1 α* ^{fllox/fllox} mouse model given an iron-deficient diet for 20 days that resulted in a 10-fold increase in hepcidin when compared with WT [36]. However, the direct role of HIF1 α on human hepcidin has come into question with subsequent studies suggesting no direct role for HIF [37].

HIF1 also indirectly regulates hepcidin through proteins involved with the previously mentioned BMP6/SMAD pathway. As previously discussed TMPRSS6 cleaves HJV decreasing the levels of membrane-associated HJV which acts to reduce hepcidin production [38]. Maurer et al. discovered a HRE within the promoter region of TMPRSS6 [39]. Lakhali et al. also demonstrated that TMPRSS6 expression increased in a HIF1-dependent manner during hypoxia [40].

Erythroblasts are responsible for utilising the largest proportion of iron within the body to produce haemoglobin [41]. Previous studies have shown that stimulated erythropoiesis suppresses hepcidin expression [41]; thus, it was long theorised that an erythroid regulator of hepcidin exists. However, the

exact molecular mechanism for this regulation is currently unclear. Several candidate molecules have been proposed as the erythroid regulator of iron homeostasis. Growth differentiation factor 15 (GDF-15) and twisted gastrulation factor 1 (TWSG1) are both cytokines produced by erythroblasts which have been found to suppress hepcidin expression in human liver cells [42,43]. However, in a *Gdf-15* knockout mouse, where erythropoiesis was stimulated via phlebotomy, there was no decrease in hepcidin expression [44]. Similarly, *Twsg1* was not increased in various mouse models of anaemia [45]. Currently, the most likely candidate for the erythroid regulator for hepcidin suppression is erythroferrone (*ERFE*). *Erfe* knockout mice have been shown to fail to suppress hepcidin following stimulated erythropoiesis, whereas heterozygotes display an intermediate degree of suppression [46]. In addition, knockout of *Erfe* in the Hbb^(Th3/+) thalassemia intermedia mouse model restored proper hepcidin expression with partial protection from iron overload [46]. However, *Erfe* knockout mice displayed no significant difference from control mice, suggesting erythroferrone plays a role as a stress erythropoiesis specific regulator and not the main erythropoiesis regulator [47].

Extrahepatic Hepcidin

In addition to the liver, hepcidin is also synthesised in a number of other organs including adipose tissue, brain kidney, heart, spleen, pancreas and stomach [48]. Within the kidney, where hepcidin is produced in the cortical thick ascending limb, hepcidin was shown to play a role in the absorption of non-haem iron through the down regulation of both divalent metal transport 1 (DMT1) and FPN [48]. The role of hepcidin within the spleen, stomach and the brain appear to be linked with its antimicrobial ability. Several macrophage cell types have been found to synthesise hepcidin when challenged with pathogens. Sow et al. infected RAW264.7 macrophages, mouse bone marrow-derived macrophages and human THP-1 monocytic cell with *Mycobacterium sp.*, which stimulated hepcidin mRNA and protein synthesis [49]. Within the stomach, hepcidin expression is regulated by bacterial infection and involved with gastric acid production [50]. Hepcidin in the brain has been found to be upregulated via inflammatory responses [51,52]. Hepcidin synthesis in adipose tissue has also been reported; however, the exact role of this type of hepcidin remains largely unknown [48]. Lastly, hepcidin production within the pancreas has been suggested to play a role in the regulation of insulin [48]. These secondary roles of hepcidin will need to be considered when drug candidates undergo animal studies and clinical trials to ensure these critical roles of hepcidin are not altered.

3. Regulation of FPN

The sole iron exporter FPN was identified by three independent research groups in early 2000 [53–55]. FPN is highly expressed in the liver Kupffer cells, periportal hepatocytes, duodenal enterocytes, splenic red pulp macrophages, and the placental syncytiotrophoblast [6]. FPN typifies the multilevel regulatory pathway of iron homeostasis (Figure 2). The primary method of FPN regulation is post-translationally via hepcidin [11]. Once hepcidin has bound to FPN, it results in its ubiquitination, internalisation and degradation [56]. Thus, this interaction between hepcidin and FPN plays a central role in the regulation of bodily iron levels.

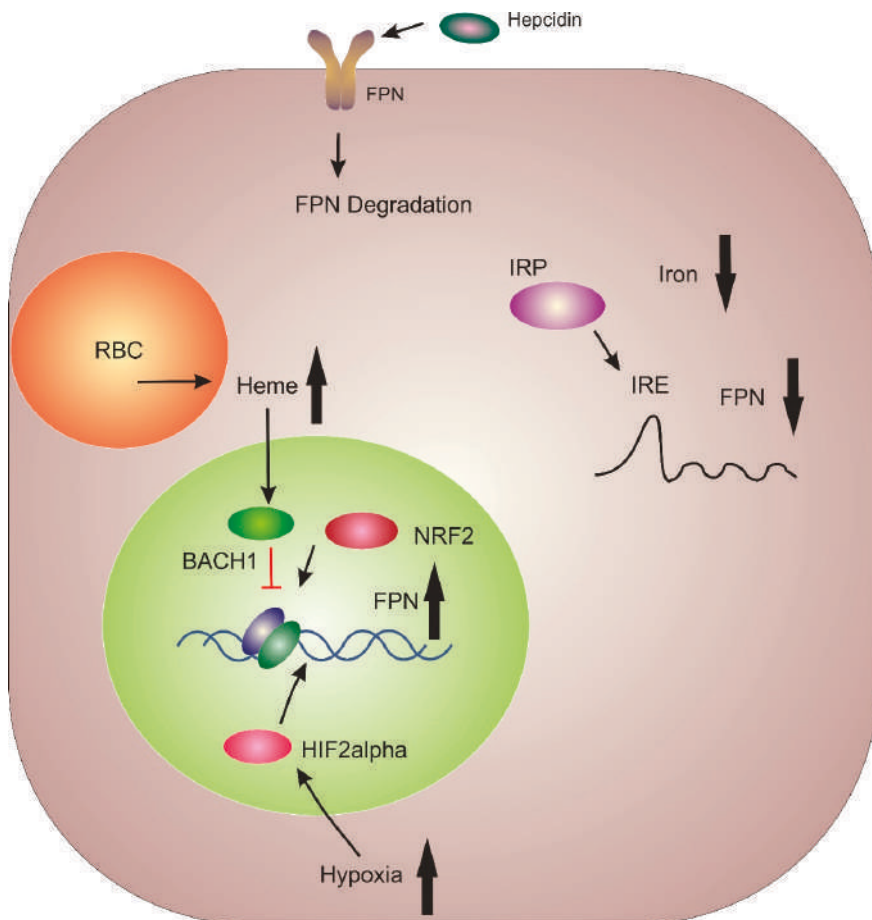


Figure 2. Major Regulatory mechanisms for ferroportin. BTB Domain and CNC Homolog 1 (BACH1) and Nuclear factor erythroid 2-related factor 2 (NRF2) act as a transcriptional repressor and activator of ferroportin (FPN) transcription, respectively. Cytoplasmic iron levels regulate the interaction between iron-responsive element-binding proteins (IRPs) and the iron response element (IRE) located on the 5' end of FPN mRNA. Decreased iron levels result in IRP binding to the IRE and reduced FPN expression. Enterocytes express a variant of FPN that lacks the 5' IRE. Hypoxia induces FPN transcription in enterocytes via hypoxia inducible factor 2 α . Lastly, FPN is controlled post-translationally through complexation with hepcidin, which results in internalisation and degradation.

Btb and cnc homology 1 (BACH1) and nuclear factor erythroid 2-like (NRF2) proteins act as transcriptional repressors and activators, respectively, for FPN, in response to cytosolic haem levels within macrophages [57]. At the post-transcriptional level, microRNA (miRNA) miR-485-3p has been shown to inhibit FPN expression during iron deficiency [58]. Translational regulation of FPN occurs via iron-responsive element-binding proteins (IRPs) binding to the iron response element (IRE) located in the 5' UTR of FPN mRNA [57]. This results in suppression of FPN mRNA translation in enterocytes and macrophages under low iron conditions [59,60]. Within the duodenum, hypoxia results in the stabilisation of HIF2 α , which has been shown to increase FPN expression [6]. Additionally, a variant of FPN which lacks the 5' UTR is predominantly expressed in enterocytes which results in sustained FPN expression in enterocytes even under iron deficiency conditions [6].

FPN expression has also been shown to be downregulated in both liver and peritoneal macrophages by lipopolysaccharide (LPS) injections [61]. This regulation of *FPN* mRNA was shown to be a result of macrophage polarisation. Wortmannin (a phosphoinositide 3-kinase inhibitor) abolished FPN deregulation in LPS-challenged macrophages while the p38-mitogen-activated protein kinase inhibitor, SB203580, intensified FPN mRNA down-regulation [61]. Toll-like receptors 2, 3 and 4 have also been found to effect FPN downregulation under inflammatory conditions. However, their mode of action is only partially understood [6]. This has led to FPN being classified as a negative acute-phase protein [62].

4. Hepcidin/FPN Axis Dysregulation

Dysregulation of the hepcidin–FPN axis has been associated with the development of cancer. This most likely results from higher rates of cell proliferation requiring greater demand for iron [63]. Thus, reduced hepatic hepcidin was found to offer a protective effect against the progression of lung [64] and breast cancer [65,66]. In addition, the over-expression of FPN also displayed a protective effect as FPN overexpression reduced cell division and colony formation *in vitro* and *in vivo* [64,65]. Transferrin receptor 1 and divalent metal transporter 1 have also been found to be upregulated in breast cancer cells [67]. Decreased FPN in triple negative breast cancer cells has also been found to stimulate cell proliferation and migration likely a result of increase cellular iron levels [68]. Hepcidin promoter DNA has been found to be hyper-methylated in human hepatocellular carcinoma resulting in its transcriptional repression [69]. In prostate cancer, the prostate epithelial cells markedly increase synthesis of hepcidin leading to cancer growth and progression [70]. Overexpression of BMPs 2, 4 and 7 have all been linked to increased hepcidin expression in a number of cancers including prostate [70] and lung [71]. Lastly, *TMPRSS6* has been found to be downregulated in triple negative breast cancer [72].

Iron levels have also been suggested to play a role in the progression of Alzheimer’s disease. Rogers et al. discovered that amyloid-beta precursor proteins ($A\beta$ PP) mRNA contains IREs in the 5’ untranslated region UTR. These IREs interact with IRPs to stabilise the mRNA [73]. In addition, $A\beta$ PP has been demonstrated to assist in retaining FPN on the cell surface of neurons. However, how $A\beta$ PP supports FPN retention is still unclear [74]. Altered function of the protein α -synuclein has been demonstrated to play a pivotal role in the pathology of Parkinson’s disease [75]. Increased iron levels within the substantia nigra region of the brain have also been associated with the pathogenesis of Parkinson’s disease [76]. This iron overload is believed to result from the dysregulated expression of ferritin, divalent metal transporter 1 and ferroxidase [75].

An understanding of how hepcidin and FPN are regulated has provided us with candidates which can be tested to target the expression of hepcidin and FPN with the aim to modulate iron levels. An increasing body of evidence suggesting that altered iron levels play a role in disease progression in multiple conditions warrants development of therapeutic strategies to modulate iron levels. One of the ways to do this is to regulate the hepcidin–FPN axis. Several promising candidates have been tested in laboratories, of which several are undergoing clinical trials. The following sections summarise the current status of these approaches.

5. Current Therapeutic Treatments for Hepcidin Deficiency

A reduction or loss of hepcidin expression or function leads to increased iron being released into the blood. The current treatment for the iron overload disorder hereditary hemochromatosis (HH) involves weekly phlebotomy as tolerated by the patient (usually totalling no more than 500 mL) until the patient reaches a plasma ferritin level of approximately 50–100 μ g/L [77,78]. Once this has been achieved, the goal of the treatment is to maintain these levels. This is normally achieved through additional phlebotomy every three to six months to maintain normal body iron levels [77,78]. However, phlebotomy is not effective for reversing arthropathy, diabetes, cardiomyopathy, cirrhosis and hypogonadism [77–79], which are some of the related symptoms/pathologies that develop in hemochromatosis patients.

In addition to phlebotomy, several iron chelators including deferoxamine (DFO), deferiprone (DFP) and deferasirox (DFX) have also been used in the treatment of hereditary hemochromatosis (HH) [80].

6. Current Therapeutic Treatments for Lowering Hepcidin

6.1. Direct Hepcidin Inhibitors

6.1.1. Anti-Hepcidin Antibodies

Since the discovery of hepcidin as a key player in the regulation of iron homeostasis, it has been the target of multiple drug candidates to prevent it from interacting with FPN. Direct hepcidin inhibition has been investigated as a possible route for normalising hepcidin levels in patients suffering from hepcidin over expression, such as the anaemia of chronic disease (ACD) and iron-refractory iron-deficiency anaemia (IRIDA). Humanised monoclonal antibodies have been developed that display high affinity towards hepcidin leading to its premature degradation. Antibody (Ab) LY2787106 was shown to be well tolerated during its phase one clinical trial, demonstrating a significant increase in serum iron levels. Unfortunately, these increases were only transient and after eight days returned to baseline [81]. Sasu et al. developed the antibody, Ab2.7 which when used in combination with erythropoietin stimulating agents (ESAs) was shown to reverse hepcidin induced anaemia in a heat killed *Brucella abortus* induced mouse model [82]. The combined treatment was also shown to increase reticulocyte numbers. However, when compared with another direct hepcidin inhibitor, short hairpin RNA (shRNA), the antibody delivered a lower level of hepcidin inhibition. This may have been due to the affinity of the antibody or due to the high turnover of hepcidin within mouse models [82].

6.1.2. Short Interfering and Short Hairpin RNA

Short interfering RNA (siRNA) causing *HEPCIDIN* gene silencing represents another area of active development into the treatment of anaemia. Short hairpin RNAs (shRNAs) targeting hepcidin (H6 and H10) developed by Amgen were demonstrated to cause a reduction in hepcidin mRNA and anaemia when used in conjunction with ESAs [82]. As discussed above, these shRNAs displayed a more robust hepcidin inhibition than the anti-hepcidin antibodies (Ab2.7). Either H6 or H10 treated anaemic mice displayed increased serum iron compared to anaemic control mice treated with shRNAs [82]. The siRNA, ALN-HPN has also been shown to decrease hepcidin mRNA (> 80%) and increase serum iron (approximately two-fold) with a single intravenous dose [83].

6.1.3. Hepcidin-Binding Molecules

Anticalins are therapeutic ligand binding proteins developed from lipocalins [84]. Lipocalins are responsible for the transport of hydrophobic and chemically sensitive molecules in the human body [84]. PRS-080 is a human neutrophil gelatinase-associated lipocalin-derived anticalin engineered for hepcidin binding which results in decreased hepcidin protein levels and subsequently increased iron and transferrin saturation [85]. PRS-080 specifically binds human hepcidin with a dissociation constant (K_d) in the subnanomolar range (0.07 ± 0.05 nM) and cynomolgus monkey hepcidin (0.07 ± 0.06 nM) [86]. To decrease the rate of kidney filtration for PRS-080, multiple (20, 30 and 40 kDa) polyethylene glycol moieties were conjugated to PRS-080, which showed no change in activity compared with unmodified PRS-080, while increasing the half-life in cynomolgus monkey (18.8 ± 1.1 , 43.5 ± 6.0 and 167.5 ± 10.6 h, respectively) [86]. PRS-080 conjugated to the 30 kDa PEG (PRS-080#22) has undergone phase one clinical trials [87]. Healthy male patients were treated with IV doses of 0.08–16 mg/kg over a 2-h period. Decreases in serum hepcidin levels were recorded 1-h post infusion, with corresponding increases in serum iron and transferrin saturation from doses 0.4 mg/kg and higher [87]. Prolonged elevated serum iron and transferrin saturation levels were demonstrated to be dose dependent with increases from 18 h (0.4 mg/kg) to 120 h (16 mg/kg) [87]. These promising results have resulted in

PRS-080 progressing towards repeat dosage clinical trials. Another phase 1 clinical trial for PRS-080#22 displayed no adverse effects when given to healthy patients (16 mg/kg) and chronic kidney disease (CKD) patients undergoing haemodialysis (8 mg/kg). In addition, serum iron and transferrin saturation were both increased with PRS-808#22 treatment with the authors suggesting this indicates that the iron was transferrin bound and therefore highly functional [88].

In parallel, the organic phosphate guanosine 5,-diphosphate (GDP) has been shown to complex with hepcidin at the active site via multiple stable hydrogen bonds [89]. This complex then prevents hepcidin from interacting with FPN, resulting in reduced internalisation of FPN in HepG2 and Caco-2 cell lines. GDP appears to be unique in this sense, as other organic phosphates do not affect FPN degradation [89].

6.1.4. Hepcidin-Binding L-RNA Aptamers (Spiegelmers)

Aptamers are an emerging class of synthetic, structured oligonucleotide therapeutics. They consist of DNA, RNA or nucleotides ordered around a modified sugar backbone which display high affinity and specificity [90]. Nox-H94, a PEGylated anti-hepcidin L-RNA aptamer, has been shown to minimise hepcidin-induced FPN degradation and ferritin expression in cynomolgus monkeys [91]. A human study involving healthy adults investigated the safety, pharmacokinetics and pharmacodynamics of Nox-H94 and showed no serious adverse health effects [92] while resulting in an increase in serum iron and transferrin saturation levels [92] (Table 1).

Table 1. Summary table of hepcidin and ferroportin agonists and antagonists.

Model	Drug	Target
Direct Hepcidin Inhibitors		
In vitro	Guanosine 5, -diphosphate (GDP) [89]	
In vivo	Ab2.7, H6 and H10 [82], ALN-HPN [83]	Hepcidin
Clinical trial	LY2787106 [81], PRS-080 [87], Nox-H94 [92]	
Inhibitors of Hepcidin Production/Synthesis		
Unfractionated heparin		
In vitro	Enoxaparin, Fondaparinux [93]	BMP6
	RO-68 and RO-82 [94]	
	SLLMWH-19 [95]	
In vivo	Dorsomorphin [96], LDN-1913189 [97]	Type 1 BMPRs
	Momelotinib [98], TP-0184 [99]	ALK2
	Imatinib, spironolactone [100]	BMP/SMAD Pathway
	sHJV.Fc [97]	BMP6
	ABT-207, h5F9-AM8 [101]	HJV
	RNAi [83]	TFR2
	AG490 [102]	JAK
	PpYLKTK [103]	STAT3
	Metformin [104]	AMPK
		Indazole [105], DS79182026 [106]
Clinical Trail	Siltuximab [107], Tocilizumab [108]	IL-6
Hypoxia-Inducible Factors (HIF) Stabilisers		
Clinical trial	Vadadustat [109], Roxadustat [110], Daproustat [111]	PHD
Ferroportin Agonists		
In vitro	Fursultiamine [112], Anti-FPN mouse antibody [113]	FPN

Table 1. Cont.

Model	Drug	Target
Hepcidin Agonists		
In vitro	Hepcidin Cys19Ser [114]	FPN
	Sorafenib, Wortmannin, Rapamycin [115]	RAS/RAF and PI3
	Epitiostanol, Progesterone, Mifepristone [116]	PGRMC1
In vivo	PR73 [117], PR65 [118], mHS17, mHS26 [119], M004, M009 [120]	FPN
	TMPRSS6-ASO#1 [121], TMPRSS6-ASO#2 [121], GalNAc-ASO [122], RNAi-GalNAc [123],	TMPRSS6
	Naringenin, Quercetin, Resveratrol [124]	NRF2
	Adenine [125]	BMP6/SMAD pathway
	Genistein [126]	BMP-RE and STAT
	Ipriflavone, Vorinostat [127]	BMP6 and STAT3
Clinical Trial	Icariin, Epimedin [128]	STAT3 and Smad1/5/8
	LJPC-401 [129]	FPN
	RNAi LNP [130]	TMPRSS6

6.2. Inhibitors of Hepcidin Production/Synthesis

One of the most important pathways involved in regulating systemic hepcidin levels in response to several stimuli is the BMP-SMAD pathway [131]. Several studies have focussed on targeting this pathway to alter hepcidin levels and normalise iron levels. This section summarises a number of these approaches.

6.2.1. Heparin-Based Targeting of the BMP/SMAD Pathway

Heparin is comprised of repeating units of uronic acid and D-glucosamine or D-glucosamine N-sulphate. Unfractionated heparin (>4 µg/mL) was shown to sequester BMPs and block SMAD phosphorylation, which then resulted in reduced hepcidin mRNA in HepG2 cells [93]. This hepcidin lowering effect was also found with low molecular weight heparin, enoxaparin and fondaparinux [93]. These types of heparins require significantly greater concentrations (40 µg/mL and > 200 µg/mL, respectively) to repress hepcidin levels. However, the anticoagulant effect of heparin makes it difficult to use in mouse models [94]. Glycol-split variants, RO-68 and RO-82 have both been shown to lack anti-thrombin binding while remaining potent hepcidin inhibitors [94]. Super-sulphated heparin SSLMWH-19 was demonstrated to exhibit an even greater degree of hepcidin inhibition than either RO-68 or RO-82. However, SSLMWH-19 still retained marginal anticoagulant properties [95].

6.2.2. Bone Morphogenetic Protein Receptor (BMPR) Inhibitors

Dorsomorphin has been shown to inhibit type 1 BMPRs, which in turn results in decreased SMAD1/5/8 phosphorylation in zebrafish and mouse models [96]. LDN-193189, a dorsomorphin derivative, has been demonstrated to inhibit BMP4-mediated SMAD1/5/8 phosphorylation in rats with ACD, followed by an increase in serum iron and FPN levels and decrease in ferritin levels in the spleen [97]. Asshoff et al. recently developed the antibody momelotinib that targets ALK2 leading to a reduction in hepcidin production in anaemic rats [98]. Lastly, TP-0184 demonstrated an inhibitory effect against ALK2 with two oral doses eight hours apart ameliorating turpentine oil mediated anaemia in mice [99]. While both momelotinib and TP-0184 appear to be specific for only ALK2, both dorsomorphin and LDN-193189 have been found to have off target effects, which reduces their potential as a therapeutic agents [132].

Two clinically approved drugs (imatinib and spironolactone) were identified that decrease hepcidin through the BMP6 pathway in a variety of cell types (HuH7 and primary hepatocytes of both human

and mouse origin) and in male wild-type C57BL/6 mice [100]. Unfortunately, imatinib is not a suitable target for drug re-purposing due to several adverse effects including fatigue, nausea, vomiting, rash, peripheral oedema and abdominal pain. In contrast, spironolactone displays generally minor side effects [100].

6.2.3. Hemojuvelin (HJV) and Transferrin Receptor 2 (TFR2) Inhibitors

The BMP co-receptor HJV has been a major focus for hepcidin inhibition with a variety of techniques developed. Soluble HJV (sHJV) has previously been implicated in hepcidin inhibition [27]. sHJV fused with immunoglobulin fragment crystallisable region (sHJV.Fc) also displays hepcidin inhibitory ability via decreased SMAD1/5/8 phosphorylation. Treatment of ACD in rats with sHJV.Fc significantly increased haemoglobin levels after a 21-day period of *Streptococcal peptidoglycan-polysaccharide* treatment [97]. Boser et al. created HJV targeting antibodies (ABT-207 or h5F9-AM8) that after a single dose have been shown to increase serum iron and decrease unsaturated iron binding capacity (UIBC) in a rat model [101].

Several studies in humans and mice have shown that TFR2 is required for the proper regulation of hepcidin [133–135]. TFR2 also plays a role in the interaction of erythropoietin (EPO) and its receptor (EPO-R) [136]. TFR2 is required for the transport of the EPO-R from the endoplasmic reticulum to the cells surface [137]. Therefore, therapeutics have been developed that target the expression TFR2 with the aim of reducing hepcidin expression. A single IV dose of RNAi against TFR2 in mice led to a marked decrease in TFR2 and hepcidin mRNA while concurrently increasing transferrin saturation [83].

6.2.4. Targeting the IL-6/STAT3 Pathway

IL-6-mediated JAK-STAT activation of hepcidin is another major pathway involved in hepcidin regulation. Hence, blocking the IL-6 pathway has also been investigated as a therapeutic treatment for ACD. IL-6 targeting chimeric antibody siltuximab demonstrated decreased hepcidin levels in 97% of multiple myeloma and Castleman disease patients, with 75% of these patients showing haemoglobin increases of > 1.5 g/dL [107]. Another IL-6 targeting antibody, tocilizumab, decreased IL-6 mediated serum hepcidin in multicentric Castleman disease with hepcidin levels falling to within the normal limit in a two-week period [108].

The small molecule AG490 has been shown to be an inhibitor of JAK2 [138]. After a single injection of AG490, a 37% reduction in hepcidin levels was evident after 24 h in an ACD mouse model [102]. The synthetic STAT3 inhibitor phosphopeptide PpYLKTK, which affects STAT dimerisation [103], decreased hepcidin mRNA expression by 35% 2 h post infection in a mouse derived hepatocyte model [103].

AMP-activated protein kinase (AMPK) has recently been demonstrated to be involved in the regulation of hepcidin through inhibition of STAT3 [104]. Wang et al. subcutaneously injected C57BL/6 mice with 100 μ L/20 g body weight turpentine for four weeks to induce anaemia of chronic inflammation [104]. Mice were injected with 250 mg/kg metformin (an AMPK activator) displayed increased serum iron and transferrin saturation while the serum hepcidin, Jak2 and phospho-Stat3 levels were reduced [104]. In addition, pre-treatment of C57BL/6 mice with metformin before treatment with IL-6 (3 μ L/20 g body weight) reduced hepcidin mRNA and protein levels and restored serum iron levels.

In a retrospective analysis of 83 Chinese type two diabetes mellitus patients, metformin therapy was associated with a decrease in serum hepcidin levels in men [104]. Previously, Fukuda et al. found that intraperitoneal administration of indazole lowered hepcidin levels in mice [105]. However, this molecule had no effect when administered orally [105]. Using this molecule as a base, a specific oral inhibitor of hepcidin, DS79182026, was generated [106]. This molecule has been shown to display low off target kinase inhibition and a dose of 30 mg/kg in an acute inflammatory mouse model significantly reduced serum hepcidin levels [106].

6.2.5. Hypoxia-Inducible Factors (HIF) Stabilisers

HIF stabilisers are another class of drugs initially designed to increase EPO levels in anaemia patients; however, they were also found to indirectly reduce hepcidin levels. These stabilisers act by inhibiting prolyl hydroxylase, which is responsible for degrading the alpha subunits of the HIF complex during normoxia [139].

Pergola et al. are currently developing an oral hypoxia-inducible factor prolyl hydroxylase inhibitor called vadadustat for use in the treatment of anaemia in CKD patients [109]. In a 20-week, double-blinded, randomised, placebo-controlled phase 2B clinical trial, vadaustat was found to significantly increase haemoglobin with decreases in hepcidin levels by six [109]. Another HIF stabiliser, roxadustat developed by Besarab et al. underwent a proof of concept phase 2B trial in 2015 on newly initiated dialysis patients who previously had not taken EPO analogues [110]. After four-week roxadustat treatment, hepcidin levels significantly decreased in all patient cohorts [110]. Recently, roxadustat has undergone two phase three trials in China undertaken with CDK patients who displayed an increase in haemoglobin levels above those of the controls [140,141]. Lastly, daprostustat a competitive reversible inhibitor of PHDs through its interactions with the catalytic iron and subsequent blocking of substrate entry [142] has also been tested as a hepcidin targeting molecule. In a phase 2A randomised trial of stage 3–5 CKD patients, a decrease in hepcidin and subsequent increase in total iron levels in the daprostustat treated cohort was observed [111].

7. Current Therapeutic Treatments Targeting Ferroportin

Ferroportin Agonists

Instead of inhibiting the expression of hepcidin, preventing FPN degradation has also been investigated as a therapeutic treatment for high hepcidin disorders. A high-throughput screen discovered that a thiamine derivative, fursultiamine, inhibited hepcidin binding to FPN, thus stabilising iron export in the HEK293-FPN-GFP cell line [112]. Unfortunately, in vivo assessment of fursultiamine showed no effect on FPN levels, as fursultiamine is a therapeutic replacement for thiamine and is thus rapidly metabolised within the body. However, the metabolite thiamine was found not to attenuate FPN internalisation [112].

In 2012, Eli Lilly filed a patent for an anti-FPN mouse antibody that inhibits hepcidin-mediated internalisation resulting in maintained FPN function [113]. However, to our knowledge, no further progress has been made with this antibody.

Ross et al. described anti-hepcidin antibodies (38G6 and 38C8) that when pre-incubated with Hek-RExTMFPN-V5/BLA cells demonstrated a marginal ability to inhibit hepcidin induced internalisation of FPN [143]. A small molecule screen conducted by Ross et al. also discovered that sulfonyl can inhibit RhoG-hepcidin at concentrations of 141 nM by interacting with cysteine 326 [143]. Further, Sulfonyl quinoxaline has been shown to form an irreversible complex with FPN and demonstrates some affinity of towards the mature FPN [143].

8. Current Therapeutic Treatments for Increasing Hepcidin

8.1. *Hepcidin Agonists*

Minihepcidins containing the first 7–9 N-terminal amino acids of hepcidin have been shown to function using a similar mechanism to full length hepcidin, reducing FPN and iron levels in mouse livers [144]. Minihepcidin molecules are rationally designed peptides generated via mutagenesis of both the hepcidin amino acid sequence discussed above and ferroportin's hepcidin binding motif [144]. The minihepcidin, PR73 caused hypoferremia and increased the survival of *Hepcidin*^{−/−} mice when infected with *Vibrio vulnificus*. In contrast, a significant number of untreated *Hepcidin*^{−/−} mice died due to infection [117]. Similarly, a single dose of PR65 in iron overloaded *Hepcidin*^{−/−} mice resulted in an 85% reduction in serum iron levels after a 24 h period, with iron levels returning to baseline after 48 h [118].

In an attempt to reduce the cost of generating minihepcidins resulting from the use of non-natural amino acids, Chua et al. developed a series of cyclic minihepcidins with mHS17 and mHS26 displaying the highest ferroportin binding affinity [119]. While the cost to manufacture these cyclic minihepcidins is significantly lower than PR73 (67% and 76% decrease in cost, respectively), both displayed a 10-fold decrease in half maximal effective concentration (EC₅₀) when compared to PR73 in vitro [119]. In addition, in vivo neither mHS17 nor mHS26 displayed any effect on serum iron levels [119].

Casu et al. recently developed two minihepcidins molecules, M004 and M009 [120]. M004 was shown to decrease FPN expression and serum iron over a 24-h period. However, it was discontinued due to the higher stability of M009. In a β -thalassaemia mouse model, *Hbb*^(th3/+) mice, treatment with low dose M009 resulted in decreased transferrin saturation and normalised red blood cell counts while high dose M009 resulted in a worsening of anaemia [120]. In a phase one study another mini-hepcidin, LJPC-401 resulted in a dose-dependent reduction in serum iron in 15 patients with iron overload [129].

Therapeutic targeting of other molecules involved in the downregulation of hepcidin production has also been tried. TMPRSS6 reduces hepcidin expression via reduced membrane HJV. Inhibiting TMPRSS6 expression has thus been an active area of research. RNAi containing lipid nanoparticles (LNP) targeting TMPRSS6 have been shown to decrease TMPRSS6 mRNA while simultaneously increasing hepcidin expression [130]. Treatment with two anti-sense oligonucleotides (ASO) (TMPRSS6-ASO#1 and TMPRSS6-ASO#2) resulted in a 90% reduction in *TMPRSS6* mRNA with a subsequent 4–5-fold increase in hepcidin mRNA expression [145]. The effectiveness of TMPRSS6-ASOs were further validated in a 2015 study by Gou et al., where they found 30% reduction in transferrin saturation and 40–50% reduction in liver iron levels in a β -thalassemia mouse model [121]. In addition, sustained dosing of TMPRSS6-ASO in a monkey model reduced serum iron levels [121]. Aghajan et al. developed TMPRSS6-ASO conjugated to triantennary N-acetyl galactosamine (GalNAc). When compared with the unconjugated ASO, the GalNAc-ASO demonstrated a 10-fold improvement in EC₅₀ and resulted in a similar decrease in serum iron [122]. Recently, Schmidt et al. conjugated siRNA conjugated to GalNAc, which resulted in a two-fold increase in hepcidin expression in splenectomised *Hbb*^(th3/+) mice [123].

Based upon the success of TMPRSS6-ASOs, Casu et al. performed a six-week study on *Hbb*^(th3/+) mice treated with TMPRSS6-ASOs or TMPRSS6-ASOs in combination with deferiprone (DFP) [146]. As expected, administration of TMPRSS6-ASO either alone or with DFP increased hepcidin levels, while DFP alone did not. TMPRSS6-ASO and DFP treatment displayed a synergistic effect on liver iron content [146]. The success of inhibiting TMPRSS6 with concomitant chelator treatment was also shown by Schmidt et al. using TMPRSS6 RNAi and DFP to reduce secondary iron overload in *Hbb*^(th3/+) mice [147]. These positive results have formed the basis for a clinical trial that commenced in 2017 [122].

8.2. Hepcidin Analogues

Mature hepcidin contains eight highly conserved disulphide bonds at positions cysteine (Cys) seven and Cys23, Cys10 and Cys13, Cys11 and Cys19, and Cys14 and Cys22 [114]. Recently, Pandur et al. created a hepcidin analogue that displayed similar FPN binding and ubiquitination characteristics as wild type (WT) hepcidin [114]. Using site directed mutagenesis, Pandur et al. probed the effect of replacing each cysteine residue with serine residues on the binding and activity of the mature peptide. They revealed that cysteine 19 is not essential, as the mutation still retained full biologic activity [114]. In addition, intracellular iron levels increased within cells treated with either WT hepcidin or the Cys19 mutant, a result not seen in any other cysteine mutants. Lastly, Pandur et al. demonstrated using ELISA that over a 96-h period the cys19ser mutant hepcidin remained in a higher active concentration when incubated in media compared to WT hepcidin [114]. As mentioned previously minihepcidins are costly to produce due to unnatural amino acids and C-terminal lipid tails. Thus, the lack of unnatural amino acids and C-terminal lipid tail should make this hepcidin mutant a promising therapeutic candidate.

8.3. Small Molecule Hepcidin Agonists

Several phytoestrogens (naringenin, quercetin and resveratrol) found in fruits and vegetables have been shown to increase *hepcidin* expression through interactions with Nrf2 and an antioxidant response element located within the hepcidin promoter within a rat model [124]. HepG2 cells treated for six hours with naringenin, quercetin and resveratrol displayed an increase in *hepcidin* expression of approximately 2.5-, 3.5- and 3.5-fold, respectively [124]. In rats treated with quercetin, *hepcidin* expression was increased by 500-fold, while naringenin and resveratrol increased *hepcidin* expression approximately 4- and 12.5-fold, respectively [124].

Mleczko-Sanecka et al. employed siRNA and small molecule screens, which highlighted the role of Ras GTPase (RAS)/RAF proto-oncogene serine/threonine-protein kinase (RAF) and phosphoinositide-3 kinase (PI3K) pathway inhibitors (sorafenib, wortmannin and rapamycin) as inducers of hepcidin mRNA in Hep3B cells [115]. Another screen of 22 water-soluble and fat-soluble vitamins conducted by Zhang et al. identified adenine (vitamin B4) as a potent activator of hepcidin which acts through the BMP6/SMAD pathway [125]. HUH7 cells treated with 50 μ M adenine for 12 h displayed an increase of 4.6-fold in hepcidin expression. When iron loaded C57/BL6 mice were fed a diet containing 0.2% (*w/w*) adenine for up to 10 days, hepcidin mRNA expression was increased with subsequent reductions in serum iron and liver iron and transferrin saturation [125].

Recently, a Kunitz-type hepatocyte growth factor activator inhibitor two (HAI-2) that targets and inhibits the proteolytic activity of TMPRSS6 was discovered [148]. HAI-2 prevented HJV cleavage by forming a series of hydrogen bonds and a disulphide bond within the TMPRSS6 active site which prevents hepcidin downregulation [148].

Zhen et al. found that the isoflavone, genistein was able to increase hepcidin expression levels in zebrafish embryos, while the inactive form of genistein, genistin and other related compounds diazdein (isoflavone) and apigenin (flavone) had no effect on hepcidin transcription [126]. This hepcidin induction requires both BMP/SMAD and JAK/STAT3 pathways as BMP-RE and STAT binding motifs are critical for proper genistein activity [126]. However, genistein appears to also act through BMP-RE independent mechanisms as HepG2 cells treated with both dorsomorphin and genistein displayed increased hepcidin expression levels over dorsomorphin treated cells alone. Unfortunately, genistein was found to inhibit cell proliferation and increase STAT3 phosphorylation in high concentrations (25–100 μ M). It also increases apoptosis at 200 μ M [149]. In addition, Chau et al. found that transgenic adenocarcinoma mouse prostate (TRAMP) mice treated with 250 mg/kg of genistein had a three-fold increase in prostate weight due to increases in telomerase activity [150].

Gaun, V. et al. employed a small molecule library screen to determine modulators of hepcidin expression. This screen of over 10,000 molecules using HepG2 cells expressing the luciferase gene under the control of the *hepcidin* promoter, identified 20 agonists and one antagonist [127]. The activity of these 21 molecules was then screened using real time PCR. Sixteen of the identified agonists increased both luciferase and endogenous hepcidin expression [127]. Meanwhile, the lone antagonist increased hepcidin transcription levels despite decreasing hepcidin-luciferase activity. The majority of chemicals identified (six) were shown to increase hepcidin expression through the BMP6 pathway, while only four chemicals increased hepcidin expression through the STAT3 pathway and the remaining six chemicals were found to work through both the BMP6 and STAT3 pathway [127]. Of note, ipriflavone and vorinostat were found to elicit an effect on hepcidin expression at concentrations 10-fold lower than those required for genistein [127]. Due to the higher potency of ipriflavone, C57BL/6 male mice were treated with increasing concentrations of ipriflavone for 50 days to determine changes in iron content, hepcidin and ferroportin expression levels [151]. The treated mice displayed a significant reduction in liver iron content of approximately 40% compared to control untreated mice. Ipriflavone also resulted in an approximate two-fold increase in hepcidin mRNA levels [151]. The hepcidin inducing effect of vorinostat was further confirmed in another study using HUH7 and primary hepatocytes from both human and mice origin [100]. Unfortunately, these effects were not seen in a C57BL/6 mouse model [100]. Another high-throughput screen conducted in zebrafish by Li et al. discovered

three steroid molecules, namely epitostanol, progesterone, and mifepristone, which increase hepcidin expression [116]. These molecules were found to activate hepcidin synthesis through the progesterone receptor membrane component 1 (PGRMC1) [116]. Zhang et al. in 2016 employed a natural product screen using traditional Chinese herbal medicinal plants that identified icariin as an inducer of hepcidin expression in HepG2 and Hepa 1–6 cells as well as in wild-type ICR mice [128]. Icariin was found to interact with the hepcidin regulatory pathway through increased phosphorylation of both STAT3 and Smad1/5/8 [128]. Similar results were seen for icariin analogues epimedin A, B and C when administered to mice, with epimedin C demonstrating the most significant increase in expression. A second compound berberine was also found to increase hepcidin expression in cell lines. However, berberine failed to increase hepcidin expression in vivo [128].

9. Conclusion

The important biological role iron plays in health and disease is exemplified by the myriad of therapeutics currently under development for the regulation of hepcidin and ferroportin. Most promisingly, some of these drug candidates are currently undergoing clinical trials, e.g., roxadustat, which has recently completed phase three trials. However, many of these developing treatments must still overcome significant challenges before they can be used as therapeutic agents. For instance, RNAi technologies still require improvements in design to avoid off-target interactions, increased stability to enable an appropriate half-life for action and a compatible delivery system for proper localisation within the body [152]. In addition, many of the aforementioned SMAD and STAT therapeutics lack the specificity to target only the pathways involved with hepcidin regulation [2,100]. Continued research into the molecular mechanism of the hepcidin/ferroportin axis will likely provide additional targets which may overcome the abovementioned limitations of the current lot of in development therapeutic treatments.

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Communication

Deregulation of Hepatic Mek1/2–Erk1/2 Signaling Module in Iron Overload Conditions

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Abstract: The liver, through the production of iron hormone hepcidin, controls body iron levels. High liver iron levels and deregulated hepcidin expression are commonly observed in many liver diseases including highly prevalent genetic iron overload disorders. In spite of a number of breakthrough investigations into the signals that control hepcidin expression, little progress has been made towards investigations into intracellular signaling in the liver under excess of iron. This study examined hepatic signaling pathways underlying acquired and genetic iron overload conditions. Our data demonstrate that hepatic iron overload associates with a decline in the activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (Erk) kinase (Mek1/2) pathway by selectively affecting the phosphorylation of Erk1/2. We propose that Mek1/2–Erk1/2 signaling is uncoupled from iron–Bmp–Smad-mediated hepcidin induction and that it may contribute to a number of liver pathologies in addition to toxic effects of iron. We believe that our findings will advance the understanding of cellular signaling events in the liver during iron overload of different etiologies.

Keywords: liver; iron; hepcidin; Mek/Erk; Hfe; Bmp/Smad

1. Introduction

In vertebrates, the liver is an essential metabolic hub. It hosts numerous biochemical processes and regulates the storage of many essential nutrients, vitamins, and minerals, as well as their release when there is a physiological need for them. The liver is the main parenchymal iron repository and at the same time the principal organ controlling systemic iron fluxes through the production of the iron hormone hepcidin [1]. High hepatic iron levels are commonly observed in many liver diseases including highly prevalent genetic iron overload disorders (i.e., hereditary hemochromatosis), hematologic disorders (i.e., thalassemia and sickle cell disease), chronic hepatitis C, alcoholic liver disease and non-alcoholic fatty liver disease I [2,3]. The main pathological effects of hepatic iron overload include liver fibrosis, cirrhosis and hepatocellular carcinoma.

The sensing of plasma iron levels (i.e., transferrin-bound iron) by liver hepatocytes involves a multiprotein membrane-bound complex consisting of transferrin receptors 1 and 2 (TfR1, TfR2), MHC I-like protein Hfe, hemojuvelin, and the bone morphogenetic protein (Bmp) receptors type I such as activin receptor-like kinase 1 (Alk2/Acvr1) and Alk3/Bmpr1a [4–12]. The Bmp–Alk2/3–Smad1/5/8 cascade is currently considered as the central intracellular relay communicating high plasma iron levels to hepcidin, since mice and patients with genetic disruption in the iron-sensing molecules show impaired Bmp/Smad signaling, low hepcidin expression and consequently develop hepatic iron overload [12–15]. Furthermore, the contribution of the extracellular signal-regulated kinases 1

and 2 (Erk1/2) to hepcidin regulation was proposed: studies in erythroleukemia K562 cells, exposed to holo-transferrin, demonstrated activation of ERK1/2 and p38 MAP kinases, a process that was dependent on Tfr2 [16]; consequently, silencing of Tfr2 and Hfe in HepG2 cells and in mice resulted in decreased ERK1/2 signaling and low hepcidin expression in the liver [8,17]. It was proposed that ERK1/2 might act in concert with Bmp-Smad signaling to control hepcidin expression [18].

The mitogen-activated protein kinases (MAPKs) are among the largest protein families in eukaryotes that transduce a variety of extracellular signals to regulate a plethora of cellular responses [19,20]. MAPKs consist of many protein kinases but three major protein kinases are extensively studied: the ERK1/2, activated by broad spectrum of extracellular ligands such as mitogens/growth factors and differentiation signals [21], and c-Jun amino terminal kinases (JNK1/2/3) and p38 kinases that are activated by stress stimuli [19,20,22]. MAPK-dependent signal transduction is required to maintain physiological metabolic adaptation while inappropriate MAPK signaling has been increasingly associated with the development of metabolic syndrome [23]. In the liver, the MAPKs play an important role in processes that regulate metabolism [23–26]. In particular, activation of stress-responsive p38MAPKs and JNKs was associated with hepatic metabolic dysfunction [20,23,26–28], whereas constitutive Erk1 or liver-specific Erk2 deficiency in mice was proposed to affect hepatic glucose and lipid metabolism, promote insulin resistance and ER stress [27,29,30].

In this study, we examined the association between hepatic iron overload in mice, caused by parenteral, nutritional and genetic iron overload, and the activity of Mek1/2-Erk1/2 signaling. Our data demonstrate decreased Mek1/2-Erk1/2 signaling output in iron overloaded conditions, suggesting that Mek1/2-Erk1/2 signaling is uncoupled from Bmp-Smad1/5/8-mediated hepcidin induction and that it may play an important role in liver diseases characterized by hepatic iron excess.

2. Results and Discussion

2.1. Classical and Stress-Induced MAPKs Activation in Iron-Loaded Livers

The aim of this study was to investigate the activity of intracellular signaling pathways in the livers under excessive iron overload. To this end, we used our previously established mouse model of parenteral iron overload, which is characterized by severe hepatic iron overload, high circulating iron levels, and increased hepcidin mRNA expression [15]. We measured the phosphorylation status of nine intracellular proteins including Mek1 (Ser217/Ser221) and Erk1/2 (Thr202/Tyr204, Thr185/Tyr187), as classical MAPK backbone components, stress-responsive MAPKs, such as JNK (Thr183/Tyr185) and p38 MAPK (Thr180/Tyr182), and effector molecules of MAPKs including p90 RSK (Ser380), Stat3 (Ser727), ATF-2 (Thr71), HSP27 (Ser78), and p53 (Ser15), using Bio-Plex Pro Cell Signaling MAPK Panel 9-plex (BioRad Laboratories, Germany). This analysis revealed a decrease in the phosphorylation levels of Mek1 and p90 RSK in iron-loaded livers, and only marginally in case of Stat3 ($p < 0.0571$); similarly, the levels of pErk1/2 showed a trend towards a decrease however the data were under the level of statistical significance (Figure 1). The levels of pJNK, p38MAPK, pHSP27, p53, and pATF-2 showed no statistically significant differences (Figure 1).

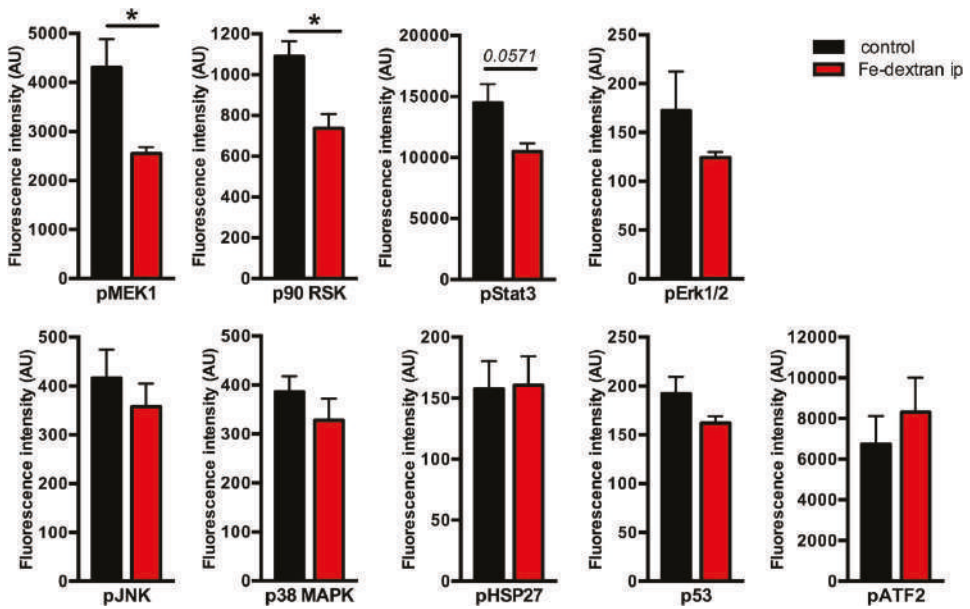


Figure 1. Identification of activated proteins in iron overloaded livers by Bio-Plex Pro Cell Signaling MAPK Panel 9-plex. Phosphorylation status of nine intracellular phosphoproteins was measured in the livers of iron-dextran injected mice and compared to controls. Data were analyzed using GraphPad Prism software and results are shown as mean \pm SEM (standard error of mean). For the statistical analysis, a nonparametric distribution and the unpaired Mann–Whitney U Test were used. * p -values < 0.05; AU: arbitrary units; n = 4; 4 per group.

Based on these results, we postulated that the presence of hepatic iron overload might associate with selective impairment of the Mek1/2-Erk1/2 pathway and its downstream pStat3 and pp90 RSK targets. This idea is supported by recent investigations showing selective activation of JNK and the p38 MAPK signaling activity under cellular iron depletion [31]. Moreover, in response to growth factors, Erk1/2, but not JNK or p38, specifically phosphorylate Stat3 at Ser727, which is also stimulated by interleukine-6 cytokine, however, in contrast to growth factors, the latter process occurs independent of Erk1/2 [32].

2.2. Association of Mitogen-activated Protein Kinases (MAPK) Activity with Hepatic Iron Overload

To test the above hypothesis we evaluated phosphorylation levels of Mek1/2, Erk1/2, Stat3 and p90 Rsk proteins in iron-loaded livers using immunoblotting analysis. This analysis revealed a significant decrease in the phosphorylation levels of Mek1/2, Erk1/2, and Stat3 (by 3.6-, 4.8-, and 3.8-fold, respectively), while the levels of p90Rsk were increased by 2.1-fold (Figure 2a).

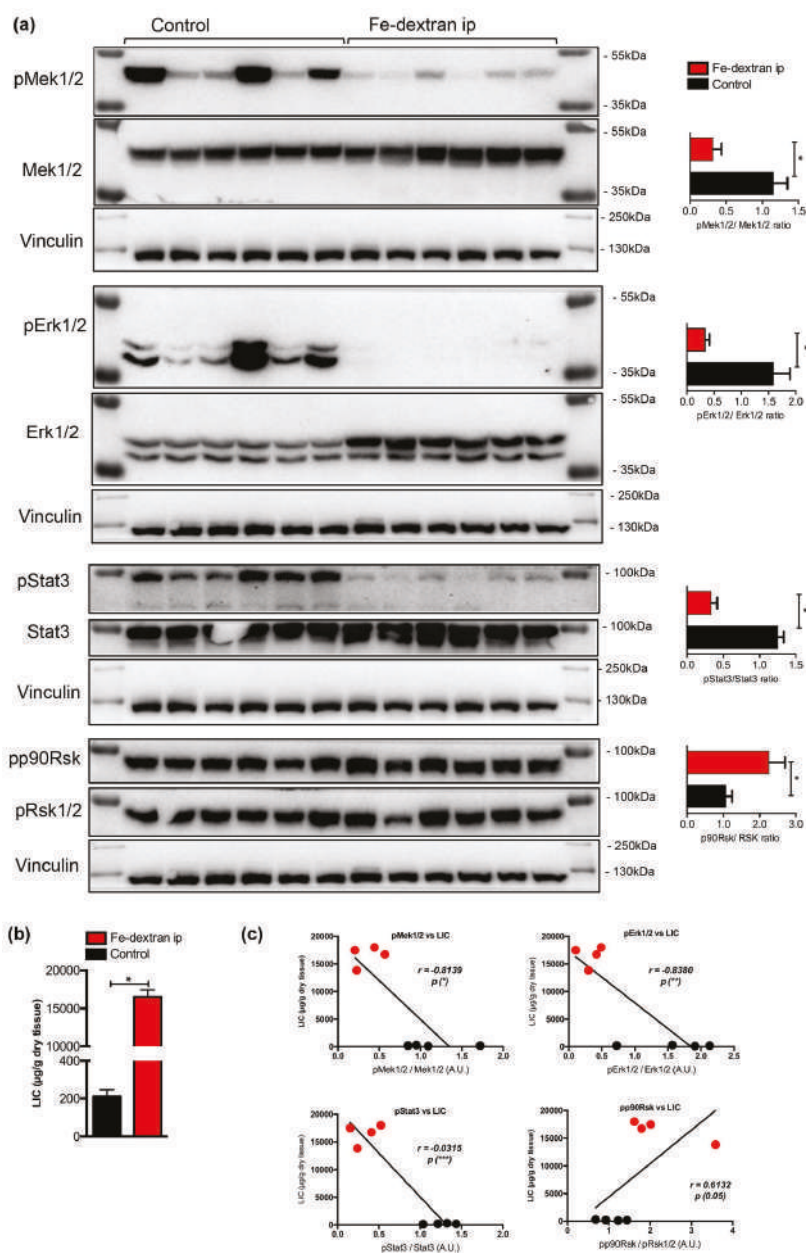


Figure 2. (a) Immunoblot analysis and relative quantification (shown in histograms on right) of pMEK1, pERK1/2, pStat3, and pp90Rsk in the livers of iron-dextran injected and control mice (n = 6; 6 mice per group). (b) Liver iron content (LIC) of control and iron-dextran injected mice. (c) Correlation analysis between LIC and the levels of pMek1/2/Mek1/2, pErk1/2/Erk1/2, pStat3/Stat3, and pp90Rsk/pRsk1/2 in the livers of iron-dextran injected and control mice. M: Page Ruler Plus Prestained Protein Ladder (Thermo Scientific). Data were analyzed using GraphPad Prism software and results are shown as mean ± SEM (standard error of mean). For the statistical analysis, a nonparametric distribution and the unpaired Mann–Whitney U Test were used. Linear regression and Pearson correlation coefficients were computed for every data set with 95% confidence intervals. * p values < 0.05.

2.3. Nutritional Iron Overload is Characterized by Low Hepatic Mek1/2-Erk1/2-Stat3 Signaling

We next questioned whether a decrease in the Mek1/2-Erk1/2-Stat3 activity, measured during parenteral iron overload, is present in conditions of nutritional iron overload. We thus examined the phosphorylation status of Mek1/2-Erk1/2-Stat3 in the livers of wild type mice undergoing nutritional iron overload, induced by feeding mice with 3% carbonyl iron containing diet for six months starting at one month of age, as we previously described [33]. We show that hepatic iron overload, induced by iron-rich diet, associated with a 2.7-, 3.4-, and 1.7-fold decrease in the phosphorylation levels of Mek1/2-Erk1/2-Stat3 proteins, respectively, while the levels of p90Rsk were unchanged (Figure 3). However the data were only marginally under the level of statistical significance ($p < 0.0571$), which is, in our opinion, caused by stringent statistical analysis we applied (unpaired, nonparametric, Mann–Whitney test; $n = 3–4$ mice per group). Collectively, our data imply that hepatic response of mice receiving parenteral or dietary iron overload associates with a selective decline in the activity of Mek1/2-Erk1/2-Stat3 branch.

Future studies are needed to determine whether a decrease in phosphorylation of Mek1/2-Erk1/2 might be caused by diminished tyrosine kinase receptors (such as epidermal growth factor receptor, EGFR) mediated signaling activity and whether its activity may correlate with changes in the levels of extracellular ligands that can bind EGFR, such as EGF, transforming growth factor alpha, amphiregulin, heparin-binding EGF, and others. In addition, it will be informative to measure the levels of growth hormone (GH), as defective GH signaling impairs activation of EGFR and ERK signaling and affects liver regeneration [34].

