Revisiting hemochromatosis: genetic *vs.* phenotypic manifestations

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Abstract: Iron overload disorders represent an important class of human diseases. Of the primary iron overload conditions, by far the most common and best studied is HFE-related hemochromatosis, which results from homozygosity for a mutation leading to the C282Y substitution in the HFE protein. This disease is characterized by reduced expression of the iron-regulatory hormone hepcidin, leading to increased dietary iron absorption and iron deposition in multiple tissues including the liver, pancreas, joints, heart and pituitary. The phenotype of HFE-related hemochromatosis is quite variable, with some individuals showing little or no evidence of increased body iron, yet others showing severe iron loading, tissue damage and clinical sequelae. The majority of genetically predisposed individuals show at least some evidence of iron loading (increased transferrin saturation and serum ferritin), but a minority show clinical symptoms and severe consequences are rare. Thus, the disorder has a high biochemical penetrance, but a low clinical prevalence. Nevertheless, it is such a common condition in Caucasian populations (1:100–200) that it remains an important clinical entity. The phenotypic variability can largely be explained by a range of environmental, genetic and physiological factors. Men are far more likely to manifest significant disease than women, with the latter losing iron through menstrual blood loss and childbirth. Other forms of blood loss, immune system influences, the amount of bioavailable iron in the diet and lifestyle factors such as high alcohol intake can also contribute to iron loading and disease expression. Polymorphisms in a range of genes have been linked to variations in body iron levels, both in the general population and in hemochromatosis. Some of the genes identified play well known roles in iron homeostasis, yet others are novel. Other factors, including both co-morbidities and genetic polymorphisms, do not affect iron levels *per se*, but determine the propensity for tissue pathology.

Keywords: Hemochromatosis; HFE; iron overload; environmental modifiers; genetic modifiers

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Introduction

Haemochromatosis can be defined in a number of ways, but, in its broadest sense, it refers to a disease associated with excess iron in the body (1,2). Where that iron comes from is an important consideration. Iron loading syndromes that result from defects in proteins directly involved with body iron acquisition (dietary iron absorption) or its regulation are known as primary iron loading disorders. Secondary iron loading usually results from blood transfusions, which are frequently used to treat haemoglobinopathies or hemolytic syndromes, but excess iron can also be associated with various chronic liver diseases, including alcoholic liver disease. Conditions that require transfusion also frequently exhibit ineffective erythropoiesis, and this also drives an increase in dietary iron absorption. Because humans have a very limited capacity to excrete iron, excess iron can remain in the body for a considerable time.

This review will concern itself largely with primary iron overload and particularly hemochromatosis resulting from mutations in the homeostatic iron regulator (*HFE*) gene (3,4). HFE-related hemochromatosis (HH) is one of a family of mechanistically related diseases characterized by reduced expression of the iron regulatory hormone hepcidin (see below), but it is by far the most prevalent of these disorders and the one encountered most frequently clinically. HH is almost exclusively a Caucasian disease of northwestern European origin, and a single mutation in the *HFE* gene is responsible for most disease (5). Homozygosity for this mutation is found at highest frequency in Ireland [1:83], but is also prevalent (usually around 1:200) in other parts of Europe and globally where there are northern European-derived populations (6-9).

In hemochromatosis, intestinal iron absorption is inappropriately high for a given body iron load (10), and this characteristic, combined with the limited capacity of humans to excrete iron (11), leads to iron accumulation and its deposition in many body tissues. Although iron is essential for normal cellular function, it is also toxic when present in excess (12), and it is iron-related free radical-mediated tissue damage that underlies the clinical consequences of the condition.

The hemochromatosis phenotype

A typical person genetically predisposed to HH will accumulate extra iron from their diet from birth (3,4). The extra iron amounts to only several milligrams each day, so it can take decades for the body to accumulate sufficient iron to lead to clinical manifestations. Indeed, many individuals do not progress to clinical disease at all. Iron demands are particularly high during the growing years, so young people with HFE mutations rarely show overt symptoms. Nevertheless, even in childhood, evidence of iron overload may be apparent in the form of increased transferrin saturation (TSAT) in genetically predisposed individuals, and this correlates with the development of iron overload in young adult life (13). With the onset of menstruation, females also regularly lose iron through menstrual blood loss which slows their rate of iron accretion. Symptomatic HH normally appears between 40 and 60 years in men, and not until after menopause in women (3,4). Some forms of hemochromatosis lead to early iron loading and tissue damage, and such juvenile hemochromatosis will be considered briefly below.

The most useful markers of body iron status are TSAT, which reflects the amount of recently absorbed iron and iron being trafficked around the body, and serum ferritin (SF), which represents body iron stores (14). A raised TSAT is the first indication of excess iron, and SF rises later after iron has accumulated in the tissues (15). Newly absorbed iron first binds to transferrin (TF) in the blood and is transported to sites of iron utilization and storage (16). Under normal circumstances, TF is approximately 30% saturated with iron, giving it a considerable buffering capacity to deal with excess iron. In HH, the TSAT is typically greater than 60% in men or 50% in women, but can become fully saturated with high iron loading (17). In this situation, the level of non-transferrin-bound iron (NTBI) in the blood increases. NTBI is normally only present in the circulation at extremely low concentrations, but in HH it can reach much higher levels (18,19). This form of iron is very readily taken up by tissues and can be particularly toxic to cells (17,20). It seems likely that NTBI is the major form in which iron is delivered to the tissues in HH (17).

