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Genetics, Genetic Testing and Management of Hemochromatosis: 15 years since hepcidin.

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Abbreviations: hemochromatosis (HC); transferrin receptor 2 (TfR2); hepcidin gene (*HAMP*); hemojuvelin (HJV); ferroportin (FPN1); LEAP-1 for *liver expressed antimicrobial protein*; bone morphogenic proteins (BMPs); repulsive guidance molecules (RGMs); soluble form of HJV (sHJV)transferrin receptor 1 (TfR1); Serum ferritin (SF); transferrin saturation: TS.

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Abstract

The discovery of hepcidin in 2000 and the subsequent unprecedented explosion of research and discoveries in the iron field have dramatically changed our understanding of human disorders of iron metabolism. Today, hereditary hemochromatosis, the paradigmatic iron-loading disorder, is recognized as an endocrine disease due to the genetic loss of hepcidin, the iron hormone produced by the liver. This syndrome is due to unchecked transfer of iron into the bloodstream, in the absence of increased erythropoietic needs, and its toxic effects in parenchymatous organs. It is caused by mutations that affect any of the proteins that help hepcidin to monitor serum iron, including HFE, and in rarer instances, transferrin-receptor 2 and hemojuvelin, or make its receptor ferroportin, resistant to the hormone. In Caucasians, C282Y HFE homozygotes are numerous, but they are only predisposed to hemochromatosis; complete organ disease develops in a minority, due to alcohol abuse or concurrent genetic modifiers that are now being identified. *HFE* gene testing can be used to diagnose hemochromatosis in symptomatic patients, but analyses of liver histology and full gene sequencing are required to identify patients with rare, non-HFE forms of the disease. Due to the central pathogenic role of hepcidin, it is anticipated that also non-genetic causes of hepcidin loss (e.g. end-stage liver disease) may cause acquired forms of hemochromatosis. The mainstay of hemochromatosis management is still removal of iron by phlebotomy, first introduced in 1950s, but identification of hepcidin has not only shed new light on the pathogenesis of the disease and the approach to diagnosis, but etiologic therapeutic applications from these advances are now foreseen.

Key words: iron; HFE; C282Y; ferroportin.

Introduction and definition

Hemochromatosis is a clinical syndrome caused by the toxic effects of excess iron in parenchymatous organs. It is due to the failure to prevent unneeded iron from entering the bloodstream. In principal, any genetic or acquired defect that, directly or indirectly, persistently causes unchecked transfer of iron into the blood (from the intestine and storage/recycling sites such spleen and liver) and toxicity in parenchymatous organs, may cause the clinical syndrome named hemochromatosis. Today, we recognize that most causes of hemochromatosis are due to partial or total loss of the activity of a small peptide hormone produced by the liver, hepcidin, which normally restrains iron entry into the circulation.

What distinctively characterizes hemochromatosis is abnormal and early elevation of serum iron coexisting with a non-impaired erythropoiesis. Iron enables hemoglobin in red blood cells to bind and transport oxygen to tissues throughout the body, but parenchymatous organs, mainly the liver, are extremely avid of iron that is essential for vital functions. An unregulated flux of iron through the blood, in the absence of increased erythropoietic needs, is diverted toward parenchymal cells of the liver and other organs where it may cause oxidative damage and lead to cirrhosis, hypogonadism, diabetes, cardiomyopathy, arthropathy, and skin pigmentation, in one word, *hemochromatosis*.

Over the past 150 years, the definition of hemochromatosis has been changing and evolving to accommodate an increasingly rapid and rich succession of the new discoveries in iron biochemistry, molecular biology and, particularly, genetics (milestones and timeline reviewed in[1]). After the description of "bronze diabetes and pigmented cirrhosis" by French physicians in the mid-1800s[2, 3], the term hemochromatosis (introduced in 1889 to generically describe the bronze stain of organs due blood-borne pigments[4]) was later generally referred to a variety of iron-loading conditions and attributed to diabetes, hemolysis, alcohol, toxins, or metabolic disturbances. Joseph Sheldon was the first to suggest in 1935 that the disease "hemochromatosis" resulted from an inherited metabolic defect[5], whereas Marcel Simon linked the syndrome to the Major Histocompatibility Complex on chromosome 6 [6], where one of the major hemochromatosis genes, *HFE*, was eventually identified in 1996[7]. Yet, even after this seminal discovery, our understanding of the essence of hemochromatosis as a unique and distinct syndromic entity and the comprehension of its pathogenic basis have been far from being clarified. Until it was finally realized that it is hepcidin, the "true" hemochromatosis gene. In this context, hemochromatosis could be finally recognized, like diabetes, an endocrine

disease[8]. This review focuses on the new concept that the loss of hepcidin, entirely or in part, (due to a genetic or acquired factors), may cause the unregulated flux of blood iron responsible for human hemochromatosis.

Hepcidin, the hemochromatosis hormone

Hepcidin, the product of the *HAMP* gene, was first isolated from human blood ultrafiltrate and named LEAP-1 for liver expressed antimicrobial peptide[9], and then identified in human urine[10] and mouse liver[11], and renamed hepcidin. Humans and rats have a single HAMP gene[11], whereas two functional genes, *Hamp 1 and 2* are present in the mouse genome[12], but only the *Hamp 1* product has a role in iron homeostasis. Hepcidin is produced in the liver as a 84 amino acid prepropeptide that is processed in a 25 amino acid bioactive circulating form[10, 11, 13].

Hepcidin is a defensin-like cysteine-rich antimicrobial peptide that likely evolved in humans as part of the innate immune defense. Innate immunity relies on a variety of effector mechanisms to defend against microbial invasion. Among them are the abundant and widely distributed disulfide-linked cationic antimicrobial peptides found in both the plant, insects and mammals[10]. In insects, cysteine-rich antimicrobial peptides are produced in the fat body (functional homologue of the mammalian liver) and transcriptionally induced in response to infection or injury.

Hepcidin responds to a variety of inflammatory signals and mediators, particularly interleukin 6 (IL6)[14], which activates hepcidin transcription through the Jak/STAT3 pathway (Figure 1)[15-17], most likely in cooperation with the BMP-SMAD signaling pathway[18], IL1[19], IL22[20, 21] and activin B[22]. Hepcidin is likely produced also in monocytes/macrophages during infection through Toll like receptors[23-25]. How does hepcidin contribute to innate immune response? While retaining some direct antifungal and antimicrobial activity in vitro,[10], its main effect is to prevent invading pathogens from using iron sources to grow and proliferate during infection. To do so, hepcidin binds and degrades the sole iron exporter in mammals, a multi-domain transmembrane protein encoded by the SLC40A1 gene, named ferroportin (FPN1)[26-28]. FPN1 transfers iron from the external milieu (i.e. maternal blood or intestinal lumen) and from internal sites of iron storage and recycling (e.g. hepatocytes and tissue macrophages, respectively) into the bloodstream. It is highly expressed in placental syncytiotrophoblasts, enterocytes, hepatocytes and reticuloendothelial (RE) macrophages. FPN1 is regulated at multiple levels, transcriptionally, post-transcriptionally, and, through

hepcidin, posttranslationally [9-11, 29, 30]. Hepcidin binding to FPN1 at the cell surface results in its internalization and ubiquitination , ultimately leading to degradation in lysosomes[31, 32]. This causes cessation of iron transfer to the bloodstream.

