



## Iron metabolism and iron disorders revisited in the hepcidin era

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### ABSTRACT

Iron is biologically essential, but also potentially toxic; as such it is tightly controlled at cell and systemic levels to prevent both deficiency and overload. Iron regulatory proteins post-transcriptionally control genes encoding proteins that modulate iron uptake, recycling and storage and are themselves regulated by iron. The master regulator of systemic iron homeostasis is the liver peptide hepcidin, which controls serum iron through degradation of ferroportin in iron-absorptive enterocytes and iron-recycling macrophages. This review emphasizes the most recent findings in iron biology, deregulation of the hepcidin-ferroportin axis in iron disorders and how research results have an impact on clinical disorders. Insufficient hepcidin production is central to iron overload while hepcidin excess leads to iron restriction. Mutations of hemochromatosis genes result in iron excess by downregulating the liver BMP-SMAD signaling pathway or by causing hepcidin-resistance. In iron-loading anemias, such as  $\beta$ -thalassemia, enhanced albeit ineffective erythropoiesis releases erythroferrone, which sequesters BMP receptor ligands, thereby inhibiting hepcidin. In iron-refractory, iron-deficiency anemia mutations of the hepcidin inhibitor TMPRSS6 upregulate the BMP-SMAD pathway. Interleukin-6 in acute and chronic inflammation increases hepcidin levels, causing iron-restricted erythropoiesis and anemia of inflammation in the presence of iron-replete macrophages. Our improved understanding of iron homeostasis and its regulation is having an impact on the established schedules of oral iron treatment and the choice of oral *versus* intravenous iron in the management of iron deficiency. Moreover it is leading to the development of targeted therapies for iron overload and inflammation, mainly centered on the manipulation of the hepcidin-ferroportin axis.

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### Introduction

Research advances in understanding the biological functions and homeostasis of iron have clarified its role in physiology and disease. Iron, essential for hemoglobin synthesis, is indispensable to all cells for the production of heme and iron-sulfur (Fe/S) clusters, which are components of proteins/enzymes involved in vital biological processes such as respiration, nucleic acid replication and repair, metabolic reactions and host defense. While essential for life, excess iron is toxic. The ability to accept/release electrons explains the propensity of iron to damage cell components and is the reason why body iron must be tightly regulated. The two-faced nature of iron is also evident in its disorders, which span from iron excess to iron deficiency and maldistribution, when some tissues are iron-loaded and others are iron-deficient.

In the new millennium studies of genetic and acquired iron disorders and the development of their corresponding murine models have identified novel iron genes, proteins and pathways and unveiled the central role of the hepcidin-ferroportin axis in systemic iron homeostasis. This review summarizes recent advances in the understanding of iron trafficking, utilization and regulation, emphasizing the implications for iron disorders of hematologic interest; for further insights readers are directed to specific reviews.<sup>1-3</sup>

## Iron trafficking

Iron trafficking is an example of circular economy. Only 1-2 mg iron are absorbed daily in the gut, compensating for an equal loss; most iron (20-25 mg/daily) is recycled by macrophages upon phagocytosis of erythrocytes. The site of regulated non-heme iron uptake is the duodenum: non-heme iron is imported from the lumen by the apical divalent metal transporter 1 (DMT1) after reduction from ferric to ferrous iron by duodenal cytochrome B reductase (DCYTB). Absorption of heme exceeds that of non-heme iron, though the mechanisms remain obscure. In enterocytes non-utilized iron is stored in ferritin - and lost with mucosal shedding - or exported to plasma by basolateral membrane ferroportin according to the body's needs (Figure 1).

### The role of transferrin and its receptors

The plasma iron pool is only 3-4 mg and must turn over several times daily to meet the high (20-25 mg) demand of erythropoiesis and other tissues. The iron carrier transferrin is central to iron trafficking. Binding to its ubiquitous receptor TFR1, transferrin delivers iron to cells through the well-known endosomal cycle.<sup>1</sup> This function is crucial not only for erythropoiesis, but also for muscle<sup>4</sup> and for B- and T-lymphocytes, as highlighted by a *TFR1* homozygous mutation that causes combined immunodeficiency with only mild anemia.<sup>5</sup> TFR1 is also essential in the gut to maintain epithelial homeostasis independently of its function of an iron importer;<sup>6</sup> in hepatocytes TFR1 is dispensable for basal iron uptake, but essential in iron loading to finely tune the hepcidin increase.<sup>7</sup>

Transferrin is emerging as a key regulator of iron homeostasis through binding to its second receptor TFR2, which has a lower binding affinity than TFR1<sup>8</sup> and whose expression is restricted to hepatocytes and erythroblasts. When plasma iron concentration is high, diferric transferrin binds TFR2 inducing upregulation of hepcidin in hepatocytes and a reduction of erythropoietin responsiveness in erythroid cells<sup>9</sup> where TFR2 binds erythropoietin receptors.<sup>10</sup> The reverse occurs in iron deficiency. The dual function of transferrin as an iron cargo and regulator seems to be dependent on the unequal ability of iron binding of the N and C terminal lobes and operates through the differential interaction of monoferric transferrin with the two receptors.<sup>11</sup>

### Iron recycling

Macrophages phagocytize senescent and damaged erythrocytes, recover iron from heme through heme oxygenase (HMOX) 1 and may utilize, conserve or recycle the metal. The relevance of their role is strengthened by the severity of conditions in which recycling is altered. *HMOX1* mutations in children cause a rare, severe disorder<sup>12</sup> and reduced recycling in inflammation causes anemia. Macrophage ferroportin is crucial for iron balance. Its expression is upregulated by heme and downregulated by inflammatory cytokines contributing to iron sequestration and its translation is repressed by iron. The protein is ultimately controlled at the post-translational level by hepcidin.<sup>13</sup>

### Cell iron import

Intracellular iron is used for multiple functions; if not utilized it is stored in ferritin, or exported by ferroportin, in order to maintain the labile iron pool within narrow limits

to avoid toxicity. Although all cells may import, export or store iron, some have specific functions:<sup>1</sup> e.g., erythroblasts are specialized in iron uptake, macrophages and enterocytes in iron export, and hepatocytes in iron storage. Within cells most iron is transferred to mitochondria for heme and Fe/S cluster production. Heme is indispensable for hemoglobin, cytochromes and enzyme activity. Biogenesis of Fe/S clusters is a process conserved from yeast to humans: this prosthetic group is essential to proteins involved in genome maintenance, energy conversion, iron regulation and protein translation.<sup>14,15</sup> In erythroblasts >80% iron is directed to mitochondria through a "kiss and run" mechanism between endosomes and mitochondria.<sup>16</sup> Mitoferrin 1 and 2 are iron transporters of the inner mitochondrial membrane, the former being essential for zebrafish and murine erythropoiesis.<sup>17</sup>

Ferritin may store up to 4,500 iron atoms in a shell-like structure formed by 24 chains, comprising both heavy (H) chains, with ferroxidase activity, and light (L) chains.<sup>18</sup> Ferritin storage of iron provides protection from oxidative damage, and also saves an essential element for future needs. H-ferritin deletion is incompatible with life and its conditional deletion in the gut deregulates the fine mechanism of iron absorption causing iron overload.<sup>19</sup> L-ferritin heterozygous mutations are rare and limited to the 5' iron regulatory element (IRE) - leading to escape from iron regulatory protein (IRP) control and constitutive high ferritin synthesis in hyperferritinemia-cataract syndrome.<sup>20</sup> Rare dominant mutations lead to elongated proteins and neuroferritinopathies, a type of neurodegeneration caused by abnormal ferritin aggregates in the basal ganglia and other areas of the brain<sup>21</sup> (Table 1).