Once within cells, any iron not immediately used for metabolic purposes is stored in ferritin, a large, multi-subunit protein that acts as a nanocage to sequester iron in a non-damaging form (21). It is synthesized on demand as the cellular iron content increases, and intracellular ferritin levels can become extremely high, particularly in specialized iron storage cells, such as hepatocytes. Ferritin-bound iron can be mobilized if the systemic demand for iron rises, and this is why phlebotomy is such as effective treatment for HH. Small amounts of ferritin can also be released into the

circulation, and the levels of this SF are proportional to body iron stores. This makes SF an extremely useful clinical marker for diagnosing and monitoring HH. The upper limit of the SF normal range is approximately 300 µg/L in men and 200 µg/L in women (14). In HH, SF usually, but not always, exceeds the normal range and can even reach several thousand. Levels of SF above 1,000 µg/L are strongly correlated with increased risk of tissue damage, and even death (22-24), but lower iron loads, giving SF values between the upper limit of normal and 1,000 µg/L, have also been shown to be clinically significant (25,26). Since SF can also be increased by inflammation (21), or released from damaged tissues, an elevated value is not specific for iron loading, although in the absence of inflammation or significant infection (e.g., metabolic syndrome, hepatitis), SF provides a reliable index of the body iron load.

Many tissues can become iron loaded in HH, with the liver, heart, pancreas, anterior pituitary, and joints being particularly severely affected (3). Consequently, clinical signs can include liver dysfunction, arthropathy, increased skin pigmentation, cardiomyopathy and diabetes mellitus. Iron loading is usually progressive, as is the development of symptoms (15), but the clinical picture can be quite variable, with different individuals manifesting different symptoms and at different times. Early symptoms can include lethargy, weakness, abdominal pain and weight loss. Since the liver is the first major organ that is exposed to newly absorbed iron, it is not surprising that liver iron levels can rise early in HH and become quite high (27). In a typical adult male, the amount of storage iron, i.e., iron in excess of immediate metabolic needs, is approximately 1 g, and in women it is approximately 300 mg (28). Much of this storage iron is in the liver. In HH, the amount of iron in the liver can reach 40 g or more (3). It is a testimony to the storage capacity of intracellular ferritin that it can sequester a large amount of iron for a long time before tissue damage becomes apparent. Advanced HH is associated with fibrosis and cirrhosis of the liver, and affected individuals are at a much higher risk of developing hepatocellular carcinoma (HCC), portal hypertension and end-stage liver disease (29). A large North American HH screening study (HEIRS) showed that men who were homozygous for the major HFE mutation had an odds ratio of 3.3 for liver disease (9). While in the past, a liver biopsy followed by the chemical measurement of its iron content was required to accurately assess the body iron load, the contemporary approach is to use magnetic resonance imaging (MRI) following by post-scan analysis of the data to generate an iron concentration (30). This method is also useful to assess body iron levels when the SF may be unreliable due to concomitant disease.

Arthropathy is another common feature of HH, and affects 25–50% of symptomatic patients (31,32). It particularly involves the second and third metacarpophalangeal joints of the hands initially, but can progress to involve other joints including the hips, wrists, ankles and knees. HH can also be associated with various bone abnormalities including reduced bone mass, altered bone microarchitecture, osteoporosis, osteopenia and fractures (33,34).

The pancreas, heart and anterior pituitary are also important sites of iron accumulation (3,4). Increased iron in the pancreatic Islets can reduce insulin production, and diabetes mellitus is a frequent complication of HH. In the heart, iron accumulates in the cardiomyocytes and this can lead to congestive heart failure and arrhythmias when iron loading is severe (35,36). Interestingly, despite these negative effects on cardiac function, HH is associated with a lower risk of coronary artery disease (37). Using a combination of human data and murine models to investigate the mechanisms underlying this phenomenon, it was demonstrated that HFE represses hepatocyte low-density lipoprotein (LDL) receptor expression, and consequently dysfunctional HFE is associated with increased receptor levels and enhanced plasma LDL-cholesterol clearance (38). Iron accumulation in the anterior pituitary can affect the production of a range of hormones and this characteristic underlies the hypogonadism that often accompanies the disease (39).