Stimulatory and inhibitory signals that control hepcidin

In order to maintain a relatively constant level of iron in the blood, in addition to inflammation and infection, hepcidin transcription responds to other distinct stimulatory and inhibitory signals (Figure 1). First of all, as an acute phase protein, hepcidin senses a number of intracellular and extracellular stress signals.

Endoplasmic reticulum (ER) stress

ER stress is associated with disruption of ER homeostasis and accumulation of unfolded or misfolded proteins in the ER[33]. The cyclic adenosine monophosphate (cAMP) response element binding protein 3–like 3, CREB3L3 (also known as CREBH), an endoplasmic reticulum (ER) stress–associated liver-specific transcription factor[34], up-regulates hepcidin transcription in response to exogenous and endogenous ER stressors leading to iron retention in vivo[30] (Figure 1). Hepcidin induction by CREB3L3 has been then confirmed by other studies in similar settings[35-38]. Because ER stress has been involved in a number of pathophysiological states, including inflammatory response, nutrient disorders and viral infection, it is anticipated that ER stress-driven hepcidin stimulation may cause systemic or intrahepatic iron retention in a variety of iron-unrelated disorders and thereby contribute to the pathogenesis of the underlying disease, including metabolic disorders associated with insulin resistance and activated gluconeogenesis (such as diabetes, obesity and non-alcoholic fatty liver disease, NADFD)[39-42]. In this context, Vecchi et al.[43], using starvation as a model of activated gluconeogenesis and insulin resistance, found that hepcidin is regulated by CREBH in cooperation with PPARGC1A, a transcriptional coactivator that controls the genes involved in energy metabolism, gluconeogenesis, mitochondrial biogenesis and respiration[44].

Chronic Liver Diseases

Hepatocellular and/or mesenchymal iron deposition, usually slight or mild, can be found in NAFLD and NASH[45]. The term of dysmetabolic iron overload syndrome (DIOS) was in fact introduced for cases of unexplained hepatic iron excess, characterized by high serum ferritin levels with normal or subnormal transferrin saturation, associated with metabolic abnormalities[46]. DIOS patients with mixed or mesenchymal iron overload seem to have more

fibrosis and definite NASH than those with pure parenchymal pattern [47] [48]. The mechanism of iron deposition in NAFLD and DIOS is likely multifactorial: gender, diet, micro-inflammatory state, disease activity, genetic background (HFE mutations) and ethnicity may account for the variability of both iron excess and its pattern. Yet, NAFLD/NASH patients with prominent insulin resistance may present hepcidin induction and predominant sinusoidal hepatic iron overload via the gluconeogenic PPARGC1A/CREBH-driven pathway described above.

During chronic liver diseases pathogenic factors related to the underlying disorder may inhibit hepcidin and lead to excess iron accumulation that contributes to progression of the underlying disease. Hepatic oxidative stress may suppress hepcidin production after alcohol abuse [49, 50] or in chronic viral hepatitis [51, 52]. Epidermal and hepatocyte growth factors (EGF and HGF), which contribute to liver regeneration after injury, also suppress hepcidin[53]. Finally gonadal hormones have been recently reported to affect hepcidin transcription, but the mechanistic details and in vivo reflections on iron status are still unclear[54-58].

Erythropoiesis

Because maintaining a constant supply of iron for hemoglobin synthesis is a priority for humans, when in the erythroid compartment demand increases, hepatic hepcidin transcription must be turned down. Hypoxia and erythropoietin inhibit hepcidin synthesis [59,61]. Hypoxia induces platelet derived growth factor (PDGF)-BB, which inhibits hepcidin transcription by downregulating CREBH protein expression[62]. Circulating factors derived from maturing erythroblasts in the bone-marrow may down-regulate hepcidin transcription in the liver, such as growth differentiation factor 15 (GDF15)[63,64], and twisted gastrulation protein (TWSG1), a BMP-binding protein[65, 66] (Figure 1). Recently a candidate hepcidin suppressor during induced erythropoiesis has been identified in a new hormone, erythroferrone (ERFE), that mediates hepcidin suppression during stress erythropoiesis [67](Figure 1).

The "iron sensing" machinery and hemochromatosis

Hepcidin is the main biological defense against uncontrolled flux of iron toward the circulation and organs. The complete loss of hepcidin in humans is responsible for rare yet severe forms of massive body iron overload (historically called "juvenile hemochromatosis"). Yet, most cases of hemochromatosis in humans are due to the genetic disruption of the "iron

sensing machinery" that the hepcidin gene uses to monitor the circulating iron pool. If this sensing process fails, the liver cannot match the raising levels of iron in the blood and produce sufficient hepcidin to block intestinal iron absorption and iron-recycling. "Iron-sensing" by *HAMP* occurs through the interaction of transferrin-iron with a multiprotein complex at the hepatocyte plasma membrane made by bone morphogenetic proteins (BMPs)[68,69], BMP receptors, co-receptor (hemojuvelin, HJV), and a number of ancillary proteins (including HFE and the second transferrin receptor, TfR2) (Figure 1 and 2).

