

Hereditary Hemochromatosis: Genetic Complexity and New Diagnostic Approaches

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Since the discovery of the hemochromatosis gene (*HFE*) in 1996, several novel gene defects have been detected, explaining the mechanism and diversity of iron-overload diseases. At least 4 main types of hereditary hemochromatosis (HH) have been identified. Surprisingly, genes involved in HH encode for proteins that all affect pathways centered around liver hepcidin synthesis and its interaction with ferroportin, an iron exporter in enterocytes and macrophages. Hepcidin concentrations in urine negatively correlate with the severity of HH. Cytokine-mediated increases in hepcidin appear to be an important causative factor in anemia of inflammation, which is characterized by sequestration of iron in the macrophage system. For clinicians, the challenge is now to diagnose HH before irreversible damage develops and, at the same time, to distinguish progressive iron overload from increasingly common diseases with only moderately increased body iron stores, such as the metabolic syndrome. Understanding the molecular regulation of iron homeostasis may be helpful in designing innovative and reliable DNA and protein tests for diagnosis. Subsequently, evidence-based diagnostic strategies must be developed, using both conventional and innovative laboratory tests, to differentiate between the various causes of distortions of iron metabolism. This review describes new insights in mechanisms of iron overload, which are needed to understand new developments in diagnostic medicine.

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Iron is involved in the function of all cells. It is able to accept and donate electrons, depending on its oxidation state: ferrous iron [Fe(II)] or ferric iron [Fe(III)]. This ability is crucial for the function of oxygen-binding molecules, mainly hemoglobin and myoglobin, and iron-containing enzymes, including the cytochrome system in mitochondria. Iron is mostly locked into iron protoporphyrin (heme) and iron-sulfur clusters, which serve as enzyme cofactors. Without iron, cells lose their capacity for electron transport and energy metabolism. However, iron can also cause damage, because Fe(II) catalyzes the generation of highly reactive hydroxyl radicals ($\cdot\text{OH}$) from hydrogen peroxide (H_2O_2), which is called the Fenton reaction (1). These hydroxyl radicals damage cellular membranes, proteins, and DNA. A large number of scavenger molecules protect cells against iron-mediated tissue damage. Proteins sequester iron to reduce this threat. Iron circulates bound to plasma transferrin, which is needed to offer the highly insoluble Fe(III) to cells via the transferrin receptor (TfR).⁴ Iron can safely be stored within cells in the form of ferritin and hemosiderin [reviewed in Ref. (2)]. Usually, only small amounts of iron exist outside this physiologic sink, although stored iron can be mobilized for reuse. Many diseases arise from imbalances in iron homeostasis. Too much iron accumulates in hereditary hemochromatosis (HH), porphyria cutanea tarda, and the iron-loading anemias (hemolytic, dyserythropoietic, myodysplastic, and aplastic anemias), which are often aggravated by multiple transfusions. In iron-deficiency anemia (IDA), insufficient amounts of iron are available for heme synthesis. In anemia of inflamma-

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⁴ Nonstandard abbreviations: TfR, soluble transferrin receptor; HH, hereditary hemochromatosis; IDA, iron-deficiency anemia; AI, anemia of inflammation; TS, transferrin saturation; NTBI, non-transferrin-bound iron; HJV, hemojuvelin; GPI, glycosylphosphatidylinositol; OMIM, Online Mendelian Inheritance in Man; HHCS, hereditary hyperferritinemia congenital cataract syndrome; DcytB, duodenal cytochrome B; DMT1, divalent metal transporter 1; HCP1, heme carrier protein 1; sTfR, soluble transfer receptor; and R/F, ratio of sTfR to the log of the ferritin concentration.

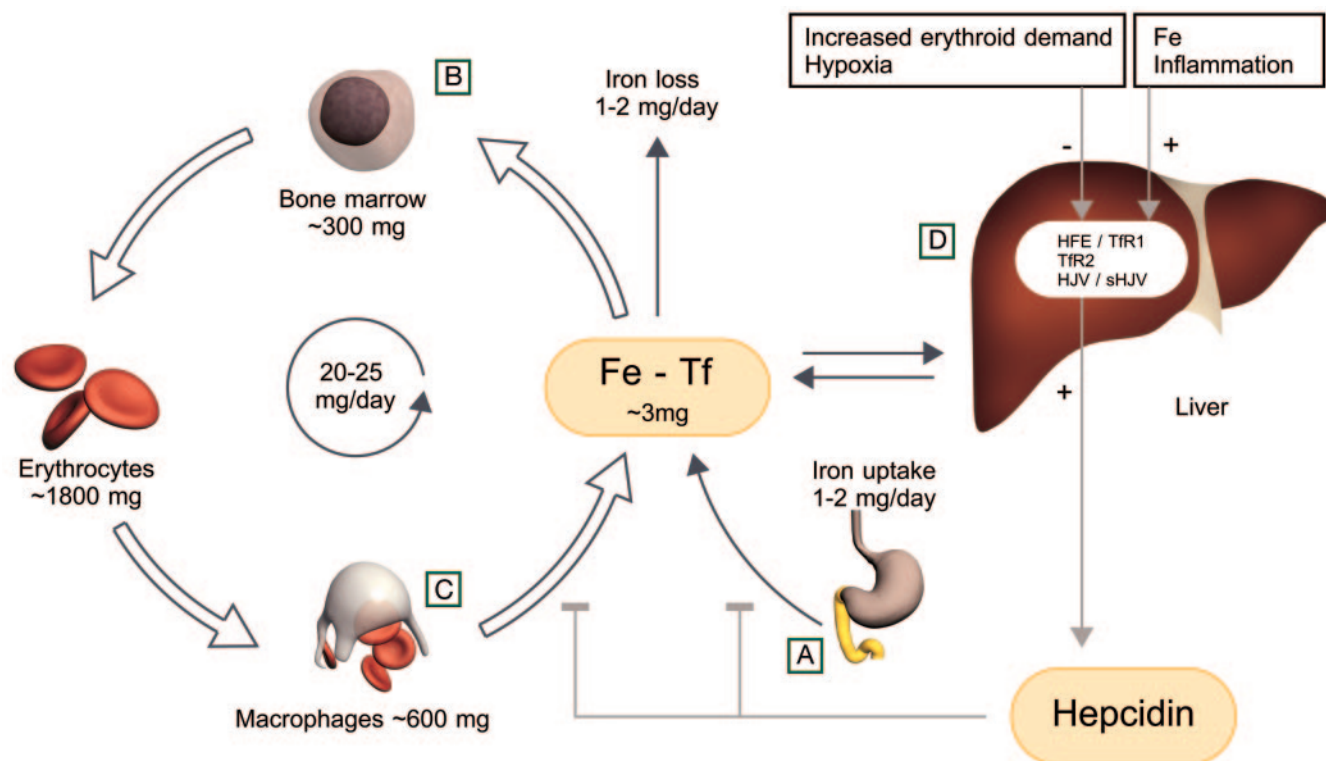


Fig. 1. Pathways of iron exchange.

The largest flux of iron takes place in the recycling of iron from senescent erythrocytes out of macrophages to incorporation in erythroid precursors. Note that values for the different tissues and fluxes are approximate. The liver and reticuloendothelial macrophages function as major iron stores. Only 1–2 mg of iron is absorbed and lost every day. Importantly, the total amount of iron in the body can be regulated only by absorption, whereas iron loss occurs only passively from sloughing of skin and mucosal cells as well as from blood loss. Hepcidin, a recently identified, antimicrobial, β -defensin-like peptide secreted by the liver, controls the plasma iron concentration by inhibiting iron export by ferroportin from duodenal enterocytes and reticuloendothelial macrophages [see also Fig. 1 of Ref. (4)]. As a consequence, an increase in hepcidin production leads to a decrease in plasma iron concentrations (92, 200). Hepcidin expression is regulated by iron concentrations in hepatocytes, by inflammatory stimuli, by erythroid iron demand, and by hypoxia via pathways involving expression of the *HFE*, *TFR2*, and *HJV* genes (Fig. 2D) (16, 82, 84, 89, 90, 104, 174, 175). In *HFE*-, *TFR2*-, and *HJV*-related HH, hepcidin production is low despite increased liver iron, leading to inappropriately increased iron absorption (16, 104, 175). A, B, C, and D refer to sites with special functions in iron metabolism as depicted in Fig. 2. *sHJV*, soluble *HJV*.

tion (AI), iron is redistributed to macrophages to promote resistance to infections (3).

