CHAPTER 20

Iron homeostasis

Carole Beaumont, Sophie Vaulont

1. Introduction

Iron is a paradoxical element in the sense that it is, at the same time, essential to any form of life, primarily to ensure the transport of oxygen or to catalyse reactions of electron transfer, nitrogen fixation or DNA synthesis, but it is also toxic due to its capacity to react with oxygen and to catalyse the production of reactive oxygen species. In solution, iron can exist under two states of oxidation, Fe (II) and Fe (III), and is very poorly soluble at physiological pH, especially when it is oxidised as Fe (III). Living organisms have thus developed many proteins to convey iron in biological fluids or through cellular membranes, and to store it in a non-toxic and easily mobilisable form (see (1, 2) for reviews). The total iron content of an adult human organism is about 4-5 g, most of it being associated with haemoglobin in circulating red cells (approximately 2.5 g). This haem iron is continuously recycled following phagocytosis and catabolism of senescent red blood cells by the tissue macrophages. This process makes it possible to recycle approximately 25 to 30 mg of iron per day, corresponding to the daily requirement of iron for erythropoiesis. The intestinal absorption of iron ensured by the mature enterocytes at the top of duodenal villi is about 1-2 mg per day which makes it possible to compensate for losses, resulting mainly from the exfoliation of epithelial cells (Figure 1). Only a fine regulation of intestinal iron absorption makes it possible to avoid iron overload of the organism since there is no means to eliminate any iron absorbed in excess. This level of absorption can be markedly increased in the event of iron deficiency, haemolysis or significant bleeding.

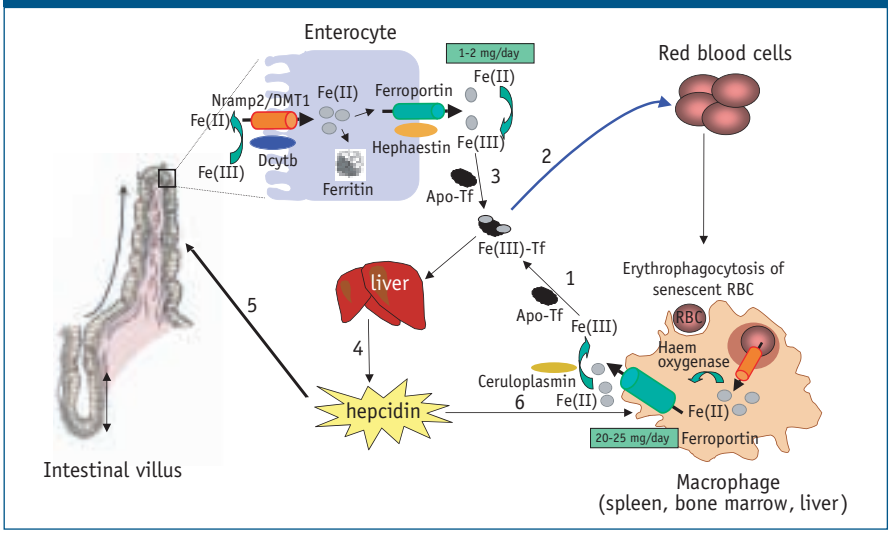
2. Iron acquisition by cells

In mammals, iron circulates in plasma bound to transferrin, an abundant protein synthesised and secreted by the liver. Transferrin has two high affinity iron binding sites specific for Fe (III), and iron binding requires the presence of carbonates or bicarbonate ion. Under normal conditions, the saturation of transferrin is about 30%. When the iron binding capacity of transferrin is saturated, iron can appear in the serum in a free form, non-bound to transferrin (NTBI). This iron penetrates easily into cells, particularly in the liver and the heart, by facilitated passive diffusion or through a transport system not yet identified, and can contribute to the onset of tissue iron overload and cause significant cellular damage.

In the liver, ZIP14, a member of the SLC39A zinc transporter family, has been recently proposed as contributing to the iron loading in the hepatocytes (3, 4). Two other plausible candidates for NTBI uptake have emerged.

First, antimicrobial neutrophil gelatinase–associated lipocalin (NGAL, 24p3), also called lipocalin-2, has been shown to carry metal in the form of a siderophore-iron

Figure 1: **Iron homeostasis**



Iron required for erythropoiesis (20-25 mg daily) is provided by the destruction of senescent red blood cells by tissue macrophages (1). Iron released to the plasma by ferroportin is oxidised by caeruloplasmin and transported by transferrin to the erythroid precursors in the bone marrow (2). Intestinal iron absorption by duodenal enterocytes compensates for daily losses (3) (1-2 mg per day). Hepcidin, a cystein-rich peptide synthesised by hepatocytes (4), negatively regulates iron export from enterocytes (5) and from macrophages (6) by binding to ferroportin and inducing its internalisation and degradation.

complex (5). This complex binds to a specific cell surface receptor for internalisation. While information to date has implicated lipocalin-mediated iron uptake only in renal development, it is possible that it functions elsewhere, particularly when normal uptake pathways are absent.

