

Molecular pathogenesis of iron overload

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Our current understanding of iron absorption under normal conditions is presented, together with an overview of the clinical disorders of iron overload and the molecular processes that contribute to increased iron deposition in iron overload. Recently, a number of new genes involved in iron metabolism have been identified which is allowing the molecular mechanisms of iron absorption to be elucidated.

deficiency.^{3,4} Iron is then stored in the enterocyte or transferred out across the basolateral membrane by a membrane bound protein called ferroportin (also known as IREG1 and MTP1).^{5–7} Extracellular ferrous iron is oxidised by the multi copper oxidase haephestin and bound by plasma transferrin.⁸

The mechanism of absorption of haem iron has yet to be elucidated. Transfer across the brush border membrane is probably mediated by an unidentified haem receptor. Once inside, enterocyte iron is released from haem by haem oxygenase and either stored or transferred out of the enterocyte by a mechanism that is likely to be similar to that for ionic iron (fig 1).⁹

Iron homeostasis is controlled by the absorption of iron from the diet. It occurs mainly in the duodenum at a rate of approximately 1–2 mg iron per day. When iron levels in the body or the diet are low, the rate of iron absorption is increased, and when iron levels are replete there is a reduction in the rate of iron absorption and excess iron is excreted when enterocytes are sloughed off every 2–3 days. Recently, a number of new genes involved in iron metabolism have been identified which is allowing the molecular mechanisms of iron absorption to be elucidated. In this review, our current understanding of iron absorption under normal conditions is presented followed by an overview of the clinical disorders of iron overload and the molecular processes that contribute to increased iron deposition in iron overload.

REGULATION OF IRON ABSORPTION

Iron absorption is regulated by a number of factors, including the level of body iron stores, the rate of erythropoiesis, and hypoxia. Enterocytes in the crypt region of the duodenal mucosa take up iron from plasma in proportion to the body's iron level, and the intracellular iron level in crypt cells reflects the body's iron status.¹⁰ Crypt cells express transferrin receptor 1 (TfR1) which mediates the uptake of transferrin bound iron (TBI).¹¹ The haemochromatosis protein (HFE) is also highly expressed in crypt cells¹² and forms a complex with β_2 microglobulin and TfR1.¹³ The role of HFE in the regulation of TfR1 mediated uptake of TBI is unclear. A number of studies in isolated cell systems have shown that HFE reduces both the affinity of TfR1 for transferrin and the uptake of iron, due either to a reduction in the cycling time of the HFE/TfR1-TBI complex through the cell or a reduced rate of iron release from transferrin intracellularly.^{14–17} Whereas when both HFE and β_2 microglobulin are overexpressed in Chinese Hamster Ovary cells, uptake of TBI was enhanced due to increased recycling of TfR1 through the cell.¹⁸

A second transferrin receptor (TfR2) has been identified.¹⁹ TfR2 mRNA is expressed at very low levels in the duodenum and does not interact with HFE in vitro.^{20,21} Its role in iron absorption is yet to be determined.

In iron deficiency, DMT1, ferroportin, and TfR1 are upregulated while ferritin is downregulated and the converse occurs when iron levels are increased.^{6,22–24} Expression of ferritin and TfR1 is regulated by post-transcriptional mechanisms.

MOLECULAR MECHANISMS OF IRON ABSORPTION

Iron is found in the diet as ionic (non-haem) iron and haem iron. Absorption of these two forms of iron occurs by different mechanisms. Absorption is a multistep process involving the uptake of iron from the intestinal lumen across the apical cell surface of the villus enterocytes and the transfer out of the enterocyte across the basolateral membrane to the plasma. Ionic iron is present in the reduced (ferrous) or oxidised (ferric) state in the diet and the first step in the uptake of ionic iron involves the reduction of iron. Recently, a putative reductase that is capable of reducing iron from its ferric to ferrous state has been identified. It is a membrane bound haem protein called Dcytb that is expressed in the brush border of the duodenum.¹ Next, ferrous ion is transported across the lumen cell surface by a transporter called divalent metal transporter 1 (DMT1) that can transport a number of other metal ions including copper, cobalt, zinc, and lead.² Evidence for the role of DMT1 in iron absorption is supported by studies in mk mice and Belgrade rat. Both of these laboratory animals have a G185R mutation in DMT1 that inhibits iron uptake across the brush border leading to iron