BMP ligands bind to type I (i.e. Alk1, Alk2, Alk3 and Alk6) and type II (i.e. BMPRII, ActRIIa or ActRIIb) receptors and turn on a signaling cascade involving the small mothers against decapentaplegic family of proteins (SMADs). In response to serum transferrin-iron, the phosphorylated SMAD1/5/8 complex (receptor-associated SMADs, R-SMADs) binds to SMAD4 (common-partner SMAD, co-SMAD), and the complex translocates to the nucleus and activates the transcription of hepcidin[70,71]. HJV, the BMP co-receptor, can be present in either a soluble or a cell-associated form that is part of the BMP signaling complex described above. Mutations involving the HJV gene cause a form of severe hemochromatosis similar to that caused by the total loss of *HAMP* [72]. A recent study has reported that HJV functions as an enhancer for iron signaling to hepcidin[73]. The iron-sensing process involves two other important proteins, HFE and TfR2 (Figure 1). HFE is a major histocompatibility class-I-like protein that interacts with transferrin receptor 1 (TfR1)[74]. Both HFE and TfR2 are each required for normal signaling of iron status to hepcidin via the Bmp6/Smad 1,5,8 pathway[75, 76]. Loss of HFE function in mice[77] and humans[78, 79] leads to inappropriately low hepcidin and hemochromatosis. Functional loss of TfR2 in mice[80] and humans[81] is also associated with blunted hepcidin expression and hemochromatosis. The details of HFE and TfR2 function in the context of the BMP/SMAD signaling pathway are still not completely understood. The C282Y mutation in HFE associated with human hemochromatosis disrupts a disulfide bond that is required for it to bind β2-microglobulin [82] and reach the cell surface where it interacts with TfR1. The H63D mutation, a common HFE polymorphism, does not impair HFE-TfR1 interaction. The pathogenic role of HFE in hemochromatosis is likely played by its physical interaction with TfR1 within the multiprotein BMP/BMPR complex in the plasma membrane/endosomal compartments of the hepatocytes (Figure 1 and 2). A recent in vitro study has shown that HFE interacts with the BMP type I receptor ALK3 to stabilize it and increase its expression thereby inducing hepcidin expression[83]. TfR2 mediates the uptake of transferrin-bound iron by hepatocytes *in vitro,*[84] possibly via receptor-mediated endocytosis,

like TfR1, but its *in vitro* affinity for transferrin is 25–30–fold lower than that of TfR1 [85]. It has been reported that, like TfR1, TfR2 interacts with HFE, so HFE and TfR2 might form an iron-sensing complex that modulates hepcidin expression in response to blood levels of diferric transferrin[86-88]. However, human studies in patients with combined TfR2 and HFE mutations[92] and studies in HFE/TfR2 double knock-out mice[76, 89, 90] have shown the additive and aggravating phenotypic effect of the contemporary loss of HFE and TfR2. All together, these data suggest TFR2 may regulate iron metabolism in an HFE-independent manner.

Various BMPs can induce hepcidin expression (i.e. BMP2, BMP4 and BMP9), but a central role has recently emerged for BMP6. In wild-type mice, *Bmp6* and *HAMP* mRNA levels are concordantly modulated by iron [91]. BMP6 is largely produced by liver sinusoidal cells and other nonparenchymal cells[92] (Figure 2). Notably, BMP6 genetic ablation leads to a hemochromatosis-like phenotype in mice[93, 94]. Finally, physical interaction between BMP6 and soluble HJV increases hepcidin expression and reduces serum levels of iron in mice [93], indicating that BMP6 is an endogenous regulator of hepcidin expression and iron metabolism *in vivo*. HFE is not required for transcriptional regulation of *BMP6* in response to dietary iron, but loss of HFE reduces BMP6 signaling, *in vitro* and *in vivo*[75, 95]. So, HFE promotes hepcidin expression via some interaction with the BMP6–SMAD signaling pathway. HFE's (and TfR2's) effects might be necessary for an optimal response to low endogenous basal levels of BMP6 (Figure 1 and 2).

At least two negative regulators of hepcidin-related BMP signaling have been identified (Figure 1 and 2). One is a membrane serine protease matriptase-2 (also called TMPRSS6) functions by inhibiting the BMP pathway, possibly by cleaving HJV[96]. Genetic loss of TMPRSS6 activity in mice or humans causes iron-refractory iron deficiency anemia (IRIDA) by stimulating excessive hepcidin synthesis that leads to sequestration of iron in macrophages and decreased dietary iron absorption[97-99]. Chronic dietary iron loading[91] and BMP-SMAD signaling pathway activity in the liver[101] induces SMAD7 that functions as a feedback inhibitor of BMP-SMAD pathway activity to down-regulate hepcidin expression. Although TMPRSS6 and SMAD7 act on hepcidin expression as feedback inhibitors, it possible that their roles are different, and while SMAD7 operates on a short-term basis to modulate acute hepcidin induction, TMPRSS6 may be involved in limiting hepcidin induction over the long-term by BMP6 and iron.

Genetics

Hepcidin and its receptor ferroportin are the central proteins in hemochromatosis. Therefore, loss of *HAMP* itself [102] or mutations that hampers the interaction of hepcidin with ferroportin [103], cause hemochromatosis in humans. But most cases of hemochromatosis in humans arise from defects in genes that regulate the *iron-sensing machinery* controlling hepcidin, including *HFE* (mutated in more than 80% of cases of hemochromatosis that are of northern European ancestry) [7], *TfR2*, [104] and *HJV* [72]. In mice, iron overload resembling hemochromatosis has been found in association with loss of additional proteins that control *HAMP* expression: C/EBP, SMAD4, BMP6, and neogenin,[105, 106] [93] but no pathogenic mutations in the relevant genes have been reported so far in humans.

HFE-Hemochromatosis

HFE-associated hemochromatosis is the most common form of the disease. It is associated with homozygosity for the 845G–A polymorphism in *HFE* that results in Cys282YTyr (C282Y) in the gene product. This polymorphism is highly prevalent in whites: the mean allelic frequency based on several screening studies is around 6%,[107] and the prevalence of C282Y homozygosity among Caucasians is 1:200–300[108]; it is much less common in other ethnicities. The polymorphism probably arose from a mutation in a single Celtic or Viking ancestor that inhabited northwestern Europe centuries ago[109] - frequencies of the C282Y allele range from 12.5% in Ireland to 0% in southern Europe. Because the genetic defect does not affect reproduction (and might even have conferred advantages against iron deficiency or pathogen infection), it spread through populations. The prevalence of C282Y is higher in certain patient groups, such as those with liver disease (5- to 10-fold higher than in the general population) and hepatocellular carcinoma- which is at least twice as frequent among patients with HFE-hemochromatosis compared with those who have other types of liver disease- type 1 diabetes, chondrocalcinosis, or porphyria cutanea tarda.

In 2010, a Y231del mutation was found in the Huh-7 hepatoma cell line (obtained from a Japanese donor) and was shown to prevent the translocation of HFE to the cell surface, similarly to the C282Y mutation [110]. More recently, this mutation has been reported in a Japanese pedigree affected by hemochromatosis[111], indicating the occurrence of a HFE-related form of hemochromatosis also in Asian populations.

Another polymorphism in HFE, H63D, has a higher prevalence in the general population (average allelic frequency ~ 14%,) and is less subject to geographic variation, but it seems to

have no clinical penetrance[112, 113]. The S65C polymorphism in HFE has also been associated with excess iron in very rare cases when it is inherited along with C282Y on one allele.

Some subjects with compound heterozygosity (H63D/C282Y) or H63D homozygosity also present with abnormal iron parameters, or even increased deposits of hepatic iron, but these patients usually have disease cofactors[114, 115].