Treatment of most cases of hemochromatosis involves therapeutic phlebotomy (40). The removal of each 100 mL of blood removes approximately 40–50 mg of iron, so aggressive phlebotomy can rapidly deplete body iron levels. Most of the adverse consequences of HH resolve following iron removal, with the extent of resolution being dependent on the level of tissue damage prior to the commencement of phlebotomy (41-43). If the disease is treated early and before severe tissue pathology, an essentially complete return to normality can be achieved. However, if tissue damage is severe, e.g., advanced cirrhosis of the liver, recovery may be more limited. Individuals diagnosed and treated early have a normal life expectancy (44,45), while those diagnosed after the development of cirrhosis have a decreased life expectancy, even if they are de-ironed by phlebotomy (9). Some clinical features of HH may not resolve following phlebotomy, including the arthropathy and increased risk of liver cancer, suggesting that early and irreversible changes can occur in some tissues (46,47).

Hemochromatosis genetics

Although hemochromatosis was named as a disease over 130 years ago (48), for most of that time its precise origin was unclear. Marcel Simon in 1976 recognized that HH was an inherited disease that was linked to the human leukocyte antigen (HLA) region on human chromosome 6 (49). However, it was not until 1996 that the affected gene was identified as *HFE* (5), and thereafter the genetics of HH became well established. HH is the most common recessive, autosomally inherited genetic disease in humans. Almost all clinical HH is associated with homozygosity for a mutation which leads to the substitution of a tyrosine residue for a cysteine at position 282 of the HFE protein. This mutation is usually simply referred to as C282Y (or pCys282Tyr). The high prevalence of this mutation indicates a very strong founder effect, and potentially there is some selective advantage for the mutation as it has been retained in the gene pool for at least 4,000 years (50). The only other prevalent mutation in *HFE* leads to the H63D substitution. This mutation on its own is rarely associated with significant iron loading (9,51,52), but when present in compound heterozygosity with C282Y, moderate iron loading has been observed in some individuals. However, they are at no or only slightly increased risk of clinical sequelae (9,37,53), and often in these cases there are comorbidities or contributing environmental factors. Multiple other mutations in HFE have been described, but these are rare (54-56). Interestingly, deletion of the *HFE* gene is the most common cause of HH in Sardinia (57). The identification of the C282Y variant as the cause of the great majority of HH greatly simplified diagnosis, and today, testing for this mutation is routine when patients present with consistently raised SF and TSAT. In some cases, a typical clinical picture of HH may be seen, but the C282Y variant may not be present. In such cases more comprehensive testing of the genome may be warranted to search for other variants in *HFE* or other iron-related genes (46,58).

Within a few years of the identification of HFE, studies into the basic regulation of iron homeostasis, mainly in mice initially, led to the identification of the liver-derived peptide hormone hepcidin as the "master" regulator of body iron homeostasis (59,60). Hepcidin is secreted predominantly by the liver and regulates iron entry into the plasma by binding to the iron export protein ferroportin (FPN) and causing its internalisation and subsequent degradation (61). The expression of hepcidin itself is inversely related to body iron requirements (59,60). When requirements are high, hepcidin levels are low, more iron is released from stores, and more iron is absorbed from the diet. When iron requirements are low, such as in an iron loading situation, hepcidin levels are high and iron entry into the plasma is reduced. One might expect that in HH, hepcidin levels would be high because of increased body iron, but in fact, hepcidin expression is considerably reduced in HH (62). This explains why people with HH continue to absorb iron even though they are already carrying a considerable iron load, as they are not able to increase their hepcidin levels to restrict iron intake. These studies demonstrate that HFE acts as an upstream regulator of hepcidin (63). Interestingly, hepcidin expression can be increased by inflammation by a pathway that is independent of HFE (64), and thus chronic inflammation has the potential to suppress the iron loading phenotype (65).

Not all forms of inherited iron loading can be explained by mutations in the *HFE* gene (type 1 HH), and genetic analyses of these disorders have led to the identification of several other key players in body iron homeostasis (*Table 1*). Patients with mutations in the gene which encodes transferrin receptor 2 (TFR2) develop hemochromatosis (type 3) that is phenotypically very similar to HFE-related disease, although perhaps a little more severe (66). There are also very severe forms of iron loading known as juvenile hemochromatosis (type 2) where iron accumulates rapidly in the body in early postnatal life, with consequent early clinical presentation. Mutations in two genes have been found to lead to juvenile hemochromatosis, *HFE2*, the gene encoding hemojuvelin (HJV) (type 2A) and *HAMP*, which encodes hepcidin itself (type 2B) (67,68). Types 1–3 hemochromatosis share the common feature of reduced hepcidin expression, which provides the mechanistic basis for the iron loading (1,2,63). In the case of HFE- and TFR2-related HH, the reduction in hepcidin is relatively small and the iron loading is gradual. In the case of HJV- and HAMP-related disease, negligible hepcidin is produced and iron loading is rapid and severe. Since the hepcidin regulatory pathway plays such an important role in determining body iron levels, it might be expected that mutations in FPN, the molecular target of hepcidin, could also result in iron loading. This is indeed the case and many mutations in FPN have been described (69,70). Some of these affect the binding of hepcidin to FPN, meaning that hepcidin is unable to effectively reduce FPN levels (61,71) and thus regulate iron absorption. These patients (HH type 4B) have an elevated SF and TSAT, like patients with HFE-associated HH. Other mutations restrict the ability of FPN to transport iron (69,70) (HH