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Abbreviations: DMT1, divalent metal transporter 1; HFE, haemochromatosis protein; HH, hereditary haemochromatosis; IRE, iron regulatory element; IRP, iron regulatory protein; NTBI, non-transferrin bound iron; TBI, transferrin bound iron; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

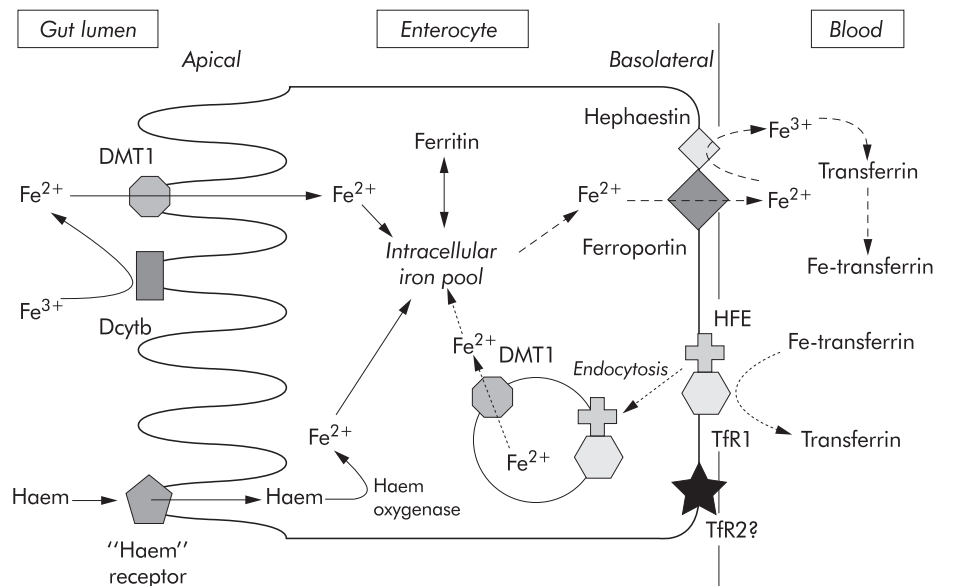


Figure 1 A model of the pathways of iron absorption by the enterocyte. The figure shows uptake of ionic iron and haem iron from the gut lumen and transfer of iron to blood. DMT1, divalent metal transporter 1; HFE, haemochromatosis protein, Tfr1, transferrin receptor 1; Tfr2, transferrin receptor 2.

The intracellular iron level controls the interaction of a cytosolic iron regulatory protein (IRP) with an iron regulatory element (IRE) in the untranslated region of the mRNA of these genes.²⁵ HFE and Tfr2 do not contain IRE and their expression is not iron regulated.^{19, 26}

IRP activity is one of the central regulators of iron absorption. IRP activity in crypt cells reflects the body's iron status. Crypt cells migrate to the villus region of the duodenum and differentiate into absorptive cells where the level of IRP binding activity, predetermined in crypts, regulates expression of iron transporters and the rate of iron absorption.²⁷ Villus cells also respond to a change in iron levels in the diet. An iron gavage reduces IRP activity, DMT1 expression, and iron absorption by villus cells within hours.²⁸

Both the rate of erythropoiesis and hypoxia regulate iron absorption.²⁹ Expression of ferroportin and Dcytb are upregulated in hypoxia and in a hypotransferrinaemic mouse which has chronic anaemia due to defective erythropoiesis.^{1, 6} Increased expression of these genes is likely to account for the increase in iron absorption.