2.4. Low Hepatic Mek1/2-Erk1/2 Signaling is Present in *Hfe*^{-/-} Mice in Spite of Low Bmp-Smad Signaling

So far, our data indicate a decrease in pMek1/2-pErk1/2-pStat3 signaling under excessive systemic and hepatic iron overload. Contrary to Mek1/2-Erk1/2, the activity of Bmp-Smad signaling was shown to increase following iron overload subsequently causing increase in the expression of hepcidin and genes known to be coregulated with hepcidin [15]. Given that previous studies suggest co-involvement of Erk1/2 and Bmp/Smad signaling for induction of hepcidin in cells [18], it seemed logical to investigate the Mek1/2-Erk1/2 signaling activity in conditions characterized by hepatic iron overload and low hepcidin expression. To this end, we employed *Hfe*-deficient mice, a well-established mouse model of genetic iron-overload, which due to the lack of *Hfe*, an upstream hepcidin regulator, showed impaired Bmp-Smad signaling, low hepcidin expression, and increased hepatic iron stores [13–15]. We detected that the activation of Mek1/2-Erk1/2, measured by phosphorylation status using immunoblot analysis, was on average 2-fold lower in the livers of *Hfe*^{-/-} mice (with marginal significance of $p = 0.0571$), whereas the levels of pStat3 and pp90Rsk were not significantly changed (Figure 4). These data suggest that a decrease in Mek1/2-Erk1/2 signaling is uncoupled from Bmp-Smad signaling activity and Smad-mediated hepcidin regulation.

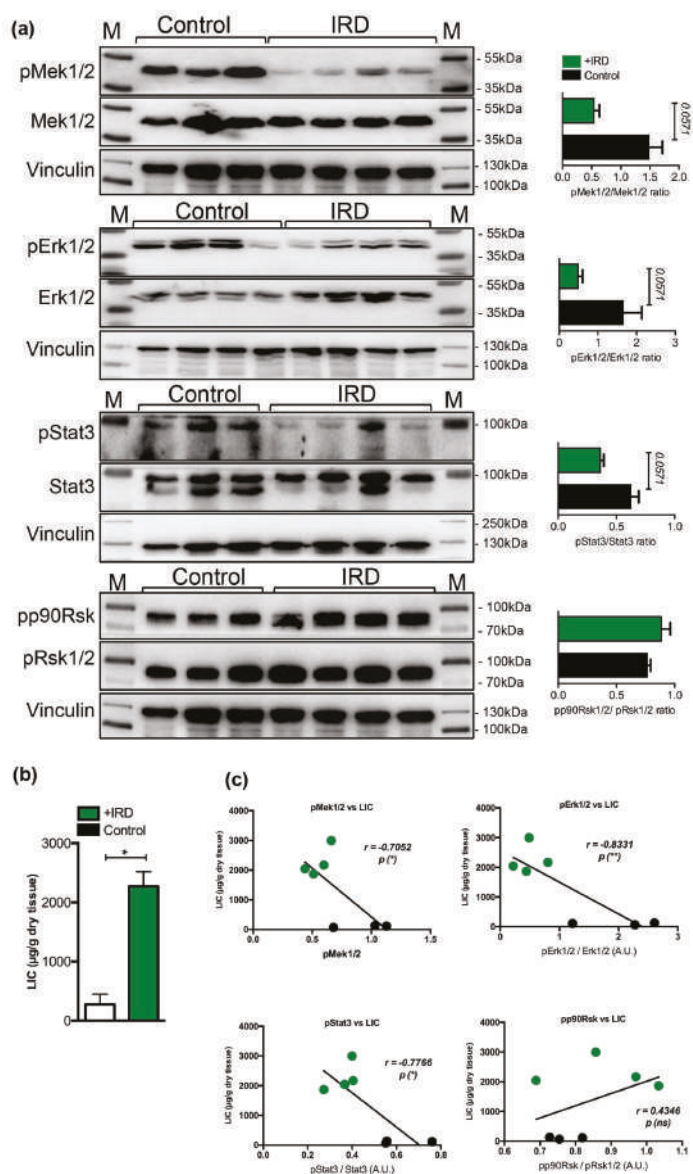


Figure 3. (a) Representative immunoblot analysis and relative quantification (shown in histograms on right) of pMek1/2, pErk1/2, pStat3, and pp90Rsk in the livers of mice maintained on an iron rich diet (IRD) and standard diet (control) (n = 3–4 mice per group). (b) Liver iron content (LIC) of control and of mice maintained on an iron rich diet (n = 3–4 mice per group). (c) Correlation analysis between LIC and the levels of pMek1/2/Mek1/2, pErk1/2/Erk1/2, pStat3/Stat3, and pp90Rsk/pRsk1/2 in the livers of mice maintained on an iron rich diet (IRD) and standard diet (control) (n = 3–4 mice per group). M: Page Ruler Plus Prestained Protein Ladder (Thermo Scientific). Data were analyzed using GraphPad Prism software and results are shown as mean ± SEM (standard error of mean). For the statistical analysis, a nonparametric distribution and the unpaired Mann–Whitney U Test were used. Linear regression and Pearson correlation coefficients were computed for every data set with 95% confidence intervals. * *p*-values < 0.05.

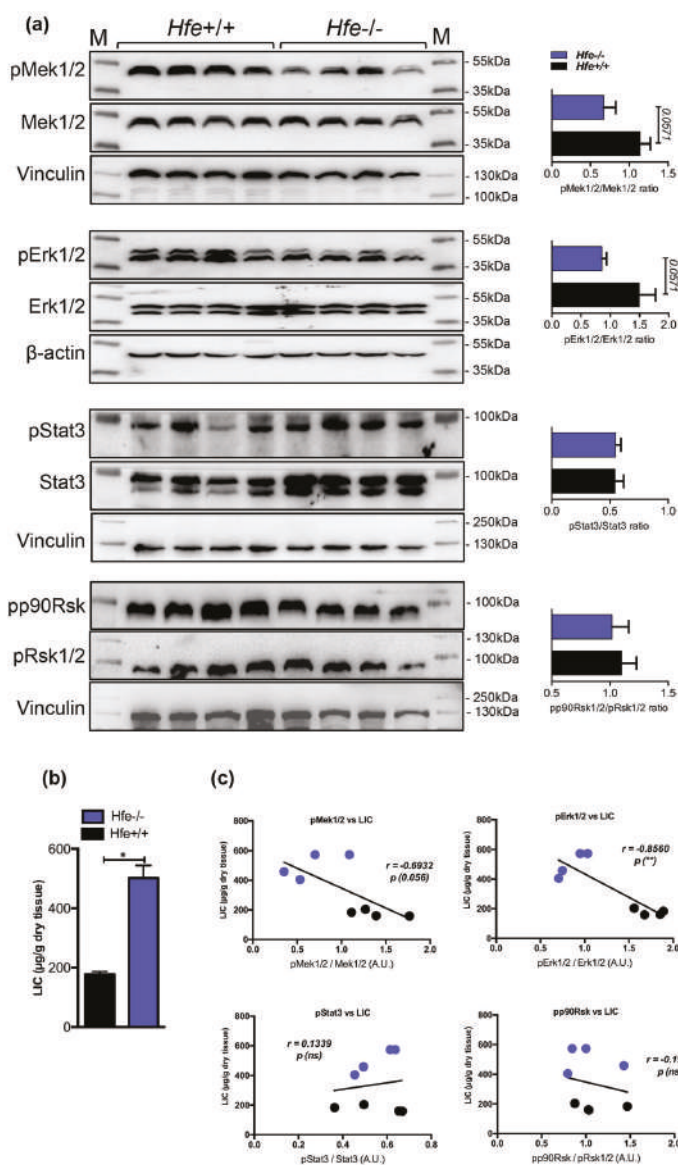


Figure 4. (a) Representative immunoblot analysis and relative quantification (shown in histograms on right) of pMek1/2, pErk1/2, pStat3 and pp90Rsk in the livers of *Hfe*^{+/+} control and *Hfe*^{-/-} mutant mice (n = 8;10 mice per group). (b) Liver iron content (LIC) of *Hfe*^{+/+} and *Hfe*^{-/-} mice (n = 4;4 mice per group). (c) Correlation analysis between LIC and the levels of pMek1/2/Mek1/2, pErk1/2/Erk1/2, pStat3/Stat3, and pp90Rsk/pRsk1/2 in the livers of *Hfe*^{+/+} and *Hfe*^{-/-} mice (n = 4;4 mice per group). M: Page Ruler Plus Prestained Protein Ladder (Thermo Scientific). Data were analyzed using GraphPad Prism software and results are shown as mean ± SEM (standard error of mean). For the statistical analysis, a nonparametric distribution and the unpaired Mann–Whitney U Test were used. Linear regression and Pearson correlation coefficients were computed for every data set with 95% confidence intervals. * p-values < 0.05.

2.5. Low Hepatic Mek1/2-Erk1/2 Signaling is Present in Hepcidin-Deficient Mice and is Further Aggravated by Iron Excess

We next investigated Mek1/2-Erk1/2 activation status in the livers of hepcidin knock-out mice (*Hamp*^{-/-}), which are deficient for hepcidin expression but maintain an appropriate Bmp-Smad signaling activity as a response to liver iron overload [15]. Similarly to the observation in the livers of *Hfe*^{-/-} mice, a 2.5- and 3.8-fold decrease in the levels of pMek1/2-pErk1/2 proteins were measured in the livers of *Hamp*^{-/-} mice (with marginal significance of $p = 0.0571$), while the pStat3 and pp90Rsk levels were not significantly changed (Figure 5a–c). Interestingly, the activation of Mek1/2, Erk1/2, and Stat3, measured by their phosphorylated levels, was suppressed by 34-, 7.5-, and 1.5-fold, respectively (with marginal significance of $p = 0.0571$), while the levels of pp90Rsk were unchanged in *Hamp*^{-/-} mice fed an iron-rich diet for six months (Figure 5d–f), which caused the development of chronic liver injury as we previously demonstrated [33].

Interestingly, the initially observed increase in pp90Rsk levels following iron-dextran injections in mice (Figure 2a,c) was not detected in the livers of mice maintained on an iron-rich diet nor in *Hfe*^{-/-} or *Hamp*^{-/-} mice. A possible explanation might be the differences in the route of iron application (i.p. injections versus nutritional/enteral iron administration) and the duration of iron loading (3-weeks of iron-dextran injections versus six months of iron-rich diet). We suspect that fast influx of iron and excessive liver iron loading following i.p. iron-dextran injections might differentially affect cellular and humoral responses than nutritional iron administration or genetic iron overload.

Taken together, our data reinforce the view that attenuation of Mek1/2-Erk1/2 phosphorylation is a function of hepatic iron overload. Given the variety of iron-overload models used in this study, we speculate that changes in Mek1/2-Erk1/2 signaling may be categorized as initiating mechanism predisposing liver cells to toxic insults. Among them, inflammation, hepatic oxidative stress including the production of highly reactive lipid peroxidation products and iron-catalyzed oxidant stress [35,36] could ultimately lead towards the progression of a chronic liver disease. This hypothesis is supported by recent studies demonstrated that inhibition of ERK1/2 signaling sensitized the cells to chemotactic stimuli [37].

The collective data let to a working model (Figure 6), in which high hepatic iron burden associates with a decrease in phosphorylation levels of Mek1/2-Erk1/2. This in turn may contribute to a number of liver pathologies in addition to toxic effects of iron.

Data presented in this study raise a number of questions as to whether (i) low Mek1/2-Erk1/2 activity occurs as a selective response to high hepatic iron levels or is triggered by high transferrin and/or non-transferrin-bound iron, (ii) in which hepatic cells (hepatocytes or nonparenchymal cells) and in which cellular compartments does Erk1/2 function, (iii) what is the level of Mek1/2-Erk1/2 cross-talk with parallel pathways such as the PI3K/AKT/mTOR pathway in iron-loaded livers, and (iv) what are the consequences of inhibition of the Erk1/2 pathway in iron-loaded livers on hepatic gene transcription?

Finally, our findings may be of relevance to other conditions where hepatic iron levels are increased such as alcoholic liver disease, characterized by low hepcidin expression, suppressed hepatic Erk1/2 activity and liver injury [27,38–43]. In addition, hepatic iron overload is present in approximately one-third of patients with nonalcoholic fatty liver disease [44–47], which is recognized as the most common chronic liver disease that can progress to non-alcoholic steatohepatitis and liver cancer [48–50]. Hepatic iron overload is also observed in patients with chronic hepatitis C virus infections (and rarely in chronic hepatitis B infections) and in end-stage liver disease [3,46,47,51–54]. Whether hepatic iron overload and the presence of suppressed Mek1/2-Erk1/2 signaling may either accelerate disease progression or whether maintaining low Mek1/2-Erk1/2 signaling may be protective from the induction of *c-Myc* and *c-Jun* as a part of increased proliferation, and therefore reduce the incidence of liver damage, are certainly important questions to address in the future. An equally interesting aspect would be to monitor Mek1/2-Erk1/2 activation from early steps of iron overload until development of liver pathologies and establish whether the activity of MAPK module is accordingly modulated.

Understanding the underlying mechanisms associated with hepatic iron overload and progressive liver failure may provide new modalities for therapeutic interventions. We believe that the data provided here will advance our understanding of cellular signaling events in the liver during iron overload of different etiologies.

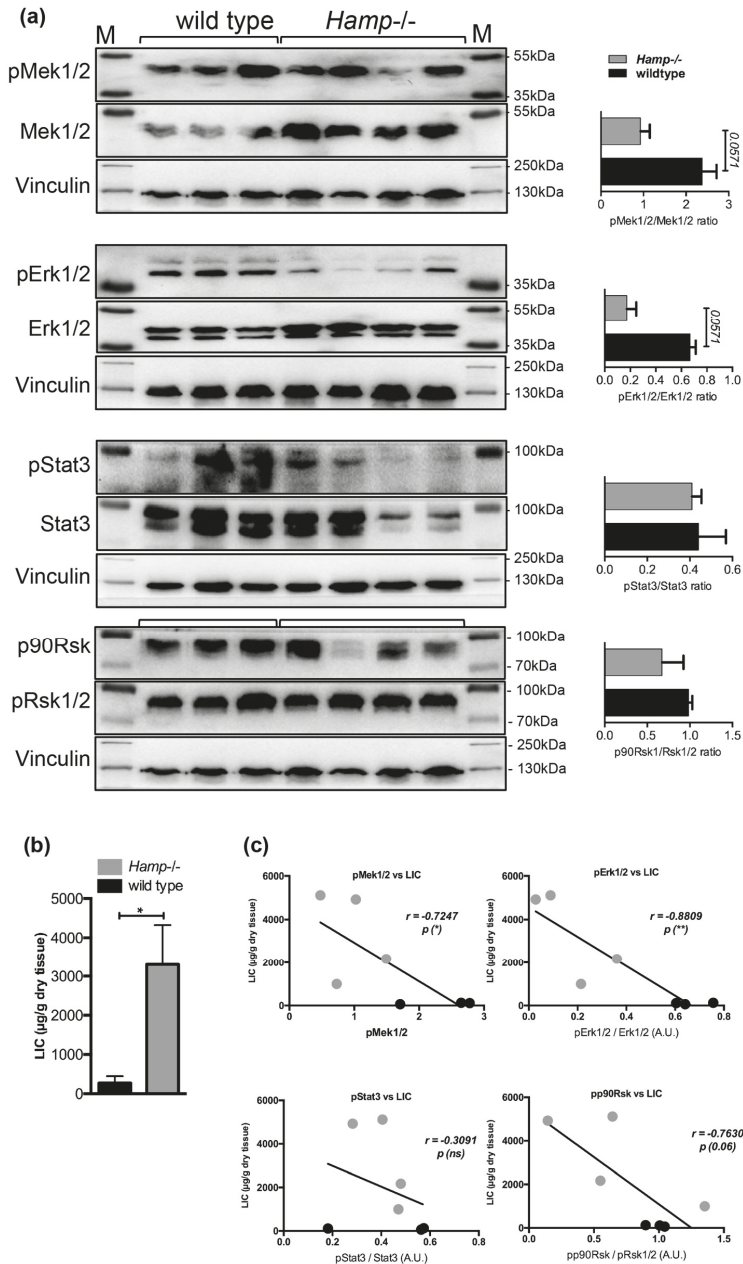


Figure 5. Cont.

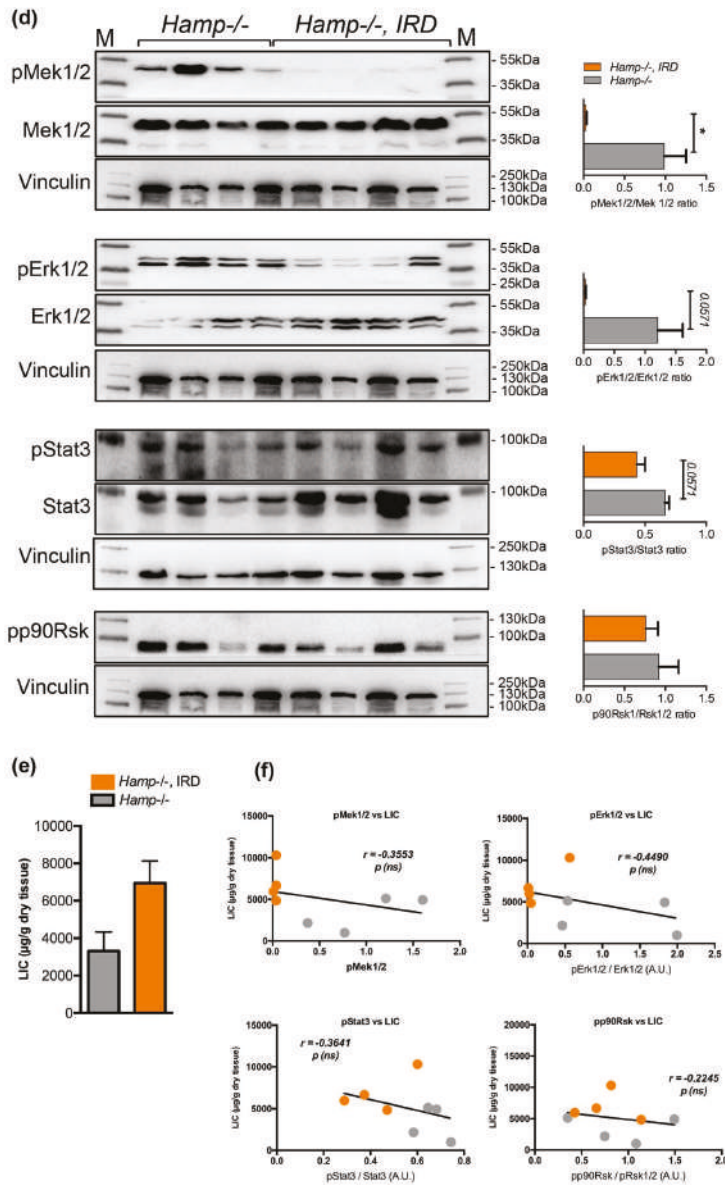


Figure 5. Immunoblot analysis and relative quantification (shown in histograms on right) of pMek1/2, pErk1/2, pStat3 and pp90Rsk in the livers of (a) control and Hamp^{-/-} mice and (d) Hamp^{-/-} mice maintained on an iron-rich diet (IRD) (n = 3;4;4 mice per group). Liver iron content (LIC) in (b) control, Hamp^{-/-} and (e) Hamp^{-/-} mice maintained on an iron-rich diet (n = 3;4;4 mice per group). Correlation analysis between LIC and the levels of pMek1/2/Mek1/2, pErk1/2/Erk1/2, pStat3/Stat3, and pp90Rsk/pRsk1/2 in the livers of (c) control, Hamp^{-/-} and (f) Hamp^{-/-} mice maintained on an iron rich diet (IRD) (n = 3; 4;4 mice per group). M: Page Ruler Plus Prestained Protein Ladder (Thermo Scientific). Data were analyzed using GraphPad Prism software and results are shown as mean ± SEM (standard error of mean). For the statistical analysis, a nonparametric distribution and the unpaired Mann–Whitney U Test were used. Linear regression and Pearson correlation coefficients were computed for every data set with 95% confidence intervals. * p-values <0.05.

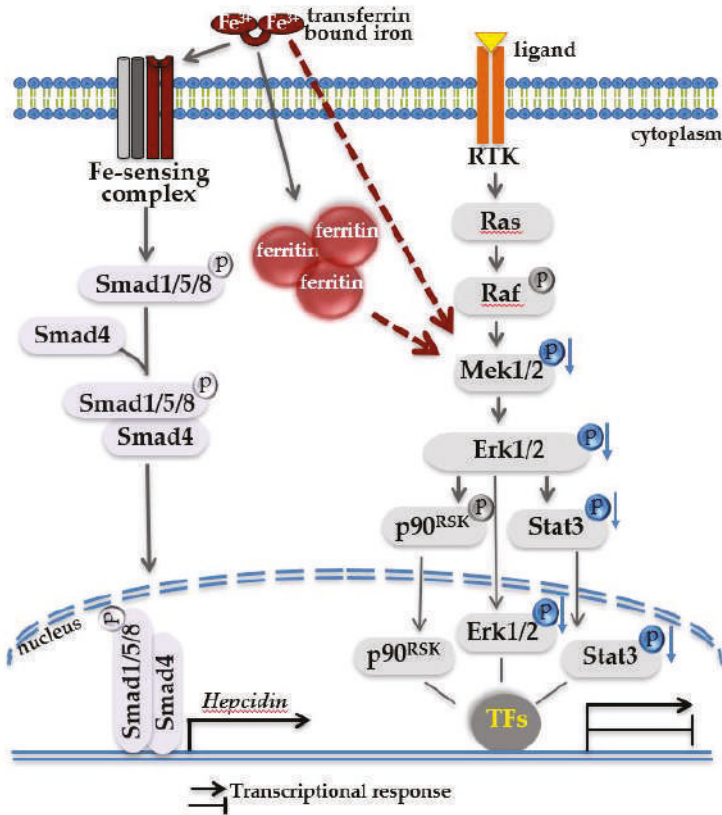


Figure 6. Proposed model of two signaling pathways operating in iron-loaded livers. Systemic iron overload results in heavy iron deposition in the liver, illustrated here in form of high ferritin. Under these conditions, high levels of circulating transferrin-bound iron is sensed by cell membrane multiprotein iron-sensing complex, resulting in the activation of intracellular Bmp-Smad signaling cascade and increased hepcidin transcription in the nucleus. Independent of Bmp-Smad-mediated hepcidin activation, through a yet unknown mechanism (proposed here by red arrows), a decrease in phosphorylation of Mek1/2-Erk1/2-Stat3 (indicated in blue) occurs which in turn may affect the property of pErk1/2 and pStat3 signaling molecules to regulate gene transcription, alone or in cooperation with other transcription factors (TFs). A decrease in the activity of Mek1/2-Erk1/2 signaling cascade can be considered as liver response to iron overload. We propose that a decrease in Mek1/2-Erk1/2 signaling activity may accelerate liver pathologies in addition to toxic effects of iron.

3. Materials and Methods

3.1. Mice and Treatments

Wild type *Hfe*^{-/-} and *Hamp*^{-/-} mutant mice, all males, were kept under a standard mouse diet containing 180 mg/kg iron (Ssniff, Soest, Germany). For the analysis livers were used from previously described wild-type mice undergoing intra peritoneal (i.p.) injection of iron-dextran solution [15,33] and from *Hamp*^{-/-} and wild type mice fed with 3% carbonyl iron (Sigma, Germany) for 6 months [15,33]. Animal experiments were approved and performed in accordance to the Ulm University Animal Care

Committee and German Law for Welfare of laboratory animals in Baden-Württemberg, Germany (Project ID: 35/9185.81-3 / 972 / 1143).

3.2. Phosphoprotein Analysis in Liver Lysates

Protein lysates (10 µg in 50 µL) were prepared from liver tissues using Bio-Plex Cell Lysis Kit (BioRad Laboratories, Munich, Germany). The levels of intracellular phosphoproteins were measured using Bio-Plex Pro Cell Signaling MAPK Panel, 9-plex (BioRad Laboratories, Munich, Germany) according to the manufacturer's instructions. The data were analyzed using Bio-Plex Manager 6.1 Software Package.

3.3. Protein Isolation and Immunoblot Analysis

Protein extracts were prepared from flash-frozen tissue after homogenization in RIPA lysis buffer (Incomplete RIPA buffer, 7× protease inhibitor cocktail, 200mM sodium orthovanadate, 1M sodium fluoride, 100mM PMSF) as previously described [15]. Total proteins (30–50 µg) were subjected to Western blot analysis with the following antibodies; anti-pMek1/2, anti-Mek1/2, anti-pErk1/2, anti-Erk1/2, anti-pp90Rsk, anti-pRsk1/2, anti-pStat3, and anti-Stat3 (all rabbit, Cell Signaling Technology, MA, USA; 1:1000 concentration). Mouse anti-vinculin (Santa Cruz, CA, USA; 1:2000) and mouse anti-β-actin (Sigma Aldrich, Missouri, USA; 1:10,000) were used as loading controls. Furthermore, membranes were washed and incubated with anti-rabbit or anti-mouse (Invitrogen, CA, USA; 1:5000) horseradish peroxidase-conjugated antibody. Western blot images were acquired using EMD Millipore Luminata HRP chemiluminescence substrate (Millipore, MA, USA) and signal acquired in Bio-Rad chemiluminescence detector (Bio-Rad Laboratories, CA, USA). The signals were semiquantified using image J (ImageJ; [www://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)).

3.4. Statistical Analysis

Data were analyzed using GraphPad Prism software and results are shown as mean ± standard error of mean. For the statistical analysis, a nonparametric distribution and the Mann–Whitney U test were used. Linear regression and Pearson correlation coefficients were computed for every data set with 95% confidence intervals. Statistically significant differences are indicated as $p < 05$ (*), $p < 01$ (**), and $p < 005$ (***)

Author Contributions: N.K.T. and N.B. performed the experiments; P.S. and I.C.C. provided resources and edited the manuscript; and M.V.S. designed the study, analyzed the data, and wrote the manuscript.

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Concept Paper

Unraveling Heparin Plasma Protein Binding: Evidence from Peritoneal Equilibration Testing

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Abstract: Peptide hormone heparin regulates systemic iron metabolism and has been described to be partially bound to α 2-macroglobulin and albumin in blood. However, the reported degree of heparin protein binding varies between <3% and \approx 89%. Since protein-binding may influence hormone function and quantification, better insight into the degree of heparin protein binding is essential to fully understand the biological behavior of heparin and interpretation of its measurement in patients. Here, we used peritoneal dialysis to assess human heparin protein binding in a functional human setting for the first time. We measured freely circulating solutes in blood and peritoneal fluid of 14 patients with end-stage renal disease undergoing a peritoneal equilibration test to establish a curve describing the relation between molecular weight and peritoneal clearance. Calculated binding percentages of total cortisol and testosterone confirmed our model. The protein-bound fraction of heparin was calculated to be 40% (\pm 23%). We, therefore, conclude that a substantial proportion of heparin is freely circulating. Although a large inter-individual variation in heparin clearance, besides patient-specific peritoneal transport characteristics, may have affected the accuracy of the determined binding percentage, we describe an important step towards unraveling human heparin plasma protein binding *in vivo* including the caveats that need further research.

Keywords: iron homeostasis; heparin; protein binding; peritoneal dialysis

1. Introduction

The peptide hormone heparin, produced by hepatocytes, regulates iron entry into the circulation and iron distribution throughout the body by degradation of the cellular iron exporter ferroportin [1]. Hormones can circulate freely or partially bound to carrier proteins in the human body [2,3]. These carrier proteins allow transport of non-soluble hormones through the blood plasma, prevent excretion, and function as a reservoir thereby regulating hormone bioavailability. The free hormone hypothesis indicates that most hormones are not capable of exerting their physiological effect in a protein-bound form [2,4–6]. Hence, for partially bound hormones, such as thyroxine, testosterone, triiodothyronine, and cortisol, assays were designed to specifically quantify the bioactive free hormone concentration [5,7–9].

It is suggested that heparin in blood is partially bound to both α 2-macroglobulin (α 2M) and albumin [10]. However, there is large controversy on the subject since the reported plasma protein-bound fraction of heparin varies between <3% and \approx 89% [10–12]. Consequently, it is uncertain to what degree currently available analytical assays quantify (only) bioactive heparin. Differences in extent of quantification of the freely circulating heparin relative to the protein bound compound between

assays may influence interpretation and comparability of hepcidin measurement and hampers recent achievements on standardization of hepcidin assays [13]. Characterization of the measured analyte will add the final aspect to the metrological traceability chain of hepcidin, which describes an unbroken calibration hierarchy from a measurement result to a defined reference in SI units. Additionally, unraveling the protein binding properties of hepcidin will improve our understanding of its biology and will allow its correct quantification, which is essential in the assessment and interpretation of the pharmacokinetic and pharmacodynamic effects of hepcidin agonist or antagonist therapies [1,14]. To address these issues, we aimed to study the degree of hepcidin protein binding in a functional human setting for the first time, rather than using previously reported *in vitro* (research) techniques [10,11], mice *in vivo* studies [11], or human *ex vivo* serum analysis [12]. Especially since current *in vitro* techniques to assess free hormone concentrations in diagnostic medicine, such as equilibrium dialysis and ultrafiltration methods, have limitations amongst which are risk for leakage, non-specific absorption of the hormone leading to low reproducibility, and bias of results, osmotic effects or the Gibbs-Donnan effect [15].

To circumvent the limitations of *in vitro* studies, we aimed to unravel hepcidin plasma protein binding with a new approach using the principle of peritoneal dialysis (PD). PD is a treatment option for patients with end-stage renal disease (ESRD), in which the peritoneal cavity is filled with dialysis fluid. During a certain dwell time, plasma components are transported into the peritoneal cavity. Transport of molecules is mainly driven by diffusion and ultrafiltration and depends on peritoneal membrane characteristics [16]. To assess these individual characteristics, all patients treated with PD frequently undergo a standardized and highly reproducible peritoneal equilibration test (PET) [17–19]. During a four-hour dialysis exchange, the peritoneal membrane transport function of a patient treated with PD is characterized to classify them as either a high, low, or average transporter in order to optimize the dwell time [17]. These peritoneal equilibration rates reflect peritoneal clearance (PC) and can be calculated using the dialysate-to-plasma ratio (D/P ratio) for any given solute that is transported from the peritoneal capillary blood into dialysate fluid. The transport rate over the peritoneal membrane into the peritoneal cavity is largely size-selective and thus the PC is inversely related to the radius or molecular weight (MW) of a molecule, regardless of molecular properties such as charge or hydrophobicity [20–24]. Therefore, the transport rate of small molecules that are bound to large plasma proteins will be determined by the size of the complex. This has been verified using the protein-bound solute *p*-cresol (MW: 108 Da), since its PC was found to be similar to β 2-microglobulin (MW: 11,815 Da) [25]. Although a high PC was expected based on the low MW of *p*-cresol, its clearance was much lower, confirming protein-binding.

In this study, we measured hepcidin, along with known freely circulating and protein-bound solutes, in blood and dialysate of patients with ESRD undergoing PET. We used this model to explore its suitability to quantify hormone binding, and therefore to unravel the extent of hepcidin binding in the circulation, by studying if the measured PC of hepcidin fits the curve describing the relation between MW and PC of free circulating solutes.

2. Results

In total, 14 patients with ESRD participated in this study. Mean age was 67 years, total creatinine clearance was between 56.4 and 129.5 L/week/1.73 m², and residual creatinine clearance was between 25.7 and 100.0 L/week/1.73 m² (Table 1). The start time of the PET after an overnight dwell was between 8:30 a.m. and noon. Four patients used prednisone (Table 1), which interferes with both total and free cortisol measurement due to metabolism into prednisolone. Therefore, cortisol measurements were not performed in these patients.

Table 1. Patient characteristics.

ID	Age (yrs)	Gender (M/F)	Underlying Disease	Use of Prednisone ¹	Creatinine Clearance (L/week/1.73 m ²) Total (residual)	Start Time PET (a.m.)
1	73	F	Primary amyloidosis	No	61.1 (32.3)	10:00
2	82	M	Renal vascular disease due to hypertension	No	56.4 (25.7)	09:30
3	55	F	Lupus erythematosus	1 × 7.5 mg	99.3 (73.7)	10:00
4	74	M	Renal vascular disease due to hypertension	No	68.2 (34.5)	08:30
5	72	M	IgA nephropathy	No	118.2 (69.6)	10:00
6	64	M	Renal vascular disease type unspecified	1 × 7.5 mg	70.5 (37.1)	12:00
7	66	M	Diabetes mellitus type 2	No	100.8 (63.9)	10:15
8	53	M	Chronic renal failure etiology unknown/uncertain	No	71.7 (43.2)	12:00
9	76	M	Renal vascular disease due to hypertension	No	114.0 (75.7)	12:00
10	72	M	Renal vascular disease type unspecified	No	128.6 (100.0)	10:00
11	55	F	IgA nephropathy	2 × 5 mg	122.6 (77.8)	11:30
12	61	F	Renal vascular disease type unspecified	1 × 5 mg	129.5 (96.7)	11:00
13	64	F	Diabetes mellitus type 2	No	101.8 (35.9)	09:30
14	77	M	Renal vascular disease due to hypertension	No	118.1 (73.1)	09:00

¹ Since use of prednisone was found to decrease the serum cortisol concentrations and interferes with measurement of both free and total cortisol, these measurements were not performed in patients using prednisone.

We measured and subsequently calculated the PC of free circulating solutes urea, creatinine, β 2-microglobulin, albumin, and IgG (Table 2), and plotted the logarithmically transformed PC of each analyte against its MW for each patient. By applying a linear mixed model, we obtained a curve with a 95% prediction interval describing the relation between MW and PC of free circulating solutes (Figure 1, solid dots). We found the equation defining the curve to be:

$$\text{Log}_{10}(\text{PC}) = 4.5744 - 0.05063 * \sqrt[3]{\text{MW}}. \quad (1)$$

Table 2. Molecular weight, measured plasma, and dialysate concentrations (mean \pm sd) and subsequent calculated peritoneal clearances (mean \pm sd) for all solutes.

Solute	MW (Da)	[Plasma] t = 0 h Mean (sd)	[Dialysate] ¹ t = 1 h Mean (sd)	Peritoneal Clearance (μ L/min) t = 0–1 h Mean (sd)	N
Urea (mmol/L)	60.0	21.5 (6.6)	15.0 (6.1)	26,959.1 (6211.8)	14
Creatinine (μ mol/L)	113.1	542.0 (172.6)	296.9 (119.9)	21,499.6 (5861.6)	14
Testosterone, total (nmol/L) ²	288.0	8.6 (7.3)	0.1 (0.1)	507.7 (260.5)	13 ³
Cortisol, total (nmol/L) ⁴	362.5	229.1 (134.4)	8.4 (5.8)	1439.0 (490.8)	10 ⁵
Cortisol, free (nmol/L)	362.5	12.7 (7.7)	8.4 (5.8)	25,030.8 (8123.9)	9 ^{5,6}
Hepcidin (nmol/L)	2789.4	10.6 (9.1)	1.2 (1.1)	4337.5 (1713.1)	13 ⁷
β 2-microglobulin (mg/L)	13,713.0	18.7 (5.0)	1.4 (0.9)	2804.0 (1587.8)	14
Albumin (g/L)	66437.0	32.1 (4.9)	0.2 (0.1)	242.3 (164.2)	14
IgG (mg/L)	150,000.0	7496.0 (1730.3)	23.1 (12.7)	129.3 (84.7)	14

¹ Average dialysate volumes were 2364 mL (range 1500–2250 mL). ² Approximately 2% of the total plasma testosterone is circulating free in both men and women [9,26]; in men 44–65% of testosterone is bound to sex hormone binding globulin and 33–54% bound to albumin; in women 66–78% of testosterone is bound to sex hormone binding globulin (MW 90 kDa), and 20–32% to albumin (MW 66,437 Da) [9]. ³ One patient was excluded since dialysate concentrations of testosterone were below the LLOD. ⁴ Approximately 5% of the total plasma cortisol is circulating free, since 80–90% of cortisol is bound to corticosteroid binding globulin (MW 52 kDa) and 10–15% to albumin (MW 66,437 Da) [6]. ⁵ Patient 3, 6, 11, and 12 used prednisone, which interferes with cortisol measurement. The results of these patients were excluded. ⁶ For one patient material was insufficient to measure free cortisol. ⁷ One patient was excluded since both plasma and dialysate concentrations of hepcidin were below the LLOD.

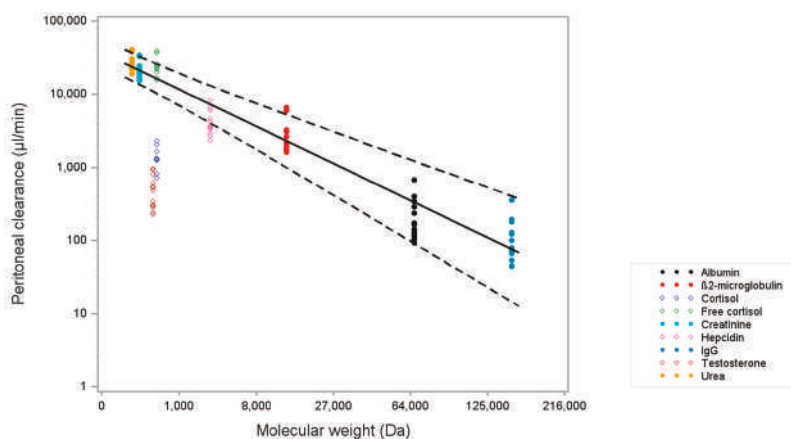


Figure 1. Peritoneal clearances ($\mu\text{L}/\text{min}$) as a function of molecular weight (Da). The logarithmically transformed peritoneal clearance (y-axis) of each specific analyte for each patient was plotted against the cube root of their molecular weight (x-axis). Based on the known free circulating analytes, i.e., urea, creatinine, β 2-microglobulin, albumin, and IgG (solid dots), a curve was established describing the relation between clearance and molecular weight (solid line) with a 95% prediction interval (dashed lines). Hepcidin, free cortisol, total cortisol, and total testosterone measurements (open dots) were plotted in the figure thereafter.

We tested the validity of our model by measuring both total (i.e., free and protein-bound) and free cortisol and total testosterone. As expected, the PC measurements of total cortisol (Figure 1, open blue dots) and total testosterone (Figure 1, open brown dots) plotted against the MW of the unbound molecules were below the free solutes curve, confirming their predominantly bound status. Their binding percentages were calculated to be 91% ($\pm 3\%$) and 97% ($\pm 1.5\%$), respectively, corresponding to what is described in literature [6,9]. The mean of free cortisol PC measurements (Figure 1, open green dots) fell within the 95% confidence interval to the free solutes curve, confirming its unbound status.

We subsequently plotted the hepcidin PC measurements against the MW of hepcidin in the graph (Figure 1, open pink dots). Interestingly, the mean PC for hepcidin was found to lie below the established free solute curve. The calculated binding percentage is 40% ($\pm 23\%$), suggesting that hepcidin circulates in a more unbound than bound status, with a large inter-individual variability. Since several hepcidin PC measurements fell within the 95% confidence interval, we can exclude a predominantly protein-bound status of hepcidin, as we found for total cortisol and testosterone.

Last, we correlated the PC of all analytes with the PC of urea (Figure 2) to ensure that the variation in PC measurement of each analyte is solely caused by patient-specific transport characteristics. We have chosen urea for this since its PC measurements showed the lowest variation. We found a strong correlation between urea and creatinine (Spearman $r = 0.9604$, $p < 0.0001$), β 2-microglobulin (Spearman $r = 0.7890$, $p = 0.0008$), free cortisol (Spearman $r = 0.9333$, $p = 0.0002$), total cortisol (Spearman $r = 0.7697$, $p = 0.0092$), and total testosterone (Spearman $r = 0.6593$, $p = 0.0142$). We observed a weaker correlation between urea and IgG (Spearman $r = 0.5516$, $p = 0.0408$) and urea and albumin (Spearman $r = 0.5077$, $p = 0.0638$). However, we did not find any correlation between urea and hepcidin (Spearman $r = 0.2198$, $p = 0.4706$). Further analysis showed that hepcidin does not correlate with any of the other analytes (Figure 3), except albumin (Spearman $r = 0.6648$, $p = 0.0132$). These results confirm a large inter-individual variation in hepcidin clearance, independent of peritoneal transport characteristics. Although this might influence the accuracy of the determined binding percentage, we conclude that a substantial proportion of hepcidin is freely circulating and exclude that circulating hepcidin is predominantly bound to plasma proteins.

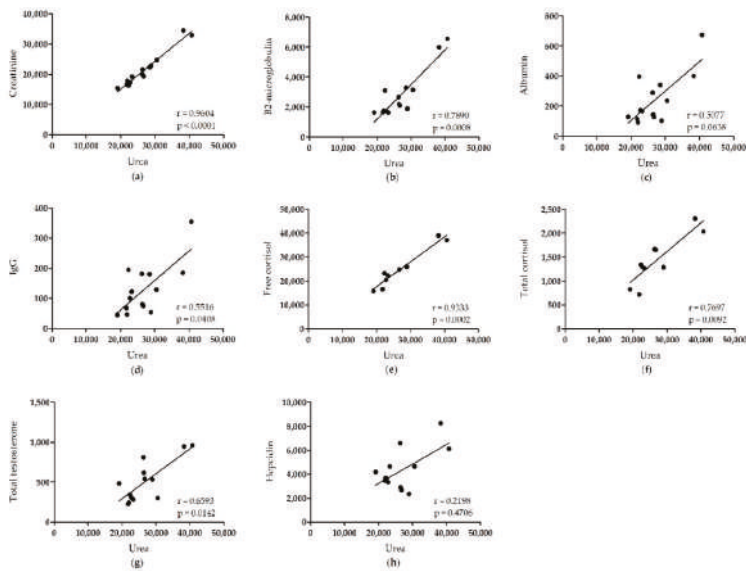


Figure 2. Correlations among the peritoneal clearance of urea with all analytes in patients with end-stage renal disease undergoing a peritoneal equilibration test. The peritoneal clearance of urea was correlated to the peritoneal clearance of (a) creatinine, (b) $\beta 2$ -microglobulin, (c) albumin, (d) IgG, (e) free cortisol, (f) total cortisol, (g) total testosterone, and (h) hepcidin. The strength of the correlation was measured using Spearman's correlation coefficient (r).

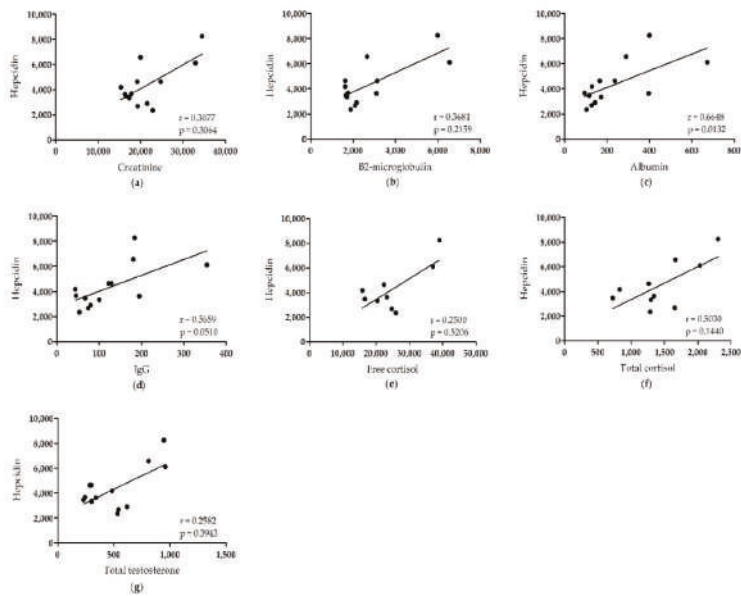


Figure 3. Correlations among the peritoneal clearance of hepcidin with all analytes in patients with end-stage renal disease undergoing a peritoneal equilibration test. The peritoneal clearance of hepcidin was correlated to the peritoneal clearance of (a) creatinine, (b) $\beta 2$ -microglobulin, (c) albumin, (d) IgG, (e) free cortisol, (f) total cortisol, and (g) total testosterone. The strength of the correlation was measured using Spearman's correlation coefficient (r).

3. Discussion

We applied the principle of PD to study the plasma protein binding of hepcidin in a functional human setting for the first time. To this end, we measured hepcidin along with known freely circulating and predominantly protein-bound solutes in blood and dialysate of patients with ESRD treated with PD undergoing PET. Our findings exclude that circulating hepcidin is predominantly bound to plasma proteins; however, the lack of correlation between hepcidin clearance and the clearance of the other analytes question the suitability of this model to determine an accurate binding percentage for this hormone.

Our finding that hepcidin is not predominantly bound is in line with other literature, in which a merely freely circulating hepcidin was observed using gel chromatography on human serum samples, followed by Liquid Chromatography-Mass Spectrometry (LC-MS/MS) [12]. In fact, other peptide hormones such as human growth hormone [27], insulin [28], and cardiac natriuretic hormones [29] circulate freely, since peptide hormones are often water-soluble and can, therefore, easily be transported via the cardiovascular system without requiring carrier proteins [30,31]. In addition, hepcidin is rapidly excreted by the kidneys, leading to a short half-life of only several hours [32–34]. Moreover, circulating hepcidin concentrations can change rapidly with subsequent effects on the circulating iron levels within 1–2 h [35,36]. Theoretically, this potential of a fast production [33,35,37] and quick turnover of hepcidin would preclude the need for protein binding [30].

Our observations differ from previous findings describing high hepcidin binding [10,11]. We believe this reported high binding percentage of $\approx 89\%$ might be attributed to the excess of hepcidin (i.e., 100 times higher than physiological conditions) that was used in the experimental set-up, in combination with physiological $\alpha 2M$ concentrations. This might have caused nonspecific binding of hepcidin, which the molecule is prone to due to its amphipathic character [38].

To the best of our knowledge, there are currently no established *in vivo* models to study protein binding and free drug or biomarker concentrations, which makes our explorative approach to study the functional protein binding of hormones unique. PET is regularly used as diagnostic test to characterize the functionality and transport properties of the peritoneal membrane in PD patients. Therefore, the strength of using PET is that dialysate sampling for research purposes provides human data with no additional burden for patients and it is a relatively simple, quick, and affordable method. Albeit we circumvent the disadvantages of *in vitro* techniques used to assess free hormone concentrations in diagnostic medicine, several limitations of our method were discovered during the study.

First, hepcidin did not correlate with urea or any of the other analytes, except albumin, suggesting that other variables besides patient-specific peritoneal transport characteristics may have affected hepcidin PC quantification. This raises the question if we can infer an accurate binding percentage for hepcidin, despite the fact that the validity of our model is confirmed by total cortisol and testosterone measurements and deduction of their binding percentages.

One of the possible explanations for the lack of correlation is the circadian rhythm of hepcidin, with plasma levels increasing during the day [39–43]. Due to practical concerns, blood and dialysate samples were not collected at the same time point, which could influence the determined hepcidin clearance differently for each patient. Nonetheless, in our previous study [43], the serum hepcidin median increased only by 2.5% between 11:00 and 13:30 amongst 24 participants. Therefore, the influence of hepcidins circadian rhythm seems unlikely since we used a time interval of only 1 h between blood and dialysate collection and PET start times were between 8:30 and noon. In addition, we did find a strong correlation between urea and both free and total cortisol, a hormone which also follows a circadian rhythm [44], suggesting the influence of a circadian rhythm is negligible.

Second, our study population suffers from ESRD and may therefore differ from healthy subjects, although there is no data to support the idea that peritoneal transport is affected by kidney function. It is described, however, that renal failure leads to the accumulation of uremic toxins in blood and tissues. These toxins can compete with other substrates for plasma protein binding to, for example, albumin [45]. As a result, the free fraction of a substrate of albumin will be increased. The strong

correlation between hepcidin and albumin could imply binding of hepcidin to albumin and, therefore, the presence of uremic toxins might explain the observed inter-individual variability between hepcidin measurements. This might have led to an overestimation of the free fraction of hepcidin. However, testosterone and cortisol are also partially bound to albumin and we see no effect on their measurements. Therefore, interference of uremic toxins appears less likely. Nonetheless, we suggest our data should be validated and confirmed in healthy individuals or patients with a normal glomerular filtration rate.

Third, we cannot exclude our results are affected by production of hepcidin by mesothelial cells of the peritoneal membrane [46]. However, we feel that this is rather unlikely due to the high volume (2000 mL) new dialysate infused at $t = 0$ and the short dialysate time of 60 min.

Fourth, it is known that macromolecules may be removed from the peritoneal cavity by lymphatic absorption, which is independent of molecular size [47]. We observed a weak correlation between the small molecule urea and both macromolecules albumin and IgG. This might indicate that transport of these molecules is not solely dependent on permeability of the peritoneum, but also on lymphatic absorption, which would influence the established curve and could possibly introduce the observed inter-individual variation.

Last, clearance measurements of both hepcidin and albumin might be affected by their adherence to the dialysis bag, since both molecules possess nonspecific binding characteristics [38,48]. Due to their amphipathic character and suggested subsequent adherence to laboratory plastics, an extra factor of inter-individual variation is introduced, independent of patient specific peritoneal transport characteristics. This could explain the lack of correlation with other analytes. Regarding the assessment of hepcidin's binding percentage, this would imply that the hepcidin concentration in dialysate, and therefore its clearance, might be underestimated and the free fraction would actually be higher.

Knowledge on the binding percentage of hepcidin is of great importance for correct quantification of the bioactive hormone, which in turn is key for both interpretation and standardization of hepcidin measurements in diagnostics of iron related disorders [1]. Characterization of hepcidin as measurand will add a key aspect to its metrological traceability chain that has recently been established, which is needed to obtain global uniform reference intervals and clinical decision limits for diagnostics [13]. Furthermore, correct analysis and interpretation of hepcidin measurement is essential in target assessment of clinical trials with hepcidin agonists and antagonists. These therapeutics are in development for treatment of several iron metabolism disorders such as hereditary hemochromatosis or anemia of inflammation [1,14]. A strong correlation has been observed between hepcidin-25 results of a wide variety of assays [13,49,50]. However, possible protein-binding could be an issue in correct hepcidin quantification when measuring target engagement of hepcidin antagonists that directly bind hepcidin in the circulation. These compounds display a high affinity for hepcidin and therefore specifically for the assessment of hepcidin antagonist efficiency, the remaining free, non-antagonist bound hepcidin should be quantified [51] rather than total hepcidin [52–54].

We can conclude that hepcidin is not predominantly bound and suggest that a substantial proportion is freely circulating, enabling direct and rapid bioactivity of the hormone. This is an important step towards unraveling human hepcidin plasma protein binding, although refinement of the model by inclusion of more patients and using more analytes with different MWs could help improve accuracy of our findings. Thereby, analytes with a high MW should preferably be excluded to circumvent influence of lymphatic absorption. Moreover, to definitively unravel hepcidin plasma protein binding, future studies must include studies on hepcidin production by mesothelial layer of the peritoneum and the prevention of nonspecific adherence of hepcidin in pre-analysis, which currently hampers (standard) dialysis techniques in diagnostic medicine.

4. Materials and Methods

4.1. Sample Collection

PET was performed according to the protocol of Isala hospital, Zwolle, The Netherlands. After an 10–12 h overnight dwell, a 2-Liter bag of 3.86% glucose solution (Baxter Healthcare Ltd., Newbury, UK) was instilled and allowed to dwell for 4 h [18]. Dialysate sampling was performed at 1 h of dwell time ($t = 1$ h), since peritoneal transport of solutes with a low MW (<200 Da) will decrease over a longer period of time due to saturation of the dialysate [22]. For sampling, all dialysate fluid was collected in a plastic (PVC) bag and mixed *ex vivo* before a sample was taken, to assure dialysate samples were representative for the fluid in the peritoneal cavity. Thereafter, the remaining dialysate was returned into the peritoneal cavity. Dialysate from 14 consecutive patients with ESRD who underwent PD was collected in tubes both with and without bovine serum albumin (1 g/50 mL, Sigma-Aldrich, Saint Louis, MO, USA), as for measurements of hepcidin and IgG, addition of albumin prevents adhesion to the tubes and subsequent loss. Blood samples were drawn prior to dialysis ($t = 0$ h) in both heparin plasma and serum tubes. All were centrifuged at 2000 g for 10 min. Blood and dialysate samples were aliquoted and immediately stored at -80 °C until measurement.

4.2. Laboratory Measurements

We measured total hepcidin concentrations (i.e., the sum of bound and unbound full length hepcidin-25), as well as freely circulating solutes urea, creatinine, β 2-microglobulin, albumin, IgG, total testosterone (free and protein-bound), total cortisol (free and protein-bound), and free cortisol in blood and dialysate.

Analytical methods are described in Appendix A. All measurements were performed in freshly thawed aliquots within 8 months after collection.

4.3. Ethics

The study was approved by the Ethics Committee and the Board of Directors of Isala hospital, Zwolle, The Netherlands, and has been in accordance with the Helsinki Declaration. All patients signed informed consent prior to participation and all samples were blinded.

4.4. Calculations and Statistical Analysis

Measurement results for solutes below the lower limit of detection (LLOD) were excluded. We calculated PC ($\mu\text{L}/\text{min}$) for each analyte using the dialysate sample taken at $t = 1$ h and blood sample taken at $t = 0$ h of each analyte, using the patient-specific dialysate volume and a dialysis time of 60 min as:

$$\text{Peritoneal clearance } (\mu\text{L}/\text{min}) = [\text{Dialysate}]/[\text{Blood}] \times (\text{Volume dialysate})/(\text{Duration of dialysis}). \quad (2)$$

Logarithmic transformation was used for PC data before statistical analysis, as the data was not normally distributed. In addition, we used a cube root scaling for MW, since transport rates are related to size and thus radius (r), and considering volume as a three-dimensional characteristic of the radius (r^3). The curve describing the relation between MW and PC of all analytes was estimated with a linear mixed model using a random slope and random intercept. The expected PC of each analyte could be calculated using its MW and the equation of the curve. We calculated the binding percentage as:

$$\text{Binding percentage } (\%) = (1 - (\text{Measured PC})/(\text{Expected PC})) \times 100\%. \quad (3)$$

In this equation, the measured PC divided by the expected PC represents the free hormone fraction. The strength of a linear association between the clearances of two analytes was measured using Spearman's correlation coefficient.

All statistical analyses were performed with SAS software, version 9.4 (SAS Institute, Inc. Cary, NC, USA) and GraphPad Prism 5.03 (GraphPad Software Inc., La Jolla, CA, USA).

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Appendix A

Laboratory Chemistry Measurements

Measurements of urea, creatinine, and IgG were performed on a Cobas C8000 random access analyzer (Roche Diagnostics, Basel, Switzerland). Albumin was measured by nephelometry on a BNII system (Siemens, München, Germany). β 2-microglobulin was measured by a direct sandwich-ELISA, using two polyclonal rabbit anti-human β 2-microglobulin antibodies, of which one was HRP labeled. The pH of all peritoneal dialysate samples was 7.5 allowing accurate measurements of β 2-microglobulin.

Measurement of Hepcidin in Serum and Peritoneal Dialysate

Measurements of hepcidin were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS) using a stable hepcidin-25⁺⁴⁰ isotope as internal standard for quantification [55]. This MS-based assay determines total hepcidin (i.e., the sum of bound and unbound fraction). Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionisation TOF MS platform (Bruker Daltonics, Hamburg, Germany). Hepcidin concentrations were expressed as nmol/L. The lower limit of quantification of this method was 0.5 nM.

