

Understanding iron homeostasis through genetic analysis of hemochromatosis and related disorders

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Genetic analysis of hemochromatosis has led to the discovery of a number of genes whose mutations disrupt iron homeostasis and lead to iron overload. The introduction of molecular tests into clinical practice has provided a tool for early diagnosis of these conditions. It has become clear that hemochromatosis includes a spectrum of disorders that range from simple biochemical abnormalities to chronic asymptomatic tissue damage in midlife to serious life-threatening diseases in young subjects. Molecular studies have identified the systemic loop that

controls iron homeostasis and is centered on the hepcidin-ferroportin interaction. The complexity of this regulatory pathway accounts for the genetic heterogeneity of hemochromatosis and related disorders and raises the possibility that genes encoding components of the pathway may be modifiers of the main genotype. Molecular diagnosis has improved the classification of the genetic conditions leading to iron overload and identified novel entities, characterized by both iron loading and variable degrees of anemia. Despite the progress in the diagno-

sis, classification, and mechanisms of iron overload disorders, the treatment of affected patients continues to rely on regular phlebotomy. Understanding the molecular circuitry of iron control may lead to the identification of potential therapeutic targets for novel treatment strategies to be used in association with or as an alternative to phlebotomy. (*Blood*. 2005;106:3710-3717)

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Introduction

The term “hemochromatosis,” introduced by von Recklinghausen at the end of the 19th century, refers to the clinical disorder that results from excess of total body iron and organ failure due to iron toxicity. The disease manifestations include cirrhosis, diabetes mellitus, hypogonadism and other endocrinopathies, cardiomyopathy, arthropathy, skin pigmentation, and, in cirrhotic patients, increased susceptibility to liver cancer. The genetic nature and the autosomal recessive type of inheritance of hemochromatosis were recognized in the 1970s.¹ As with other genetic disorders, the last few years brought spectacular advances in our understanding of the molecular basis of the disease. Complementing cellular and animal models, studies of iron-loaded patients have provided new insights into the molecular regulation of iron metabolism.² The proteins altered in hemochromatosis are components of a pathway that controls iron homeostasis according to the body needs. These are signaled to duodenal cells and to iron recycling macrophages by regulators, historically defined “store” and “erythroid” regulators.³ Although signals between distant tissues (such as bone marrow, liver, and duodenum) remain to be identified, we now know that both regulators converge on the liver peptide hormone hepcidin,⁴ which on interaction with its putative receptor ferroportin⁵ controls the export of iron from target tissues (principally the duodenal enterocytes the placenta, iron-storing hepatocytes, and iron-recycling macrophages).

The identification of the genes that are mutated in hemochromatosis has revolutionized the diagnosis of primary iron overload, by introducing molecular tests that allow early, presymptomatic, and accurate diagnosis. For this reason hemochromatosis is now considered a classic paradigm in molecular medicine.⁶

In this review the major advances resulting from the molecular studies of genetic iron overload are outlined in the light of their relevance to the iron regulatory pathway.

Primary or genetic iron overload

Hemochromatosis

For a comprehensive discussion of the disease manifestations, penetrance, diagnosis, and treatment, the reader is referred to a recent review.⁷ Early symptoms of hemochromatosis include asthenia, abdominal pain, and arthralgia; the organ involvements with hemochromatosis are shown in Table 1. The biochemical abnormalities of iron parameters include a transferrin saturation of greater than 45% and a serum ferritin concentration of greater than 300 $\mu\text{g/L}$ in men and greater than 200 $\mu\text{g/L}$ in women. The diagnosis of hemochromatosis, once based exclusively on iron quantitation in liver biopsies, now in most cases relies on genetic tests. Nowadays the indication for liver biopsy is restricted to patients who do not have an informative genotype or is used for prognostic evaluation in cases of severe iron loading.⁸ Most individuals undergo molecular diagnostic testing when they present with only biochemical signs of iron overload, such as high transferrin saturation or elevated serum ferritin concentration. This approach has drawbacks. First, it implies a differential diagnosis with all the numerous conditions characterized by these common biochemical abnormalities, such as secondary iron overload, alcohol excess, dysmetabolic syndrome, and others. Second, it poses

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Table 1. Organ involvement in hemochromatosis

| Organ | Manifestations | Complications |
|---------------|---|--|
| Liver | Enzyme alterations | Fibrosis, cirrhosis, hepatocellular carcinoma* |
| Heart | Arrhythmia, failure | Cardiomyopathy |
| Pancreas | Hyperglycemia | Diabetes mellitus |
| Pituitary | Decreased libido, impotence in men; amenorrhea in women | Hypogonadism |
| Thyroid gland | Decreased thyroid hormone | Hypothyroidism |
| Joints | Arthralgia | Arthropathy |
| Skin | Pigmentation | None |

All the possible hemochromatosis complications are listed; however, patients presenting with all these clinical findings are exceptional, and most have nonspecific symptoms, or abnormalities of iron parameters and of liver enzymes.