In the clinical setting serum ferritin is a marker of iron deficiency when its level is low, and of iron overload/inflammation when its level is increased, reflecting macrophage ferritin content. However, both the origin and the function of serum ferritin remain largely unexplored. One hypothesis is that the secreted ferritin<sup>22</sup> may be re-uptaken by cells as an alternative mechanism of iron recycling, e.g., when iron release from macrophages is limited in inflammation.

The cytosolic chaperon Poly (rC) binding protein 1 (PCBP1) delivers iron to ferritin,<sup>23</sup> and *Pcbp1* null mice have microcytic anemia.<sup>24</sup> Ferritin turnover occurs through "ferritinophagy", an autophagic process orchestrated by nuclear receptor co-activator (NCOA)4, a cargo molecule that directs ferritin to lysosomal degradation, to recover iron when needed.<sup>25,26</sup> PCBP1 also delivers iron to prolyl-hydroxylase (PHD2) the enzyme that induces degradation of hypoxia inducible factors (HIF), one of the several links between iron and the hypoxia pathways.<sup>27</sup>

### Iron export

The ubiquitous iron exporter ferroportin cooperates with the oxidases ceruloplasmin or hephaestin, to release ferric iron to transferrin. Enterocytes, macrophages, hepatocytes and trophoblasts express high ferroportin levels for their specific functions in iron homeostasis. Blocking iron export may be dangerous in some cells. For example, conditional ferroportin deletion in murine cardiomyocytes leads to local iron overload and cardiac failure;<sup>28</sup> furthermore, specific deletion of ferroportin in erythroblasts and erythrocytes leads to hemolytic anemia, due to the toxicity of iron derived from hemoglobin oxidation in an environment (red blood cells) with limited antioxidant capacity.<sup>29,30</sup>

Erythroblasts may also export heme through feline leukemia virus C receptor (FLVCR).<sup>31,32</sup> The latter two export mechanisms seem counterintuitive in cells that need iron/heme for the production of hemoglobin, but are likely biological safeguard mechanisms that protect erythroid cells from iron/heme excess.

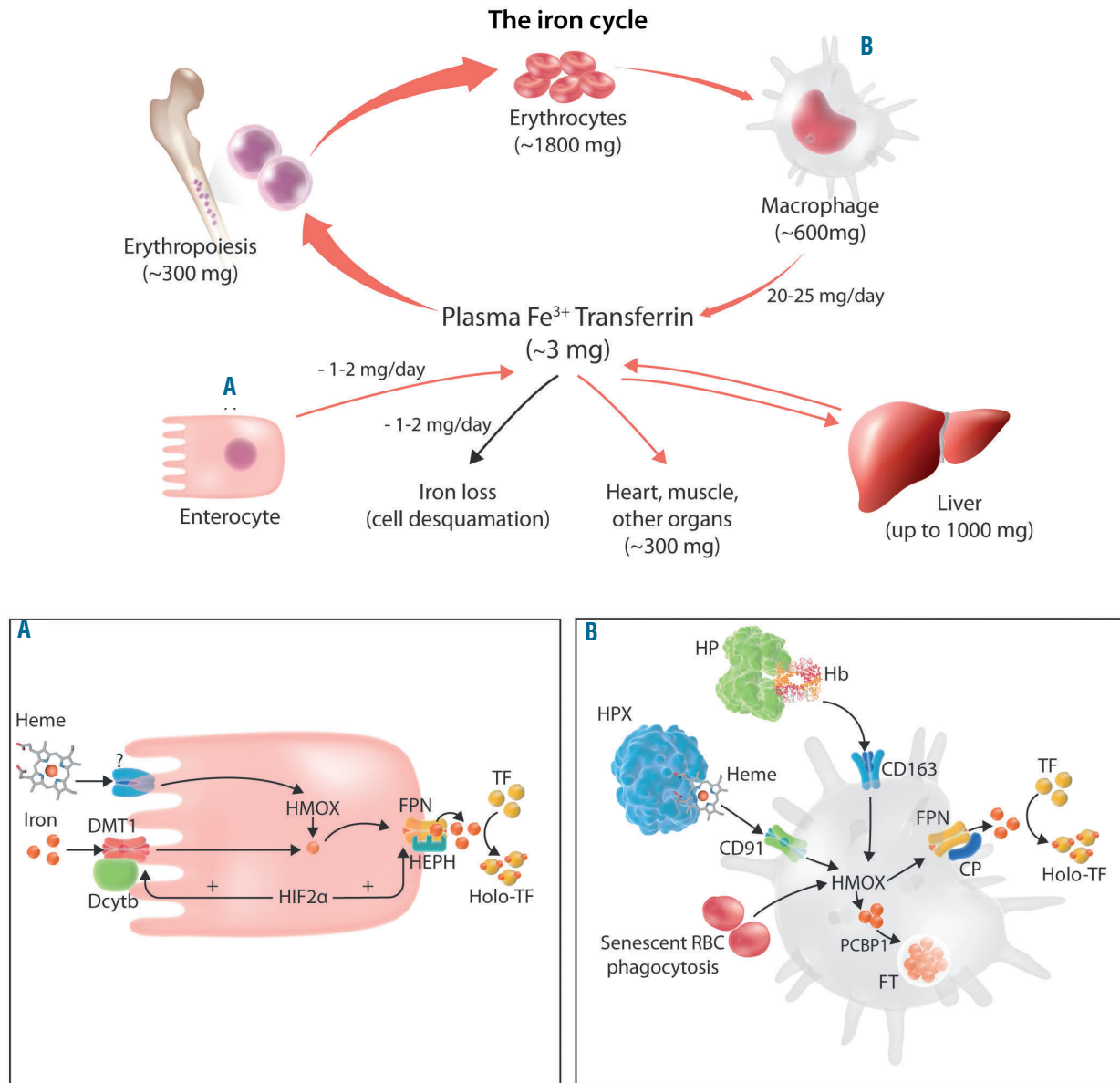
## Iron homeostasis

Maintaining iron balance requires tight regulation at cellular, systemic and tissue levels.