The control of iron homeostasis acts at both the cellular and the systemic level and involves a complex system of different cell types, transporters, and signals. To maintain systemic iron homeostasis, communication between cells that absorb iron from the diet (duodenal enterocytes), consume iron (mainly erythroid precursors), and store iron (hepatocytes and tissue macrophages) must be tightly regulated. The recently identified β -defensin-like antimicrobial peptide hepcidin is thought to be the long-anticipated regulator that controls iron absorption and macrophage iron release. Hepcidin is synthesized in the liver when changes occur in body iron needs, such as in anemia, hypoxia, and inflammation, and is secreted in the circulation. Recently, light was also shed on how hepcidin exerts this regulatory function; it was reported to counteract the function of ferroportin, a major cellular iron-exporter protein in the membranes of macrophages and the basolateral site of enterocytes, by inducing its internalization and degradation (4–6).

Background information on these major pathways of iron exchange and the role of hepcidin in iron regulation

is provided in Fig. 1, and Fig. 2 displays key sites in iron homeostasis in more detail. Also shown in Figs. 1 and 2 are the many proteins involved in iron metabolism. Most of these proteins have been identified during the last few years, during which molecular genetics have added substantially to the understanding of iron metabolism [see Refs. (7–16) and the reviews in Refs. (17–19)]. Since the discovery of the hemochromatosis gene (*HFE*)⁵ in 1996, the identification of numerous additional genes that are mutated in genetic hemochromatosis has revolutionized the diagnosis of primary iron overload by introducing molecular and protein tests that allow early, presymptomatic, accurate diagnosis. However, many links are still incomplete, leaving even more intriguing questions. In this review, we summarize the major advances from the

⁵ Human genes: *HFE*, hemochromatosis; *HJV* (alias, *HFE2*), hemochromatosis type 2 (juvenile); *HAMP*, hepcidin antimicrobial peptide; *SMAD4*, mothers against DPP homolog 4 (*Drosophila*); *TFR2*, transferrin receptor 2; *SLC40A1*, solute carrier family 40 (iron-regulated transporter), member 1; *FTL*, L-ferritin; and *SLC11A2*, divalent metal transporter 1 [(aliases, *DMT1*, *DCT1*, and *NRAMP2*); solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2].

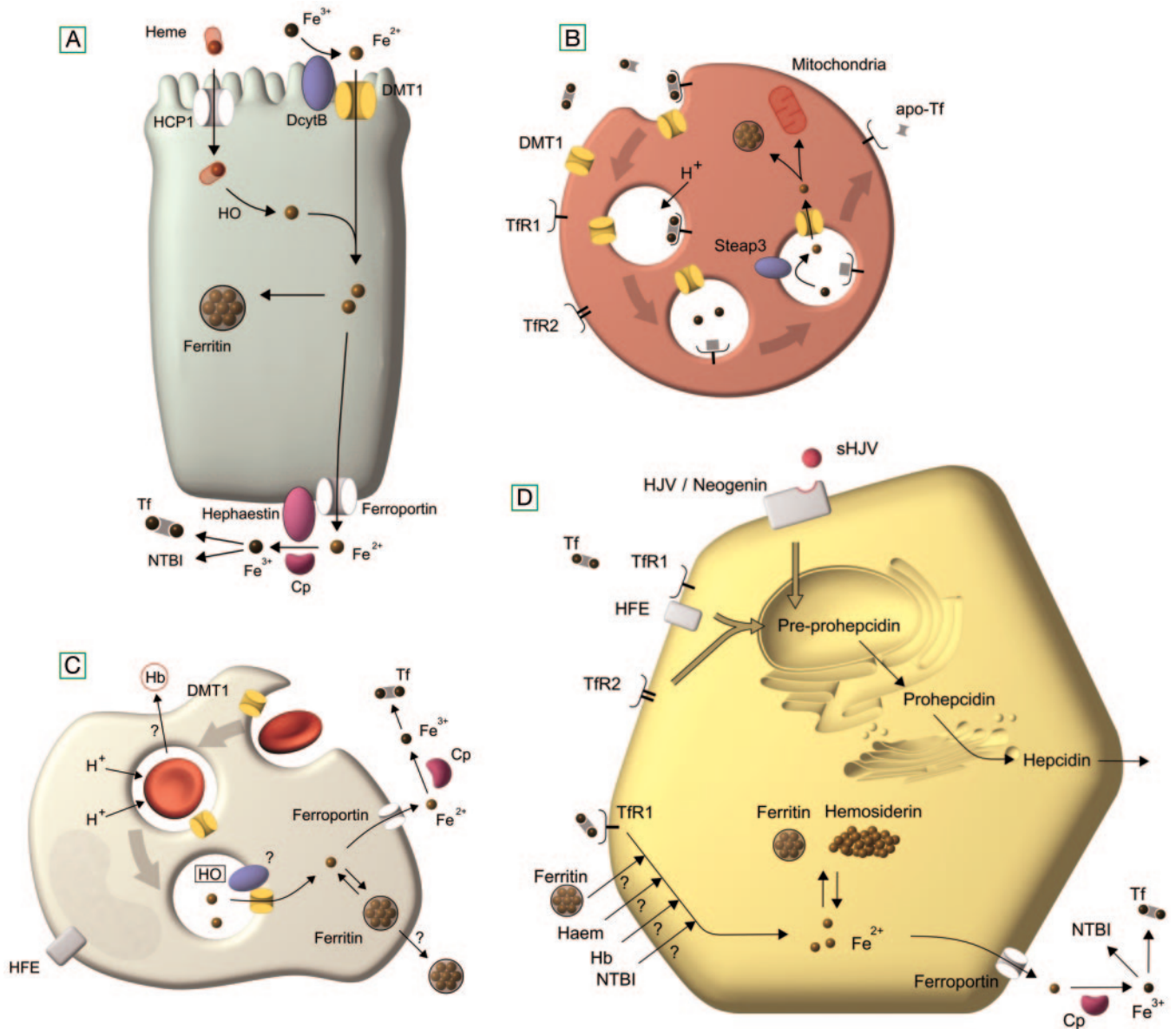


Fig. 2. Key sites of iron metabolism.

There are 4 sites with special functions in iron handling: enterocytes (A), erythroid precursors (B), macrophages (C), and hepatocytes (D). (A), enterocyte. Iron in food can be present in an inorganic form (Fe^{3+}) or as hemoglobin or myoglobin. Fe^{3+} in soluble iron complexes is reduced to Fe^{2+} by DcytB in the brush border and is transported into the duodenal enterocytes by DMT1. Heme enters the enterocyte after enzymatic digestion of hemoglobin and myoglobin, presumably through a heme cell transport protein, HCP1 (166). Within the enterocyte, heme is degraded by heme oxygenase (HO), and Fe^{2+} iron is released. From there, iron is either stored as ferritin or transported across the basolateral membrane to enter the circulation. This transport across the basolateral membrane is mediated by the iron transporter ferroportin, which transports Fe^{2+} to the plasma, where it is oxidized to Fe^{3+} by hephaestin, a membrane-resident multicopper oxidase very similar to ceruloplasmin (Cp), facilitating binding to transferrin (Tf). Evidence is accumulating that the peptide hormone hepcidin inhibits ferroportin function (4). Consequently, in case of high plasma hepcidin concentrations, most iron absorbed by the enterocyte is trapped as ferritin and lost in the feces. (B), erythroid precursor. Erythroid precursors take up iron through the transferrin cycle. Transferrin binds to TfR1 on the cell surface. The complexes localize to clathrin-coated pits, which invaginate to initiate endocytosis. In specialized endosomes, a decrease in pH induces the release of iron from transferrin. Fe^{3+} is converted to Fe^{2+} , presumably by STEAP3 (168), which enables iron transport out of the endosomes via DMT1. Subsequently, apotransferrin (apo-Tf) and the TfR1 both return to the cell surface. Both proteins participate in further rounds of iron delivery. Iron is then either stored as ferritin or exported out of the cell by ferroportin and ceruloplasmin (Cp). A considerable amount of iron is incorporated into hemoglobin. (C), macrophage. Reticuloendothelial macrophages carry out iron recycling. They ingest senescent erythrocytes and lyse them in a phagolysosomal compartment. Hemoglobin (Hb) is degraded, and iron is liberated from heme. The enzyme heme oxygenase (HO) participates in this process. Iron is then either stored as ferritin or exported out of the cell by ferroportin and ceruloplasmin (Cp). A considerable amount of iron is released as ferritin or hemoglobin (201). (D), hepatocyte. Hepatocytes take up iron through multiple pathways. The molecules involved in transport of NTBI, hemoglobin (Hb), heme (Haem), and ferritin have not yet been identified. As in macrophages, iron in hepatocytes is either stored as ferritin and hemosiderin or exported out of the cell by ferroportin and subsequently oxidized by ceruloplasmin (Cp) before binding to transferrin (Tf). Available data do suggest that HFE in interaction with TfR1 and parallel to TfR2 is implicated in the iron-sensing pathway of hepatocytes that controls hepcidin synthesis (84). A GPI-linked cell-associated HJV may interact with the transmembrane neogenin receptor to induce changes in hepcidin synthesis in the hepatocyte. A soluble circulating form of HJV (sHJV) derived from skeletal muscle is hypothesized to serve as an antagonist to disrupt these interactions (89, 90). Knowledge on the cellular processes regulating this hepcidin production and secretion in the hepatocyte is limited. The hepcidin gene (HAMP), located on chromosome 19q13.1, encodes a precursor protein of 84 amino acids. The production and localization of pre-prohepcidin in hepatocytes is assumed to be intracellular in the secretory pathway (202). During its export from the cytoplasm, this full-length pre-prohepcidin undergoes enzymatic cleavage of a 20-amino acid amino-terminal endoplasmic reticulum-targeting signal peptide, producing a 64-amino acid prohepcidin peptide (203). Next, the 39-amino acid proregion peptide is removed by a furin-like proprotein convertase (14, 204), most likely in the trans-Golgi network, producing mature hepcidin (25-amino acid form), which can be purified from human blood ultrafiltrate (171). Question marks indicate that either the pathway or the transport has not been elucidated.

molecular studies of iron overload in the light of their relevance to the understanding and design of diagnostic strategies for defects in iron regulatory pathways.