Measurement of Total Cortisol and Total Testosterone in Serum

Cortisol and testosterone were analyzed by Liquid Chromatography-Mass Spectrometry (LC-MS/MS) after protein precipitation and solid-phase extraction. Internal standard [²H₄]-cortisol and [¹³C₃]-testosterone (Isoscience, Ambler, PA, USA) was added to 100 μ L serum. Subsequently, 300 μ L acetonitrile with 0.1% formic acid was added for protein precipitation and 300 μ L H₂O was added to 200 μ L supernatant followed by solid phase extraction (Oasis HLB 1cc, Waters, Milford, MA, USA). The eluate (methanol:isopropanol 95:5) was dried under a stream of N₂ gas, reconstituted in methanol:water (3:7) and injected (10 μ L) into an Agilent Technologies 1290 Infinity Ultra High Performance Liquid Chromatography (UHPLC)-system (Agilent Technologies, Agilent, Santa Clara, CA, USA) equipped with a BEH C18 (1.7 μ m 2.1 \times 50 mm) analytical column (Waters Corp.) at 60 °C. Mobile phase A (methanol:water 20:80 + 2 mM NH₄CH₃COO + 0.1% formic acid) and B (methanol:water 98:2 + 2 mM NH₄CH₃COO + 0.1% formic acid) were run in a gradient (0.4 mL/min). Start gradient 70:30 A:B for 2.5 min; then to 40:60 A:B in 3.5 min; followed by a gradient in 0.5 min to 2:98 to remain such for 0.5 min and thereafter to 70:30 A:B in 0.5 min and remain such for 0.5 min. Retention time was 1.41 min and 4.37 min for cortisol and testosterone, respectively. Total run time was 8 min. A 9-point calibration curve was used (cortisol and testosterone, Steraloids). An Agilent 6490 tandem mass spectrometer (Agilent Technologies, Agilent, Santa Clara, CA, USA) was operated in the electrospray positive ion mode, with a capillary voltage 3.5 kV, fragmentor voltage 380 V, sheath gas temperature 350 °C, and gas temperature 150 °C with N₂ collision gas. Two transitions (qualitative and quantitative) were monitored. Transitions (Q1 > Q3) were m/z 363.4 > 97.1 (34 V) and m/z 363.4 >

121.1 (25 V) for cortisol; m/z 367.4 > 97.1 (34 V) and m/z 367.4 > 121.1 (25 V) for [2H₄]-cortisol. m/z 289.2 > 109.1 (30 kEV) and m/z 289.2 > 97.1 (30 kEV) for testosterone; m/z 292.3 > 112.1 (30 kEV) and m/z 292.3 > 100.1 (30 kEV) for 13C₃-testosterone. Dwell time was 100 ms and 50 ms for cortisol and testosterone, respectively. The method was linear assessed by CLSI EP6 protocol [56]. For cortisol total CV is 3.6% at 300 nmol/L and 3.1% at 1080 nmol/L. For testosterone total CV is 6.0% at 0.9 nmol/L and 5.3% at 19 nmol/L.

Measurement of Free Cortisol in Serum by Equilibrium Dialysis—Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Serum was dialyzed in a multi-cell equilibrium dialyzer using Teflon dialyzing cells consisting of two identical parts between which a flat membrane is fitted (Dianorm-Geräte). The dialysis cells are incubated in a water bath with constant agitation by a rotating apparatus and temperature during dialysis was maintained at 37 ± 0.5 °C, by constant monitoring with an immersion thermostatic system (Tinytag TGP-4020 logger and PB5001-1M5 sonde; Gemini data loggers). The dialysis membranes were prepared from Dianorm (Diachema dialysis membranes 10.14) with a cut-off of 5 kDa. Equilibrium dialysis was performed for 5 h with 180 μ L serum against 180 μ L HEPES buffer; while rotating the dialysis apparatus at 20 rpm. The HEPES buffer used for equilibrium dialysis was composed to reflect the electrolyte composition and pH of serum and contained in addition to 12.570 g/L N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid (HEPES), 5.353 g/L NaCl, 0.200 g/L KCl, 0.224 g/L KH₂PO₄, 0.275 g/L MgSO₄·7 H₂O, 0.300 g/L urea, 0.275 g/L CaCl₂·2 H₂O, 0.900 g/L NaOH, and 0.520 g/L NaN₃ (Merck, Darmstadt, Germany). The pH was adjusted with HCl to pH 7.40 (at 37 °C). The buffer was diluted by ultrapure water produced with a Milli Advantage A10 and 100 μ L dialysate was subsequently used for total cortisol measurement by LC-MS/MS as described above.

Measurement of Total Cortisol and Total Testosterone in Peritoneal Dialysate

Testosterone and cortisol in peritoneal dialysate were analyzed by LC-MS/MS after solid-phase extraction. Sample preparation consisted of internal standard addition [13C₃]-testosterone (Isoscience, Ambler, PA, USA) and [³H₄]-cortisol (Isoscience) to 1000 μ L dialysate and solid phase extraction (SPE) using Oasis[®] MCX 1cc cartridges (Waters Corp, Milford, MA, USA).

Columns were pre-equilibrated with 1 mL MeOH:IPA (95:5) and subsequently washed with 1 mL H₂O. After application of the sample, columns were washed with 1 mL H₂O:NH₄OH (95:5) and 1 mL MeOH:H₂O (20/80) + 2% HCOOH. The 300 μ L eluate (MeOH) was dried under a stream of N₂ gas, reconstituted in MeOH:H₂O (30:70). A nine-point calibration series was used (using testosterone and cortisol both from Steraloids). Calibrators, quality controls, and samples (reconstituted in 100 μ L) were injected (10 μ L) into an Agilent Technologies 1290 Infinity VL UHPLC-system (Agilent Technologies, Santa Clara, USA), equipped with a BEH C18 (1.7 μ m 2.1 X 50 mm) analytical column (Waters Corp., Milford, MA, USA) at 60 °C. Mobile phase A (MeOH/H₂O (20:80) + 2 mM NH₄CH₃COO + 0.1% HCOOH) and B (MeOH/H₂O (98:2) + 2 mM NH₄CH₃COO + 0.1% HCOOH) were run in a gradient (0.4 mL/min). The gradient program was as follows: Start gradient A:B (70:30) for 2.5 min; followed by a gradient in 2 min to A:B (60:40) and subsequent 2.5 min A:B (35:65). For column washing, a gradient 0.5 min A:B (98:2) was established to remain such for 0.5 min. The column was re-equilibrated to A:B (70:30) in 0.5 min and remained such for 0.5 min. Retention times were 1.5 min and 4.5 min for cortisol and testosterone, respectively. An Agilent 6490 tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was operated in the electrospray positive ion mode. Operating conditions were as follows: Capillary voltage 3.5 kV, fragmentor voltage 380 V, sheath gas temperature 350 °C, and gas temperature 150 °C. The collision energy was optimized between 25 and 34 eV for all solutes and Nitrogen was used as collision gas. Two mass transitions were monitored for each analyte and the internal standards. The first transition was used for quantification, the second for confirmation. The transitions (Q1 > Q3) were m/z 289 > 97 and m/z 289 > 109 for testosterone; m/z 292 > 100 and m/z

292 > 112 for [13C3]-testosterone; m/z 363 > 121 and m/z 363 > 97 for cortisol; m/z 367 > 121 and m/z 367 > 97 for [3H4]-cortisol. Total run time was 9 min.

For both total and free cortisol PC calculations, we used total cortisol measurements in dialysate, since we assume that all cortisol present in dialysate after 1 h of dwell time is free considering the large MW of its main binding protein corticosteroid binding globulin (MW 52 kDa).

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Article

L-Ferritin: One Gene, Five Diseases; from Hereditary Hyperferritinemia to Hypoferritinemia—Report of New Cases

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Abstract: Ferritin is a multimeric protein composed of light (L-ferritin) and heavy (H-ferritin) subunits that binds and stores iron inside the cell. A variety of mutations have been reported in the L-ferritin subunit gene (*FTL* gene) that cause the following five diseases: (1) hereditary hyperferritinemia with cataract syndrome (HHCS), (2) neuroferritinopathy, a subtype of neurodegeneration with brain iron accumulation (NBIA), (3) benign hyperferritinemia, (4) L-ferritin deficiency with autosomal dominant inheritance, and (5) L-ferritin deficiency with autosomal recessive inheritance. Defects in the *FTL* gene lead to abnormally high levels of serum ferritin (hyperferritinemia) in HHCS and benign hyperferritinemia, while low levels (hypoferritinemia) are present in neuroferritinopathy and in autosomal dominant and recessive L-ferritin deficiency. Iron disturbances as well as neuromuscular and cognitive deficits are present in some, but not all, of these diseases. Here, we identified two novel *FTL* variants that cause dominant L-ferritin deficiency and HHCS (c.375+2T > A and 36_42delCAACAGT, respectively), and one previously reported variant (Met1Val) that causes dominant L-ferritin deficiency. Globally, genetic changes in the *FTL* gene are responsible for multiple phenotypes and an accurate diagnosis is useful for appropriate treatment. To help in this goal, we included a diagnostic algorithm for the detection of diseases caused by defects in *FTL* gene.

Keywords: ferritin; hereditary hyperferritinemia; hereditary hypoferritinemia; iron metabolism; cataracts syndrome; neurodegenerative disease

1. Introduction

Ferritin is an iron-binding protein that stores and releases iron and thus contributes to maintaining and controlling iron homeostasis. Iron is stored in ferritin in the Fe^{+3} form and released in the Fe^{+2} form. Tissue ferritin is a multimeric protein formed from the assembly of 24 peptide subunits, known as the light (L) and heavy (H) ferritin subunits. The H subunit has ferroxidase activity and converts iron from Fe^{+2} to Fe^{+3} , which enables iron storage; it also serves to regulate pH and increase concentrations of free radicals in the body, which can be extremely damaging to cellular structures and proteins [1]. The L-ferritin subunit helps with electron transport in and out of the ferritin core protein and plays a role in iron release, as Fe^{+2} , from ferritin. Unlike tissue ferritin, serum ferritin is partially glycosylated and nearly completely iron-free [2,3] and is mainly composed of L-ferritin subunits [4–6].

Focusing on the L-ferritin (*FTL*) gene, five diseases have been identified as directly resulting from mutations in this gene. These diseases include hereditary hyperferritinemia with cataract syndrome (HHCS), neuroferritinopathy, benign hyperferritinemia (or hyperferritinemia without iron overload), autosomal dominant L-ferritin deficiency and autosomal recessive L-ferritin deficiency.

HHCS (OMIM#600886, ORPHA163) is associated with mutations located in the iron responsive element (IRE) at the 5' untranslated region (UTR) of the L-ferritin mRNA, which results in the disruption of binding with iron regulatory proteins (IRP1 and 2), this is known as the IRP-IRE post-transcriptional regulatory system [7–9]. Mutations in this RNA motif result in the loss of ferritin translation repression and excess ferritin production, even though iron levels remain normal. Ferritin overproduction leads to deposits in the lens of the eye, resulting in the development of cataracts [10]. Up to now, there are 47 known mutations associated with HHCS [11].

In 2001, Curtis and collaborators [12] described for the first time neuroferritinopathy (OMIM#606159, ORPHA:157846), an autosomal dominant condition characterized by normal to low serum ferritin levels, progressive chorea or dystonia, and subtle cognitive deficits. Neuroferritinopathy is classified as a member of the group of diseases known as neurodegeneration with brain iron accumulation (NBIA). So far, there have been ten reported mutations causing this condition, mostly located at the C-terminal region of the *FLT* gene [13]. *FTL* mutations diminish ferritin's ability to store iron so, in an attempt to control free iron levels, neurons produce more ferritin, resulting in iron and ferritin accumulation in the basal ganglia of the brain and leading to movement and cognitive disabilities [14].

Benign hyperferritinemia or genetic hyperferritinemia without iron overload (OMIM#600886, ORPHA:254704) is another *FTL* mutated disorder where patients have high (greater than 90%) glycosylated serum ferritin levels. There are three known mutations in the N-terminal region of the *FTL* gene that cause benign hyperferritinemia [15]. Despite serum ferritin hyperglycosylation, no other harmful effects have been detected in patients with this disorder [16].

Finally, two variants in *FTL* have been reported causing L-ferritin deficiency, i.e., hypoferritinemia (OMIM#615604, ORPHA:440731). Mutation Glu104Ter was described in a single patient with inheritance in autosomal recessive mode and consists of a G > C nucleotide substitution in exon 3 (c.310G > T). This mutation causes a complete lack of translation of the *FTL* gene with subsequently undetectable levels of serum ferritin. This patient presented with seizures and restless leg syndrome [17]. The *FTL* mutation Met1Val, resulting in a change at the start codon (c.-1A > G), has also been described in a single case; however, this mutation was inherited in an autosomal dominant manner. The patient presented with decreased levels of serum ferritin, but no history of iron deficiency anemia or neurologic dysfunction [18].

In this study, we report the identification of two novel mutations in the *FTL* gene detected by gene sequencing. One mutation is associated with a diagnosis of HHCS and the other with a diagnosis of dominant L-ferritin deficiency. We also describe an additional dominant L-ferritin deficiency case with a previously described (Met1Val) mutation in the *FTL* gene. Moreover, we have performed an extensive review of all reported variants in the *FTL* gene linked with the previously described five conditions to help in the understanding of the phenotypes.

2. Results

We completely sequenced the entire coding region, intron-exon boundaries and 5' and 3' regulatory regions for the *FTL* gene either by Sanger sequencing or by next generation sequencing (NGS).

2.1. Case Studies

2.1.1. Family 1—A Case with Autosomal Dominant L-Ferritin Deficiency

Proband II.1 from family 1 (Figure 1A and Table 1) is a four-year-old female of Spanish origin referred to the department of Pediatric OncoHematology Service of the Clinic University Hospital Virgen de la Arrixaca because of refractory hypoferritinemia (serum ferritin 4–9 ng/mL) unresponsive to oral iron supplementation, without any accompanying sign or symptoms. Physical examination was normal with normal weight and size for age. At the age of six, the proband complained of recurrent severe headaches. Cerebral CT and MRI were normal except for a small (non-specific) subcortical area of gliosis in the right frontal lobe. This was considered an incidental finding by pediatric neurologists who established a provisional diagnosis of primary headache and recommended treatment with flunarizine.

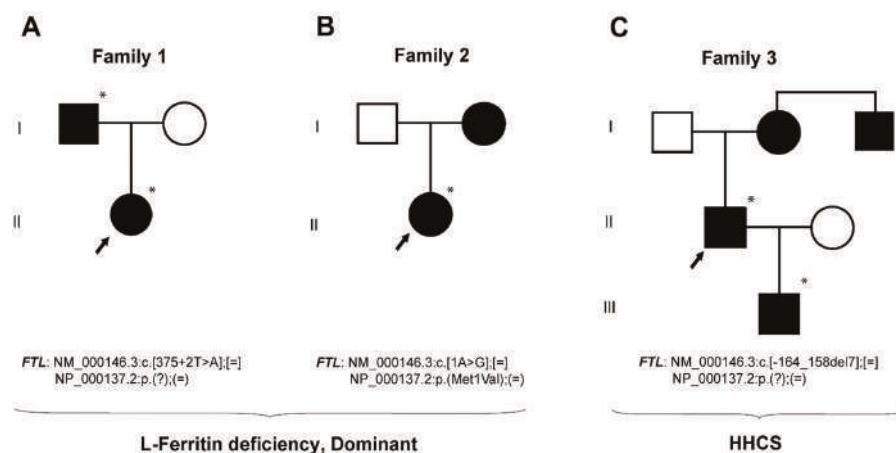


Figure 1. Pedigree trees from three studied families affected from dominant L-ferritin deficiency and HHCS. Squares indicate males and circles females. Probands are pointed with an arrow. Filled symbols indicate affected members and asterisks indicate subjects with genetic studies done at BloodGenetics SL. Mutations are named according the HGVS nomenclature.

Mutation analysis revealed the presence of T > A change in the intron 3 of the *FTL* gene, at position 375 + 2 (NM_000146.3:c.[375 + 2T > A];[=], HGSV nomenclature). This variant was also found in a heterozygous state in the father of the proband (I.1). Human Splicing Finder software predicted that this variant alters the wild type splicing donor site, affecting mRNA splicing. This mutation is novel and has not been previously reported either in the literature or in any public database (ENSEMBL, NCBI, 1000Genomes, public HGMD). However, another variant without a known clinical significance exists at the same position, c.375 + 2T > C, reported in the SNP database as rs1371561306 and with a very low allele frequency (MAF = 0.000008 reported in TOPMED database).

Table 1. Genetic and clinical features of the probands.

Case	Family 1	Family 2	Family 3	Reference Values
Patient	II.1	II.1	II.1	-
Gender	F	F	M	-
Age at diagnosis (years)	4	2	67	-
Hb (g/dL)	12.2–13.3	13.1–13.7	14.0	13.5–17.5 (M), 12.1–15.1 (F)
MCV (fL)	78–84	80	90.2	80–95
Ferritin (ng/mL)	4–9	2–7	3037	12–300 (M), 12–200 (F)
Transferrin sat (%)	12.9	17.2–26.2	22.0–41.0	25–50
Iron (µL/dL)	n/a	61.95	46	49–226
Mutation	c.375 + 2T > A	p.Met1Val	c.-164_158del7	-
Disease	Novel	Previously reported [18]	Novel	-
Inheritance	L-ferritin deficiency	L-ferritin deficiency	HHCS	-
	AD	AD	AD	-

The following abbreviations were used: HB, hemoglobin; MCV, mean corpuscular volume; TF sat, transferrin saturation; F, female; M, male; n/a, not available. The mutation nomenclature used follows the HGVS guidelines.

2.1.2. Family 2—A Case with Autosomal Dominant L-Ferritin Deficiency

Proband II.1 from family 2 (Figure 1B and Table 1) is an asymptomatic two-year-old girl evaluated at the department of Pediatric OncoHematology Service of the Clinic University Hospital Virgen de la Arrixaca for further investigation of mild neutropenia and eosinophilia. The patient had been previously diagnosed with a small ventricular septal defect. During her follow-up, we did not find neutropenia or other hematological anomalies, but rather marked hypoferritinemia without anemia. Hypoferritinemia was unresponsive to oral iron supplementation. She occasionally complained of mild asthenia and an occasional mild headache, which were found to be tension headaches after a full evaluation by a pediatric neurologist. The proband's mother had low serum ferritin levels (<6 ng/mL), low transferrin saturation (9.6%) with normal levels of transferrin.

Sequencing analysis of this proband (Figure 1B, II.1) showed an A > G substitution at position 1 in the heterozygous state, causing the start codon methionine to change into valine. This mutation was previously reported in 2004 [18] and was described in the SNP database as rs139732572 with a very low allele frequency (MAF = 0.000008 reported in the ExAc database). This variant has been classified in ClinVar database as pathogenic (Variation ID 96689), causing L-ferritin deficiency in dominant inheritance mode. Here we report the second case of a patient with hypoferritinemia and this same mutation in the *FTL* gene (NM_000146.3:c[1A > G];[=], NP_000137.2:p(Met1Val);(=), HGVS nomenclature).

2.1.3. Family 3—A Case with HHCS

The proband I.1 in family 3 (Figure 1C and Table 1) is a 65-year-old man with a history of enolism and dyslipidemia, showing high levels of serum ferritin (>3000 ng/mL), motive for what he was referred to the Hematology Service at the University Hospital Germans Trias i Pujol (HGTiP). At age of 45, he underwent cataract surgery. Initially, he underwent three therapeutic phlebotomies, but they were suspended due to the development of anemia. Magnetic resonance (MR) imaging showed normal deposits of liver iron (30 μ mol/g). The family history suggests the presence of HHCS due to the existence of additional cases of hyperferritinemia (proband's son and uncle) and cataracts (proband's mother). The proband's son (II.1) is a 39-year-old male with history of stage 1 orchiectomized and disease-free seminoma and no surgical removed cataracts. He was contacted by the same hematology service (HGTiP) under suspicion of HHCS because of hyperferritinemia (>2000 ng/mL) and cataracts. The hematological evaluation was normal except for the high ferritin levels, and liver magnetic resonance showed normal levels of hepatic iron (20 μ mol/g).

The genetic studies performed on family 3 showed the presence a heterozygous deletion c.[-164_-158del7] located in the 5' *FTL* IRE in the proband (I.1) and his son (II.1), both affected with hereditary hyperferritinemia with cataracts syndrome (Figure 1). Genetic analyses were not available for the mother and the uncle of the proband. This variant consists of a deletion of seven nucleotides (CAACAGT), excising part of the hexanucleotide loop and upper stem of the *FTL* IRE (Figure 2). Following the traditional nomenclature for *FTL* IRE mutations, we refer to these mutations as Esplugues +36_42del7 mutation (HGVS nomenclature as NM_000146.3:c.[-164_158del7];[=]). This deletion is predicted to impair the IRE structure. RNA secondary structure modelling of WT and mutated *FTL* 5' IRE sequences was performed using the Sfold web server, which predicted that -164_-158del7 mutation, located at the hexanucleotide loop, is likely to disturb the WT IRE conformation (Figure 3). In addition, the SIREs web server prediction [19] indicate loss of the IRE structure, as the mutated query returned no results. This mutation has not been previously described in the literature, but other similar IRE deletions have been previously demonstrated to be pathogenic for HHCS [11]. The location and severity of this mutation, together with the clinical manifestations of HHCS present in the affected individuals of this family, indicates that this variant is most probably the genetic cause of disease in this family.

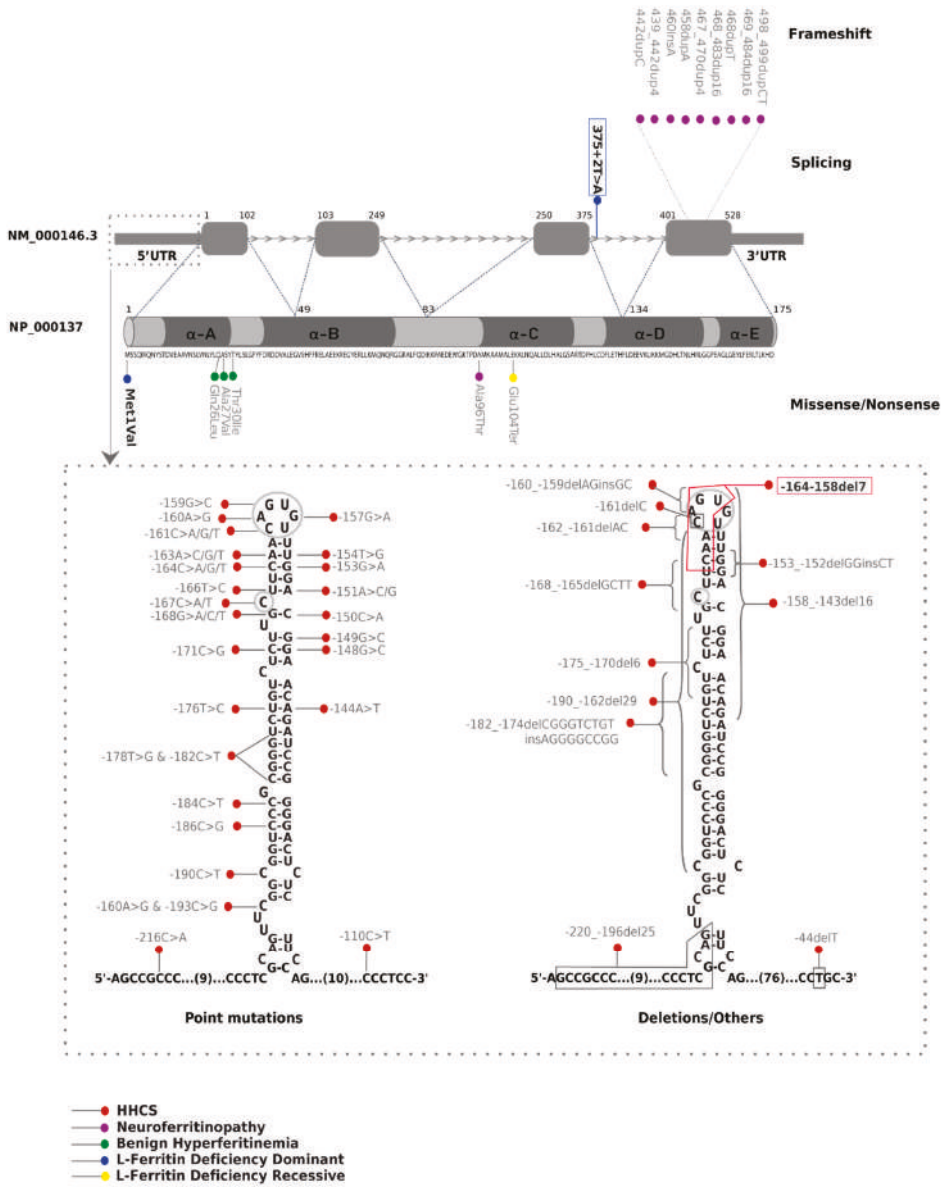


Figure 2. Schematic localization of literature reported and new *FTL* mutations. Mutations described in this work are in bold and new mutations are boxed. The domains of the five alpha helices (A to E) are represented in the protein (NP_000137.2). Mutations are classified as nonsense, frameshift, missense, or splicing. Here we report *FTL* protein changes using the three-letter amino acid code.

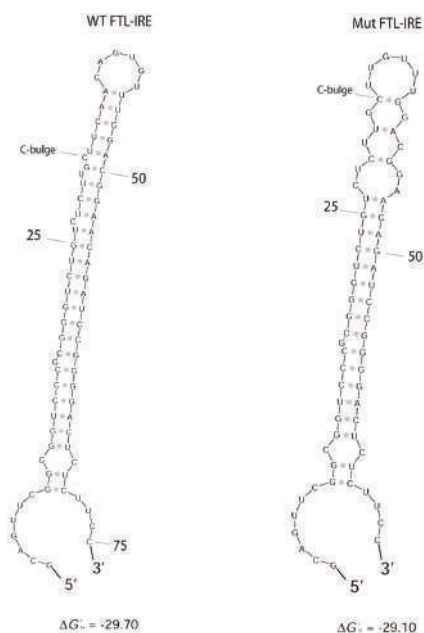


Figure 3. WT and mutated *FTL*-IRE fold prediction. Predicted secondary structure of WT and mutated *FTL*-IRE using Sfold web server [20]. Deletion in hexanucleotide loop (c.-164_158del7) is expected to disturb completely the IRE structure. Nucleotides are numbered from the transcription starting site. Free energy (ΔG) is detailed.

2.2. Update on L-Ferritin Mutations and Diseases

Mutations in L-ferritin gene causes five different phenotypic diseases, as summarized in Table 2. After an extensive literature search, we have collected all 63 different mutations reported so far for these five diseases, including the two novel mutations reported in the present work (Figure 2 and Supplementary Material: Table S1).

Most of the *FTL* mutations described correspond to hereditary hyperferritinemia with cataract syndrome (HHCS), including 36 point mutations, 9 deletions, and 2 insertion-deletions. All the mutations for HHCS are located at the 5'UTR of the *FTL* gene (chr19:49468566–49468764), affecting the primary sequence and structure of *FTL*-IRE (Figure 2). HHCS is an autosomal dominant disorder, and all reported variants are in heterozygous state except for three cases [11,21,22] where homozygous mutations have been described. Patients with HHCS do not present clinical manifestations other than high serum ferritin levels and congenial bilateral nuclear cataracts.

Neuroferritinopathy is the only NBIA disorder with an autosomal dominant inheritance. It is caused by mutation in the *FTL* gene. Up to now, nine insertions from one to 16 nucleotides located in the exon 4 of *FTL* gene have been reported to cause this neurodegenerative disorder (Figure 2). These insertions alter the reading frame of the C-terminal region, generating a longer protein with additional aberrant amino acids. In addition, a missense Ala96Thr mutation (rs104894685) has been also described to cause neuroferritinopathy; amino acid 96 is predicted to be situated at the same tertiary structure region as the pathogenic insertions [13]. Mutations in the C-terminal region of *FTL* disrupt α -helix D or E, which seems to be essential for the stability of the peptide [23,24]. Clinical and biochemical manifestations of this disease include low serum ferritin levels, iron accumulation in the basal ganglia and progressive and severe neurological dysfunctions with subtle cognitive deficits in some cases.

Table 2. Summary of diseases caused by mutations in *FTL* gene.

Disease	Hereditary Hyperferritinemia Cataract Syndrome	Benign Hyperferritinemia	Neurodegeneration with Brain Iron Accumulation ³	L-Ferritin Deficiency, Dominant	L-Ferritin Deficiency, Recessive
First publication	[7,8]	[15]	[12]	[18]	[17]
Inheritance	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal recessive
Mechanism	LOST OF IRP REGULATION	(DO NOT PROCEED)	DOMINANT NEGATIVE EFFECT	HAPLOINSUFFICIENCY	TOTAL LOSS OF FTL
Mutation/s	Many in the 5' UTR	Missense in exon 1	Frameshift in exon 4	p.(M1V; =)	p.(E104X; E104X)
Type	5' UTR	Affects the A α-helix near the N-terminus	Predicted to cause loss of the C-terminal secondary structure	Start loss	Nonsense
Hematological features	High serum ferritin Normal serum iron Normal transferrin saturation Normal red cell counts Normal hematologic parameters	High serum ferritin Serum ferritin hyperglycosylation Normal hematologic parameters	Low serum ferritin	Low serum ferritin Low transferrin saturation (17%) Normal serum iron Normal hematologic parameters	Undetectable serum ferritin Normal transferrin saturation Normal hematologic parameters
Other features	Congenital bilateral nuclear cataract		Severe neurological dysfunction Gastrointestinal dysphagia		Idiopathic generalized seizures Atypical RLS Progressive hair loss

The following abbreviations were used: IRP, iron regulatory protein; IRE, iron responsive element; UTR, untranslated region; RLS, restless leg syndrome; A α-helix, first domain alpha helix of FTL protein.

Three heterozygous mutations in *FTL* exon 1 have been associated with hyperferritinemia without iron overload where cataracts were absent; this condition has also been named benign hyperferritinemia. The Thr30Ile mutation (rs397514540, ExAc MAF = 0.000008) has been identified in French and British families [15,25]. Two further pathogenic mutations—p.(Ala27-Val) and p.(Gln26Ile)—have been reported in two additional patients [26]. Mutations in exon 1 of *FTL* are associated with higher than normal serum ferritin glycosylation. These three variants alter the A α -helix near the N terminus of L-ferritin; it has been hypothesized that the aberrant peptide extends the length of the hydrophobic cluster of amino acids at the N terminus, increasing the secretion of L-ferritin [15]. However, the reason for the development of hyperferritinemia and hyperglycosylation associated with these mutant ferritin forms is still not fully elucidated [25].

We have described in this report a novel intronic splicing mutation in the *FTL* gene (NM_000146.3:c.[375 + 2T > A];[=]) associated also with autosomal dominant L-ferritin deficiency. Including this novel splicing mutation, two heterozygous mutations have been described to cause L-ferritin deficiency with autosomal dominant transmission. Cremonesi and collaborators identified in 2004 a heterozygous A > G substitution in the first nucleotide of *FTL*, which change the ATG start codon (methionine) into a valine [18]. This mutation is predicted to encode a non-functional and unstable protein. Despite the low serum levels of L-ferritin, the proband presenting this mutation does not show either serious neurological problems (other than headaches) indicating that the molecular mechanism of L-ferritin deficiency with autosomal dominant inheritance is haploinsufficiency.

Finally, only one Italian case has been reported to cause L-ferritin deficiency with autosomal recessive inheritance; a homozygous substitution at nucleotide 310 G to T that produces a premature stop codon (E104X) [17]. This amino acid change is predicted to be located in the middle of the α -helix C domain, a critical region for the stability of the protein. In silico analysis predicted that a stop codon at this position produces a truncated protein unable to fold into a functional peptide and, therefore, leads to the generation an L-ferritin subunit with a complete loss of function. This homozygous mutation is associated with a more aggressive phenotype, which is characterized by undetectable ferritin levels, idiopathic generalized seizures and atypical restless leg syndrome.

3. Discussion

L-ferritin disease is a clear case of Mendelian disease-related genes that are associated with multiple diseases, including hyperferritinemia linked with cataracts in HHCS, isolated hyperferritinemia (benign hyperferritinemia), hypoferritinemia associated with other symptoms (neurological symptoms as in neuroferritinopathy, or muscular symptoms in autosomal recessive L-ferritin deficiency), or isolated hypoferritinemia (autosomal dominant L-ferritin deficiency). For improving the diagnosis of these different diseases, we have created a clinical diagnostic algorithm of diseases caused by mutations in *FTL* gene (Figure 4).

The underlying mechanisms for these disease patterns are not fully elucidated for all these diseases. In HHCS, it is known that the pathological molecular mechanism is linked to the disruption of the IRP-IRE post-transcriptional regulatory system, with the de-repression of L-ferritin mRNA and the subsequent overproduction of L-ferritin protein that precipitates and deposits in the lens of the eyes, producing cataracts. In HHCS, authors have argued that an association exists between the clinical severity of the disease and the location of the mutation in *FTL*-IRE [11,27,28]. Higher serum ferritin levels are mostly associated with mutations in the apical loop or the C-bulge area, compared with mutations in the upper or lower stems. These findings are consistent with our cases in family 3, i.e., a father and son with a seven-nucleotide deletion in the IRE of the *FTL* gene (5'-CAACAGU3'). The deletion partially affects the hexanucleotide loop of the IRE, and patients show considerably high serum ferritin levels (2071–3037 ng/mL) and develop early bilateral cataracts (Table 1).

Mutations in exon 4 of *FTL* gene that alter the C-terminal sequence and the length of the protein have been reported to cause neuroferritinopathy. These mutations affect the D- or E α -helix (see Figure 2), leading to significant disruption of the tertiary and quaternary structure of the *FTL* protein

and producing an unstable and leaky ferritin. Studies have shown that mutant ferritin maintains the normal spherical shell structure and size [29]. However, mutant FTL C-termini, rich in amino acids with iron-binding properties, may be extended and unravel out of the shell. These mutant structures could initiate aggregation, forming ferritin inclusion bodies/precipitates [29,30]. Previous in vitro functional analysis and in vivo mouse models have revealed that these molecular-level defects have two main consequences: (1) the loss of normal protein function, reducing iron incorporation; (2) and the acquisition of toxic function through radical production, ferritin aggregation, and reactive oxygen species (ROS) generation [14,31–33]. It has been demonstrated that mutations in the C terminus have a dominant-negative effect, which explains the dominant transmission of the disorder [34].

In benign hyperferritinemia, missense variations in exon 1 of *FTL* increase the hydrophobicity of the ferritin N-terminal site. The A- α -helix of the protein is altered and hyperglycosylated, although the precise molecular mechanism is not clear yet.

L-ferritin deficiency is characterized by haploinsufficiency of the FTL protein in autosomal dominant L-ferritin deficiency, or complete loss of function in autosomal recessive L-ferritin deficiency. Mutations associated with this condition are predicted to affect protein expression. The inactivation of one allele of *FTL*, which occurs in the two described heterozygous cases of autosomal dominant L-ferritin deficiency, produces a significant reduction of L-ferritin in serum. The phenotype of this dominant condition is normal apart from low levels of serum ferritin and low transferrin saturation. However, the homozygous mutation Glu104Ter causes a total loss of function of L-ferritin and leads to ferritin missing the FTL chain. Previous mutational studies have suggested that the presence of H homopolymer ferritin in fibroblasts is associated with reduced cellular iron availability and increased ROS production, which triggers cellular damage. These findings were also found in neurons derived from patient fibroblasts and correlate with the neurological phenotype of this more severe condition [17].

In this study, we have identified two new cases of autosomal dominant L-ferritin deficiency, one due to a new mutation in intron 3 of the *FTL* gene that most probably affects splicing. The T > G substitution in nucleotide 375 + 2 (genomic coordinates g.49469665 in Assembly GRCh37.p13) modifies a highly conserved dinucleotide GT donor site, a key sequence recognized by the spliceosome during splicing. Mutations in splice site junctions are likely to lead to exon skipping or total or partial intron retention in the mRNA transcript in most cases [32]. Our in silico analysis using Human Splicing Finder suggests that our variant would activate a new alternative donor site onwards (375 + 5G and +6T), leading to the insertion of four additional nucleotides in the mRNA sequence between exon 3 and 4 and altering the reading frame of the transcript [33]. Therefore, this change would lead to a premature stop codon formation and the putative generation of a truncated FTL protein of 128 amino acids, if the aberrant mRNA is not detected and degraded by the nonsense mediated decay (NMD) system. According to our bioinformatics prediction, the c.375 + 2T > A mutation will not generate a dominant-negative version of FTL protein, but a truncated FTL protein completely missing the E- α -helix domain and partially lacking the D- α -helix domain. Supported by the clinical manifestations found in this family (low serum ferritin levels, low transferrin saturation and lack of serious neurological or movement abnormalities), the molecular mechanism in this case is most probably due to the loss of function of FTL and it will be not expected that the disease derives in neuroferritinopathy. However, we cannot totally and completely exclude the later development in life of brain iron overload and neuroferritinopathy in these patients by a yet unknown and novel mechanism.

Patients with hereditary hyperferritinemia could be misdiagnosed as patients suffering from hereditary hemochromatosis, liver dysfunction or inflammation. Some patients have received unnecessary invasive diagnostic techniques, such as liver biopsy, and are inappropriately treated with venesections and phlebotomies that can cause severe iron-deficiency anemia. On the other hand, clinical manifestations of neuroferritinopathy including tremor, parkinsonism, psychiatric problems, and abnormal involuntary movements, the presence of ferritin-iron precipitation in glia cells

and neurons [30], are often misdiagnosed and treated as Huntington's or Parkinson's disease. Potential treatment targets for neuroferritinopathy may include an optimized iron chelator to induce the re-solubilization of iron aggregations, in combination with radical scavengers to prevent oxidative ferritin damage [35]. Patients with benign hyperferritinemia could be misdiagnosed as patients with HHCS due to the presence of high ferritin levels and the possible late-onset appearance of cataracts. Apart from the surgical removal of cataracts in HHCS, HHCS and L-ferritin deficiency have no specific therapy.

These facts emphasize the importance of a correct and early genetic diagnosis for the subsequent implementation of proper treatment, avoiding detrimental or inappropriate treatments. Following clinical algorithms, such as the one included in this publication (Figure 4) and also HIGHFERRITIN Web Server (<http://highferritin.imppc.org/>) [36] will surely help in this goal.

4. Materials and Methods

4.1. Patients

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee on 10th July 2015.

4.2. DNA Extraction, PCR Amplification, and DNA Sequencing

Genetic studies were performed with minor differences for all the pedigrees. Genomic DNA was extracted from peripheral blood using the FlexiGene DNA kit (Qiagen) according to manufacturer's instructions.

Ferritin gene regions (exonic, intron–exon boundaries, and untranslated regions) were sequenced using the Sanger method or next generation sequencing (NGS).

For the Sanger method, the *FTL* gene was amplified using 50 ng of genomic DNA. Primer sequences and PCR conditions are available upon request. The resulting amplification products were verified on a 2% ethidium bromide agarose gel. The purified PCR products were sequenced using a conventional Sanger method. Sequencing results were analyzed using Mutation Surveyor software (SoftGenetics LLC, PA, USA).

For NGS methods, patients were analyzed using a targeted NGS gene panel (v14) for hereditary hemochromatosis and hyper/hypoferritinemia that included the following nine genes: *HFE*, *HFE2*, *HAMP*, *TFR2*, *SLC40A1*, *BMP6*, *FTL*, *FTH1*, and *GNPAT*.

Briefly, the capture of genomic regions was conducted starting from 225 ng of gDNA using a custom design HaloPlex™ Target Enrichment 1–500 kb kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Library quality was determined using the Agilent 4200 TapeStation System. The quantity was measured using a QubitdsDNA assay kit with a Qubitfluorometer (Life Technologies, Carlsbad, CA, USA) and fluorescence was detected using a SpectraMax Gemini EM microplate reader (Molecular Devices). Libraries were sequenced using MiSeq reagent kit v2 (300 cycles) (Illumina, San Diego, CA, USA) on an Illumina or a MiSeq or MiniSeq sequencer (Illumina, San Diego, CA, USA), generating 150-bp paired-end reads. Samples were aligned with the reference human genome GRCh37/hg19 and data analysis was performed using our algorithms. Mutations detected by NGS were confirmed by conventional Sanger sequencing.

Genetic variants are reported following the official Human Genome Variation Sequence (HGVS) nomenclature and refer to NM_000146.3 for the *Homo sapiens FTL* transcript variant and NP_000137.2 for the *Homo sapiens FTL* protein.

Reported mutations in this study have been submitted to the Leiden Open Variation Database (<http://www.lovd.nl>) or to ClinVar (<http://www.ncbi.nlm.nih.gov/clinvar>).

Algorithm for differential diagnosis of L-Ferritin diseases

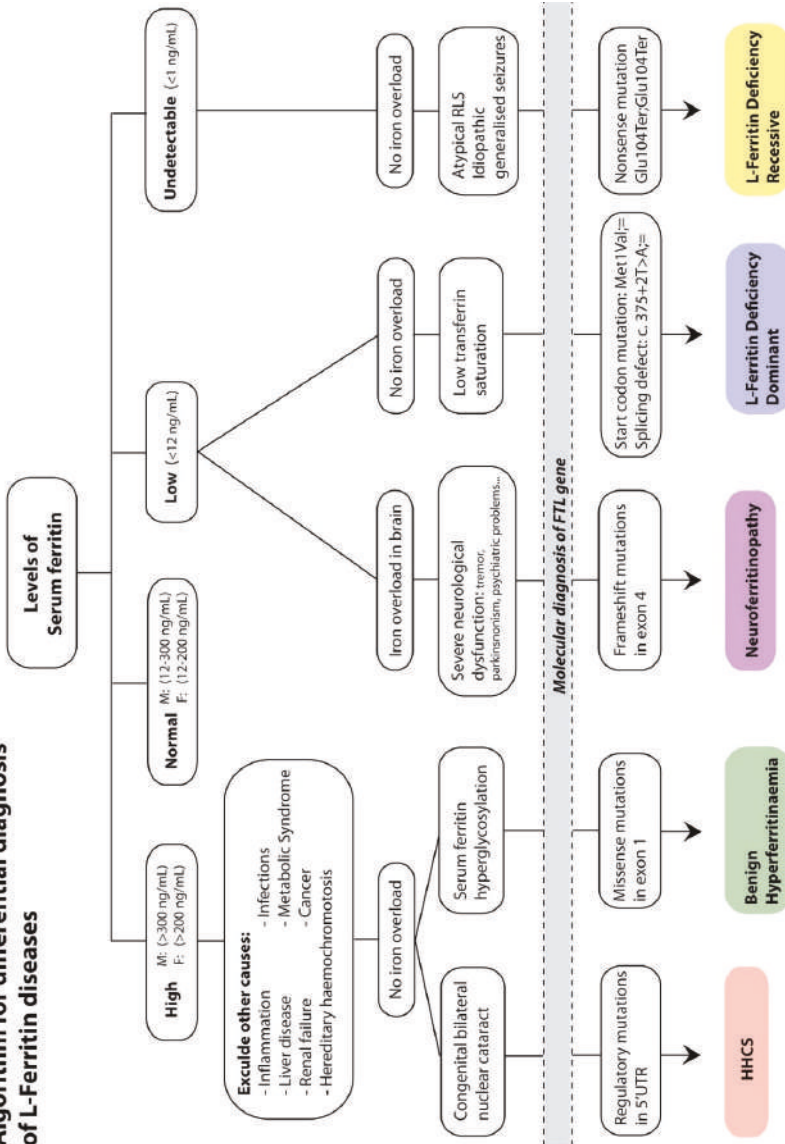


Figure 4. Algorithm for diagnosis of diseases caused by defects in *FTL* gene. The following abbreviations were used: F, female; M, male. The mutation nomenclature used follows the HGVS guidelines.

4.3. FTL RNA Fold Predictions

RNA folding analysis was carried out to predict the IRE structure of wild-type (WT) and mutated FTL-IRE using the Sfold web server (<http://sfold.wadsworth.org/>) [20]. DNA sequences used for folding predictions are shown in Supplementary Material Table S2. The SIREs web server tool was also used to predict iron-responsive elements [19].

Supplementary Materials: The following are available online at <http://www.mdpi.com/1424-8247/12/1/17/s1>, Table S1: Table summarizing all FTL mutations identified up now in the literature, including the two novel mutations described here. Table S2: DNA sequence for FTL RNA fold predictions.

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Review

Emerging and Dynamic Biomedical Uses of Ferritin

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Abstract: Ferritin, a ubiquitously expressed protein, has classically been considered the main iron cellular storage molecule in the body. Owing to the ferroxidase activity of the H-subunit and the nucleation ability of the L-subunit, ferritin can store a large amount of iron within its mineral core. However, recent evidence has demonstrated a range of abilities of ferritin that extends well beyond the scope of iron storage. This review aims to discuss novel functions and biomedical uses of ferritin in the processes of iron delivery, delivery of biologics such as chemotherapies and contrast agents, and the utility of ferritin as a biomarker in a number of neurological diseases.

Keywords: ferritin; iron; iron delivery; nanotechnology; nanocage; drug delivery; inflammation; serum biomarker

1. Ferritin Introduction

Ferritin, a protein originally identified in 1937 by Vilém Laufberger [1], is a ubiquitously expressed iron storage protein most commonly characterized by its ability to accumulate and store up to 4500 atoms of iron [2]. Ferritin consists of 24 subunits, typically comprised of different ratios of the H and L chain subunit. The ratios vary by organ and even by cell type. Importantly, the different subunits have divergent functions—H-ferritin utilizes ferroxidase activity that is necessary for the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron while L-ferritin contains acidic residues on the surface cavity of the protein that facilitate ferroxidase turnover and are crucial for the nucleation of ferric iron within the core of the fully formed protein. Historically, the primary function of the 24-mer protein has been to sequester extracellular and cytosolic iron and store this iron within its core for future use by the cell. This functionally prevents the formation of harmful reactive oxygen species (ROS) created through Fenton chemistry. Many reviews in the past few decades have focused on various aspects of the normal biological function of ferritin and its regulation pertaining to iron homeostasis [3–6]. In this review, we will outline novel and dynamic functions of ferritin that have recently come to light, specifically focusing on the novel uses for ferritin as an iron delivery protein, a delivery agent for numerous other biologics, and as a biomarker for various diseases.

Although traditionally characterized as a cytosolic protein, ferritin has also been found in mitochondria [7], plant plastids [8], the nucleus [9–11], and extracellularly in serum [12] and cerebrospinal fluid [13]. The different subcellular localizations of ferritin suggest a unique function for it in a variety of cell types. For example, nuclear ferritin reportedly binds to and protects DNA from UV-induced damage [9] as well as iron-induced damage [11]. The presence of ferritin in nuclei led to a series of studies by our group on the role of ferritin in cancer cells that revealed knockdown of ferritin increases sensitivity to radiation and chemotherapy in cancer cells [14]. Mitochondrial ferritin, while coded by a different gene than either L- or H-ferritin, shares 79% homology to H-ferritin and forms the classic ferritin shell [15]. In the mitochondria, free iron can cause potentially devastating effects; mitochondrial ferritin serves to sequester potentially harmful iron using ferroxidase activity [4].

2. Ferritin as an Iron Delivery Protein

Iron delivery throughout the body is a vitally important process that is very tightly regulated. Iron deficiency may result in a number of longstanding and severe phenotypes such as anemia [16]. Similarly, iron overload, known as hemochromatosis, may result in organ failure [17] or significant inflammatory conditions [18]. Moreover, iron dyshomeostasis in the brain may result in significant neurodegeneration [19–21]. Excess organ and cellular accumulation in disease states occurs because the regulation of iron uptake is altered. The primary mechanism for iron uptake has historically been thought to be through the action of transferrin (Tf) delivering iron through transferrin receptor (TfR) (Figure 1). However, this paradigm may not address times of extreme iron need, such as during development and growth. While transferrin typically delivers two atoms of iron at a time, ferritin can potentially deliver a much more substantial amount of iron at a single time, allowing for rapid growth and iron utilization.

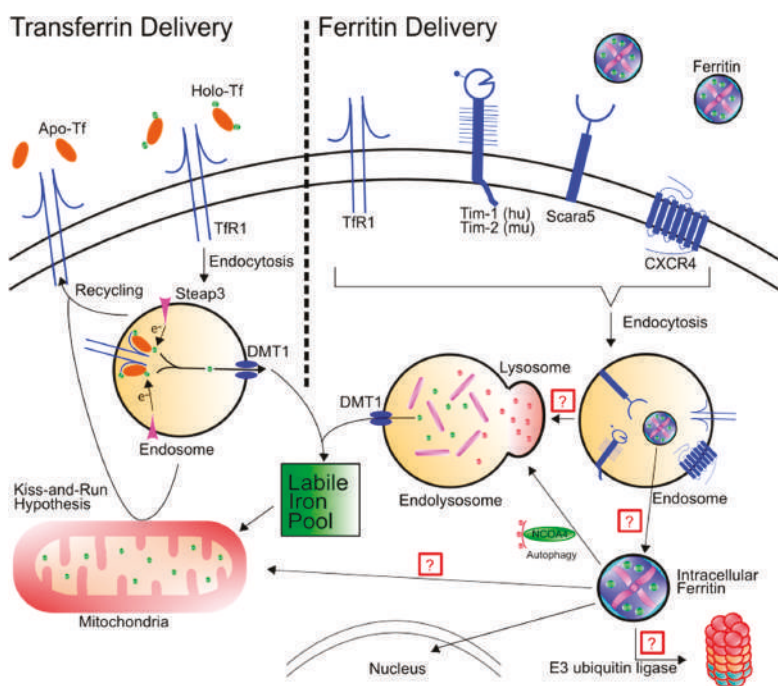


Figure 1. Schematic of transferrin and ferritin uptake and intracellular destiny. Left: Holo-Tf can bind to TfR1 and deliver iron into the labile iron pool through the endosome via DMT1. TfR1 can also be recycled to the cell surface, depositing apo-Tf into the extracellular matrix. An alternative hypothesis termed “kiss-and-run” results in endosomal delivery of iron to the mitochondria by brief interactions between the endosome and the mitochondria. Right: Ferritin binding has been shown for TfR1, Tim-1, Tim-2, Scaras5, and CXCR4. After binding, ferritin is endocytosed. Note, while not all receptors are simultaneously required for endocytosis of ferritin, the schematic is demonstrating that all of the receptors in question have been found in the endosome. Ferritin trafficking after endocytosis has yet to be fully elucidated. Hypotheses include trafficking to the lysosome for protein degradation or for ferritin to leave the endosome through another means. Once ferritin is cytosolic, it can be shuttled to the lysosome via NCOA4 or it may be transported to the nucleus. There are a number of other potential hypotheses as well: (1) Ferritin may be poly-ubiquitinated and degraded by the proteasome or (2) ferritin may be shuttled to the mitochondria to deliver iron.

In particular, serum ferritin represents an important consideration in iron delivery; saturation of serum ferritin can range wildly depending on a number of factors, such as inflammation or development, with values ranging between 5% and 50% saturated [22]. It remains to be determined the extent to which serum ferritin functions to deliver iron; iron saturation of ferritin is largely ignored in the clinic. This is particularly important as around 90% of patients that present in the clinic with hyperferritinemia do not have iron overload [23]. While serum ferritin reportedly has a lower level of iron saturation than liver ferritin [24], the iron contained within serum ferritin was still a relatively higher amount than transferrin iron. Cohen et al. found that while liver ferritin contained 2074 atoms of iron per molecule of ferritin, serum ferritin contained 689 atoms of iron per molecule of ferritin [24]. Thus, despite the common tendency to refer to serum ferritin as “iron poor” that one consistently sees in the literature, it stands that if serum ferritin participates in iron delivery it is a significant source of iron.

In order for ferritin to serve as an iron delivery protein, it must be released from cells (Figure 2). Secretion of ferritin has been demonstrated in many cell types, such as macrophages [18,25,26], hepatocytes [27], and Kupffer cells of the liver [28], in both healthy and disease settings. While the exact mechanism of ferritin secretion is still very much open for debate, the lack of glycosylation of extracellular ferritin suggests a non-classical secretion route. Cohen et al. have demonstrated the secretion of ferritin through the lysosomal secretory pathway, a cell-specific directed pathway that is not the product of damaged cells [24]. In this pathway, specialized secretory lysosomal granules are created both as degradative/modulatory compartments as well as storage areas, eventually followed by secretion of its contents to the extracellular milieu [29]. Subsequently Truman-Rosentsvit et al. found that in addition to the lysosomal secretory pathway, ferritin can also be secreted via the multivesicular body-exosome pathway [30]. Consistent with the secretion in membrane bound vesicles, Mrowczynski et al. has demonstrated the presence of H-ferritin in exosomes [31]. Recently, Kimura et al. have further described this secretion mechanism as dependent upon the tripartite motif containing 16 (TRIM16) secretory autophagy receptor, avoiding autolysosomal degradation by pivoting ferritin cargo to the plasma membrane for secretion [32]. Thus, the data appear to support an active rather than passive release mechanism for ferritin from cells. Consequently, the amount of serum ferritin may be reflective of cellular processes and not a by-stander to inflammation and non-specific product of cell damage. This indicates that ferritin secretion may be an active response to physiological conditions and identifying those conditions that promote ferritin secretion are an area that could lead to new knowledge about ferritin function and iron requirements.

Recent studies have demonstrated ferritin ability for iron delivery. In particular, studies have provided evidence for H-ferritin uptake into the brain parenchyma [33], regulation of H-ferritin trafficking across the blood-brain barrier (BBB) [34–38], and H-ferritin uptake into the developing mouse brain (Unpublished Observations). In these experiments, radioactive ^{59}Fe loaded into H-ferritin was used to track the transport of iron directly across the BBB in vitro [34] or directly into the brain in vivo [33]. This was further corroborated by inductively coupled plasma mass spectrometry (ICP-MS) studies of iron delivery by H-ferritin [35]. Todorich et al. demonstrate the importance of H-ferritin-mediated iron delivery to the brain, demonstrating that H-ferritin can replace transferrin as an obligate iron source for oligodendrocytes in vitro [39]. Further evidence has demonstrated both H- and L-ferritin binding and endocytosis in a number of different cell types, including hepatocytes [40], reticulocytes [41], lymphoid cells [42], and erythroid precursors [26,43,44]. In all of these cell types, binding and endocytosis of ferritin results in increased intracellular iron levels. The ability of ferritin to deliver a significantly higher amount of iron relative to transferrin suggests the existence of a more efficient iron delivery system perhaps especially in times of high iron demand, such as neural development and rapid growth [45].

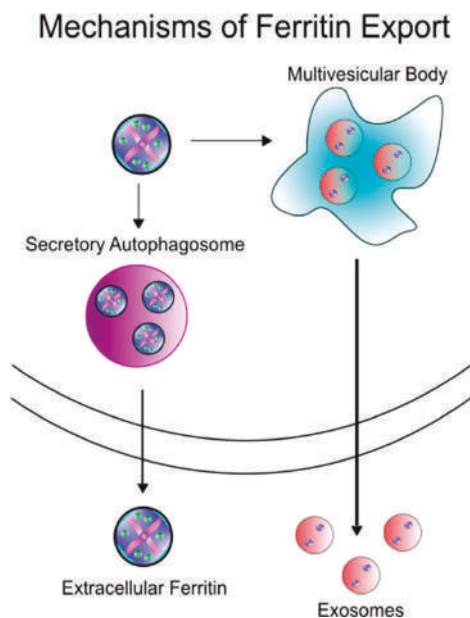


Figure 2. Schematic of iron export mechanisms. Ferritin has been hypothesized to be secreted in two different paradigms: (1) Ferritin is trafficked from the cell via secretory autophagy or (2) ferritin encapsulated in exosomes is released extracellularly from multivesicular bodies.