*Increased risk in cirrhotic patients.

the challenge of identifying, among the at-risk genotypes, the proportion of individuals who will develop overt disease. This involves the recognition of both the environmental and the genetic determinants that can modify the genotype expression.

Molecular genetics. Hemochromatosis is a heterogeneous genetic disease that may result from mutations in at least 4 genes (Table 2).

The HFE gene. The era of molecular genetics in hemochromatosis started almost 30 years ago with the identification of the disease linkage to HLA class I and of the linkage disequilibrium to HLA haplotype A3.¹ The positional cloning is more recent history since the first gene, *HFE* (previously called *HLA-H*),⁹ was cloned in 1996.

As expected on the basis of the observed HLA-A3 disequilibrium, most patients (about 80%) with adult-onset hemochromatosis have a single point mutation that results in the substitution of a tyrosine for cysteine at position 282 (C282Y) of the HFE protein.⁹ Due to a founder effect, the C282Y homozygous genotype is particularly common in Northern Europe (where 1 in 300 to 1 in 400 individuals are C282Y homozygous) whereas a decreasing frequency gradient is observed for this genotype towards Southern Europe.¹⁰ A recent multiracial screening of more than 99 000 individuals has shown that the C282Y mutation is most common in whites, but extremely rare in nonwhites.¹¹ A second mutation is characterized by the replacement of histidine by aspartic acid at position 63 of the protein (H63D). In animal models this substitution confers a mild risk of iron loading.¹² In humans it is a frequent polymorphic change in all ethnic populations, but its contribution to the clinical disease is negligible, except in compound heterozygosity with C282Y.⁹ Other HFE mutations are rare, often reported in combination with C282Y.

The cloning of the *HFE* gene was expected to lead to rapid advances in understanding the regulation of iron homeostasis. However, the molecular function of *HFE* in iron metabolism has

remained obscure, even after HFE was linked to the transferrin receptor (*TFR1*) cycle.¹³ HFE is an HLA class I atypical protein, which binds β_2 -microglobulin and competes in vitro with transferrin for TFR1 binding. However, studies of the HFE effect on iron uptake in cell lines transfected with *HFE* have provided conflicting results.^{13,14} The coexpression of HFE and TFR1 on the basolateral membrane of the cells of duodenal crypts, demonstrated by immunohistochemical studies, led to the fascinating “crypt” hypothesis.¹⁵ According to this view, the HFE-TFR1 complex in the crypt stem cell was the sensor of the total body iron, aimed at programming the expression of iron transporters of absorptive cells in the villi. This hypothesis has been challenged by the discovery of hepcidin (see “Hepcidin deficiency: a unifying pathogenetic cause”), which induces changes of duodenal absorption that are too rapid to be compatible with the time required for the maturation of cells from the crypt stem cell.¹⁶ Also, the increased iron transport occurs on the basolateral membrane and not on the luminal side. Because *HFE* encodes an atypical HLA-like molecule, it has been proposed that it might have some immune-related function.¹⁷ Emerging data relate the HFE role to hepcidin activation in the liver, but the underlying molecular mechanisms remain unknown.

Clinical observations suggest that the HFE protein is not the only inhibitor of iron absorption because its inactivation causes a disease of late onset that predominantly affects males and has low penetrance. The penetrance of hemochromatosis is a controversial issue^{18,19}; it is influenced by sex, age, environmental factors, and modifier genes. Studies of the identification of modifiers are promising in animal models,^{20,21} but the identification of human modifiers remains complex.

Some ability to regulate iron absorption is retained in *HFE* hemochromatosis and the absorption can even be further increased, as shown in *HFE*-deficient mice after phlebotomy or iron restriction.²² Also, the iron burden and clinical phenotype is more severe in C282Y homozygotes with associated β -thalassemia trait than in C282Y homozygotes without thalassemia.²³ Thus, it seems that HFE plays a role in the down-regulation, but it does not influence the up-regulation of intestinal iron uptake. Accordingly HFE appears to be a component of the “store” regulator. Consistent with this interpretation, the presentation of *HFE*-related disease in middle age indicates that the role of HFE protein is irrelevant during growth and development, when iron stores are low and iron requirements are high.