Table 1 Forms of hemochromatosis and some other iron loading disorders

Disorder	Gene symbol	OMIM	Type of disorder	Hepcidin level	Iron phenotype and clinical features
HFE-related hemochromatosis (type 1)	*HFE*	235200	Primary iron overload	Low	Late onset (4th–5th decade of life) parenchymal iron overload; liver disease; arthropathy; hypogonadism; diabetes; cardiomyopathy
Juvenile hemochromatosis (HJV-related) (type 2A)	*HFE2 (HJV)*	602390	Primary iron overload	Very low	Severe early onset (childhood) parenchymal iron overload; cardiac disease; liver cirrhosis; hypogonadism; diabetes; arthropathy
Juvenile hemochromatosis (hepcidin-related) (type 2B)	*HAMP*	613313	Primary iron overload	Very low to absent	
TFR2-related hemochromatosis (type 3)	*TFR2*	604250	Primary iron overload	Low	Parenchymal iron overload; liver disease; arthropathy; hypogonadism; diabetes; cardiomyopathy; "intermediate" age of onset
FPN-related hemochromatosis (transport defective) (type 4A) (ferroportin disease)	*SLC40A1 (FPN1)*	606069	Primary iron overload	Low to normal[†]	Reticuloendothelial iron overload (spleen and liver); raised SF but normal or reduced TSAT; fatigue, arthralgia
FPN-related hemochromatosis (hepcidin resistance) (type 4B)	*SLC40A1 (FPN1)*	606069	Primary iron overload	High	Parenchymal iron overload; increased TSAT and SF; similar clinical features to HFE-related hemochromatosis
β-thalassemia	HBB	613985	Hemoglobinopathy	Low to normal	Parenchymal and reticuloendothelial iron overload; anemia; reticulocytosis; cardiac diseases; liver disease; transfusion worsens the phenotype
Sickle cell anemia	HBB	603903	Hemoglobinopathy	Low to normal	
X-linked sideroblastic anemia	ALAS2	300751	Hemoglobinopathy	Low[†]	
Pyruvate kinase deficiency	PKLR	266200	Hemolytic anemia	Low	Parenchymal iron overload; anemia
Hereditary spherocytosis	Heterogenous	182900	Hemolytic anemia	Low	Parenchymal iron loading; anemia; jaundice; splenomegaly
Some other iron-loading disorders					
Friedreich ataxia	FXN	229300	Mitochondrial iron overload	Unknown[†]	Mitochondrial iron overload; neurological and heart disease
Hereditary atransferrinemia	TF	209300	Plasma protein deficiency	Low	Severe hemosiderosis of the heart and liver; microcytic anemia
Hereditary aceruloplasminemia	CP	604290	Plasma protein deficiency	Low	Iron loading in the brain, retina, liver and pancreas; increased SF but low TSAT; movement disorders and cognitive impairment; retinal degeneration; cirrhosis; diabetes mellitus; microcytic anemia

[†], predicted levels based on current knowledge of hepcidin regulation. ALAS2, delta aminolevulinate synthase 2; CP, ceruloplasmin; FPN, ferroportin; FXN, frataxin; HAMP, hepcidin anti-microbial peptide; HBB, hemoglobin beta; HJV, hemojuvelin; OMIM, Online Mendelian Inheritance in Man; PKLR, pyruvate kinase, liver and red blood cell; SLC40A1, solute carrier family 40 (iron-regulated transporter), member 1; TF, transferrin; TFR2, transferrin receptor 2; TSAT, transferrin saturation.

type 4A or FPN disease). Affected individuals accumulate iron in various organs, but they do not absorb dietary iron efficiently, so SF is increased, but not TSAT. Interestingly, such patients develop relatively few clinical symptoms despite high tissue iron (70).