There is considerable debate regarding the mechanism for ferritin uptake. While previous studies have demonstrated clathrin-dependent endocytosis of ferritin [46], there is not yet a consensus on the specific receptor mediating the uptake. Further complicating matters is the report that both H-ferritin and L-ferritin utilize different receptors. As previously stated, ferritin typically exists in a ratio of H- and L-ferritin; the ratio of H- to L-subunits may dictate the specific receptor preferred. While L-ferritin has been shown to bind to Scara5 [47], H-ferritin has thus far been shown to bind to Tim-2 in rodents [48–50], Tim-1 in humans [34,51], as well as CXCR4 [52] and TfR1 [53] (Figure 1). There is discrepant literature describing the ability for ferritin binding to saturate to Tim-2; one study found ferritin binding to be saturable [49] while another found it is not [50]. Importantly, we are not aware of any literature testing for respective contributions of ferritin binding between the TfR1 and Tim receptors. To this point, most cells in the studies studying TfR1 binding to ferritin did not assay for Tim-1/2 as a second uptake receptor. It is likely that both systems exist side-by-side and display different expressions for TfR1 and Tim-1/2 depending on cell type. This suggests that in cells that do not have TfR1, such as mature oligodendrocytes [54], ferritin internalization is dependent on Tim-1/2. Furthermore, it is well known that Tf receptors are saturated to almost 100% capacity when Tf saturation is normal (30%)—further suggesting that ferritin binding to Tim-1/2 may represent the preferred method for uptake of ferritin into the cell.

While binding and endocytosis of ferritin have been previously demonstrated, the eventual downstream fate of endocytosed ferritin has yet to be studied. An important consideration for ferritin uptake is that ferritin can transport anywhere from 100 to 1000 times as much iron as transferrin and therefore improper delivery and regulation of this delivery may have devastating consequences for the cell. As such, it is clearly critical to cell viability to have a route for handling iron release after exogenous ferritin uptake. For example, does ferritin get moved to the endosome and/or lysosome after uptake? If so, what happens to the iron contained within ferritin after ferritin degradation? Li et al. have previously demonstrated H-ferritin delivery to a human T lymphoblast cell line (MOLT-4),

showing H-ferritin trafficking to the endosome as well as to the lysosome [53]. It remains to be seen if this delivery route is the same for all cell types, especially in cells lacking TfR1 such as mature myelinating oligodendrocytes [54]. It may also be possible for ferritin to leave the endosome without being transported to the lysosome; the diameter of ferritin is only 12 nm, leading to a hypothesis that ferritin may leave the endosome through pores created under cellular stress [55] (Figure 1).

Previous characterization of the Tf-TfR axis has shown the ability for endosomal divalent metal transporter 1 (DMT1) to facilitate exit of iron from the endosome to be utilized by the cell [36,38]. Furthermore, previous studies have also demonstrated that H-ferritin transport is affected by pharmacological inhibition of DMT1 [34]. A second possibility for the fate of ferritin after uptake can be seen again in the Tf-TfR axis. Das et al. have shown that Tf may deliver iron to the mitochondria after uptake while still in the endosome, a process that has been termed “endosomal kiss-and-run” [56]. It is likely that this process, namely the delivery of extracellular iron to the mitochondria, is highly upregulated especially in times of high iron demand. Thus, the possibility exists for ferritin to perform a similar function as Tf to deliver a substantially higher amount of iron directly to the mitochondria either through “kiss-and-run” activity or through a directed transport mechanism (Figure 1).

Once ferritin is within the cytosol either via exogenous (uptake) or endogenous (translation) means, the fate of the protein itself and the iron contained within has been the subject of many studies, but the literature is inconsistent. Some studies suggest that intracellular ferritin is transported to the lysosome to be degraded and the iron inside the ferritin core to be recycled [27]. In particular, nuclear receptor coactivator 4 (NCOA4) was found to be essential in the transport of cytosolic ferritin to the lysosome, a process termed “ferritinophagy” [57]; NCOA4 knockout cells had reductions in ferritin degradation as well as lower intracellular labile iron [57,58]. These studies demonstrated that NCOA4 binds specifically to H-ferritin, but not L-ferritin, at the Arg23 residue [58]. Furthermore, NCOA4 is importantly recognized by the HECT-type E3 ubiquitin ligase HERC2 which itself is iron-sensitive, suggesting NCOA4 is degraded under conditions of iron excess. However, since NCOA4 only recognizes H-ferritin, there is still the question of how L-ferritin is degraded. Other literature suggests that ferritin in general is largely degraded in the proteasome and this degradation can be regulated based on proteasomal activity [59]. Still, others suggest that both lysosomal-mediated and proteasome-mediated degradation of cytosolic ferritin are indeed important contributors to overall ferritin homeostasis [60]. In 2010, Zhang et al. examined the degradation pathway by inhibiting either the lysosome or the proteasome and found that lysosomal proteolysis is the major contributor to ferritin degradation, with proteasomal-mediated degradation conferring a smaller proportion of the degradation [61]. Whether this finding is relevant to all cell types or only the mouse B cell line used in the Zhang et al. study is unclear.

3. Ferritin as a Delivery Agent

In addition to iron, ferritin has the unique ability to encapsulate and deliver other molecules. Indeed, ferritin is a highly preferred molecule in the field of nanotechnology owing to its self-assembly properties, precise cage alignment, and the ability to modify the surface of the fully formed protein with various conjugates to increase specificity and functionality. Importantly, despite the rigid structure under normal physiological conditions, ferritin can be disassembled when the pH becomes very acidic (pH 2–3) or very basic (pH 11–12) [62]. This process can be reversed by changing the pH back to neutral conditions, spontaneously forming ferritin into the full 24-mer [62] (Figure 3). Many researchers have utilized these assembly properties to load various compounds and molecules within the ferritin core. This flexibility in loading and assembly allows for numerous non-classical uses of ferritin. Moreover, even though ferritin has a high molecular weight (~474 kDa), the total diameter of the assembled 24-mer is roughly 12 nm. This diameter is easily smaller than many nanoparticles that are currently in development as drug delivery vehicles; the small diameter of ferritin permits wide movement across membranes, including into the nucleus and potentially out of endosomes [10]. Furthermore, while typically containing a negative surface charge, the charge on ferritin can vary depending on

subunit composition, which allows for further flexibility when targeting ferritin molecules across different membranes [63]. Taken together, the unique characteristics of ferritin allow for robust nanocage assembly and targeted delivery.



Figure 3. Schematic of encapsulation paradigms for ferritin. Fully formed 24-mer ferritin molecules can be disassembled using 8 M urea or acidic (pH 2–3) or basic (pH 11–12) conditions. A number of different molecules can be mixed with the disassembled ferritin, including chemotherapies, siRNAs/miRNAs, and contrast agents. Upon returning the ferritin mixture to neutral conditions (pH 7.0) coupled with dialysis, ferritin will spontaneously form the 24-mer around the molecule of interest. Free molecules can be dialyzed away from solution.

One prominent area of investigation in the application of ferritin nanocages is in the treatment of cancer. Taking advantage of the fact that cancer cells require a higher amount of iron, many researchers have begun to use ferritin nanocages with encapsulated chemotherapies as delivery agents both *in vitro* [64,65] and *in vivo* [65,66] to great effect. Typically, chemotherapeutics such as doxorubicin, are modified by either forming a complex of the drug with transition metals such as Cu(II) [67] or by adding a charged accessory molecule such as poly-L-aspartic acid [68]. Not only does this complex facilitate entry into the ferritin core, but it also allows the transition metal or accessory molecule to stay associated with the ferritin protein while the drug is gradually released from ferritin [68]. For several chemotherapies that target the nucleus or DNA, the ability of ferritin to translocate into the nucleus represents a potential added benefit therapeutically [9,10]. Though the mechanism for drug release from ferritin either in the cytosol or in the nucleus has not yet been elucidated, there is strong evidence that delivery of chemotherapies via ferritin is an effective method that results in reduced non-specific cytotoxicity [69,70]. These reduced off-target effects suggest that cancer cells preferentially take up the ferritin nanocages in an attempt to obtain more iron from circulating ferritin. To further promote the uptake of ferritin by cancer cells, Li et al. conjugated epidermal growth factor (EGF) to H-ferritin as a way to target epidermal growth factor receptor (EGFR)-overexpressing breast cancers in mice [71].

While many studies of ferritin delivery of chemotherapies reveal strong efficacy in non-central nervous system (CNS) cancers [72–74], the use of ferritin nanocages is even more promising for therapeutics targeting brain cancers. Owing to the BBB, effective chemotherapeutic treatment of brain cancers has been extremely difficult. However, as previously described, new evidence suggests ferritin can traffic across the human BBB through Tim-1 [34], potentially allowing for the delivery of directed therapeutics into the brain parenchyma. In diseases such as neuroblastoma or glioblastoma that are extremely aggressive and have a severe phenotype with limited treatment options, ferritin nanocages represent a highly exciting and novel delivery route (Figure 2). As an example, Chen et al. have recently demonstrated that apoferritin loaded with doxorubicin can reliably cross the BBB and deposit the chemotherapy specifically within the tumor in a mouse model resulting in increased survival [75]. This example again demonstrates the preferential uptake of ferritin by cancer cells, likely in an attempt to obtain more iron.

In addition to loading the ferritin molecule with chemotherapies such as doxorubicin, the ferritin molecule may allow for delivery of small interfering RNAs (siRNAs) in a number of different biological paradigms. For example, Li et al. have demonstrated a simple method for encapsulating siRNA and using this ferritin-siRNA combination to perform high efficiency transfections of human primary mesenchymal stem cells (hMSCs) and peripheral blood mononuclear cells (PBMCs) in vitro when compared to commercially available Lipofectamine [70]. This study demonstrated that ferritin-siRNA could achieve high gene expression silencing with a low siRNA concentration of 10 nM. This work has large implications in the treatment of various immune-related disorders, as in vivo delivery of this ferritin-siRNA complex did not show any immune activation of the PBMCs but was preferentially taken up by activated B- and T-cells. Furthermore, it has been shown that delivery of H-ferritin siRNA sensitizes glioma to radiation [14,76] which brings up the possibility of using ferritin containing H-ferritin siRNA in order to sensitize various cancers to radiation.

In 2013, Kanekiyo et al. detailed an exciting use for ferritin as a natural delivery agent for vaccines [77]. In this study, the authors fused the influenza virus haemagglutinin (HA) to the ferritin protein and immunized mice with this fusion protein. As a result, mice produced broad-scale neutralizing antibodies against common and vulnerable targets of universal influenza vaccines. Furthermore, the HA-ferritin fusion protein elicited ten times as high of a response as the naïve vaccine, demonstrating increased potency and breadth of influenza immunity. Kanekiyo and colleagues have also extended this approach to the creation of a potential vaccine for Epstein-Barr Virus [78]. This seminal study spawned other studies such as one by Han and colleagues—priming of dendritic cells using ferritin nanocages conjugated to ovalbumin antigenic peptides OT-1 and OT-2 [79]. In this study, the dendritic cell-based vaccine was developed by putting OT-1 and OT-2 on the exterior surface and interior cavity, respectively, resulting in effective priming of CD4⁺ and CD8⁺ T cells after antigen presentation by the dendritic cell. Immunization of mice with this vaccine resulted in differentiation and proliferation of CD4⁺ and CD8⁺ T cells specifically targeting OT-1 and OT-2.

4. Ferritin as a Mineralization Chamber

Magnetic resonance imaging (MRI) is a powerful diagnostic technique with extensive uses in the detection of tumor development [80], white matter development and injury [81,82], and aberrant vascularization [83,84]. To create a clinically useful image that is both highly sensitive and accurate, contrast agents are typically used to aid the visual interpretation of MRI scans. The most common contrast agents used are different formulations of gadolinium (Gd), owing to the strong paramagnetism of Gd from seven unpaired electrons. However, a significant problem with Gd is that it nonspecifically labels all highly vascularized tissues, resulting in false positives and a loss of spatial resolution.

Ferritin represents a viable alternative to Gd labeling for MRI. Both endogenous and exogenous ferritin have found great utility in improving MRI contrast capabilities and disease monitoring [85]. In particular, an increased amount of iron within the ferritin core directly correlates with T2* contrast power. It has been shown that Gd can also be loaded within the ferritin core and used as a more prominent imaging agent [72,86] (Figure 2). This function of improved contrast has many applications, especially in the diagnosis or monitoring of cancer progression. Other functionalities of ferritin use in MRI rely on preferential uptake of ferritin by resident macrophages within a cancer tumor [73] or by the tumor cells themselves either by native uptake or by enhancing uptake with further conjugations [87].

An interesting use of ferritin on an industrial process-scale level is the use of thermostable ferritin from *Pyrococcus furiosus* as a water treatment biologic [88]. In this example, ferritin is used to sequester excess phosphate and arsenate due to the high phosphate and arsenate content of seawater and industry waste. While current phosphate removal methods use a system that is heavily reliant on calcium and aluminum sulfates or bacteria that adsorb calcium, the removal of phosphates by ferritin represents a lower maintenance cost and a method that can be recycled [88].

5. Ferritin as a Marker for Inflammation

There are three main regulatory pathways for the expression of ferritin: The iron response proteins (IRPs)/iron responsive element (IRE) system [89], the regulation of transcription through NF- κ B [90], and transcriptional regulation through the hypoxia inducible factor 1 alpha (HIF-1 α) binding to a HIF responsive element (HRE) upstream of the IRE system [91]. The IRP/IRE system functions by the binding of IRPs to the IRE at the 5' untranslated region (UTR) of ferritin mRNA under conditions of low iron, inhibiting its translation. However, in conditions of high iron, IRP binding to the IRE is decreased, leading to increased ferritin expression. Similarly, ferritin gene transcription is upregulated in conditions of inflammation where inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-2 (IL-2) signal to increase binding of NF- κ B to the transcription enhancer FER2 upstream of the IRE and coding region [90]. It has been shown that the NF- κ B pathway is highly responsive to inflammation [92]. Evidence for this pathway stems from activation of toll-like receptor 2 (TLR2) in macrophages resulting in IRP-independent upregulation of H-ferritin, as well as direct pharmacological activation of the NF- κ B pathway [93]. Similarly, it has been shown that H-ferritin expression is also responsive to tumor necrosis factor alpha (TNF- α) [94], interleukin-2 (IL-2) [95], and IL-10 [96]. Consequently, the elevated presence of ferritin in serum has long been considered in clinical settings to be an acute phase inflammatory marker, as elevated serum ferritin levels have been correlated with increased levels of pro-inflammatory cytokines [97,98]. Potential sources of serum ferritin during inflammation include secretion by macrophages [24,25] and/or release from cells due to tissue damage [99], both indicators of inflammation or infection. Importantly, there is disagreement in the literature about whether serum ferritin is a pro-inflammatory marker [100], an anti-inflammatory marker [101,102], or is simply a byproduct of release during the course of infection by damaged cells [99]. Understanding mechanisms of release of ferritin and how those mechanisms are influenced at the cellular level could provide insights into the biological and clinical meaning of ferritin in serum.

Serum ferritin has also been routinely interpreted in the clinic as a surrogate measure of iron storage in the body [103]. There are numerous studies that examine the correlation between serum ferritin and iron deficiency anemia or hemochromatosis in which serum ferritin levels are decreased or elevated, respectively [104]. However, inflammation or infection is known to drastically change the amount of serum ferritin present [105]. Accordingly, in an individual with acute or chronic inflammation, the extent to which serum ferritin levels reflect inflammation may reflect the total body iron stores. To further complicate this understanding, there are studies demonstrating that serum ferritin is not the most reliable marker for total body iron stores, especially when there are other complicating factors such as liver or kidney damage [12]. Put together, it seems that clinically, serum ferritin may be used as a marker for total body iron stores in the healthy individual, however, in a disease setting serum ferritin may be more indicative of the underlying pathophysiology such as organ damage or infection.

Another important consideration is while serum ferritin may be used clinically to suggest inflammation, serum ferritin measurements are focused largely on the L-ferritin subunit and as such, remain a poor indicator of iron status of the patient in this inflammatory state [97], as described earlier it is H-ferritin that is mostly responsive to the inflammatory process. H-ferritin has been detected in serum [106] and has the potential to be much more dynamic and informative than L-ferritin. A critical question that has yet to be answered clinically is: Is the iron saturation of serum ferritin in any given individual more informative to the total body iron stores than serum ferritin levels itself? Currently, it is rather trivial to measure serum ferritin levels but to measure the iron contained within serum ferritin is considerably more difficult. One recent study has looked at ferritin secretion from macrophages and determined that iron saturation of the ferritin was about a third as saturated as liver ferritin [24]. This is important as many consider serum ferritin to be iron-free or iron-poor, but the reality is that it contains a significant amount of iron. Thus, ferritin saturation may be the better measure of total body iron stores rather than the serum ferritin molecules themselves.

In addition to the utility of serum ferritin clinically, intracellular ferritin has also been implicated in the host response to microbial infection. Recent evidence demonstrates that H-ferritin confers tolerance to malaria and sepsis during infection by limiting reactive oxygen species (ROS) and overall oxidative stress. For example, Gozzelino et al. shows evidence that the activity of H-ferritin confers a metabolic adaptation to *Plasmodium* infection [107]. This activity is through the chelation ability of H-ferritin to limit labile iron from activating the pro-apoptotic c-Jun N-terminal kinase (JNK). Furthermore, the authors found that JNK activity inhibits H-ferritin expression and causes increased susceptibility of malaria coupled with tissue damage and oxidative stress. To extend this study, Weis et al. also looked at this metabolic adaptation in sepsis, a devastating syndrome resulting from a maladaptive host response to infection [108]. Much in the same way as Gozzelino et al., Weis and colleagues demonstrated a disease tolerance to sepsis that was facilitated by H-ferritin. Importantly, activity of H-ferritin to sequester cytosolic iron was critical for the setup of disease tolerance. Weis et al. showed that H-ferritin iron chelation prevents iron-driven oxidative inhibition of the glucose-6-phosphatase to sustain glucose production via liver gluconeogenesis. Another recent study that demonstrates the importance of H-ferritin in response to microbial infection was by Reddy et al. [109]. In this study, the authors infected H-ferritin knockout mice or wild-type mice with *Mycobacterium tuberculosis*, demonstrating that knocking out H-ferritin specifically in myeloid cell populations decreased survival after infection, increased the overall inflammatory response, and increased the total bacterial loads. These studies highlight the general importance of ferritin in the inflammatory response as well as for normal host iron homeostasis.

In contrast, ferritin has also been shown to have a role as an anti-inflammatory molecule. Ferritin in serum reportedly binds to high molecular weight kininogen (HK) [110]. Normally, HK is a co-factor in the intrinsic coagulation pathway; HK cleavage by the serine protease kallikrein leads to release of bradykinin and two-chain high molecular weight kininogen [111]. This particular pathway has large implications in the progression of a number of inflammatory diseases such as asthma [112], inflammatory bowel disease [113], and rheumatoid disorders [114,115]. Ferritin binding to HK serves as an anti-inflammatory signaling pathway, as the direct interaction of ferritin with HK prevents HK cleavage and a concomitant reduction in bradykinin release [102]. This overall interaction diminishes the total inflammatory response. In a similar paradigm, Fan and colleagues demonstrated that L-ferritin can play an important anti-inflammatory role in lipopolysaccharide (LPS)-stimulated macrophages [101]. In this study, Fan et al. show that overexpression of L-ferritin in macrophages can inhibit LPS-mediated transcription of TNF- α and IL-1 β , both critical pro-inflammatory cytokines. Conversely, they showed that L-ferritin is significantly downregulated after LPS stimulation. From this study, the authors conclude that L-ferritin has an underappreciated role in the anti-inflammatory resolution cascade. Importantly, both of these studies are direct evidence of ferritin playing an anti-inflammatory role.

6. Ferritin as a Disease Biomarker

While the amount of iron has been shown to increase throughout the body with age [116–118], abnormal iron homeostasis can lead to devastating diseases. Typically, a concomitant increase in ferritin levels are paired with increased iron levels [119], attempting to ensure safe handling of this increase in iron. However, a failure of ferritin levels to concomitantly increase with iron may impact normal physiology and induce pathology as a result of iron-mediated induction of oxidative stress. This role for ferritin and a mishandling of systemic iron has been shown to be implicated in a number of diseases such as Still's disease, hemophagocytic syndrome, and sideroblastic anemia—implications of serum ferritin in these diseases and others have been critically reviewed in numerous other reviews [12,104,106]. Similarly, serum ferritin as a bioindicator for cancer has been extensively reviewed as well [120,121]. Here we will focus specifically on neurological disorders and the impact that ferritin has on diagnosis as a biomarker for disease or progression. One interesting new development has been proposed in Friedreich's Ataxia where the authors discuss the hypothesis

that the mitochondrial protein frataxin may oligomerize like ferritin and perform functions redundant with mitochondrial ferritin, acting as another iron storage molecule [122]. Loss of frataxin and this iron storage property may result in Friedreich's Ataxia and subsequent neurodegeneration.

6.1. Neuroferritinopathy

Neuroferritinopathy (NF), an autosomal dominant disease resulting from mutations in L-ferritin, is classified as belonging to a group of movement disorders known as Neurodegeneration with Brain Iron Accumulation (NBIA). This disease is an extrapyramidal syndrome typically characterized by excessive iron accumulation in the basal ganglia. NF was originally characterized as a disease arising from an additional adenine at c.460 of exon 4 in the L-ferritin gene, but other mutations around the same region were quickly identified as contributing to disease pathology [123]. These mutations cause a frameshift in the C-terminus of the L-ferritin subunit resulting in reduced or ablated iron storage and subsequent iron-mediated damage, as this mutation creates a larger than normal pore [123]. In 2010, Barbeito et al. showed that fibroblasts from NF patients have higher levels of H- and L-ferritin as well as increased levels of total iron stores [124]. Furthermore, Maccarinelli and colleagues showed in 2014 that mice with the same L-ferritin mutation had a progressive impairment in motor coordination and a propensity to accumulate iron within post-natal hippocampal neurons [125]. Interestingly, this mutation in NF patients is present in all cells, but there is no evidence for iron mismanagement or dyshomeostasis by the rest of the body. It is currently unclear whether brain cells are simply more sensitive to iron-dependent toxicity or if there is a separate function for L-ferritin within the brain parenchyma. It is likely that the high oxygen and glucose consumption of the brain leads to an increase in iron utilization and therefore ROS/oxidative stress. Without functional L-ferritin, brain cells are rendered unable to survive this oxidative damage.

6.2. Parkinson's Disease

One of the most prominent neurodegenerative diseases with marked neurodegeneration as well as substantial iron accumulation in the substantia nigra pars compacta (SNpc) is Parkinson's disease (PD). In general, there is agreement that iron accumulation in the SNpc increases with age, but the additional increase in iron in the SNpc as a result of PD depends heavily on the stage and progression of PD [126]. With regard to ferritin in PD, there are a number of conflicting reports regarding the expression of ferritin within the SNpc; biochemical studies have shown both increased [126] and decreased [127] levels of ferritin within the SNpc in PD. In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD, H-ferritin overexpression in neurons is protective against disease progression [128]. Although there seems to be altered iron management specifically within the SNpc, cerebrospinal fluid (CSF) levels of ferritin and serum ferritin levels were not significantly different between PD and non-PD patients [127]. In contrast however, there is a recent study detailing how serum ferritin may serve as a potential biomarker for diagnosis in PD [129]. In this study, the authors found an increased level of serum ferritin in PD patients when compared to non-PD patients. Unfortunately, the sample size of the patient cohort used in this study was relatively small (40 healthy vs. 40 PD patients) and was limited to a cross-sectional analysis. Furthermore, there is a lack of consensus about the reliability of using serum ferritin as an indicator of brain iron status or even, as previously discussed, systemic iron status. Another consideration in the utilization of serum/CSF ferritin as a biomarker in PD is that peripheral inflammation plays a large role in PD disease progression [130]. As such, serum/CSF ferritin levels will likely be altered as a result and may not be informative of brain iron status.

6.3. Restless Legs Syndrome

In Restless Legs Syndrome (RLS), a disorder characterized by an irresistible urge to move the limbs and unpleasant sensations in the legs, a well-established neurobiological abnormality is low brain iron despite normal peripheral iron levels [131]. However, RLS is about five times more prevalent in iron deficient populations [132,133]. It is clear that ferritin levels are significantly impacted by and

are important in RLS pathology, but it is not yet known how ferritin may specifically contribute to disease progression or disease symptoms. We have previously shown that Tf, TfR, and H-ferritin levels are decreased in the endothelial cells of RLS patients, indicating iron mismanagement at the level of the BBB, suggesting decreased brain iron acquisition in RLS brains [134]. Furthermore, studies have also demonstrated a decrease in H-ferritin and iron levels specifically in the SNpc of RLS brains compared to controls [135]. Currently, serum ferritin is used to guide treatment, but we have discussed the concerns regarding the utility of serum ferritin both as a measure of total body iron stores and serum ferritin levels are of no value in predicting response to treatment. Lammers et al. studied serum ferritin, bodily iron, and RLS, finding no association between early-onset RLS and longitudinal measures of iron stores [136]. Similarly, while serum ferritin is currently used clinically to guide treatment, it has been shown that serum ferritin levels have no predictive value in the response to treatment [131]. This is an important consideration as it underlines the fact that serum ferritin levels are not necessarily predictive of brain ferritin levels or total brain iron status.

6.4. Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS), a progressive and devastating disease, is characterized by loss of motor neurons in the cortex, brainstem, and spinal cord. While ALS is a relatively common disease, the exact mechanisms for this neurodegeneration is currently unknown, with a number of factors such as oxidative stress, excitotoxic stimulation, and genetic factors all implicated to play a role. It has long been known that in the ALS iron homeostasis is disrupted; there is higher iron in the CSF and spinal cord of ALS patients [137]. While there are a large number of studies that demonstrate elevated levels of serum ferritin in ALS [137–139], there is not yet a consensus regarding whether serum ferritin is predictive [139–141] or unrelated [137,138] to disease progression and survival rates.

7. Conclusions

Classically, ferritin has been described as a critical iron storage protein whose dual roles as both an antioxidant and an iron sink were essential to cellular survival. The importance of ferritin can directly be seen in knockout mouse models; both H and L homozygous knockout mice are embryonic lethal [128,142]. While not disputing the importance of these intracellular roles, it is clear that ferritin has highly dynamic and novel functions outside of the cells. Recent evidence has demonstrated the ability of ferritin to participate in iron delivery, delivery of macro/micromolecules, and as a potential biomarker in many neurologic diseases. Though serum ferritin is largely comprised of the L subunit, iron levels in ferritin remain much higher than transferrin; there is significant potential for serum ferritin to deliver anywhere between 10 to 100 times higher iron than transferrin [35]. Furthermore, while it is clear that ferritin can deliver iron to various organs throughout the body, it is still unclear as to why the body needs multiple iron delivery routes, especially with such drastic differences in amount of iron delivered. Besides iron, the delivery of ferritin-encapsulated macro- and micromolecules has important implications in the treatment and diagnoses of various disorders. While serum ferritin has been implicated in a number of diseases, the exact role and mechanism for serum ferritin in these disorders has not yet been fully characterized. There is a large volume of literature dedicated to exploring ferritin biology, but a number of questions and topics remain to be explored regarding ferritin delivery, downstream fate, role of ferritin in many diseases, and the utility of ferritin clinically.

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Review

The Role of NCOA4-Mediated Ferritinophagy in Health and Disease

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Abstract: Nuclear receptor coactivator 4 (NCOA4) is a selective cargo receptor that mediates the autophagic degradation of ferritin (“ferritinophagy”), the cytosolic iron storage complex. NCOA4-mediated ferritinophagy maintains intracellular iron homeostasis by facilitating ferritin iron storage or release according to demand. Ferritinophagy is involved in iron-dependent physiological processes such as erythropoiesis, where NCOA4 mediates ferritin iron release for mitochondrial heme synthesis. Recently, ferritinophagy has been shown to regulate ferroptosis, a newly described form of iron-dependent cell death mediated by excess lipid peroxidation. Dysregulation of iron metabolism and ferroptosis have been described in neurodegeneration, cancer, and infection, but little is known about the role of ferritinophagy in the pathogenesis of these diseases. Here, we will review the biochemical regulation of NCOA4, its contribution to physiological processes and its role in disease. Finally, we will discuss the potential of activating or inhibiting ferritinophagy and ferroptosis for therapeutic purposes.

Keywords: NCOA4; ferritinophagy; iron homeostasis; erythropoiesis; ferroptosis; cancer

1. Introduction

Iron is required in multiple biological processes [1] including oxygen binding and transport [2], ATP production (as a cofactor in the citric acid cycle and electron transport) [3], and DNA biosynthesis and repair [4,5]. Iron deficiency is the main cause of anemia [6], which causes significant morbidity and mortality worldwide. Likewise, when iron deficiency affects other tissues (skeletal muscle, heart or brain) it can have fatal consequences [4]. Conversely, iron overload can generate Reactive Oxygen Species (ROS) by the Fenton reaction of the Haber-Weiss cycle thereby contributing to the pathophysiology of iron overload in diseases such as hereditary hemochromatosis [7,8]. Thus, cells must establish a delicate balance between iron availability and storage. This is accomplished through the orchestrated action of a network of proteins involved in iron transport/import (transferrin: Tf, transferrin receptor: TfR, DMT1), export (i.e., the iron export transporter, ferroportin: FPN), storage (ferritin: FTH1/FTL), and a host of additional iron homeostasis regulatory circulating and intracellular proteins [3]. Recently, our lab and others identified a role for Nuclear Receptor Coactivator 4 (NCOA4) in regulating iron homeostasis by binding to ferritin and facilitating its autophagic degradation in a process termed “ferritinophagy [9].” This process is itself regulated by intracellular iron levels that promote or inhibit ferritinophagy depending on iron availability and demand [10]. Here, we will review the biochemical regulation of NCOA4, its role in intracellular and systemic iron homeostasis, and its importance in physiological processes (such as erythropoiesis) and in disease. We will discuss the role of ferritinophagy in ferroptosis, a new type of cell death triggered by iron accumulation and lipid peroxidation. Finally, we will explore the therapeutic potential of modulating NCOA4-mediated ferritinophagy.

2. NCOA4 Mediates Ferritin Transport to the Lysosome for Degradation

Cellular iron is mainly stored in ferritin, a protein complex composed of 24 subunits of ferritin light and heavy chains (FTL, FTH1). Ferritin can chelate up to 4500 iron atoms in its ferric-Fe(III) form [11]. Iron is incorporated into ferritin via ferritin iron pores as Fe(II), which is further oxidized to Fe(III) by FTH1 inside the ferritin cage, leading to inert deposits of Fe(III) that are unavailable for intracellular use or generation of ROS [12]. For utilization, iron needs to be released from ferritin and reduced again to Fe(II) [13]. While there are studies showing iron can exit ferritin via the ferritin pore, the most likely mechanism of iron release involves proteolytic degradation of the ferritin shell. This degradation process takes place primarily in the lysosome [14] although some minor proteasomal contribution has been observed [15].

Multiple groups reported ferritin degradation as dependent on autophagy, a conserved catabolic cellular process that degrades cellular proteins and damaged organelles via the lysosome as part of a recycling and protective process to ensure cellular homeostasis basally and in response to stress [14,16,17] (for in-depth reviews on the molecular mechanisms of autophagy we point the reader to several excellent reviews on the subject [18–20]). Canonical autophagy requires the coordinated action of approximately 30 autophagy-related (Atg) proteins to form double-membrane vesicles (autophagosomes) that capture cellular cargo in both non-selective and selective manners for eventual lysosomal degradation. Autophagy initiation requires activation of the ULK complex followed by production of phosphatidylinositol-3-phosphate (PI3P) by the class III PI3K complex (VPS34, Beclin1, and p150) and membrane contribution from ATG9 vesicles. Pro-MAP1LC3B ('LC3B' and related ATG8 family members) is cleaved to LC3B-I by ATG4B and further conjugated to phosphatidylethanolamine by ATG7, ATG3 and the ATG5-ATG12-ATG16L1 complex. Lipidated LC3B-II binds to the growing membrane of autophagosomes and contributes to its elongation and fusion and also acts as a recruitment platform for autophagic molecular machinery [16,21]. Autophagosomes fuse with lysosomes thereby delivering cargo for degradation via lysosomal enzymes. Autophagy was initially described as a survival response to nutrient starvation; however, it is now appreciated that autophagy is active basally in all cells and can be induced by multiple stressors, such as hypoxia or ROS [22,23]. Recently, selective autophagic processes have been reported involving specific receptor proteins that target select cargo for autophagic degradation including mitochondria (mitophagy), endoplasmic reticulum (ER-phagy) or pathogens (xenophagy) [16]. Using autophagy-deficient MEFs (Atg7^{-/-}), Asano et al. showed that in iron-depleted cells, autophagy is required for ferritin degradation via the lysosome. Based on their work they hypothesized a selective ferritin autophagic process dependent on an as yet undetermined cargo receptor [17]. We recently identified NCOA4 as the cargo receptor targeting ferritin to the autophagosome in a selective autophagic process (ferritinophagy) [9].

We used a combination of autophagosome enrichment, stable isotope labelling by amino acids in cell culture (SILAC), and liquid chromatography–mass spectrometry (LC–MS) to profile proteins enriched in autophagosomes. One of the most robustly and reproducibly enriched autophagosomal proteins was NCOA4 [9]. While NCOA4 was originally identified as a nuclear receptor coactivator (discussed below), we demonstrated that NCOA4 localized to autophagosome and lysosome structures. To understand the role of NCOA4 in autophagy, we performed affinity purification-mass spectroscopy and identified ferritin subunits as interacting partners (FTL, FTH1) [9]. We also identified HERC2 and NEURL4, subunits of an E3 ubiquitin ligase complex, but this interaction was not seen in FTH1 immunoprecipitates, suggesting distinct NCOA4-ferritin and NCOA4-HERC2/NEURL4 complexes [9]. NCOA4 colocalized with ferritin in autophagosomes and lysosomes. Critically, NCOA4 depletion inhibited delivery of ferritin to the lysosome and thereby ferritin degradation. Reduced ferritin turnover led to a significant decrease in bioavailable intracellular iron that altered the intracellular iron homeostasis network of proteins [9]. In parallel, Dowdle et al. also identified NCOA4 as an autophagy receptor for ferritin [24].

There is limited structural information for NCOA4 except for a N-terminal coiled-coil domain that has been reported as a protein interaction [25] and self-oligomerization domain [26]. Phylogenetic

studies show no paralogs for NCOA4, although the ARA70-I family domain (which overlaps with the N-terminal coiled coil domain) is conserved in NCOA4 orthologs through metazoans [27]. NCOA4 has multiple splice variants in rodents and two transcript variants in humans (Figure 1a): NCOA4 α (614 Aa, 70 kDa) and the splice isoform NCOA4 β (286 Aa, 35 kDa) [28]. The isoforms share the N-terminal coiled-coil domain (Aa 1–238) and a small portion of the C-terminus (Aa 566–614). In vitro assays with recombinant NCOA4 and FTH1 mapped the interaction to the NCOA4 C-terminal domain (Aa 383–509), a domain only present in NCOA4 α and not in NCOA4 β , and a conserved surface arginine (R23) on FTH1 [10]. An independent study confirmed this interaction and demonstrated that FTH1 can bind up to 24 NCOA4 fragments (Aa 383–522) [29]. Site-directed mutagenesis on NCOA4 identified W497, I489, S492, L494, and L498 as residues important for ferritin binding. Importantly, NCOA4^{I489A/W497A} expression in HCT116 NCOA4 knockout (KO) cells abolished FTH1 binding and ferritin delivery to autophagosomes. Likewise, FTH1^{R23A} mutants prevented an interaction with NCOA4 in cells thereby inhibiting ferritin delivery to the lysosome even under conditions of iron deprivation when ferritinophagy is normally activated.

Flux through the ferritinophagy pathway is dependent on NCOA4 levels which are tightly regulated by intracellular iron levels (Figure 1b,c) [10]. Under iron-replete cellular conditions, NCOA4 binding to HERC2, an E3 ubiquitin ligase, is increased, leading to proteasomal degradation of NCOA4. Decreased NCOA4 levels inhibit ferritinophagy and increase ferritin iron storage [10]. NCOA4-FTH1 binding appears to be inhibited by high levels of iron, which would similarly decrease ferritinophagy and favor ferritin iron storage [29]. Interestingly, the site for HERC2 binding on NCOA4 (Aa 383–509) overlaps with the site for FTH1 binding raising the possibility that HERC2 and FTH1 binding are mutually exclusive. Future experiments are necessary to determine whether NCOA4 can simultaneously bind HERC2 and FTH1 or whether there is competitive binding. While NCOA4 co-purifies with iron, the mechanism of iron binding and the iron-binding pocket is unclear at this time. Under iron-deficient conditions, presumably when iron occupancy of NCOA4 decreases, HERC2 binding to NCOA4 is decreased, leading to NCOA4 stabilization and induction of ferritin degradation and iron release [10].

The mechanism by which NCOA4-FTH1 reaches the lysosome is not entirely clear. Despite NCOA4 association with multiple ATG8 proteins in in vitro binding assays, no canonical LC3-interaction region (LIR) motif has been identified in NCOA4, although the presence of other non-canonical ATG8-binding motifs may suggest the use of this alternative motif for interaction [31]. While classical autophagy appears to play a prominent role in ferritin delivery to the lysosome, other reports suggest that multiple pathways, including the ubiquitin-proteasome system, can contribute to ferritin degradation. Contribution of each degradation pathway may be different according to cell type or even type of iron deprivation, e.g., one report suggests that ferroportin-mediated iron export promotes ferritin degradation by the proteasome while iron chelation agents induce degradation in the lysosome [32]. Recent studies have suggested an ESCRT complex-mediated endosomal sorting pathway as an alternative, ATG8-independent, lysosomal trafficking pathway for NCOA4 and ferritin. Goodwin et al. report a highly regulated alternative pathway for ferritin degradation involving TAX1BP1, FIP200, ATG9A, VPS34, and components of the ESCRT machinery that is independent of the classical ATG8 macroautophagy pathway [33]. Likewise, the ESCRT-III endosomal pathway has been shown to mediate rapid starvation-induced degradation of autophagy receptors such as NCOA4, p62, NBR1, TAX1BP1, and NDP52 [34]. Further studies are required to explore the mechanism that directs NCOA4 complexes to the lysosome as well as the contribution of different pathways to ferritinophagy.

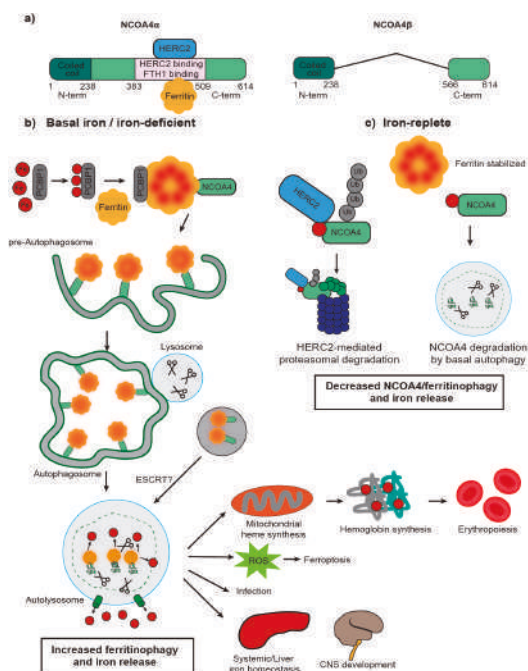


Figure 1. NCOA4-mediated ferritinophagy pathway is regulated by intracellular iron. (a) Schematic of NCOA4 transcript variants in humans: NCOA4 α and NCOA4 β . NCOA4 α is a 614 Aa protein (70 kDa) with an N-terminal coiled coil domain and a C-terminal domain that contains a FTH1 and HERC2 binding domain (Aa 383–509). NCOA4 β (286 Aa, 35 kDa) shares with NCOA4 α the N-terminal domain and a portion of the C-terminal domain. (b) Poly rC-binding protein 1 (PCBP1) binds to iron (Fe) and delivers it to ferritin. Fe is stored in ferritin complexes containing ferritin heavy and light chains. NCOA4 binds to ferritin through its C-terminal domain and delivers it to the nascent autophagosome. The mechanisms involved in NCOA4 delivery to the autophagosome are unclear but could involve non-canonical LIR motifs. Fusion of the autophagosome with the lysosome leads to degradation of ferritin and iron release in a process known as “ferritinophagy.” Some studies also suggest alternative pathways for lysosomal delivery of NCOA4-ferritin complexes involving the endosomal pathway (endosomal sorting complex required for transport: ESCRT). Iron is exported to the cytosol where it can be used in several physiological processes that involve iron such as mitochondrial heme synthesis and erythroid differentiation. Increased ferritinophagy flux correlates with reactive oxygen species (ROS) production and a type of cell death known as ferroptosis. Increased ferritinophagy flux has also been observed in certain *E. coli* urinary tract infections [30]. Finally, iron is also likely required for other physiological processes like liver iron homeostasis and central nervous system (CNS) development, although further research is required to clarify NCOA4’s role in the CNS disease under normal and pathological conditions such as neurodegeneration. (c) NCOA4 levels and ferritinophagy are regulated by intracellular iron levels. In iron-replete conditions, HERC2 (an E3 ubiquitin ligase) binds NCOA4 in an iron-dependent manner and mediates its proteasomal degradation. NCOA4 can also be degraded by basal autophagy. Decreased NCOA4 levels lead to stabilization of ferritin by decreased ferritinophagy flux and decreased intracellular levels of iron.

While the role of NCOA4 in mediating ferritin degradation and cellular iron homeostasis appears to be a conserved function of NCOA4 across cell types, earlier reports suggested NCOA4 acts as a nuclear receptor coactivator. NCOA4 was originally identified in a yeast-two hybrid screen as a coactivator of the androgen receptor (AR), enhancing its transcriptional activity in prostate cancer [35]. Subsequent studies suggest that NCOA4 also regulates a number of additional

nuclear receptors [36–40]. A recent study suggests that NCOA4 can be regulated by thyroid hormone, promoting NCOA4 recruitment to chromatin regions that induce a transcriptional program supporting erythropoiesis [41]. Finally, Bellelli et al. suggest NCOA4 inhibits DNA replication origin activation by binding to the MCM2-7 helicase complex via the NCOA4 N-terminal domain [42]. Further work is required to reconcile these various reported functions of NCOA4.

3. NCOA4 Mediates Ferritin Iron Release to Support Erythropoiesis

Given the importance of NCOA4 in intracellular iron homeostasis, several studies have addressed the role of NCOA4 in physiological cellular processes that depend upon iron availability, such as erythropoiesis. Erythropoiesis consists of several differentiation steps between the hematopoietic stem cell progenitor stage and the mature enucleated red blood cell (RBC) that are characterized by gradual degradation of cellular content [43] and production of hemoglobin [12]. Hemoglobinization requires large amounts of iron for heme synthesis with approximately 2 g of iron from the total 3–5 g in the human body incorporated in RBC hemoglobin [3]. Iron is delivered to RBCs by circulating transferrin (Tf) that is internalized via TfR-mediated endocytosis [2]. Next, iron is reduced to its Fe(II) form and exported from the endosome by DMT1. Two different mechanisms by which iron is transported to mitochondria for heme synthesis have been proposed: (1) by direct delivery from the endosome [44] or (2) by export to the cytoplasmic labile iron pool followed by intermediate storage in ferritin, lysosomal ferritin degradation, and delivery to the mitochondria [45,46]. NCOA4 depletion in the K562 human erythroleukemia cell line, an *in vitro* model of erythroid differentiation, impaired hemoglobinization and differentiation [10], supporting a role for ferritinophagy in mitochondrial iron delivery and heme synthesis. This finding has been confirmed in a murine cellular model of erythropoiesis (G1E-ER4 cells), showing defects in hemoglobinization after NCOA4 depletion albeit without affecting differentiation [47]. These studies support a model whereby iron exported from the endosome enters the cytoplasmic iron pool and is stored in ferritin prior to NCOA4-mediated ferritinophagy and subsequent delivery to the mitochondria for heme synthesis. Based on temporal evaluation of hemoglobinization in their model system, Philpott and collaborators described a model that involves sequential binding of poly rC-binding protein 1 (PCBP1), an iron chaperone, and NCOA4 to ferritin to balance iron storage or use depending on the erythroid differentiation stage [47]. In early erythroid stages, PCBP1 delivers iron to ferritin for storage while low levels of NCOA4 decrease ferritinophagy to further favor iron storage. At the midpoint of erythroid differentiation during the orthochromatic erythroblast stage, a period that correlates with high iron demand and hemoglobin synthesis, PCBP1 and NCOA4 coordinate to increase iron flux through ferritin and ferritinophagy to provide sufficient iron for heme synthesis (Figure 1). Finally, at late stages of erythrocyte development (reticulocyte) as cells lose organelles, iron import is decreased, ferritin levels decrease, and any remaining endosomes may transfer iron directly to mitochondria for ongoing heme synthesis [12,47].

Initial connections between NCOA4 and erythropoiesis on an organismal level were made in zebrafish developmental studies where NCOA4 mRNA expression was found to be upregulated at sites of erythropoiesis [48]. Consistent with this, transcriptional profiling of erythroblasts at sequential stages of differentiation showed that NCOA4 was one of the top upregulated genes in orthochromatic erythroblasts, where hemoglobin synthesis is maximal [49]. NCOA4 depletion in a zebrafish model revealed defects in globin synthesis and hemoglobinization [10]. Using a NCOA4 KO mouse model, Bellelli et al. identified NCOA4-mediated ferritinophagy as critical for iron availability for heme synthesis [50]. NCOA4 KO mice developed a mild hypochromic microcytic anemia together with ferritin and iron accumulation in tissues, indicating inefficient iron mobilization from ferritin. Mice fed an iron-deficient diet developed severe anemia with a compensatory activation of the Erythropoietin (Epo) pathway. Mice fed an iron-enriched diet developed significant accumulation of iron-laden ferritin and excess cytoplasmic iron that precipitated ROS-associated toxicity in the liver and premature death in comparison to controls [50]. This suggests that mice unable to efficiently degrade ferritin may eventually develop an ‘overflow’ of iron into the cytoplasm leading to higher susceptibility to oxidative

damage as discussed in greater detail below. NCOA4 appears to be more important at various stages of development as NCOA4 KO mice examined at the immediate postnatal period showed a more severe anemia [41]. This study suggests a differential temporal requirement of NCOA4-mediated ferritinophagy in periods of high iron demand. As the observed anemia is less severe in adult NCOA4 KO mice compared to young mice, this could suggest cell-autonomous or non-autonomous mechanisms that compensate for NCOA4 loss over time.

NCOA4 KO mice had higher ferritin in serum [50], suggesting that tissues overloaded with iron “leak” ferritin to the blood. This data suggests that there is a mechanism independent of NCOA4-mediated lysosomal delivery and non-classical lysosomal-mediated secretion that promotes ferritin secretion. In agreement with the NCOA4 KO mouse model, NCOA4 depletion in a monocytic cell line did not impair ferritin secretion but in fact increased it, suggesting that NCOA4 is not required for ferritin secretion [51]. Recently, Meyron-Holtz and collaborators demonstrated ferritin secretion via two independent pathways: a non-classical secretory-autophagy pathway and a multivesicular-body-exosome pathway [52].

To date, multiple studies have evaluated the role of NCOA4 in erythropoiesis *in vivo* but all of them have evaluated it in a systemic KO setting. Despite *in vitro* evidence in K562 and G1E-ER4 cells suggesting NCOA4 has a cell-autonomous role in erythropoiesis, it remains to be elucidated whether there are non-autonomous contributions of NCOA4 to erythropoiesis. Since NCOA4 knockout is non-fatal in these models, compensatory mechanisms may develop in erythroid cells or other cell types to overcome NCOA4 loss to support hemoglobin synthesis. Identifying these mechanisms will be of interest in the future. Finally, these data support study of NCOA4 in the context of multiple pathological conditions with imbalances in iron homeostasis like hemochromatosis or iron deficiency anemia [53].

4. NCOA4-Mediated Ferritinophagy Modulates Ferroptosis

Ferroptosis is an iron-dependent form of regulated cell death characterized by lipid peroxidation [54,55]. Given the central role of NCOA4-mediated ferritinophagy in regulating intracellular iron levels, ferroptosis sensitivity has recently been shown to be modulated by NCOA4 [56,57]. Ferroptosis is distinct from other identified types of cell death (apoptosis, necrosis or autophagy) at the morphological level, with absence of plasma membrane rupture or blebbing, presence of small mitochondria with reduced cristae, and lack of chromatin condensation [58]. In general, the main ferroptosis-inducing event is lipid peroxidation, which is triggered by inactivation of the lipid repairing phospholipid peroxidase, GPx4 (directly by compounds such as RAS-selective lethal 3 (RSL3) and indirectly by blocking cystine metabolism and glutathione synthesis (erastin, sorafenib and BSO)), or by iron accumulation leading to ROS and lipid peroxidation production (Figure 2). Here, we will focus on how ferroptosis is regulated by alteration in iron homeostasis pathways and specifically NCOA4-mediated ferritinophagy. For in-depth details on the ferroptosis pathway and biochemical regulation, comprehensive reviews are available [54,55,59].

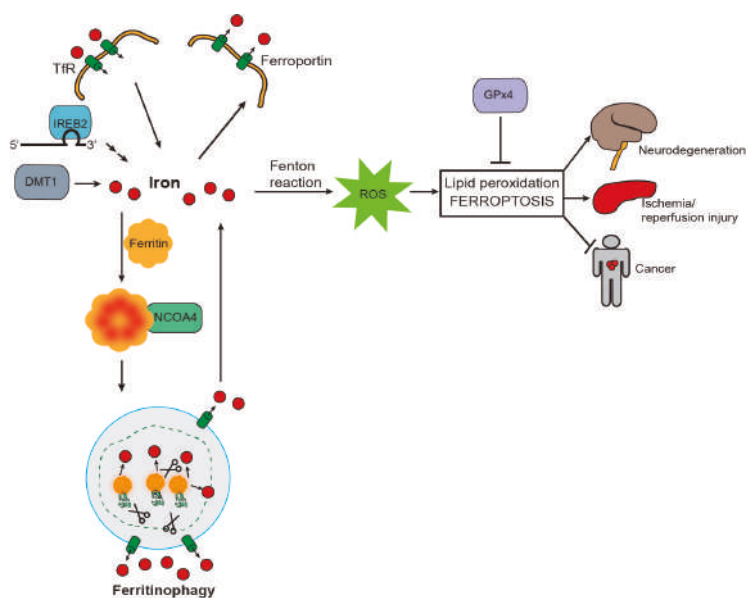


Figure 2. Iron and ferritinophagy are required for ferroptosis induction. Iron release in the cytosol can generate Reactive Oxygen Species (ROS) by the Fenton Reaction. ROS leads to lipid peroxidation and ferroptosis. Iron increase in the cytosol is mediated by increased import (by the transferrin receptor: TfR), decreased export (by ferroportin), increased translation of iron-metabolism related mRNAs via Iron Response Element Binding Protein 2 (IREB2) and increased cytosolic flux by Divalent Metal Transporter 1 (DMT1). Iron can also be stored in ferritin and delivered to the lysosome for NCOA4-mediated iron release from the lysosome. Increased ferritinophagy flux contributes to ferroptosis. Glutathione Peroxidase 4 (GPx4) repairs lipid peroxidation and inhibits ferroptosis. Ferroptosis is a promising therapeutic target. Inhibiting ferroptosis could protect from iron-induced cell death in neurodegeneration and ischemia/reperfusion injury while triggering ferroptosis could be effective in cancer patients.

As iron is necessary for the generation of lipid peroxides and thereby initiation of ferroptosis, ferroptosis sensitivity is impacted by compounds that decrease iron availability (iron chelation: deferoxamine (DFO)) and alterations in proteins involved in iron homeostasis that ultimately affect the cellular labile iron pool [58]. For instance, ferroptosis sensitivity is increased with overexpression of TfR (higher import of iron) and decreased expression of FTH1 and FTL (less ability to store iron) [60,61]. As NCOA4 directly regulates ferritin levels and thereby bioavailable levels of iron in the cell, it is not surprising that NCOA4-mediated ferritinophagy has recently been shown to modulate ferroptosis. While early studies were equivocal with respect to the role of autophagy in modulating ferroptosis [49,50], initial reports of NCOA4 depletion showed a decrease in intracellular free iron with a concomitant decrease in oxidative stress [9] accompanied by an increase in glutathione production [56]. Accordingly, NCOA4 deletion inhibited ferroptosis by blocking ferritinophagy and ferritin degradation [56,57]. Conversely, NCOA4 over-expression increased sensitivity to ferroptosis [56]. In fact, treatment with sorafenib, a ferroptosis-inducing drug [54,62], increased NCOA4 expression correlating with its role as mediator of ferroptotic cell death by promoting ferritinophagy and ROS production [63]. While the authors report upregulation of ELAVL1, a transcriptional regulator, as necessary for induction of autophagy/ferritinophagy, it is unclear whether there is a direct relationship between sorafenib, ELAVL1 function, NCOA4 expression levels, and flux through the ferritinophagy pathway [63].

Interestingly, Bellelli et al. showed the *in vivo* relevance of altering ferritinophagy to the ferroptosis pathway. They showed that NCOA4 depletion in mice fed with an iron-rich diet increased expression of GPx and SOD, likely due to overloaded FTH1 leading to iron leakage in tissues followed by upregulation of GPx and SOD as a compensatory mechanism to cope with oxidative stress [50]. Additional studies are required to address the *in vivo* regulation of autophagy/ferritinophagy as it relates to their contribution to ferroptosis. Also, it remains an open question whether ferroptosis occurs as part of a normal physiological process, perhaps during development.

5. Ferritinophagy and Ferroptosis in Disease

Ferroptosis has been linked to several pathological conditions including neurodegenerative disease, ischemia/reperfusion injury and cancer. As such, there is significant interest in identifying pharmacologic means of blocking (neurodegenerative disease, reperfusion injury) or triggering (cancer) ferroptosis for therapeutic purposes. Given NCOA4 can modulate ferroptosis sensitivity, understanding whether NCOA4 has a role in the pathophysiology of these various diseases is critical.

5.1. Neurodegenerative Disease

Neurodegenerative diseases (ND) have historically been associated with inappropriate iron accumulation and oxidative stress. More recently, ferroptosis has been shown to play a part in neurodegeneration pathophysiology [64]. In Parkinson's Disease (PD), increased iron accumulation and ROS lead to dopamine oxidation, contributing to an oxidative state that promotes dopaminergic neuronal cell death [65]. Elevated DMT1 expression [66] and activating mutations in Tf both lead to increased cytosolic iron and are correlated with worse outcomes for PD patients. Conversely, mutations in TfR2 that decrease cellular iron import correlate with better disease outcomes [67]. Similar observations have been made in Alzheimer's Disease (AD) [55], where iron is found to co-aggregate with pathogenic amyloid deposits [68]. Indeed, immunofluorescence of brains from patients with AD showed decreased expression of ferroportin, the iron export transporter [69]. Direct evidence that ferroptosis can modulate neuronal cell death initially came from *in vitro* rat organotypic hippocampal slice cultures treated with glutamate to mimic the pathophysiology of stroke and ND [58]. Glutamate induced ferroptosis that could be prevented by iron chelators and ferrostatin [58]. Studies with genetic [70] or pharmacological inhibitors of ferroptosis [71,72] in a variety of *in vitro* models and multiple ND disorders (PD, AD, Huntington's) subsequently confirmed that inhibition of ferroptosis is a potential strategy to prevent neuronal cell death.