## Cell iron homeostasis

### The IRP-IRE system

This system is based on the post-transcriptional control of iron genes mediated by the interaction of IRP with IRE of their mRNA untranslated regions. In iron-deficiency states, IRP1 and 2 increase iron uptake by stabilizing *TFR1* mRNA and blocking iron storage and export by suppressing ferritin and ferroportin translation. In iron-replete cells, Fe/S clusters convert IRP1 into cytosolic aconitase, while IRP2 undergoes iron-dependent proteasomal degradation. The IRP1/aconitase interconversion on the one hand links iron to tricarboxylic acid and cell metabolism, and on the



**Figure 1. The iron cycle.** Iron (Fe) circulates bound to transferrin to be released to all organs/tissues through transferrin receptor 1. Most iron (20-25 mg) recycled by macrophages, which phagocytize senescent red blood cells (RBC), is supplied to the bone marrow for RBC production. The daily uptake of dietary iron by duodenal enterocytes is 1-2 mg; the same amount is lost through cell desquamation and blood loss. Excess iron is stored in the liver and macrophages as a reserve. Arrows indicate directions. Numbers (in mg) are a mean estimate. (A) Focus on intestinal iron absorption. The metal transporter DMT1 takes up ferrous iron, reduced by DCYTB, on the luminal side of the enterocyte. Iron not used inside the cell is either stored in ferritin (FT) or exported to circulating transferrin (TF) by ferroportin (FPN), after ferrous iron is oxidized to ferric iron by hephaestin (HEPH).<sup>2</sup> Hypoxia inducible factor (HIF)-2 $\alpha$ , stabilized by local hypoxia, stimulates the expression of the apical (DMT1) and basolateral (FPN) transporters.<sup>63</sup> Heme, after entering the cell through an unknown mechanism, is converted to iron by heme oxygenase. (B) Focus on the iron recycling process. Macrophages recover iron from phagocytized RBC after heme is degraded by heme oxygenase. They also recover heme from hemoglobin (Hb)-haptoglobin (HP) or heme-hemopexin (HPX) complexes.<sup>2</sup> Iron not used inside the cells is either stored in FT or exported to the circulation by FPN with the cooperation of ceruloplasmin (CP). The latter is the preferential route in normal conditions.

other hand highlights that, through Fe/S clusters, iron controls its own availability. IRP2 binds IRE at normal tissue oxygen, IRP1 acts in hypoxic tissues, such as the duodenum and kidneys.

Murine models of total and tissue-specific IRP inactiva-

tion are providing further insights into the local function of these proteins. Deletion of both *Irps* in mice is incompatible with life; loss of *Irp2* results in mild anemia, erythropoietic protoporphyria and adult-onset neurodegeneration in mice<sup>33</sup> and in patients,<sup>34</sup> likely because of function-

**Table 1.** Genetic and acquired iron disorders.

|   | Inheritance                            | Gene                               | Phenotype   |
|---|--|------------------------------------|---|
| <b>Genetic iron overload without anemia</b>           |  |                                    |   |
| HH type 1   | AR                                     | <i>HFE</i>                         | Inappropriate low hepcidin<br>Adult-onset iron overload   |
| HH type 2   | AR                                     | <i>HJV</i><br><i>HAMP</i>          | Low hepcidin<br>Juvenile iron overload  |
| HH type 3   | AR                                     | <i>TFR2</i>                        | Low hepcidin<br>Early-onset iron overload   |
| HH type 4<br>gain-of-function FPN mutations           | AD                                     | <i>SCLA0A1</i>                     | Hepcidin resistance<br>Severe iron overload   |
| Ferroportin disease<br>loss-of-function FPN mutations | AD                                     | <i>SCLA0A1</i>                     | Macrophage iron overload  |
| <b>Iron-loading genetic anemias</b>                   |  |                                    |   |
| Thalassemia syndromes                                 |  |                                    |   |
| α-thalassemia   | AR                                     | <i>HBA</i>                         | Microcytic anemia   |
| β-thalassemia   | AR                                     | <i>HBB</i>                         | + iron overload   |
| Congenital sideroblastic anemia (non-syndromic)*      | X-linked                               | <i>ALAS2</i>                       | Microcytic anemia   |
|   | AR                                     | <i>SLC25A38</i>                    | Ringed sideroblasts   |
|   |  | <i>GLRX5</i><br><i>HSPA9</i>       | Iron overload   |
| Congenital sideroblastic anemia (syndromic)*          |  |                                    |   |
| SA and ataxia   | X-linked                               | <i>ABCB7</i>                       | SA and ataxia   |
| SIFD  | AR                                     | <i>TRNT1</i>                       | SA, immunodeficiency and developmental delay  |
| Congenital dyserythropoietic anemia                   |  |                                    |   |
| Type 1  | AR                                     | <i>CDAN1</i><br><i>C15orf41</i>    | Anemia, splenomegaly, jaundice, erythroblasts<br>multinuclearity, iron overload                             |
| Type 2, HEMPAS  | AR                                     | <i>SEC23B</i>                      |   |
| Type 3  | AR                                     | <i>KIF23</i>                       |   |
| Hypotransferrinemia                                   | AR                                     | <i>TF</i>                          | Microcytic anemia, iron overload  |
| <i>DMT1</i> mutations                                 | AR                                     | <i>SLC11A1</i>                     | Microcytic anemia, iron overload  |
| <b>Genetic iron deficiency</b>                        |  |                                    |   |
| IRIDA   | AR                                     | <i>TMPRSS6</i>                     | Iron-deficiency anemia<br>Refractoriness to oral iron   |
| <b>Genetic regional iron-FT accumulation</b>          |  |                                    |   |
| Hyperferritinemia-cataract syndrome                   | AD                                     | <i>FTL</i> promoter ( <i>IRE</i> ) | High serum ferritin in the absence of iron overload<br>Congenital cataract due to FT deposition in the lens |
| Ferritinopathy  | AD                                     | <i>FTL</i>                         | Brain iron accumulation   |
|   | AR                                     | <i>FRDA</i>                        | Neurodegeneration<br>+ cardiac iron overload  |
| <b>Acquired iron overload</b>                         |  |                                    |   |
| Chronic blood transfusions                            |  |                                    | Iron overload requiring chelation therapy   |
| <b>Acquired iron-loading anemias</b>                  |  |                                    |   |
| RS MDS  | Clonal disorder with somatic mutations | <i>SF3B1</i>                       | Macrocytic anemia. Ringed sideroblasts.<br>Iron overload  |
| <b>Acquired absolute iron deficiency</b>              |  |                                    |   |
| Iron deficiency                                       |  |                                    | Low body iron ± microcytic anemia   |
| <b>Acquired functional iron deficiency</b>            |  |                                    |   |
| Anemia of inflammation                                |  |                                    | Low serum iron. Normocytic anemia<br>Macrophage iron accumulation   |

HH: hereditary hemochromatosis; AR: autosomal recessive; AD: autosomal dominant; FPN: ferroportin; SA: sideroblastic anemia; \*only forms of hematologic interest are shown. SIFD: congenital sideroblastic anemia, B-cell immunodeficiency, periodic fevers, and developmental delay; HEMPAS: hereditary erythroblastic multinuclearity with positive acidified serum lysis test; DMT1: divalent metal transporter 1; IRIDA: iron-refractory, iron-deficiency anemia; FT: ferritin; RS MDS: ringed sideroblast myelodysplastic syndrome.

al iron deficiency. Adult *Irf1*-knockout mice have a normal phenotype in basal conditions. Intestinal epithelium *Irf1* and 2 deletion in adult mice leads to impaired iron absorption and local iron retention because of ferritin-mediated mucosal block.<sup>55</sup>