The second candidate is the L-type calcium channel that has been shown to contribute to cardiac iron loading in mice (6). While these channels have typically been studied in excitable cells, they are widely expressed and might also function in hepatocyte iron uptake.

The internalisation of the iron-transferrin complex by the cells requires specific membrane receptors present at the surface of many cellular types. The transferrin receptor (TfR) is a dimer of two identical subunits of 95kDa molecular weight, bound by two disulphide bridges. There are two different genes coding for the transferrin receptor, *TfR1* and *TfR2*. The expression and the regulation of the *TfR2* gene is very different from that of *TfR1*, and limited mainly to the liver (7, 8). In addition, the

affinity of the transferrin for TfR2 is approximately 30 times weaker than for *TfR1*. Although *TfR2* can bind and internalise transferrin, this receptor probably does not serve a primary role in cellular iron uptake. Mutations in *TfR2* gene are responsible in humans for hereditary haemochromatosis (HH) with liver iron accumulation (9), reinforcing the idea that this receptor, in contrast to *TfR1*, is not involved in iron uptake but may contribute to signalling between iron stores and the duodenum (10). The erythroid precursors of the bone marrow can express up to a million molecules of *TfR1* on their surface. Once bound to the receptor, the transferrin-iron complex is internalised by endocytosis. Following acidification of the endosome, iron is released from transferrin, reduced to Fe (II), possibly by Steap3, a recently identified ferrireductase (11) and transferred to the cytosol by Nramp2/DMT1 (also called SLC11a2), a cotransporter of Fe (II) and protons (12). Several isoforms of Nramp2/DMT1, resulting from an alternative splicing or the use of two tissue-specific promoters, have been reported (13). One isoform is mostly expressed at the apical side of duodenal enterocytes and constitutes a very specific route for dietary Fe (II) acquisition by these cells (14). The other form is present in almost all tissues and represents the endosomal iron transporter (15). DMT1 mutations have been shown to be responsible for a hypochromic microcytic anaemia, in both the Belgrade rat (16) and in *mk* mice (17). Interestingly, three cases of homozygous DMT1 mutations have been recently described in humans, presenting with severe neonatal hypochromic microcytic anaemia and liver iron overload (18-20). Taken together, these DMT1 mutations highlight the role of the transferrin receptor-mediated iron uptake pathway in erythropoiesis. In mice, tissue-specific deletion of DMT1 has confirmed the preponderant role of DMT1 in intestinal iron absorption and in erythropoiesis (21).

Tissue macrophages, which have a specific function in iron recycling, express very few transferrin receptors. These specialised cells acquire iron mainly in the form of haemoglobin through erythrophagocytosis of senescent red blood cells (22).

3. Erythrophagocytosis and haem iron recycling by the macrophages

The majority of iron in the organism is present associated with haemoglobin, and phagocytosis of senescent erythrocytes by tissue macrophages ensures an efficient recycling of iron (Figure 1). The amount of iron recycled daily by macrophages is about 20-25 mg and is sufficient to ensure the iron requirements for erythropoiesis (22, 23). This mechanism relates mainly to spleen and bone marrow macrophages and, to a lesser extent, to Küpffer cells in the liver. The biochemical modifications of red blood cell membranes during ageing (externalisation of phosphatidyl-serine, peroxidation of the membrane lipoproteins, loss of sialic acid residues and formation

of neo antigens of senescence) constitute essential signals for the macrophage to identify the red cells to be eliminated. After the initial stage of recognition ensured by the interaction of the red cells with specific receptors, the red cell is internalised by phagocytosis and the maturation of the phagosome (which can include the recruitment of the endoplasmic reticulum (24)) will allow the degradation of red cell components. Under the action of an enzymatic complex anchored in the membrane of the endoplasmic reticulum and containing an NADPH-cytochrome C reductase, haem oxygenase 1 and biliverdin reductase, the intracellular catabolism of haem produces CO, iron and bilirubin.