CLINICAL SYNDROMES OF IRON OVERLOAD

Hereditary haemochromatosis

Clinical disorders of iron overload are classified in table 1. In populations of Northern European ancestry, hereditary haemochromatosis (HH) is the most common disorder of primary iron overload. This autosomal recessive disorder usually results from a homozygous mutation in the HFE gene of Anglo-Celtic populations. The clinical features of HH have been reviewed extensively.³⁰

"In populations of Northern European ancestry, hereditary haemochromatosis is the most common disorder of primary iron overload"

The HFE gene was discovered in 1996 by Feder and colleagues²⁶ who described a novel gene containing two missense mutations. One of these mutations (Cys282→Tyr; C282Y) was found to be homozygous in 83% of 178 patients with typical HH and has probably arisen in the last 2000 years, as estimated from ancestral haplotype studies.³¹ Studies from other groups of HH patients from Europe, the UK, and Australia have demonstrated that, on average, 85–90% of patients with HH are homozygous for the C282Y

Table 1 Classification of iron overload states

Familial or hereditary forms of haemochromatosis
Hereditary haemochromatosis (HH, HFE1)
C282Y homozygosity
C282Y, H63D heterozygosity
Other HFE gene mutations
Juvenile haemochromatosis (HFE2)
Transferrin receptor 2 mutation (HFE3)
Ferroportin mutation (HFE4)
Acaeruloplasminaemia
Atransferrinaemia
Neonatal iron overload
Autosomal dominant haemochromatosis (Solomon Islands)
Acquired iron overload
Iron loading anaemias
Thalassaemia major
Sideroblastic anaemia
Chronic haemolytic anaemias
Dietary iron overload
Chronic liver disease
Hepatitis C
Alcoholic liver disease
Non-alcoholic steatohepatitis
Porphyria cutanea tarda

mutation.^{32–34} A second mutation (His63→Asp; H63D) was also identified but was not associated with the same degree of iron overload as the C282Y mutation.²⁶ Lower frequencies of homozygosity for the C282Y mutation (64%) are found in Southern European patients with HH.³⁵ The C282Y mutation is rare in African, Asian, Polynesian, and indigenous Australian chromosomes.³⁶

Other mutations within the HFE gene have been found that are associated with iron overload, usually in combination with C282Y heterozygosity: Mura *et al* reported enrichment of S65C missense substitution in patients with mild HH.³⁷ A novel splice site mutation causing skipping of exon 3 in the HFE locus has also been reported (IVS3+1G→T).³⁸ Piperno *et al* found two novel missense mutations in five unrelated HH patients with C282Y heterozygosity.³⁹

The role of mutations in the HFE gene as a cause of HH was further strengthened in 1998 when a knockout mouse model for the HFE gene was described.⁴⁰ This model exhibited

changes in iron metabolism and developed iron overload that were similar to humans with HH.

Clinical and biochemical expression of HFE gene mutations

There is variable clinical expression of haemochromatosis protein (HFE) mutations in terms of the development of iron overload and clinical disease. Previous HLA based family studies suggested that almost all patients who had inherited the underlying genetic defect would develop iron overload.⁴¹ However, more recent studies of patients with HH have shown that up to 26% of subjects homozygous for the C282Y mutation may not develop iron overload.⁴² A large, systematic, Australian population based study has shown that 15 of 16 C282Y homozygotes are detected with an elevated transferrin saturation $\geq 45\%$; 50% of homozygotes had typical clinical features and 25% had significant hepatic fibrosis or cirrhosis.

“One in 700 individuals in an Anglo-Celtic population will have clinically significant iron overload but not possess mutations in the HFE gene”

Progressive iron overload occurs in the majority of C282Y homozygotes.⁴³ However, the frequency of biochemical expression varies with different populations. A study in blood donors in San Diego found that 36% of C282Y homozygotes had a transferrin saturation less than 45%.⁴⁴ Up to 20% of patients heterozygous for both mutations (C282Y, H63D compound heterozygotes) demonstrate a clinical syndrome identical to that observed in C282Y homozygotes.⁴⁵ Likewise, 1 in 700 individuals in an Anglo-Celtic population will have clinically significant iron overload but not possess mutations in the HFE gene.⁴³ Cirrhosis and significant hepatic fibrosis are rarely observed in C282Y homozygotes or compound heterozygotes under the age of 40 years, provided that no other hepatotoxins or hepatotropic viruses are present and serum ferritin level is less than 1000 ng/ml.⁴⁵⁻⁴⁶