Non-HFE hemochromatosis

The non-HFE-related forms of hemochromatosis are rarer as compared to HFE-hemochromatosis but unlike HFE, are not restricted to northern European descent. A list of gene mutations responsible for non-HFE hemochromatosis forms is reported in[116].

Adult onset forms. Most reported cases of non-HFE hemochromatosis are due to TfR2 mutations, found largely in inbred families with high consanguinity, identified in different ethnicities, including southern Asian populations [116].

Most mutations in FPN that result in an iron overload syndrome are associated with ferroportin disease [117], which is pathogenically distinct from hemochromatosis (see below). However, distinct missense heterozygote mutations of ferroportin can result in a hemochromatosis syndrome- the only known form of "classic" hemochromatosis with a autosomal dominant trait- due to resistance of ferroportin to hepcidin inhibition. Even though hepcidin is produced at normal or even higher levels,, the mutations in FPN cause hyper-absorption of iron from the diet and hepatocellular iron overload as in classic hemochromatosis[116]

Juvenile onset forms. The form of hemochromatosis characterized by early onset and severe phenotype, known for decades and historically referred to as juvenile hemochromatosis[118,119], is due to loss of *HAMP* itself and, more frequently, to pathogenic mutations of *HJV,* reported in more that fifty pedigrees, with the G320V mutation found in nearly 50% of juvenile hemochromatosis families[118, 119]. The concept and spectrum of juvenile hemochromatosis has been further extended by the identification of patients with combined mutations for hemochromatosis proteins, such as HFE and TFR2[120], presenting with a severe hemochromatotic syndrome identical to the juvenile forms.

Genetic testing and diagnosis

Hereditary hemochromatosis is a genetically heterogeneous disease that results from the complex interaction between genetic and acquired factors. If the altered gene plays a dominant role in hepcidin synthesis/activity (e.g., HAMP itself or HJV), iron overload occurs rapidly. In these cases, the modifying effects of acquired environmental and lifestyle factors is negligible and the clinical presentation is invariably dramatic, with early onset (usually second decade) of a full-blown organ disease. In contrast, p. Cys282Tyr HFE homozygosity results in a genetic predisposition that requires the concurrence of host-related or environmental factors to produce disease (Figure 3). The transferrin saturation (TS) is almost always increased in patients with hemochromatosis. Later, serum ferritin levels also begin to increase, indicating the accumulation of iron in tissues. In HFE C282Y homozygotes who underwent liver biopsy, excess tissue iron was detected in 52% of the female and 75% of male patients [107]. Increased levels of liver enzymes are present at diagnosis of 24%–32% of C282Y homozygotes identified by screening, fibrosis in 30%–42 % of the male and 2.7%–4.0% of the female patients, and cirrhosis in 4.4%–11.8% of the male and up to 2.7% of the female patients[121, 122].

Ernest Beutler was the first to draw the attention on the low penetrance of the C282Y HFE mutation[123]. Three longitudinal population screening studies in which patients were followed for more than 20 years have then shown that disease progresses in only a minority of untreated patients with HFE C282Y[124-126-131]. As many as 38%–50% of patients homozygous for HFE C282Y will develop iron overload and 10% to 33% will eventually develop hemochromatosis-associated morbidity[122,127]. Penetrance is usually higher among male patients homozygous for HFE C282Y than female patients, probably because of menstruation, pregnancy and lactation. An additional reason for more severe iron phenotypes typically detected in men than women with HFE hemochromatosis is the reported inhibitory effect of testosterone on hepcidin transcription and the resulting stimulation of intestinal iron absorption[56-58].

Apart from male sex, alcohol abuse is likely an important host “modifier” associated with hemochromatosis-related cirrhosis (Figure 3). Hemochromatosis subjects who drink more than 60 g alcohol per day are approximately 9 times more likely to develop cirrhosis than those who drink less than this amount[128]. Combinations of mutations in genes involved in the iron-sensing process, such as in *HAMP*, *HJV* and *TfR2* have been associated with more severe phenotype, but patients with these are rare[120, 129-131,132-134135 , 136-139] Polymorphic variants of BMP-2 are reportedly associated with higher penetrance of HFE-HC[140] (Figure 3). In recent

years, next generation gene sequencing studies have provided new information on the role of genetic modifiers in HFE-hemochromatosis. Novel loci affecting iron homeostasis in individuals at risk for hemochromatosis have been recently reported, some including known iron-related genes (such *HFE*, *FPN*, Tf, Tfr2, TfR1, TMPRSS6) and others novel (including the aryl hydrocarbon receptor nuclear translocator 1 gene, ARNT1)[141]. SNPs at ARNT1, Tf, and TFR2 affect iron markers in HFE C282Y homozygotes at risk for hemochromatosis . A genome-wide association study (GWAS) identified the rs3811647 Tf polymorphism as the only SNP significantly associated with iron metabolism through serum transferrin and iron levels, indicating an indirect link with the phenotypic presentation of HFE-HH[142]. More recently, in an exome sequencing study, a p.D519G variant in the glyceronephosphate O-acyltransferase (GNPAT) gene showed the most significant association with severe iron overload [143]. Yet, this association has not been confirmed in a different hemochromatosis population[144]. In Italian patients the *patatin-like phospholipase domain containing- 3* gene (*PNPLA3*), the I148M polymorphism was found to associate a with the risk of higher stage of fibrosis, and possibly cirrhosis[145], whereas a more recent retrospective GWAS study in German/Swiss/Austrian patients has reported that only variant rs236918 in the proprotein convertase subtilisin/kexin type 7 (PCSK7) gene was associated with cirrhosis or advanced fibrosis [146]. Debate continues over the roles played by fatty liver, high BMI[147, 148], and oxidative stress-related gene polymorphisms[149](Figure 3).

HFE-hemochromatosis should be suspected in a middle-aged Caucasian presenting with unexplained cirrhosis of the liver, bronze skin, diabetes and other endocrine failure, or joint inflammation and heart disease. A symptomatic untreated hemochromatosis patient with such a clinical presentation invariably has high rates of TS and levels of SF. This patients should be tested for HFE. However, this classical syndromic presentation is rare. Today diagnosis is made at earlier stages as an effect of screening and enhanced case detection due to greater clinician awareness and higher index of suspicion. There exist therefore distinct scenarios in which hemochromatosis could be suspected and a diagnostic work-up implemented (Figure 4).

Widespread use of HFE testing has increased the chance of detecting C282Y homozygosis in asymptomatic individuals. Clinical diagnosis of hemochromatosis is guided by the serum ferritin concentration. If the serum ferritin is elevated, diagnosis of hemochromatosis is made and full clinical work-up should be initiated (Figure 5). For the staging of liver fibrosis, liver

biopsy should be considered in patients with serum ferritin >1000 lg/L, unless cirrhosis is obvious. If the serum ferritin concentration is normal, follow-up once a year is sufficient.