Iron released by the catabolism of the senescent red cells is recycled towards the plasma or stored in the macrophage associated with the ferritin molecule, a highly conserved iron-binding protein (see (25) for review). The egress of iron out of the macrophages is ensured by ferroportin (also called IREG1, MTP1 or SLC40a1) a membrane exporter of Fe (II) (see (26) for review). This protein is expressed mainly in the macrophages of the liver and spleen, in duodenal enterocytes and in placenta (27). Ferroportin has now been well characterised as the receptor for the iron regulatory hormone, hepcidin (see below). The early conditional inactivation of the ferroportin gene during mouse development induces iron deficiency anaemia due to cellular iron retention in macrophages and duodenal enterocytes, showing that ferroportin is probably the sole iron exporter in these tissues (28). In humans, several mutations in ferroportin were recently described in an autosomal dominant form of haemochromatosis (the ferroportin disease) (29). This pathology is characterised by elevated serum ferritin levels generally with normal transferrin saturation, reflecting iron overload mainly at the level of the liver macrophages (Küpffer cells). Accordingly, a missense mutation was recently characterised in mouse ferroportin that affects its iron export capacity. Similar to human patients, the heterozygote mice present iron loading in the Küpffer cells, high serum ferritin and low transferrin saturation (30). Various ferroportin mutations have now been reported and shown to affect either the iron transport capacity of the protein or its capacity to respond to the hepcidin systemic signal (31-33). The Fe (II) transported towards plasma by ferroportin is oxidised by caeruloplasmin, a plasmatic copper-dependant ferroxidase synthesised by the liver. Interestingly, the presence of caeruloplasmin was also shown to be directly required for the stability of the cell surface ferroportin (34). Fe (III) is then bound by transferrin. The inactivation of the caeruloplasmin gene in mouse induces an excessive iron accumulation in hepatocytes and macrophages (35). It is likely that caeruloplasmin is also involved in the exchanges of iron between various tissues, and patients with hereditary acaeruloplasminaemia gradually develop iron overload often associated with diabetes, retinal degeneration and neurological symptoms (36).

4. Intestinal iron absorption

Intestinal iron absorption is limited to the duodenum and is ensured by the mature enterocytes present at the top of the villi. Iron is absorbed at the apical side, transferred to the baso-lateral side of the enterocyte, and then exported towards plasma (Figure 1). Part of the absorbed iron may remain into the enterocyte associated with ferritin. In this case, iron will be eliminated at the time of exfoliation of the cells. In humans, a normal daily diet contains approximately 13-18 mg of iron, of which only 1-2 mg will be absorbed. The molecular mechanisms of inorganic iron absorption have recently been clarified (37, 38). The first step consists in mobilising iron by reducing it from the ferric to the ferrous state. This step is believed to be catalysed by Dcytb, a membrane-bound reductase of the b561family of cytochromes whose expression is strongly induced by iron deficiency. However, mutant mice with Dcytb deficiency do not present iron deficiency nor any abnormalities of erythropoiesis (39), suggesting that, at least in the mouse, other ferric reductase enzyme or other factors can function in dietary iron absorption. Ferrous iron Fe (II) is then transported through the membrane of the enterocyte by the previously mentioned Nramp2/DMT1 transporter, whose synthesis is, as for Dcytb, strongly induced by iron deficiency. Haem iron represents a significant source of iron in the diet and seems to be better absorbed than inorganic iron. The mechanism of haem absorption is still poorly understood but may depend on the recently identified haem carrier, HCP1 (40). This protein was demonstrated to be also a high affinity folate transporter and was thus renamed PCFT/HCP (41). After catabolism of haem by haem oxygenase 1, iron probably combines with the pool of iron imported by Nramp2/DMT1 and is exported towards plasma via ferroportin present at the baso-lateral side of the enterocytes. Binding of iron by plasma transferrin requires its preliminary oxidation into Fe (III) and this stage is catalysed by the transmembrane protein hephaestin. This enzyme shares 50% identity with caeruloplasmin and belongs to the family of multi-copper oxidases. A partial deletion of the hephaestin gene present on X chromosome was found in the *sla* (sex linked anaemia) mice, which have microcytic hypochromic anaemia due to a deficit in intestinal iron absorption, and iron overload of duodenal enterocytes (42).