To date there is no direct evidence linking ferritinophagy, ferroptosis, and ND. While elevated ferritinophagy sensitizes cells to ferroptosis, the data connecting autophagy and ND instead suggest that decreases in global autophagy activity are correlated with development of ND. Indeed, defective autophagy can cause ND in genetic mouse models of autophagy loss [73,74]. Mutation in *WDR45*, leading to decreased autophagy, has been identified in a number of patients with Neurodegeneration with Brain Iron Accumulation (NBIA) [75]. In general, as autophagy levels decrease with age [76], iron levels increase [77]. As defective autophagy is associated with ND, this suggests a more complex relationship of ferritinophagy activity to ND. Interestingly, in long term NCOA4 KO, mice have increased serum iron and were more sensitive to iron overload [50]. This could suggest that long-term ablation of ferritinophagy *in vivo* leads to an iron overload phenotype, albeit with decreased utilization of iron for erythropoiesis leading to anemia. Additional studies are necessary to clarify the expression, regulation and function of NCOA4 in the central nervous system (CNS). Given the possible role of ferritinophagy in mediating neuronal cell death, further studies will clarify NCOA4's role in ferroptosis in the CNS and establish the potential of ferroptosis inhibition in ND, either by iron chelation or by modulating ferritinophagy flux.

5.2. Cancer

Many cancers accumulate large amounts of iron to support increased proliferation. This is accomplished in a cell-autonomous manner by increasing iron import or decreasing export [78–82] and in a non-autonomous manner by increasing iron supply from other cell types like macrophages [83,84]. Initial data suggests NCOA4 may be relevant to tumorigenesis and that the intersection of the ferritinophagy and ferroptosis pathways may represent a therapeutic avenue. A positive correlation between NCOA4 mRNA and NCOA4 α protein levels and transformation has been described in ovarian carcinoma [85,86]. Here, overexpression of several oncogenes (MYC, H-Ras, p53 inactivation) in normal endometriotic cells to induce transformation led to an upregulation of NCOA4 α and NCOA4 β expression. Interestingly, NCOA4 α knockdown in transformed cells decreased survival whereas NCOA4 β overexpression decreased colony formation. On the other hand, in studies of prostate cancer, NCOA4 α acts as a tumor suppressor while NCOA4 β expression correlated with proliferation and invasion [87]. Similar roles were identified for the two isoforms in MCF7 breast cancer cells [88]. Whether NCOA4 functions differently depending on the cancer context and understanding whether the ferritinophagy specific function of NCOA4 drives tumor effects are critical questions for future study.

While robust evidence for the role of ferritinophagy in tumorigenesis is lacking, there is a significant evidence for the importance of autophagy in cancer with recent links between ferroptosis sensitivity and tumor autophagy dependence. The role of autophagy in cancer is exceedingly complex with autophagy acting in a tumor suppressive or tumor promoting manner depending on the tumor context (reviewed in detail [21]). Autophagy is activated in a variety of cancers [21,89–93] to support metabolism, redox homeostasis and probably iron turnover. In particular, many Kras-driven tumors, such as pancreatic cancer, depend on high autophagic flux [89] and have significant iron accumulation due to elevated TfR expression [94]. Notably, pancreatic cancer cell lines have increased NCOA4 expression and a corresponding high flux through the ferritinophagy pathway [9]. These baseline characteristics would suggest a high sensitivity to ferroptosis. In fact, ferroptosis inducers erastin and RSL3/5 were originally discovered as part of a synthetic lethality screen to determine synergies with the Ras oncogene [60,95]. More direct evidence for the role of NCOA4 in modulating ferroptotic cell death in pancreatic cancer cells came from Yang et al. who showed that artesunate-mediated ferroptotic cell death is attenuated by NCOA4 depletion [96]. In general, autophagy (and therefore ferritinophagy) has been shown to be a positive regulator of ferroptosis [56,57]; therefore, triggering ferroptosis in cancers with high autophagy levels might reveal a potential vulnerability. One possible future therapeutic strategy would be to increase iron flux through the ferritinophagy pathway, leading to increased labile iron and ROS, thereby sensitizing cancer cells to ferroptosis-inducing agents.

5.3. Infectious Disease

Ferritinophagy is also involved in modulating susceptibility to infectious diseases. Uropathogenic *Escherichia coli* survive by forming reservoirs within urothelial cell autophagosomes. These bacteria traffic with ferritin-bound iron to the autophagosome to support their proliferation. The authors show that ferritin trafficking is NCOA4-dependent and that NCOA4 depletion reduces bacterial load [30]. Autophagy inhibitors and iron chelators were able to reduce bacterial burden and host cell death, suggesting a therapeutic potential to modulating NCOA4-dependent ferritinophagy in certain bacterial infections. Human cytomegalovirus (HCMV) protein pUL38 blocks the function of USP24, a deubiquitinase, to prevent an iron-dependent, endoplasmic reticulum (ER)-stress induced premature cell death [97]. USP24 deubiquitinase activity stabilizes NCOA4 protein thereby promoting ferritinophagy, which in turn increases cellular iron levels promoting iron-dependent ER stress-induced cell death. Therefore HCMV protein pUL38, via inhibition of USP24, decreases ferritinophagy in order to protect HCMV-infected cells from a premature cell death [97]. These studies suggest that modulating NCOA4-mediated ferritinophagy levels may be an effective strategy to inhibit certain bacterial or viral infections.

6. Conclusions and Future Directions

NCOA4-mediated ferritinophagy is integral to iron homeostasis in normal and pathological conditions. Significant progress has been made identifying the molecular mechanisms that regulate NCOA4 activity. However, a number of outstanding questions regarding the biochemical regulation of NCOA4 remain. First, how NCOA4 is regulated on a transcriptional and post-transcriptional level has yet to be determined. Importantly, NCOA4 mRNA does not appear to have a 5' or 3' Iron Regulatory Element (IRE) that would engage the iron responsive IREB1/2 system of post-transcriptional regulation. NCOA4-FTH1 and NCOA4-HERC2 binding appears to be regulated by iron levels; however, the mode of NCOA4 iron binding as well as the structural requirements for FTH1 and HERC2 binding are unclear. Further, where and when NCOA4 first interacts with FTH1 or HERC2 in the cell is unclear. The precise mechanism of NCOA4-FTH1 delivery to lysosomes is unclear and there may be multiple routes including a canonical autophagic route and/or an ESCRT complex dependent endosomal-lysosomal transport pathway [33,34]. NCOA4 has been reported to localize to and function in the nucleus as a nuclear receptor coactivator and as a regulator at DNA replication origins. How cytoplasmic versus nuclear NCOA4 localization is determined is an unanswered question, especially given NCOA4 lacks a canonical nuclear localization sequence. Interestingly, the two NCOA4 isoforms, NCOA4 α and NCOA4 β , differ in that NCOA4 β does not contain the C-terminal elements for binding FTH1 and HERC2; however, their relative functions and expression levels in cells is undetermined at this time.

The role of NCOA4-mediated ferritinophagy in physiological processes has so far been assessed mainly in the context of erythropoiesis in zebrafish and murine models of systemic NCOA4 depletion. However, these studies have been unable to identify if the impact on erythropoiesis is due to cell-autonomous effects on iron metabolism in erythrocytes or due to impairment of whole body iron availability. Further studies are required to address this question using conditional knockout mouse models that interrogate NCOA4 in red blood cells or in other cell types that contribute to erythropoiesis. Furthermore, results from Gao et al. suggest there might be a temporal variation in NCOA4 dependency [41], with a higher reliance at early developmental stages. Defining the importance of NCOA4 at different developmental stages as well as comparing acute vs. long-term KO will be key to understanding the mechanisms triggered in erythrocytes or other cell types to compensate for NCOA4 loss. In addition, understanding the relative importance of NCOA4-mediated ferritinophagy in other organs and cell types, especially those highly involved in systemic iron homeostasis, such as the liver and the macrophage system, is critical and will depend on conditional NCOA4 KO mouse models.

The role of ferritinophagy in disease is an underexplored area. Based on the importance of NCOA4 in erythropoiesis and systemic iron homeostasis, future studies of NCOA4 function in the context of anemia and hemochromatosis and whether inhibition or upregulation of ferritinophagy can impact these and other disease processes where iron homeostasis is dysregulated will be informative. The recent discovery that ferritinophagy can modulate sensitivity to ferroptosis, an iron-dependent form of cell death linked to neurodegeneration, cancer and ischemia/reperfusion injury, suggests ferritinophagy may be similarly important in these diseases. Understanding how ferritinophagy contributes to ferroptosis sensitivity in each pathological setting *in vivo* will be key to designing therapeutic interventions that could trigger or block ferroptosis. The recent discovery of NCOA4 has opened a new area of biology with connections to a wide range of physiologic and pathophysiological processes. Future studies will clarify the above described unanswered biochemical and *in vivo* functional questions fundamental to our understanding of NCOA4 biology.

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Conflicts of Interest: J.D.M. is an inventor on a patent pertaining to the autophagic control of iron metabolism.

Abbreviations

NCOA4	Nuclear Receptor Co-Activator 4
ATP	Adenosine Triphosphate
DNA	Deoxyribonucleic acid
ROS	Reactive Oxygen Species
TfR	Transferrin Receptor
DMT1	Divalent Metal Transporter 1
FPN	Ferroportin
FTH1	Ferritin Heavy Chain 1
FTL	Ferritin Light chain
Fe	Iron
PI3P	Phosphatidylinositol-3-phosphate
MEFs	Mouse Embryonic Fibroblasts
Atg7	Autophagy-related protein 7
SILAC	Stable Isotope Labeling by Amino-acids in Cell culture
LC-MS	Liquid Chromatography-Mass Spectrometry
HERC2	HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2
NEURL4	Neutralized E3 Ubiquitin Protein Ligase 4
PCBP1	Poly rC-binding protein 1
LIR motif	LC3-interaction region
ESCRT	Endosomal Sorting Complex Required for Transport
CNS	Central Nervous System
AR	Androgen Receptor
RBC	Red Blood Cell
GPx4	Glutathione Peroxidase 4
RSL3	RAS-selective lethal 3
BSO	Buthionine Sulfoximine
DFO	Deferoxamine
SOD	Superoxide Dismutase
IREB2	Iron Response Element Binding Protein 2
ND	Neurodegenerative Disease
PD	Parkinson's Disease
AD	Alzheimer's Disease
NBIA	Neurodegeneration with Brain Iron Accumulation
HCMV	Human Cytomegalovirus

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Review

Impairment of the Developing Human Brain in Iron Deficiency: Correlations to Findings in Experimental Animals and Prospects for Early Intervention Therapy

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Abstract: Due to the necessity of iron for a variety of cellular functions, the developing mammalian organism is vulnerable to iron deficiency, hence causing structural abnormalities and physiological malfunctioning in organs, which are particularly dependent on adequate iron stores, such as the brain. In early embryonic life, iron is already needed for proper development of the brain with the proliferation, migration, and differentiation of neuro-progenitor cells. This is underpinned by the widespread expression of transferrin receptors in the developing brain, which, in later life, is restricted to cells of the blood–brain and blood–cerebrospinal fluid barriers and neuronal cells, hence ensuring a sustained iron supply to the brain, even in the fully developed brain. In embryonic human life, iron deficiency is thought to result in a lower brain weight, with the impaired formation of myelin. Studies of fully developed infants that have experienced iron deficiency during development reveal the chronic and irreversible impairment of cognitive, memory, and motor skills, indicating widespread effects on the human brain. This review highlights the major findings of recent decades on the effects of gestational and lactational iron deficiency on the developing human brain. The findings are correlated to findings of experimental animals ranging from rodents to domestic pigs and non-human primates. The results point towards significant effects of iron deficiency on the developing brain. Evidence would be stronger with more studies addressing the human brain in real-time and the development of blood biomarkers of cerebral disturbance in iron deficiency. Cerebral iron deficiency is expected to be curable with iron substitution therapy, as the brain, privileged by the cerebral vascular transferrin receptor expression, is expected to facilitate iron extraction from the circulation and enable transport further into the brain.

Keywords: developmental; iron deficiency anemia; neonatal; transferrin receptor; treatment

1. Introduction

Iron deficiency is the most common type of malnutrition in humans [1–4]. Iron deficiency occurs due to an inadequate intake, excess loss, or increased need, and gradually leads to insufficient functions of many organs, including the bone marrow, and as a consequence, iron deficiency leads to iron deficiency anemia [5–7]. According to the WHO, anemia affects 1.8 billion people worldwide, equivalent to approximately 25% of the world’s population. Among this group, approximately 0.5 billion are women of a reproductive age, and in developing countries, the incidence of anemia is even higher

and varies during pregnancy from 35% to 56% for Africa, 37% to 75% in Asia, and 37% to 52% in Latin America.

Women of a reproductive age are particularly at risk for developing iron deficiency due to menstrual bleeding and pregnancy. The prevalence of iron deficiency anemia in pregnant women is high, even in industrialized countries with well-established iron supplementation policies, e.g., in Denmark, where iron deficiency with anemia affects roughly 12% of pregnant women. The state of pregnancy greatly increases the demand of iron used in foetal organogenesis and growth. The pregnant body prioritizes the fetal iron supply over the maternal utilization up until a threshold of the maternal iron stores being adequate, with a maternal plasma ferritin concentration of approximately 14 µg/L [8]. When the maternal iron stores exceed this threshold, the placenta actively transports iron into the fetal circulation to ensure adequate iron supply to the fetus [9].

Iron deficiency can adversely affect brain development in fetuses and infants. Whereas most knowledge on the fetal needs for iron during pregnancy has been obtained within hematological, nephrology, and gastrointestinal disciplines, the impact on the fetal brain of maternal iron deficiency during pregnancy remains quite understudied. A higher turnover of iron in the developing brain [10], in addition to the widespread expression of iron-containing proteins, nonetheless dictate the importance of iron for the developing brain [11–14]. This is particularly underscored by the profound expression of transferrin receptor 1 by dividing neuroprogenitor cells [15,16] and signifies that cells of the developing brain with respect to their need for iron share a range of common conditions with precursor cells participating in bone marrow erythropoiesis and the formation of duodenal enterocytes of the fully developed organism. This review aims to summarize the current evidence on the significance of iron for the developing brain, how iron deficiency may impair functioning of the central nervous system (CNS) in the human brain and brains of experimental animals, and which therapeutic advances available can prevent damage to the developing CNS.

1.1. Transport of Iron into the Brain

The brain acquires iron during life in a privileged manner in that its capillary endothelial cells are the only endothelial cells of the entire body that express transferrin receptors [17]. Iron circulates in blood exclusively bound to transferrin, unless pathological conditions like hemochromatosis occurs, which will result in the presence of low-molecular-weight forms of non-transferrin bound iron. The brain capillary endothelial cells form the blood–brain barrier (BBB) that prevents paracellular, non-specific entry of the iron-containing transferrin into the brain [17]. Rather, the brain capillary endothelium regulates iron transport into the brain via the expression of transferrin receptors [9,13,17]. Iron-transferrin attaches to the transferrin receptor, which results in the formation of endocytic vesicles. Recent studies have also shown that the transferrin receptor of the BBB can bind and take up circulating ferritin [18–21]. These vesicles are slightly acidic, and the lower pH reduces the binding affinity of iron to transferrin, which loosens their binding. The iron, present on its ferric form, is reduced to ferrous iron, which can be transported out of the endosome and into the cytosol by divalent metal transporter 1 (DMT1) [22]. The release of iron from transferrin within the endosome causes the iron-free apo-transferrin to detach from the transferrin receptor, which allows unbound apo-transferrin to recycle to the luminal surface [17].

Unbound ferrous iron is a potent pro-oxidative molecule that needs immediate oxidation [23,24]. Consequently, ferrous iron is either oxidized within the cytosol by ferrous oxidases, e.g., ceruloplasmin, or gets transported into the brain's extracellular space via the efflux transporter ferroportin, while undergoing oxidation during passage of the cellular membrane [23]. The iron transported across the brain endothelium accordingly occurs in a non-transferrin bound iron form and thereby is a candidate for binding to transferrin present within the brain extracellular space [17].

Iron in the cytosol participates in essential metabolic processes, e.g., participation in mitochondrial respiration via incorporation mitochondrial enzymes. Many cell types of the body also store residual iron as ferritin-iron, as ferritin also has pro-oxidant activity and is capable of oxidizing ferrous iron

to store around 4500 iron atoms [25,26]. Of note, however, is that brain capillary endothelial cells hardly express ferritin, except for during development [27], suggesting that virtually all iron present within the brain capillary endothelial cells is immediately directed toward transport across the BBB to ensure its function further inside the brain.

Iron is also transferred to the brain via transfer across choroid plexus epithelial cells that form the blood–cerebrospinal fluid (CSF) barrier [17]. Like the endothelial cells forming the BBB, the epithelial cells of the choroid plexus also express transferrin receptors [24], but the quantitative relevance of the choroid plexus for iron transport into the brain is of less significance due to their much lower surface than that of brain endothelial cells of the BBB. The choroid plexus nonetheless very likely makes an important contribution to cerebral iron homeostasis, as transferrin of the blood plasma is filtered through the blood–CSF barrier and enters the brain ventricles, while transferrin in parallel is also synthesized and secreted from the choroid plexus to enter the brain ventricles [24]. In sum, this suggests that transferrin of the brain ventricle, and likely also elsewhere in the brain’s extracellular space, is derived from the choroid plexus. In the extracellular compartment of the brain, transferrin is needed to capture non-transferrin-bound iron transported across the BBB or released from neurons and glia. The need for transferrin in the brain’s extracellular space is further underscored by the presence of transferrin receptors on neurons [28]. Surprisingly, transferrin receptors and DMT1 are hardly detected on major glial cells like astrocytes, oligodendrocytes, and microglia [23,28], which suggests that iron enters glial cells as non-transferrin-bound iron, possibly via specific transporters like ZIP14 [29].

1.2. Transport of Iron into the Developing and Iron-Deficient Brain

The uptake of iron-containing transferrin at the BBB and blood–CSF barriers and the further transportation of iron into the brain are dramatically upregulated in the developing brain [30–32]. The higher iron uptake strongly correlates to a higher expression of transferrin receptors by brain endothelial cells in the developing brain, as evidenced from studies on the rodent brain [30,31]. The upregulated iron transport is attributable to a generally higher need for iron as the progenitor cells of the brain proliferate and differentiate into their final phenotypes [15,16,32,33]. Interestingly, as the cerebral turnover of iron is extremely low and ceases with increasing age, virtually all the iron transported across the brain barriers during development is believed to remain within the brain [10–12].

Correspondingly, when the events take place during development, the brain also adapts to conditions with deprivation in iron accessibility by the upregulation of transferrin receptors [28]. When cerebral iron deficiency occurs, the brain profoundly increases the internalization of transferrin receptors in the capillary endothelial cells. The brain also increases the expression of transferrin receptors in neurons in iron deficiency, whereas glial cells, even in stages with robust iron deficiency, fail to express transferrin receptors [28].

Combining iron deficiency with stages of development produces the maximal demand for the brain to mobilize transferrin receptors, but in this context, it is of note that there seems to be an upper limit for the extent to which the brain can adapt. Supporting this notion, the brain failed to increase the expression of transferrin receptors when iron deficiency was subjected to experimental animals during development [31,34]. Therefore, the failure to further increase transferrin receptor expression suggests that the developing brain is particularly vulnerable to severe iron deficiency.

1.3. The Significance of Iron for Precursor Cells of the Developing Brain

The availability of iron for the brains’ cells must be adequate to undertake several iron-dependent processes, not only to ensure important functions such as cellular division and differentiation, but also the development of the entire brain [9,11,12], e.g., (i) the complex cellular architecture consisting of neuronal axons ensheathed with myelin synthesized by oligodendrocytes, (ii) the complicated brain–barrier interface supported by astrocytes and pericytes to regulate transport in and out of the brain, and (iii) the establishment of an innate immune system in the brain via the formation of microglia.

The significance of iron for maintaining cellular functions has been covered in former reviews [11,12,35–37]. Iron denotes an essential part as the co-factor of several proteins that can be organized into four groups: Non-enzymatic iron-containing proteins; enzymes that use iron-sulfur as a co-factor; enzymes with an iron-containing heme group; iron-containing enzymes without heme or an iron-containing sulfur group. Together, these four groups of proteins undertake essential iron-dependent cellular events, i.e., electron transfer in the mitochondria, regulation of the expression levels of several genes, regulation of cellular division and differentiation, the binding and transport of oxygen, the synthesis of neurotransmitters (in particular serotonin, norepinephrine, and dopamine), the packaging of neurotransmitters in the axon terminal, the reuptake and degradation of neurotransmitters, and the co-factor function for peroxide- and nitrous oxide-generating enzymes for the functioning of immune cells and the intracellular killing of pathogens [11,12,35–37]. More specifically, for the developing brain, these above-mentioned cellular iron-dependent processes make their contribution to adequately ensure series of important events ranging from early formation of the neuronal tube to later differentiation of neuronal precursor cells into neurons and glial cells. Iron is very important for the formation of the neuronal tube, of which the formation is abrogated during a conditional lack of transferrin receptors [15,16]. Severe iron deficiency early in life is also expected to impair the forming brain due to the loss of function of the iron-containing enzyme ribonucleotide reductase that is essential for cellular division [38]. This leads to the major concern that remains unexploited, which predicts that developmental iron deficiency during early gestation can cause a permanent reduction in the number of neuronal and glial cells in spite of iron being supplied later in life, e.g., by admitting iron to the neonate [39]. Concerning glial cells, their formation in the developing brain depends on iron-containing enzymes to ensure cellular division and differentiation. Regarding oligodendrocytes, the lack of iron availability during development is thought to significantly affect their capability to form myelin [11,12,40], and current hypotheses concern whether the formation of myelin is permanently affected, even if the iron supply is restored later in life, hence hinting towards a certain time-window during development where iron availability must be adequate to promote myelination [41].

1.4. Translational Models of the Brain Development

The effects of iron deficiency on the brain will likely manifest, with the earlier the impacts taking place representing the worst condition. The gestational ages vary dramatically between mammalian species, which is very important to notify when comparing experimental data on the effects of iron deficiency.

The normal brain development of different mammalian species can be compared (<http://www.translatingtime.org/translate>) [42]. Events like neurogenesis and myelination in the brains of rats and mice time wise are almost identical (Figure 1), but differences dramatically occur when comparing rodents and humans. Figure 1 also shows that myelination of the human brain compared to myelination in rats and mice is clearly different regarding both timing and the time period of gestation, i.e., myelination of the human brain takes places around post-conception (PC) day 250, which translates to PC day 30 in the rat. The latter is very important to emphasize, because it shows that opposed to the human brain, myelination takes places after birth in the rodent (around P7), which must be taken into account when designing an experimental study on brain development with the purpose of detecting correlations between species.

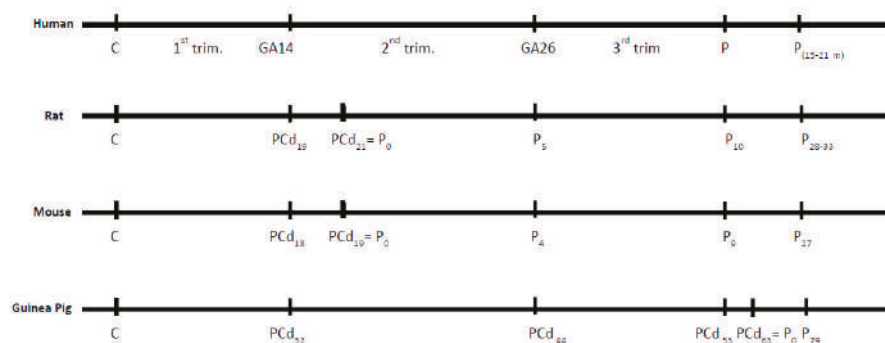


Figure 1. Comparison of the developmental ages of the human, rat, mouse, and guinea pig with respect to myelination of the entire brain. C = day of conception. Psuffixnr = postnatal day. PCd = post-conception day. P = partus. GA = human gestation week corresponding to PCd. trim. = Trimester.

1.5. Evidence of Deleterious Effects of Iron Deficiency on the Developing Brain

The effects of iron deficiency on the gastro-intestinal tract and hematological system are well-described, with the effects being reversible. In contrast, the developing CNS differs from many organs of the body as the impacts are much more prone to be irreversible, even when iron supplies are restored, because the neurons are post-mitotic from the time of birth. The following paragraphs will outline the studies that have been conducted to delineate the effects of iron deficiency on the developing brain, with an emphasis on whether effects were reversible or irreversible. To facilitate the translational relevance, the different species are reported separately based on changes in neuronal and glial functioning, and probable changes in behavior. The selected studies were identified based on a search strategy using PubMed to identify primary research on experimental animal studies and human studies using the following MESH words: iron deficiency, development, brain, or neuro, which revealed approximately 60 relevant studies on experimental animals and observations of the developing human brain during the most recent two decades.

1.6. Experimental Animals

The experimental animal data point towards significant effects on the brain during periods of iron deficiency during both the gestational period and after birth (Table 1). The data on small rodents like rats and mice clearly show that the most dramatic effects on the brain development occur when iron deficiency is introduced in pregnancy, whereas data from larger animals like the domestic pig and non-human primates show an influence when iron deficiency is introduced to the offspring (Table 1).

Rodents, especially the laboratory rat, denote the most popular experimental animal for studies of iron deficiency. The gestational period in the rat principally covers the first two trimesters in humans, with the transition between 1st and 2nd trimesters occurring only two days before delivery, whereas the third trimester in the human is reflected in the first weeks after birth in the rat (Figure 1).

The latter points towards a significant difference in the possibilities to compare human and rodent studies, as feeding of this early postnatal rodent no longer occurs via the transfer of nutrients across the placenta, but instead relies on a functioning gastrointestinal system of the neonate. The absorption of iron mainly occurs in the proximal duodenum and is regulated by the iron availability of the duodenal enterocytes. These are mainly under regulation of the general iron status in the neonate via signaling via circulatory levels of hepcidin, which is a hormone synthesized and released from the liver in response to inflammatory stimuli and high circulatory levels of iron [43]. However, inflammation in the neonate may lead to increased levels of hepcidin, as this will negatively affect iron uptake from the gut [6], and hence the rodent as a model of development equal to the third semester in the human fetus

represents a model of potential risk. On the other hand, for the study of the effects of iron deficiency during development, the early postnatal rodent represents an accessible model with many possibilities for intervention.

Table 1. Studies in experimental animals showing cerebral effects of iron deficiency (ID) subjected to pregnant females or their offspring. References are listed chronologically after species rather than after specific topics, as the many studies addressed more than a single objective. Most data were obtained from studies on rats. Abbreviations: ABR, auditory brainstem responses; DPOAE, distortion product of otoacoustic emissions; IHC, immunohistochemistry; PUFA, long-chain polyunsaturated fatty acids.

Species	Study Design	Method	Offspring Age	Conclusion	Reference	
Rat	ID before conception + Gestational ID	Electrophysiological recordings	P15–P30, P65	Late term effects on synapses in hippocampus in spite of cerebral iron repletion	[44]	
	ID before conception	Behavior	P10–Adult	Some persistent effects in spite of iron repletion	[45]	
	ID before conception + Gestational ID	Brain iron Neurotransmitters	P35	Behavioral impairments related to persistent loss in dopamine in spite of brain iron reversal	[46]	
	Gestational ID + Lactational ID	mRNA	P6–P21	ID from E15 leads to alteration in tyrosine hydroxylase and reversibility in behavior	[47]	
	Gestational ID + Lactational ID	mRNA, proteins morphology	P7–P15; P30	Lower BDNF, impaired neuronal differentiation	[48]	
	ID before conception + Gestational ID	Behavior	Adult	Detrimental effects of behavioral tasks, sex dependency	[49]	
	Gestational ID	Myelination	P25	Impaired myelination with correlation to impairment	[50]	
	Gestational ID	mRNA, proteins	P32–P69	Effect of behavior, no effects on motor skills in hippocampus	[51]	
	Gestational ID + Lactational ID	Behavior	P65	Permanent changes in behavioral tasks	[52]	
	Gestational ID + Lactational ID	mRNA morphology	P7–P65	Permanent changes in mRNA of neuronal markers and dendritic branching in spite of postnatal reversal to normal diet	[53]	
	Gestational ID	mRNA, T3, T4	P12	Marked reduction in T3, T4	[54]	
	Rat	ID before conception + Gestational ID	ABR, DPOAE	P0–P45	First trimester displays profound changes in auditory brain stem response	[55]
		Gestational ID + Lactational ID	MRI, NMR	P7–56	Restoration of brain iron, permanent size reduction in hippocampus and neurochemical hall-markers in spite of postnatal reversal to normal diet	[56]
Gestational ID		mRNA	P7–P56	Impaired formation of neuronal network and impaired neuronal plasticity in spite of postnatal reversal to normal diet	[57]	
Gestational ID		Morphology	P21–P40	25% reduction in dendritic length 20% reduction in axonal diameter	[58]	
ID before conception + Gestational ID		ABR	P40	Increased ABR latencies in ID depending on stage of ID	[59]	
Gestational ID + Lactational ID		mRNA	P10–P15	Elevated angiogenic/vasculogenic signaling with increased blood vessel complexity	[60]	
ID before conception + gestational ID		mRNA, T3, T4	E13–P10	Marked reduction in T3, T4 Lowering of thyroid hormone responsive genes	[61]	
Embryonic brain		mRNA	Not available (Cultures at E16)	DFO-induced ID lowers expression of series of markers of dendritic and synaptic development, and mitochondrial function	[62]	

Table 1. Cont.

Species	Study Design	Method	Offspring Age	Conclusion	Reference
	Gestational ID	Tactile stimuli	P1–P32	Tactile stimuli reverse defect myelination and alteration in oligodendrocytes and microglia, but not astrocytes	[63]
Rat	Gestational ID	Pro/anti-oxidant	P0–P70	Age- and iron-dependent levels of oxidative stress profiling	[64]
	Gestational ID	mRNA, IHC	P21, P35	Defect myelination, alteration in glial cells	[65]
Mouse	Gestational ID Brain iron	Hematology	E17–E18	Effect of brain weight, lower brain iron	[66]
	ID in offspring	Brain iron	Adult	Correction of cerebral ID with parenteral iron	[67]
Guinea Pig	Gestational ID + Lactational ID	ABR	P9–P24	Effect of ABR in ID Part restoration with PUFA treatment	[68]
	Gestational ID + Lactational ID	ABR	P24	Effect of ABR in ID	[69,70]
Domestic Pig	Gestational ID + Lactational ID	Cognitive tasks	0–4 weeks after birth	No cognitive deficits	[71]
	ID in offspring	MRI	0–6 weeks after birth	Cerebral ID, alteration in brain tissue composition persists in spite of iron repletion	[72]
	Lactational ID	RNA analysis	4 weeks after birth	Change in hippocampal DNA methylation and gene regulation	[73]
	Gestational ID	MRI, IHC	PD 2–30	ID after PD 14 detriments white matter	[74]
Monkey	ID in offspring	1H NMR	Infancy	Change in metabolomic profile in CSF	[75]
	ID in offspring	Proteomic	Infancy	Persistent change in proteomic profile in CSF	[76]
	ID in offspring	1H NMR	Infancy	Metabolomic profile in CSF predicts effects of ID on brain iron metabolism	[77]
	ID in offspring	Cognitive tasks	Infancy	Only initial cognitive + behavioral deficits	[78]
	Gestational ID + Lactational ID	Cognitive tasks	Infancy	Cognitive and emotional effects present, but vary with protocol	[79]

The studies pertained on the developing rodent brain all point towards a deleterious effect of dietary iron deficiency subjected to the mother during pregnancy (Table 1). The effects range from observations based on a direct comparison with normal fed mothers to reports on permanent effects on the brain of the offspring in spite of iron being admitted even early after birth. The effects on the developing rat brain concern structural, biochemical, and behavioral impairments (Table 1). Structurally, influences include structural defects in general brain development [48], and more specifically, the development of dendritic length and arborization, and effects on the formation of synapses [44,53,57,58]. A particular focus in many studies has been the effects of changes in the expression level of genes related to the functioning of synaptic transmission [55,59], vascularization [55], and hormones improving metabolism [61]. Studies have also reported on defects in the synthesis of monoaminergic neurotransmitters [46,47,57], and growth factors [48]. Additionally, studies have reported on behavioral disturbances [44,49,51,52]. A single study has reported on the impaired development of glial cells [65] and the impaired formation of myelin has also been reported [44,63,65], suggesting that earlier studies demonstrating that changes in the profiles of fatty acids in phospholipids are present in iron deficiency relate back to the functioning of the developing oligodendrocytes [11–13,40].

The significance of dietary iron deficiency on the developing mouse has gained less attention than that of the rat. Iron deficiency negatively affects the brain weight, iron content, and formation of oligodendrocytes and their myelination [66,80]. As previously mentioned, genetic depletion

of the transferrin receptor in the mouse results in severe fetal effects and impaired neurotransmitter formation [15,16]. In the guinea pig, a series of studies have been made on neural transmission in the brain stem, and reportedly deleterious effects of iron deficiency were partly restored by dietary supplementation with polyunsaturated fatty acids, indicating a beneficial effect on otherwise impaired myelination [72–74].

Most studies of larger animals have been conducted in postnatal animals, which, for practical reasons, make this approach durable [8]. Studies of gestational iron deficiency performed in the domestic pig report on impaired myelination, but without cognitive effects [72]. Impaired myelination was also reported in piglets, who were only subjected to postnatal iron deficiency [75]. Interestingly, epigenetic regulation is also affected when iron deficiency is present in the piglet brain [74]. Another intriguing study using MRI reports on permanent changes in the brain of the domestic pig in spite of the reversal of brain iron with dietary treatment [73].

In terms of the non-human primate brain, a single study has reported on cognitive effects following gestational iron deficiency, but the effects were not consistent and were largely dependent on the induced dietary regimen [79]. Studies on iron deficiency induced in the offspring have demonstrated that this led to significant changes in nuclear magnetic resonance (NMR)-detectable metabolites and proteomic profiles in CSF, clearly hinting towards impaired cerebral metabolism [76,77]. Another study has concluded that iron deficiency subjected to the offspring led to behavioral deficits that were compensable, suggesting that the effects of iron deficiency were less deteriorating [78].

1.7. The Developing Human Brain

In humans, the brain forms very early, and maternal iron deficiency is likely to impair the developing brain during the entire period of pregnancy. Compiling the studies reporting the negative impact of iron deficiency on the formation of the developing brain in experimental animals, the following factors stand out as being particularly important: The timing and the severity of the iron deficiency regimens. These factors are also very important to keep in mind when considering the impact of iron deficiency or iron deficiency with anaemia in the human brain, as they are likely to be the most determinant concerning whether damage is at risk of being irreversible [81,82]. The effects of iron deficiency on brain development were suggested to include the genesis of dendrites and synapses, hence clearly addressing the effects of iron deficiency on differentiation during formation of the human brain, and specifically suggesting an impact on particular brain regions such as the cerebral cortex (i.e., frontal cortex, prefrontal-striatal network, auditory cortex), hippocampus, and striatum [3,82]. Prior studies were clearly limited in access to measurements on brain functioning in real-time and merely relied on correlations between the iron status measured in blood and putative changes in behavior. Infants with low cord-blood s-ferritin and haemoglobin were prone to negative emotions, and they were less alert and difficult to sooth, and in a 5-year follow-up, the children had poorer behaviour and development outcomes, trouble with auditory language skills, and fine motor skills [82–84]. A single trial showed that maternal anaemia in pregnancy could be linked to 14% of cases of mental retardation at a 7-year follow-up. It should, however, also be kept in mind that iron deficiency in humans is not likely to be as extreme as can be instituted in experimental animals, and this should indeed be kept in mind when translating data from animal models to hypotheses in human physiology. A valid indicator of the severity in humans is seen when iron deficiency is complicated with anemia. In this situation, the iron transport to the fetus will be prioritized over the maternal iron need unless a certain threshold (ferritin $\approx 14 \mu\text{g/L}$) is met [8,83]. Furthermore, in severe cases of iron deficiency with anemia, fetal erythropoiesis is more highly prioritized than neurodevelopment [33,58].

The data obtained from several studies in humans all congregate towards the conclusion that there are significant effects on brain development, both pre-and postnatally (Table 2). Compared to the more extreme situations that invariably relate to the experimental animals, the impairment in iron statuses is not so dramatic in the human brain, and hence also the reported results [83–106]. The studies of humans have mainly involved structural analyses on brain growth in real-time [83–89], neurophysiological measurements

of basic functions related to myelination and cranial nerve development [90–96], and neuropsychological tests of cognition, memory, and personal traits [97–106].

Table 2. Studies of humans showing cerebral effects of iron deficiency (ID) or iron deficiency with anemia (IDA) subjected to pregnant females or their offspring. References are listed after specific topics. Abbreviations: ABR, auditory brainstem responses; LBW, low birth weight; No. F/O, numbers of patients (females/offspring); PND, postnatal day; VEP, visually evoked potentials.

Study Objective	Evidence of ID	Infant Age	No. F/O	Conclusion	Reference
Fetal brain development					
Normal development	Maternal IDA	PND 3–5 days	/70	Maternal IDA adversely affects 1 hippocampal morphogenesis and fetal production of BDNF	[83]
Normal development	Maternal IDA	18 months	331/	Maternal ID at 34 weeks associated with lower motor scores	[84]
Normal development	Normal iron status	7–11 years	/39	MRI iron content in basal ganglia influences spatial intelligence	[85]
Brain connectivity	Infant IDA	Mean 21.5 years	/31	Different patterns of functional connectivity between former IDA and control young adults	[86]
Risk of schizophrenia	Maternal IDA	Prospective study	/6872	Maternal ID as risk factor for schizophrenia in offspring	[87]
Cerebral functions	IDA in adults	Adult	/2957	IDA associated with increases in psychiatric disorders	[88]
Autism	Infant IDA	2–7 years	/102	No evidence between IDA and autism	[89]
Basic cerebral functions					
ABR	LBW	PND 42–6 months	/285	Iron supplements did not improve ABR, but ABR was discarded as measure of impairment in ID	[90]
ABR	Maternal IDA	PND 2, 3 months		ABR closely related to severity of maternal and neonatal iron status	[91]
ABR	Infant IDA	6–24 months		Prolonged latencies in ABR traces in IDA	[92]
ABR	Infant IDA	<48 h	/90	Latent iron deficiency associated with abnormal ABR	[93]
VEP	Infant IDA	6–24 months	/50	Negative correlation between severity of IDA and VEP latencies	[94]
Eye-blinking rates	Infant IDA	9–10 months	61	Increased eye-blink rates consistent with low dopamine function in IDA	[95]
EEG recordings	Infant IDA	0, 9 months	/80	ID associated with EEG asymmetry	[96]
Memory					
Memory	Infant IDA	8–10 years	/201	Iron supplementation substantially restores cognitive capabilities	[97]
Execution, memory	Infant IDA	19 years	/114	Chronic impairment of functions related to frontostriatal-connections (executive functions), and hippocampus (recognition memory)	[98]
Recognition memory	Infant IDA	6–18 months	/209	Sustained effects on memory in 10-year follow-up in spite of oral supplement in early life	[99]
Higher cerebral functions					
Social-emotional behavior	Infant IDA	9–10 years	/77	Social-emotional behavior associated with ID	[100]
Behavior	Normal	6–8 years	/264	Fe supplementation in pregnancy without consistent effect on behavior	[101]
Cognition	Infant IDA	1–3 years	/3	Improvement in cognition once iron stores were restored	[102]
Cognition	Infant IDA	Mean age 12.0		Reduced cognitive performance	[103]
Cognition	Infant IDA	12 months	828/828	No effect of IDA on cognition or motor development	[104]
ADHD symptomology, IQ	Infant IDA	2.5–5 years	/123	Effects of early deprivation and ID on ADHD symptoms and IQ years after adoption	[105]
ADHD symptomology	Infant IDA	Mean age 11.0		IDA associated with ADHD	[106]

Concerning the studies on the development of the brain that took morphological approaches, reports indicate that brain volumes, neurogenesis, and iron content are reduced [83–85]. This also led to permanent defects in neuronal connectivity years after developmental iron deficiency was recovered [91], which was further associated with a higher risk for psychiatric disorders [87,88], but not for causing autism [89].

Influences of impaired sensory function and neuronal development were also attributed to developmental iron deficiency using auditory brainstem responses (ABR) [90–93]. However, at least one study brought the reliability of this measure into question [90] and hinted that changes in ABR can have other explanation. Other studies taking a neurophysiological approach showed impairment in the visual input and general brain activity [94–96], suggesting that, e.g., dopaminergic neurotransmission is affected [95].

Examining higher functional tasks, the impact on structures and their function in the forebrain have been reported in many studies of the hippocampus (various memory tasks) [97–99] and cerebral cortex (social behavior, cognition, association with ADHD) [100,102,103,105,106]. Conversely, behavior was reportedly not affected in two other studies [101,102], which suggest that effects on the developing brain could be subtle unless dramatic maternal iron deficiency occurs.

2. Conclusions

The human brain develops throughout the gestational period, ranging from the formation and proliferation of neuroprogenitor cells, to their later migration, and later differentiation into fully developed neurons and glial cells. Severe iron deficiency can negatively impact cell division, neurotic outgrowth and formation of the neuronal network, and myelination in glial cells. Experimental studies in animals, especially the laboratory rat, clearly support that these cellular events can be impacted by developmental iron deficiency. In the human brain, where events in the third semester are reflected in the initial postnatal weeks, reports also point towards the negative impact of iron deficiency during development. The quality of the identified studies reported here, including the number of involved subjects, appears valid, but some limitations subtract the possibilities for overall conclusions. The translational value of the result of the experimental animal is high, but more data obtained in higher animals with a longer gestation than the rodent brain would be appreciated. Concerning the human data, a certain shortage in the number of available studies prevails and more studies monitoring the cerebral function postnatally are needed. With respect to the validity of the results, it must also be emphasized that publication bias may exist towards the demonstration of effects of iron deficiency on brain development. This would leave out negative results that may remain unpublished, and this may play an important role as scientific results on the developing human brain are rather scarce. Investigations on the brain in the gestational period are obviously very complicated, so research on biomarkers from the umbilical cord or chorion villus biopsy would be highly appreciated.

In terms of the prevention of iron deficiency, strategies have not yet been developed to specifically address the developing brain. Supplementation with oral or parenteral iron is possible in pregnancy and postnatally [2], and strategies to halt iron deficiency anemia will likely also improve cerebral iron deficiency as the brain is able to extract iron from the blood due to the expression of transferrin receptors on brain capillaries [39]. Parenteral iron supplementation is being assayed in pregnant women and women with post-partum hemorrhage to generally improve their iron status [2,4,107,108], and this will likely also improve the cerebral iron status.

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Abbreviations

BBB	blood–brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid

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Article

Iron Release from Soybean Seed Ferritin Induced by Cinnamic Acid Derivatives

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Abstract: Plant ferritin represents a novel class of iron supplement, which widely co-exists with phenolic acids in a plant diet. However, there are few reports on the effect of these phenolic acids on function of ferritin. In this study, we demonstrated that cinnamic acid derivatives, as widely occurring phenolic acids, can induce iron release from holo soybean seed ferritin (SSF) in a structure-dependent manner. The ability of the iron release from SSF by five cinnamic acids follows the sequence of Cinnamic acid > Chlorogenic acid > Ferulic acid > *p*-Coumaric acid > *Trans*-Cinnamic acid. Fluorescence titration in conjunction with dialysis results showed that all of these five compounds have a similar, weak ability to bind with protein, suggesting that their protein-binding ability is not related to their iron release activity. In contrast, both Fe²⁺-chelating activity and reducibility of these cinnamic acid derivatives are in good agreement with their ability to induce iron release from ferritin. These studies indicate that cinnamic acid and its derivatives could have a negative effect on iron stability of holo soybean seed ferritin in diet, and the Fe²⁺-chelating activity and reducibility of cinnamic acid and its derivatives have strong relations to the iron release of soybean seed ferritin.

Keywords: cinnamic acid derivatives; soybean seed ferritin; iron release; binding ability; Fe²⁺-chelating activity; reducibility

1. Introduction

Iron plays an essential role in living organisms, such as oxygen transfer, DNA synthesis, electron transport, and tricarboxylic acid and nitrogen fixation. Actually, iron deficiency is one of the most serious global nutritional problems, which affects about two billion people in the world [1]. Although the prevalence of iron deficiency anemia (IDA) is higher in developing countries, iron deficiency is still common among women and young children in industrial countries. More importantly, IDA can cause a series of consequences, such as reduced cognitive, motor development in infants and poor pregnancy outcomes. Ferritin is a natural and ubiquitous iron storage protein occurring widely in plants, animals, bacteria and fungi [2]. It consists of 24 subunits that assemble in a highly symmetric manner to a hollow protein shell with the outside diameter of about 12–13 nm and inner diameter of about 7–8 nm [3]. One ferritin molecule contains six four-fold, eight three-fold, and twelve two-fold channels (Figure 1a), which connect the inner cavity to bulk solution and serve as multiple pathways for iron entry and exit. Ferritin can store up to 4500 Fe³⁺ in its cavity, and therefore, natural ferritin, especially plant ferritin from legume seeds, has been considered as a novel alternative dietary iron supplement against IDA [1]. As compared to other iron supplements with a small size, ferritin has three major advantages: the protection of protein shell from interaction with other dietary factors, the safer form of iron stored as ferric cores rather than ferrous iron, and the possible intact absorption by receptor-mediated endocytosis [1].

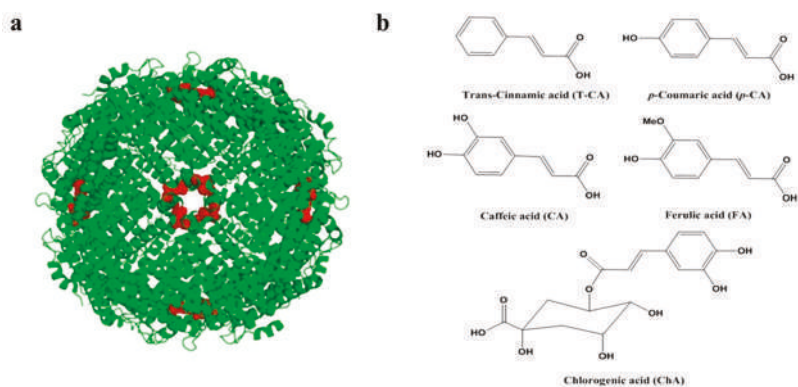


Figure 1. (a) Crystal structure of plant ferritin with views down the four-fold axes (channels) of the protein shell. The tryptophan residue of each subunit is highlighted in red; (b) Chemical structure of cinnamic acid derivatives: *Trans*-cinnamic acid (T-CA); *p*-Coumaric acid (*p*-CA); Caffeic acid (CA); Ferulic acid (FA); Chlorogenic acid (ChA).

So far, Fe²⁺ oxidation and mineral deposition in ferritin have been studied intensively and extensively [1,4]. Although some reductants such as ascorbate, 6-hydroxydopamine, 5-aminolevulinic acid, superoxide anion radical, 1,2,4-benzenetriol, benzene metabolites, NADH and anthocyanins have been shown to induce iron release from ferritin [5,6], and several iron chelators including 2, 2'-bipyridine, salicylate, citrate, nitritotriacetate, and desferroxamine B can also induce iron release from ferritin at a slower rate [7–9]. However, there has been less attention paid to the iron release of ferritin induced by nutrients and the stability of ferritin iron core in food systems.

Phenolic acids are secondary metabolites, which are widely distributed in various plant foodstuffs. Among these phenolic acids, cinnamic acid derivatives (Figure 1b), such as caffeic, chlorogenic, *p*-coumaric and ferulic acids, are reported to possess functional activities like cancer prevention, antituberculosis, antileukaemic, hepatoprotective, antidiabetic, antioxidative, and hypocholesterolemic activities [10–12]. However, to date, whether these phenolic acids have an effect on the function of protein through intermolecular interaction has received much less attention. Based on the fact that phenolic acids such as cinnamic acid derivatives and ferritin co-exist in plant foodstuffs, and that cinnamic acid derivatives have a strong reducing activity, while ferric iron within phytoferritin exhibits a relatively strong oxidative activity, it is of special interest to know if the two kinds of molecules could interact with each other, and if so, what is the consequence of such interaction, which is the focus of this work.

2. Results and Discussion

2.1. Isolation and Characterization of SSF and rH-2

Ferritin can stay in two forms, holo and apo ferritin. Naturally occurring ferritin is a holo form which contains hydrous ferric oxide nanoparticles as iron cores within its cavity. Holo plant ferritin usually consists of two H type subunits of 26.5 (H-1) and 28.0 kDa (H-2), while their ratios vary among species. In this study, natural holo soybean seed ferritin (SSF) was used for iron release experiments because it is most extensively studied among all known plant ferritins [1,13]. However, our recent studies have showed that SSF is unstable during storage, because the extension peptide (EP) of the H-1 subunit exhibits significant serine protease-like activity, which is located at the N-terminal extremity. Consequently, recombinant soybean seed H-1 ferritin (rH-1) is prone to degradation, whereas its analogue, recombinant soybean seed H-1 ferritin (rH-2), becomes very stable under

identical conditions [14]. Therefore, apo rH-2 was used to study the interaction between cinnamic acid derivatives and protein.

After purification, these two kinds of ferritins were analyzed by PAGE and TEM. Nondenaturing gel electrophoresis (native PAGE) revealed the purified SSF and rH-2 as a single complex (Figure 2a), suggesting that they have been purified to homogeneity. SDS-PAGE indicated that SSF consists of nearly 28.0 and 26.5 kD subunits, a result consistent with previous observations [15]. As expected, rH-2 was composed of only one 28.0 kD subunit (Figure 2b). TEM analyses revealed that rH-2 molecules were well dispersed with an outside diameter of ~12 nm (Figure 2c) after being negatively stained with uranyl acetate. In contrast, without negative staining, iron cores can be clearly observed in holo SSF (Figure 2d) as isolates contains ~1800 g atom of iron [1].

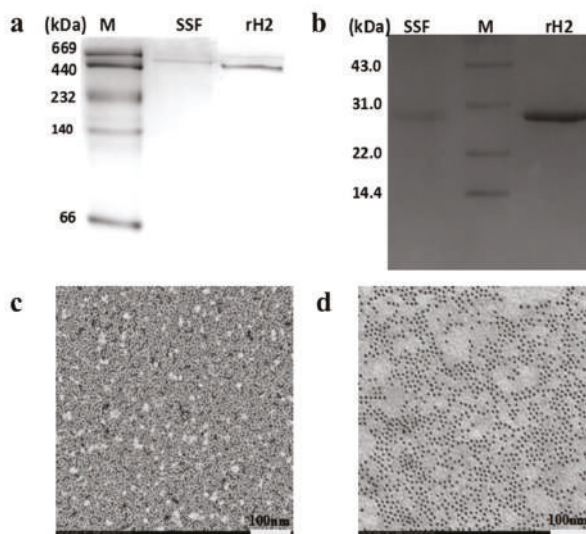


Figure 2. (a) Native PAGE analyses and (b) SDS-PAGE of soybean seed ferritin (SSF) and recombinant H2 ferritin (rH-2); (c) TEM picture of purified rH-2 after being negatively stained by 2% uranyl acetate; (d) TEM picture of holo soybean seed ferritin (SSF) without negative staining of uranyl acetate.

2.2. Iron Release from SSF Induced by Cinnamic Acid Derivatives

Subsequently, we analyzed the possibility of all cinnamic acid derivatives to induce iron release from SSF at a concentration of 25 μ M [5], and results are displayed in Figure 3. It was found that all of them not only were able to cause iron release from protein shell, but also such iron release showed a structure-dependent manner. For example, CA induces iron release from SSF at the initial rate of 0.104 ± 0.006 mM/min, which is fastest among all tested compounds. The rate of iron release follows the sequence CA > ChA > FA > *p*-CA > T-CA in Mops buffer (pH 7.0) at 25 °C. CA has the higher ability to induce iron release from ferritin as compared to its analogue, ChA (0.092 ± 0.021) mM/min. This might be derived from the fact that ChA contains a sinapic acid group in its structure, thereby its large size prevents it from diffusion into the protein shell to some extent. These results suggest that the rate of iron release is inversely proportional to the size of phenolic acids, which is in agreement with a previous report [16]. Thus, it seems that the molecular size of phenolic acids plays an important role during the iron release from SSF induced by reductants.

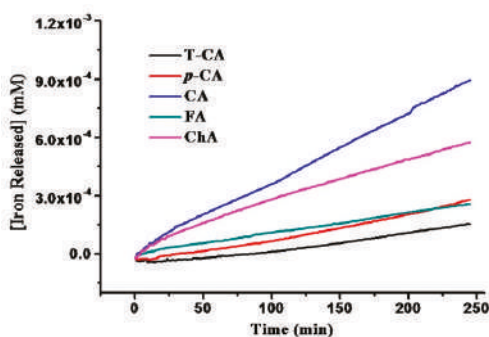


Figure 3. Kinetics of iron release from holo SSF induced by cinnamic acid derivatives. Iron release from SSF in the presence of reductants was followed by measuring the increase in absorbance at 562 nm about 250 min due to the chelation of Fe^{2+} by ferrozine. Conditions: 0.15 μM SSF, 25 μM cinnamic acids, 50 mM Mops, pH 7.0, 0.15 M NaCl, 500 μM ferrozine, 25 $^{\circ}\text{C}$.

Besides, it was observed that the number of HO groups of cinnamic acid derivatives has a marked effect on the rate of iron release from ferritin. For example, with an increase in the number of HO from 0, 1, to 2, the rate of the iron release from ferritin increased from 0.002 ± 0.001 mM/min for T-CA, 0.014 ± 0.004 mM/min for *p*-CA, to 0.104 ± 0.006 mM/min for CA, respectively, suggesting that the number of HO group in cinnamic acid derivatives is closely associated with their ability to induce iron release from ferritin. Consistent with this idea, the rate of iron release from ferritin induced by CA was greatly decreased by ~50% after one HO group was replaced by OMe in FA (0.046 ± 0.011 mM/min) as shown in Figure 3.

2.3. Fluorescence Quenching Analyses

The above results demonstrated that all of these cinnamic acid derivatives are able to facilitate iron release from ferritin to different extents. To shed light on the mechanism by which iron release from ferritin was induced by these phenolic acids, we investigated the binding activity of these small molecules to ferritin through their interaction with protein. Protein intrinsic fluorescence mainly from Trp residues has been widely used to study their interaction with small molecules because of its sensitivity to microenvironment surrounding the fluorophore residue. As shown in Figure 1a, there are 24 Trp residues in one soybean seed ferritin molecule [17]. By taking advantage of this, we used intrinsic emission spectroscopy to study the interaction between these small molecules and apo rH-2, and results were shown in Figure 4. It was observed that all of cinnamic acid and its derivatives were able to quench the protein fluorescence, showing similar fluorescence quenching curves, indicative of similar interaction between cinnamic acid derivatives and apo rH-2. Thus, it seems that their interaction with apoferritin is independent of chemical structure, inconsistent with the above iron release results.