The most common symptoms at presentation now include fatigue, malaise, and arthralgia, and hepatomegaly (Figure 4). If any of the symptoms are related to hemochromatosis, the TS and serum ferritin level are both increased and an indication for HFE gene testing is then mandatory. If the patient is a C282Y homozygote, the diagnosis of HFE-related hemochromatosis is confirmed. In the presence of any other HFE genotype, comorbidities (eg, obesity, chronic alcohol consumption etc.) have to be considered first. In the absence of these comorbidities, or if the iron abnormalities persist after these conditions have been effectively treated, tissue iron overload must be confirmed, ideally by liver biopsy, before considering non–HFE-related forms of hemochromatosis. Liver iron content can also be assessed, noninvasively, by magnetic resonance imaging (MRI) over a wide range of concentrations.

In symptomatic patients with combined heterozygosity for C282Y/H63D or H63D homozygosity, the actual pathogenic factors are usually unrecognized comorbidities. In the absence of these, they can present with increased iron measures and modest periportal hepatic iron overload, which can be reversed by phlebotomy. Parenchymal iron overload in the absence of hematologic disorders or advanced cirrhosis is typical of TfR2-related hemochromatosis or rarer forms of HFE-related hemochromatosis: these forms require gene sequencing for final diagnosis. Hemochromatosis that is associated with mutations in TfR2 usually presents at an earlier age than HFE-hemochromatosis and the phenotype is usually more severe. Most patients already have symptomatic organ disease (liver disease, diabetes, cardiomyopathy) at the time of diagnosis. Unlike HFE-hemochromatosis, this form strikes Caucasians and non-Caucasians.

Before *HFE* was identified, hemochromatosis was diagnosed based on results of liver biopsies and hepatic iron content and distribution. Hemochromatosis-related iron accumulation typically affects the hepatocytes; Kupffer cells are usually spared until late stages of disease progression[150]. In patients displaying hepatic iron deposition in their liver biopsy, further diagnostic considerations depend on the cellular and lobular distribution of iron and on the presence or absence of associated findings (Figure 4)[151]. In patients with pure parenchymal (i.e. hepatocellular) iron overload, the two main differential diagnoses are: (i) early hemochromatosis in the absence of cirrhosis after excluding compensated iron loading anemia; and (ii) end-stage cirrhosis in which iron distribution is heterogeneous from one

nodule to the next, and there are no iron deposits in fibrous tissues, biliary walls, or vascular walls. HFE gene testing should be then performed and direct the diagnostic strategy. In the presence of pure parenchymal iron overload and negative HFE testing, non-HFE hemochromatosis should be considered and investigated by gene sequencing (Figure 4). This is particularly true among non-Caucasian patients, because HFE-hemochromatosis is extremely rare. If genetic test is not available but clinical and histological features indicate the presence of a non-HFE hemochromatosis from, phlebotomy should be considered. Severe hepatic iron overload can also be caused by the hereditary disorders aceruloplasminemia and hypo- or atransferrinemia[116]. Both are extremely rare and can easily be distinguished from hemochromatosis by their clinical features. Aceruloplasminemia causes neurological manifestations (progressively severe extrapyramidal signs, cerebellar ataxia, dementia), and hypo- or atransferrinemia causes life-threatening anemia.

In patients with pure non-parenchymal (Kupffer cell) iron overload the most common form of hereditary hyperferritinemia, the so called ferroportin disease, should be suspected. Ferroportin disease was clinically described in 1999 [153] and associated with *FPN* mutations in 2001[154]. As opposed to HFE- and non-HFE-HC, the pattern of inheritance of FD is autosomal dominant. Therefore, either parent carries the pathogenic mutation of FPN and presents with unexplained hyperferritinemia. Numerous mutations of the FPN gene have been identified so far worldwide[116]. Yet, a few common FPN mutations have been independently reported in different countries (e.g., p.Val162del; p.Ala77Asp; p.Gly80Ser). Ferroportin disease is caused by loss-of-function mutations in FPN that impair iron export, particularly by reticuloendothelial macrophages. The result is iron accumulation in macrophages (reflected by high levels of SF) (Figure 4). Iron deposits in parenchymal cells of these organs are also increased, but less visible at histology, and the disorder is associated with mild visceral disease. Levels of iron in the bloodstream are low (reflected by low-normal TS), and under certain circumstances (e.g. aggressive phlebotomy regimens), this phenomenon can also lead to iron-restricted erythropoiesis and anemia. Therefore, the hall-marks of this disorder and leading key elements for diagnosis are reticuloendothelial iron overload (differently from hemochromatosis, MRI typically shows spleen and bone marrow iron accumulation)[152] hyperferritinemia with normal transferrin saturation (Figure 4).

In symptomatic patients with abnormal TS and normal serum ferritin level, hemochromatosis can be formally excluded. Conversely, in symptomatic patients with increased serum ferritin levels and a normal TS, the workup should first focus on other common causes of

hyperferritinemia, such as metabolic disorders, inflammation, cancer etc (Figure 4). If they are not found, or if the hyperferritinemia persists after treatment, the next step depends on whether or not the liver iron content is increased on magnetic resonance imaging or liver biopsy. If so, hereditary non–HFE-related iron overload such as FPN disease can be considered.

Patients with juvenile-onset forms of hemochromatosis differ considerably from HFE-hemochromatosis with respect to age, an almost equal ratio between sexes, greater frequency of cardiac and endocrine disturbances (Figure 4). Lamon[153] first reviewed all published cases and described the main clinical features of juvenile hemochromatosis. The patient usually presents in the second decade, typically with hypogonadism that manifests as primary infertility in the female. A dilated cardiomyopathy that often becomes refractory to treatment is a common complication; the untreated patient usually dies of cardiac disease by the 30th year. The hepatic complications of iron overload may seem not as common as in the case of adult forms of hemochromatosis (HFE-, TFR2, and FPN-HC), but this may be simply because the clinical picture is dominated by the endocrine and cardiac failure. The hepatic pathology may be profound, with histologically diagnosed cirrhosis developing even at a young age in up to 40% of patients.

The low penetrance of HFE C282Y is the main argument against the use of genetic screening among the general population. However, biochemical screening (followed by genetic testing when indicated) should be considered in groups with a high prevalence of this polymorphism (patients with liver disease, porphyria cutanea tarda, and/or or chondrocalcinosis; hemochromatosis family members; northern European populations).