TFR2: the second gene. *TFR2* hemochromatosis²⁴ is often reported as a disorder similar to *HFE* hemochromatosis.⁷ In general terms the clinical complications, the pattern of liver iron storage, and the response to phlebotomy are identical in the 2 disorders. *TFR2* patients are rare, have different age, sex, and clinical complications,²⁴⁻³⁰ making difficult the phenotypic comparison with the other types of hemochromatosis. The disease is reported in

Table 2. The hemochromatosis genes and proteins

| Gene | Protein | Protein class | Expression | Interaction | Disease type by OMIM |
|----------------|-------------|-----------------------|-------------------------------------|-------------|----------------------|
| <i>HFE</i> | HFE | HLA class I, atypical | Ubiquitous | TFR1 | 1 |
| <i>TFR2</i> | TFR2 | TFR family | Hepatocytes | Transferrin | 3 |
| <i>HAMP</i> | Hepcidin | Antimicrobial peptide | Hepatocytes, skeletal muscle, heart | Ferroportin | 2b |
| <i>HJV</i> | Hemojuvelin | RGM homologue | Heart, liver, skeletal muscle | Neogenin* | 2a |
| <i>SLC40A1</i> | Ferroportin | Iron exporter | Ubiquitous | Hepcidin | 4† |

OMIM indicates Online Mendelian Inheritance in Man¹⁰¹; *HJV*, hemojuvelin-encoding gene; *SLC40A1*, solute carrier family 40, member 1; HLA, human leukocyte antigen; TFR1, transferrin receptor 1; RGM, repulsive guidance molecule.

*See “Note added in proof.”

†Also called ferroportin disease.

whites and Japanese.^{29,30} Comparing the clinical phenotype of small series of patients we concluded that the clinical picture of *TFR2* hemochromatosis is less severe than that of the juvenile form but more severe than that of the *HFE*-related disease.³¹ Iron accumulation may occur in the second to third decades of life²⁵⁻²⁷ and an iron-loaded child, aged 3.5 years, was reported.²⁸ These features are shared with juvenile hemochromatosis. However, the progression of iron overload is slow in *TFR2* patients.²⁶ A constant feature is the persistently high transferrin saturation, even after phlebotomy.

At the time of its cloning the function of *TFR2* was even more obscure than that of *HFE*. Based on the sequence homology with *TFR1* and its ability to bind transferrin, it was initially considered an iron uptaker.³² However, *TFR2* is not iron-regulated because it has no iron-responsive elements (IREs), that is, elements typical of genes that are regulated by cellular iron, in its untranslated (UTR) mRNA region. *TFR2* does not bind *HFE* and shows a restricted pattern of tissue expression, predominantly in hepatocytes.³³ The hypothesis that *TFR2* mediates iron uptake was difficult to reconcile with the high iron stores in patients with *TFR2* inactivation.²⁴ Targeted disruption of the murine *TFR2* (Y245X), orthologous to the first mutation recognized in humans, led to the same pattern of iron accumulation as observed in patients.³⁴

TFR2 murine promoter has consensus sequences for erythroid/liver-related transcription factors as GATA, CCAAT/enhancer-binding protein (C/EBP), implying tissue-specific regulation.³⁵

Recent data obtained in hepatoma-HepG2 cell line show that exposure to the ligand, diferric transferrin, up-regulates and stabilizes *TFR2* protein, increasing its half-life. This suggests that *TFR2* senses the body iron status by sensing the saturation of transferrin.^{36,37} According to other experimental data, *TFR2* promotes the intracellular movement of transferrin, modulating its serum concentration³⁸ and therefore influencing the amount of iron available for erythropoiesis.

Clinical observations such as the young age at presentation of *TFR2* hemochromatosis point to a role for *TFR2* during growth, when iron requirements and absorption rate are high and iron stores limited. Taking into account all these findings it plausible to suggest that *TFR2* protein is involved in the “erythroid regulator” pathway.

Juvenile hemochromatosis: the hepcidin link. Juvenile hemochromatosis was described as a separate entity from hemochromatosis in several anecdotal reports.³⁹ The disease shares numerous features with *HFE* hemochromatosis, but all the clinical manifestations develop earlier because intestinal iron absorption is greater and the rate of iron accumulation faster than in the classic form. Clinical symptoms are similar to *HFE* hemochromatosis, but hypogonadism and cardiac disease are more frequent presenting symptoms.⁴⁰ Premature deaths because of heart failure or major arrhythmias are reported in untreated or undiagnosed patients with cardiac involvement.³⁸ The early onset, the huge iron burden, the clinical severity, and the involvement of both sexes suggest that the “juvenile” gene product exerts an inhibitory effect on iron absorption stronger than *HFE*,³¹ a hypothesis confirmed by molecular genetic studies.

The first gene identified in this disorder was *HAMP*-encoding hepcidin,⁴¹ a liver peptide hormone that is the central regulator of iron homeostasis in animal models,⁴²⁻⁴⁴ where it controls both duodenal iron absorption and macrophage iron release.⁴ We found that the hepcidin gene was inactivated by homozygous mutations in probands of 2 unrelated families of Mediterranean origin.⁴¹ The mutations reported so far, all in the homozygous state, cause either a complete inactivation of the protein⁴⁰ or a substitution of one of the invariant cysteines⁴⁵⁻⁴⁷ of the peptide. Another mutation

introduces a new out-of-frame ATG initiation codon.^{48,49} The identification of hepcidin gene as responsible for a subset of juvenile hemochromatosis conclusively linked hepcidin to iron metabolism in humans. Hepcidin-related juvenile hemochromatosis is a rare condition, accounting for less than 10% of the known “juvenile” families worldwide. However, as it often occurs for orphan rare disorders, its molecular study was the clue to understanding the role of hepcidin in human iron metabolism and in the pathogenesis of hemochromatosis (see “Hepcidin deficiency: a unifying pathogenetic cause”).