Other genetic defects of iron metabolism

Well defined kindreds exist with mutations outside the HFE gene. Juvenile haemochromatosis is an autosomal recessive disorder characterised by iron loading in the same pattern as adult HH but in the second or third decade of life. Roetta *et al* studied nine affected families and identified a locus on the long arm of chromosome 1 not known to correspond to a gene involved in iron metabolism.⁴⁷ Juvenile haemochromatosis is now also termed HFE2. Camaschella *et al* reported a new locus on 7q22 and showed that a homozygous nonsense mutation in the gene encoding Tfr2 is found in some individuals with non-HFE related iron overload.⁴⁸ The disorder described by Camaschella *et al* is now also termed HFE3.

“Juvenile haemochromatosis is an autosomal recessive disorder characterised by iron loading in the same pattern as adult HH but in the second or third decade of life”

Pietrangelo *et al* studied a large Italian family in which 15 of 53 members had iron overload and none had C282Y mutations. Microsatellite analysis of the HH phenotype showed no linkage to the HFE gene.⁴⁹ The iron overload disorder in these patients has now been linked to a mutation in the ferroportin gene and is termed HFE4.⁵⁰⁻⁵¹ The mechanisms underlying the development of iron overload in the HFE2-HFE4 disorders are not clear.

Acaeruloplasminaemia is a rare genetic iron overload disorder that results in diabetes and neurodegenerative disease due to a mutation in the caeruloplasmin gene causing non-expression of the gene product. Lack of caeruloplasmin

reduces plasma ferroxidase activity and cellular release of iron causing progressive accumulation in the liver, pancreas, and brain.⁵² Atransferrinaemia is a very rare inherited disorder in which there is no plasma transferrin. Patients have enhanced iron absorption but the erythroid precursor cells are unable to utilise non-transferrin bound iron (NTBI) and thus respond as though there is severe iron deficiency. Excess NTBI accumulates in the liver, pancreas, and heart as the rate of iron release from these tissues is also reduced due to lack of transferrin.⁵³ These patients require transferrin infusion for survival.

Regulation of iron absorption in hereditary haemochromatosis

In HH patients there is an increase in the rate of iron absorption. Enterocytes of HH patients have increased IRP activity and reduced ferritin levels.⁵⁴ Furthermore, recent studies have shown that expression of the iron transporters DMT1 and ferroportin are both upregulated in subjects with HFE-HH, non-HFE HH, and iron deficiency but not secondary iron overload.⁶⁻⁵⁵⁻⁵⁶ Increased expression of DMT1 mRNA and protein has also been observed in the intestine of the HFE knockout mouse model of HH inducing enhanced iron absorption.⁵⁷⁻⁵⁸ However, this finding is controversial as others found no increase in DMT1 mRNA and protein levels in the intestine of C282Y HFE and β_2 microglobulin knockout mouse models of iron overload.⁵⁹

These observations indicate that there is an incorrect sensing of the body's iron level by the intestinal crypt cells in HH. In a recent study we have reported that HFE regulation of TBI uptake by the duodenum is impaired in the HFE knockout mouse model of HH.⁶⁰ This would lead to reduced intracellular iron levels in crypt cells of the duodenum that do not reflect correctly the high plasma iron levels found in HH. Thus enterocytes would be incorrectly programmed to absorb iron from the diet.

Exactly how C282Y HFE impairs the uptake of TBI from plasma by the duodenum is unknown. However, these findings are consistent with results obtained using Chinese Hamster Ovary cells described earlier where overexpression of wild-type HFE and β_2 microglobulin enhanced the uptake of TBI by increasing the rate of TfR1 recycling through the cell.¹⁸ As it is known that the C282Y HFE does not associate with β_2 microglobulin and TfR1 and its cell surface expression is reduced,⁶¹⁻⁶² it is unlikely that C282Y HFE could stimulate TBI uptake by Chinese Hamster Ovary cells as the wild-type HFE does. Therefore, a relative reduction in TBI uptake in the presence of C282Y HFE by Chinese Hamster Ovary cells would be consistent with reduced uptake of TBI by the duodenum of HFE knockout mice and by macrophages from patients with HH.⁶⁰⁻⁶³

Molecular mechanisms of iron loading of the liver

The liver is the main site of iron storage and most of the iron is deposited in hepatocytes as ferritin or haemosiderin. In iron overload the rate of iron uptake exceeds the rate of iron release by hepatocytes resulting in increased hepatic iron levels. Usually iron is transported in plasma by transferrin. However, in iron overload, transferrin becomes saturated with iron and excess iron or NTBI is also present. The hepatocyte can take up both NTBI and TBI and both sources are likely to contribute to elevated hepatic iron deposition in iron overload.