To determine whether these cinnamic acid derivatives can interact with proteins through binding, dialysis experiments were carried out wherein apo rH-2 (0.55 μM) was firstly incubated with these phenolic acids (22 μM), followed by dialysis against buffer for four times to remove free small molecules, respectively, and results are given in Figure 5. It was found that after dialysis for 5 h, the fluorescence of apo rH-2 could return to an original state upon treatment with these phenolic compounds, respectively (Figure 5). These results indicated that the fluorescence quenching of apo rH-2 caused by these compounds is dynamic, resulting from collisional encounters between the fluorophore and quencher. Instead, it is difficult to form a complex between ferritin and each of these cinnamic acid derivatives under the present experimental conditions; at most, the binding of the small

compounds to apo rH-2 is very weak, and therefore, dialysis can remove them from their mixture with apo rH-2.

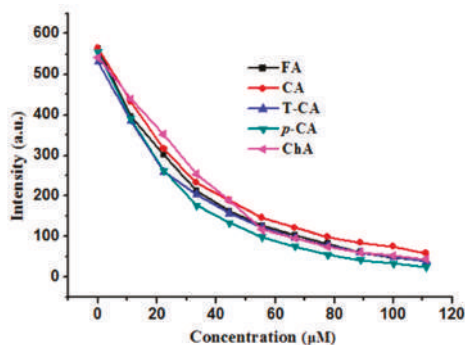


Figure 4. Comparison of cinnamic acids on the fluorescence quenching of apo rH-2. Conditions: 0.55 μM rH-2 in 50 mM Tris-HCl, [phenolic acids] = 0–110 μM , pH 7.0, 25 $^{\circ}\text{C}$. λ_{Ex} = 280 nm, slits for excitation and emission are 5 nm and 10 nm.

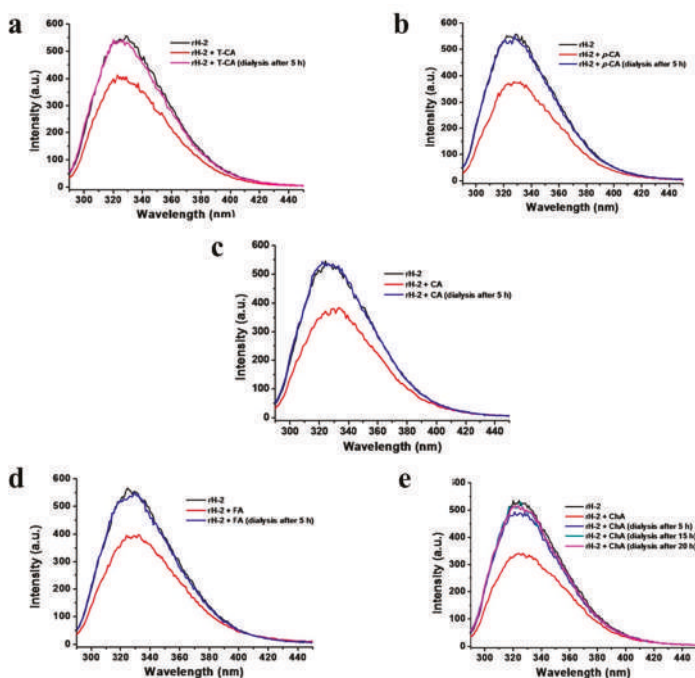


Figure 5. Dialysis study of rH-2 with cinnamic acids at pH 7.0. (a) *Trans*-Cinnamic acid; (b) *p*-Coumaric acid; (c) Caffeic acid; (d) Ferulic acid; (e) chlorogenic acid. Conditions: 0.55 μM rH-2 in 50 mM Tris-HCl, [cinnamic acids] = 22 μM , 25 $^{\circ}\text{C}$. λ_{Ex} = 280 nm, slits for excitation and emission are 5 nm and 10 nm.

The weak binding activity of the cinnamic acid derivatives to ferritin might be derived from the fact that they contain less than three hydroxyl groups in the structure. Consistent with this view, our recent study showed that gallic acid, methyl gallate and propyl gallate having three HO groups

can bind to rH-2 tightly, while their analogues with two HO groups cannot [18]. Thus, the number of the hydroxyl groups is closely associated with the interaction mode between phenolic acids and protein, and more hydroxyl groups in the structure favors the binding of phenolic acids to protein. Further support for this idea comes from a recent study showing that tannic acid with many hydroxyl groups can facilitate ferritin association through strong hydrogen bonds [19].

2.4. Effect of the Fe^{2+} Chelating Activity of Cinnamic Acid Derivatives on the Iron Release from Ferritin

It was previously reported that the chelating activity of reductants on iron ion was an important factor which is related to iron release from the ferritin shell [20]. Therefore, in this study, the iron chelating activity of cinnamic acid derivatives was determined, respectively, to gain insight into the mechanism of iron release from ferritin. As shown in Figure 6a, the chelating activity of cinnamic acid derivatives on ferrous ions follows the sequence of CA > ChA > FA > T-CA \approx *p*-CA. The chelating activity of CA and ChA with catechol moiety was ~63% and 58%, respectively, which was almost six-fold stronger than their analogue, FA (~11%) where one hydroxyl group was replaced with OMe. The large difference in the chelating activity between CA/ChA and FA suggested that two hydroxyl groups in the benzene ring of CA and ChA were required for Fe^{2+} chelating, while one hydroxyl group in the benzene ring of FA is not enough to chelate iron. Agreeing with this idea, both T-CA (~7.0%) and *p*-CA (~6.5%) with zero or one hydroxyl group exhibited even much weaker chelating activity than CA or ChA. Additionally, it was found that CA and ChA exhibited nearly the same chelating activity, suggesting that the carboxyl group might not be involved in iron chelating, and at most it contributed much less to iron chelating as compared to the hydroxyl group.

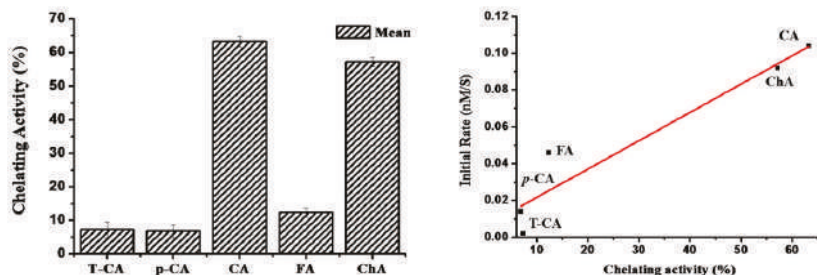


Figure 6. Chelating activity of cinnamic acid derivatives on ferrous ion. Vertical bars represent the standard error from means of three separate tests.

More importantly, it was observed that the chelating activity of these phenolic acids was in good agreement with the above iron release results (Figure 3); namely, the stronger the chelating activity, the faster rate of the iron release from ferritin. Consistent with this conclusion, there is a linear relationship between the rate of iron release from ferritin induced by these phenolic acids and their chelating activity as shown in Figure 6b. Based on these findings, it is reasonable to believe that the chelating activity of cinnamic acid derivatives has an important effect on the rate of iron release from ferritin induced by these compounds. This might be because these cinnamic acid derivatives can act as iron chelators to help iron to move out of SSF.

2.5. Effect of Reducibility of Cinnamic Acid Derivatives on the Iron Release from Ferritin

To better understand the mechanism of iron release from ferritin, we also studied the voltammetric oxidation of these phenolic acids in pH 7.0 phosphate buffer by cyclic voltammetry, and results are shown in Figure 7a. Cyclic voltammograms of CA, FA, *p*-CA and ChA at a sweep rate of 100 $mM s^{-1}$ exhibited one anodic peak and one cathodic peak, suggesting that the oxidation process for these four

investigated compounds was reversible. However, cyclic voltammograms of T-CA showed no peak, indicating that T-CA has no reducibility [21]. As shown Table 1, ChA (0.31 V) has the largest anodic peak potential among all of the five compounds.

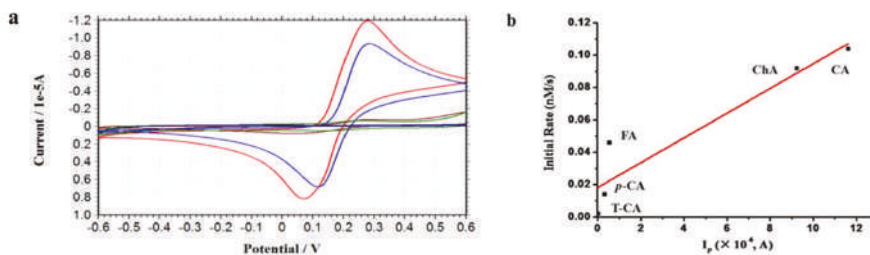


Figure 7. Cyclic voltammograms taken with a 3-mm glassy carbon electrode at 100 mVs^{-1} of: 0.5 mM cinnamic acid (mazarine line), *p*-coumaric acid (green line), caffeic acid (red line), ferulic acid (carmine red), and chlorogenic acid (blue red) in 50 mM phosphate buffer of pH 7.0.

Table 1. Evaluation of the anodic peak potentials of the studied cinnamic acid derivatives (E_p —anodic peak potential (V)) at pH 7.0, as well as the anodic peak currents at pH 7.0 (I_p —anodic peak current (A)) during the cyclic voltammetry experiment.

Phenolic Compound	E_p	$I_p (\times 10^{-6})$
T-CA	0	0
<i>p</i> -CA	0.27	0.31 ± 0.12
CA	0.28	11.61 ± 0.35
FA	0.24	0.53 ± 0.07
ChA	0.31	9.23 ± 0.46

Interestingly, the oxidation peak current of the cinnamic acid derivatives follows the sequence of $\text{CA} > \text{ChA} > \text{FA} > \text{p-CA} > \text{T-CA}$ (Table 1). This trend is in good agreement with the initial rate of iron release from SSF induced by these compounds. For reversible systems, the oxidation peak current, I_p could represent the reducibility of the analyte in the solution bulk [22]. Caffeic acid and chlorogenic acid with two hydroxyl groups in their benzene ring structure show the stronger oxidation peak current as compared with chlorogenic acid. In contrast, FA and *p*-CA with only one hydroxyl group have weaker oxidation peak current, while T-CA with no HO group has no reducibility. These results indicated that the reducibility of cinnamic acids can greatly influence the rate of iron release from SSF. Indeed, there is also a linear relationship between the oxidation peak current of the cinnamic acid derivatives and their ability to induce iron release from ferritin as shown in Figure 7b. Therefore, the reducibility of phenolic acids also is another important factor affecting their ability to induce iron release from the ferritin shell.

3. Materials and Methods

3.1. Chemicals

Five cinnamic acid derivatives used in this study were purchased from J&K Chemical (Beijing, China). Ferrous ion chelator 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) was obtained from Sigma-Aldrich Chemical Co. (Beijing, China). Sephacryl S-300, DEAE Sepharose Fast Flow, native electrophoresis marker, and SDS electrophoresis marker were purchased from GE Healthcare Bio-Sciences AB (Beijing, China). Sodium citrate, terephthalic acid (TA) and magnesium chloride hexahydrate were obtained from Beijing Chemical Reagents Co. (Beijing, China). All other reagents used were of analytical grade or purer.

3.2. Preparation of Soybean Seed Ferritin (SSF) and Recombinant Soybean Seed H-2 Ferritin (rH-2)

SSF and apo rH-2 were purified as previously described with some modification [23,24]. Typically, approximately 1 kg of soybean seeds was soaked in distilled water for 8 h and mixed with three volumes of extraction buffer (50 mM KH_2PO_4 , pH 7.5, 1% polyvinylpolypyrrolidone). After filtration, the filtrate was incubated for 15 min at 60 °C and then was centrifuged at $5000\times g$ for 5 min to separate the insoluble material. The supernatant was adjusted to 0.5 M MgCl_2 and the mixture stood for 30 min at 4 °C followed by addition of sodium citrate (final concentration of 0.7 M) to complex the magnesium. After 10 h, the resultant supernatant was centrifuged at $12,000\times g$ for 20 min at 4 °C. The brown precipitate thus obtained was dissolved in 50 mM $\text{KH}_2\text{PO}_4\bullet\text{Na}_2\text{HPO}_4$ buffer (pH 7.5) and was dialyzed against the same buffer three times.

Escherichia coli strain BL21 (DE3) which contained rH-2 expression plasmids was grown at 37 °C. Protein expression was induced with 1 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) for 7 h. The system was centrifuged and re-suspended in buffer solution (50 mM Tris-HCl, pH 8.0), followed by disruption by sonication. The supernatant of the crude extract was collected by centrifugation and further purified by ammonium sulfate fractionation (40% saturated fraction). After washed by distilled water to remove other proteins, the sediment was dissolved by solution buffer. Next the solution was dialyzed against the same buffer three times to remove the ammonium sulfate. SSF and rH-2 protein were further purified by Sephacryl S-300 gel filtration chromatography (GFC) and DEAE-Sephacryl Fast Flow column, respectively. Finally, protein purity was analyzed by SDS-PAGE and Native-PAGE analysis. Protein concentrations were determined according to the Lowry method as previous reported [25].

3.3. Kinetic Measurement of Iron Release from Holo Soybean Seed Ferritin

The assay system (1 mL of total volume) contained 0.21 μM ferritin, 500 μM ferrozine, 0.15 M NaCl, and 25 μM cinnamic acid derivatives in 50 mM Mops buffer, pH 7.0 as previously described [5]. The same solution but just using buffer instead of cinnamic acid derivatives was used as control. Experiments were conducted at 25 °C. The iron release was measured with the development of $[\text{Fe}(\text{ferrozine})_3]^{2+}$ at 562 nm using a Varian Cary 50 spectrophotometer, and $\epsilon_{562} = 27.9 \text{ mM}^{-1}\text{cm}^{-1}$. The absorbance at 562 nm from the control was deducted from that of all sample mixtures. The initial rate (v_0) of iron release measured as $[\text{Fe}(\text{ferrozine})_3]^{2+}$ formation was obtained from the linear A_1 term of third-order polynomial fitted to the experimental data as reported previously: $Y = A_0 + A_1t + A_2t^2 + A_3t^3$ and $dY/dt = A_1 + 2A_2t + 3A_3t^2$ (at $t = 0$, $(dY/dt)_0 = v_0$). Here t is the time in minutes and Y is the concentration of $[\text{Fe}(\text{ferrozine})_3]^{2+}$ at time t in minutes [16].

3.4. Fluorescence Titration Analysis

Fluorescence titration measurements were recorded on a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA), using quartz cuvettes of 1 cm path length at room temperature. In this measurement, apo rH-2 (0.55 μM , 150 mM NaCl and 50 mM Tris-HCl, pH 7.0) was titrated with 1 μL cinnamic acid derivatives (5 mM, 50 mM Tris-HCl, pH 7.0), respectively. The excitation wavelength was 280 nm, and the emission wavelength was 325 nm. Furthermore, dialysis measurements were conducted to analyze the binding affinity of cinnamic acids to apo rH-2. Typically, a mixture of apo rH-2 (0.55 μM) plus cinnamic acid derivatives (22 μM) was dialyzed (100 kDa cutoff) for 5 h against the solution buffer (50 mM Tris-HCl and 150 mM NaCl, at pH 7.0) to detect this kind of interaction [18].

3.5. Chelating Activity of Cinnamic Acid Derivatives on Ferrous Ion

The chelating activity of cinnamic acid derivatives on ferrous ion was measured as described previously with some modifications [5]. The reaction system (1 mL) contained 20 μM FeSO_4 , 60 μM ferrozine, and 60 μM cinnamic acid derivatives in 50 mM Mops buffer (pH 7.0). After reaction for

5 min at 25 °C, the absorbance of system was detected at 562 nm spectrophotometrically. The ability of cinnamic acids to chelate Fe²⁺ was calculated as follows: chelating activity (%) = $\frac{A_0 - A}{A_0} \times 100$, where *A* and *A*₀ are the absorbance in the presence or absence of the cinnamic acid derivatives, respectively.

3.6. Cyclic Voltammetry

Cyclic voltammetry experiments were performed using a potentiostat (microAutolab Type III with an Autolab Faraday Cage) and voltammograms were obtained with a scan rate of 100 mV s⁻¹ with an increment potential of 2.4 mV, between -0.6 V and 0.6 V. The working electrode was a 3 mm glassy carbon disk in combination with a Metrohm tipholder, cleaned by polishing with 3 μm alumina powder during 30 s followed by fixing the potential in ultrasonication during 5 s, between acquisitions. A saturated calomel electrode was used as a reference electrode in conjunction with a platinum counter electrode. Each acquisition required 40 mL of sample. Oxygen was removed with a N₂ current flow during 5 min prior to analysis. Cyclic voltammetry experiments were controlled by the CHI600E Electrochemical Workstation. Cyclic voltammograms were taken in the absence and in the presence of cinnamic acid derivatives.

3.7. Statistical Analysis

All data analyses were performed using Origin 8.0 software and the structural formula was processed by ChemDraw 7.0. All experiments were carried out in triplicate.

4. Conclusions

The present study demonstrates that cinnamic acid derivatives, a class of naturally occurring phenolic acids, can induce iron release from holo soybean seed ferritin (SSF) in a structure-dependent manner for the first time. The ability of iron release from SSF by five cinnamic acids follows the sequence of CA > ChA > FA > *p*-CA > T-CA. Although the five phenolic acids exhibit a weak ability to bind with ferritin, they are able to induce iron release from holo ferritin through their Fe²⁺-chelating activity and reducibility. These studies indicate that cinnamic acid and its derivatives could have a negative effect on iron absorption in humans, because they can result in loss of a certain amount of iron from holo ferritin through interactions. Therefore, the interactions between cinnamic acid derivatives and holo SSF should be avoided as much as possible during food processing.

Author Contributions: G.Z. and X.S. conceived and designed the experiments; X.S. performed the experiments; X.S., H.C. and Z.J. analyzed the data; X.S. and H.C. wrote the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

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Review

The Functional Versatility of Transferrin Receptor 2 and Its Therapeutic Value

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Abstract: Iron homeostasis is a tightly regulated process in all living organisms because this metal is essential for cellular metabolism, but could be extremely toxic when present in excess. In mammals, there is a complex pathway devoted to iron regulation, whose key protein is hepcidin (Hepc), which is a powerful iron absorption inhibitor mainly produced by the liver. Transferrin receptor 2 (Tfr2) is one of the hepcidin regulators, and mutations in *TFR2* gene are responsible for type 3 hereditary hemochromatosis (HFE3), a genetically heterogeneous disease characterized by systemic iron overload. It has been recently pointed out that Hepc production and iron regulation could be exerted also in tissues other than liver, and that Tfr2 has an extrahepatic role in iron metabolism as well. This review summarizes all the most recent data on Tfr2 extrahepatic role, taking into account the putative distinct roles of the two main Tfr2 isoforms, Tfr2 α and Tfr2 β . Representing Hepc modulation an effective approach to correct iron balance impairment in common human diseases, and with Tfr2 being one of its regulators, it would be worthwhile to envisage Tfr2 as a therapeutic target.

Keywords: Tfr2; iron metabolism; hepcidin; erythropoiesis; SNC

1. Tfr2 Gene and Proteins

Tfr2 is a type II transmembrane glycoprotein, a member of the transferrin receptor family and homologous to Tfr1 [1].

It is encoded by *TFR2*, a 2471 bp long gene localized on the long arm of human chromosome 7 (7q22.1) that consists of 18 exons, and gives origin to two main variants regulated by different specific promoters: Tfr2 α and Tfr2 β (Figure 1).

Tfr2 α results from the transcription of all exons, and is prevalently and highly expressed in hepatocytes and erythroid cell lines. Tfr2 α cDNA is 2.3 kb long (AF067864), and the Tfr2 α is a protein of about 89 kDa encompassing 801 amino acids [2]. As Tfr1, Tfr2 α has a short cytoplasmic tail (aa 1–80) that contains a consensus sequence YQRV for endocytosis, a transmembrane domain (aa 81–104) with four cysteines (aa 89–98 and 108–111), involved in disulphide bonds, likely responsible for *TFR2* homodimerization, and a large extracellular domain (aa 105–801) comprising a protease-associated domain and two RGD motifs that bind di-ferric Tf (Fe2Tf). Furthermore, an N-terminal mitochondrial targeting sequence (MTS) has been found in Tfr2 intracellular domain [3]. In vitro analysis demonstrated that Tfr2 α on cell membranes can be shed and give origin to a soluble form, and that this process is inhibited by Fe2Tf [4]; however, this form could not be found in animal or human sera.

Tfr2 α transcription is upregulated in mouse embryonic fibroblast cells (NIH3T3) by erythroid GATA1, EKLF, and cEBP/ α transcriptional factors, while FOG1 seems to inhibit GATA1 enhancement [5]. Also, hepatic Hnf4 α stimulates Tfr2 α transcription, since it is significantly decreased

in liver-specific HNF4 α -null mice [6]. There is no Tfr2 α IRE/IRP-dependent post-transcriptional regulation [7], while the hepatic tetraspanin CD81 is able to interact with Tfr2 α and induce its degradation [8].

Tfr2 β has an in-frame transcription start site in exon 4, so the Tfr2 β cDNA (NM_001206855.1) transcript lacks exons 1–3, and presents 142 additional untranslated base pairs at its 5' end. Tfr2 β is ubiquitously expressed at low level, and mostly expressed in spleen, heart, and brain. The resulting protein lacks the cytoplasmic and the transmembrane domain [2]. Since no signal peptide involved in the secretory pathway could be evidenced in Tfr2 β isoform, it is supposed to be a cytosolic 60 kDa protein identical to the Tfr2 α extracellular domain. At the moment, no transcriptional/translational regulatory pathway is known for Tfr2 β isoform (Figure 1).

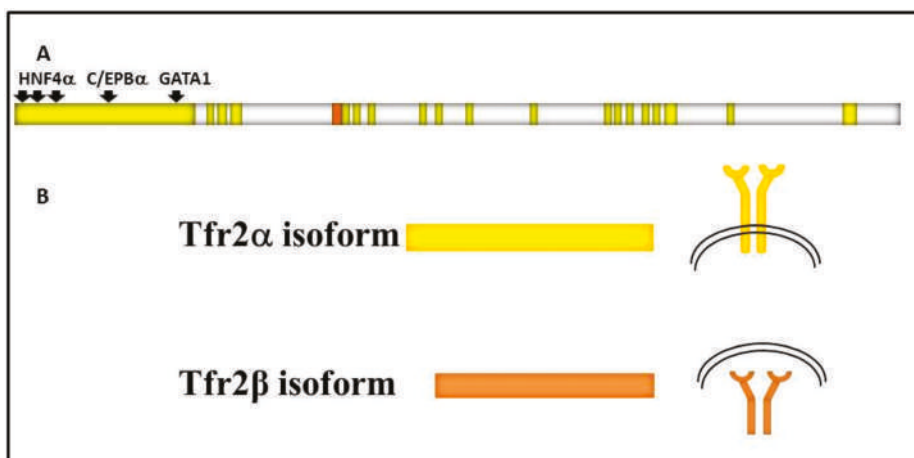


Figure 1. Schematic representation of: (A) *TFR2* gene structure. *TFR2* 18 exons are in bright yellow, *TFR2* α promoter is shown upstream of the gene, and transcriptional factors binding sites are highlighted by black arrows. 5' untranslated region (142 bp long) of *TFR2* β transcript is shown in orange; (B) the two main transcripts and of Tfr2 α and Tfr2 β isoforms, that are identical in the common sequence, and the two protein localizations, on the cell surface or in the cytosol, respectively.

1.1. *Tfr2* and *HFE3*

Inactivating mutations of *TFR2* gene (OMIM: 604720) lead to type 3 hereditary hemochromatosis (*TFR2*-HHC or *HFE3*), a rare recessive disorder characterized by increased transferrin saturation and serum ferritin concentration and iron overload [9].

HFE3 is one of the 5 different forms of hereditary hemochromatosis, a genetically heterogeneous disorder due to the deregulation of iron protein hepcidin (Hepc) [10] (Table 1). *TFR2*-HHC presents an earlier age of onset than type 1 hereditary hemochromatosis (*HFE*-HHC), and some pediatric patients have been reported so far. However, the majority of the affected individuals are young adults with abnormal serum iron indices [11].

Most of the mutations involved in *HFE3* pathogenesis cause an inactivation of both Tfr2 isoforms, but some of them, occurring in exons from 1 to 3, impair the production of the Tfr2 α isoform only [12–14]. Three patients with homozygote mutation M172K, that impairs Tfr2 β translation initiation codon, were identified, all presenting typical hemochromatosis symptoms (cirrhosis, hypogonadism, cardiomyopathy, arthritis) at an average age of 38 ± 5 years [13,15].

Unfortunately, few clinical data are available on patients with these mutations to allow an exhaustive genotype/phenotype analysis.

Table 1. Hereditary hemochromatosis (HHC types) and their relationship with hepcidin.

HH type	Acronym	Inheritance	Gene	Protein	Function
HFE1	HFE-HHC	AR	HFE	Hfe	Hepc regulator
HFE2a	HJV-HHC	AR	HJV	Hemojuvelin	Hepc regulator
HFE2b	HEPC-HHC	AR	HAMP	Hepc	Fe absorption inhibitor
HFE3	TFR2-HHC	AR	TFR2	Tfr2	Hepc regulator
HFE4	FPN1-HHC	AD	SLC40A1	Fpn1	Hepc receptor

1.2. Systemic Iron Metabolism: The Hepc-Fpn1 Axis and the Proteins Involved in Hepc Regulation

In mammals, the hepatocyte-secreted hormone Hepc regulates systemic iron homeostasis [16]. Hepc is codified by *HAMP* gene, which encodes for an 84 amino acids precursor protein, from which active 20–25 amino acids peptides are generated [17]. It is expressed primarily in the liver, although low levels of Hepc transcripts have been also reported in other organs [18].

How *HAMP* gene expression is regulated is mostly unknown. There are no IRE elements in its transcript, but the transcriptional factor CCAAT/enhancer binding protein- α is highly expressed in the liver, and seems to stimulate *HAMP* expression, while the hepatocyte nuclear factor 4- α (HNF-4) represses Hepc expression [19].

The molecular processes involved in hepatic Hepc regulation are quite complex. Basal Hepc expression is regulated through the bone morphogenetic protein 6 (Bmp6) and Smad protein signaling pathway. In iron excess condition, Bmp6, produced and secreted by liver sinusoidal endothelial cells (LSECs) [20], binds to bone morphogenetic protein receptors, ALK2 and/or ALK3 [21], activin receptor type 2A (Actr2a) [22], Hemojuvelin (Hjv) and Neogenin [23]. The protein complex activates signals transducers Smad1/5/8, leading to their interaction with the common mediator Smad4. As a consequence of this interplay, Smad4 translocates into the nucleus and promotes Hepc transcription [16].

More recently, it has been demonstrated that bone morphogenetic protein 2 (Bmp2), expressed in LSECs, can also trigger Hepc transcription increase [24].

A second Hepc regulatory pathway involves di-ferric Tf (Fe2Tf) as the signaling of increased iron availability, transferrin receptor 1 (Tfr1), hemochromatosis type 1 protein (Hfe), and transferrin receptor 2 (Tfr2). It has been demonstrated that Fe2Tf competes with Hfe for binding Tfr1 then, when circulating, Fe2Tf increases as a consequence of iron raising, Hfe dissociates from Tfr1 and binds Tfr2 [25]. Hfe/Tfr2 complex is then responsible for Hepc response to iron increase, through the activation of Erk1/2 and MAPK cascade that has been proposed to potentially converge on the Bmps/Smad1/5/8-mediated pathway [26].

The hierarchy of the two pathway activations, and their relationship, are still not completely defined. In vitro data support the hypothesis that the complex Hfe/Tfr2 interacts with membrane Hjv (mHjv) on cell surface, thereby, the link between the two signaling pathways occurs [27]. It has been found, in vivo, that both Hfe and Tfr2 knock-out (KO) mice present lower pErk1/2 [28] and pSmad1/5/8 proteins [29,30], meaning that these two proteins regulate both signal translation pathways. Hepatic Hepc upregulation is inhibited by matriptase 2 (MT-2 or Tmprss6), that acts as Hepc inhibitor cleaving mHjv expressed on the plasma membrane [31]. *TMPRSS6* gene expression has been found to be induced by chronic dietary iron loading and Bmp6 injection [32], and its interaction with Neogenin facilitates mHjv cleavage and inactivation in transfected cells [23] (Figure 2).

On the contrary, in iron deficient conditions, this signaling pathway is inhibited by soluble Hjv (sHjv) and Tmprss6, which physically interacts with mHjv, causing its fragmentation.

Hepc expression in hepatocytes is systemically regulated by multiple signals: body iron availability, such as iron-loaded transferrin and hepatic iron stores, erythropoietic activity, hypoxia, and inflammation [33]. Hepc secreted by hepatocytes regulates iron release from duodenal enterocytes, splenic macrophages, and hepatocytes, which are responsible for dietary iron absorption, contain large amounts of iron from erythrocyte recycling, and act as an iron reservoir and export iron when

needed, respectively. Hepc exerts its function, binding the iron exporter ferroportin 1 (Fpn1) [34] and stimulating complex internalization and degradation, leading, de facto, to cellular retention of iron [35]. Elevated plasma Hepc, as in inflammatory state, downregulates iron efflux from several cell types, and this leads to an overall reduction in plasma iron. On the contrary, low Hepc, as seen in iron-depleted or erythropoietic expansion conditions, causes an increased iron release by macrophages and by the basolateral site of villi duodenal cells.

A potent Hepc inhibitor signal is iron demand for erythropoiesis, mediated by three Hepc modulators (Gdf15, Twgs1, Erfe). Their roles and precise mechanisms in Hepc regulation are still not completely clear, but Erfe, in particular, has emerged as a potent Hepc negative regulator in conditions of acute erythropoietic demand, acting in conjunction with erythropoietin (Epo) signaling, as well as in anemia of inflammation (AI) condition [36].

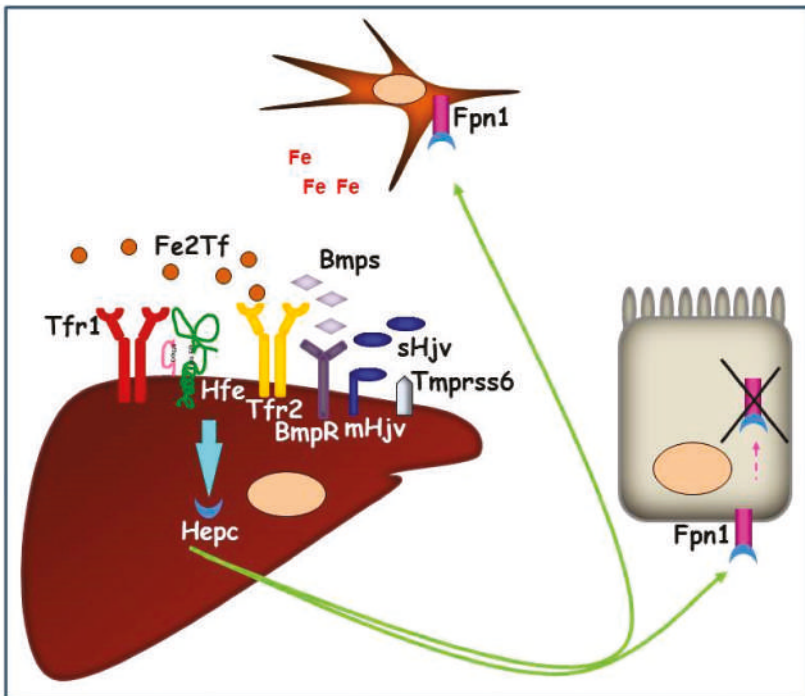


Figure 2. Graphic representation of the hepatic Hepc pathway in conditions of systemic iron increase. The iron signaling proteins (Fe2Tf and Bmps) interact with iron sensors (Tfr1, Hfe, Tfr2, BmpRs) and their co-activator (mHjv) to promote Hepc production. Hepc, secreted by hepatocytes, is transported in plasma and binds iron exporter Fpn1 on duodenal and reticuloendothelial cell surfaces causing its internalization and intracellular degradation. As a consequence, iron remains entrapped in these cells, systemically reducing the metal availability.

HAMP expression is induced by inflammation and infection. This acute phase response involves a different pathway from the ones described above, and is mainly mediated by interleukin 6 (IL-6) inflammatory cytokine, and requires the signal transducer and activator of transcription 3 (STAT3) activation, and the binding of STAT3 to a STAT3-binding motif in the Hepc promoter [37]. In addition, cytokine IL-22, involved in immunological response to extracellular infections, as well as Toll-like receptor 5 (TLR5) agonist flagellin, seem to upregulate Hepc, strengthening the hypothesis of a possible Hepc role in innate immunity [38].

Conversely, *HAMP* expression is repressed by hypoxia both in vitro and in vivo in animal models [33] and humans [39,40]. The mediator of Hepc response to hypoxia seems to be the hypoxia inducible factor (HIF), even though it is not clear if it acts directly or indirectly on Hepc regulation [16]. The fact that Hepc inhibitor, *Tmprss6*, presents a hypoxia responsive element (HRE) in its promoter [41] might make *Tmprss6* the linking protein between hypoxia and iron homeostasis.

Mutations in *HAMP* gene and in genes codifying for Hepc regulatory proteins (*HFE*, *TFR2*, and *HJV*) cause the lack of upregulation of Hepc as a response to increased liver iron stores. In fact, inappropriately low levels of liver Hepc are observed in patients and mouse models of hereditary hemochromatosis [42,43]. These conditions promote a continuous dietary iron absorption that leads to iron overload. On the contrary, inappropriately high Hepc has been found in animal models and patients with iron resistant iron deficient anemia (IRIDA), a genetic disorder due to mutations in *TMPRSS6* gene [44–46].

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2. Tfr2 in Liver

In the liver, *Tfr2 α* is a sensor of circulating iron, but the knowledge about the *Tfr2 α* hepatic function is still incomplete. It is known that *Tfr2 α* localizes in caveolar microdomains [47], membrane structures involved in the recruitment of receptors that can be activated by ligand binding [48]. Also, *Tfr2 α* localizes in lipid raft domains on the exosomal cell membrane, where it is internalized by clathrin-mediated endocytosis, if transferrin saturation (TS) is low [49].

Tfr2 α protein regulation occurs mainly through its stabilization on the cell membrane as a consequence of the binding to *Fe2Tf* [50,51]. An in vitro study showed that, in the presence of *Fe2Tf*, *Tfr2 α* has an increased half-life and is recycled, while in presence of apo-Tf membrane, *Tfr2 α* is mainly subjected to lysosomal degradation [52]. It has been recently demonstrated that CD81 is also able to induce *Tfr2 α* degradation, but the correlation between this *Tfr2 α* regulatory route and Hepc pathway is still obscure [8].

Therefore, increased TS has an opposite effect on the two *Tfrs* via two different mechanisms: it causes a decrease of *Tfr1*, regulated by the IRE/IRP system, but a stabilization of *Tfr2 α* on the cell surface [1].

This supports the hypothesis that *Tfr2 α* exerts its function(s) as a signaling receptor more than as an iron importer.

According to the available in vitro data, hepatic *Tfr2 α* interacts, on the cell membrane, with two main iron proteins, *Tfr1* and *Hfe*.

The current model assumes that *Tfr2 α* , in conjunction with *HFE* and *Tfr1*, is a partner of a sensor complex of circulating iron that activates Hepc in response to elevated TS [53]. In physiological conditions (TS 30–35%) *Hfe* and the complex *Tf/Tfr1* are bound on the plasma cells; when TS increases in response to increased iron availability, loaded *Tf* impairs *Hfe* binding to *Tfr1/Tf* complex, leading it to bind *Tfr2 α* , that is stabilized on the membrane by the same *Fe²Tf*. The resulting complex *Tfr2 α /Fe2Tf/Hfe* causes the activation of Hepc transcription [25]. On the HuH7 hepatoma cell surface, this *Tfr2 α /HFE* interaction occurs within a multiprotein complex, that also includes *mHjv* [27]. It remains to be demonstrated if this complex activates the intracellular signaling to upregulate Hepc expression, also, in vivo.

In the presence of *Fe2Tf*, *Tfr2 α* is able to activate *Erk1/2* and *p38 MAPK* kinase signaling transduction pathway [47], since *Tfr2* KO mice present a decrease of *pErk1/2* [28]. Furthermore,

the Smad1/5/8 pathway also seems to be involved in Tfr2 α -mediated signal transduction, since pSmad1/5/8 is decreased in Tfr2 KO animals, as well [29].

Erk 1/2 phosphorylation could be increased also by Hfe overexpression, and both Tfr2 and Hfe cause an increase of the pro-hormone convertase furin [29], previously demonstrated to be involved in Hepc regulation [54]. Whether it is the sole and/or the main Tfr2 dependent Hepc regulatory pathway is still not clear.

3. TFR2 Mouse Models

The first Tfr2 KO animal model was generated by targeted mutagenesis, introducing a premature stop codon (Y245X) in the murine Tfr2 coding sequence [55]. This mutation is homologous to the Y250X variant, originally detected in humans and responsible for HFE3 [9]. Even young homozygous Y245X mice maintained on a standard diet had high liver iron concentration, in agreement with the observation of early iron overload in HFE3 patients [11]. As in humans, heterozygous animals were normal. The histological distribution of iron resembles the features of HFE3, with the typical liver periportal accumulation.

Subsequently, different murine models of Tfr2 inactivation were developed, including Tfr2 total (Tfr2 KO) and liver-specific (Tfr2 LCKO) knockouts [56,57] as well as a Tfr2/Hfe double KO [28]. All these models are characterized by an inadequate hepatic Hepc expression and liver iron overload with variable severity. However, when generated in the same genetic background, Tfr2 KO mice were shown to have a more severe iron overload than Hfe KO, although less severe than the Tfr2/Hfe double KO [58]. These observations are in agreement with the model of Tfr2/Hfe proteins' cooperation in the liver.

In a double Tfr2/Hjv KO mouse model, plasma Hepc and Hepc transcription was lower than in Tfr2, and similar to Hjv single KOs, respectively. The same was true for the Tfr2/Hfe double KO [59]. Also, a recent study on a mouse model with inactivation of both Bmp6 and Tfr2 (Tfr2/Bmp6 double KO) demonstrated that loss of functional Tfr2 further represses Hamp expression, Smad5 phosphorylation, and plasma Hepc amount in Bmp6 KO mice. The same results were obtained in the Hfe/Bmp6 double KO, and the Hfe/Bmp6/Tfr2 triple KO [60]. All these data support the hypothesis that Tfr2 and Hfe act downstream Bmp6 and upstream Hjv in Hepc regulatory pathway.

Last, Tfr2 germinal vs liver-specific KO animals highlighted a distinct function of Tfr2 outside the liver in maintaining iron balance. In fact, Tfr2 KO mice have less severe iron overload, slightly higher hemoglobin (Hb) levels [57,61], and moderate macrocytosis than Tfr2 LCKO [56,57].

To study the specific function of Tfr2 β isoform in iron metabolism, a specific mouse model was generated, introducing the M167K substitution in the Tfr2 protein [57]. This mutation, homologous to the one found in naturally mutant individuals with HFE3, substitutes the start codon methionine of the Tfr2 β isoform, with a lysine. Interestingly, this knock-in mouse model (Tfr2 KI), specifically lacking the Tfr2 β -isoform ($\alpha^+ \beta^0$), is characterized by normal transferrin saturation, liver iron concentration, Hepc, and Bmp6 levels, but shows transient anemia at a young age. In addition, adult Tfr2 KI animals accumulate iron in the spleen, due to a significant reduction of iron exporter Fpn1 mRNA, thus suggesting a possible regulatory effect of Tfr2 β isoform on splenic Fpn1 expression. These data are further supported by the results obtained in Tfr2 macrophage-specific KO mouse model. These animals present normal systemic iron parameters, but lower Fpn1 transcript and protein in peritoneal macrophages [62]. Recent studies demonstrated that Tfr2 β is well expressed in reticuloendothelial cells of different tissues, where it exerts its role in modulating iron availability in these tissues, acting on Fpn1 transcription (see below). Since Fpn1 protein has several regulatory systems both at the transcriptional [63,64] and post-transcriptional level through IRE/IRP system [7], and origins from different Fpn1 transcripts with or without IREs [65], it remains to be clarified how and when Tfr2 β acts on Fpn1 regulation.

4. Tfr2 in Extrahepatic Tissues

4.1. Tfr2 in the Erythropoietic Compartment

A Tfr2 α erythropoietic role was firstly hypothesized in genome-wide association studies that identified Tfr2 α polymorphisms affecting hematologic parameters [66,67]. These data were further strengthened by the identification of Tfr2 α as a component of the erythropoietin receptor (EpoR) complex in erythroid progenitor cells. Tfr2 α was shown to be crucial for efficient transport of EpoR to the cell surface and for its terminal differentiation, since human erythroid progenitors with silenced Tfr2 α showed a delayed differentiation [68]. Another hint was provided by the increased Hb content present only in Tfr2 germinal KO, but not in liver-specific KO mice. Since both mouse models manifest comparable iron overload, the lack of enhanced hemoglobinization in Tfr2 LCKO mice suggests that the erythroid function of Tfr2 α is preserved [68]. Also, double Tmprss6/Tfr2 KO mice develop erythrocytosis while, in double Tmprss6/Tfr2 LCKO mice, where Tfr2 α is functional in erythroid cells, red blood cells (RBC) number is normal [61].

Recently, a mouse model lacking Tfr2 in bone marrow cells (Tfr2^{BMKO}) was developed injecting BM cells from Tfr2 KO mice in lethally irradiated C57/BL6 mice. Tfr2^{BMKO} mice manifest reduced mean corpuscular value (MCV) and low Hepc levels as a typical response to iron deficiency, but an enhanced terminal erythropoiesis, demonstrated by increased RBC and Hb content [69]. Interestingly, erythropoiesis and Epo level in these mice do not change in a mild dietary restriction setting, as happens for WT animals, where the Epo level is drastically increased.

As a whole, these data suggest that the lack of Tfr2 confers increased Epo sensitivity to erythroid progenitor cells, a hypothesis that is further supported by the induction of Epo target genes, like Hamp regulator Erfe [70], in these animals.

A similar animal model was recently developed crossing Tfr2 floxed mice with Vav-Cre expressing mice to obtain Tfr2 silencing in erythroid compartment [62]. Results differed from previous work since decreased RBC and splenomegaly were observed, but these discrepancies might be explained by the different procedures used to create the two mouse models since, in the first case, Tfr2 is silenced in all bone marrow (BM) cell lines after a BM transplant procedure while, in the latter, only the erythroid cell lines are Tfr2 null.

In another study, Tfr2 erythropoietic role was further investigated studying the erythropoiesis of two Tfr2 mice with one or both Tfr2 isoforms silenced (Tfr2 KI and Tfr2 KO), and with normal or increased iron availability [57]. The evaluations were performed in bone marrow and spleen, in young and adult animals to unravel the erythropoietic role of Tfr2 isoforms at different ages, and in the two main erythropoietic organs. It resulted that the lack of Tfr2 in Tfr2 KO mice leads to macrocytosis with low reticulocyte number and increased Hb value, together with an anticipation of erythropoiesis in young mice both in BM and in the spleen [71], probably because the increased systemic iron amount present in these animals allows them to reach mature erythropoiesis even at a young age.

Although different animals and approaches were used in these studies, and partially contradictory results were obtained, they all demonstrate that erythropoiesis is impaired by a lack of Tfr2 in BM, independently from its activity in hepatic tissues.

Moreover, results obtained studying Tfr2 KI ($\alpha^+\beta^0$) mice [57] demonstrated, for the first time, the involvement of Tfr2 β in favoring iron availability for erythropoiesis. In fact, the sole lack of Tfr2 β , in normal systemic iron condition, causes an increased but immature splenic erythropoiesis seen only in young mice, as if they had insufficient iron availability during animal growth, that is normalized in animal adult age. Decreased iron availability for erythropoiesis in Tfr2 KI young mice is demonstrated by increased ferritin (Ft) and decreased divalent metals transporter 1 (DMT1) in their splenic monocyte, the increase of Erfe transcription in BM and spleen, and the low hepatic Hepc transcription that could, in turn, be responsible for the increased splenic Fpn1 amount in these animals [71].

This effect, due to Tfr2 β absence, in aged matched Tfr2 KO ($\alpha^0\beta^0$) mice, was compensated by the increased amount of circulating iron available that may be used for erythrocyte production (Figure 3A).

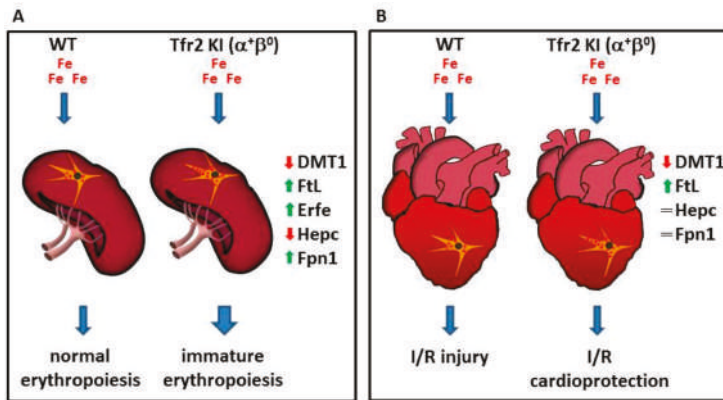


Figure 3. Schematic model illustrating Tfr2 β function in regulation iron export from reticulo-endothelial (REL) cells. Lack of Tfr2 β causes an increased iron retention in REL cells that (A) causes the onset of an immature erythropoiesis in the spleen, and (B) induces a cardioprotection against the effect of the reperfusion of oxygenated solutions after an ischemic event in heart (see text).

4.2. Tfr2 β in the Heart

The cardiac muscle is a major site of oxygen consumption, so an adequate intracellular iron pool is essential to its aerobic activity. This is demonstrated by the finding that deletion of cardiac Tfr1 in mice causes fatal energetic failure in cardiomyocytes [72]. Cardiomyocytes express relatively high levels of Hepc and Fpn1, despite the fact that these cells have no role in systemic iron control [73].

Studies on mouse models demonstrated that the cardiac Hepc/Fpn1 axis is essential for heart cells' autonomous control of the intracellular iron pool that guarantees a normal cardiac functionality [73], and that Hepc/Fpn1 appears to protect the heart from the effects of systemic iron deficiency [74].

On the other side, cardiomyocytes are particularly susceptible to ROS-mediated damage because they are rich in mitochondria and consume large amounts of oxygen [75]. Therefore, when labile iron pool (LIP) expansion occurs, oxidative stress can affect cardiac functions, as it happens in severe juvenile HHC forms [10].

Although ubiquitously expressed, Tfr2 β is highly transcribed in heart [2], such that a role for Tfr2 β isoform in cardiac iron management has been postulated.

Indeed, in the hearts of two Tfr2 β null mice with normal or increased systemic iron amount, Tfr2 KI and Tfr2 LCKO [57], the silencing of Tfr2 β induces a selective activation of different proteins involved in cell survival, antioxidant enzymes, and kinases involved in cardioprotective pathways that are usually activated by stressful stimuli.

In particular, Tfr2 KI and Tfr2 LCKO mice develop a greater resistance against acute ischemia/reperfusion (I/R) challenge, irrespective of animals' systemic iron content, via the activation of the RISK or SAFE/GSK3 β cardioprotective pathways, respectively. The iron imbalance present in these mice hearts was demonstrated by the finding that both models present the activation of antioxidant proteins, pro-apoptotic markers, and catalase, even before I/R [76]. They also have a slightly increased synthesis of cardiac ferritins, similarly to what happens in ischemic preconditioning, in which a small increase of ferritin protects cardiac cells from iron-mediated oxidative damage associated with ischemia/reperfusion injury [77].

Since previous data demonstrated a significant decrease of Fpn1, and an increased iron deposit in splenic macrophages in Tfr2 β -null mice [57,71], one might hypothesize that Tfr2 β isoform inactivation, in the heart, causes an iron retention in cardiac reticuloendothelial cells that is able to induce cardioprotective pathways activation and to reduce iron availability to form free oxygen radicals during the reperfusion phase (Figure 3B).

4.3. *Tfr2* in the Central Nervous System (CNS)

Iron levels in the brain vary during life. The iron amount increases with aging in the striatum and the brain stem [78] and it is present in most CNS cell types: neurons, oligodendrocytes, microglia, and astrocytes [79]. A well-regulated iron homeostasis is important for brain development and function. Iron deficiency negatively impacts neurodevelopmental processes [80], and is also implicated in a number of psychiatric and neurological conditions, learning disabilities, attention deficit hyperactivity disorder (ADHD), and pediatric restless legs syndrome (RLS) [81,82]. On the contrary, brain iron overload is present in Alzheimer's and Huntington's neurodegenerative disorders, as well as in Parkinson's disease (PD) [83]. Nevertheless, the exact role of iron in these diseases' onset/worsening is still debated, and it remains to be clarified whether brain iron overload is directly involved in their pathogenesis, or it is a secondary effect that contributes to their clinical symptoms' progression.

The main sites of brain iron uptake are the brain vascular endothelial cells (BVECs) present in the blood–brain barrier (BBB) [84]. As in other organs, there are two main pathways responsible for CNS cells' iron uptake, the Tf-TfR1 pathway, and the NTBI transport pathway. Traditionally, Tf-TfR1 is considered a major pathway, and works as in all the other cell types of the organism, through a receptor-mediated endocytosis of plasma Tf circulating in the ventricles [85].

The NTBI transport pathway has been recently reevaluated as a significant way to introduce iron in CNS, and it could be done through vesicular or non-vesicular mechanism. In the first case, Tf homologues, such as lactoferrin and melanotransferrin, might be involved in TfR-mediated iron transport; moreover, the newly characterized Ft receptors, Tim 2 and Scara5, can introduce iron inside the cells through a Ft-FtR pathway. Non-vesicular iron uptake can be exerted by iron importer DMT1, that is present in endothelial cells of the brain microvasculature, as well as other importers like IN4/5/6 [86].

In CNS, the iron exporter Fpn1 is found in BVECs, neurons, oligodendrocytes, astrocytes, the choroid plexus, and ependymal cells and microglia, together with ceruloplasmin (CP) or hephaestin (Hp), the two ferroxidases that cooperate with Fpn1 to facilitate iron export [87]. Fpn1 could be the main protein responsible for iron release from CNS cells, even if other proteins and mechanisms have been brought into play for these processes [86].

Inside brain cells, the majority of iron is bound to ferritin heteropolymers (Ft H/L) [88]. Their cellular distribution and ratio varies with iron status, age, and disease conditions [89].

CNS iron homeostasis is intracellularly modulated by (IRE/IRPs) system [90], and by local and systemic Hepc. Injection of Hepc into the mouse lateral cerebral ventricle decreases Fpn1 protein levels and treatment of primary cultured rat neurons with Hepc decreases Fpn1 expression and reduces these cells release of iron [91]. More recently, it was demonstrated that injection of adenovirus expressing Hepc (ad-hepcidin) in brain ventricles reduces brain iron in iron-overloaded rats through the downregulation of iron transporter [92]. This data indicates that Hepc/Fpn1 axis is present and acts in CNS, as in the other districts of the organism.

It remains a matter of debate whether Hepc acting in brain is locally produced or comes from the systemic circulation crossing the BBB or both [93].

Similar to other Hepc regulatory proteins, *Tfr2* gene expression has been shown in total brain extracts [2,94], in brain tumor cell lines [95], or in specific neuronal subtypes as dopaminergic neurons [3]. Furthermore, a transcriptome study on *Tfr2*-null mice revealed that several genes involved in the control of neuronal functions are abnormally transcribed [96]. Of note, the same experimental approaches, applied to *Hfe*-null mice, revealed that a consistent percentage of transcripts are modified in the same way in the two models [96]. This highlights the possibility of a cooperation between *Tfr2* α and *Hfe* protein in CNS iron regulation, as in the rest of the organism.

Immunofluorescence studies using a *Tfr2* α -specific antibody demonstrate that the protein is significantly produced in mouse hippocampus, amygdala, central nucleus, and in the hypothalamic paraventricular nucleus [97].

A recent study assessed the situation of iron in the brain of Tfr2 KO mouse model vs WT sib pairs subjected to an iron-enriched diet (IED). They both are iron overloaded animals, so one could distinguish the effects of Tfr2 silencing from those due to Tfr2-independent iron load modifications.

It has been demonstrated that Tfr2 causes a lack of brain Hepc response to the systemic rise of iron levels, with altered iron mobilization and/or cellular distribution in the nervous tissue [98].

Moreover, Tfr2 KO mice present a selective over activation of neurons in the limbic circuit and the emergence of an anxious-like behavior.

Also, microglial cells showed sensitivity to iron perturbations of Tfr2 KO mice, being more reactive, dystrophic, and with a high level of apoptosis [97]. In light of these data, Tfr2 appears to be a key regulator of brain iron homeostasis, and could have a role in the regulation of the brain regions that are involved in the anxiety onset, mainly, the basolateral and central nucleus subregions of the amygdala [98].

5. Tfr2 in Intracellular Iron Trafficking

It is still under debate if Tfr2 α contributes to iron introduction inside the cells. When the protein was characterized, it was reported that, *in vitro*, it was able to introduce iron inside cells [2], but its contribution to intracellular iron amount *in vivo* seems to be quite negligible, since Tfr1-deficient mice present severe iron deficiency not compensated by the presence of Tfr2 [1].

Conversely, Tfr2 α seems to have a role in intracellular iron trafficking, at least in specific cell types. The first evidence about it was found in dopaminergic neurons, where a novel Tf/Tfr2 α -mediated iron transport pathway to the mitochondria has been reported [3]. Disruption of this Tf/Tfr2 α -dependent system has been associated with PD, and this finding highlights the role of iron accumulation in this movement disorder [3]. In this regard, a protective association between some Tf and Tfr2 α genetic haplotypes and PD was reported, suggesting that Tf or a Tf/Tfr2 α complex may play a role in the etiology of these disorders [99].

More recently, a similar Tfr2 α function in iron delivery to mitochondria has been convincingly demonstrated in erythroid cells. In an intermediate stage of human erythroid cell maturation, Tfr2 α was present in cytoplasmic multi-organellar complexes, formed by lysosomes surrounded by mitochondria, and found to be co-regulated with several proteins, among which, ionic channels and proteins involved in lysosomal modification and in mitochondrial membrane contacts to other intracellular organelles [100]. Therefore, Tfr2 α in lysosomes has been proposed to be involved in iron delivery from these organelles to mitochondria, for Hb synthesis. Considering the abovementioned evidence about a specific role for Tfr2 α in erythropoiesis, this data might represent one of the molecular processes at the basis of Tfr2 α erythropoietic function/s (Figure 4).

In light of the above data, Tfr2 α involvement in iron delivery to the mitochondria, notably, seems to work at least in the two compartments in which this protein is significantly produced: brain and bone marrow. Although a similar mechanism has been evidenced also in cell lines derived from other organs (HeLa and hepatoma cell lines) [52,101], it remains an open question if this Tfr2 α function is present also in other cell types.