5. Intracellular homeostasis of iron: the IRE/IRP system

The synthesis of a number of key proteins of iron metabolism involved in transport, storage and utilisation of iron is controlled in a coordinated way at the post-transcriptional level by intracellular iron (see (43) for review). This regulation depends on the interaction between cytoplasmic proteins named “iron regulatory proteins” (IRP), which act as sensors of iron, and “iron responsive elements” (IRE), which

are highly conserved 30-nucleotide mRNA motifs with a stem-loop structure. Single IRE motifs are present in the 5′ non-coding region of mRNA, coding for H and L ferritin subunits, ferroportin and the erythropoietic form of delta aminolaevulinic acid synthase (eALA-S). One or more IREs are also found in the 3′ non coding region of mRNA coding for proteins implicated in iron transport (transferrin receptor TfR1, isoform I of Nramp2/DMT1). These iron-mediated post-transcriptional regulations allow the cell to adapt its capacity of iron acquisition to its immediate requirements. This is achieved by modulating the stability of TfR1 mRNA and the translation of the ferritin iron-storage protein following iron entry in the cell.

There are two distinct molecular forms of IRP, IRP1 and IRP2, which present a high binding-affinity for IREs in the native state. The entry of iron into cells induces a change in IRP1 conformation by acquisition of an iron-sulphur cluster [4Fe-4S] or oxidation of IRP2 followed by its degradation by the proteasome. The recognition of an IRE motif by an IRP molecule induces repression of ferritin and eALA-S synthesis, by preventing the formation of the translation initiation complex and stabilisation of TfR1 mRNA by protection against endonuclease cleavage. The function of the IRE found in ferroportin or Nramp2/DMT1 mRNAs seems more complex and still remains poorly defined. However, recent elegant studies by Galy and collaborators have highlighted the involvement of the IRPs in the duodenum in controling key iron absorption molecules (44) and allowed the definition of the respective roles of IRP2 in the determination of critical body iron parameters such as organ iron loading and erythropoiesis (45).

A pathological condition called “hereditary hyperferritinaemia-cataract syndrome” (HHCS) is due to mutations in the IRE of the L ferritin mRNA (46, 47). This autosomal dominant syndrome is characterised by both hyperferritinaemia in the absence of other signs of iron overload and bilateral early onset cataract. Several mutations or partial deletions of IRE structures were described (48), resulting in constitutive expression of L ferritin in the absence of iron overload. The crystal formation of ferritin in the dehydrated environment of the lens is the prime cause of the cataract (49). This syndrome is one of the rare examples of a translational pathology. It represents a differential diagnosis of haemochromatosis, classically diagnosed on the basis of hyperferritinaemia.

6. Iron homeostasis at level of the organism: the role of hepcidin

There is no specific mechanism by which organisms can eliminate iron absorbed in excess, and iron overload can only be avoided by the fine tuning of intestinal iron absorption and iron recycling by macrophages (50). The regulation of intestinal iron absorption has remained elusive for a long time, but notable progress has been

achieved recently with the identification of genes responsible for genetic forms of haemochromatosis (HFE, TfR2, Haemojuvelin, HJV see (51, 52) for reviews as well as Chapters 22 and 23 of this book) and lately by the discovery of hepcidin, a circulating peptide which plays a major role in iron homeostasis. Hepcidin was isolated and purified in 2001 simultaneously by two groups who were trying to identify new antimicrobial peptides (53, 54). Hepcidin was found to have antimicrobial activity *in vitro* but this activity, as compared to other antimicrobial peptide of the defensin family, is effective at much higher concentrations and requires a much longer time of action. In contrast to the inferior vertebrates, where the activity of hepcidin seems to play a very significant role in the innate immune response, the role of hepcidin in superior vertebrates has evolved towards a role in iron homeostasis. However, in particular conditions, when produced by macrophages infected with *Mycobacterium tuberculosis,* hepcidin may have antimycobacterial activity (55). The hormonal role of hepcidin was initially revealed thanks to the study of two transgenic murine models. Hepcidin deficient mice were shown to develop tissue iron overload, especially in liver, pancreas and heart, with depletion in macrophages iron stores (56). Conversely, hepcidin transgenic mice which overexpressed hepcidin throughout development were severely anaemic at birth and rapidly died of microcytic hypochromic anaemia (57). It is now established that hepcidin reduces the quantity of circulating iron by preventing its exit from the cells, especially from enterocytes and macrophages. To limit cellular iron egress, hepcidin binds to ferroportin, thereby inducing its internalisation and degradation (58, 59). The molecular mechanisms of hepcidin-induced ferroportin degradation have now been well established (60) and the exact binding domain of ferroportin required for hepcidin activity has been characterised (61). In the absence of hepcidin, increased intestinal iron absorption associated to increased iron efflux from macrophages lead to parenchymal iron overload (56, 62). It is likely that ferroportin expressed on placental cells is also the target of hepcidin produced by embryonic liver. This mechanism of hepcidin action accounts for the rapid reduction in serum iron levels which follows direct hepcidin injection into mouse (63), transgenic hepcidin gene induction (64) or hepcidin stimulation by IL-6 perfusion (65, 66).