Hemojuvelin: the common juvenile player. The most common gene of juvenile hemochromatosis mapping to the pericentromeric region of the long arm of chromosome 1 was recently identified.⁵⁰ The gene (*HFE2* or *HJV*) is highly expressed in the liver, but also in skeletal muscle and heart and encodes a new protein, called “hemojuvelin.”⁵⁰ The gene is transcribed in several isoforms because 5 alternative splicing variants have been recognized. The transcript of larger size is predicted to encode a 426-amino acid, glycosylphosphatidylinositol (GPI)-linked protein, characterized by an RGD motif and a von Willebrand type D domain. The protein is homologous to a repulsive guidance molecule (RGM),⁵⁰ which does not appear to be involved in iron metabolism. RGM interacts with an ubiquitously expressed receptor, neogenin, and this interaction may regulate neuronal survival.⁵¹ More than 30 mutations have been detected in *HJV* gene clustered in exons 3 and 4. These mutations are usually private,^{50,52-54} except G320V, which has been repeatedly reported in different ethnic groups.^{50,52} The finding of low/unmeasurable hepcidin levels in patients carrying mutations in the hemojuvelin-encoding gene indicates that the protein is hepcidin-related⁵⁰ and not a component of a distinct pathway. Thus, severe hepcidin deficiency is the hallmark of juvenile hemochromatosis.

Acquired conditions that suppress hepcidin production and allow a similar maximal rate of iron absorption are anemia, hypoxia, and iron deficiency.⁴⁴ The genetic defect in juvenile hemochromatosis converts a normal physiologic response into a sustained pathologic process (inappropriate and persistent absence of hepcidin). Along this vein the hypothesis may be raised that juvenile hemochromatosis is a disease of the “erythroid regulator” pathway.

Hepcidin deficiency: a unifying pathogenetic cause. Urinary hepcidin is extremely low or undetectable in all juvenile cases studied.^{50,55} Patients with *HFE* disorder have low/normal hepcidin levels, measured either as RNA on liver biopsies^{56,57} or as amount of peptide excreted in the urine. However, normal hepcidin levels are inappropriate and reduced, if related to the degree of iron loading.⁵⁸ Similar findings characterize the *HFE*-deficient mice, which are unable to increase hepcidin expression after iron loading.^{59,60} The observation that the constitutive expression of hepcidin prevents iron accumulation in *HFE*-deficient mice⁶¹ supports its role in the pathogenesis of the disease. Low hepcidin liver RNA was documented in *TFR2*-deficient mice⁶² and low hepcidin urinary levels (especially low hepcidin-ferritin ratios) were found in patients with *TFR2* mutations.⁶³ Given the key role of hepcidin in iron homeostasis, the severity of juvenile hemochromatosis that results from hepcidin inactivation, and the relative deficiency of hepcidin in all forms of the disease, it becomes evident why the insufficient production of hepcidin regardless of iron overload is now considered the key pathogenetic feature of hemochromatosis.

These findings indicate that all hemochromatosis proteins are interrelated and that both *HFE* and *TFR2* are modulators that operate upstream of hepcidin (Figure 1). However, their function is not redundant. Disruption of both *HFE* and *TFR2* genes produces a