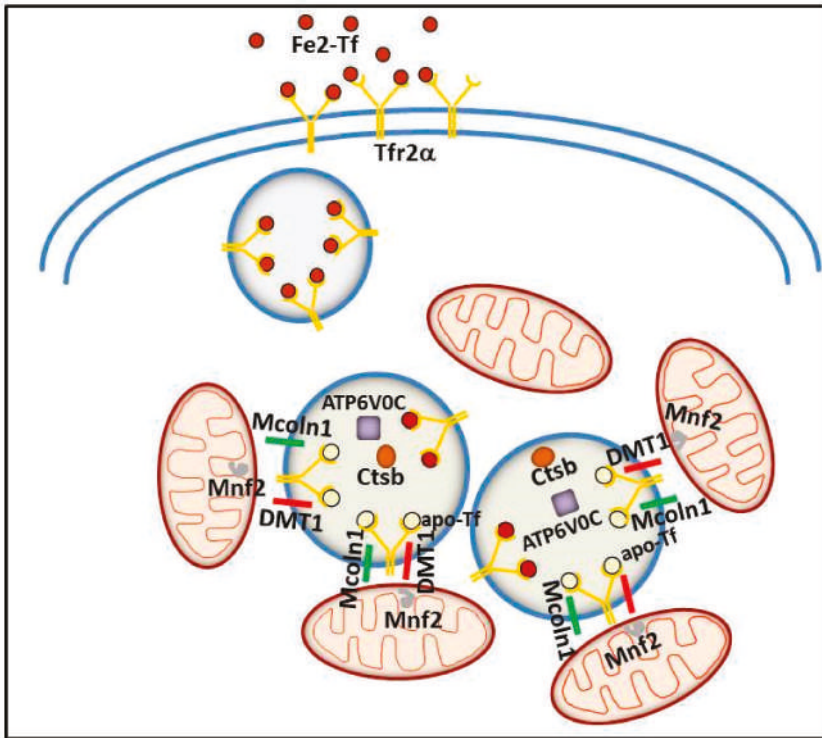


Figure 4. Schematic model illustrating the putative intracellular Tfr2 α role in erythroid cells. Tfr2 α —together with ATPV0C, a V-ATPase that contributes to vesicular acidification and lysosomal activity; Mcoln1, a lysosomal divalent cation channel; lysosomal cathepsin B (CTSb); and iron importer DMT1—could be involved in iron delivery from lysosome to mitochondria, with the collaboration of Mitofusin-2 (MFN-2), a mitochondrial outer membrane protein involved in mitochondria–endoplasmic reticulum contacts.

6. Tfr2 in Other Diseases

Being a “regulator of the iron regulator” hepcidin, Tfr2 transcriptional analysis was attempted to unravel if the Tfr2 isoforms could be reliable markers for some disorders in which iron perturbations occur.

6.1. Tfr2 in Cancer

Cancer cells need an increased amount of iron for their growth, and iron importers upmodulation confer a selective advantage to these cells.

Since its characterization, the significant transcription of *TFR2* gene appeared evident in BM cancer cells, in particular, erythroid leukemic cells [5], but also in myeloid malignant cells [2], while Tfr2 β seemed much more prevalent than the Tfr2 α isoform in chronic B cells lymphocytic leukemia (B-CLL) cells [102].

Due to high *TFR2* expression in erythroid lineage, and to its functional relationship with the erythropoietin receptor (EpoR) [68], *TFR2* transcription was evaluated in patients with myelodysplastic syndromes (MDS), a hematopoietic disorder with a variable risk to evolve in acute myeloid leukemia (AML), and in which chronic anemia can be corrected by Epo injection [103]. It has been found that Tfr2 α and Tfr2 β isoforms, as well as EPOR transcript, have a lower level of transcription in BM from high risk MDS patients, such as RAEB2, compared to controls and low risk MDS cells [104]. Likewise,

AML patients with high level of Tfr2 α and Tfr2 β present an increased survival [105]. Therefore, Tfr2 isoforms might represent good predictive markers for MDS/AML prognosis.

Calzolari et al. 2007 [106] demonstrated *TFR2* expression in colon and ovarian cancer cell lines, as well as in lymphoma and glioblastoma (GBM). Moreover, in glioblastoma TB10 cell line under hypoxic condition, a marked increase of *TFR2* transcription was observed. In these cell lines, *TFR2* high expression is probably correlated with cell proliferation, since Tfr2 silencing inhibited GMB cell growth. Surprisingly, tumor cells from GBM patients with high *TFR2* transcriptional levels present a better prognosis compared to patients with low transcripts. Although, this is probably due to the fact that Tfr2-expressing cells have a highly increased proliferation, so they are more sensitive to temozolomide, the anti-proliferative drug used in GBM therapy, more than to a direct involvement of Tfr2 in the disease course [107].

6.2. Tfr2 in Alzheimer's Disease (AD)

Alzheimer's disease (AD) is another common degenerative disorder in which iron perturbation has been demonstrated [108].

A recent genetic study from 116 AD patients has found that a Tfr2 single nucleotide polymorphism (rs 7385804) and a Tfr2 haplotype, composed by two SNPs (rs 7385804 and rs 4434553) are associated with a decreased AD susceptibility [109].

In the same study, a significant decrease of Tfr2 transcription was found in peripheral blood mononuclear cells (PBMC) from AD patients, compared to healthy controls ($p < 0.001$) [110].

7. Tfr2 as a Therapeutic Target

In our opinion, three might be the major application of Tfr2 α as a therapeutic target: (a) as Hepc regulator, it could be a target in disorders in which Hepc amount is, in some way, inadequate to body iron availability; (b) since Tfr2 α production is selective in specific organs and cell types, it could represent a selective target to correct iron perturbation in these organs; (c) Tfr2, being a membrane protein that is able to bind plasma Tf and to be internalized with it, this property could be utilized as a vector for drugs.

As mentioned above, TFR2 α is involved in regulation of Hepc and, in consequence, in iron modulation according to body/organs needs. Among the Hepc-related disorders there are all the hereditary hemochromatosis forms (Table 1) and the secondary iron overload disorders, like hemoglobinopathy, where abnormally low Hepc amounts lead to iron overload.

In this regard, just published data demonstrate that Tfr2 KO BM transplantation in beta-thalassemia intermedia (β -TI) mouse models significantly improved these animals' erythropoiesis, opening a new way to the therapy of this very common disorder [111].

In IRIDA and anemia of chronic disease (ACD), where abnormally high Hepc causes the onset of an iron deficiency condition [111], Tfr2 downmodulation might be beneficial to decrease Hepc hyperproduction. A preliminary study on mice revealed that Tfr2 silencing, through small interfering RNAs (Tfr2-siRNA) in a single dose, led to a significant Hepc downmodulation, and increased transferrin saturation within 24 h post-administration, persisting for more than two weeks, and to a recovery from anemia in animal models of ACD [112].

Beside the liver, in brain, Tfr2 regulates the production of local Hepc and iron amount in the CNS, since Tfr2 KO mice brain have a blunted Hepc response to brain iron overload [97]. In recent time, it has emerged that brain Hepc production is altered in several neurodegenerative disorders: downmodulated in Alzheimer's and Parkinson's disease [113,114] and upmodulated in restless legs syndrome (RLS) [115]. It might be worthwhile to further investigate if Tfr2 is involved in these Hepc variations, and consider using anti Tfr2 antibodies or siRNA-based therapy to rescue Hepc physiologic values in RLS. Nowadays, siRNA delivery to brain is quite difficult, due to the presence of the BBB, but the ongoing studies on nanoparticles' use, to target siRNA in specific sites, could allow an increase in the efficacy of this therapy [116].

An alternative therapeutic approach aims to deliver blocking antibody in the brain, exploiting the BBB physiologic activities. This is based on the use of anti-Tfr1 antibodies, since Tfr1 is well expressed in BBB endothelial cells and is involved in receptor-mediated transcytosis. Indeed, it has been demonstrated that the bispecific Tfr1/BACE1 (β -amyloid cleaving enzyme-1) antibody resulted in being effective in decreasing β -amyloid concentration in the brain [117].

Due to high Tfr1/Tfr2 homology and the common cellular internalization through a receptor-mediated endocytosis pathway, one could hypothesize a parallel Tfr2-based approach for CNS, blocking antibody delivery. Nevertheless, it should be further confirmed, the presence of Tfr2 on BBB endothelial cells and its involvement in transcytosis.

Recent studies have aimed at exploiting the Tfr1 as a vehicle for drug delivery inside the cells through endocytosis, often utilizing Tfr1 natural ligand, Tf, conjugated with different synthetic molecules [1]. Moreover, since Tfr1 is able to bind and internalize FtH also [118], FtH nanocages conjugated with a PARP inhibitor, olaparib, were developed for breast cancer therapy [119].

Due to its strict homology to Tfr1, one might hypothesize that Tfr2 α could also be utilized to deliver drugs inside cells. This approach would be particularly useful for two main Tfr2 α features; Tfr2 α -selective expression in particular tissues (hepatic, erythroid, and in CNS) and Tfr2 α high expression in several tumor cells, sometimes with Tfr1 and sometimes without it. High expression of Tfr1 and Tfr2 α were, in fact, detected in tumor and para-cancerous normal liver tissues collected from 41 patients with hepatocellular carcinoma (HCC) [120]. Tfr2 α is also highly expressed in brain tumor cells in several cases of anaplastic astrocytoma and glioblastoma, but not in normal brain or endothelial brain cells [121].

Furthermore, in the light of the new data on Tfr2 α function in delivery iron to mitochondria, Tfr2 α could, possibly, represent a good vehicle for drug delivery in these organelles [122], paving the way to tailored therapies for mitochondrial iron disorders, notably Friedreich's ataxia [123].

Unfortunately, too few functional data are available at the moment on Tfr2 β isoform to foresee possible therapeutic applications.

8. Conclusions

The process of body iron homeostasis is complex, and since there is no apparent significant excretory pathway, the amount of iron absorbed from mature duodenal enterocytes and that is recycled by macrophages needs to be tightly regulated. From the last decades, iron pathways have been enriched of new regulatory proteins whose functions remains to be elucidated from the molecular point of view. Each of them could represent a potential target for a focused pharmacological therapy of disorders with iron unbalance, that still represents the most prevalent of diseases all around the world.

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Review

What Is Next in This “Age” of Heme-Driven Pathology and Protection by Hemopexin? An Update and Links with Iron †

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Abstract: This review provides a synopsis of the published literature over the past two years on the heme-binding protein hemopexin (HPX), with some background information on the biochemistry of the HPX system. One focus is on the mechanisms of heme-driven pathology in the context of heme and iron homeostasis in human health and disease. The heme-binding protein hemopexin is a multi-functional protectant against hemoglobin (Hb)-derived heme toxicity as well as mitigating heme-mediated effects on immune cells, endothelial cells, and stem cells that collectively contribute to driving inflammation, perturbing vascular hemostasis and blood–brain barrier function. Heme toxicity, which may lead to iron toxicity, is recognized increasingly in a wide range of conditions involving hemolysis and immune system activation and, in this review, we highlight some newly identified actions of heme and hemopexin especially in situations where normal processes fail to maintain heme and iron homeostasis. Finally, we present preliminary data showing that the cytokine IL-6 cross talks with activation of the c-Jun N-terminal kinase pathway in response to heme-hemopexin in models of hepatocytes. This indicates another level of complexity in the cell responses to elevated heme via the HPX system when the immune system is activated and/or in the presence of inflammation.

Keywords: hemopexin; heme homeostasis; iron homeostasis; hemolysis; haptoglobin; ferroptosis; inflammation; biomarker; heme oxygenase; liver; microbiome; trauma; hemorrhage

1. Introduction

The first direct evidence that hemopexin (HPX)-mediated heme transport links heme (iron-protoporphyrin IX) and iron metabolism was published in 1988 [1]. In that study, heme-HPX complexes were as effective as diferric-transferrin as a sole source of iron for the growth of mouse hepatoma cells that were used as models of liver parenchymal cells (i.e., hepatocytes). The rate of heme uptake via HPX is linked with cell growth and shows highest uptake in the period just before exponential growth and slowest in the stationary phase. Also, the iron status of hepatoma cells affects the extent of heme uptake. For example, decreasing intracellular iron with the chelator deferoxamine increased HPX-mediated heme transport, as did inhibiting iron-dependent ribonucleotide reductase (required for DNA synthesis).

Plasma HPX rapidly targets heme to hepatocytes [2] that respond with a cytoprotective program involving safe trafficking of heme, its iron, and, uniquely, also copper [3]. This copper is needed

for the coordinate induction of heme oxygenase-1 (*HO1*) and metallothionein genes for antioxidant protection [4–7]. Within cells, heme delivered by heme-HPX endocytosis is quickly transported to the nucleus relieving Bach 1 repression, thus activating *HO-1* gene transcription [8,9]. Heme also travels to the smooth endoplasmic reticulum for degradation by heme oxygenases and into mitochondria, presumably via the mitochondrial heme exporter, FLVCR1b [10]. The heme-iron is utilized for the Fe/iron regulatory protein (IRP)/iron response element (IRE) system to regulate proteins of iron homeostasis at the translational level in part via storage on ferritin, thus keeping intracellular iron levels low [6]. Overall, the regulated delivery of heme by HPX maintains cell redox homeostasis. This is not the case when cultured cells are incubated with free “heme”, which is rapidly and extensively taken up compared with heme-HPX endocytosis (at approximately a five-fold higher rate on a molar basis) [11]. Heme generates ROS and, thus, is readily toxic [12]. Intracellular heme levels of 3 μM are rapidly reached, equivalent to 1 million heme molecules in the volume of the mitochondria [13].

The liver is the principle organ that responds to changes in systemic iron signals in order to maintain body iron homeostasis. Iron stores are regulated solely at the level of absorption not excretion. Interestingly, the bioavailability of heme from the diet as an iron source is superior to that of inorganic iron. However, iron, not heme, is exported into the systemic circulation because after [^{59}Fe]heme is placed in the lumen of isolated rat duodenum, iron-transferrin not heme-HPX is present in the mesenteric vein [13]. This supports extensive catabolism of heme by HOs in duodenal enterocytes.

In addition, the liver is the first site of defense against dietary antigens and pathogens from the gut. Furthermore, low levels of heme are normally present in bile providing a source of heme and iron for gut bacteria and, thus, biliary heme is poised to influence the composition of the microbiome. In fact, using intravenous hemin to mimic a heme overload in the plasma of mice leads to the secretion of “black” bile [14].

The liver is an immune-responsive organ. Although, hepatocytes comprise ~90% of the liver mass, it also has many different types of cells including immunologically active Kupffer cells, stellate cells, and trafficking monocytes. As reviewed by Crispe [15], hepatocytes act in both innate and adaptive immunity. Hepatocytes synthesize and secrete several proteins needed for cell defenses during distinct pathologies ranging from ischemia, physical trauma, infections, and sepsis. These cells act not only by providing acute phase proteins in response to the cytokine IL-6 but also to direct T- cells [15]. They also respond to factors such as hepatocyte growth factor to synthesize IL-6. Rapid, short-term elevations in IL-6 are part of the early warning signals to activate the immune system in response to infections and injury. However, when IL-6 levels are sustained chronic inflammation occurs that can become life threatening, as in sepsis. Additionally, IL-6 is often dramatically elevated in patients receiving chimeric antigen receptor T cell therapy (CAR-T) and has been associated with both cytokine release syndrome (CRS) and neurotoxicity in these patients. Importantly, increased CRS grade was associated with peak IL-6 levels, peak ferritin (Ftn), and peak C-reactive protein [16]. Predictive models of CRS based on various cytokines and biomarkers are currently being researched [17].

Here, we provide preliminary data showing that IL-6 alters the response of models of liver cells to heme-HPX significantly limiting the extent of activation of the mitogen-activated protein kinase C-Jun kinase activation (JNK) pathway and changing levels of its substrates that are transcription factors. While the gene targets have not yet been identified completely, our data raise the possibility that when IL-6 is present in the short term, such cross talk may reprogram cell responses to heme-HPX that are normally beneficial. On the other hand, if certain protective signaling cell responses set in motion by the HPX system are attenuated by sustained IL-6, then there may be dire consequences. Such interactions between IL-6 and HPX may contribute to the worsening of sepsis including the development of septic shock. In the brain, these responses might exacerbate the pathology of intracerebral hemorrhage, traumatic brain injury or stroke where tissue and red blood cells injury is accompanied by inflammation.

The extent of damage by heme to the brain is beginning to be understood and several studies in mice provide evidence that HPX is protective in intracerebral hemorrhage (ICH), subarachnoid hemorrhage, and stroke. Hemopexin is present in human and rat CSF [18–20] and HPX mRNA has been

located in ependymal cells [21], neurons [22], and glial cells [23]. Studies using HPX-null mice have revealed a role for HPX in myelin basic protein expression by oligodendrocyte and oligodendrocyte differentiation [23]. Hemolysis in the brain leads to disruption of the blood–brain barrier. However, a recent study [24] on patients with brain hemorrhage (intracerebral and subarachnoid) provides evidence that iron toxicity may be principally responsible for pathology rather than heme clearance. A review on heme in the context of neurodegeneration, where there are known mitochondrial defects, has recently been published in this *Pharmaceutical* special issue [25].

The purpose of this review was to assess from recently published research the biochemical role for HPX in protecting against heme toxicity in hemolysis and the accompanying inflammation in order to better define how heme- and iron-driven pathology develops in patients. Evidence supports that HPX is protective in both the presence and absence of bacterial/pathogen infection [26]. Hemopexin has two functions: firstly, as an extracellular antioxidant by sequestering heme and thereby protecting molecules in biological fluids from oxidation by heme and secondly by regulating heme uptake via heme-endocytosis which ensures redox metal homeostasis after heme and iron metabolism without leading to oxidative stress. Our focus was predominantly on recent clinical studies. Children are vulnerable to depletion of HPX because HPX is a developmentally regulated protein. Several studies included have investigated HPX as a candidate biomarker, generally in a panel with additional proteins; however, it is not always clear if the observed changes in HPX metabolism are linked solely to hemolysis or to inflammation or, as we contend here, to both. A more thorough understanding of the biochemistry of the HPX system may lead to novel therapeutic approaches for ameliorating or preventing heme-related pathologies. For example, the physiologically relevant role of the scavenger receptor LRP1/CD19 in the endocytosis of heme from Heme-HPX varies with cell type and is not fully understood in the context of the HPX system.

2. Mechanisms of Heme Toxicity

The cell damage by hemoglobin (Hb), by its heme (and by heme's iron) are often ascribed to “oxidative stress”—an all-encompassing term for many different events encompassing inflammation and cytotoxicity. Both Hb and heme damage lipoproteins in the circulation [27,28]. Several recent studies show that high levels of heme consistent with that seen in patients with hemolytic disorders have several effects on cells. For example, heme inhibits the proteasome; generates aggresome-like induced structures (ALIS); activates the unfolded protein response (UPR); and activates several plasma systems including the coagulation cascade, binding to immunoglobulins and activating the alternative pathway of complement (see reviews by Roumenina et al. [29,30]).

Understanding these various aspects of heme toxicity for cells is of interest as well as assessing differences among various cell types (e.g., hepatocytes, macrophages, neurons, glia, endothelial cells, myocytes, kidney cells, and certain stem cells) in terms of their cell defenses. This information is also needed to better define panels of biomarkers in hemolysis, oxidative stress, and inflammation to aid in the diagnosis, prognosis, and response to therapy of patients with various hemolytic diseases and conditions.

2.1. Heme-Related Inhibition of the Proteasome

To identify the proteins and processes involved in heme stress, changes in the proteome phenotype of HO1^{+/+} and HO1^{-/-} mouse fibroblasts were investigated using mass spectrometry (MS) in combination with stable isotope labeling by amino acids of cultured fibroblasts in response to a range of heme concentrations [31]. The fibroblasts were incubated for 12 or 24 h with non-toxic levels of heme (5 or 10 μ M) or with 40 μ M that was deemed toxic and led to impaired mitochondria as evident by low ATP levels. Toxic heme caused nuclear condensation together with disorganization of the cytoskeleton. Furthermore, heme at levels greater than 10 μ M induced caspase 3/7 activity, suggesting activation of apoptosis. It should be noted that 10 μ M heme is often used in many in vitro studies in the literature. These heme-related toxicities were prevented by adding HPX to the cell

culture medium. The MS analyses revealed 2068 proteins that responded to heme. Mass spectrometry MetaCore analyses showed protein networks involved in the UPR, general protein folding, and, as expected, anti-oxidant defenses. Also, heme stress, perhaps not surprisingly, had clearly made an impact on iron metabolism because the most strongly induced proteins were the proteins of iron storage H- and L-Ftn. Specifically, L-Ftn increased independent to initial HO1 protein levels in the two sets of fibroblasts. Heme had an impact on the proteasome because ubiquitin and the ubiquitin adapter protein sequestosome 1 (SQSTM1)/P62 were induced by heme. This is a scaffold protein with multiple domains central to a variety of functions. Principally, it is part of a signaling hub to control cell viability when there is cytotoxicity [32]. Various heat shock proteins and proteins involved in antioxidant defenses (e.g., peroxiredoxin, glutathione (GSH)-S transferase, and thioredoxin reductase) were also increased but to a lesser extent. As expected, heme levels were higher in the HO1^{-/-} cells but changes in response to toxic levels of heme were also found in cells expressing functional HO1, as well as in a murine macrophage cell line and human embryonic kidney cells. Intriguingly, heme bound the 19S ATPase subunit of the 26S complex of the proteasome. However, proteasome inhibition may not be due to the redox activity of heme because cobalt-protoporphyrin IX, which is redox inert, also inhibited the proteasome. The cell toxicity from heme was proposed to be due to the changes in proteasome structure likely from the effects of other reactive molecules such as lipid peroxides as well as from chemical reactions involving heme.

Consistent with these observations on the effects of high intracellular heme, it is not surprising that genetic hemolytic diseases are protein disaggregation disorders. In β -thalassemia, the erythrocyte precursors use protein quality control to protect themselves [33]. Due to the mutations in the β -globin gene in β -thalassemia, α -globin chains accumulate in erythrocyte precursors and precipitate due to the fact of their inherent instability. However, they are polyubiquitinated and degraded by the proteasome. Enhanced proteasome activity due in part to the induction of protein subunits of the proteasome is found in cells from β -thalassemic patients. This was shown by transcription profiling to be due to the stress response transcription factor Nrf1. Brief inhibition of the proteasome led to α -globin chain accumulation; however, α -globin did not accumulate in the erythrocyte precursors of β -thalassemic mice treated with the proteasome inhibitor bortezomib. Thus, the means to regulate protein quality control may differ between erythroid precursor cells in vitro and in vivo in mice perhaps due to the differences in activity of the Hb stabilizing protein.

2.2. Heme-Related Aggresome-Like Structure Formation

In 2016, Travassos and colleagues [34] were the first to report that heme, at high levels (30–100 μ M), would produce protein aggregates intracellularly. These aggregates also known as aggresome-like induced structures (ALIS) had a distinct composition, which included ubiquitinated proteins and SQSTM1/P62 in RAW macrophages [34]. The transcription factor NF-E2-related factor (NRF2) was required, which increases the transcription of SQSTM1/P62 raising protein levels. By using the HO1 inhibitor tin-protoporphyrin and cells derived from mice lacking FtnH, these ALIS were shown to be formed in response to oxidative stress from the intracellular heme and its iron. Furthermore, the response to heme was reversible because there were changes in the number of the ALIS over time. Interestingly, the maximum formation occurred 12 h after heme and declined over the following 12 h. Importantly, this indicates that there is an active cell recovery response to clear these structures. Even at these high levels, heme did not activate autophagy—the cell survival mechanism that allows the controlled degradation and replacement of cell components and can induce cell cycle arrest and inhibit apoptosis.

2.3. Heme-Related Unfolded Protein Response (UPR)

During the course of atherosclerosis, plaques undergo defined changes as the pathology develops. Ultimately, this can lead to a high risk of acute ischemic events. Plaque instability is increased depending upon the lipid content and the presence of a thin fibrous cap. Intraplaque hemorrhage

occurs after activation of neovascularization and new blood vessels enter the plaques. When these plaques (i.e., atheromas) rupture, both smooth muscle cells and resident macrophages are exposed to high heme levels, to oxidized forms of cell-free Hb, and to the accompanying oxidative stress.

Addition of heme to cells *in vitro* increases intracellular reactive oxygen species (ROS) [35] and evidence supports that oxidatively damaged proteins are likely to be partially misfolded, thus generating endoplasmic reticulum (ER) stress. When this occurs, the UPR (for reviews, see References [36,37]) is activated to prevent ER overload, restore homeostasis in the ER, and to promote cell survival. The UPR consists of the activation of several intracellular signal transduction pathways and operates at both transcriptional and translational levels. For example, mobilization of the transcription factors ATF6, ATF6B, CREB3, and the inositol-requiring enzyme 1 leads to increased transcription of genes encoding chaperones such as HSP90, HSP70 and GRP94. Mobilization of the pancreatic ER kinase-like ER kinase (PERK, pancreatic eIF2 α kinase, EIF2AK3) leads to phosphorylation of the translation initiation factor eIF2 α , leading to a global downregulation of protein synthesis.

The analysis of human carotid artery specimens from patients undergoing carotid endarterectomy showed significant amounts of heme in atherosclerotic plaques. Human aortic smooth muscle cells are key players in the development of atherogenesis. To evaluate how high levels of heme cause ER stress and activate the UPR, the levels of markers of ER stress were measured after human aortic smooth muscle cells were incubated with heme for up to 6 h [38]. In cultured cells isolated from complicated lesions with hemorrhage, even those obtained some distance from the border of intraplaque bleeding, had increased expression of glucose-regulated protein 78kDa (Grp78) and CCAAT-enhancer binding protein homologous protein (CHOP) in response to heme compared with atheromas or healthy arteries. The CHOP is a multi-functional transcription factor that down regulates the anti-apoptotic mitochondrial protein BCL2 which is an apoptosis regulator supporting pro-apoptotic activities of the mitochondria; and, thus, a robust marker for apoptosis. Low levels of heme (1 μ M) did not activate the UPR; however, heme at 10 or 25 μ M, activated all three “arms” or “sensors” of the UPR. Inositol-requiring enzyme activation, PERK activation, and ATF6 activation were detected after 3 h of incubation. These data support that, in complicated lesions from patients with atherosclerosis, heme triggers ER stress and cell death.

Based on these changes in UPR marker proteins in response to the high heme, a model was presented that heme, depending upon its concentration, activates both pro-apoptotic and pro-survival pathways. Significantly, incubating human smooth muscle cells *in vitro* with heme caused a transitory pro-apoptotic response and permanently activated the pro-survival responses (ATF6, GRP78). There was also a high level of induction of heme-responsive genes including *HOI1*, which is cytoprotective. This research provides evidence for interaction between heme and iron metabolism together with the UPR activating pathways to help cells survive heme-induced stress. Furthermore, α -1 microglobulin and HPX, which both bind heme in plasma, significantly decreased the effects of heme on ER stress markers when added to the medium of human smooth muscle cells *in vitro*. Thus, patients with atherosclerosis may benefit from plasma replenishment with HPX.

2.4. Heme-Related Activation of Complement

The complement system consists of ~70 proteins and is another part of the human immune system that contributes to immune defenses but can also exacerbate inflammatory diseases and conditions. Complement proteins rapidly sense tissue changes and the antigens presented by invading pathogens. By binding to these molecular signals on the surface of pathogens, they facilitate pathogen destruction by bringing in phagocytic macrophages and neutrophils that also produce cytokines [39]. Complement signaling and activation pathways have now been linked with T cells, which act in homeostatic responses.

Merle and colleagues [29] showed that the complement system is activated in sickle cell disease (SCD) patients and is the likely cause of nephropathy because deposits of complement components C3 and C5b were present within the tissues of kidney biopsies taken from patients. Using a combination of

in vitro and in vivo approaches with a mouse model of SCD, the mechanism of complement activation triggered by intravascular hemolysis was shown to be due to the effects of heme on endothelial cells. Furthermore, intravenous (IV) HPX was protective limiting the complement activation.

In a mice model of phenylhydrazine (PHZ)-induced intravascular hemolysis (IVH), C3 deposits were detected predominantly in the kidney and in the glomeruli. The role of C3 was confirmed because the renal injury was markedly less in C3 null mice. In WT mice, IV heme or IV purified human Hb also led to activation of complement with extensive C3 deposits in the kidney. Significantly, there were no detectable deposits after HPX was given IV providing strong evidence that HPX effectively prevented complement activation in response to heme and, importantly, also to Hb. As an avid heme-binding protein, this protection by HPX also supports that the pathological effects of Hb are due in part to the release of heme with activation of complement. In contrast, human serum albumin that can bind two molecules of heme but significantly lower affinity than HPX, was not protective. Phenylhydrazine causes a huge IV Hb/heme load with extensive acute kidney injury as shown by tissue and vascular markers of inflammation (e.g., IL-6, P-selectin), as well as ultrastructural changes in the renal tubules. Surprisingly, this kidney damage was resolved and renal function restored without treatment. In contrast, these changes in the kidney did not occur in response to IV heme. In addition, HPX was not protective in the PHZ-treated mice that were dying possibly from acute pancreatitis [40]. While the overall conclusion was that HPX is a sentinel for the kidney in IVH against heme toxicity, it was suggested that events generating hemolysis-derived products before the release of heme might be the damaging species. Degradation of RBCs produced heme-loaded microvesicles and heme induced both the alternate and complement pathways and activated complement either in serum in vitro or on endothelial surfaces. Complement proteins can be inactivated with blocking antibodies, although this type of treatment is still under development. Nevertheless, the identification of a role for complement in vascular injury and organ pathology raises the possibility that such antibodies would be useful as adjunct therapeutics with plasma protein replenishment to minimize or preclude development of the pathology of heme toxicity.

2.5. Hb-Related Globin Toxicity

It has been known for some time that globin is potentially toxic to cells. Hb (from RBCs) and myoglobin (from myocytes) are the principal sources of globin in plasma. There are clear differences in the structure of these two molecules that drive their chemical responses to agents such as CO, which is released only from heme catabolism [41] and from lipid peroxidation. Due to the fact of its inherent instability, α -globin aggregates within red blood cells at various stages of their development and sites in the body causing much of the pathology in β -thalassemia (see Section 2.1). An excess of α -globin chains without sufficient β -globin due to the point mutations in this disease is potentially toxic and to counteract this, an α -globin chain stabilizing protein is expressed in erythroid precursor cells solely, which is a chaperone for the α -globin [42].

The mechanism by which heme itself and Hb contribute to cell and protein damage in hemolysis has been extensively investigated during the past ten years or so and is well understood. Early studies on the transfer of heme to HPX from Hb in vitro revealed denaturation of the globin, apparent in the absorbance spectra [28]. In vivo studies with mice or rats or in vitro studies with non-neuronal cells have not yet pinpointed globin as a toxic agent. However, a recent in vitro study with mixed mouse neurons and glia in culture incubated with hemoglobin (Hb) and HPX (i.e., in the absence of haptoglobin (Hp)) led to white precipitates of globin protein in the medium on the surface of cells and within cells [43]. This was associated with significant levels of neurotoxicity, based on lactate dehydrogenase release from neurons, although not glia, after incubation with Hb (4 μ M, i.e., 16 μ M heme) and HPX (1mg/mL, i.e., ~17.5 μ M). When the mixed cells were incubated with heme, HPX completely protected both types of cells from heme toxicity. Protective effects of HPX were not considered due to the extracellular “scavenging” but rather were ascribed to intracellular protection

against heme toxicity via heme breakdown by HOs and protection against iron toxicity by Ftn, which was shown to be induced [44,45].

Published studies on Hp knockout mice have not recorded obvious globin precipitation, not even after PHZ-induced hemolysis, which is extensive, and not even after IV HPX. Also, Hb clearance from the plasma occurs at essentially the same rate in Hp-null mice and wild-type mice without obvious toxicity [46]. However, Hb clearance in mice differs from humans because although human Hp binds to CD163, mouse Hp does not and mouse Hb binds more tightly to CD163 than human Hb [46]. Without Hp, the kidney and muscle of mice [47] are the tissues most affected by oxidative damage in response to Hb. The globin toxicity detected in neuronal cells *in vitro* may be due to the very defined medium that may not contain the proteins and other molecules normally present in CSF (or at their normal concentrations). Perhaps further research on Hp-null mice and human brain cells will help determine if there are protective factors in cells and biological fluids *in vivo*, for example, other heme binding molecules that mitigate against globin precipitation or oxidation reactions, or if there are additional differences to be discovered between heme and Hb clearance in humans and mice.

2.6. Heme and Iron-Dependent Cell Death by Ferroptosis

There are clearly defined programs and mechanisms for cell death such as apoptosis or necroptosis in response to toxic agents and/or a variety of environmental conditions and stresses. Distinct morphological changes including abnormal mitochondrial size may also be manifest. An iron-dependent cell death pathway termed ferroptosis has been investigated and defined [48–50]. This requires intracellular oxidative stress that increases lipid peroxidation, decreases GSH levels, thus changing GSH/GSSG and increasing γ -glut amyl transferase, together with the instigator iron. Ferroptosis, as has been pointed out in Reference [49] has a distinct biochemical, morphological, and genetic “fingerprint”. Clearly, heme catabolism by HOs raises intracellular ferrous iron presumably predominantly in the cytosol. Several studies carried out with primary neuronal mouse cells, immortalized neuronal cells, and mice models of intracranial hemorrhage (ICH) [51] provide evidence that heme-mediated ferroptosis is part of heme toxicity in human hemolytic disorders. The responses of cells, including primary human hepatocytes [35], to heme-HPX where the intracellular redox state is maintained without ROS production have therefore been proposed to be anti-ferroptotic [3]. Also, studies with immortalized proximal renal tubule cells from HO1^{+/+} and HO1^{-/-} mice have provided strong evidence that it is not the generation of ferrous iron from heme catabolism solely that leads to ferroptosis. Furthermore, HO1 can have an anti-ferroptotic role in these kidney cells [52].

Heart attacks and stroke occur after blood vessel injury that activates platelets causing occlusion and thrombosis. Also, platelet micro particles are seen in patients with hemolysis including SCD. Intriguingly, heme increased P-selectin expression that drives vaso-occlusion in SCD is evident by not only endothelial cells but also by platelets (see Sections 4.1 and 4.2).

When human platelets suspended in Tyrode’s buffer were incubated with heme, filopodia-like structures appear at the cell surface in the presence of 5–10 μ M heme [53]. Further changes in the cell surface consistent with damage—“blebbing”—were seen in response to 25 μ M heme. Using sets of biochemical inhibitors and activators for various death pathways in conjunction with heme, the data supported that the platelets were neither undergoing apoptosis nor necrosis. There was a 5 fold increase in ROS and in markers of lipid peroxidation together with elevated HO1 and cytosolic iron in response to heme. In addition, oxidative stress was generated including effects on GSH with evidence for a role for the amino acid antiporter system Xc, which transports cystine into cells that is needed and used for GSH synthesis. The ROS production (i.e., increased hydroxynonenal) by heme was controlled when the iron-chelator deferoxamine, the ferroptosis inhibitor ferrostatin-1 or the HO inhibitor tin-PPIX were present. Thus, 10–25 μ M heme generated ferroptosis in platelets *in vitro*.

Protection of neurons against ferroptosis may come from the development of inhibitors of double-stranded RNA-dependent protein kinase, as shown recently in mouse hippocampal H22 cells [54]. This kinase responds to oxidative stress and ER stress and may be part of the pathology of

neurodegeneration with mitochondrial impairment as in Parkinson's disease and Alzheimer's disease that are both associated with increased brain iron [55–57].

Brain damage after ICH includes irreversible damage to neurons in primary and secondary injury, the latter is generally considered to be due to the Hb and heme from lysed RBCs. Apoptotic cells and necrotic cells have both been identified in the perihematomal region after ICH. Both ferroptosis and necrosis were identified as being involved in Hb- and heme-induced toxicity in primary cultured neurons, immortalized hippocampal neurons (HT22 cells), and in male mice after ICH [58].

Human monocytes may also be susceptible to ferroptosis induced by heme in transfusion-dependent patients, who often develop iron-overload. When human monocytic THP-1 cells in serum-free medium were incubated for 2 h with 20 μ M heme, there was an increase in ROS production together with an increase in cells with markers of necrosis but not markers of apoptosis [59]. The ROS production was prevented by N-acetyl cysteine (which can chelate metals), by iron chelators deferoxamine and deferasirox (the latter being more effective), or an inhibitor of NADPH oxidase that generates ROS. Ferrostatin-1 decreased cell death in response to heme, whereas erastin, which induces ferroptosis by inhibiting the system Xc, increased cell death implicating ferroptosis as the cause. The protective effects of serum proteins against heme toxicity by binding heme was clearly shown because the addition of albumin or fetal bovine serum decreased the amount of heme associated with the cells and heme toxicity even at very high concentrations of heme (20–80 μ M).

Overall, these studies demonstrate the potential that heme toxicity can kill cells by ferroptosis opening up an additional therapeutic approach in the clinic especially for neurodegenerative conditions and ischemic and hemorrhagic stroke. A review on the nexus between iron dyshomeostasis, excitotoxicity, and ferroptosis in stroke with a focus on ferroptosis has recently been published [60].

3. Potential Therapies to Replete HPX Levels for Protection against Heme Toxicity

As previously reviewed, HPX plasma levels can become depleted in patients with genetic hemolytic anemias (e.g., SCD, β -thalassemia) and hyper-hemolytic states for example after extensive blood transfusions [61,62]. In mice models of these hemolytic conditions, HPX replenishment by intraperitoneal (I.P.) or I.V. injection or by hepatic over-expression of HPX is often lifesaving. Thus, these animal studies formed the foundation for the concept of plasma protein replenishment therapies with HPX to combat the various damaging effects of heme in biological fluids. Unfortunately, Hp is often virtually undetectable in patients with hemolysis and so the current view is that a combination of HPX with Hp may prove most beneficial. Given the information on the effects of heme that include complement activation and activation of ferroptosis, additional combinations of therapies may prove even more beneficial to certain groups of patients.

3.1. Plasma Protein Replenishment Therapy

There are some unique aspects to the lipid profiles of patients with SCD, who overall have hypocholesterolemia, with low levels of both low- (LDL) and high-density (HDL) lipoproteins. In mice models of SCD, heme activates endothelial cells via toll-like receptor 4 driving events leading to vaso-occlusion [63]. Two independent studies of SCD patients describe differences in heme and HPX content of LDL and HDL [64,65]. Both addressed that oxidation of lipoproteins by heme and Hb is due to the ongoing hemolysis. In addition to HPX, α -1 microglobulin, albumin, and lipoproteins all bind heme. Vendrame and colleagues [64] considered that the varying levels of HPX would determine how much heme might intercalate in the lipoproteins in SCD patients. The LDL fraction had a higher heme concentration than did HDL, but the HDL fraction had a higher concentration of HPX. Thus, HDL is considered an important defense with potentially anti-inflammatory activity against heme toxicity but appears to be insufficient to protect endothelial cells from heme. Nevertheless, and intriguingly, HPX is linked with lipid metabolism because the cholesterol levels in the SCD patients were inversely correlated with circulating plasma HPX concentrations.

In the second study [65], the level of lipid peroxidation in SCD patients undergoing regular transfusions was measured using a sensitive assay—the end product of lipid peroxidation malondialdehyde. In SCD patients, Hp is essentially absent; however, HPX is present albeit significantly decreased. Consequently, as might be expected, both LDL and HDL oxidation were increased demonstrating the loss of protection against heme-mediated damage by the plasma proteins. At the postmortem of an SCD patient, oxidized LDL was found in the pulmonary artery. These observations reinforce the conclusion from other studies on SCD that replenishment therapy with HPX together with Hp may prove useful therapeutically; and this is also supported by studies with mice models of SCD (see Section 8).

3.2. Plasma Exchange

Plasmapheresis or therapeutic plasma exchange may provide another means to raise HPX and Hp plasma levels and may be more cost effective than recombinant proteins. Plasmapheresis is an established therapy and has been described as a symptomatic treatment for critically ill patients. Whole blood is removed, the plasma and cells separated, and the plasma replaced with another solution that may be specially prepared plasma from another person. Plasma is normally rapidly frozen after separation from RBCs and used after thawing within 24 h. The plasma is often used to remove and replace a patient's plasma when they have autoimmune disease. Plasma exchange was effective in reversing organ dysfunction in children with thrombocytopenia-associated multiple organ failure [66]. However, while generally considered safe, it can cause bleeding, allergic reactions, and also potentially increase the chances of developing a bacterial infection. Using a small group of three patients, plasma exchange was found to successfully replete HPX and Hp in two SCD patients that were refractory to RBC exchange [67]. There were significant increases in both HPX and Hp while heme decreased ~30%. Unfortunately, the condition of one patient deteriorated, their circulating HPX and Hp levels were not increased by plasmapheresis presumably because their liver was not able to sustain and synthesize plasma proteins.

3.3. The Scavenger Receptor LRP1/CD91 as a Target for Therapies When There Is Hemolysis in the Brain

In a murine model of ICH, the administration of an antagonist (a potential inhibitor of agonist action) and agonists (producing a known effect) of TLR7 led to changes in mRNA and protein levels of the scavenger receptor LRP1/CD91 that binds heme-HPX tightly ($K_d \sim 4$ nM, [68]). The expression of LRP1/CD91, HPX itself, and TLR7 were all increased when mice were treated after ICH induction with the TLR agonist imiquimod and decreased by TLR7 inhibitor ODN2088 [69]. The authors proposed that these studies reveal the potential for a role for TLR7 in the uptake of heme-HPX, although this was not shown directly. (See also Section 8.5.)

4. Protective Functions of HPX in Various Pathologies

The published clinical research on HPX within the past two years covers diverse clinical states: SCD, β -thalassemia, thrombolytic conditions, trauma with hemorrhage, intracerebral hemorrhage, hemorrhagic stroke, spinal cord injury, carotid artery disease, tuberculosis and diabetes, liver disease, kidney injury, pre-eclampsia, and microbial invasion of the amniotic cavity. In several studies, HPX has been investigated as a biomarker, used as a parameter to establish hemolysis or, in some cases, to assess the extent of hemolysis (“hemolytic index”). Depending upon the cause, extent, and duration of hemolysis there are significant differences in the amount of heme load from Hb that HPX counteracts. Additional factors arise when the immune system has been activated. Intriguingly, there are compensatory changes in the immune system in depression, bipolar disorder, chronic widespread pain and fatigue, and HPX expression in the CSF is increased in these patients. Notably, HPX is not an acute phase reactant in humans unlike Hp [70]. Furthermore, even when there is extensive or chronic hemolysis, as in SCD, high levels of Hb readily deplete plasma Hp but HPX is often still present

albeit at lower than normal levels. Evidence from both clinical and animal research now supports that changes in HPX may differ depending upon the source of Hb, i.e., IV Hb or RBC lysis.

In patients with traumatic hemorrhage, high levels of heme and Hb (four times greater than heme) were present in plasma and heme levels were overall greater than 5 μM . Both were higher than plasma non-transferrin bound iron. Interestingly, the plasma heme levels were proportional to the number of units transfused rather than to the length of storage time of the RBCs. Thus, plasma transfusions are anticipated to provide significant HPX and Hp for protection against the Hb/heme from RBCs. Nevertheless, in the face of high levels of Hb, HPX levels rapidly and extensively decline. Information on HPX's recovery time is needed in a variety of clinical situations and historical studies show that it may take several days [62]. In contrast to the recycling of HPX from liver, Hb–Hp complexes are degraded after endocytosis via the scavenger receptor, CD 163, in macrophages predominantly in the spleen. The extent of uptake by tissue resident macrophages including Kupffer cells in the liver is not known. When this Hb–Hp scavenger system is impaired or saturated, Hb–Hp remains in the plasma [71]. It is anticipated that in the clinic, information on the collective levels of heme, Hb, HPX, and Hp will prove important for assessing and determining patient treatments [72].

When using HPX as a biomarker, it is important to consider that HPX expression and its activity as a cytoprotectant differ with development, aging, and gender; as well as in response to the various diseases and conditions investigated. The age-dependent changes in HPX levels make newborns and young children vulnerable to heme-related pathology including oxidative stress and inflammatory responses, and can, under certain circumstances, drive inflammation. Furthermore, inflammatory responses generally are more prevalent with age and are exacerbated by genetic factors and by auto-immunity.

As described above (Section 2.4), the complement system is an integral part of the immune system and is composed of a plethora of plasma proteins that interact to fight infection in a variety of ways. Significantly, heme from Hb activates the complement system *in vivo* and in plasma *in vitro*. As with other immune system cells and proteins, this provides a means to protect in the early stages of disease including infections, and also to exacerbate inflammation and accompanying heme-driven pathology. These inflammatory conditions as well as the source of heme (IV heme, IV Hb or RBC lysis), determine which major organs and cells are affected. The lung is a more recently identified site of hemorrhage and heme injury, in addition to the liver, heart, kidney, and brain. Hemorrhagic brain damage weakens the integrity of the blood–brain barrier and heme is deleterious for the vascular system because of toxic effects on endothelial cells, smooth muscle cells, and, thus, blood vessel integrity.

4.1. HPX in Sickle Cell Disease

Unbiased analyses of the plasma proteome revealed several novel proteins that correlated with a therapeutic response (i.e., decreased hemolysis in young patients with SCD) during treatment with hydroxycarbamide (HC). Hydroxycarbamide is also known as hydroxyurea (an inhibitor of ribonucleotide reductase). As a long-time treatment for SCD, HC is thought to help prevent vaso-occlusion and vasculopathy by increasing γ -globin synthesis, altering adhesion factor expression by the endothelium, and by reducing the population of neutrophils. Plasma HPX levels significantly increased as the rate of hemolysis declined in response to HC, demonstrated by an increase in HbF% [73]. In addition, the levels of α -, β -, and γ -globin chains, the Hp-related protein, and complement C9 decreased. Thus, these protein changes were all associated with decreased hemolysis. Two other biomarker panels were investigated: one for inflammation (including ceruloplasmin, lower α 1 acid glycoprotein, CD5 antigen-like protein, and factor XII among others), and another for decreased coagulation (including lower factor XII, carboxypeptidase, and platelet basic protein). Overall, the changes in these biomarkers also support significant improvements in these children in response to HC, which may prove useful therapeutically in other conditions with extensive hemolysis.

4.2. HPX Status in Platelets in Hemostasis and Thrombosis

When normal hemostasis is overwhelmed by pathological factors, there is uncontrolled clot formation that leads to blocked arteries or veins. Platelets together with endothelial cells and coagulation factors are crucial mediators of both vascular hemostasis and thrombosis. The thrombi that develop in arteries are rich in platelets and form at the sides of or around thrombotic plaques even where there is a high shear flow. Thrombi in veins have fibrin and RBC, but form on intact endothelial cell wall only in areas where there is a low shear force. Hemopexin may have specific roles in trauma injury with hemorrhage, which is one example of a thrombotic condition. A clot is also an example of thrombosis where there is a local obstruction of blood flow. Clots can also form on wounds and in those circumstances aid healing. Thus, depending upon the site of injury monitoring changes in HPX in plasma and other biological fluids may be helpful in diagnosis, prognosis, and to predict the response to therapies in these patients.

In the setting of hemorrhage, hemostasis may occur by normal vasoconstriction and narrowing of blood vessels by an abnormal obstruction that includes atherosclerotic plaque via activation of the coagulation cascade (clot formation) or by physical ligation during surgery. These processes regulate vascular integrity and, thus, blood flow. The responses involve complex biochemical systems with multi-factorial processes that may even drive pathology in cancer. Hemopexin has been linked to various cancers (reviewed in Reference [74]) and approximately half of deaths of cancer patients with malignant tumors are associated with thrombotic events. Vascular occlusive diseases include atherosclerosis and lead to carotid artery disease and heart attacks, which is a leading cause of death in developed countries.

Evidence supports that the risk for thrombosis increases when there are larger platelets. Recent presumptive evidence supports that there are different populations of platelets, and two populations of human EDTA platelets were separated and identified based upon their mean volumes. Not only do platelets vary in size; but based on their diverse protein content that includes differences in HPX content, platelets have different functions [75]. Consistent with the link to thrombosis, large platelets were found to have more glycoproteins expressed on their cell surface and were found to be able to adhere better to collagen surfaces. Proteomic analyses revealed that 80/894 proteins differed in abundance between the large and small platelets. The differences among them are beginning to be defined. For example, the activation of integrin by ADP was greater in small platelets. Intriguingly, the proteins most relevant for heme and iron homeostasis including HPX, Hp and α -1 anti-trypsin, transferrin, and vitronectin, as well as immunoglobulins, were all more abundant in small platelets. It will therefore be interesting to understand the physiological relevance of these observations.

5. Hemopexin as a Biomarker for Concomitant Changes in Hemolysis, Inflammation, Heme, and Iron Metabolism—Some Challenges Include Age, Gender, and Type of Infection

Due to the developmental expression of HPX and of Hp and transferrin, the potential exists for key differences in the changes in these proteins as biomarkers (among other parameters of disease progression) between children and adults during therapy. This will be important especially in conditions complicated by inflammation in the presence of heme- (and iron-) toxicity known to be related to pathology. There will also be gender differences not solely because of estrogen-related effects in women but also to documented differences in iron-metabolism between men and women. Men with coronary artery disease have lower levels of plasma iron-transferrin and HPX but higher levels of inflammatory markers, macrophage infiltrates, and iron stores. There are increased levels of plasma Hb, increased numbers of Hb CD68^{+/+} macrophages, together with ferritin and transferrin receptor 1 in atheromas [76]. Thus, there are differences in both heme and iron metabolism in men and women with atherosclerosis. Such differences need to be fully established to help physicians better assess the progress of their patients from diagnosis through therapy.

Women are more prone to inflammatory diseases and are three times more likely than men to develop rheumatoid arthritis. Once more, this is not solely due to the presence of estrogen because

several disease-associated genes reside on the X chromosome. Increases in inflammatory markers were recently found in the CSF of seven rheumatoid arthritis patients, which also correlated with fatigue. Fatigue is considered to be related to changes in the central nervous system. For over twenty years it has been known that tumor necrosis factor (TNF), which is an inflammatory cytokine, plays a major role in arthritis [77] and that targeting TNF (for example with a monoclonal antibody such as infliximab) is effective and improved the symptoms of fatigue. An “arthritis proteome” identified that TNF blockade with infliximab decreased 35 proteins in the CSF. Hemopexin was one out of seven candidate proteins and changes in HPX are known to be associated with arthritis [78]. Contactin 1 and complement factor B, both known to increase with systemic inflammation, were also decreased. Both fibrinogen γ -chain and complement factor B decreased in response to the TNF inhibitor. The decrease in HPX in response to infliximab was considered to be due to the amelioration of both systemic and CNS inflammation because markers of inflammation were decreased in the CSF. Overall, these data support a relationship between arthritis symptoms and CNS inflammatory pathways as targets of the TNF inhibition. Although the infliximab is stated to not cross the blood–brain barrier, TNF α crosses via receptor transport. Thus, systemic TNF α can damage the endothelial cells of the barrier creating localized permeability and infliximab peptides were detected in the CSF. Intriguingly, the circumventricular organs were proposed as an alternative route into the brain. These are brain structures with extensive and permeable capillaries, which allow the passage of molecules both into and from the brain (e.g., certain hormones [79]), and which contain cells with TLR4, a target of LPS and heme [80].

Pregnant women are at risk for heme-related toxicity when they develop the potentially lethal condition of preeclampsia, typically in the third trimester. Preeclampsia is associated with life-threatening high blood pressure among other symptoms and occurs in about 3–7% of all pregnancies. The pathology is not well understood but is thought to originate from changes in the placenta. It has been associated with autoimmune disorders and blood vessel problems, and there are several other risk factors including a history of diabetes, high blood pressure or kidney disease. Several studies have provided evidence that changes in the level of the heme-binding protein alpha-1 microglobulin (A1M) may correlate with the severity of this condition. As this condition becomes more severe, A1M levels increase and may reflect increased endogenous oxidative stress [81]. Hemopexin levels also changed in women with preeclampsia and may be related to changes in cardiac function. Hemopexin was proposed to become depleted in the early stages of pregnancy and to rise in the third trimester when the high blood pressure symptoms of preeclampsia become apparent. However, confirmation of this will require further analyses to fully elucidate. Nevertheless, biomarkers for Hb sequestration and degradation pathways may prove informative in assessing the mother’s status in preeclampsia.

HPX as a Biomarker for Sepsis

Evidence from both clinical and animal studies [35,61] shows that HPX has potential as a biomarker in sepsis, as reviewed previously [62]. A panel of biomarkers is urgently needed for sepsis and septic shock patients due to the prevalence and high incidence of mortality and health issues. Patients who recover from sepsis endure long-term effects that include both physical and mental problems.

Vimentin may prevent lymphocyte apoptosis and be anti-inflammatory and, thus, may be a useful new target in sepsis [82]. Vimentin is one of the main constituents of the intermediate filament proteins that maintain cell shape, in part, by stabilizing the interactions of the cytoskeleton and perhaps more importantly by helping cells resist damage including apoptosis. Both serum and lymphocyte levels of vimentin were significantly increased in these two groups of patients. Hemopexin was one of 56 proteins identified in plasma, finally ranking fourth in a protein network of 12 based upon interactions with eight other proteins in the network (defined as “betweenness centrality”). Thus, HPX has been linked in a co-expression protein network with vimentin in patients diagnosed with sepsis and septic shock [82].

Ekregbesi and colleagues [83] tested the hypothesis that inflammation- or infection-associated hemolysis contributes to sepsis-associated anemia and also leads to detectable HO1 in plasma. Hemopexin levels were used as one of several parameters of hemolysis. The prevalence and extent of anemia, the presence of inflammation and levels of HO1 were determined in a cohort of ICU patients within 12 h of admission. Most patients (~83%) were deemed anemic and hemolysis was evident because of heme in the plasma (median levels ~21 μ M), low Hp, decreased HPX (median levels 9.6 mg/dL, normal levels ~77 mg/dL) together with elevated HO1. Hemolysis was accompanied by IL-10-associated inflammation and the presence of IL-6 and TNF α . In this study, HPX was neither associated with the anemia nor with morbidity. However, all of the cytokines that were increased, including IL-6 and IL-10, were weakly inversely correlated with HPX. The IL-6 levels correlated with IL-10 levels and HO1 induction was associated with IL-10 levels rather than the extent of hemolysis. In contrast to other studies on sepsis [35,84], the severity of sepsis was considered better indicated by the rise in plasma IL-10 and the HO1 levels rather than the low HPX levels on admission.

Iron is needed by pathogens for cell growth and to establish an infection. Circulating ferritin light chain (L-ferritin, FtL), needed for iron homeostasis, has recently been shown to play a protective role in the cecal ligation and puncture model in mice that produces, as the gut contents leak, polymicrobial sepsis. [85]. There are known gender differences in iron markers and, in the clinic, serum ferritin levels together with several other markers are used to assess iron stores. Ferritin light chain is a secreted form of ferritin and low in iron content, which predominates in the circulation and whose function has been enigmatic for decades. The increase in serum ferritin in response to infection indicates a role in the acute phase response and this study supports such a link. The role of myeloid cell specific ferritin, using ferritin H (FtH) deleted cells, was also addressed. The FtH deficiency dampened the inflammatory response in vivo and decreased the response in vitro of bone marrow derived macrophages to LPS. However, it did not alter phagocytosis or bacterial clearance. Hemopexin levels have been implicated as protective in a previous study with this model of sepsis [35] but were not apparently affected by the loss of FtH in the myeloid cells (perhaps due in part to the wide range of individual values among the mice compared with those of Hp). FerritinH and FtL chains were both increased. FerritinL is anti-inflammatory because when it was elevated there were lower levels of liver injury, decreased inflammatory cytokine levels and increased inducible nitric oxide synthase, which is protective in the recovery from post-ischemic inflammation.