Some knowledge of the hepcidin regulatory pathway (*Figure 1*) is helpful in understanding disease penetrance in hemochromatosis (59,60). To modulate body iron homeostasis, hepcidin must respond to multiple signals, including body iron load, changes in erythropoiesis,
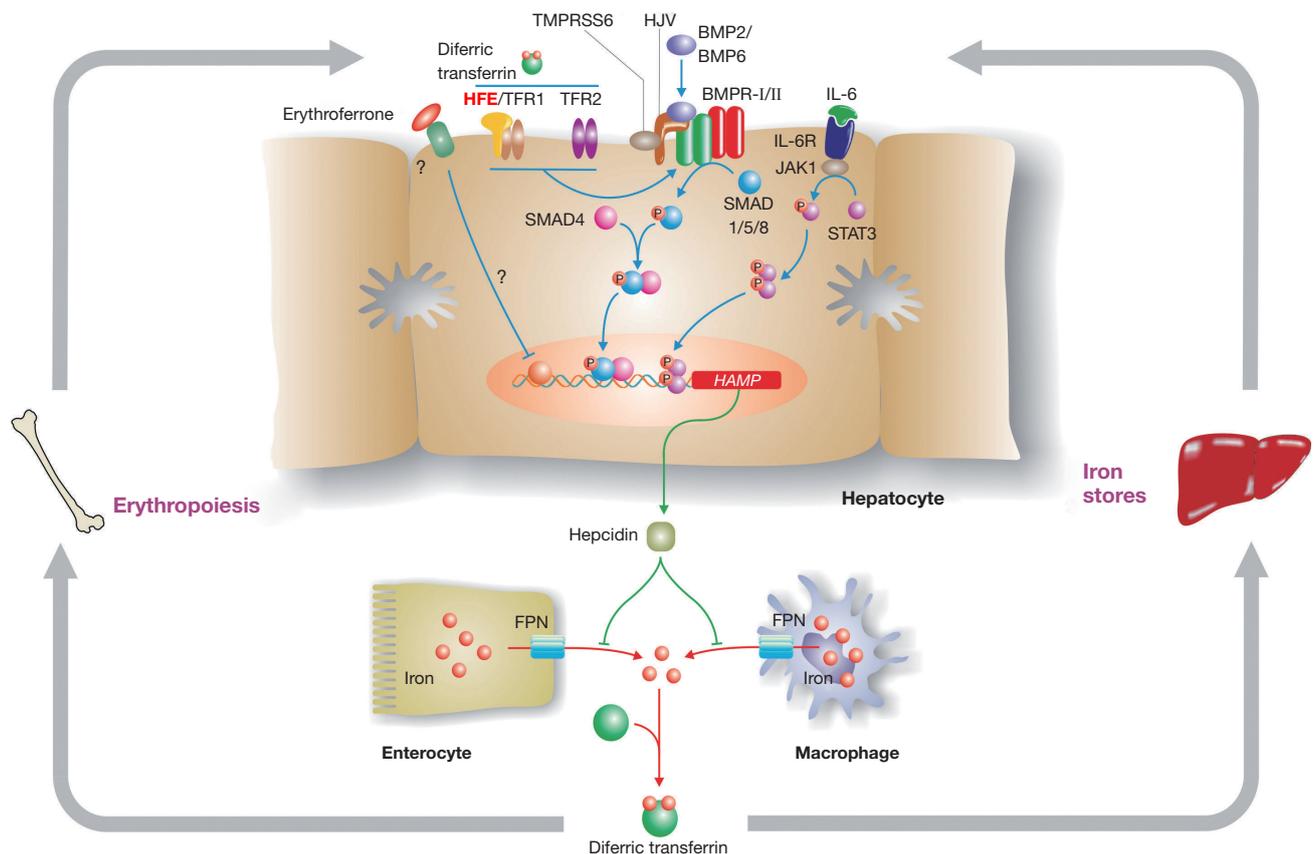
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Figure 1 The hepcidin pathway and the regulation of body iron homeostasis. Hepcidin is the master regulator of body iron homeostasis. It is synthesized predominantly by hepatocytes and, after secretion, binds to the iron export protein ferroportin (FPN) and removes it from the surface of target cells. This in turn decreases iron export from these cells. Macrophages and intestinal enterocytes are prime targets, but most cells express FPN on their surface. The *HAMP* gene, which encodes hepcidin, is regulated by a complex series of upstream signalling pathways. The bone morphogenetic protein (BMP)/SMAD pathway is the core regulatory pathway that responds to body iron requirements. Mutations in various proteins that modulate signalling through this pathway lead to hemochromatosis by reducing hepcidin expression. These include hemojuvelin (HJV) which acts as a BMP co-receptor, and homeostatic iron regulator (HFE) and transferrin receptor 2 (TFR2), which modulate signalling through the BMP/SMAD pathway via mechanisms that are not yet fully understood. Increased body iron levels normally stimulate hepcidin expression via the BMP/SMAD pathway, while proinflammatory cytokines increase hepcidin by signalling through the JAK/STAT pathway. The suppression of hepcidin by enhanced erythropoiesis is, at least in part, mediated by erythroferrone.

inflammation, hypoxia and other stimuli. The bone morphogenetic protein (BMP)/SMAD signalling pathway is the central pathway modulating hepcidin expression. BMPs act through their cell surface receptors to phosphorylate SMAD1/5/8 which binds to SMAD4. The resulting complex enters the nucleus where it binds to and activates the *HAMP* promoter. Several BMPs have been shown to stimulate this pathway, including BMP2 and BMP6, and at least BMP6 is responsive to cellular iron levels. For hepcidin to be regulated by inflammation, an intact BMP/

SMAD signalling pathway is necessary, but not sufficient. Pro-inflammatory cytokines, notably IL-6, will stimulate *HAMP* expression via the JAK/STAT3 signalling pathway. Several signals appear to be involved in communicating the iron requirements of the erythroid marrow to hepcidin, with erythroferrone being the best studied (72). This remains an area of active investigation.