Hereditary Hemochromatosis

Iron-overload disease can be primary (hereditary) or secondary (inborn or acquired) (20). The latter disorders have in common the fact that the patient is anemic (Table 1). When anemia is accompanied by increased erythroid activity and/or ineffective erythropoiesis, e.g., in case of thalassemia and sideroblastic anemia, congenital dyserythropoietic anemia, and some myelodysplastic disorders, there is an appropriately increased absorption of iron from the diet because of higher needs of iron for hemoglobin synthesis (20,21). These patients develop iron overload even without transfusions of erythrocytes. If transfusions are needed, they will add to the body iron excess. Primary causes of hemochromatosis usually stem

from inherited abnormalities of proteins implicated in iron transport and regulation that may lead to excessive absorption of iron from the gastrointestinal tract. The disease was first described at the end of the 19th century by von Recklinghausen, but also by Trousseau and Troissier. It was von Recklinghausen who originally introduced the term hemochromatosis. In 1935, Sheldon wrote his classic review in which hemochromatosis was regarded as a very rare disease that results from excess total body iron and organ failure attributable to iron toxicity (22). By the 1980s, a higher prevalence was suggested, probably because of the widespread availability of serum iron, iron-binding capacity, and ferritin assays by that time. In the 1970s, hemochromatosis was recognized as an autosomal recessive disorder linked to the short arm of chromosome 6, which contains the gene that encodes HLA-A (23). However, it was only in 1996, that Feder et al. (7) identified the hemochromatosis (*HFE*) gene (previously called HLA-H gene). These authors attributed the most common form of hereditary hemochromatosis (HH) to homozygosity for the C282Y sequence variation of this gene. The so called *HFE*-related HH is characterized by an increase in iron absorption inappropriate to body iron stores, which leads to iron deposition in parenchymal organs such as the liver and the pancreas. Initial clinical symptoms of tissue iron overload typically occur at adult age and are often nonspecific and vague. In later stages, disease manifestations may include arthropathy, diabetes mellitus, hypogonadism and other endocrinopathies, liver cirrhosis, cardiomyopathy, skin pigmentation, and in cirrhotic patients, increased susceptibility to liver cancer (24–35). Early diagnosis and therapeutic phlebotomy can prevent the development of tissue damage, reducing morbidity and mortality and providing long-term survival similar to the general population (25, 26, 31, 33, 34, 36–38).

The identification of the *HFE* gene was the start of rapid advances in the understanding of iron homeostasis. It has since become obvious that other genetic entities can cause clinical pictures identical to that of the *HFE* gene defect [reviewed in Ref. (19)]. On the other hand, some of these new gene abnormalities are associated with markedly different clinical and biochemical pictures of iron overload. The large number of genes in which sequence variants are associated with iron overload complicates the diagnostic approach. Unfortunately, an increasing number of patients undergo molecular testing just because plasma ferritin and transferrin saturation (TS) are increased. Often this leads to an unnecessary search for hereditary defects in individuals with various common, nonhereditary conditions that are characterized by similar abnormalities in serum ferritin and/or TS, such as hepatitis, excessive alcohol consumption, several conditions co-occurring in the metabolic syndrome [glucose intolerance, obesity, hypertension (39)], and secondary forms of iron overload (2, 20, 21, 25, 40–54) (Table 1 and Fig. 3). Furthermore, without a well-defined indication, molecu-

Table 1. Differential diagnosis of iron overload in humans.

Hereditary hemochromatosis
HFE-associated HH (type 1) (7)
C282Y homozygosity
C282Y/H63D compound heterozygosity
Non-HFE-associated HH
Type 2A HJV variants (16)
Type 2B hepcidin variants (15)
Type 3 TfR variants (9)
Type 4 ferroportin variants (11)
Other
HHCS (133–135, 162)
Heme oxygenase deficiency (205)
Neonatal iron overload (206)
Aceruloplasminemia (119, 136)
Congenital atransferrinemia or hypotransferrinemia (160, 161)
DMT1 variants (163)
Secondary iron overload
Iron-loading anemias (20, 21)
Ineffective erythropoiesis
Thalassemic syndromes
Sideroblastic anemia
Myelodysplastic syndrome
Congenital dyserythropoiesis
Increased erythropoiesis
Chronic hemolytic anemia
Parenteral iron overload (including multiple blood transfusions)
Other
Metabolic syndrome (43, 50)
Obesity (42, 47, 54)
Hypertension (48)
Insulin resistance (49–52)
Chronic liver disease
Hepatitis (193, 194)
Alcohol abuse (45)
Nonalcoholic steatohepatitis (44)
Porphyria cutanea tarda (207)
Iron overload in sub-Saharan Africa (118–120)

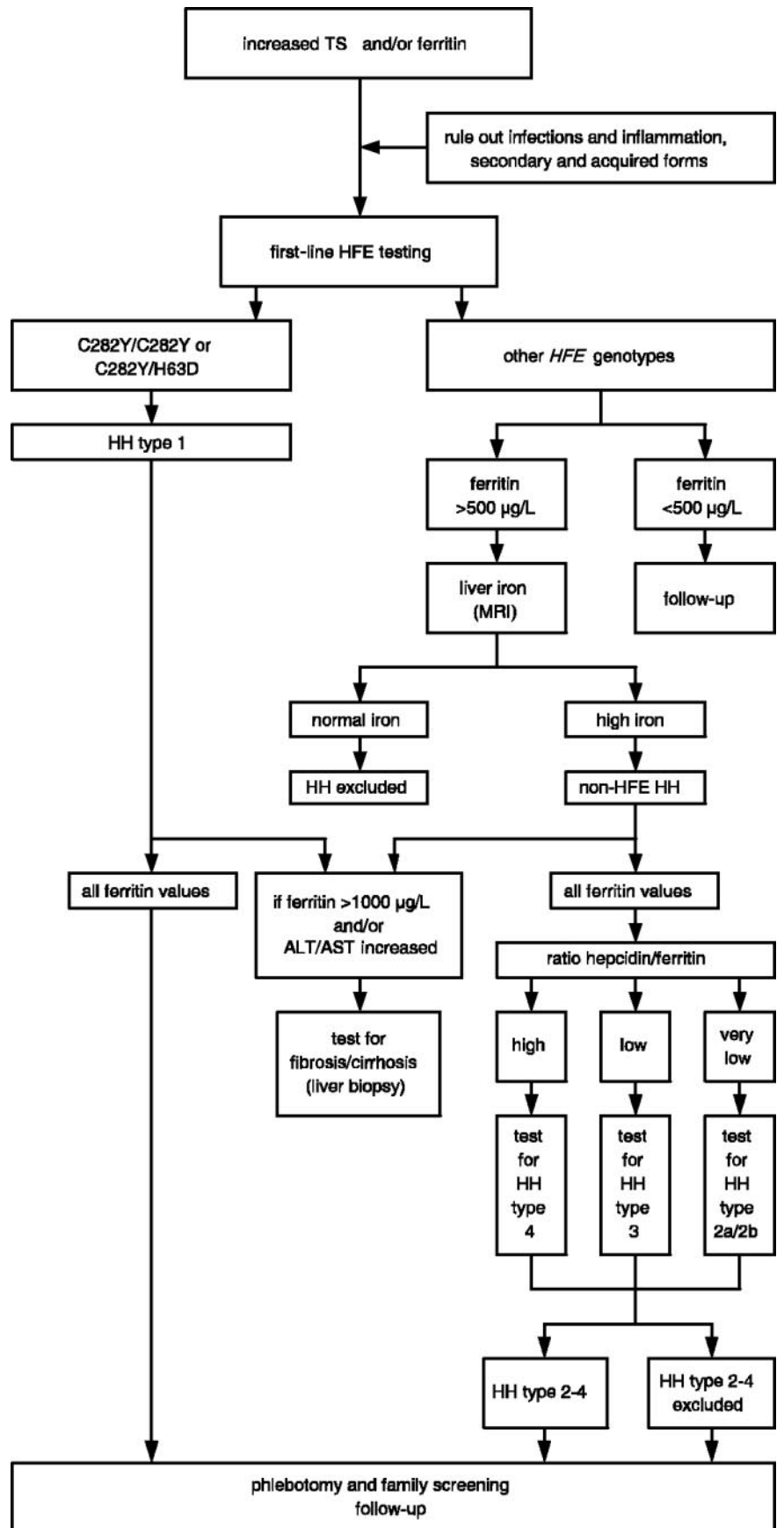


Fig. 3. Flow chart proposed for the diagnosis of the various forms of HH.