7. Regulation of hepcidin gene expression

Hepcidin synthesis takes place mainly in the liver (hence the name “hep” for hepatocyte together with “idine” for its antimicrobial activity). However, recent studies indicate that hepcidin could also been synthesised in macrophages, in response to bacterial pathogens (55, 67), in activated splenocytes (68), in pancreatic beta cells (69), in kidney (70) and in adipocytes (71). However, the contribution of these

hepcidin-expressing tissues to the circulating levels of hepcidin has not been addressed so far. The hepcidin gene is of small size; it comprises 3 exons that code a pre-pro-peptide of 84 AA, including a N-Terminal signal peptide, a pro-region, and the C-terminal mature peptide of 25 AA, as purified in blood and urine (see (72) for review). The pro-hormone was shown to lack any biological activity (73). Posttranslational processing of hepcidin is mediated predominantly by the prohormone convertase furin (74-76). Hepcidin expression can be followed either by quantification of liver mRNA levels in animal models or by assaying serum or urinary hepcidin content. Only one group so far has been able to develop antibodies against hepcidin and to measure hepcidin in urine (77) and more recently in the serum (78). The difficulty in obtaining anti-hepcidin antibodies lies in the complexity of the three-dimensional structure of the peptide, its small size and its conservation among species. Like any antimicrobial peptide, hepcidin is a cysteine-rich peptide, with 8 residues out of 25 which are committed in four disulphide bridges. A number of studies have reported SELDI-TOF based assays to measure hepcidin to overcome the difficulty of developing an immuno-assay (see (65) for review). Secretion of bioactive hepcidin-25 by liver cells (76) and urinary hepcidin measured by ELISA (79) were shown to correlate with hepcidin gene transcription. There is also a commercially available ELISA for serum pro-hepcidin but the physiological significance of this assay, and thus its interest in clinical studies, is not validated and no relatioship between pro-hepcidin and iron absorption was demonstrated (see (65) for review).
Hepcidin expression, as could be expected from its dual properties, is controlled by iron and by inflammation (see (72) for review).

7.1 Hepcidin regulation by inflammation

The fact that the hepcidin gene is sensitive to inflammatory stimuli probably reflects the ancestral bactericidal properties of the peptide. Injection of LPS or turpentine into mice stimulates the production of hepcidin (80) and high levels of hepcidin were detected in patients developing anaemia of chronic diseases (76, 77). The proinflammatory cytokines play a central part in the induction of the hepcidin gene. IL6 stimulates hepcidin expression *in vivo* with concomitant reduction in serum iron (injection into mouse or perfusion in healthy volunteers (66)), as well as *in vitro* in hepatocytes in primary culture (77). The molecular mechanisms responsible for hepcidin regulation by IL-6 have been very well studied and the classical JAK/Stat3 pathway has been characterised (81-83). Importantly, the consequence of an increase in the production of hepcidin is compatible with all the symptoms characteristic of the anaemia of inflammation: reduction in serum iron, retention of iron in macrophages and blocking of intestinal iron absorption (see (84) for review). The anaemia of inflammation, commonly observed in patients with chronic

infections, malignancy, trauma, and inflammatory disorders, is a well-known clinical entity. Until recently, we understood little about its pathogenesis. It now appears that the inflammatory cytokine IL-6 induces production of hepcidin, which may be responsible for most or all of the features of this disorder. As for the cytokines such as IL-1 and TNF-α, their ability to activate or repress hepcidin gene expression remains under investigation (66, 85, 86).

7.2 Hepcidin regulation by iron status

Iron overload induces an increase in the synthesis of hepcidin (87). This response of hepcidin to iron is to limit iron excess, which can cause, when it accumulates, irreversible tissue lesions, secondary to the production of free radicals. Conversely, iron deficiency results in the reduction in the production of hepcidin which ensures a better availability of iron to the developing erythrocytes in the bone marrow (88). Thus, hepcidin appears as the "ferrostat" of the organism, adjusting the quantities of circulating iron according to body requirements.

The mechanisms of hepcidin gene regulation by iron are not yet fully elucidated but yet constitute an area of active research and impressive breakthroughs have been made the last three years. Many arguments suggest that the three haemochromatotic proteins HFE, TfR2 and HJV could, individually or in association, contribute to the regulation of hepcidin synthesis by iron. These proteins, when mutated, are responsible for HH, a prevalent iron disorder hallmarked by intestinal hyperabsorption of iron, hyperferraemia, and hepatic iron overload (52).