While the roles of HJV, hepcidin and FPN are now well-defined, precisely how HFE and TFR2 influence *HAMP* expression is not fully understood. However,

there is compelling evidence that both proteins are required for optimal signalling through the BMP/SMAD pathway (73). The proteins may form part of a large multi-protein complex with BMP receptors and co-receptors that enhances HJV-mediated BMP-SMAD signalling (74), but this has yet to be proven *in vivo*. Interestingly, mice lacking both HFE and TFR2 have a more severe phenotype than mice lacking either gene alone (75), suggesting that the roles of the two proteins are not fully interchangeable. A similar situation has been observed in humans (76).

Penetrance of HFE-associated hemochromatosis

The phenotypic presentation of individuals who are homozygous for the C282Y variant can be quite variable. Some individuals never accumulate significant amounts of iron nor show clinical manifestations, others show biochemical evidence of iron loading (increased SF and TSAT) but no clinical consequences, whereas others show iron loading in multiple tissues and clinical sequelae (3,4). The majority of C282Y homozygotes will not develop significant disease, even if they have raised iron indices, so HH is a disorder with low clinical penetrance. In a study published by Beutler and colleagues in 2002 that stimulated discussion in this area, only 1 of 152 C282Y homozygotes met their definition of "frank clinical hemochromatosis" (77). However, many considered this figure to be unrealistically low. The reconciliation of these two views comes with the understanding of how penetrance is defined. If a biochemical definition is used, the penetrance of C282Y homozygosity is quite high (in the Beutler study, SF was raised in 76% of men and 54% of women), but if a clinical definition of phenotype is used, penetrance is much lower. The clinical phenotype itself can vary from very subtle changes (e.g., fatigue, reduced quality of life) to the more overt consequences of pathological damage to organs. If mortality is used as an end point, then penetrance is very low indeed. Most clinicians would agree that severe iron-related disease is very infrequent in HH, but many C282Y homozygotes will develop some clinical signs, even if subtle. There is certainly evidence that iron loading does not need to be at a level that precipitates severe disease and organ damage to lead to adverse health outcomes (25,26).

Biochemical penetrance in HH is simple to ascertain as it is based on objective measurements such as SF, TSAT or body iron load. In most HH patients, the TSAT is often well above the normal range, and the SF concentration is also usually increased. The latter can range from mildly

(300–1,000 μg/L) to grossly (>1,000 μg/L) elevated. In two large population-based studies of HH, up to 84% of male and 73% of female C282Y homozygotes had a raised TSAT, and 88% of male and 57% of female homozygotes had a raised SF (9,52). As would be expected from their limited physiological loss of iron, biochemical penetrance of HH is much higher in men than it is in women (9,52). Even though HH is a progressive iron loading disorder, the rate of body iron acquisition can vary. It is usually greatest in the first few decades of life, but thereafter diminishes and the SF level may remain steady or even decline (9,52,78). This is not unduly surprising. In HFE-related HH, hepcidin levels are reduced, but not absent, so the body retains some capacity to regulate its iron intake, and it will decline as the iron load increases (10).

Only a relatively small number of studies have objectively looked at clinical penetrance in HH. Allen *et al*. (52) genotyped 31,192 Australians and identified 203 C282Y homozygotes. All homozygotes, and a selection of other genotypes were examined by clinicians who were unaware of the genotype. This study showed that 28.4% of male, but only 1.2% of female, C282Y homozygotes developed iron overload-related disease, with disease being defined as biochemical evidence of iron overload in the presence of at least one clinical symptom (e.g., biopsy-proven fibrosis, HCC, arthritis) (52). Nevertheless, in the same study 82% of men and 54% of women had a raised SF (and 73% and 69% respectively had a raised TSAT), consistent with the observation that a predisposing genotype or raised body iron does not lead to overt disease in most individuals. An earlier study by Whitlock *et al*. (79) analyzed several longitudinal, population-based studies and found that 38–50% of C282Y homozygotes had a raised SF, but only 10–33% developed HH-related symptoms. These analyses appear representative of the true clinical picture. Consistent with their higher iron load, men are by far the most likely to develop end-organ damage in HH (9,52,80). In a more recent study, Pilling *et al*. examined the large UK Biobank population (451,243 participants; 2,890 C282Y homozygotes) and found that liver disease, diabetes mellitus and rheumatoid arthritis and osteoarthritis were over-represented (with odds ratios ranging from 1.5 to 4.3) in participants homozygous for the C282Y variant (37).

A SF of greater than 1,000 μg/L confers a clearly increased risk of tissue damage and clinical sequelae (23), but are individuals with more modest iron accumulation at increased risk of developing the complications of iron overload? Recent evidence suggests this is the case, with

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C282Y homozygotes with a SF value between the upper limit of normal and 1,000 µg/L having a lower mortality than the general population following phlebotomy therapy (25). Another study showed that there was a beneficial effect of phlebotomy in C282Y homozygotes with SF vales less than 1,000 µg/L, and in this case the effect was observable after only a few months of iron depletion (26). These studies demonstrate that there is a benefit in treating HH patients with mildly elevated iron indices as it will reduce their risk of subsequent clinical problems.