Based on the model proposed for genetic causes of hepcidin loss, it is also understood that any non-genetic cause that chronically and consistently prevents the synthesis/activity of hepcidin will also lead to hemochromatosis (Figure 5). This is the case of massive liver iron overload resembling hemochromatosis associated with end-stage liver disease due to loss of hepcidin-producing liver mass[154]. Seemingly, it is anticipated that circulatory iron overload associated with acute or subacute liver failure due to toxic or immune mediated insults [a paradigmatic example is the dramatic neonatal hemochromatosis syndrome associated with alloimmune gestational liver disease[155]] may be contributed by a loss of hepcidin function. In all these acquired conditions the uncontrolled raise in the vascular system of nontransferrin bound forms or iron with high propensity to induce reactive oxygen species and oxidant

damage (labile plasma iron, LPI, and labile cellular iron, LCI, respectively[155]), will damage cells and organs, particularly those which display high rate of reactive oxygen species production, due to robust mitochondrial energetic activities, and less antioxidants (endocrine glands and myocardium, to name a few)(Figure 5). In a recent study, serum iron and ferritin levels were markedly elevated, hepcidin levels were lower in patients with acute-on-chronic liver failure (ACLF) and multiorgan failure (MOF): TS was higher and correlated with poor outcome[156].

Management

Understanding hepcidin regulation in the liver has not only shed new light on the pathogenesis of hemochromatosis and the approach to diagnosis but therapeutic applications from these advances are also foreseen[157]. The notion that hepcidin excess or deficiency may contribute to the dysregulation of iron homeostasis in hereditary and acquired iron disorders, raises the possibility that hepcidin-lowering or enhancing agents may be an effective strategy for curing classic iron-related diseases (e.g. hereditary hemochromatosis or anemia of chronic disease, respectively). Phlebotomy is the standard treatment for all forms of hemochromatosis since 1950, when it was first introduced [157]. The treatment is effective and safe, and, although systematic studies have never been conducted to determine when it should be started, how frequently it should be performed, or therapeutic endpoints, practical strategies for treatment and maintenance have entered clinical practice[107]. Early diagnosis and prompt initiation of phlebotomy increases survival times of patients with hemochromatosis, whereas hypogonadism, cirrhosis, destructive arthritis, and insulin-dependent diabetes due long-standing tissue iron-overload, are usually irreversible. It is anticipated that phlebotomy will remain for long time the standard treatment for hemochromatosis, but if phlebotomy is contraindicated because of severe anemia, cardiac failure in severe juvenile forms, or poor tolerance, other therapeutic strategies could be considered and, in the near future, include also hepcidin agonists. A hormone (hepcidin)-replacement therapy in hemochromatosis could be useful to prevent excess iron deposition, particularly in severe forms of juvenile hemochromatosis. Long-acting hepcidin peptides (which overcome the problem of the short-lived hepcidin molecule in vivo) could be developed. Alternatively, the use of hepcidin agonists could be a valuable strategy aimed at either raising serum hepcidin levels, or favoring FPN internalization/ degradation. The former, could be achieved by targeting the BMP/SMAD signaling pathway, the latter by using small-molecules able to trigger the internalization, ubiquitination or degradation of FPN.

Minihepcidins[158] TMPRSS6 inhibitors[159, 160] and BMP6[161] have already shown some potential in pre-clinical settings and have provided proof of concept that modulators of the BMP-SMAD pathway can cure hemochromatosis.

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Figure Legends

Figure 1. Stimulatory and inhibitory signals and pathways controlling hepcidin transcription.

The main hepcidin stimulatory signals identified include iron, inflammation/infection, and ER/nutrient stress. The iron signal converge on a membrane-associated hetero-tetrameric signaling complex, composed of transferrin-iron, BMP ligands, two type I and two type II serine threonine kinase receptors, a co-receptor (hemojuvelin, HJV) and ancillary proteins (including HFE and TfR2) that trigger a common signal transduction cascade involving r-SMADs and co-SMADs and activate transcription of the hepcidin gene (see text for details). Both HFE and TfR2 act as sensors for the iron-signal and are necessary for optimal induction of the BMP/SMAD pathway. A key mediator of hepcidin response to inflammation is interleukin 6 (IL-6) which stimulates hepcidin transcription through STAT3, possibly by interacting with the BMP/SMD pathway. Activin B likely uses the BMP/SMAD pathways to induce hepcidin during inflammation. Hormonal and nutrient signals during activated gluconeogenesis induce hepcidin through cAMP and involve the transcriptional co-activator PPARGC1A and CREBH, a transcription factor also responsible for hepcidin regulation by a variety of ER stressors (see text for details). The main signals for hepcidin inhibition arise in the bone marrow during active erythropoiesis and include growth differentiation factor 15 (GDF15), a member of the TGF-β superfamily, twisted gastrulation protein (TWSG1), a BMP-binding protein, and erythroferrone (ERFE) (see text for details). Three "negative modulators" of the BMP/SMAD signaling pathway have also been identified: the soluble form of HJV (sHJV), a membrane serine protease matriptase-2 (TMPRSS6) that possibly cleaves HJV, and SMAD7 (see text for details). Neogenin, a membrane receptor for RGM, has been proposed to stabilize HJV, and participate in HJV shedding , but its role is still controversial.

Figure 2.The iron-sensing machinery in the liver.

Iron-transferrin from the portal vein enters the sinusoids and induces the local production of BMPs, such as BMP6, by sinusoidal cells, Kupffer cells and hepatic stellate cells. Both iron-transferrin and BMPs engage the membrane-associated hetero-tetrameric signaling complex, composed of two type I and two type II serine threonine kinase receptors, the HJV coreceptor and a number of ancillary proteins that activate a common signal transduction cascade leading to induction of hepcidin transcription.

Figure 3. *HFE*-associated Hereditary Hemochromatosis: host and genetic factors affecting clinical expressivity

Most C282Y homozygotes present progressive expansion of the plasma iron compartment, as reflected by increasing saturation of transferrin, followed by progressive accumulation of iron in parenchymal cells of the liver and other organs, as heralded by rising serum levels of ferritin. However, only a limited percentage of cases presents signs and symptoms of target-organ impairment, due to a number of genetic and non-genetic factors that may affect the rate of iron accumulation and/or the extent of organ damage and disease (see text for details).

Figure 4. Algorithm for the diagnosis and management of hemochromatosis.

Different clinical scenarios in which hemochromatosis can be suspected and relevant diagnostic work-up are shown (see text for details). HC: hemochromatosis; S-Ft: serum ferritin; Tf-Sat: transferrin saturation; I.O.: iron overload; LIC: liver iron concentration; FD: ferroportin disease; LB: liver biopsy; MRI: magnetic resonance imaging.

Figure 5 Genetic and non-genetic causes of hemochromatosis.