To escape IRP control, both enterocytes and erythroid cells also have a ferroportin isoform that, lacking the 5' IRE, ensures iron export in iron deficiency while remaining sensitive to degradation by hepcidin.<sup>36</sup>

#### Ferritinophagy

In iron deficiency, cells may recover iron through ferritinophagy, a process mediated by the multifunctional protein NCOA4.<sup>25,26</sup> First described as a transcriptional co-activator of androgen nuclear receptor, this protein is iron-regulated at the post-translational level. In iron-replete cells NCOA4 is bound by the E3 ubiquitin ligase HERC2 and degraded by the proteasome.<sup>37</sup> In iron-deficient cells NCOA4 binds ferritin inducing its degradation. NCOA4 also controls DNA duplication origins and its loss *in vitro* predisposes cells to replication stress and senescence,<sup>38</sup> coupling cell duplication and iron availability. *Ncoa4*-knockout mice accumulate ferritin in the liver and spleen, have reduced iron recycling and demonstrate increased susceptibility to iron-deficiency anemia.<sup>39</sup> The high *NCOA4* expression in erythroblasts<sup>24</sup> suggested a role for ferritinophagy in hemoglobinization *in vitro*,<sup>24</sup> in zebrafish embryos<sup>37</sup> and in mice.<sup>40</sup> However, the major relevance of the process is in iron-storing macrophages (Nai A. *et al.*, unpublished data) contributing to systemic homeostasis.

#### Systemic iron homeostasis: the hepcidin-ferroportin axis

The identification of hepcidin was a breakthrough in understanding how the liver is the central regulator of iron homeostasis and how its deregulation leads to iron disorders. The 25 amino acid mature hepcidin peptide controls iron export to the plasma by inducing lysosomal degradation of the iron exporter ferroportin in enterocytes, macrophages and hepatocytes;<sup>15</sup> moreover, hepcidin also occludes the central cavity that exports iron in ferroportin.<sup>41</sup>

Hepcidin transcription is upregulated in hepatocytes by circulating and tissue iron, through a crosstalk with liver sinusoidal endothelial cells, which produce the ligands (BMP6 and 2) that activate the hepatocyte BMP-SMAD pathway. *BMP6* expression is regulated by iron,<sup>42</sup> possibly in the context of an antioxidant response, controlled by NRF2.<sup>43</sup> *BMP2* is less iron sensitive and is highly expressed in basal conditions.<sup>44,45</sup>

Inflammatory cytokines such as interleukin (IL)-6 upregulate hepcidin expression by activating the IL-6R-JAK2-STAT3 pathway. High hepcidin levels induce iron retention in macrophages, high serum ferritin levels and iron-restricted erythropoiesis, all features of anemia of inflammation. For full hepcidin activation the IL-6 pathway requires functional BMP-SMAD signaling.<sup>46</sup>

Hepcidin expression is inhibited by iron deficiency, expansion of erythropoiesis, anemia/hypoxia, testosterone and possibly other factors.<sup>1,47</sup> The most powerful inhibitor is the liver transmembrane serine protease matrilysin 2, encoded by *TMPRSS6*,<sup>48</sup> which cleaves the BMP co-receptor hemojuvelin,<sup>49</sup> thereby attenuating BMP-SMAD signaling and hepcidin transcription. The relevance of *TMPRSS6* is highlighted by iron-refractory, iron-deficiency anemia (IRIDA), which results from

*TMPRSS6* mutations in patients<sup>50</sup> and inactivation in mice.<sup>48</sup> Deregulated, persistently high hepcidin blocks iron entry into the plasma and leads to iron deficiency. Another local inhibitor, the immunophilin FKBP12, binds the BMP receptor ALK2, suppressing the pathway activation.<sup>51</sup>

Erythroferrone (ERFE) is released by erythroid precursors stimulated by erythropoietin to suppress hepcidin expression and favor iron acquisition for hemoglobin synthesis.<sup>52</sup> In hypoxia hepcidin is also suppressed *in vitro* by soluble hemojuvelin, released by furin,<sup>53</sup> an effect unclear *in vivo*, and by platelet-derived growth factor-BB in volunteers exposed to hypoxia.<sup>54</sup> Proposed models of hepcidin regulation in different conditions are depicted in Figure 2A-D.

Macrophages produce hepcidin in inflammation, potentiating the systemic effect on iron sequestration.<sup>55</sup>

#### Local effects of hepcidin

As an antimicrobial peptide hepcidin is induced in the skin of patients with necrotizing fasciitis caused by group A streptococcal infections. Mice without hepcidin in keratinocytes fail to block the spread of infection because of a reduction of the neutrophils recruiting chemokine CXCL1.<sup>56</sup>

Cardiomyocytes produce hepcidin with local effect on ferroportin. Conditional cardiomyocyte hepcidin deletion in mice does not affect systemic iron homeostasis but leads to excess iron export, severe contractile dysfunction and heart failure.<sup>57</sup>

Emerging evidence links iron with lipid and glucose metabolism. Genome-wide association studies found overlapping associations for iron and lipid traits.<sup>58</sup> Adipocytes produce hepcidin in severe obesity<sup>59</sup> and hepcidin and gluconeogenesis are concomitantly upregulated in conditions of insulin-resistance.<sup>60</sup> Finally *Tmprss6*-knockout mice with high hepcidin levels are protected from obesity induced by a high-fat diet.<sup>61</sup>