C282Y homozygosity is responsible for the vast majority of HH, but do other genotypes (particularly C282Y/+, C282Y/H63D, H63D/H63D and H63D/+) confer an increased risk of iron loading? In general, these other genotypes have only a very limited effect. Some C282Y or H63D heterozygotes have increased TSAT and SF, but they do not develop the complications of iron overload (9,51,81). For example, Pedersen and Milman (82) demonstrated that TSAT was elevated in 9% of C282Y heterozygotes, and 8% of H63D heterozygotes, with corresponding SF values of 9% and 12%. Very few in either group had both indices raised. Similarly, H63D homozygotes show very low biochemical penetrance. A small number may have raised iron indices, but clinical consequences are rare (83). The situation is a little different for C282Y/H63D compound heterozygotes. These individuals are more likely to have raised iron indices than simple heterozygotes (9,52), and in one study, 0.5–2.0% of compound heterozygotes developed clinical evidence of iron overload (53). In the HEIRS study, male compound heterozygotes were also more likely to report a history of liver disease (9), but a more recent meta-analysis did not show a link between compound heterozygosity and liver cirrhosis (84). Many compound heterozygotes who do show clinical sequelae will present with a co-morbidity (e.g., fatty liver, viral hepatitis), so it is often difficult to attribute their disease symptoms solely to increased iron (15).

Environmental, physiological and genetic modifiers of the HFE-hemochromatosis phenotype

A range of genetic, physiological and environmental factors can contribute to the variable penetrance in HH, and these can also operate at different levels. There may be variation in components of the iron homeostatic machinery such that different individuals take up and/or store different amounts of iron. Alternatively, the amount of iron accumulated by two individuals may be similar, but the pathological consequences of that iron may differ. For example, one individual may be particularly susceptible to the development of hepatic fibrosis. It is the net effect of these influences that determines whether someone genetically predisposed to HH will develop disease and how severe that will be.

Environmental and physiological factors play an important role in determining the severity of HH. This is perhaps best illustrated by the sex difference in the prevalence of iron loading and HH-related disease (9,37,52). Women and men do not fundamentally differ in their iron absorption mechanism, but women lose much more iron than men during their lifetime through both menstrual bleeding and childbirth (85,86), and testosterone suppresses hepcidin, favouring higher iron intake in men (87). Thus, it takes women much longer to accumulate iron to clinically significant levels, and this never happens at all in many women. After the menopause, women and men accumulate iron at similar rates (88). Other forms of blood loss, usually pathological, can also ameliorate the HH phenotype, and regular blood donation can limit body iron accumulation and hence disease expression, although this is not always the case (89-91).

Another physiological modulator to iron loading is the immune system. Interest in this area was piqued soon after the cloning of the *HFE* gene with the recognition that HFE was a non-classical major histocompatibility complex (MHC) class I-like protein (5), and is consistent with the earlier association of HH with the HLA system (49). However, even before HFE was identified, it was recognized that HH patients with more severe iron loading had abnormally high CD4/CD8 lymphocyte ratios (92) and that this reflected constitutively low CD8+ lymphocyte numbers (93-96). Although the mechanisms are not fully understood, HFE may act as a suppressor of CD8+ T cell activation and differentiation (97,98). In addition, primary defects in the immune system per se can lead to iron loading, and this could influence the hemochromatosis phenotype. Not only do mice lacking both HFE and β2-microglobulin have more severe iron overload than mice lacking only HFE (99), but mice lacking classical MHC class I proteins also accumulate excess iron (100,101). Indeed, extended HLA haplotypes have been associated with variations in iron loading in HH (95,96,102). The mechanisms linking both the adaptive and innate immune systems to iron homeostasis are only partly understood and this represents a fruitful area for further investigation.

In HFE-related HH, age is an important consideration

as iron levels progressively increase over time in many, but not all, individuals. The older a person is, the more likely they are to have accumulated a sufficiently large amount of iron to show the clinical manifestations of the disease (103). Symptoms are rarely observed in younger patients and it is usually the 4th or 5th decade of life before significant health problems arise (3,4).

In most cases, the iron content of the diet is unlikely to exert a major influence on iron loading in HH, but it can be a contributing factor (104,105). Vegetarians and vegans will be relatively protected from iron loading and may take longer to manifest signs of HH, whereas C282Y homozygotes who eat a large amount of red meat may load relatively quickly (91). In addition, factors which influence the efficiency of iron absorption, either positively or negatively, could contribute in a small way to body iron load. For example, proton pump inhibitors may reduce dietary iron absorption, and individuals taking iron supplements or substances that increase iron absorption (such as large doses of vitamin C) may take up relatively more iron (106,107). C282Y homozygotes do not generally need to be too restrictive about their diet, but they should limit their intake of foods containing large amounts of iron or substances that may stimulate its absorption (89).