The diagram includes innovative molecular, hepcidin, and magnetic resonance imaging (MRI) tests. Future studies are needed to collect evidence to validate this flow chart. Except for the information included in this diagram, rational gene targeting is also based on information on clinical presentation, hemoglobin (low in secondary forms of iron overload and in some cases with ferroportin disease), family history (hereditary disease), concomitant diseases (e.g., hepatitis and alcohol abuse), and age at presentation (young age in juvenile forms of HH) (53). HH types 1–4 refer to the OMIM classification (Table 2). ALT, alanine aminotransferase; AST, aspartate aminotransferase.

lar diagnostics carry the risk of detecting abnormalities in genes of which the clinical consequence in time is unknown at present.

Currently there are 5 major forms of HH, each caused by sequence variations in a different gene (Table 2).

Classic Hemochromatosis: The *HFE* Gene

The C282Y sequence variant may have originated in a single Celtic ancestor in northern Europe approximately 2000 years ago (55). Rapid dissemination of this C282Y sequence variant has subsequently caused HH to be one of the most common inherited disorders in the Caucasian population of northern European descent, with a carrier frequency of the main C282Y sequence variant of 1 in 10 and homozygosity in 1 in 200. Apparently this genetic defect has no negative adverse consequences for reproduction. It may even have conferred some advantages, for which 2 main hypotheses have been proposed. The first has been mentioned frequently and is based on the lower prevalence of iron deficiency and a subsequent positive effect on the reproduction of young C282Y heterozygous

women in ancient times, when dietary iron was scarce (56). To date, however, evidence is lacking that the presence of *HFE* sequence variants also protects against (iron deficiency) anemia. In fact, there is good evidence that the prevalence of iron deficiency is no higher in carriers than in wild-type individuals (57). An alternative hypothesis is based on a protective effect against some pathologic agents. This can be related to the *HFE* protein as a cell-surface receptor for some infectious agents (58) or to the relative iron deficiency that occurs in the macrophages in the presence of the C282Y sequence variant, and offers a possible protection against many virulent species of bacteria that multiply mainly in iron-rich macrophages, such as *Mycobacterium tuberculosis* and *Yersinia pestis* (59). Although this latter theory is tantalizing, it remains to be tested at both the molecular level and in epidemiologic studies.

During the past few years, the understanding of the prevalence of clinically overt C282Y-homozygous HH has undergone a revision. In contrast to most of the earlier studies, recent surveys involving *HFE* genotyping of

Table 2. Characteristics of the hereditary forms of iron overload.

HH	Gene	OMIM type	Interaction	Frequency	Hepcidin ^a	Severity ^b	Clinical findings
Classic	<i>HFE</i>	1	TfR1? ^c	Frequent	↓	++	<p>Clinical: Symptoms start after 4th decade. Chronic fatigue, joint pain, impotence, infertility, hepatomegaly, diabetes mellitus, skin pigmentation, liver cirrhosis, decompensation cordis, arrhythmias.</p> <p>Biochemical: Increased serum TS and ferritin concentrations.</p> <p>Morphologic: Iron overload predominantly in hepatocytes.</p> <p>Inheritance: Autosomal recessive, prevalent male expression</p>
Juvenile							
HJV-related	<i>HJV</i>	2a	Neogenin	Rare	↓↓	++++	<p>Clinical: Symptoms start after the 1st decade. Abdominal pain, hypogonadotropic hypogonadism, cardiac arrhythmias and intractable heart failure, diminished glucose tolerance.</p>
Hepcidin-related	<i>HAMP</i>	2b	Ferroportin	Very rare	↓↓	++++	<p>Biochemical: Increased TS and ferritin concentrations.</p> <p>Morphologic: Iron overload in liver, heart, endocrine glands, and skeletal muscle.</p> <p>Inheritance: Autosomal recessive, both sexes equally affected.</p>
TfR2-related	<i>TFR2</i>	3	Transferrin	Very rare	↓	+++	<p>Clinical, biochemical, and morphologic: Similar to <i>HFE</i>-related hemochromatosis.</p> <p>Inheritance: Autosomal recessive.</p>
Ferroportin disease	<i>SLC40A1</i>	4	Hepcidin	Rare	↑ ?	+	<p>Clinical: Symptoms of iron overload comparable to <i>HFE</i>-related hemochromatosis, mild anemia early in life, reduced tolerance to phlebotomy.</p> <p>Biochemical: Marked increase in serum ferritin with relatively mildly increased TS.</p> <p>Morphologic: Iron overload predominantly in reticuloendothelial cells.</p> <p>Inheritance: Autosomal dominant.</p>

^a ↓, mildly decreased; ↓↓, strongly decreased; ↑, increased.

^b +, mild; ++, moderate; +++, severe; +++, very severe.

^c ?, not known.

nonclinically selected populations found that most C282Y homozygotes had no symptoms of disease (60–63). The incomplete penetrance of the C282Y sequence variant raises questions about the cost-effectiveness of population screening. Instead, early case detection by family (cascade) screening and increased awareness for the disease in the presence of symptoms that are consistent for HH is more likely to have a significant effect (64–66).

HFE genotypes other than C282Y homozygosity rarely cause clinically significant iron overload [reviewed in Ref. (67)]. C282Y heterozygotes usually do not develop iron overload unless they have associated conditions, such as environmental factors (alcohol, viruses, hepatic disease) or variant forms of other genes (see below). These C282Y carriers not only have increased mean TS and ferritin concentrations compared with their wild-type counterparts, but also increased concentrations of non-transferrin-bound iron (NTBI) (68). NTBI is thought to be the potentially toxic form of iron responsible for tissue damage in cases of iron overload (Fig. 2) (69, 70). The plasma concentration of NTBI, which is a rather unknown species of iron, highly correlates with that of TS in HH patients (71).

Case-control studies on the relationship of the C282Y heterozygous genotype with diseases, however, have not consistently supported heterozygosity as a risk factor for diabetes, arthritis, cancer, liver disease, and cardiovascular disease [reviewed in Ref. (72)].

A particular group of *HFE* genotypes consists of persons who are compound heterozygous for C282Y and H63D. These individuals have been described as being at higher risk to develop iron overload, but in a generally much milder form than in C282Y homozygotes (73, 74). However, given the fact that the clinical penetrance of C282Y homozygosity is very low (62), compound heterozygotes with clinical disease will be scarce. A third sequence variant, S65C, with an allele frequency as low as 1.6%–2.0%, was found to exert a consistent but small effect on serum iron indices, particularly when present in combination with other *HFE* genotypes, such as C282Y and H63D (75–77). However, to date there are no convincing data that S65C is associated with HH. Therefore, there is insufficient evidence to include testing for S65C to confirm the presence of HH in patients with increased serum iron indices. Other *HFE* sequence variants of clinical relevance are rare, and most of them are private (78).

The molecular function of *HFE* in iron metabolism has long been attributed to the crypt hypothesis (79, 80). According to this widespread view, the *HFE* protein linked to transferrin receptor 1 (TfR1) in the duodenal crypt stem cell is the sensor of total body iron, aimed at programming the expression of iron transporters in absorptive cells in the villi. In this model, the relative iron deficiency of mature absorptive enterocytes and increased intestinal absorption at the apical membrane of the enterocyte are attributed to abnormal interaction between

TfR1 and mutant *HFE* in the basolateral membrane. This theory has been opposed by the recent work of Enn's group (81), who showed that the association of *HFE* with TfR1 is not essential to its function. However, it is mainly since the discovery of hepcidin that the crypt model has been replaced by the "hepcidin model" as the prevailing hypothesis (Fig. 1). Sequence variations in *HFE* were shown to lead to inappropriately low concentrations of hepcidin, suggesting that *HFE* is involved upstream in the regulation of hepcidin expression (82, 83). Because hepcidin synthesis is restricted to liver cells, this provided strong evidence that *HFE* in the liver and/or macrophages was exerting its effects in hepatic cells and not in crypt cells (84). Indeed, available data do suggest that *HFE*/TfR1 in parallel to transferrin receptor 2 (TfR2) is implicated in the iron-sensing pathway of hepatocytes that controls hepcidin synthesis (Fig. 2D) [reviewed in Ref. (84)]. Taken together, it is not known yet exactly how *HFE* senses body iron. Nevertheless, by controlling serum concentrations of the proposed central iron regulator hepcidin, *HFE* appears to be involved in the maintenance of body iron homeostasis. Hepcidin has been reported to exert this regulatory role by disrupting the function of ferroportin, a major transmembrane iron exporter on enterocytes and macrophages (Fig. 1) (4). This model fits the hypothesis that plasma iron loading in HH stems from inappropriately excessive release of iron not only by enterocytes but also by hepatocytes and macrophages (85). Recently, De Almeida et al. (86) proposed that *HFE* encodes for an HLA-like molecule that has some immune-related function. This provides molecular evidence that a protein shown to affect iron metabolism also clearly affects immunologic functions.