7.2.1 HJV

HJV belongs to the family of Repulsive Guidance Molecules, of which some isoforms are expressed in the central nervous system, and others, such as HJV, are expressed in the skeletal muscle and in the liver. HJV was shown to act as a Bone Morphogenic Protein (BMP) co-receptor which is involved in the regulation of hepcidin expression through the Smad1,5,8/Smad4 pathway (89, 90). Accordingly, liver-specific Smad4 knockout mouse manifest nearly complete deficiency of hepcidin together with systemic iron overload (91). The BMPs, belonging to the TGF-β family, are among the most potent inducers of hepcidin. However, while many ligands of the BMP family are able to positively regulate hepcidin expression *in vitro* (89, 91, 92) and *in vivo* (90), it is only recently that BMP6 has emerged has being the putative *in vivo* candidate responsible for the iron-dependent activation of the Smad signaling pathway (93). BMP6 gene expression is directly regulated by the amount of iron and increasing amount of phosphorylation of Smad1,5,8 could be directly related to iron-dependent expression of hepcidin *in vivo* (93, 94). In addition, a recent report

demonstrates that BMP6 knockout mice have severe haemochromatosis due to hepcidin deficiency related to an alteration of the Smad signaling pathway (Roth MP and colleagues, Nat Genetics, in press).
HJV proteins are membrane-bound through a GPI anchor and can also exist in a soluble form (sHJV). The soluble form is in competition with the membrane form, inducing the repression of hepcidin synthesis (95). sHJV was shown to inhibit holotransferrin-induced hepcidin gene expression in hepatocytes (96) and to lead to an increase in serum iron and a decrease in liver phospho-Smad1,5,8 when injected into mice (90). The prohormone convertase furin is responsible for the release of sHJV (97) and secretion of HJV has been reported to be regulated by iron and hypoxia (90, 95, 97). HJV was found to interact, like the other Repulsive Guidance Molecule, with the membrane protein neogenin (98). However, HJV-induced BMP signaling and hepcidin expression was shown to be independent of the presence of neogenin (99).

7.2.2 HFE

HFE is a non-classical HLA class I membrane molecule, whose function is not yet completely elucidated. Early experiments of co-crystallisation or co-immunoprecipitation clearly showed that the HFE protein was able to interact with TfR1 (100, 101). These two proteins are expressed at the baso-lateral side of the undifferentiated cells of the crypt located at the base of the duodenal villi and their interaction in the intestine has led to the proposal of several scenarios for the role of HFE in iron homeostasis (see (102) for review). However, recent data obtained with transgenic and knockout mouse models have clarified the role of HFE. First, intestinal HFE is not essential for the physiologic control of systemic iron homeostasis under steady state conditions (103) excluding a primary role for duodenal HFE in the pathogenesis of haemochromatosis. Second, HFE is required for appropriate expression of hepcidin which then controls intestinal iron absorption (104, 105). Accordingly, HFE deficiency in hepatocytes fully reproduces the murine HH phenotype (106). This observation, defining a new key role for HFE in hepatocytes, is in good agreement with the finding that haemochromatosis patients who have undergone liver transplantation do not demonstrate reaccumulation of excess liver iron posttransplantation (107). Finally, it was shown that HFE induces hepcidin expression when it was not in complex with TfR1 (108).

7.2.3 TfR2

For TfR2, it is now generally agreed that this receptor does not serve a primary role in cellular iron uptake but rather may act as an iron sensor (10). Like TfR1, TfR2 has been shown to interact with HFE (109). Schmidt et al. have recently proposed a model of serum iron sensing by the liver. At low transferrin saturations, HFE is

sequestered by TfR1. As serum iron saturation increases, HFE is dislodged from its overlapping binding site on TfR1 by Fe-Tf. HFE is then free to interact with TfR2 and to signal, in some manner, for the upregulation of hepcidin (108). If either HFE or TfR2 is mutated or absent, the complex is unable to sense increased serum transferrin saturation, and dysregulation of iron homeostasis occurs.

7.3 Hepcidin regulation by iron deficiency, anaemia and hypoxia

Iron deficiency, anaemia and hypoxia inhibit the synthesis of hepcidin allowing iron supply to match erythropoietic demand (80). This response is most likely multifactorial and some of the signals require active erythropoiesis (110). Several candidates able to repress hepcidin gene expression have been proposed. In iron deficiency, both decreased BMP6 levels (93) and increased sHJV (111) could contribute to hepcidin repression.