Other clinical conditions can also influence disease expression in HH. For example, excess alcohol consumption is frequently associated with an increased body iron load and this reflects a reduction in hepcidin expression (108,109). Also, C282Y homozygotes who consume excess alcohol have more severe liver disease and are more likely to progress to cirrhosis (110,111). The incidence of non-alcoholic fatty liver disease (NAFLD) is rising globally and it too can contribute to disease expression in HH (112,113). Even in the absence of HFE mutations, many patients with NAFLD develop mild iron overload (dysmetabolic iron overload syndrome) (114). Hepcidin levels are inappropriately high in NAFLD, likely reflecting increased inflammation, but why this does not limit iron absorption is unclear, and suggests the possibility of hepcidin resistance (115). Increased iron indices have also been demonstrated in autoimmune and viral hepatitis, and these correlate with inappropriately low hepcidin expression and potentially increased iron absorption, but hepcidin regulation in these conditions appears to be complex (113). Experimental studies have shown that the combination of iron and other hepatic toxins increases the severity of liver pathology (116-118).

Although environmental factors or co-morbidities appear to account for the majority of variation in the penetrance of HH, genetic modifiers also play a role. Perhaps the clearest demonstration that genetics contributes to variations in body iron status comes from studies with inbred mouse strains which vary widely in their capacity to accumulate and store iron (119,120). Any mutations/polymorphisms that affect the activity of proteins involved in iron homeostasis, and notably the hepcidin regulatory pathway, have the potential to alter body iron levels in HH.

A number of studies have specifically looked for genetic modifiers of HFE-related HH, while others have sought genetic explanations for variations in iron status in the general population that could also influence the HH phenotype. Some of these are summarized in *Table 2*. Some of the modifying variants are in genes encoding proteins of iron metabolism, so their involvement is not surprising. These include the hepcidin regulators BMP2 (134) and TMPRSS6 (133), and the iron reductase CYBRD1 (122,123), which is predicted to be involved in iron absorption, and the iron transport protein TF (132), but others are novel (121). One of these is *GNPAT* which encodes an enzyme involved in peroxisomal lipid metabolism (124,125). Precisely how *GNPAT* contributes to iron homeostasis is unclear, but in vitro studies have shown it to be a potential regulator of hepcidin expression (124). The examination of *GNPAT* variants in other populations has shown mixed results, some supporting its involvement in modulating HH risk, but others not (126-128). Polymorphisms in yet other genes (such as *PCSK*7 and *PNPLA3*) have been associated with liver disease risk (129-131), and these also may modulate the HH phenotype. The number of genetic modifiers of HH identified will undoubtedly increase in time, but it is most likely we are dealing with multiple polymorphisms having small effects rather than a few modifiers with large effects.

Summary and conclusions

There is extensive clinical experience with HH and, in most cases, the disorder can be easily diagnosed, particularly since the advent of genetic testing, and readily treated by phlebotomy. Biochemical penetrance in *C282Y* homozygotes is relatively high, but clinical penetrance is relatively low, and clinical sequelae are much more likely to be seen in men than in women. Variations in penetrance between individuals reflect a combination of physiological, environmental and genetic factors, and co-morbidities. The physiological and environmental factors are broadly appreciated, but studies

Table 2 Some genes containing polymorphisms/mutations that may potentially modify the HFE-related hemochromatosis phenotype

Gene symbol	Gene product	Function	Reference
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like	Linked to TF expression; involved in circadian rhythm generation	(121)
BMP2	Bone morphogenetic protein 2	Upstream positive regulator of hepcidin	(121)
CYBRD1	Duodenal cytochrome B	Iron reductase that may be involved in dietary iron absorption	(122,123)
FADS2	Fatty acid desaturase 2	Linked to TF expression; involved in lipid metabolism; changes in lipid and iron homeostasis are frequently associated	(121)
GNPAT	Glyceronephosphate O-acyltransferase	Peroxisomal protein involved in the production of plasmalogens, a type of lipid	(124-128)
NAT2	N-acetyltransferase 2	Linked to TF expression; involved in xenobiotic metabolism; link to iron homeostasis unclear.	(121)
PCSK7	Proprotein convertase subtilisin/kexin type 7	Serine protease involved in processing proproteins in the constitutive secretory pathway	(129,130)
PNPLA3	Patatin like phospholipase domain-containing protein 3 (or 1-acylglycerol-3-phosphate O-acyltransferase or adiponutrin)	A multifunctional enzyme with both triacylglycerol lipase and acylglycerol O-acyltransferase activity; involved in lipid metabolism in adipocytes	(131)
TF	Transferrin	The major plasma iron transport protein	(121,132)
TMPRSS6	Transmembrane serine protease 6	Upstream negative regulator of hepcidin	(133)

of genetic modifiers are in their relative infancy. In time we will learn more about polymorphisms that affect iron loading and/or disease outcome, but we are likely looking at a genetic landscape where multiple loci each contribute a small effect. In the great majority of cases, HH patients will continue to be monitored using conventional blood iron status parameters, with the use of non-invasive imaging to monitor organ iron load and tissue pathology, and phlebotomy to deplete accumulated iron. However, with an increasingly advanced understanding of iron homeostasis mechanisms and disease modifiers, and bespoke therapeutic options on our doorstep, we now have the tools available to diagnose and treat unusual iron overload cases when they present.

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