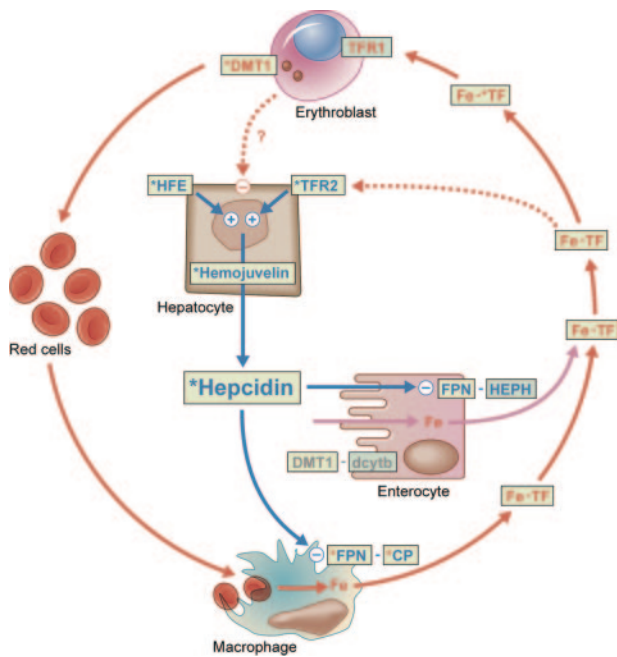


Figure 1. Genetic disorders affect different steps of the iron regulatory loop. Schematic representation of iron homeostasis. The hepcidin regulatory pathway is indicated in blue; the erythroid pathway, in red. Activation is indicated by plus (+) and inhibition by minus (-). The question mark on the dotted arrow indicates an uncertain effect. Proteins whose gene is affected by mutations are in light green rectangles. They are marked with a blue asterisk if their alteration causes hemochromatosis and with a red asterisk if it leads to iron overload and iron-deficient erythropoiesis/anemia. DMT1 indicates divalent metal transporter 1; TFR1, transferrin receptor 1; Fe-TF, diferric transferrin; TFR2, transferrin receptor 2; FPN, ferroportin 1; CP, ceruloplasmin; dcytb, duodenal cytochrome b; and HEPH, hephaestin. Illustration by Kenneth Probst.

juvenile phenotype,⁶⁴ pointing out that the effect of HFE and TFR2 on hepcidin modulation is additive. TFR2 could be a checkpoint when erythropoiesis is fully active, as occurs during growth; in this situation a high rate of iron absorption is permitted by the low levels of hepcidin and the role of TFR2 might be to up-regulate hepcidin when transferrin becomes oversaturated. On the other hand, HFE might increase hepcidin in conditions characterized by stable erythropoiesis as occurs in adult life, when iron balance is positive.

The finding that the simultaneous inactivation of *HFE* and *TFR2* produces a juvenile phenotype,⁶⁴ identical to that resulting from hepcidin mutation, does not favor the existence of other hepcidin modulators of the response to iron.

Digenic inheritance and modifiers. The genetic heterogeneity of hemochromatosis and the putative role of the hemochromatosis proteins in the hepcidin pathway raise the possibility that the disease may be the result of mutations in multiple genes. The first case of digenic inheritance with the simultaneous presence of *HFE* (C282Y) and hepcidin (ATGG deletion in exon 2) heterozygous mutations was reported in a subject with a severe, juvenile phenotype.⁶⁵ In animal models, *Hfe*^{-/-} mice that also lack a single hepcidin allele have greater liver iron accumulation than *Hfe*^{-/-} mice.⁶⁶

The biochemical phenotype resulting from the interaction of hepcidin or hemojuvelin heterozygous mutations with the homozygous C282Y genotype was more severe than that observed in C282Y homozygous patients matched for age and sex.^{67,68} Such modulatory effects may occur also in the interaction of hepcidin or hemojuvelin heterozygous mutations with the C282Y/H63D compound heterozygosity, whereas the effect of the interaction with C282Y heterozygosity is unpredictable.^{65,67-70} Although extensive

screening for mutations of hemochromatosis-associated genes has not been reported, the available data indicate that multiple mutations are rare and do not explain most of the variation in the penetrance of *HFE* hemochromatosis.

A clear example of digenic inheritance is the juvenile phenotype that results from the association C282Y/H63D in *HFE* with a homozygous missense mutation of *TFR2*.⁶⁴ Although serum hepcidin measurements were inconclusive in these patients, the phenotype is identical to that resulting from full hepcidin inactivation.

Common polymorphic changes of hemochromatosis-related genes could represent modifiers of the main genotype and either accentuate the iron burden or exert a protective effect.⁷¹ Milder genotypes, such as C282Y homozygosity, are more susceptible to the effect of modifiers than severe mutations. Multiple polymorphisms could have a small additive effect, changing the classic monogenic hemochromatosis disorder into an oligogenic disease. In this vein, we may speculate that some unexplained cases of iron overload are the result of multiple gene effect. The identification of modifiers in humans is a complex undertaking because it entails functional or large population studies.

Multiple genes and modifiers pose diagnostic challenges. In practical terms it is reasonable to search for mutations of all hemochromatosis genes only in selected cases. These are represented by patients whose severe phenotype is not adequately explained by the *HFE* genotype, as C282Y/H63D compound heterozygotes or C282Y heterozygotes (Figure 2). It is presently unclear whether measurements of the plasma or urinary concentration of hepcidin, possibly related to a quantitative measure of total