As noted by Chaim Hershko [86] in a review entitled "Iron, Infection and Immune Function", the term "nutritional immunity" was first used in 1973 to demonstrate the importance of preventing human pathogens from multiplying and dividing to establish an infection [87]. It is well known that both iron and heme (the latter presumably as an iron source), are necessary for the growth of pathogenic bacteria. The need for heme is apparent from the plethora of uptake systems that bacteria employ to ensure the capture of heme, many of which are redundant ready to bind: heme itself, Hb, heme-HPX or Hb-Hp [88]. Therefore, HPX by sequestering heme acts in nutritional immunity and to overcome this heme/iron limitation many human pathogens express receptors for heme-HPX uptake. HPX has recently been linked to host immunity and the anti-microbial action of IL-22 because, in mice, limiting infection depends upon HPX but not on Hp, in spite of the fact that the expression of both proteins in the liver is induced by IL-22. Mice were infected with either *Escherichia coli* that causes sepsis in humans or *Citrobacter rodentium* that normally resides in the gut of mice and can also cause a wide range of infections in humans. *Citrobacter rodentium* shares several features with *E. coli* in how it establishes infections. After WT and IL22^{-/-} mice were infected, C-reactive proteins, serum amyloid A1, α 2-macroglobulin as well as HPX and Hp were increased in plasma. Hepatic levels of HPX and Hp mRNA rose after infection in WT mice and in the IL22^{-/-} mice, but to a significantly lesser extent; consequently, Hb accumulated in the plasma due to the ongoing hemolysis caused by the infection. The presence of HPX was sufficient to clear *C. rodentium* in WT mice but not in HPX^{-/-}, Hp^{-/-}HPX^{-/-} or Hp^{-/-} mice. Furthermore, HPX reduced the level of infection (judged by decreased numbers of circulating bacteria) in IL22^{-/-} mice regardless of whether it was from an intravenous or oral infection.

6. Organs That Endure the Worst Heme Toxicity Include the Lung, Liver, Kidney and Brain

6.1. Lung Disease/Injury

Lung injury comes from exposure to many different kinds of inhaled compounds and can lead to pulmonary hemorrhage. Blood gets into the alveolar spaces when there is disruption of the alveolar capillary membrane (diffuse alveolar hemorrhage) [89]. The heme levels in plasma of patients with chronic obstruction pulmonary disorder (COPD) was significantly increased compared with non-smokers [90]. As described in Section 2.3, high intracellular heme levels lead to oxidative damage of proteins, which in turn activates the UPR. Consistent with this, the levels of one key regulator of the UPR, namely, GRP78, were extensively increased in the lung of COPD patients.

Chemical weapons were first used in World War 1; furthermore, chemical weapons such as chlorine may have been used in Syria within the past two years, while mustard gas and nerve agents were used against residents in Northern Iraq in 1988. The toxic gases chlorine and bromine damage lungs. Hemolysis developed in mice exposed to bromine by inhalation with heme-mediated lung damage that was ameliorated by a single IV treatment with recombinant human HPX [91]. Abnormally high levels of plasma heme were detected in the mice up to 14 days post bromine exposure. This was associated with increased lung elastase activity, which may induce the UPR via the PERK/CHOP arm [90]. The protection by exogenous human HPX was due to the counteracting high heme levels in part by sequestering heme, thus relieving RBC lysis after bromine exposure but also by stimulating the level of endogenous HPX circulating in plasma of these mice. The mechanism of the induction of endogenous HPX is currently unknown and may be occurring, but unrecognized or detected, in other mice models of hemolysis and HPX replenishment. Incidentally, bromine is present in cigarette smoke and, thus, represents a risk factor for chronic smokers.

Both these studies on patients support that HPX plasma replenishment is of potential therapeutic value in different kinds of lung injury that produce lung fibrosis and emphysema; and, especially, in combination with drugs that relieve the UPR and ER stress in lung cells.

6.2. Liver Disease/Injury

Many different types of liver pathology ultimately lead to liver cancer including chronic liver disease scarring such as cirrhosis. These pathologies can be caused by hepatitis B or C infection, by alcoholism or by fat accumulation (i.e., fatty liver). Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and there is a greater prevalence of HCC in men. Hepatocellular carcinoma can be cured if the liver disease is identified early enough. Hepatocellular carcinoma can develop when there is cirrhosis and the only cure is a liver transplant. Thus, identifying biomarkers of liver disease and especially HCC is of crucial importance.

Potential biomarkers including those for liver disease may be discovered in the content of exosomes, which are very small endocytic membrane bound vesicle derived from all cells (as far as is known). Exosomes were first discovered in maturing mammalian reticulocytes [92]. Their content of proteins and miRNA provides a window on the cells that release them providing information not only on the cell of origin but also potentially that of the tissues and organs from which the cell is derived. Thus, exosomes are providing useful biomarkers consisting of proteins and peptides that are readily being identified and quantitated by proteomic profiling. For example, HPX levels together with properdin were decreased in exosomes of patients with HIV with active drug use compared with HIV or with HIV patients with alcoholism. Properdin is the only known positive regulator of complement that stabilizes the alternative pathway convertases C3bBb [93]. Thus, HPX and properdin are poised to be potential markers for co-morbidity in drug abusers who were HIV positive, [94] indicating both liver disease and complement activation.

Several changes in carbohydrate structure of glycoproteins have been linked to liver disease [74] and another means to monitor liver disease utilizes identification of aberrant core fucosylation of plasma glycoproteins as biomarkers. Such alterations are readily assessed from patient serum samples.

Human HPX has one O-linked oligosaccharide chain at its N-terminal threonine and five additional N-linked chains [95]. Core fucosylation of glycoproteins is site specific and clearly identifies a protein. A multiplex LC-MS/MS array was used for the serological assessment of liver disease and to determine the extent of fibrosis of the liver. Key and specific changes (both increases as well as decreases in fucosylation of several N-linked glycoproteins) were detected at the stage of fibrosis in a comparison of the data garnered from healthy control, fibrotic, and cirrhotic livers. While five proteins were identified that marked liver fibrosis including transferrin, ceruloplasmin, α -1 antitrypsin, and vitronectin, there were also additional changes found in cirrhosis including HPX. Of relevance here, the changes included aberrant fucosylation at N630 of transferrin, N187 of HPX, and both N138 and N762 of ceruloplasmin, and N354 of clusterin [96]. Thus, detectable changes in fucosylation levels of several key plasma proteins including HPX, either increase or decrease as the liver disease worsens.

Additional monitoring of plasma proteins via glycan screening, including HPX [97], may also provide evidence of changes in liver function, thus aiding in the early detection and progress of HCC. This technique involves protein extraction using lectin-affinity binding of plasma samples. Preliminary data using MALDI-MS or ESI analysis show that glycan screens may prove useful to detect changes disease-related changes in HPX, Hp, and kininogen as liver function deteriorates.

6.3. Kidney Disease/Injury

It is well established that acute kidney injury occurs in hemolysis and that the kidney is susceptible to damage by Hb and heme. Thus, this organ is particularly vulnerable when plasma Hp and, eventually, HPX are both decreased.

Severe malaria pathology is associated with inflammation, endothelial cell activation and hemolysis. Furthermore, acute kidney disease is a known complication of malaria, especially in infection by *Plasmodium falciparum* and contributes to a high rate of mortality. One potential biomarker of acute kidney injury is chitinase-3 like 1 protein (CH3L1 YKL-40, HCgp39). It has also been associated with burn injury and bacterial infection. In spite of its name, CH3L1 is not an enzyme and does not act on chitin, which is an N-acetyl glucosamine polysaccharide that is expressed by endothelial and immune cells. However, CH3L1 on epithelial cells may bind chitin binding proteins expressed by bacterial strains that are potentially pathogenic. Thus, CH3L1 has been proposed to enhance the adhesion and binding of pathogenic bacteria. A high ratio of heme to HPX in children with malaria indicated severe disease with adverse clinical outcomes, and 46% of the children had acute kidney injury [98]. Interestingly, children with the highest heme levels (and lowest HPX) had significantly lower levels of parasitemia and the heme to HPX ratio decreased with recovery demonstrating a useful parameter to assess the severity of malaria.

Following up on these studies, Conroy et al. [99] investigated in a clinical trial if inhaled NO was an effective adjunct therapy for severe malaria in children in Uganda and investigated changes in CH31L as a potential biomarker for kidney damage. The rationale and hypothesis that children with malaria might benefit from exposure to nitric oxide (NO) as an adjunct therapy was first proposed in 2011. This was based on the response of adults with malaria to nitric oxide as a therapy, where NO minimized endothelial cell activation, data from animal models of malaria, and the improvement of adults with severe malaria in response to the NO precursor arginine. Plasma biomarkers were measured for 4 days and, finally, at day 14. There was an increase in CH3L1 in pediatric severe malaria (0–18 years of age) consistent with acute kidney injury. Several panels of plasma biomarkers were investigated including those for endothelial activation (Ang-2, sICAM-1). Hemopexin was included in the panel for intravenous hemolysis together with lactate dehydrogenase (LDH) and heme. The levels of CH31L correlated with markers of inflammation (e.g., surface receptor triggering receptor-expressed myeloid cells, [100]).

Kidney transplants are carried out worldwide and generally have a high success rate, although they do have risks. Nevertheless, markers for rejection after kidney transplantation are needed. Some research supports that in certain cases due to low abundance in the circulation, proteins may

be more readily detected in urine than in plasma. Hemopexin, together with tetraspanin-1, has the potential to be a novel urinary exosome marker in adult T-cell-mediated rejection (TCMR) in kidney transplant recipients detected by nanoscale liquid chromatography coupled to tandem mass spectrometry. Tetraspanin-1 is a member of a family of 4-pass transmembrane proteins implicated in cell adhesion, migration and proliferation, which interact with membrane proteins including integrins. Large multimolecular complexes organize cell–cell interactions and matrix–cell interactions and consequently activate signaling pathways. Tetraspanin-1 is overexpressed in several different cancerous cells and plays a role in carcinogenic progression including the migration and invasion by malignant cells [101]. Intriguingly, both HPX and tetraspanin-1 were significantly increased in the TCMR patients [102].

6.4. Brain/Injury and Neurotoxicity

The brain is protected by the integrity of the blood–brain barrier that prevents toxic plasma components like heme, intact blood cells, and pathogens from reaching the brain. However, most brain injuries including stroke and hemorrhage (intracerebral, subarachnoid, stroke) cause damage within the brain that leads to damage of the protective blood–brain barrier and leakage of molecules across these cells. Neurotoxicity comes from the presence of Hb, heme, and iron derived from its catabolism, as well as inflammatory events all with consequences for the different types of brain cells. In brain cells, the HO2 isozyme predominates although the heme-inducible HO1 is also present. The role for the various cell types in the response to brain injury is under active investigation. To better assess brain injury and the response to both the brain damage, markers in the CSF are being defined not just for hemolysis and heme toxicity but also for inflammation and the activation of endothelial cells. Such information will hopefully guide and improve therapeutic approaches. In this regard, iron uptake via transferrin and, intriguingly, via ferritin into the brain are influenced by gender and genotype [103].

Hemopexin is present in the CSF, likely produced by neurons and ependymal cells [22,23]. Several studies have revealed that CSF HPX levels change in response to neurological diseases including Alzheimer's [104] as well as brain hemorrhage [24]. Once more, HPX's role as a potential therapeutic was reinforced in a recent review on subarachnoid hemorrhage, which compared the pathophysiology in humans and rodents [105], and Hp is active in outcomes in patients after subarachnoid hemorrhage [106] or after ICH [107]. When there is impairment of the blood–brain barrier both brain and systemic HPX and Hp systems may interact. However, it is most likely that brain inflammation drives the local synthesis of HPX. Emerging treatment strategies for clearing hemoglobin/heme from the brain after intracranial bleeding were presented in a comprehensive review by Galea and colleagues [108]. Also, the clearance of heme through the inducible HO1 pathway as a treatment for ischemic stroke has recently been reviewed [109]. We refer the reader to these publications and have not presented these topics in detail here.

Further insight into the development of neurotoxicity and inflammatory conditions in the brain has now come from using the neurotoxic effects of lymphodepletion chemotherapy. Endothelial cell activation by cytokines leads to disseminated intravascular coagulation, capillary leakage, and increased blood–brain barrier permeability. When the blood–brain barrier is permeable, it no longer protects the CSF from exposure to high concentrations of systemic cytokines including IL6 and IL4 [110]. Such severe endothelial activation may lead to multiple areas of hemorrhage. Interferon- γ (IFN- γ) induces brain vascular pericytes to secrete cytokines that activates endothelial cells. Most patients developed cytokine release syndrome initiated by T cell activation with fever and hypotension that was associated with high IL-6. As described in Section 12, IL-6 can alter the response of models of liver and neuronal cells to cytoprotective heme-HPX signaling.

One of the most detailed studies on the relationship between protection of human brain cells via heme clearance by the HPX and Hp systems and activation of localized inflammatory response comes from a study by Righy and coworkers [24] on patients after either intracerebral or subarachnoid hemorrhage. Hemorrhagic stroke depletes the CSF of HPX and Hp as does subarachnoid hemorrhage

as heme and iron increased in the CSF [24]. A comparison of the levels of heme, iron, HPX, and Hp in the plasma and CSF at 24, 48, and 72 h post-ictus revealed that iron levels were extremely high throughout. Plasma HPX levels were essentially maintained and Hp was lower at the 24 h time point, but the values of both varied widely including some patients who had undetectable protein levels. The CSF HPX decreased over the first 48 h post ictus, but then appeared to increase by 72 h. The Hp appeared to increase over this three-day period as it did in the plasma. Significantly, these changes were linked to survival in that plasma iron, and heme over the first 48 h were highest in the non-survivors. These data indicate that iron overload dominated perhaps more so than toxicity from heme per se. Nevertheless, extracellular Hb is clearly toxic, and when the defenses are overwhelmed, then some protection is derived from HPX. Furthermore, 3 days after admission, IL-4 levels in the CSF were higher in the survivors, whereas both IL-6 and IL-8 in the circulation were significantly increased in the non-survivors. These cytokine responses suggest that some anti-inflammatory protection was taking place in the brain. Thus, treatments for brain hemorrhage using local anti-inflammatory agents may have a protective role in addition to heme toxicity attenuation.

7. Hemopexin Metabolism Is Altered When Inflammation Occurs in the Brain in Non-Hemorrhagic Conditions

There is a novel compensatory immune-regulatory reflex system (CIRS) in a large population of patients diagnosed with depression and bipolar disorders who are particularly affected. Markers to demonstrate that there was activation of an immune-inflammatory response system (IRS) are being sought. In this study, there is an associated increase in pro-inflammatory M1 macrophages and T-helper (Th)-1 pro-inflammatory cytokines including IL-6 trans signaling, together with positive acute phase proteins and complement. In fact, evidence for activation of the immune system during major episodes comes from the findings of increases in T-helper type 2 cells that protect and secrete several interleukins (IL-4, -5, -9, -13, and -17). These cells are required for humoral immunity as well as regulatory T cells that act against extracellular pathogens and facilitate cell repair but can contribute to chronic disease such as allergies and asthma. A larger increase was observed in IL-4 and IL-10 production with IL-6 signaling and transcription of the sIL-1 receptor antagonist soluble IL-2, TNF α receptors, and HPX together with four acute phase reactants Hp, α -1 acid glycoprotein, α 1-antitrypsin, and ceruloplasmin. (Based on clinical data, HPX is not an acute phase reactant in humans [62], although the human *HPX* gene contains an “active” IL-6 response element [111]). Therefore, this is evidence of the primary immune-inflammatory response with spontaneous recovery or in response to treatment with anti-depressants. Unfortunately, after each acute episode, the patients developed a sensitized IRS and CIRS response. Thus, there remains a need for composite biomarker(s) to estimate the relative ratio of these two responses in these and other mood disorders that clearly have a biochemical basis [112].

There is also a lack of biomarkers for chronic widespread pain. This occurs in chronic fibromyalgia syndrome that manifests as general muscle pain, tiredness, together with anxiety and depression. Even cognitive disabilities may exist. A preliminary proteome was analyzed using 2D electrophoresis of plasma samples to assess if different patterns in plasma proteins could be found that changed in response to pain or to the psychological distress. Hemopexin together with two complement components and clusterin were proteins associated with the psychological aspects of this condition. These were considered to represent immunity, iron, and lipid metabolism. On the other hand, proteins associated with metabolic and immunity process (kininogen, fibrinogen γ -chain, and ceruloplasmin) were linked to pain intensity. It seems that several psychiatric disorders have immune activation, where changes in HPX metabolism help to identify them [113]. Further study may provide new knowledge of factors that influence HPX metabolism and its levels in plasma and CSF.

8. Animal Models of Human Hemolytic Diseases and Conditions

Animal models of human diseases and potential therapies for them allow investigation of target tissues and cells that are not always possible in patients. As briefly summarized in the Introduction, the

extracellular antioxidant protective role of HPX is only one means whereby the HPX system protects against heme toxicity. In a variety of animal models of hemolysis, the protective effect of HPX has been recently linked to increased HO1 activity in hepatocytes and other cell types. Importantly, it is the rate at which heme builds up in cells, the intracellular concentration of heme, and cofactors for HOs, which affect the extent of heme metabolism by HOs and, thus, the potential for toxicity either in response to heme or to increased ferrous iron from heme catabolism, as recently described [3]. When cells are incubated with “free” heme *in vitro*, even when albumin is present in culture medium, heme very rapidly accumulates in cells—within seconds and minutes. Also, because this process is unregulated compared with the endocytosis of heme-HPX, toxicity readily develops (see Section 2 above for the toxic effects of heme at ~10–25 μM or higher). Heme oxygenase enzymes are quickly induced upon entry of heme across the plasma membrane and into the nucleus. Under these conditions, we do not know what limits HO enzymatic activity or the rate of increases in ferrous iron in the cytosol or, simultaneously, carbon monoxide (CO) levels. What is a toxic threshold for intracellular heme concentrations? Does CO from heme catabolism inhibit the intracellular heme-proteins such as those in the electron transport chain as it does cytochrome P-450? Clearly, a certain amount of cytosolic ferrous iron from HOs can readily be dealt with by cells in response to the changes in the proteins of iron homeostasis via the Fe/IRP/IRE system. However, what might be a “breaking point” for toxicity? The cytosolic “labile” ferrous iron pool is ~1 μM whereas iron stored on ferritin is 0.7–0.36 mM. Certainly, defense against iron toxicity may be equally or perhaps more important than heme sequestration, for example, after brain hemorrhage in patients, ([24], see Section 6.4).

Protection of cells against heme involves both sequestration of extracellular heme as well as intracellular events that include induction of HO1 activity. Also, HO1 responds to many stimuli not heme solely. In animal and cell studies, evidence for a role for HO1 in cytoprotection often comes from the use of metal porphyrins (e.g., cobalt-, tin- or zinc-protoporphyrin IX) that have been shown to inhibit HO enzymatic activity *in vitro* (but also induce HO1 mRNA to very high levels). Thus, HOs have been linked to the cytoprotection of the HPX system because protective effects of HPX are “lost” in the presence of these heme analogs.

8.1. Hemopexin Reduces Inflammation and Decreases Oxidative Stress in Models of SCD

In mice models of SCD [114], a single infusion of human HPX (1 $\mu\text{mole/kg}$; ~14 μM) together with equimolar amounts to Hb increased HO1 in liver, skin, and kidney within 1 h. Hemopexin inhibited stasis from the Hb (less than 1% at 24 h) compared with untreated mice (10–11%). Plasma Hb and heme levels were unchanged 1 h after infusion of HPX. Also, and importantly, HPX did inhibit the induction of markers of inflammation such as inflammatory cytokines (chemokine 5), but did not affect the plasma levels of TNF α , IL-10 or IFN γ . Furthermore, HPX inhibited NFkBphosphoP65, considered to promote stasis via activation of pro-inflammatory adhesion factors. Furthermore, the increased levels of hepatic oxidative stress indicated by the presence of hydroxynonenal were ameliorated by HPX. In previous studies from Vercellotti, Belcher, and colleagues [63], heme sequestration by HPX mobilization to the surface of endothelial cells of Weibel–Palade body protein P-selectin and von Willebrand factor that drive vaso-occlusion, a potentially lethal pathology of SCD. Because induction of hepatic HO1 and engineered hepatic over expression of HO1 inhibited stasis in these mice, the inhibitor tin-PP was administered at levels to block HO1 activity, which prevented the protection by HPX. Furthermore, CO administration to the mice before Hb fully restored protection by HPX. To support the relevance of the mice model to clinical SCD and to show directly that heme induces proteins such as P-selectin needed for vaso-occlusion, human umbilical vein endothelial cells were incubated with heme (10 μM for 30 min or histamine as a positive control), which readily induced P-selectin.

Overall, HPX was considered to inhibit stasis by inducing HO1 and, thus, raising CO levels. One challenge with mice as a model for human hemolytic conditions including SCD is that, unlike in humans, Hb is cleared via CD163 in macrophages and, thus, does not require Hp. Intriguingly, the authors point to novel hepatic uptake of Hb–Hp complexes into hepatocytes as an additional clearance

mechanism that will need defining. It may be related to the process in rats documented in 1972, where 85–95% of the radioactivity from ^{59}Fe -Hb was present with liver parenchymal cells whether injected IV alone or with Hp [115]. The conclusion was that plasma protein replenishment therapies with both HPX and Hp might be best for SCD patients, especially those with acute chest syndrome.

Evidence is accruing for differences in the response of HPX, Hp, and HOs to intravenous Hb or Hb from intravascular hemolysis compared with Hb release after the administration of RBCs. Graw et al. [116] showed that an infusion of stored red blood cells increased hepatic HO1 mRNA and also in kidney and spleen. In addition, HO1 levels in all these organs were increased by co-infusion with HPX (or albumin or Hp).

The excessive heme from erythrophagocytosis causes the loss of resident red-pulp macrophages. In this context, there are some unexpected relationships in mice between HO1 activity and hepatic HPX expression (that drives plasma levels), which is altered when spleen function is impaired. Spleen macrophages may die due to the toxicity of heme when HO1 is inhibited or when heme export via plasma membrane transporter FLVCR1b [117] is impaired because proteins heme such as HPX or albumin are needed as extracellular heme acceptors to drive transport down the concentration gradient. Hepatic HPX is increased significantly when HO1 is inhibited or absent as shown in studies with *HO1* knock out mice [118]. These studies showed that the status of spleen macrophages affects HPX levels both in the liver (HPX mRNA) and in the circulation. The implication of this includes whether some of the effects in mice given Hb, RBCs or in hyper-hemolytic states (after phenylhydrazine) are due to the changes in endogenous levels of HPX, especially when inhibitors of HO1 are administered. Thus, when both spleen macrophages and Kupffer cells are involved in RBC clearance, there may be changes in the regulation and activity in the various defenses against plasma Hb/heme including the HPX system compared with low levels of hemolysis (or IV heme or Hb in animal studies). In the light of observations on toxicity from globin, when HPX is present with Hb in $\text{Hp}^{-/-}$ mice or in humans with hemolysis sufficient to have depleted Hp, there must be mechanisms *in vivo* that mitigate extracellular globin toxicity in Hp deficiency. Alternatively, globin precipitation is a key missing part of the complicated puzzle that is the mechanism of Hb/heme toxicity.

8.2. Hemopexin Protects the Blood–Brain Barrier

Using a model of cerebral ischemia-reperfusion injury in rats, focal cerebral ischemic and reperfusion, Dong and colleagues [119] showed that HPX alleviated cognitive dysfunction rapidly when injected intracerebro-ventricularly once reperfusion was initiated. This protective effect was also linked to HO1 activity because it was reversed by the HO1 inhibitor, zinc-PPIX [119]. In a similar experimental approach, HPX was protective after cerebral ischemia/reperfusion injury and protection was lost when zinc-PPIX was given [119].

Importantly, HPX in the cerebral spinal fluid (CSF) helps to maintaining the integrity of the blood–brain barrier, in part, because HPX and the induction of HO1 helps new blood vessel formation by supporting both the migration and differentiation of endothelial progenitor cells [120]. The administration of HPX allowed the rats to recover after cerebral ischemia, as assessed by the magnitude of their synaptic plasticity, but this was blocked by administration of the HO1 inhibitor ZnPPiX. Thus, HO1 activity is implicated in the protection by HPX of both the endothelial cells and blood–brain barrier integrity in rats with cerebral ischemia [120].

The HPX, LRP1/CD91 positive cells, and heme were detected from day 1 in both the clot and in the tissue surrounding the brain hematoma, and perihematoma in piglets were injected with autologous blood into the right frontal lobe of the brain [121]. The administration of the iron-chelator deferoxamine significantly decreased all of these indicators of the HPX system. Unfortunately, the full significance of protection by HPX in these studies is not yet clear because deferoxamine does not cross the blood–brain barrier. Therefore, when this chelator is reported to be effective, it is presumptive evidence for an impairment of the blood–brain barrier allowing molecules from the plasma to reach the brain and potentially vice versa.

Importantly, data from a rat model of the sports-related head injury supports that HPX is a potential diagnostic for a frequent form of traumatic brain injury—diffuse axonal injury. Biomarkers for brain damage are urgently needed because such injuries, as is now evident, occur even in recreational and professional sports. They represent a serious and complex brain injury with significant morbidity and mortality. Low linear and angular accelerations to the head [122] replicate axonal injury and hemorrhagic tears that represent the histology and neurological changes of axonal injury. Both HPX and glyceraldehyde-3-phosphate were 2 out of 58 proteins in plasma deemed potentially diagnostic for this type of brain injury.

A role in olfactory system development is perhaps one of the most intriguingly recent discoveries about HPX. Neurogenesis relies on neural stem cells of the subventricular zone that reside in a specialized niche. Abnormalities develop in the SV2/olfactory bulb pathways of HPX-null mice that impair neuroblast migration in the subventricular zone as well as the rostral migratory stream. Exogenous HPX inhibited apoptosis and promoted the migration and differentiation of cultured neural stem cells [123].

Furthermore, HPX is protective against bleeding and blood loss in trauma injury, which is the leading cause of death for the young, including infants, and in the middle aged. Trauma includes the severe injury from automobile accidents, industrial accidents, and on the battleground. These severe injuries lead to significant blood loss and internal bleeding may damage the lungs. Both blood loss and hemorrhagic shock after trauma are routinely treated with massive blood transfusions to maintain Hb oxygen levels. This may well be life saving for patients but also presents a hazard to their health because of extensive RBC lysis from blood transfusions. It is well established that RBCs deteriorate; the longer that blood and packed RBCs are stored, the more likely RBCs will lyse and release Hb leading to heme toxicity. Thus, extensive transfusions are associated with poor recovery due to the Hb/heme load for the patient and, significantly, there is an increase in organ injury. Furthermore, bacterial infection becomes more prevalent in part because nutritional immunity is overcome by the systemic heme and iron loads.

8.3. Hemopexin Is a Part of Nutritional Iron Defense System in the Lung

Mice have provided novel information on the dangers from heme leading to the development of pathology in the lung, which are exacerbated by bacterial infection of the lungs following, for example, trauma-induced hemorrhage [72]. Most human pathogens have multiple ways to acquire heme via surface receptors and transport systems for heme itself, or for Hb, Hb-Hp, heme-HPX and for iron-transferrin. After trauma hemorrhage, the mice were resuscitated with plasma and leukocyte-reduced RBCs that were either fresh or stored for 14 days. Two days later, their lungs were infected by administering the opportunistic pathogen, the K-strain of *Pseudomonas aeruginosa* that has many virulence genes. This Gram-negative rod bacterium is associated with bronchial infections in patients with cystic fibrosis and is predominantly responsible for the nosocomial infections in hospital intensive care units. Also, it has developed resistance to many antibiotics. As expected and linked with hemolysis, infusion of stored RBCs increased lung injury and the severity of bacterial infection determined by a higher bacterial count in the lung and increased pulmonary edema. These changes were greater than the responses to fresh RBC infusion. Changes in plasma Hb, heme, and non-transferrin bound iron were measured longitudinally over 48 h after trauma hemorrhage. When IV HPX (1 mg/kg) was administered immediately before resuscitation, the mice had significantly lower levels of Hb compared with mice exposed to stored RBCs. These data support that the increased lung injury (pulmonary edema and lung bacterial count) after resuscitation with stored RBCs is due to the heme. Following up on observations that heme can activate TLR4 driving the pathology of SCD in mice [63], it was shown that administration of HPX or pharmacological inhibition of TLR4, during the traumatic hemorrhage and resuscitation completely prevented the *P. aeruginosa*-induced mortality following resuscitation with stored RBC. The TLR4^{-/-} mice were similarly “protected”. In addition, using murine alveolar macrophage line (MS1) heme inhibited bacterial phagocytosis of

macrophages via the release of the damage-associated molecular pattern molecule HMGB1, a death high mobility group box transcription factor, which is released from apoptotic cells. Antibody to HMGB1 protected against death following the *P. Aeruginosa* infection. It was shown from a group of 50 patients with trauma hemorrhage that they received sufficient heme from resuscitation therapy transfusions to overwhelm HPX. However, it remains to be established how changes over time of the different products generated during hemolysis, including heme levels, are linked to adverse outcomes in after transfusions in these types of patients.

In mice, HPX has been shown to be involved in the nutritional iron defense system in the lungs. Pneumonic plague is highly contagious and the causative agent is *Yersinia pestis*. After mice received a lethal dose of pneumonic plague, both HPX and Hp increased 48 h later in the lung and serum. However, when this lethal plague dose was given together with a single injection of EV76 live attenuated *Y. pestis*, there was a more rapid increase, within 24 h, of HPX and Hp in both lung and in serum. Some broad range antibacterial drugs are available to treat outbreaks of multi-drug resistance *Y. pestis*, but while they are effective in vitro and can be given intranasally (ideal for rapid absorption and incidentally a useful route for drug administration in humans), they are not effective in mice leading to an ~86% rate of fatality. However, a combination treatment revealed that antibiotic treatment 48 h after a previously lethal dose of *Y. pestis* treatments was extremely effective—all the mice survived. When the mice were checked 21 days later, there were no signs of *Y. pestis* infection in the blood or in key organs such as the spleen, liver, and lungs. These studies are exciting because they reveal that live vaccine strains can activate not only lung immunity but also systemic immunity. They also show the potential for treatment via inhalants for acute lung infections and for patients whose lungs are rapidly deteriorating as in pneumonic plague. Finally, this research shows the importance of iron and heme sequestration for nutritional immunity that is protective and demonstrates a combination therapy that is effective to treat antibiotic resistant strains of pathogenic bacteria [124].

8.4. Hemopexin Is Neuroprotective in Models for Intracerebral Hemorrhage

Secondary brain injury is the term used for the damage produced by extracellular heme and leads to irreversible brain damage and enduring neurological deficits. The first studies showing that HPX protects the brain were published in 2009 and used a rat ischemia-reperfusion stroke model [125]. Recently, data from mice have revealed the therapeutic potential for a clinical grade HPX in neuroprotection for intracerebral hemorrhage (ICH) [107]. Brain HPX levels were increased using a CNS-targeted recombinant adeno-associated viral vector. This organ-specific expression of HPX resulted in smaller lesions and improved functional recovery from ICH including significantly reduced hematoma volumes compared with the control rats. Furthermore, although HO1 and iron levels were not elevated, there was increased microgliosis and decreased astrogliosis and lipid peroxidation. Importantly, the elevated HPX in brain improved both central and peripheral clearance mechanisms indicating some communication and interactions between the systemic and brain clearance systems. This may represent a situation where the blood–brain barrier is “leaky”, as shown when the iron chelator deferoxamine, which does not cross the blood–brain barrier, is effective at reducing brain damage and symptoms. A role for the circumventricular organs (see Section 5) is also a possibility.

8.5. Hemopexin Is Induced in Response to TLR7 Activation in Models of ICH

Toll like receptors are expressed by all cells of the immune system and are integral to the innate and adaptive immune systems. Thus, TLRs are logical targets for interference by pharmacological means for development and maintenance of inflammatory conditions including inflammatory bowel disease, rheumatoid arthritis, and SLE. As described above (Section 8.3), when heme binds to toll-like receptor 4 on endothelial cells, P-selectin is induced leading to the assembly of structures on the cell surface that causes vaso-occlusion as seen in SCD [63]. The Bruton tyrosine kinase (BTK), which is needed for B-cell receptor signaling and the survival of B-cells in humans, has been identified downstream of five TLRs including TLR4 and TLR7. A BTK–LRP1 pathway has been proposed to aid

in heme clearance by heme-HPX uptake into brain cells in a mouse model of ICH [126]. In this model, the stereo striatal injection of type VII collagen [69] causes traumatic brain injury that is hemorrhagic with the development of inflammation. The TLR7 activation improved neurological deficits including neurological scores for spatial learning and memory. Improvement was also seen in the physical changes to the brain such as edema, blood–brain barrier permeability and the hematoma volume. Compared with sham-operated mice, TLR7 activators increased the protein expression of HPX, the scavenger receptor LRP1/CD91 that binds heme-HPX, as well as BTK and other pathway components. Furthermore, all of these proteins were decreased in response to TLR7 inhibitors. Investigation of LRP1 as a therapeutic target in ICH is described in Section 3.3. The role of BTK in the TLR-mediated regulation of innate immunity and differences among how information from mice models and humans may affect the application to human therapeutics has recently been reviewed [126].

9. Divergent Evolutionary Fate of Hp and HPX Reveals the Potential for Additional Roles for HPX

The only vertebrates known to lack Hb and red blood cells are, among the notothenioids, Antarctic ice fishes. Ice fishes live in the Southern Ocean that encircles Antarctica and, to survive, they produce anti-freeze proteins, thus preventing ice crystals from forming in their thin blood. Thin blood is another adaptation because at cold temperatures, thin blood is easier to circulate. Also, ice fishes have developed large hearts and their blood vessel have large diameters [127]. A genetic accident allowed ice fish to survive when a gene needed for the assembly of Hb was completely corrupted. Although cold water offers more oxygen, fish living in cold waters do have fewer RBCs in their blood over the winter months to save energy. Sadly, now these species and the icefish are threatened by climate change and warming oceans; however, they provide a unique situation to gain insights into the function of HPX.

Compared with hundreds of red blooded notothenioids, these ice fish have very little Hp and its translation into a functional protein has actually been silenced [128]. Overtime, a degeneration of the Hp genotype manifested in a separate lineage. This was due to the distinct nonsense mutations including a deletion frame shift and a mutated poly A sequence. Significantly, the loss of Hb effectively reduced the selective constraints on Hp maintenance. Importantly, this took place without affecting either the HPX gene or its expression. The HPX genotype is preserved and the transcription rate of the HPX gene is comparable to that in the red-blooded notothenioids. The authors of this study speculate that HPX persists due to the selective pressure to maintain mitochondrial function and to capture heme released from dead and dying cells. This had been previously proposed as a function for HPX, but to what extent there exists such intracellular sources of heme-proteins from dying and apoptotic cells is unknown. In addition, these fish have little bone but do have an extensive muscular system; and, thus, HPX may be conserved because it is needed for heme reclamation in myoglobin biology.

10. Immune Cells as Therapeutic Targets in Intravenous Hemolysis

Evidence supports that a calcium sensor “calprotectin” on the surface of immune cells may be an important target for reducing heme-mediated inflammation in patients with IVH. Human neutrophils and monocytes constitutively express this sensor, which is a heterodimer (S100A8 and S100A9). These proteins are currently possible candidates for both diagnostics and therapeutics when inflammation is causing disease-related pathology. They participate in cytoskeletal rearrangement and arachidonic acid metabolism. When the calprotectin dimer is released from cells, they modulate the inflammatory response by stimulating leukocyte recruitment and inducing cytokine secretion. Mechanistically, S100A8 drives the NLRP3 inflammasome and leads to IL-1 β secretion. Myeloid-derived alarmin and S100A8 (AKA calgranulin A) have been shown to propagate hemolytic inflammation via leukocyte priming [129]. Thus, S100A8 alone may represent a novel target to reduce inflammation in hemolytic disorders.

Human CD4+ cells were differentiated into human monocytes and used to address the question of whether heme-induced inflammation is amplified under sterile conditions. When these cells were incubated with S100A8, there was the expected increase in pro-IL1 β , which required the activation of NF κ B, in turn partly dependent upon signaling via the TLR4. In addition, when these cells were incubated with heme, pro-IL1 β secretion was secreted and the effect of heme was enhanced by addition of S100A8. Furthermore, S100A8 levels increased in plasma when IVH was induced in mice; and heme sequestration by HPX administration prevented this S100A8 production.

The S100A8 levels were found to be increased in human hemolytic and sickle cell anemias. With the evidence that some heme effects are amplified by autocrine S100A8 production, these studies provide a mechanism whereby leukocyte priming in the absence of bacterial infection could be harmful. Thus, S100A8 represents a potential therapeutic target to reduce heme-mediated inflammation in hemolytic disorders.

The presence of CNS trauma such as spinal cord injury (SCI) has limited therapy and the pathology of SCI is linked to the polarization of microglia to the M1 or M2 state. Heme is known to activate mouse macrophages to the pro-inflammatory M1 state [130]. Also, HPX increases at the lesion site after crush injury to the spinal cord during the time when intramedullary spinal cord hemorrhage is alleviated and is synchronously correlated with the M2 marker Arginase 1 in microglia. Loss of HPX *in vivo* decreased the number of “protective” M2 microglia relative to increased M1 microglia in the lesion site. This is consistent with a role for the M1 microglia in delaying recovery and exacerbating the behavioral dysfunction after SCI. In LPS-stimulated primary cultured murine microglia, the heme-binding activity of HPX led to a rapid increase in the cells in the M2 state and was associated with improvements such as less neuronal degeneration, less demyelination together with increased numbers of mature oligodendrocytes [131].

11. Novel Metabolite Regulation of Tissue Resident Macrophages and Bone Marrow Macrophage Populations by Heme for Iron Recycling and Body Iron Homeostasis

Senescent red blood cells are degraded daily by almost half a million macrophages and their heme iron recycled for all of the body’s iron needs (see citations within Reference [132]). This link between heme and iron metabolism is important for normal iron homeostasis but also provides a special example of how heme, derived from citric acid cycle precursors, is involved in novel metabolite regulation in pathological hemolytic diseases and conditions. For example, in hemolysis, red pulp macrophages in the spleen not only phagocytose red blood cells but likely also take up Hb–Hp complexes and heme-HPX complexes via surface receptors CD163 and LRP1/CD91, respectively. Intracellular heme levels in macrophages are therefore assumed to be normally limited by heme export by FLVCR and/or degradation by HOs. This exporter is a member of the major facilitator family of transporters and, as such, moves heme (the solute) down a concentration gradient. However, FLVCR heme export *in vitro* requires a heme-binding protein such as albumin or HPX to be present extracellularly to accept the heme. Presumably this is also needed *in vivo* and could be compromised in patients with hemolysis and decreased HPX or when heme-binding by circulating albumin is compromised. (Many pharmaceuticals occupy the hydrophobic site on albumin that accommodates heme.) Heme catabolism generates iron and this must be exported via ferroportin to transferrin or in macrophages via secretion on L-ferritin.

In pathological hemolysis, the increasing heme is toxic and excessive heme induces the loss of resident red-pulp macrophages. As macrophages die, the population of pulp macrophages and bone marrow cells is replenished by the relief by heme of Bach1-mediated transcriptional repression of the gene encoding the transcription factor Spi-C in monocytes [132] that drives their differentiation into macrophages. Circulating monocytes enter the spleen and upregulate *SpiC*, leading to macrophage differentiation, reestablishment of the splenic red-pulp macrophage compartment, and restoration of iron homeostasis. Thus, local signaling within tissues might be sufficient to promote the acquisition of specialized macrophage functions irrespective of their lineage. This further demonstrates a critical

role of monocytes to repopulate the body with splenic red-pulp macrophages and bone marrow macrophages. Furthermore, this regulation is likely to take place at other sites of hemorrhagic damage including macrophages at atherosclerotic plaques and alveolar macrophages that regulate surfactant production. Other tissue-specific macrophages are found in bone (osteoclasts) and induction of HO1-inhibited osteoclast differentiation into osteoclasts in vitro and in vivo and may affect bone in rheumatoid patients who had increased serum bilirubin levels [133].

12. Evidence for Cross Talk between IL-6 and the HPX System in Models of Liver and Neuronal Cells

Evidence is mounting to show that HPX protects against heme toxicity from Hb in both sterile and infectious inflammation [26]. The cytokine IL-6 contributes to host defense via stimulation of the hepatic acute phase response, immune cell responses, and hematopoiesis. However, when IL-6 synthesis is sustained due to the perturbed regulation, chronic inflammation ensues, which is life threatening. IL-6 is elevated in several hemolytic states and in mice studies to model human sepsis [134]. Interleukin-6 interacts with specific receptors on many different cell types including hepatocytes and lymphocytes but also on bone marrow cells, synovial fibroblasts, CD4 and CD8T cells, and B cells. Interleukin-6 can activate the MAP3K upstream kinase MKK4 for the Jun N-terminal kinase (JNK) in human HepG2 hepatoma cells [135], a signaling pathway well known to regulate several normal biological processes, not solely stress-related responses. A substrate of phospho-JNK, the transcription factor c-Jun, is phosphorylated in mouse hepatoma cells in response to heme-HPX providing evidence for activation of the JNK pathway [136]. Because the HPX system is protective, we propose that JNK activation by heme-HPX is part of JNK's important role in normal biological processes needed for the maintenance and/or restoration of cell homeostasis in hemolysis and oxidative stress.

The purpose of this research was to determine if signaling pathways activated by the inflammatory cytokine IL-6 affect, and possibly override, the activation of the JNK pathway by heme-HPX, and if so, to what extent. Our hypothesis was that if there is cross talk between the IL-6 and heme-HPX activation of the JNK pathway, then the target for inhibition by IL-6 will be MKK4 rather than JNK. Elevated IL-6 cross talk in the early stages of inflammation when there is hemolysis may reprogram the cytoprotective cell response(s) to heme-HPX and/or perhaps impair them.

Our preliminary data showed that incubation of heme-HPX with mouse Hepa cells as models of hepatocytes (Figure 1A) and with rat PC12 cells that are models of cholinergic neurons (Figure 1B, [137]) led to the rapid phosphorylation of MKK4 and JNK. Furthermore, two JNK substrates c-Jun(Ser63) and c-Jun(Ser73) were also phosphorylated. These phosphorylations occurred very rapidly with very high levels of p-MKK4(Thr261), p-JNK(Thr183/Tyr185), and p-c-Jun(Ser63, Ser73) detectable in cell extracts within 2 min after addition of 5 μ M heme-HPX to the cell medium. The time course data in Figure 1 show that they remain high for at least 30 min relative to total JNK levels supporting activation of the JNK pathway by heme-HPX.

When Hepa cells were incubated with increasing concentrations of IL-6 simultaneously with heme-HPX, the extent of phosphorylation of MKK4, JNK, and of c-Jun (Ser 63 and 73) was significantly decreased (Figure 2). The effect of IL-6 was dose-dependent. The IL-6 consistently decreased on average to 54.3% the levels of p-MKK4 and also decreased p-JNK levels to 60.4% (further details are in the legend of Figure 2). We used correlation analyses to assess for a possible association first between the IL-6 and p-MKK4 levels as variables and, second, between the IL-6 and p-JNK levels as variables to validate our hypothesis. Statistical analysis using Kendall's Tau and Spearman's rho rank correlation analyses show there was a strong negative correlation between the IL-6 concentration and p-MKK4 levels and also the IL-6 and p-JNK levels. Applying both types of correlation analyses the correlation was significant at the 0.01 level (2 tailed). The Kendall's tau correlation coefficients were -0.683 , significance 0.008 (2 tailed), and -0.730 , significance 0.005 (2 tailed) for p-MKK4 and p-JNK, respectively, and the Spearman's rho correlation coefficients were -0.775 , significance 0.008 (2 tailed), and -0.825 , significance 0.003 (2 tailed) for p-MKK4 and p-JNK, respectively. Thus, IL-6

significantly reduced JNK pathway activation by heme-HPX demonstrating for the first time cross talk on a heme-HPX signaling pathway with an inflammatory cytokine and confirming our hypothesis.

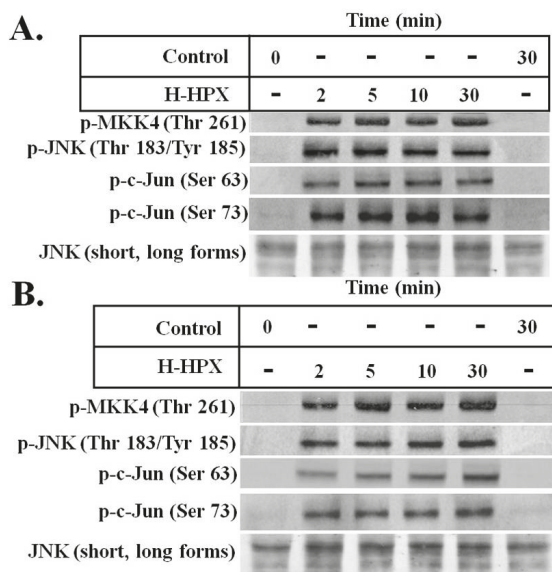


Figure 1. Heme-hemopexin activates the JNK pathway via MKK4 in hepatic and neuronal cells. In panel (A) Mouse Hepa cells were incubated with PBS (control) or heme-HPX (5 μ M, H-HPX in PBS) for up to 30 min as indicated. The levels of p-MKK4 and p-JNK—both kinases of the JNK pathway and of p-c-Jun, a substrate of JNK, together with total JNK levels—were detected by immunoblotting of cell extracts (40 μ g protein/lane), as described briefly below. (B) Data shown are from a similar time course experiment to determine the effects of heme-HPX on the JNK pathway and c-Jun phosphorylation in rat PC12 cells. The data shown in both panels are from a representative experiment of two independent biological replicates. Hepa cells are minimal deviation hepatoma cells from a mouse solid tumor line BW 7756 and were grown and maintained as previously described [1]. Western blots were performed by loading the same amount of protein from the sample of cell lysate per lane (40 μ g protein/lane), and after electrophoresis on SDS-PAGE gels, the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Primary antibodies (Thermo-Fisher Scientific, Waltham, MA, USA) used at 1:1000 dilution, were detected by binding a secondary antibody of goat anti-rabbit IgG horseradish peroxidase (generally 1:5–10,000) and the peroxidase enzyme activity detected using the chemiluminescence ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Data were collected using a multifunction phosphoimager/fluorimager laser scanner (Molecular Dynamic Storm Molecular Imager, LICOR, Lincoln, NE, USA).

Although, MKK4 is a master regulator against liver damage and for liver regeneration [138], the implications of this initial *in vitro* study for the effects of IL-6 on the HPX system *in vivo* in clinical hemolytic and inflammatory conditions and in rodent models of HPX replenishment remain to be established. Many stimuli of hepatocytes regulate the acute phase production of IL-6, both at the autocrine and endocrine/paracrine levels [139]. For example, when the liver itself is injured, the Kupffer cells are the primary source of IL-6 that stimulates IL-6 production by parenchymal cells for the acute phase response. There may be differences in the upstream events in different cell types because IL-6 alone did not phosphorylate MKK4 in the mouse hepatoma cells as it did in human HepG2 cells. In addition, there may be differences in the response to heme-HPX in the presence of IL-6 between transformed cells and non-transformed cells. Preliminary data from PC 12 cells (not shown) revealed that IL-6 decreased pMKK4 levels in response to 5 μ M heme-HPX more effectively when added to the

medium 30 min before heme-HPX. This suggests some differences in response to IL-6 cross talk with heme-HPX between liver and neuronal cells.

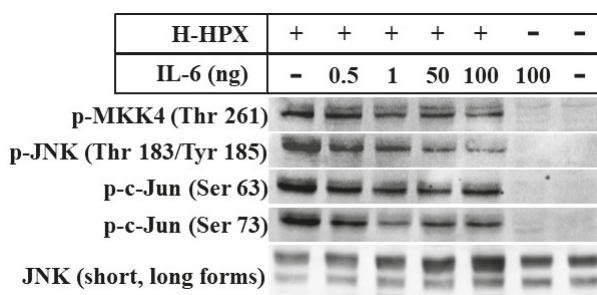


Figure 2. IL-6 crosstalk decreases JNK pathway component levels induced by heme-HPX. The data are from immunoblot analyses of cell extracts (40 μ g protein/lane, as described in the legend to Figure 1). After incubation of Hepa cells with 5 μ M heme-HPX in the presence or absence of IL-6 at the concentrations shown, for 30 min, IL-6 (1.0–100 ng/mL) consistently decreased to 54.3% the levels of p-MKK4 (mean \pm SD of three highest IL-6 concentration datasets: 58.63 \pm 6.21; 49.97 \pm 9.66). The IL-6 also decreased p-JNK levels to 60.4% (mean \pm SD of three highest IL-6 concentration data sets 56.25 \pm 11.56 and 64.5 \pm 4.96). These data show that IL-6 decreases the levels of key kinases of the JNK pathway activated by heme-HPX in Hepa cells. The data shown are from a representative experiment of two independent biological replicates. Kendall's tau and Spearman's rho rank correlation analyses were used for the statistical analyses of the relationship between IL-6 concentrations and the levels of p-MKK4 and p-JNK as described in the text.

Nevertheless, and overall, this research shows that further investigations of the interaction between IL-6 and the HPX system in different cell types including primary human hepatocytes are warranted. Our data provide a basis to gain a better understanding of the consequences of cross talk between the IL6 and other signaling pathways that may ultimately be used to improve patient diagnosis, therapy, and prognosis when there is hemolysis together with immune and inflammatory responses.

13. Conclusions

It is apparent from the research covered here, which builds on many previous studies in this area, that we now have a more detailed understanding of how the hemopexin system protects against both heme toxicity and iron toxicity. Many biological events are involved and include different types and sources of hemolysis; heme-driven activation of cells of the immune system and of endothelial cells; heme- and cytokine-mediators, among others, of inflammation; and specific parameters of heme and iron homeostasis, such as the activity of HOs, the induction of FtH, and the increase in plasma FtL. Many of these events and processes are depicted in Figure 3. Furthermore, identification of the activation of different kinds of cells reveals a significant number of sites in the body that can be perturbed by heme and, hence, the number of biological processes affected by hemolysis. For example, endothelial cells help to maintain the integrity of the blood–brain barrier and blood vessel walls and minimize the development of atheromas on the wall of blood vessels that can develop into hemorrhagic plaques.

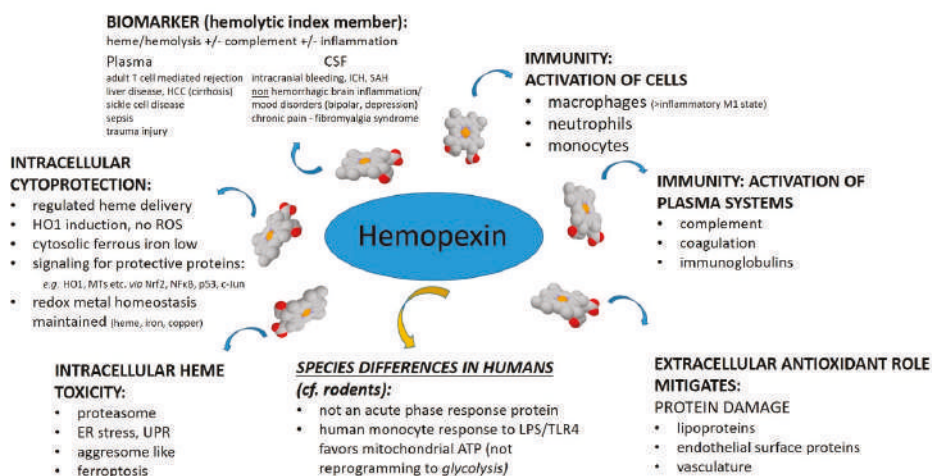


Figure 3. Summary of the current status of the hemopexin (HPX) system and the role it plays in the biological processes that protect the human body against heme- and iron-mediated toxicity. This depiction of the processes that are associated with the HPX system as it protects against heme toxicity provides a sense of the extensive nature of the biological systems and cells that are deleteriously affected by heme. Furthermore, in humans, C-reactive protein 1 and Hp levels rise when there is an acute phase reaction but HPX does not [62]. In addition, lipopolysaccharide-(LPS) stimulated human peripheral blood monocyte-derived macrophages rely on oxidative phosphorylation for ATP production and do not undergo the metabolic reprogramming to glycolysis as do mouse bone marrow-derived macrophages [140]. Such differences require assessing when using mice as pre-clinical models [141].

Experimental evidence shows that HPX protects against all identified damage by heme in more instances than not. After endocytosis of heme-HPX by hepatocytes, HPX recycles from the liver after delivering heme [2], but because the hepatic concentration of heme alters HPX turnover, this can change plasma HPX levels [142,143]. Heme-HPX taken up into non-hepatic tissues by LRP1/CD91 a receptor that directs its ligands to lysosomes, may thus deplete HPX without generating signals for compensatory hepatic synthesis and secretion of HPX to plasma. Clearly, HPX levels decrease quite quickly after massive hemolysis (reflected by the number of heme molecules and the duration of the heme load) and the heme can take about 6 days to clear in humans before returning to normal levels [62].

Plasma exchange therapy appears poised to be a useful means to increase HPX and Hp and reduce heme toxicity. Perhaps such treatment could be a means of “respite care” for cells and organs. There is already evidence from studies on proteasomes, ER stress, and UPR as well as ALIS formation that heme-toxicity related responses can be short- or long-lived or are reversible. It will be important to establish the amount of intracellular heme from which cells can recover, how long a period of high intracellular heme and/or iron can be tolerated, and how cells return safely to homeostasis. In the future, what will be needed to counteract heme and iron toxicity are treatments that combine raising HPX (perhaps with Hp depending upon Hb levels) with certain targeted iron chelators (or other means to prevent ferroptosis) and/or a means to increase plasma FtL. We draw the reader’s attention to some challenges in the design of in vitro experiments to “mirror” heme toxicity in humans; as well as the differences between the response to heme/Hb toxicity of humans and mice (see Figure 3). It is perhaps worth remembering that experimental animals generally live out their lives in sterile conditions. Also, there are numerous differences between the response of human and mouse immune cells as pointed out in 2004 [141], and include both the innate and adaptive immune systems.

Analyses of the number and level of biomolecules in patient samples are currently extremely sensitive, thus generating a plethora of data. The plasma proteome contains thousands of proteins compared with those isolated by C18 chromatography including the normally abundant HPX [144]. Thus, future multiplexing analyses will generate so much data that very sophisticated software will be required for analysis, which may not be available to all researchers and physicians in the field. As the means to develop pathway analyses, pattern recognition networks are increasingly available through online bioinformatics programs. Ideally, a public repository for all this information is needed, which is accessible to researchers and physician–scientists to share and compare their patient data, especially for prognosis and response to treatments. In this review, juxtaposing a wide variety of studies will help researchers to more readily extend their sets of target molecules in the clinic, thus helping in the diagnosis, prognosis, and response to therapies of patients.

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