The clinical syndrome of hemochromatosis, characterized by inappropriate hepcidin synthesis/activity, progressive appearance in the serum of non-transferrin bound iron, including redox-active forms such labile plasma iron, followed by tissue iron overload in parenchymatous organs is associated with genetic loss of proteins involved in hepcidin regulation (genetic or hereditary hemochromatosis) or hepatic disorders leading to persistent and prolonged loss of hepcidin-producing liver mass (acquired hemochromatosis)(see text for details).

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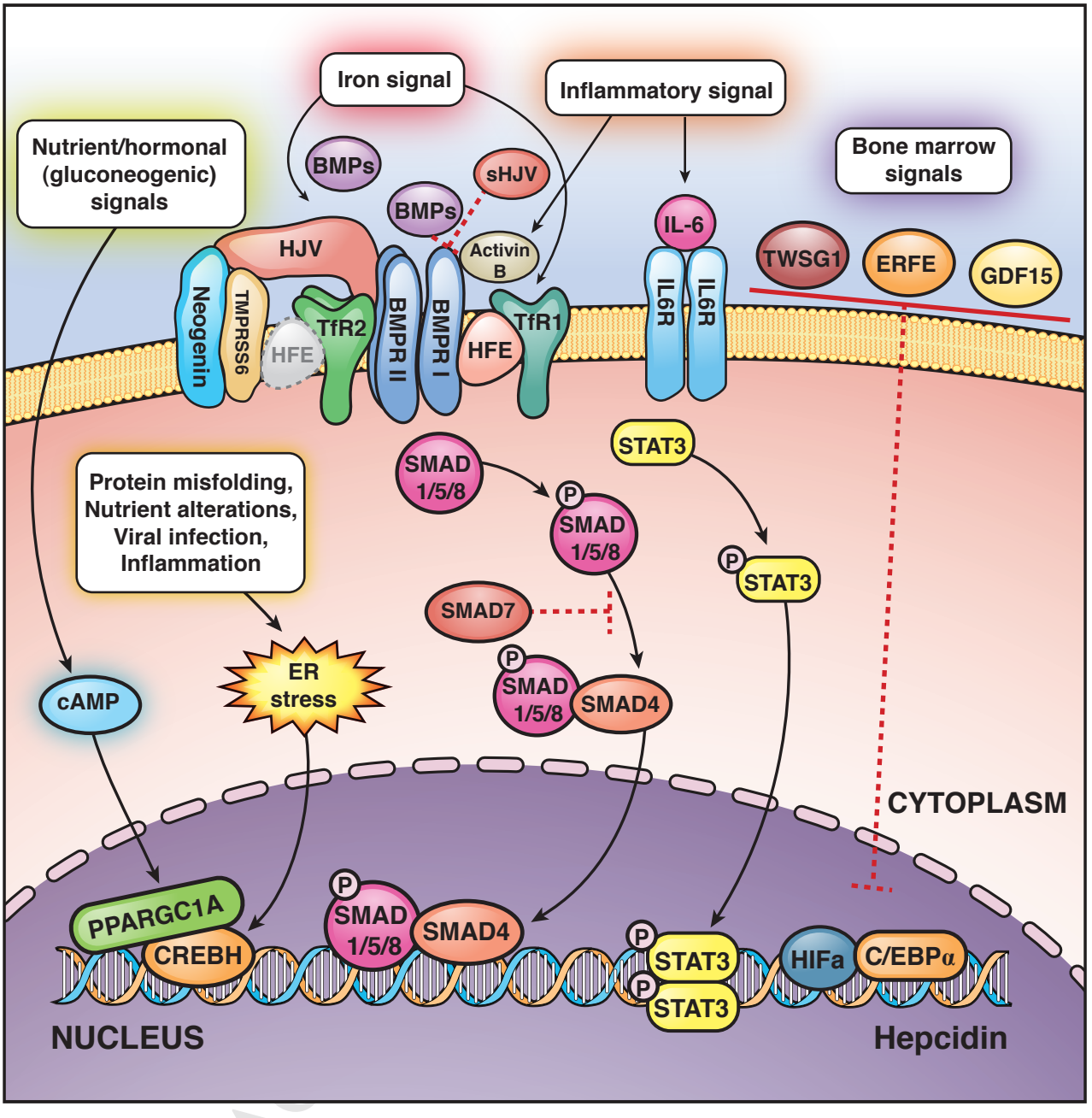
Iron signal
Inflammatory signal
Nutrient/hormonal (gluconeogenic) signals
Bone marrow signals
BMPs
sHJV
BMPs
Activin B
HJV
Neogenin
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HFE
TfR2
BMPR II
BMPR I
HFE
TfR1
IL-6
IL6R
IL6R
TWSG1
ERFE
GDF15
STAT3
SMAD 1/5/8
P
SMAD 1/5/8
Protein misfolding, Nutrient alterations, Viral infection, Inflammation
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STAT3
SMAD7
ER stress
P
SMAD 1/5/8
SMAD4
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CYTOPLASM
PPARGC1A
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NUCLEUS
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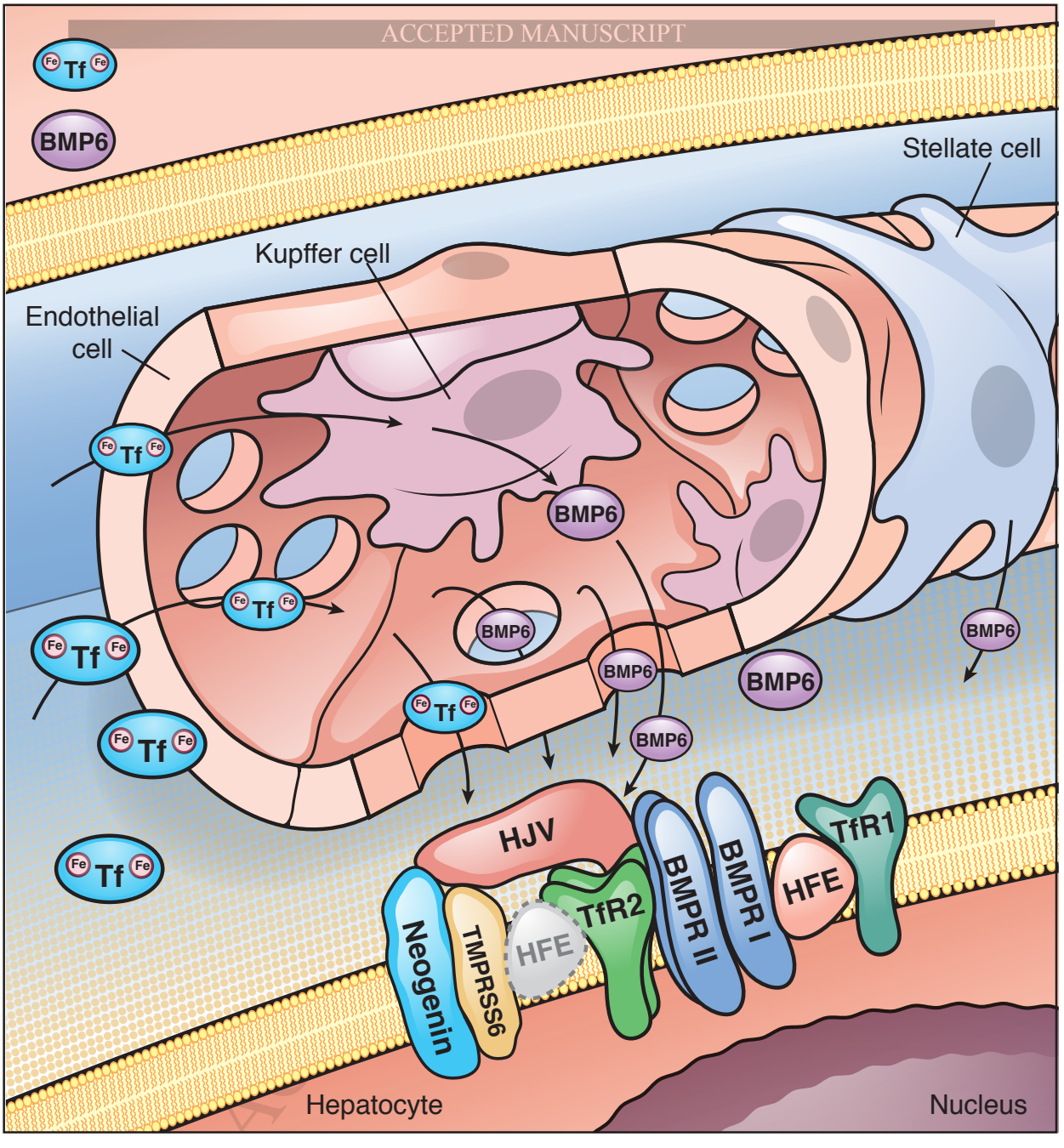