Recently, GDF15, a member of the TGF-β family, was reported to mediate the suppression of hepcidin in thalassaemia (112). On the basis of the very high concentrations of GDF15 observed by the authors in the blood of the β-thalassaemia patients they analysed, they proposed that GDF15 overexpression arising from an expanded erythroid compartment can contribute to iron overload in thalassaemia syndromes by inhibiting hepcidin expression. Indeed, in dyserythropoietic states such as thalassaemia, hepcidin expression was shown to be repressed in spite of the presence of iron overload (113, 114). The authors further provided evidence that GDF15 was able to directly inhibit hepcidin gene expression in hepatoma cells (112). Further investigation is needed to determine whether the pathogenic role of GDF15 is limited to anaemias with ineffective erythropoiesis. Pinto et al. recently proposed that erythropoietin (Epo), the primary signal that triggers erythropoiesis in anaemic and hypoxic conditions, could also directly mediate hepcidin repression in hepatocytes through Epo-R signaling and the regulation of the transcriptional factor C/EBPα (115). It has been suggested that hypoxia inducible factor (HIF)-1, a heterodimer whose expression is regulated post-translationally, can downregulate hepcidin in response to hypoxia. In the presence of oxygen, HIF-1 α is modified by iron-dependent prolyl hydroxylases (PHD) and is then degraded through the ubiquitin-proteasome pathway via its interaction with vHL. In the presence of hypoxia or following iron chelation, prolyl hydroxylase activity is inhibited. HIF-1 α then accumulates and translocates into the nucleus, where it binds to ARNT/HIF-1 β, which is constitutively expressed. The heterodimer HIF-1 binds to the hypoxic response elements (HREs) of target gene regulatory sequences, resulting in the transcription of genes implicated in the control of metabolism and angiogenesis, as well as apoptosis and cellular stress (see (116) for review). Erythropoietin is one of these direct target genes. HIF-1 was

recently shown to bind to the promoter of hepcidin *in vivo* and to reduce its expression in the liver (117). In support of a role for the HIF/vHL axis in coupling iron sensing to iron regulation, hepatic deletion of the vHL gene causes decreased hepcidin levels and increased ferroportin expression (117). Finally, an increase in reactive oxygen species (ROS) levels in hypoxic conditions has also been proposed as a possible mechanism responsible for hepcidin repression via alteration of C/EBPα and STAT-3 activities (118). Of note, oxidative stress and increased ROS levels have been postulated to be responsible for hepcidin down-regulation during alcohol loading (119) and in a mouse model expressing the hepatitis C virus and demonstrating hepatic iron loading (120).

Very recently a genetic inability to downregulate hepcidin has been recognised which was found to be due to TMPRSS6 mutations. The affected "Mask" mice have abnormal hair distribution and iron-deficiency anaemia, due to decreased iron absorption because of inappropriately high hepcidin levels (121). Similarly, TMPRSS6 KO mice display an overt phenotype of alopecia and a severe iron deficiency anaemia accompanied by a marked up-regulation of hepcidin (122). Finally, in humans, mutations of TMPRSS6 lead to iron-refractory iron-deficient anaemia (IRIDA) and IRIDA patients have remarkably high hepcidin levels, in spite of iron deficiency (123-125).

What is TMPRSS6? TMPRSS6, also called matriptase-2, is a type II transmembrane serine proteases (TTSPs) family member, primarily expressed in the liver. These proteolytic enzymes are characterised by a short N-terminal cytoplasmic tail, a membrane-spanning region, and an ectodomain encompassing potential ligand binding domains and a C-terminal trypsin-like serine-protease domain (see (126) for review). The inappropriate elevated levels of hepcidin observed in mutants mice an in humans who harbour TMPRSS6 mutations suggest that TMPRSS6 functions normally to down regulate hepcidin expression at the transcriptional level to maintain systemic iron level. Accordingly, Du et al. reported that in hepatoma cells, transfected TMPRSS6 was able to inhibit hepcidin activation by multiple stimuli and that the serine protease domain was necessary for this inhibitory activity (121). The molecular mechanisms responsible for TMPRSS6-mediated hepcidin suppression have been recently unravelled. Matriptase-2 interacts with HJV through the ectodomain and cleaves HJV on the plasma membrane releasing soluble small peptide fragments. This TMPRSS6-induces HJV cleavage is responsible for hepcidin inhibition by blocking the BMP/HJV activating pathway. Matriptase-2 «Mask» mutant shows no cleavage activity and the human mutant only partial cleavage capacity (127). How the proteolytic activity of TMPRSS6 responds to iron deficiency to activate HJV cleavage remains to be elucidated.