#### Crosstalk between iron, oxygen and erythropoiesis

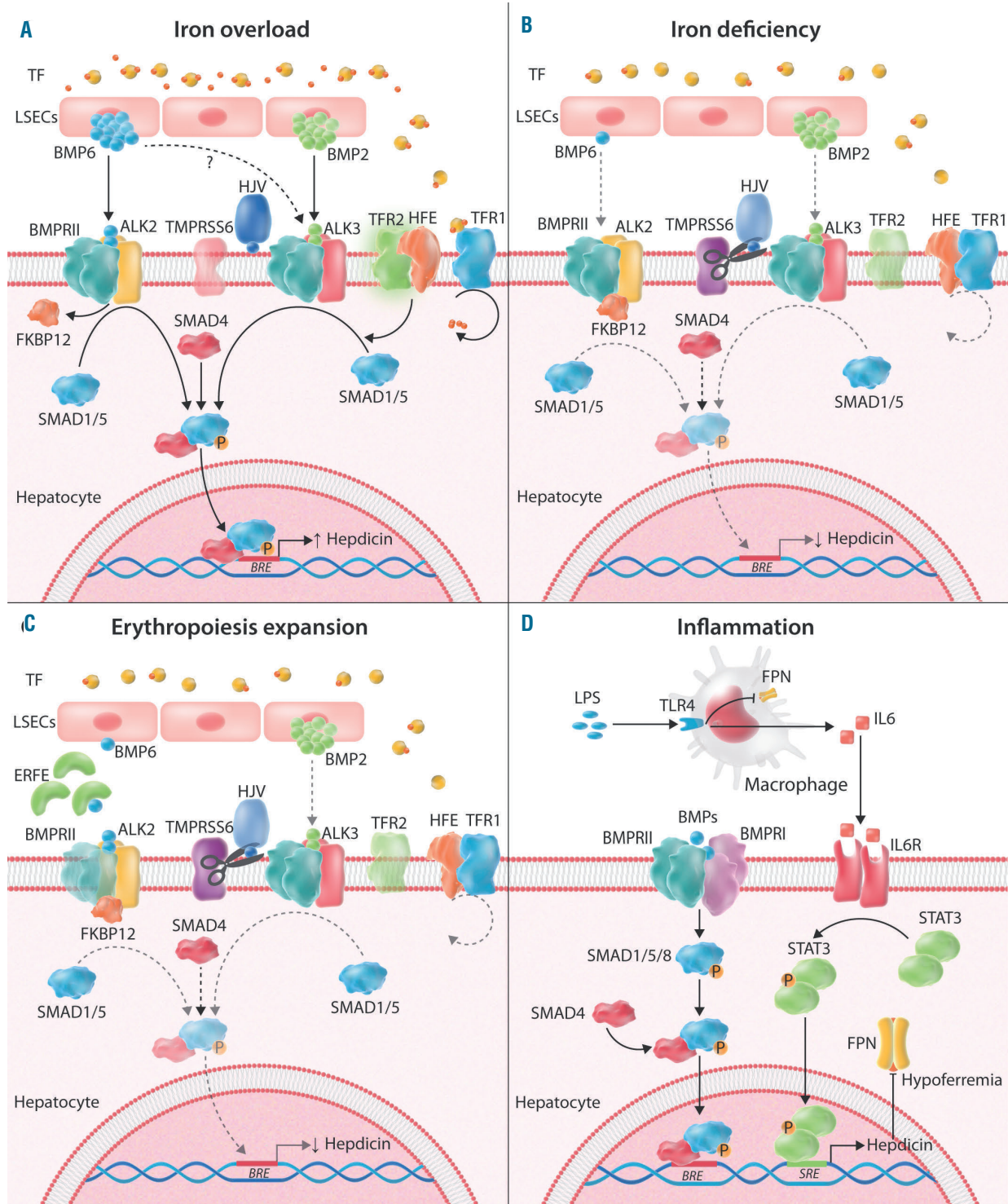
The hepcidin-ferroportin axis intersects other biological systems, such as IRP, hypoxia responsive pathways and erythropoietin signaling.

#### Iron absorption revisited

Iron absorption is a physiological example of crosstalk between IRP-hypoxia and the hepcidin-ferroportin axis. In the hypoxic duodenal environment, IRP1 controls translation of hypoxia-inducible factor 2 $\alpha$  (HIF-2 $\alpha$ ) which, stabilized by prolyl hydroxylase, upregulates the expression of apical (DMT1) and basolateral (ferroportin) enterocyte iron transporters.<sup>62</sup> In iron deficiency, absorption is enhanced by hepcidin downregulation, which, favoring export, depletes enterocytes of iron, further stabilizing HIF-2 $\alpha$ <sup>63</sup> (Figure 1). In iron overload, high hepcidin increases enterocyte iron and impairs luminal uptake. In addition, the rapid cell turnover with shedding of ferritin-loaded enterocytes further limits iron absorption. In this way the interaction between local (hypoxia, IRP) and systemic (hepcidin) mechanisms optimizes iron balance.

#### Crosstalk between iron and erythropoiesis

Iron and erythropoiesis are interconnected at multiple levels and are reciprocally regulated. First, iron tunes renal production of erythropoietin, the growth factor essential for proliferation and differentiation of erythroid cells, The



**Figure 2. The regulation of hepcidin expression** (adapted from Silvestri et al.<sup>147</sup>). Schematic representation of a model of hepcidin regulation by two branches of the bone morphogenetic protein (BMP)-SMAD pathway, based on BMP knockout models,<sup>44,45,148</sup> in (A) iron overload, (B) iron deficiency, (C) erythropoiesis expansion and (D) inflammation. According to a recent study BMP2 and BMP6 work collaboratively *in vivo*, possibly as a heterodimer.<sup>153</sup> (A) BMP2 produced by liver endothelial cells (LSEC) binds BMP receptor type II, which phosphorylates the BMP receptors type I (ALK3) to activate SMAD1/5/8. The latter associated with SMAD4 translocate to the nucleus to bind BMP responsive elements (BRE) in the hepcidin promoter. In iron overload increased diferric transferrin displaces HFE from transferrin receptor (TFR) 1 to enable iron uptake and stabilizes surface TFR2 potentiating ALK3 signaling. Hemojuvelin (HJV) acts as a BMP co-receptor, while the function of the other hemochromatosis HFE and TFR2 proteins in the pathway activation is still unclear. Iron increases the production of BMP6 by LSEC, thereby activating ALK2 and likely also ALK3 (indicated by a dotted arrow) in the pathway. (B) In iron deficiency TMPRSS6, stabilized on the cell surface,<sup>149</sup> cleaves HJV<sup>49</sup> and inactivates the BMP2-ALK3 branch of the pathway. TFR2 is destabilized by the lack of diferric transferrin and HFE binds TFR1.<sup>150</sup> The BMP6 pathway is inactive in the absence of the ligand and also because ALK2 is inactivated by FKBP12 binding.<sup>51</sup> Together with epigenetic regulation,<sup>151</sup> this suppresses hepcidin expression. (C) Erythropoiesis enhanced by erythropoietin consumes a large amount of iron: low serum iron (and diferric transferrin) suppresses the BMP2-ALK3 pathway,<sup>152</sup> while BMP6 and other BMP are sequestered by erythroferrone (ERFE) released by erythroid cells.<sup>72,154</sup> The result is hepcidin inhibition. (D) Schematic representation of hepcidin regulation by inflammatory cytokines and of the pathogenesis of anemia of inflammation. Stimulated by inflammation [here indicated by lipopolysaccharide (LPS) through toll-like receptor 4 (TLR4)], macrophages produce interleukin-6 (IL6), which stimulates Janus kinase 2-signal transducer and activator of transcription 3 (JAK2-STAT3) signaling to upregulate hepcidin in association with the BMP-SMAD pathway.

synthesis of erythropoietin is mediated by HIF-2 $\alpha$ . In iron deficiency, IRP1 binding to HIF-2 $\alpha$  5'IRE represses the latter's translation and decreases erythropoietin production, to limit erythropoiesis and iron consumption. When the mechanism fails, as in *Irf1*-knockout mice, a transient polycythemia occurs in the relatively iron-deficient young animals, which reverts in adult mice with iron sufficiency.<sup>64,65</sup> In this context prolyl hydroxylase, the enzyme that induces degradation of HIF-2 $\alpha$ , is iron-dependent and is thus inactive in iron deficiency.