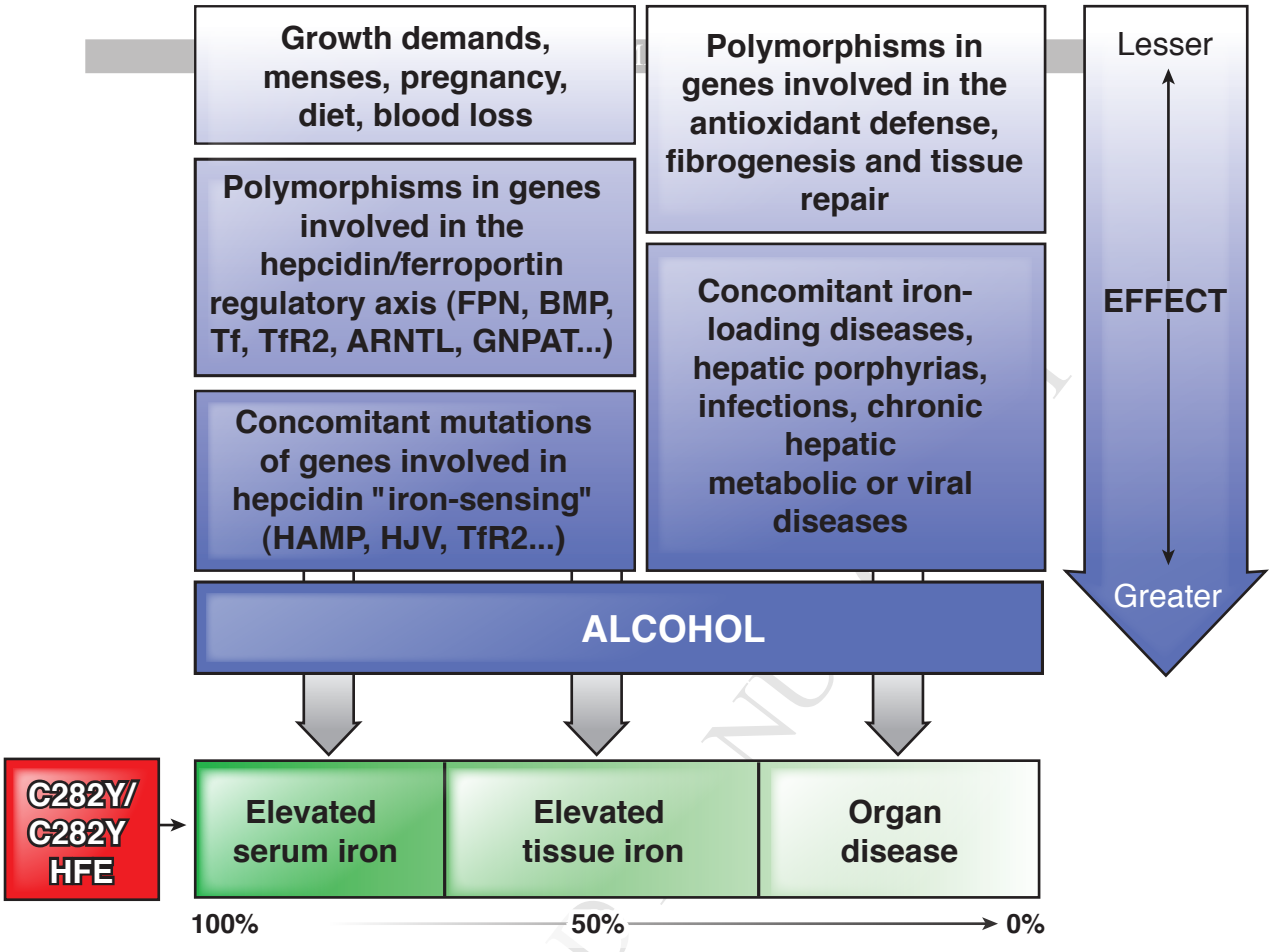
Growth demands, menses, pregnancy, diet, blood loss
Polymorphisms in genes involved in the antioxidant defense, fibrogenesis and tissue repair
Polymorphisms in genes involved in the hepcidin/ferroportin regulatory axis (FPN, BMP, Tf, TfR2, ARNTL, GNPAT...)
Concomitant iron-loading diseases, hepatic porphyrias, infections, chronic hepatic metabolic or viral diseases
Concomitant mutations of genes involved in hepcidin "iron-sensing" (HAMP, HJV, TfR2...)
Lesser
EFFECT
Greater
ALCOHOL
C282Y/ C282Y HFE
Elevated serum iron
Elevated tissue iron
Organ disease
100%
50%
0%