Since the identification of the hemochromatosis (*HFE*) gene, remarkable developments have occurred in our understanding of iron transport and storage molecules, with the description of hepcidin, hemojuvelin (HJV), TfR2, and ferroportin proteins, in which alterations can lead to various types of HH (Table 2 and Fig. 2) (19).

Juvenile Hemochromatosis

Juvenile hemochromatosis shares most features with adult hemochromatosis, but the clinical manifestations develop earlier because intestinal iron absorption is higher and the rate of iron accumulation is faster. Individuals with the juvenile phenotype are more likely to present with cardiomyopathy and/or endocrine diseases, although liver cirrhosis is also part of the syndrome.

JUVENILE HEMOCHROMATOSIS ATTRIBUTABLE TO HJV DEFICIENCY

The most common gene causing juvenile hemochromatosis was recently identified, mapping to the pericentromeric region of the long arm of chromosome 1 (Table 2) (16). The gene *HJV* [hemochromatosis type 2 (juvenile); also known as *HFE2*] encodes a newly identified protein, called hemojuvelin (HJV), and is highly expressed in

skeletal muscle and heart and only to a limited extent in liver (Fig. 2D) (16, 87). HJV contains a C-terminal putative transmembrane domain characteristic of a glycosylphosphatidylinositol (GPI)-linked membrane anchor. To date, ~25 different sequence variants have been detected in the *HJV* gene clustered in exons 3 and 4 (78, 88). The finding of low and even unmeasurable hepcidin concentrations in patients carrying sequence variants in the *HJV*-encoding gene indicates that the protein is related to hepcidin and not a component of a distinct pathway (16). This relationship between HJV and hepcidin synthesis is further strengthened by recent reports (89, 90). In short, there appear to be 2 forms: a GPI-linked cell-associated HJV, which may interact with a transmembrane neogenin receptor to induce changes in hepcidin synthesis in the liver; and a soluble, circulating form derived from skeletal muscle that can serve as an antagonist to disrupt these interactions. It is hypothesized that the amount of shedding of soluble HJV is down-regulated by increased iron supply to the muscle or oxygen tension in the circulation. The subsequent binding of soluble HJV to the neogenin receptor in hepatocytes is suggested to down-regulate hepcidin mRNA through as yet unidentified pathways (Fig. 2D) (89, 90). These pathways need further experimental confirmation; however, if confirmed, soluble HJV could be a useful biomarker in disorders of iron metabolism.

JUVENILE HEMOCHROMATOSIS ATTRIBUTABLE TO HEPCIDIN DEFICIENCY

The first gene identified in juvenile hemochromatosis was hepcidin antimicrobial peptide (*HAMP*), which encodes hepcidin. The sequence variants reported to date, all in the homozygous state, all cause juvenile onset of the clinical disease (Table 2) [(15) and reviewed in Ref. (78)]. Identification of the *HAMP* gene as being responsible for a subset of juvenile hemochromatosis conclusively linked hepcidin to iron metabolism in humans (12–14, 91–93). Recent experimental data on iron accumulation in organs of mice with disrupted *SMAD4* [mothers against DPP homolog 4 (*Drosophila*)] gene in the liver indicate that much remains unknown concerning the factors and condition involved in hepcidin regulation. In this latter study, for example, the authors found that growth signals (transforming growth factor- β /bone morphogenetic proteins) that are involved in diverse developmental processes also induce hepcidin synthesis, requiring *SMAD4* as a well-known tumor suppressor gene to transcriptionally activate the hepcidin promoter (94).

HEMOCHROMATOSIS ATTRIBUTABLE TO TFR2 DEFICIENCY

The gene coding for transferrin receptor 2 (TfR2) was described in 1999 (8). This protein has a high degree of homology with the classic transferrin receptor (TfR1) but is expressed predominantly in the liver and in erythroid precursors (Fig. 2) (95, 96). Compared with TfR1, TfR2

binds diferric transferrin with ~30-fold lower affinity. Genetic hemochromatosis linked to sequence variations in the *TFR2* gene is rare and seems, with a few exceptions, to be clustered to families of southern European origin, mainly from Italy and Portugal, and to families in Japan (Table 2) (9, 97, 98). It has been suggested that in hepatocytes HFE/TfR1 and TfR2 act in the same or converging pathways upstream from hepcidin. In this hypothesis, TfR2 is thought to be a sensor of plasma transferrin saturation in the signaling pathway that controls hepcidin synthesis (Fig. 2D) (84, 85, 99–103). This is in line with the low hepcidin concentrations found for patients carrying TfR2 variants (104, 105), and a more severe HH phenotype when both HFE and TfR2 variants are present (106). In general terms, clinical complications, the pattern of liver iron storage, and the response to phlebotomy are identical in HFE-related HH [Online Mendelian Inheritance in Man (OMIM) type 1] and TfR2-related HH (OMIM type 3; Table 2). The onset in the latter, however, is generally somewhat earlier in adult life (107, 108).

Ferroportin Disease: The *SLC40A1* Gene

Ferroportin 1 is a protein implicated in the efflux of iron out of cells such as enterocytes or macrophages (Fig. 1 and Fig. 2, A, C, and D) (10, 109). Sequence variants in the *SLC40A1* [solute carrier family 40 (iron-regulated transporter), member 1] gene (previously called *SLC11A3*), which encodes ferroportin/iron-regulated transporter 1/metal transporter-1 protein, have been demonstrated to be linked to a dominant form of hereditary iron overload (Table 2) (5, 11, 110). The ferroportin protein, predicted to have several transmembrane domains, is expressed mainly in Kupffer cells and splenic macrophages, at the basolateral membrane of enterocytes, and to a lesser extent in hepatocytes, where it coordinately plays the role of iron exporter (109, 111–113). This export appears to be tightly regulated by hepcidin to prevent either iron deficiency or iron excess (114). Hepcidin was shown to bind to ferroportin in epithelial cells and macrophages and induces internalization and degradation of ferroportin, thereby blocking iron export (4–6).

Several *SLC40A1* sequence variants have been described and are distributed worldwide. A common deletion (162delVal), has been found in unrelated families (78, 115, 116). Other sequence variations are private, with the exception of the Q248H substitution, which has been detected in populations of African descent but for which contribution to African iron overload is a matter of debate (117–121). Recent studies have shown that most *SLC40A1* sequence variants are unresponsive to hepcidin-mediated internalization (5, 122, 123).

Ferroportin disease has distinctive features compared with HFE-related hemochromatosis. Young patients generally present with isolated hyperferritinemia and normal or slightly increased TS (Table 2) (124). Marked hepatic iron overload progressively develops, and histologically, iron accumulates predominantly in liver macrophages

(Kupffer cells). Therapeutic phlebotomies may be poorly tolerated and lead to IDA. Therefore, during treatment, TS values and hemoglobin concentration must be closely monitored. Usually, ferroportin disease is less severe than HFE-related hemochromatosis (124).

Since the description of the first families with ferroportin disease attributable to either 162delVal and N144H changes in *SLC40A1* (11, 110), different phenotypic presentations have been noted in the *SLC40A1* gene. It appears as though there are several classes of ferroportin variants depending on the causative ferroportin mutants. These variants differ mainly in the extent of increase in TS values, the tendency to (borderline) anemia, and the distribution of iron deposits among macrophages and hepatocytes (11, 110, 117, 122, 123, 125, 126). Recent attempts to subdivide the phenotypes have led to 2 classes, described as a loss-of-function and a gain-of-function phenotype, respectively (126). This first classification is based on recent publications on the *in vitro* behavior of the different variants, which for this purpose is correlated with clinical data from the numerous publications since 2001 on families carrying *SCLC40A1* sequence variations (5, 117, 122).