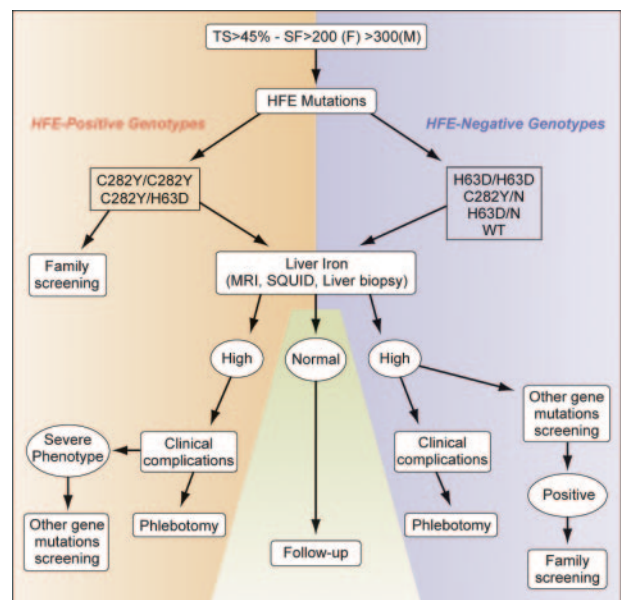


Figure 2. Flow chart for hemochromatosis diagnosis based on genotype at risk and liver iron content. Starting from abnormal iron parameters (which should be obtained at least twice) and after exclusion of conditions leading to secondary iron overload, the genetic test is performed. The approach for *HFE*-positive and -negative genotypes is shown. Assessment of liver iron is useful for subsequent therapeutic decision: liver biopsy can be done on an individual basis; superconducting quantum interference device (SQUID) and magnetic resonance imaging (MRI) can be done if available. If liver iron concentration is normal, only follow-up is proposed in all genotypes. When liver iron content is high, clinical complications should be assessed and phlebotomy is recommended in all genotypes. Selected cases of C282Y or C282Y/H63D with a severe phenotype could be screened for mutations in other genes. Among patients with *HFE*-negative genotypes, screening for other gene mutations is advisable only if iron overload is present. Family screening is indicated when causal mutations are recognized either in *HFE* or in other hemochromatosis-associated genes. TS indicates transferrin saturation; SF, serum ferritin. Illustration by Kenneth Probst.

body iron, might in the future substitute for the cumbersome procedure of screening for mutations in multiple genes.

Ferroportin disease: a hemochromatosis-related dual face disorder

A dominant form of iron overload was initially reported as Solomon Island hemochromatosis.⁷² Subsequently it was shown that the dominant disorder is usually characterized by iron accumulation in macrophages but normal/low transferrin saturation. These atypical features indicate a different pathogenesis than in other forms of hemochromatosis. Patients with dominant hemochromatosis have heterozygous mutations of the *SLC40A1* (previously called *SLC11A3*) gene, encoding ferroportin/iron-regulated transporter 1 (IREG1)/metal transporter protein-1 (MTP1) protein.^{73,74} This protein, predicted to have several transmembrane domains, is expressed in Kupffer cells and at the basolateral membrane of enterocytes, macrophages, placental cells, and hepatocytes, where it plays the role of iron exporter.⁷⁵⁻⁷⁷ Recent *in vitro* data suggest that hepcidin binds to ferroportin and induces its internalization and degradation.⁵

Several mutations of *SLC40A1* have been described, all infrequent, but distributed worldwide.⁷⁸ A recurrent deletion (162delVal) has been reported in unrelated families.^{79,80} A *SLC40A1* mutation has also been characterized in a single patient from Solomon Islands.⁸¹ A disease caused by heterozygous mutations in the iron exporter ferroportin generated a controversy as to whether these mutations might cause a gain or a loss of the metal exporter function. Iron accumulation in macrophages observed in liver biopsies of patients suggests some loss of function. Consistent with a pure disorder of iron release (Table 3) some patients have low transferrin saturation, iron-deficient erythropoiesis, and even mild anemia or impaired tolerance of phlebotomy.^{79,80} These patients do not develop iron-related clinical complications. However, patients who carry other mutations accumulate iron in hepatocytes and develop clinical signs of iron-induced damage, as in classic hemochromatosis.⁸²

Data obtained by transfecting the kidney epithelial HEK293 cell line with distinct ferroportin mutants confirm and further strengthen the concept of the dual nature of this disorder.⁸³ In some mutants, such as 162delVal, ferroportin is retained in the cytosol and does not reach the plasma membrane, with a consequent loss of function.⁸³ Interestingly, high levels of hepcidin have been reported in 2 carriers of 162delVal.⁵⁵ Considering that ferroportin is the hepcidin receptor, elevated hepcidin levels could induce the internalization and degradation of normal ferroportin, produced by the *trans* allele with the final result of a dominant-negative effect rather than a haploinsufficiency. Elevated hepcidin would also reduce intestinal iron absorption and explain the lack of parenchymal iron accumulation in this subset of mutants. At the opposite extreme, other ferroportin mutants reach the membrane but are resistant to the hepcidin-induced degradation.⁸⁴ In this case the

homeostatic regulation is lost and deregulated ferroportin activity results in enhanced and inappropriate iron export.⁸⁴ The hepcidin-resistant heterogeneous mutants show a gain of function, leading to a hemochromatosis-like phenotype. Hepcidin levels, measured in a few cases of the latter mutants, are normal, but disproportionate to the degree of iron loading (E. Nemeth and T. Ganz, unpublished results, April 2005), as in hemochromatosis.⁵⁸ Some *in vitro* studies have documented the multimeric nature of ferroportin,⁸⁵ a finding that would explain the gain of function effect of heterozygous mutants, but other studies do not report this finding.⁸³

The variable phenotype resulting from mutations of the ligand (hepcidin) or its receptor (ferroportin) is understandable when considering their interaction and their respective roles in iron homeostasis (Figure 1).