8. Involvement of hepcidin in iron overload disorders

Since the discovery in 1996 of the first gene implicated in the most frequent form of HH (128), the *HFE* gene, the list of genes responsible for haemochromatosis has increased (*HJV*, *TfR2*, *hepcidin* and *ferroportin*) making of HH a heterogeneous disease (see (29) for review). Affected patients with HH have parenchymal iron deposition in the liver, heart, and endocrine tissues but a paucity of iron in intestinal epithelial cells and tissue macrophages. In severe cases, tissue iron leads to cirrhosis, cardiomyopathy, diabetes, and other endocrinopathies. All forms of HH (excluding haemochromatosis related to ferroportin) have in common the fact that the expression of the hepcidin gene is inappropriate in face of the iron overload, with the severity and the precocity of the disease being directly related to the residual levels of hepcidin. Thus, in juvenile haemochromatosis (related to mutations in the haemojuvelin (129, 130) or hepcidin (131, 132) genes), which is a rare form of haemochromatosis with an early and severe aggravation of the iron burden, both hepcidin mRNA and urinary hepcidin levels are either completely lacking or very strongly decreased.

In haemochromatosis related to HFE (the most prevalent form of HH) and TfR2, the levels of hepcidin are not low but they do not increase in spite of the constitution of the iron overload (105, 133, 134). The causal link between hepcidin deficiency and haemochromatosis has been reinforced by the observation that, in a mouse model of HFE-dependent haemochromatosis, iron overload is prevented by overexpressing hepcidin.

Apart from HH, they are several conditions associated with secondary iron overload in which iron deposition is rather mild but could accelerate liver injury and the development of fibrosis, cirrhosis and hepatic carcinoma. Decreased hepcidin gene expression has been recently proposed as being responsible for hepatic siderosis in patients with porphyria cutanea tarda (135), in patients with hepatitis C virus (120) and in alcoholic liver diseases (119, 136). Hepcidin suppression was also proposed to account for the hepatic parenchymal siderosis observed in haem oxygenase deficiency (137).

9. Conclusion

In less than ten years, our understanding of iron metabolism has evolved considerably. It has changed from a simplified model based on a single uptake mechanisms relying on the transferrin receptor pathway and a storage mode based on ferritin, to a complex protein network highlighting the specialised function of certain cell types towards iron acquisition and transport. Identification of the haemochromatotic proteins HFE, TfR2, HJV, the iron regulatory peptide, hepcidin, iron transporters and enzymes with ferro-oxidase or ferri-reductase activity as well as new iron sensing

partners such as TMPRSS6, as major regulators of iron metabolism are hallmarks of these past few years and have made iron metabolism a fascinating field to study. New genetic disorders have been identified, due to mutations in one or the other of these new genes and iron has turned out to be an aggravating factor of several pathological conditions such as anaemia of chronic disorders, infections, cardiovascular diseases, diabetes, renal insufficiency or neurodegenerative diseases. Importantly, the liver appeared as a central element that contributes to the maintenance of iron homeostasis. Signalling from tissue iron stores or erythropoietic activity of the bone marrow ends up by regulating hepcidin synthesis, revealing unexpected pathways and much complexity. However, a significant amount of work remains to be carried out to precise the molecular mechanisms of hepcidin action, its synthesis, maturation, secretion and transport and to understand the crosstalk between all the regulators identified by genetics. There is no doubt that hepcidin measurements will be used for the diagnosis, classification and follow-up of disorders of iron metabolism. Lastly, hepcidin and its regulators constitute considerable potential therapeutic targets for treatment of iron disorders with, at the two extremes, iron overload and anaemia of inflammation.

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Multiple Choice Questionnaire

To find the correct answer, go to http://www.esh.org/iron-handbook2009answers.htm

1. Indicate the clinical situation where high plasma hepcidin levels are expected:

a) Iron-deficiency anaemia ☐
b) Inflammation ☐
c) Congenital dyserythropoietic anaemia ☐
d) Hereditary haemochromatosis ☐

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2. What is the molecular target of hepcidin?

a) The transferrin receptor ☐
b) Ferroportin ☐
c) Ferritin ☐
d) TMPRSS6/matriptase 2 ☐

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3. What is the major tissue producing hepcidin?

a) Pancreas ☐
b) Heart ☐
c) Liver ☐
d) Kidney ☐

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4. Hepcidin gene expression is inhibited by?

a) Epo ☐
b) Holotransferrin ☐
c) BMPs ☐
d) Leptin ☐

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5. What is the principal pathway regulating hepcidin?

a) AKT/PKB ☐
b) BMP/HJV ☐
c) ErbB/HER ☐
d) Wnt/β-catenin ☐

NOTES