Second, in *in vitro* studies, iron deprivation induced a block of early erythroid progenitors, by inactivating mitochondrial aconitase;<sup>66</sup> this block could be overcome by isocitrate supplementation.<sup>67</sup> Finally the erythroid response to iron restriction is optimized by the iron sensor TFR2, a partner of erythropoietin receptor.<sup>10</sup> Loss of bone marrow TFR2 in mice increases the sensitivity of erythroblasts to erythropoietin, which causes erythrocytosis, especially in iron deficiency.<sup>68</sup> Liver TFR2 upregulates hepcidin and *TFR2* mutations cause hemochromatosis.<sup>69</sup> As a sensor of iron-bound transferrin, erythroid TFR2 regulates erythropoiesis, while liver TFR2, controlling hepcidin, modulates iron acquisition according to erythropoietic needs. The recently demonstrated *TFR2* expression in osteoclasts and osteoblasts<sup>70</sup> places this iron sensor at the crossroads of red cell production, iron homeostasis and bone turnover.

While iron regulates erythropoiesis, the reverse is also true. The old hypothesis that the erythropoietic drive controls iron absorption through an erythroid regulator<sup>71</sup> was confirmed by the discovery of ERFE, the best example of tissue-mediated regulation of hepcidin. ERFE is a member of the tumor-necrosis factor (TNF)- $\alpha$  family, produced by several tissues, but increased in response to erythropoietin only in erythroid precursors. ERFE sequesters BMP receptor ligands, especially BMP6,<sup>72</sup> inhibiting BMP-SMAD signaling and hepcidin. However, ERFE fails to suppress hepcidin when the BMP pathway is overactive.<sup>73</sup> *Erfe*-knockout mice are not anemic, indicating that ERFE has a modest effect on hepcidin repression in steady state. However, ERFE contributes to iron loading in mice with  $\beta$ -thalassemia.<sup>74</sup>

## Iron disorders

The improved understanding of iron physiology has profoundly changed the modern approach to iron disorders, known historically for centuries as iron deficiency (chlorosis) in young females and hemochromatosis (bronze diabetes) in middle-age males. We now suspect hemochromatosis based on iron parameters and confirm the diagnosis by genetic testing well before the development of iron overload and organ damage. We are aware that anemia is a complication of iron deficiency, though not the only one, since other tissues/organs may be iron-depleted before anemia develops, as occurs in chronic heart failure.<sup>75</sup>

Hepcidin is tightly controlled to maintain body iron balance. Loss of this control leads to opposite genetic or acquired disorders (Table 1).

### Genetic disorders

#### Hereditary hemochromatosis

The pathophysiology and diagnosis of hemochromatosis were profoundly influenced by the cloning of the *HFE*

gene<sup>76</sup> and the definition of the genetic heterogeneity of the disease (Table 1). Overall hemochromatosis is due to “insufficient hepcidin production” or exceptionally to “hepcidin resistance”.<sup>77</sup> Mutations in genes of the hepcidin-ferroportin axis disrupt iron homeostasis, leading to increased iron absorption, high transferrin saturation and increased toxicity from non-transferrin bound iron (NTBI) species.<sup>78</sup> The commonest form of hemochromatosis in Caucasians is due to homozygous *HFE*(C282Y) mutations. Genetic tests allow early diagnosis so that individuals with the affected genotypes show high biochemical penetrance (increased transferrin saturation  $\pm$  increased serum ferritin) but low clinical expression. Loss-of-function mutations of *HJV*, *TFR2* and *HAMP* (encoding hepcidin) lead to more severe diseases, collectively called “non-*HFE* hemochromatosis”. In all the recessive forms hepcidin is inappropriately low in comparison with iron excess and the onset and severity of iron overload correlate with the hormone deficiency.<sup>79</sup> The implementation of next-generation sequencing in familial and isolated cases of iron overload<sup>80</sup> has enabled the identification of mutations in more than one gene and provided examples of digenic inheritance. Both genetic and acquired modifiers contribute to the penetrance of *HFE*-hemochromatosis, interfering with hepcidin expression: e.g., alcohol aggravates the iron burden, whereas blood donations attenuates it.<sup>77</sup>

Ferroportin mutations are inherited in a dominant manner (Table 1). The different effects of these mutations account for the controversy in disease nomenclature. Loss-of-function mutations impair iron export, are associated with iron accumulation in K upffer cells and require no or reduced phlebotomy therapy, representing the true “ferroportin disease”.<sup>81</sup> Gain-of-function mutations lead to hepcidin resistance and the release of too much iron, as occurs in hemochromatosis.

The clinical severity of hemochromatosis is related to NTBI, a toxic iron species bound to low molecular weight molecules, easily taken up by hepatocytes and pancreatic cells via ZIP14 transporter<sup>82</sup> and by cardiomyocytes through other transporters.<sup>83</sup> While iron uptake by transferrin receptor is tightly regulated, the uptake of NTBI is not and persists in iron overload. NTBI leads to the generation of reactive oxygen species and cell damage, causing liver fibrosis (which may progress to cirrhosis and hepatocellular carcinoma), chronic heart failure, diabetes, hypopituitarism and other complications of iron loading.<sup>77,79</sup>

#### Iron-refractory iron-deficiency anemia

This form of iron deficiency anemia (IRIDA) was recognized after the discovery of hepcidin as being due to mutations of its inhibitor *TMPRSS6*.<sup>50</sup> High hepcidin levels lead to a phenotype opposite to that of hemochromatosis, reminiscent of anemia of inflammation.<sup>84</sup> The anemia is refractory to oral iron and may require intravenous therapy, especially when iron demand is high.<sup>85</sup> *TMPRSS6* genetic variants modulate iron and hematologic traits in several genome-wide association studies,<sup>58</sup> alter hepcidin levels in normal subjects<sup>86</sup> and might confer susceptibility/resistance to iron deficiency, as observed in blood donors.<sup>87</sup>

Other rare recessive disorders of the transferrin receptor pathway – such as hypotransferrinemia and *DMT1* mutations – lead to “atypical microcytic anemia” with

increased transferrin saturation and iron stores, because of decreased iron utilization by blunted erythropoiesis.<sup>85</sup>

#### **Congenital sideroblastic anemia**

Ringed sideroblasts are erythroblasts with iron-loaded mitochondria that, clustering around the nucleus, confer the appearance of a ring at Perls iron staining. Hereditary sideroblastic anemias are usually due to heme deficiency: X-linked sideroblastic anemia is caused by mutations in *ALAS2*, the first, rate-limiting enzyme of heme biosynthesis, while recessive forms are associated with mutations of mitochondria glycine importer solute carrier family 25 member 38.<sup>88</sup> Rare severe cases result from mutations of Fe/S cluster proteins, such as *GLRX5*<sup>89</sup> or *HSPA9*,<sup>90</sup> which decrease Fe/S groups and the activity of ferrochelatase, the last enzyme of the heme pathway. Another cause of low heme in *GLRX5* deficiency is the overactive IRP1 that, not being converted to aconitase because of the Fe/S cluster deficit, blocks *ALAS2* translation, thereby preventing heme formation. These disorders reveal the tight connection of heme-Fe/S metabolism. Among syndromic forms, the X-linked *ABC7* deficiency reduces export of Fe/S clusters to the cytosol,<sup>91</sup> while others are associated with immunodeficiency<sup>92</sup> strengthening the need of Fe/S clusters in other cell types (Table 1). Those due to mitochondrial protein mutations<sup>91</sup> are not discussed here.