“In iron overload the rate of iron uptake exceeds the rate of iron release by hepatocytes resulting in increased hepatic iron levels”

Functional studies have shown that uptake of TBI by the hepatocyte occurs by TfR1 and TfR2 independent pathways. Hepatocytes express only a low number of TfR1 receptors and the main pathway of TBI uptake by hepatocytes and hepatoma

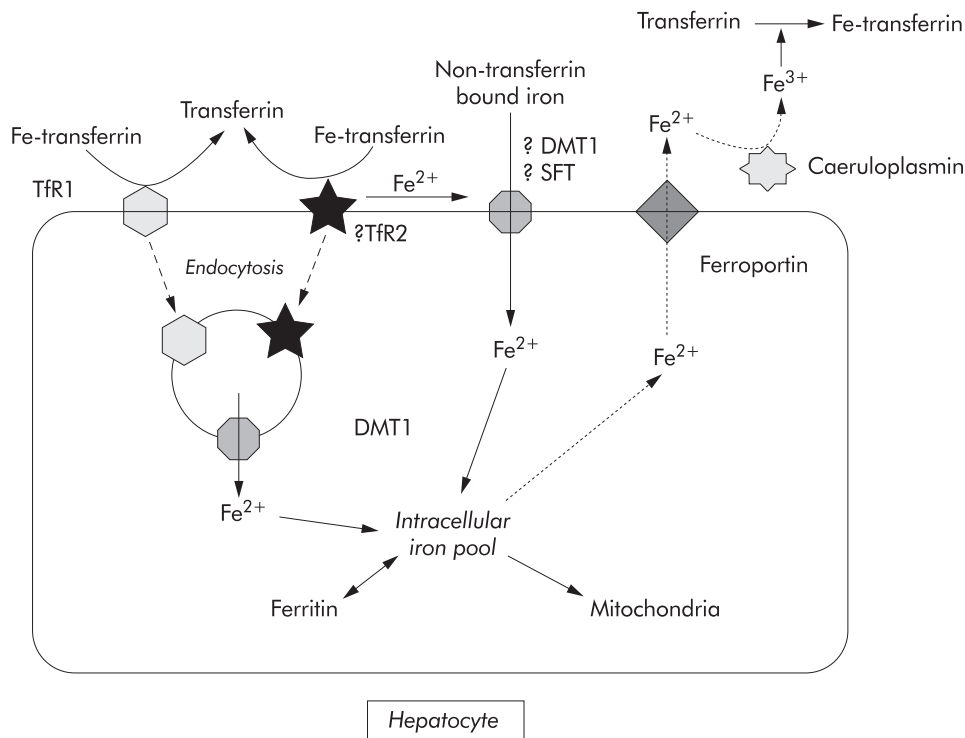


Figure 2 Diagrammatic representation of the pathways of uptake of transferrin bound iron and non-transferrin bound iron by hepatocytes (see text for explanation). Tfr1, transferrin receptor 1; Tfr2, transferrin receptor 2; DMT1, divalent metal transporter 1; SFT, stimulator of iron transport.

cell lines is thought to be mediated by low affinity Tfr1 independent pathways.^{64–65} The first of the Tfr1 independent pathways involves endocytosis of TBI while the second involves release of iron from transferrin at the cell surface and transport into the cell by an iron transporter that also mediates uptake of NTBI.^{66–67} Tfr2 is highly expressed in human liver.¹⁹ Its role in hepatic iron transport is yet to be established but it is likely that Tfr2 mediates uptake of iron by the Tfr1 independent pathway (fig 2).