Clinical diagnosis

Once a molecular diagnosis of hemochromatosis/ferroportin disease is established, further work-up should include assessment of liver iron and of clinical complications. Quantitation of liver iron can be obtained by measuring liver iron concentration (LIC) in liver biopsy. Elevated hepatic iron index (LIC related to patient age > 1.9 y) was, before cloning the *HFE* gene, the *conditio sine qua non* for hemochromatosis diagnosis (for a review, see Pi-trangelo⁷). At present these levels are taken as a measure of the iron stores but have lost most of their diagnostic significance. Liver biopsy, however, provides additional information on histology (presence of fibrosis, cirrhosis) as well as on the distribution of iron (hepatocytes versus Kupffer cells) and can still be useful in some patients. Liver iron can be noninvasively measured using superconducting quantum interference device (SQUID), a very sensitive technique, but its use is limited by the very small number of available machines.⁸⁶ Alternatively magnetic resonance imaging (MRI) can be adapted for iron measurements and was recently validated to noninvasively determine LIC.^{87,88} Patients with significantly increased liver iron should be screened for other clinical complications, especially heart disease, diabetes, and hypogonadism.⁷

In patients with a genotype at risk, family screening should be carried out by means of iron parameters and genetic testing. A practical diagnostic approach to hemochromatosis, based on *HFE* genotype and the new techniques for liver iron quantitation is proposed in Figure 2. Diagnosis of ferroportin disease is more complex because it requires that all the various conditions that can cause isolated hyperferritinemia be ruled out.

Treatment

Hemochromatosis treatment is mandatory in symptomatic patients or when serum ferritin levels are significantly increased, because of the risk of liver fibrosis. C282Y homozygous patients are at risk of fibrosis when ferritin levels are more than 1000 ng/mL.⁶ Phlebotomy remains the most effective treatment. The recommended protocol is to remove one (or 2) 400- to 500-mL units of blood (each containing approximately 200-250 mg iron) weekly, until serum ferritin level is less than 50 ng/mL and transferrin saturation less than 30%. A variable time, depending on the iron burden, may be required to achieve this goal. After phlebotomy, iron is easily mobilized from the liver in all hemochromatosis types, indicating that a marrow signal is sensed by the hepatic iron export system despite inactivation of the genes of the hepcidin pathway. Once iron depletion is reached, lifelong therapy with 3 to 4 phlebotomies per year maintains the iron stores at an adequately low level. If started in the precirrhotic phase of the disease, phlebotomy prevents organ

Table 3. Differences between disorders characterized by increased iron absorption and decreased iron recycling

| Increased iron absorption | Decreased iron recycling |
|--|---------------------------------------|
| Early increased transferrin saturation | Reduced/normal transferrin saturation |
| Late increase of serum ferritin | Early increase of serum ferritin |
| Iron accumulation in hepatocytes | Iron accumulation in macrophages |
| Normal hemoglobin levels | Anemia, iron-deficient erythropoiesis |
| Autosomal recessive | Autosomal dominant* |

*Except aceruloplasminemia.

damage and improves survival. Hypogonadism and insulin-dependent diabetes require lifelong hormonal replacement therapy. Arthritis is independent of iron loading and is not improved by phlebotomy.

The treatment experience is limited in ferroportin disease. It is reasonable to apply phlebotomy protocols less stringent than in hemochromatosis, both because macrophage iron is less prone to cause tissue damage and because some patients have impaired tolerance of phlebotomy. At present, the only therapeutic alternative to phlebotomy is iron chelation by subcutaneous deferoxamine, which is used in anemic patients or in severe cardiac disease. Protocols are similar to those established for secondary iron overload. Serum ferritin and urinary iron excretion are used to monitor the treatment efficacy. Oral iron chelators are or will be available for secondary iron overload but are not approved for the treatment of hemochromatosis. Erythropoietin has been used in selected patients to increase hemoglobin levels and to allow concomitant phlebotomy.⁴⁰

Once the molecular mechanisms of hepcidin function are clarified in detail it is not unrealistic to expect that suitable hepcidin agonist molecules will become available as a supportive treatment of iron overload to modulate iron absorption.

Rare genetic defects of iron loading

Rare genetic conditions that may lead to iron overload pose diagnostic and therapeutic challenges to physicians involved in the diagnosis and treatment of hemochromatosis (Table 4).

Hypotransferrinemia is a rare recessive disease, characterized by an extremely low transferrin level (< 0.1 g/L [10 mg/dL]), the total absence likely being incompatible with life), severe iron deficiency anemia, and iron loading of the liver and other parenchymal organs.⁸⁹ The condition emphasizes the key role of the transferrin-TFR1 pathway for iron uptake in erythroid cells and indicates the existence of alternative mechanisms for hepatocyte iron uptake. In the model of hypotransferrinemic *hpx* mice,⁹⁰ hepcidin levels are extremely low, which explains the intestinal iron hyperabsorption. This could be related to the anemic condition or, considering that transferrin might be a signal for the hepcidin modulator TFR2, to the lack of TFR2 signalling.^{36,37} Treatment is based on transferrin replacement by plasma.