One of these phenotypes is consistent with a loss-of-function hypothesis, and haploinsufficiency has been attributed to mutant ferroportin (A77D, 162delVal, G490D, and G323V) that is retained inside the cell (5, 110, 117, 122). The decrease in iron efflux causes a bottleneck in cells that generate the largest iron flows, i.e., macrophages involved in the recycling of iron from senescent erythrocytes. Thus, consistent with a disorder of pure iron release, these patients experience iron retention, mainly in macrophages, which leads to the combination of high ferritin concentrations, low TS, iron-deficient erythropoiesis, and even mild anemia or impaired tolerance of phlebotomy. Because the iron accumulated in macrophages has low toxicity, these patients rarely develop iron-related clinical complications. The findings of high urinary hepcidin concentrations in patients carrying the 162delVal variant might explain the internalization and degradation of the nonmutated *trans* allele, which leads to an additional decrease in intestinal iron uptake (127, 128).

On the other hand, in patients who carry other sequence variants (Y64N, N144D, N144H, C326Y, and C326S), iron accumulates in the hepatocytes; these patients thus develop clinical signs of iron-induced damage, as in classic hemochromatosis (5, 11, 117, 122, 129, 130). This gain-of-function phenotype has been postulated to be the consequence of hepcidin resistance of the mutated ferroportin. As a result, an inappropriately high number of mutant ferroportin molecules is displayed on the cell surface, leading to increased iron efflux from enterocytes and macrophages. This lack of regulation appears to mimic hepcidin deficiency and to yield a phenotype similar to HFE-related hemochromatosis, in which intestinal iron absorption is increased, TS is increased, and excess iron is deposited in hepatocytes and other tissues.

Here the presence of a surplus of iron in the circulatory compartment may be related to better tolerance of phlebotomies. In particular, patients with ferroportin variants that completely obstruct hepcidin sensitivity (Y64N and C326S) (122) exhibit a more severe phenotype, with predominant iron overload in the hepatocytes. Dysfunction appears to be less severe in patients carrying the N144D and N144H variants, in whom the hepcidin sensitivity of ferroportin is only partially hindered and iron accumulates in both the hepatocytes and macrophages (117, 122, 131, 132). Hepcidin concentrations in some of these cases with a gain-of-function phenotype are increased, whereas others are normal but relatively too low to the degree of iron loading (108).

Diagnosis of ferroportin disease is complex because it requires that all various conditions causing isolated hyperferritinemia are ruled out. Ferroportin disease should always be suspected in familial forms of hyperferritinemia or in sporadic cases of high ferritin in the absence of known secondary causes, such as infection, metabolic syndrome, inflammation, and malignancy (124, 133). Differential diagnosis should also include familial hyperferritinemia congenital cataract syndrome (HHCS), which is a rare disease without iron overload but with high ferritin concentrations (134, 135); aceruloplasminemia, which manifests predominately with neurologic symptoms (136, 137); and the increasingly prevalent metabolic syndrome present in obese, hypertensive, insulin-resistant, or dyslipidemic individuals (Table 1) (39, 43).

Because clinical data will accumulate with time and *in vitro* and experimental studies in mice and humans will be performed, it will undoubtedly become clear whether this first classification of ferroportin disease is still useful or needs to be adjusted based on new insights. This classification might be of help in prescreening for diagnosis based on hepcidin concentrations and in determining the prognosis and optimal treatment. To date, the experience with treatment of ferroportin disease has been limited. It is possible that treatment protocols will become less stringent than those for the other forms of HH because macrophage iron is less likely to cause tissue damage and some patients suffer from impaired tolerance of phlebotomy. Alternatively, chelation therapy might be considered in those cases with substantial parenchymal iron overload and low tolerance of phlebotomy.

Modifiers of Hemochromatosis Type I

The majority of C282Y-homozygous individuals will present with abnormal iron indices (138), whereas only a small number will manifest clinical features (60, 61, 139, 140). "Mild" genotypes such as C282Y homozygosity are relatively susceptible to the effect of environmental and genetic modifiers. As more modifiers are identified, our ability to predict those C282Y-homozygous patients with an increased risk for developing severe iron overload and, consequently, clinical complications will improve. The identification of more modifiers will therefore contribute

to the debate on whether population screening for HH should be undertaken or whether alternative strategies should be implemented to improve early detection (140). Among these approaches is the screening of relatives of a clinically overt C282Y-homozygous proband (141). This family (cascade) screening approach might appear to be even more cost-effective after the dominant modifying factor in the index case is identified.

The influence of factors such as excessive alcohol intake (142, 143), viral hepatitis (144), dietary iron (145), or body mass index in women (146) likely explains some of this phenotypic heterogeneity in C282Y homozygosity. The influence of modifier genes is an attractive additional explanation. Indeed, in addition to the background strain, several genes involved in iron metabolism act as modifiers, as shown in *HFE*-knockout mice (147–149). Support for genetic modifiers also comes from several reported cases in which patient homozygous for the C282Y genotype and heterozygous for *HAMP* or *HJV* developed more severe iron overload than in C282Y-homozygous patients matched for age and sex (150, 151). Similar interactions might also occur in persons who are heterozygous for *HJV* and *HAMP* and carry the C282Y/H63D genotype, but the evidence for a modulatory effect on heterozygous C282Y sequence variations appears inconsistent (150–154). Another recent example of digenic inheritance is the juvenile phenotype that results from compound C282Y/H63D heterozygosity in combination with homozygous *TFR2* missense variants (106).

In general, however, multiple sequence variations in the recently identified genes involved in iron homeostasis are rare and do not appear to explain most of the variation in the penetrance of *HFE* hemochromatosis (151, 153, 155). Nevertheless, there is evidence that persons with the Hp 2-2 sequence variant of the gene coding for the haptoglobin protein have higher iron stores than controls (156, 157), although this is not found consistently (158). In addition, some centers have reported that the presence of 2 copies of the ancestral HLA haplotype on chromosome 6 leads to more iron loading. However, this could not be confirmed by detailed analysis of chromosome 6p21.3 and the *HFE* gene (155, 159).

Finally, the penetrance of C282Y homozygosity could also be the result of multiple small additive effects that change the classic monogenic hemochromatosis into an oligenic or even a multifactorial disease in which homozygosity for the C282Y variant is only a predisposing factor.

The OMIM Classification

The OMIM database (<http://www.ncbi.nlm.nih.gov/omim/>) lists 4 types of HH, each caused by sequence variations involving a different gene (Table 2). However, most workers in the field of iron metabolism do not approve with this classification because (a) it is neither genotypic nor phenotypic, but rather a mixture of the two; (b) it leaves no space for recently identified atypical cases that are related to combinations of sequence variations

(106, 148–152); (c) it sets up hypothetical genes, e.g., *HFE1*, *HFE2*, *HFE3*, and so forth, when its not really a gene family at all; and finally (d) there are also other forms of hemochromatosis that are not included in the OMIM classification (Table 2). Therefore, among others, Pi-trangelo (19) recently pled for a definition that is based on the pathophysiologic entity instead of on the responsible genes. Nevertheless, the associations of the various genes with either an adult (*HFE*, *TFR2*, ferroportin) or a juvenile onset (*HJV* and *HAMP*) are helpful in selecting the starting point for genetic testing. If a patient also has a marginally decreased hemoglobin, increased hepcidin, or poorly tolerates phlebotomy, ferroportin disease should be considered. Probably in the future hepcidin concentrations in urine or serum might be of help in prescreening patients with a clinical and biochemical presentation consistent with non-*HFE*-related forms of HH (a) to differentiate between the 3 additional types or their combinations (Table 2 and Fig. 3); (b) to determine prognosis in time; and (c) to optimize individual treatment by choosing among no treatment, phlebotomy, chelation therapy, or to treat with hepcidin agonist or antagonists. If confirmed, urinary or serum hepcidin concentrations might even form the basis of a revised classification of HH.

Rare Genetic Defects of Iron Loading

The vast majority of hereditary iron overload reported to date seems to be caused by *HFE*, *TFR2*, ferroportin, *HJV*, or *HAMP* sequence variations. However, there are other, more rare genetic defects that appear to lead to tissue iron accumulation (Table 2).

In hereditary cases of iron overload in the presence of isolated hyperferritinemia, the differential diagnosis should include hypotransferrinemia and aceruloplasminemia.

Hypotransferrinemia is a rare recessive disease, characterized by an extremely low transferrin concentration, severe IDA, and iron loading of the liver and other parenchymal organs (160, 161). Aceruloplasminemia is a late-onset recessive disease attributable to sequence variations in the ceruloplasmin gene. Ceruloplasmin is a copper-dependent ferroxidase that likely cooperates with ferroportin to export iron from macrophages, hepatocytes, and intestinal mucosal cells (Fig. 2). The disease is characterized by diabetes mellitus, iron loading of the liver and pancreas, retinal degeneration, and neurologic signs and symptoms attributable to stimulation of hydroxyl radical formation by the persistent presence of Fe^{2+} in the circulation. Anemia is usually present early in life because of insufficient iron supply to the erythron. The combination of low serum iron and transferrin but high serum ferritin should raise suspicion of this disease (136, 137).