Tfr1 expression by hepatocytes is downregulated by iron loading.^{20–68} In fact there is complete absence of Tfr1 expression in HH patients⁶⁹ and in the HFE knockout mouse,²⁰ and therefore Tfr1 mediated uptake of TBI is unlikely to contribute significantly to iron loading of the liver. Tfr2 does not contain an IRE and its expression in the liver is not iron regulated. In the HFE knockout mouse, Tfr2 expression remains high and Tfr2 mediated uptake of iron could contribute to loading of the liver in HH.²⁰

NTBI is extremely toxic, can generate free radicals, and is rapidly cleared from plasma by the liver.⁷⁰ Hepatocytes take up NTBI by a process that involves iron reduction and transport across the cell membrane by a carrier mediated process^{71–73} which may involve the iron transporters DMT1 or stimulator of iron transport.⁷⁴ Both the uptake of NTBI and expression of DMT1 in the liver have been shown to be enhanced by iron loading and this process may contribute to iron loading of the liver.^{23–71}

The mechanism of iron release by hepatocytes is not well understood. Ferroportin is localised to the hepatocyte cell membrane and is a likely candidate for the transporter of iron out of cells.⁷ Iron is then oxidised by caeruloplasmin and bound by plasma transferrin (fig 2).⁷⁵ Further studies need to be undertaken to establish if ferroportin expression and the rate of iron release are altered with iron loading.

HFE is not expressed in hepatocytes and therefore is not likely to play a role in the regulation of iron transport in hepatocytes.^{12–76} In HH, iron metabolism of hepatocytes is not directly affected by mutations in the HFE protein but rather, increases in plasma TBI and NTBI contribute to elevated iron levels and subsequent liver damage.

“Recently, the hepatic antimicrobial peptide hepcidin has been identified as a potential new player in iron metabolism”

Recently, the hepatic antimicrobial peptide hepcidin has been identified as a potential new player in iron metabolism. Interestingly, a transcription factor USF2 knockout mouse which lacks hepcidin expression developed iron overload that was similar to HH.⁷⁷ However, others have shown that hepatic hepcidin levels are upregulated in iron overload.⁷⁸ Thus the role of hepcidin in iron homeostasis is unclear but it may act as a signalling molecule that regulates hepatic iron levels and the rate of iron absorption.

Mechanisms of iron induced liver injury

The mechanisms by which iron may cause liver disease have been reviewed.⁷⁹ Studies that have examined collagen gene expression in iron overload have indicated that iron deposition in hepatocytes is necessary since, if iron is not present in hepatocytes, collagen gene expression is not increased.⁸⁰ These data suggest that either iron loaded hepatocytes directly release profibrogenic substances, which activate hepatic stellate cells (the principal cellular sources of collagen and other matrix proteins in chronic liver disease) or release substances which stimulate Kupffer cells to produce profibrogenic substances which activate hepatic stellate cells. Iron overload can induce lipid peroxidation of organic membranes leading to cell injury and cell death. Lipid peroxidation products have been shown to stimulate collagen production in activated hepatic stellate cells and cultured human fibroblasts.⁸¹ Alternatively, lipid peroxidation products may increase production of transforming growth factor β or other profibrogenic substances by Kupffer cells which might then stimulate hepatic stellate cell activation.⁸² Hepatocellular carcinoma could result from DNA damage from iron induced adduct formation and chromosomal damage⁸³ or proliferation and dedifferentiation of hepatic stem cells termed “oval cells”.⁸⁴

CONCLUSION

There have been significant advances in recognising the evolving phenotype of iron overload syndromes and their associated genotypes over the last five years, although there are likely to be further candidate genes identified. However, reconciling the mechanisms by which the various iron transport genes and proteins contribute to normal homeostasis of iron metabolism is far from complete. Candidate mutations in iron transport genes and abnormal protein functions have been described predominantly at the molecular and isolated cell levels. Attempts to confirm these observations at the whole organism level have either proved difficult or have not been attempted. Clearly, the key to "ironing out" the molecular pathogenesis of iron overload disorders depends on the ability to correlate events at the gene, RNA, and protein levels with functional outcomes in the whole organism.

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