Aceruloplasminemia, first described in Japan, is a late-onset recessive disease due to mutations of ceruloplasmin gene. Ceruloplasmin (CP) is a copper-dependent ferroxidase that likely cooperates with ferroportin to export iron from macrophages and hepatocytes. The disease is characterized by diabetes, iron loading of the

liver and pancreas, retinal degeneration, and neurologic signs and symptoms.⁹¹ Anemia is usually present early in life due to insufficient iron supply to the erythron. Serum iron and transferrin saturation are low, but high serum ferritin should raise the suspicion. Serum CP is low/undetectable. Studies of mice deficient in CP indicate that the iron excess is due to defective cellular iron efflux.⁹² For this reason the disease is unresponsive to both phlebotomy and iron chelation.

Hyperferritinemia-cataract syndrome (HHCS) is caused by heterozygous mutations of the IRE of L-ferritin that lead to a deregulated, constitutive synthesis of L-ferritin. The only pathologic consequence is early bilateral cataracts.⁹³ The molecular diagnosis requires sequencing of a short DNA fragment encompassing the L-ferritin IRE element. A correct diagnosis is important; HHCS patients are not iron loaded and should not be erroneously treated by phlebotomy.

Divalent metal transporter 1 (DMT1 or DCT1 or NRAMP2 or SLC11A2) is a metal transporter that takes up dietary iron at the brush border of the duodenal enterocytes. It also operates in the erythroblast transferrin cycle to transport iron from the endosomes to the cytosol. Spontaneous mutations of *DMT1* in rodents cause severe iron deficiency anemia.⁹³⁻⁹⁵ Recently the first example of *DMT1* mutation in humans has been reported. As expected, the patient had a hypochromic-microcytic anemia, but, at variance with animal models, anemia was associated with significant hepatic iron overload.⁹⁶ The increased iron absorption likely bypasses the DMT1 defect in the gut and could be related to the up-regulation of the heme-iron absorptive pathway.⁹⁶ Defects of this type, although rare, likely remain undiagnosed.

A reticuloendothelial iron overload with both a genetic and an acquired background, initially described in Bantu populations, is present also in African American individuals and it is due to an undefined susceptibility gene. No causal mutations in hemochromatosis genes have been described in this condition. A polymorphic change of ferroportin (Q248H), absent in whites, is present in a minority of patients who have increased serum ferritin and a slight degree of anemia,^{97,98} but its role in the pathogenesis of the disease is unclear.⁸⁴

Neonatal hemochromatosis is characterized by massive hepatic iron overload and liver failure, usually fatal unless liver transplantation is performed. Its hereditary nature is uncertain, although the occurrence of multiple cases within the same family⁹⁹ has been documented. Recently it has been proposed that recurrent cases may be the result of an alloimmune process and not of an inherited condition, because of the improved outcome after treatment of at risk pregnancies with high-dose immunoglobulin.¹⁰⁰

Table 4. Rare genetic defects leading to iron overload

| Disease | Inheritance | Gene | Clinical features |
|-----------------------------|-------------|----------------|--|
| Hypotransferrinemia | AR | <i>TF</i> | Anemia, iron overload |
| Aceruloplasminemia | AR | <i>CP</i> | Anemia, iron overload |
| DMT1 defects | AR | <i>SLC11A2</i> | Anemia, iron overload, neurologic symptoms |
| Hyperferritinemia-cataract* | AD | L-ferritin | Bilateral cataract |
| African iron overload | ? | ? | Iron overload |
| Neonatal hemochromatosis | ? | ? | Liver failure, iron overload |

DMT1 indicates divalent metal transporter 1; AR, autosomal recessive; *SLC11A2*, solute carrier family 11, member 2; AD, autosomal dominant; ?, uncertainty of the genetic condition (see "Rare genetic defects of iron loading" for details).

*The condition is not characterized by systemic iron overload.

Conclusions

Genomic medicine applied to the study of hemochromatosis has opened new perspectives in our understanding iron homeostasis. Genetic iron overload may result from heterogeneous mutations in several genes. Phenotype differences characterize the disorders related to the ligand (hepcidin) or the receptor (ferroportin) and these differences must be considered when planning genotype assessment. In addition, conditions characterized by the concomitant presence of anemia and iron overload have been identified and pose diagnostic and therapeutic challenges.

The correct diagnosis of these diseases has therapeutic implications because phlebotomy protocols for patients with iron-recycling defects should not be as aggressive as in hemochromatosis. Treatment for

several newly defined rare disorders is not yet established. Understanding in detail their molecular basis may lead to the development of targeted treatments.

Note added in proof: The findings related to neogenin were reported in a recently published paper.¹⁰²

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