In hereditary cases of isolated hyperferritinemia in the absence of iron overload, the presence of HHCS should be considered. In this condition, sequence variations in the 5'-untranslated region of the L-ferritin (*FTL*) gene can

cause increased serum ferritin (133–135, 162). The absence of an increased TS suggests that, in this syndrome, hyperferritinemia is not attributable to iron overload, but to a dysregulation of the production of L-ferritin light chain in relation to mutations in the iron-responsive element of the L-ferritin mRNA.

Lately, the first example of sequence variants for *SLC11A2*, the gene that encodes for divalent metal transporter 1 (DMT1; also known as DCT1 or NRAMP2), was shown in a human with hepatic iron overload in the presence of hypochromic anemia (163, 164). DMT1 is a metal transporter that transports dietary Fe^{2+} at the brush border of duodenal enterocytes in concert with duodenal cytochrome B (DcytB), a ferrireductase (Fig. 2A). DMT1 is also responsible for the recovery of iron from recycling endosomes during TfR-associated cellular uptake in erythrocyte precursor cells and other cells needing iron (Fig. 2, B and C). The human presentation surprisingly differs from that of rodents with variant DMT1, which do not develop iron overload but do present with severe forms of hypochromic, microcytic anemia with diminished erythrocyte survival (165). The increased iron absorption in humans likely bypasses the DMT1 defect in the gut and could be related to up-regulation of the recently identified mammalian heme transporter, heme carrier protein 1 (HCP1; Fig. 2A) (166, 167).

Defects in proteins known to be involved in iron metabolism, such as hephaestin or DcytB, have not been identified in humans (Fig. 2A). In addition, several new proteins of the iron-regulatory pathways are still being discovered, e.g., the ferrireductase 6-membrane epithelial antigen of the prostate 3 (Steap3; Fig. 2B) (168) and the hemoglobin deficit (hbd) sequence variation in the mouse gene *Sec15l1* (169). It is reasonable to expect that in time previously undiagnosed defects in iron homeostasis could be ascribed to defects in these newly identified genes.

Role of Hepcidin

Hepcidin is shown to be the principal regulator of systemic iron homeostasis (Fig. 1). We will therefore elaborate on the formation of hepcidin and its diagnostic assessment in more detail. In concordance with its dual function, hepcidin expression is modulated by systemic iron requirements and infectious and inflammatory stimuli (Fig. 1). Its discovery has changed our understanding of the pathophysiology of iron disorders (170). Hepcidin acts as an iron hormone to control iron absorption and macrophage iron release (Fig. 1). Recent reports indicated that hepcidin does so by diminishing cellular iron efflux by binding to the transmembrane iron exporter ferroportin on enterocytes and macrophages and subsequently inducing its internalization (4–6).

Hepcidin is produced by hepatocytes and is secreted into plasma as a 25-amino acid mature form (12, 14, 171) (Fig. 2D). In addition to the 25-amino acid form, additional amino-terminal processing events produce 2 smaller hepcidin forms of 22 and 20 amino acids, which

can be isolated from urine (14). In vivo studies in mice have demonstrated that only the full-length 25-amino acid hepcidin induces significant hypoferrremia when injected intraperitoneally (172). Recently, these findings were confirmed by in vitro studies from the same group that showed that compared with the 25-amino acid hepcidin, the truncated 22-amino acid form has greatly diminished activity, whereas the deletion of 5 N-terminal amino acids, in the case of 20-amino acid hepcidin, led to almost complete loss of activity (173).

HEPCIDIN IN HEMOCHROMATOSIS

HH. Urinary hepcidin is extremely low or even undetectable in all juvenile cases of hemochromatosis studied (Table 2) (16, 127). Similar findings of low hepatic hepcidin RNA expression or low urinary hepcidin concentrations characterize men with HFE deficiency and *HFE* sequence variants (82, 174, 175) and patients with *TFR2* sequence variants (104). Concentrations in ferroportin disease appear to vary with the sequence variations in the *SCL40A1* gene and the ways they influence the activity of the ferroportin protein. High urinary hepcidin concentrations were reported for patients with the 162delVal variant. This might explain the internalization and degradation of the nonvariant *trans* allele, which lead to additional decreases in intestinal iron uptake (127). In contrast, hepcidin concentrations are within reference values in some cases with a gain-of-function variant (108), but are relatively too low for the degree of iron loading.

The digenic inheritance of variant HFE with variant forms of HAMP, HJV, and TfR2, found in some patients with clinically overt HH, might be consistent with a synergistic effect that these encoded proteins exert on the main regulator of iron metabolism, hepcidin (Fig. 2D).

The ratio of urinary hepcidin to serum ferritin could be a useful index for assessing inadequate hepcidin responses to iron loading in hemochromatosis. For example, patients with HH attributable to TfR2 variants have a low hepcidin/ferritin ratio (104). Thus, the lack of appropriate hepcidin response to iron loading could be a unifying diagnostic test for all of these disorders (Fig. 3). In addition, the hepcidin concentration could correlate with the severity of the phenotype and determine the prognosis and need for stringency of the treatment protocol.

Secondary hemochromatosis. The hepcidin/ferritin ratio is also low in several secondary syndromes of iron overload characterized by ineffective erythropoiesis, including thalassemia and congenital dyserythropoiesis type 1. In pilot-scale studies, hepcidin is found to be suppressed for both disorders, with a median hepcidin/ferritin ratio in thalassemia syndromes that is only 5% of that of controls (176), indicating that the erythroid iron needs appears to be dominant over the inhibitory signals of iron stores (80, 127, 177, 178). In addition, available data, although limited, suggest that sickle cell anemia and hereditary spherocytosis, both disorders in which altered erythrocyte

physiology leads to accelerated hemolysis and increased erythropoiesis (which implicates an increased iron demand), also lead to relatively low hepcidin concentrations (176). The above results are in line with the theory that iron absorption is increased in a variety of refractory anemias characterized by erythroid hyperplasia in conjunction with ineffective erythropoiesis [reviewed in Ref. (21)]. Thus, even without transfusions, these patients will accumulate iron. However, most of these disorders are transfusion dependent; therefore, the iron burden in these disorders is mainly attributable to multiple transfusions. In thalassemia patients, transfusion can lead to increased urinary hepcidin concentrations after 3–4 days, most likely as a result of relief of anemia and, thus, suppression of erythropoiesis and a decreased demand for hepatic iron (176). Hepcidin concentrations may also predict effects on iron absorption. In patients with hemolytic anemia receiving erythrocyte transfusions, increasing hepcidin concentrations will decrease iron absorption.

These findings highlight hepcidin as a diagnostic tool in both hereditary and secondary forms of hemochromatosis and may also contribute to the development of innovative therapeutic interventions. Further optimization and increased access to assays for urinary and serum hepcidin as well as analysis of hepcidin in various forms of hemochromatosis are needed, however, to further enlarge our understanding of the contribution of hepcidin in these disorders. This will be of value as a substitute for the cumbersome procedures for detecting genetic variants in these non-HFE-related forms of HH (Fig. 3). Additionally, this might be relevant for the thalassemia syndromes, for which there is an ongoing search for noninvasive measures of iron burden and improved therapeutic interventions.

HEPCIDIN IN AI

AI, or anemia of chronic diseases, represents an important and highly prevalent clinical problem. Recent studies indicated that hypoferrremia in inflammation is caused by a cytokine-mediated (particularly interleukin-6 and -1) increases in hepcidin production (179–182). The axis that produces AI leads from interleukin-6 and -1 to hepcidin, via blocking of ferroportin, to hypoferrremia, and then to AI. These findings are in line with characteristics of AI: sequestration of iron in macrophages and hepatocytes and decreased iron absorption, all of which decrease plasma iron concentrations. The purpose of these events is proposed to be to limit the availability of iron for microbial growth. On the other hand, this leads to insufficient iron for erythropoiesis, with consequent development of anemia. Thus, the development of AI can be considered a side effect of the hepcidin-induced hypoferrremic response to infection and inflammation.