#### **Acquired iron disorders**

##### **Iron deficiency**

Iron deficiency, both isolated and associated with anemia, represents one of the five major causes of disability burden worldwide, especially in women.<sup>93</sup> For discussions of the etiology, clinical presentation and treatment of iron deficiency with or without anemia readers are directed to specific reviews.<sup>94-96</sup> In absolute iron deficiency low total body and serum iron fully suppress hepcidin, a mechanism of adaptation to increase iron absorption. In functional iron deficiency (e.g., in inflammation) total body iron is not decreased, but iron is sequestered in stores by the high hepcidin levels.<sup>84,97</sup> This distinction strongly influences the route of iron administration required to treat iron deficiency, as discussed below.

##### **Anemia of inflammation**

Proinflammatory cytokines such as IL-6 and IL-1 $\beta$ , produced in chronic infections, autoimmunity, cancer, renal failure and other chronic disorders activate hepcidin expression leading to iron-restricted erythropoiesis and anemia of inflammation, once named anemia of chronic diseases.<sup>84,97,98</sup> By withholding iron in macrophages, extracellular Gram-negative microorganisms are deprived of this essential nutrient.<sup>99,100</sup> This is an innate defense mechanism known as 'nutritional immunity'.<sup>101</sup> A recent interpretation is that hypoferrremia prevents the generation of NTBI that potentiates the pathogenicity of Gram-negative bacteria.<sup>102</sup> Anemia, usually moderate and normocytic, is multifactorial, because of concomitant insufficient erythropoietin production and impaired early erythroid commitment.<sup>98</sup> Microcytosis occurs in longstanding severe inflammation such as in Castleman disease, a lymphoproliferative disorder in which high IL-6 production strongly enhances hepcidin synthesis<sup>103</sup> or in patients with ectopic hepcidin expression by liver adenomas.<sup>104</sup> Anemia reverts after anti-IL6 receptor treatment in Castleman disease or after surgical removal of the tumor

in the case of adenoma.

Anemia of inflammation regresses with control of the disease. In selected cases intravenous iron or erythropoiesis-stimulating agents are used. Since treatment is often unsatisfactory, manipulation of the hepcidin pathway (blocking either its production or function) is proposed as a novel therapeutic opportunity.<sup>97</sup>

##### **Iron-loading anemias**

Low hepcidin levels explain the iron overload that develops in the absence of blood transfusions in "iron-loading anemias", i.e., anemias with ineffective erythropoiesis (Table 1). ERF, released by erythropoietin-stimulated erythroblasts, inhibits hepcidin, despite iron overload. In non-transfusion-dependent  $\beta$ -thalassemia patients, serum ERF levels are high,<sup>105</sup> to ensure iron acquisition for the expanded erythropoiesis.<sup>106</sup> However, since the erythropoiesis is inefficient, excess iron interferes with erythroblast maturation aggravating anemia in a vicious cycle.<sup>107</sup> In patients with transfusion-dependent thalassemia, hepcidin increases following transfusions which partially suppress erythropoiesis.

ERF contributes to the iron loading of some clonal myelodysplastic syndromes. Patients with the ringed sideroblasts subtype of myelodysplastic syndrome (once called refractory anemia with ringed sideroblasts) carry a somatic mutation in the spliceosome gene *SF3B1*.<sup>108</sup> Among other abnormally spliced products, an elongated variant of ERF is more efficient than the wildtype hormone in hepcidin repression.<sup>109</sup>

#### **Diagnostic implications**

Notwithstanding spectacular advances in our understanding of iron metabolism and homeostasis our diagnostic approach to iron disorders still relies mainly on three historical tests: serum iron, transferrin (or total iron binding capacity) and ferritin. Transferrin saturation (Tsat), i.e. the ratio of serum iron/total iron binding capacity and serum ferritin coupled with genetic testing and non-invasive magnetic resonance imaging measurements of liver iron content, define the nature and severity of iron loading in both hemochromatosis<sup>77</sup> and thalassemia.<sup>110</sup> Other useful markers are the level of serum soluble transferrin receptor (sTfRC), related to the expansion of erythropoiesis or iron deficiency, the sTfRC/log ferritin ratio for the diagnosis of iron deficiency in inflammation<sup>98</sup> and the Tsat/log hepcidin ratio to suspect IRIDA.<sup>111</sup>

Enzyme-linked immunosorbent assay kits can measure serum hepcidin levels. However, this does not provide any information additional to serum ferritin, since the two variables are tightly related.<sup>112,113</sup> Some researchers propose determining hepcidin levels in order to choose the better therapeutic route of administration of iron supplementation (oral vs. intravenous),<sup>114</sup> as well as its correct timing<sup>115</sup> and schedule.<sup>116</sup> However, besides being subject to circadian oscillations, hepcidin levels change rapidly in response to activating and inhibitory signals, making their measurement useful in only a limited number of conditions.<sup>47</sup> A kit to measure human serum ERF concentration is available for research purposes. Whether the elongated ERF identified in individuals with *SF3B1* mutations will become a biomarker of ringed sideroblast myelodysplastic syndrome<sup>109</sup> remains to be tested.



## Therapeutic implications

Hepcidin levels favor response (when low) or resistance (when high) to oral iron administration, explaining part of iron refractoriness.<sup>117</sup> The dynamics of the increase in hepcidin levels after oral iron therapy have suggested that alternate-day administration of iron salts is an alternative to daily refracted doses, with the former being a protocol that increases both efficacy and tolerability, at least in women with iron deficiency without or with mild anemia.<sup>116,118,119</sup> The availability of more tolerated, iron-stable and efficacious preparations has increased the use of intravenous iron, especially of the high-dose single-injection compounds.<sup>120</sup> However, when used to correct iron deficiency in inflammation, intravenous iron may lead to macrophage iron accumulation whose long-term effects are unknown.

Manipulation of the hepcidin-ferroportin axis is the most logical experimental approach to iron disorders. The rationale is to use hepcidin agonists for iron overload disorders caused by inappropriate/low hepcidin and hepcidin antagonists to release sequestered iron in IRIDA and in anemia of inflammation (Table 2).