Hepcidin is low in IDA (175, 179). The correct diagnosis of iron deficiency, however, is essential for successful patient management. Nevertheless, in some patients, typically those with inflammatory problems, diagnosis can

be difficult. Measurement of ferritin is currently the most accepted laboratory test for diagnosing body iron deficiency, but persons with AI may have normal or increased ferritin values even when iron deficient. The plasma concentrations of iron and transferrin, however, are decreased (183, 184). Other markers that have been developed in recent years for this purpose include the soluble TfR (sTfR) and new erythrocyte indices provided by advanced hematology analyzers (185–187). The ratio of sTfR to ferritin (R/F ratio) has even been suggested as the gold standard. A drawback of the use of this ratio in medical practice, however, is the lack of appropriate standardization of the current sTfR assay (188). This necessitates that each laboratory calibrate the R/F ratio by quantitative phlebotomy measurements (189). Additional studies are needed to assess whether hepcidin measurements aid in the selection of patients with chronic inflammation who are also iron deficient and therefore need further investigation to explain their IDA and to guide treatment with iron supplements.

Hepcidin Assays

Assays for hepcidin detection and quantification in plasma or urine have not been widely available, and the development of reagents has been hampered by technical difficulties. The development of immunochemical methods based on the production of specific anti-hepcidin antibodies is difficult because of the small size of hepcidin (25 amino acids), that fact that the sequence is conserved among animal species, and the limited availability of antigen. Therefore, at present, only a limited number of tools are available to investigate human hepcidin protein production, maturation, and excretion. For serum, only the measurement of prohepcidin is possible, by a commercially available ELISA that uses antibodies directed against amino acid residues 28–47, which encompass the propeptide region of the 64 prohepcidin precursor (Fig. 2D) (190, 191). This propeptide is removed by proprotein convertases, most likely during the secretion process, and is therefore not present in the mature (bioactive), 25-amino acid form of hepcidin that is released into the circulation. For urine, 2 assays have been reported to measure mature hepcidin. One of them consists of purification of hepcidin from the urine by column chromatography, with subsequent concentration of the antigen followed by a specific immunodot method (179). More recently, we described a surface-enhanced laser desorption/ionization time-of-flight mass spectrometry-based assay on a platform provided by CIPHERGEN Biosystems (175). Urinary hepcidin concentrations determined by both methods highly correlate. Notably, only the latter mass spectrometry-based method can distinguish between the 25-, 22-, and 20-amino acid forms of hepcidin in urine (175). The clinical utility of hepcidin measurements in urine was recently confirmed by the fact that urinary hepcidin concentrations significantly correlated with he-

patic hepcidin mRNA concentrations in liver transplant patients (192).

The relationship between hepcidin and prohepcidin remains controversial. One question is whether the prohepcidin detected by the prohepcidin antibody actually reflects the amount of bioactive hepcidin in serum. At present, the biological importance of the 64-amino acid prohepcidin in regulating iron status is not known. Moreover, it can be anticipated that the prohepcidin antibody also detects the 39-amino acid propeptide that is released from hepcidin by proprotein convertase-mediated processing. In addition, it is not clear yet whether the prohepcidin detected in serum is actively and specifically excreted by vital liver cells or is simply the result of the aspecific and well-known physiologic phenomenon of cell leakage by damage or death of a small percentage of cells. Finally, it is possible that serum concentrations of the 25-amino acid hepcidin and the 64-amino acid prohepcidin or 39-amino acid propeptide differ through variation in their half-lives. Experimentally, using a human endotoxemia model, we observed increased hepcidin in the presence of invariable serum prohepcidin concentrations (180). On the other hand, a report of a patient with iron accumulation attributable to ferroportin disease showed substantially and significantly increased serum concentrations of prohepcidin that correlated positively with urinary hepcidin concentrations (128). Possibly, serum prohepcidin and urinary hepcidin measurements correlate only in severe iron metabolism disorders, whereas this correlation is absent in inflammation, in which iron homeostasis is less severely disturbed. Nevertheless, discrepancies between body fluid concentrations of prohepcidin and the bioactive mature forms of hepcidin raises questions concerning the clinical utility of the currently available prohepcidin assay. For the moment, urinary hepcidin measurements most accurately reflect hepatic hepcidin production.

In summary, preliminary studies indicate that hepcidin assays clearly differentiate between relevant clinical iron disorders such as between IDA and AI and between iron overload attributable to multiple transfusions and ineffective erythropoiesis (Table 3). Furthermore, a hepcidin assay might have a role as a screening test for the presence of HH, thereby reducing the workload and costs of the cumbersome procedures of screening for sequence variations in the multiple genes responsible for HH (Fig. 3).

Table 3. Hepcidin concentrations in the various diseases.

Increased hepcidin	Decreased hepcidin
High iron stores	Increased and/or ineffective erythropoiesis
Anemia of chronic disease	Iron deficiency
Ferroportin disease	Classic, juvenile, and TfR2 HH

Clinical and Molecular Diagnosis of Hereditary Forms of HH

The combined measurements of the serum iron concentration, transferrin concentration, or total iron-binding capacity (and calculation of the TS) and the ferritin concentration provide a simple and reliable first assessment of body iron concentrations. Once hyperferritinemia and/or increased TS have been found, acquired causes of increased serum iron should be excluded (Fig. 3 and Table 1). Among these are some common diseases, such as liver diseases and diseases that are part of the metabolic syndrome: diabetes mellitus (insulin resistance), obesity, hypertension, and hyperlipidemia (2, 39, 42–45, 47–54, 193, 194). Obesity, for example, tends to increase ferritin, whereas it decreases serum iron and TS in wild-type persons of either sex (42, 47, 54). TS aids in the diagnosis of iron overload and its origin. Normal or slightly increased TS in combination with increased serum ferritin concentrations suggest HHCS, aceruloplasminemia, or ferroportin disease (53).

When iron overload is suspected and acquired causes have been excluded, diagnosis of hereditary causes of hemochromatosis should be considered (Table 2). Currently, a liver biopsy is used for assessment of cirrhosis, which increases the risk for hepatocellular carcinoma, or to identify concomitant diseases. Determination of the hepatic iron concentration by magnetic resonance imaging is increasingly recognized as a valuable diagnostic tool in patients with increased serum iron indices (195–199) and might be useful to establish iron overload in those patients without a pathognomonic *HFE* genotype and substantially increased ferritin concentrations (>500 $\mu\text{g/L}$). This technology is now available in many medical centers and might improve the diagnostic procedure by allowing a reliable and noninvasive assessment of hepatic iron, provided that the proper software and calibration techniques are used.

A search for non-*HFE* hereditary defects is indicated in selected cases in the presence of confirmed iron overload in the absence of C282Y homozygosity in the *HFE* gene.

A severe phenotype (severe loading at a relatively young age) points to the presence of variant forms of hepcidin or HJV, whereas normal or only moderately increased TS makes the presence of ferroportin disease more likely. The measurement of urinary hepcidin may contribute to the differential diagnosis of the non-*HFE* hereditary forms of HH, hepcidin being very low in the presence of *HJV* and *HAMP* sequence variants, low in case of homozygosity for *TFR2* sequence variants, and likely to be increased in ferroportin disease (Table 2). In patients with an at-risk genotype, family screening should be carried out by assessment of iron indices and genetic testing. A practical approach, based on the high prevalence of *HFE* sequence variants among patients with HH and new techniques for liver iron quantification, is proposed in Fig. 3.

Thus, rational gene targeting is based on information on clinical presentation, family history, serum iron indi-

ces, concomitant diseases, age at presentation, confirmation of iron overload or presence of the *HFE* genotype, and hepcidin measurements. Ongoing improvements in molecular biology techniques and their automation will further assist in efficient gene diagnosis.

Conclusions and Recommendations

Since the discovery of the *HFE* gene in 1996, several novel gene defects have been detected, adding to the understanding of the mechanism and diversity of iron-overload diseases. At least 4 main types of HH have been identified. Surprisingly, genes involved in HH encode for proteins that all affect pathways centered around liver hepcidin synthesis and its interaction with ferroportin, an iron exporter in enterocytes and macrophages. Hepcidin concentrations in urine are negatively correlated with the severity of HH. Cytokine-mediated increases in hepcidin concentrations appear to be an important causative factor in AI, which is characterized by sequestration of iron in the macrophage system.

For clinicians, the challenge is now to diagnose *HFE*-related HH before irreversible tissue damage appears and at the same time to distinguish HH from increasingly common diseases that lead to only moderately increased body iron stores, such as the metabolic syndrome. The other challenge is to optimally use both conventional and innovative laboratory tests to differentiate between the various causes of iron overload.

We have described a systematic diagnostic strategy for patients with suspected HH and for their relatives. This includes rational targeting of the gene variations to be screened. Such a careful approach allows correct assignment of the patient's diagnosis. This has important implications for prognosis, follow-up, and treatment of the patient and his or her relatives. We therefore recommend that after initial clinical and laboratory investigations and exclusion of acquired causes of hyperferritinemia, atypical patients are sent to specialized centers that can perform investigations with an up-to-date, targeted approach. However, the strategy proposed may change in time with advances in noninvasive techniques for the assessment of hepatic iron and tissue damage, the availability of hepcidin measurements in both urine and serum, and the identification of new key players in iron homeostasis.

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