### Increasing hepcidin levels /decreasing ferroportin activity

In preclinical studies, increasing hepcidin levels prevented iron overload or redistributed iron to sites of safe stor-

age. Potentially useful in hemochromatosis, whose treatment is still based on phlebotomy,<sup>77</sup> hepcidin agonists are of interest in disorders with ineffective erythropoiesis, such as  $\beta$ -thalassemia.<sup>107</sup> Agonists include hepcidin analogues, minihepcidins, inhibitors of hepcidin repressors such as anti-TMPRSS6 molecules or compounds that block ferroportin activity. By inducing iron restriction hepcidin agonists ameliorated anemia and iron overload in preclinical studies of thalassemia models,<sup>106,121</sup> a few hepcidin agonists are currently being investigated in phase I-II clinical trials (Table 2). Hepcidin mimics could also be useful to induce iron restriction in polycythemia.<sup>122</sup> According to recent findings hepcidin might have a role as an antimicrobial peptide in the treatment of Gram-negative sepsis<sup>102</sup> and streptococcal necrotizing fasciitis.<sup>56</sup>

### Other approaches

In non-transfusion-dependent  $\beta$ -thalassemia (*Hbb<sup>th1/hh1</sup>* and *Hbb<sup>th3/4</sup>*) mice transferrin infusions improve the phenotype, increasing hepcidin and hemoglobin levels, improving erythrocyte survival and limiting splenomegaly,<sup>123,124</sup> effects similar to those observed when *Tfr1* expression is decreased.<sup>125</sup> Selective inactivation of bone marrow *Tfr2* improves anemia in a non-transfusion-dependent *Hbb<sup>th3/4</sup>* model, enhancing the sensitivity of erythroid cells to erythropoietin.<sup>126</sup>

Short interfering RNA against *DMT1*, administered in

**Table 2.** Targeted therapeutic approaches for disorders with low and high hepcidin.

| Compounds  | Mechanism  | Effect                                     |
|--|--|--|
| <b>IA. TO INCREASE HEPCIDIN OR REDUCE FERROPORTIN ACTIVITY<sup>122</sup></b>   |  |  |
| <b>Hepcidin analogues</b> and minihepcidin <sup>121</sup>                      | Replacement therapy                                  |  |
| BMPs   | Activating the hepcidin signaling pathway            | Increased hepcidin                         |
| <b>Anti-TMPRSS6 (ASO, siRNA)</b> <sup>137</sup>                                | Counteracting hepcidin inhibition                    | Reduced iron overload                      |
| <b>FPN inhibitor VIT-2763</b> <sup>155</sup>                                   | Blocking the hepcidin receptor                       | Increased Hb in ineffective erythropoiesis |
| <b>IB. OTHER APPROACHES</b>  |  |  |
| Transferrin injections <sup>123</sup>  | Decreasing transferrin receptor 1                    | Reduction of iron uptake                   |
| Protoporphyrin IX <sup>138</sup>   | Inhibiting heme oxygenase 1                          | Reduction of iron recycling                |
| <b>IIA. TO DECREASE HEPCIDIN OR INCREASE FERROPORTIN ACTIVITY<sup>97</sup></b> |  |  |
| Anti cytokines (IL-6, IL-6R) <sup>103</sup>                                    |  |  |
| <b>Anti-BMP6 MoAb</b> <sup>139</sup>   |  |  |
| BMP receptor inhibitors <sup>140</sup>   | Reducing the hepcidin signaling pathway              | Reduced hepcidin                           |
| Anti-hemojuvelin MoAb <sup>141</sup>   |  | Reduced macrophage iron sequestration      |
| Heparins <sup>142</sup>  |  | Correction of hypoferrremia                |
| Anti-hepcidin MoAb <sup>143</sup>  |  | (Partial) correction of anemia             |
| <b>Anti-hepcidin Spiegelmer</b> <sup>144</sup>                                 | Hepcidin binders                                     |  |
| <b>Anti-hepcidin anticalin</b> <sup>145</sup>                                  |  |  |
| Anti-ferroportin MoAb <sup>139</sup>   | Interfering with hepcidin-ferroportin interaction    |  |
| GDP <sup>146</sup>   | Blocking iron export and decreasing Stat3 activation |  |
| <b>IIB. OTHER APPROACHES</b>   |  |  |
| Prolylhydroxylase inhibitors <sup>134</sup>                                    | Increasing EPO                                       | Correction of EPO defect                   |
|  | Increasing iron absorption                           | Correction of hypoferrremia                |

I. Compounds potentially useful in hereditary hemochromatosis and  $\beta$ -thalassemia; II. Compounds potentially useful in anemia of inflammation. Compounds tested in clinical trials are indicated in bold. BMP: bone morphogenetic protein; ASO: antisense specific oligonucleotides; siRNA: short interfering RNA; FPN: ferroportin; VIT 2763: small molecule oral ferroportin inhibitor; Hb: hemoglobin; IL: interleukin; MoAb: monoclonal antibodies; GDP: guanosine 5'-diphosphate encapsulated in lipid vesicle; EPO: erythropoietin.

nanoparticles to target intestinal absorption,<sup>127</sup> established a proof of principle of reducing dietary iron uptake. Another approach might be to block intestinal HIF-2 $\alpha$  by specific antagonists.

Clinical trials are showing that correcting ineffective erythropoiesis by activin ligand traps<sup>128</sup> not only improves anemia but, in the long-term, also iron loading in both non-transfusion-dependent and transfusion-dependent thalassemia<sup>129</sup> and ringed sideroblast myelodysplastic syndrome.<sup>130</sup> Some thiazolidinones have been shown to stimulate hepcidin activity in preclinical studies.<sup>131</sup> The use of proton pump inhibitors reduced the need for phlebotomy in patients with hemochromatosis.<sup>132</sup>

### Decreasing hepcidin levels/increasing ferroportin function

In preclinical models of anemia of inflammation, hepcidin antagonists decreased hepcidin expression, an effect verified in clinical trials for some compounds.<sup>133</sup> Another option is to interfere with the hepcidin-ferroportin interaction (Table 2). However, targeting the hepcidin-ferroportin axis may not fully correct this multifactorial anemia characterized by low erythropoietin and a blunted erythropoietic response.<sup>97,98</sup> Another approach is based on manipulation of the hypoxia-responsive pathway.<sup>65</sup> Prolyl hydroxylase inhibitors or HIF stabilizers, now tested in chronic kidney disease, by increasing HIF-2 $\alpha$  might target two abnormal processes enhancing both erythropoietin synthesis and iron absorption.<sup>134</sup>

### Unresolved issues

Notwithstanding significant advances many questions about iron metabolism and homeostasis remain un-

answered. The mechanisms of intestinal heme absorption are mysterious, as are the roles of secreted ferritin and soluble transferrin receptor. We have just started exploring the autonomous regulation of iron in the heart and vascular wall; the role of iron (deficiency or excess) as a cofactor of metabolic disorders, chronic liver disease, heart failure, pulmonary hypertension and neurodegeneration still requires elucidation. We need to be able to diagnose isolated tissue iron deficiency better and to increase the limited number of iron status markers.

In hematology we need to clarify the relationship between iron and platelet production considering that iron deficiency directs the common erythroid-megakaryocyte precursor towards the platelet lineage.<sup>135</sup> More information is required on the role of iron in B-lymphocyte development and function, in B-cell malignancies, such as multiple myeloma,<sup>136</sup> and in response to infectious diseases. We have to explore better how iron/TFR2 intersects the erythropoietin signaling pathway and bone metabolism.

We need novel protocols of iron supplementation and clear indications regarding high-dose intravenous iron to optimize iron therapy. Targeted approaches, now in clinical trials, have the potential to change traditional treatment – such as the time-honored phlebotomy-based regimen – for disorders such as hemochromatosis. Repurposing commercially available compounds, developed for other conditions, to iron/erythroid disorders is another option. All these approaches will, it is hoped, enable a more personalized treatment of iron disorders in the near future.

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