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## **Non *HFE* Hemochromatosis: Pathophysiological and diagnostic ASPECTS**

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**Running Title:** Non HFE HH

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## Abstract

Rare genetic iron overload diseases are an evolving field due to major advances in genetics and molecular biology. Genetic iron overload has long been confined to the classical type 1 hemochromatosis related to the *HFE* C282Y mutation. Breakthroughs in the understanding of iron metabolism biology and molecular mechanisms led to the discovery of new genes and subsequently new types of hemochromatosis. To date four types of hemochromatosis have been identified: *HFE*-related or type1 hemochromatosis, the most frequent form in Caucasians, and four rare types, named type 2 (A and B) hemochromatosis (juvenile hemochromatosis due to *hemojuvelin* and *hepcidin* mutation), type 3 hemochromatosis (related to *transferrin receptor 2* mutation), and type 4 (A and B) hemochromatosis (ferroportin disease). The diagnosis relies on the comprehension of the involved physiological defect ,that can now be explored by biological and imaging tools, which allow non invasive assessment of iron metabolism. A multidisciplinary approach is essential to support the physicians in the diagnosis and management of those rare diseases.

## Introduction

Since the description of Hemochromatosis by Trousseau in 1865, and the demonstration of its genetic nature <sup>1</sup>, many studies have shed light on its putative pathophysiological mechanism. The first major breakthrough was the discovery of the *HFE* gene <sup>2</sup>. This made it possible to diagnose the most common form of hereditary hemochromatosis (*HFE* or Type 1 hemochromatosis) due to the *p.Cys282Tyr* mutation (C282Y) in the *HFE* gene. Further discoveries unraveled iron metabolism regulation and its molecular mechanism, leading to the description of new and rarer form of hemochromatosis which are referred as non *HFE* hemochromatosis. Hepcidin, which is coded by the *HAMP* gene <sup>3</sup>, plays a central role <sup>4,5</sup>. Mainly secreted by the liver <sup>6,7</sup>, this small peptide was shown to interact with ferroportin<sup>8</sup> (coded by the *SLC40A1* gene), the only known cellular iron exporter, this interaction inducing ferroportin internalization and degradation. Thus, through its regulation of ferroportin, hepcidin can reduce iron export from macrophages and enterocytes into the bloodstream. The Transferrin Receptor 2<sup>9,10</sup> (TFR2, coded by the *TFR2* gene) and Hemojuvelin <sup>11-13</sup>(coded by the *HJV* gene) are critical cofactors in hepcidin secretion regulation. Each of these genes can have mutation leading to different peculiar forms of Hemochromatosis, whose phenotypical expression can share common signs or have specific features. These discoveries, and the broader availability of genetic testing, enabled a better discrimination of *HFE* and non-*HFE* related Hemochromatosis from various secondary causes of iron overload. However, if physiology needs to be known for enlightening the expression of these conditions, it makes the diagnosis workup more complex since the phenotype can be mixed and the appropriate tests to perform difficult to choose. This emphasizes the relevant role of referral centers who can provide guidelines, genetic advice, and in-house genotyping testing to support physicians for proper evaluation of their patients with suspected rare genetic iron overload syndromes.

## PHYSIOLOGY AND PATHOLOGY

### IRON METABOLISM

#### IRON UPTAKE AND EXPORT

Iron uptake occurs in the proximal part of the duodenum where two forms of iron are available: heme iron, mainly found in meat from the degradation of myoglobin and hemoglobin, and non heme iron found in vegetable and grains. Heme iron is carried out by endocytosis through the apical membrane of enterocytes, possibly by the Heme Carrier Protein <sup>14</sup>; the subsequent catabolism pathways are not yet definitely demonstrated. Non heme iron is transported into the cytoplasm of enterocytes by Divalent Metal Transporter 1(DMT1)<sup>15</sup>. Ferroportin (*SLCA40A1*)<sup>16-18</sup> is the only known cell iron exporter, located at the basal membrane of enterocytes and at the membrane of macrophages, where it allows iron egress from the cytoplasm to the bloodstream with subsequent oxidation by hephaestin<sup>19</sup> and binding to transferrin (Figure 1).

#### HEPCIDIN

Hepcidin (HAMP) is a small peptide, first identified as an antimicrobial peptide<sup>4-6</sup>. Mainly synthesized in the liver by hepatocytes, it is also produced at a lower level by adipocytes<sup>20</sup> and macrophages<sup>21</sup>. Highly expressed in iron overload and inflammation<sup>4,22</sup>, hepcidin was later shown to be the key hormone of iron metabolism regulation<sup>7,23,24</sup>. Hepcidin interacts with ferroportin: circulating hepcidin binds to membrane ferroportin and causes subsequent ferroportin internalization and degradation<sup>8</sup> (Figure 1). As a consequence, cellular iron egress is impaired. Hepcidin causes hypoferremia by decreasing cellular release into the plasma<sup>25</sup>, therefore regulating body iron availability through influencing iron release by both enterocytes and macrophages.

## HEPCIDIN REGULATION

The regulation of hepcidin expression has been mainly studied at a transcriptional level <sup>26</sup>. In inflammation, hepcidin induction results from the signaling of interleukin-6 through its receptor and STAT3 <sup>27-30</sup> (signal transducer and activator of STAT3). Basal expression is regulated through a bone morphogenetic protein (BMP) /SMAD pathway <sup>12,31-33</sup>. BMP6 <sup>34,35</sup> is thought to play a major role in association with its coreceptor hemojuvelin<sup>11-13</sup> (HJV), and is also involved in the response of hepcidin expression to iron stores <sup>36</sup>. Although molecular mechanisms remain not fully elucidated, it is currently accepted that HFE, TFR1, TFR2 and HJV form a complex at the hepatocyte membrane <sup>37-39</sup>, which is thought to play a major role in the sensing of iron stores according to serum transferrin saturation, with subsequent regulation of hepcidin expression<sup>9,40,41</sup> (Figure 1). TFR2 has been reported to activate signal transduction involving MAP kinase pathway <sup>42</sup>. A post-traductional level of hepcidin activity control, which implicates the furin dependent cleavage process, has been recently highlighted <sup>43,44</sup>.

## IRON OVERLOAD

Iron overload arising from mutations in genes involved in iron metabolism are induced by two types of mechanisms

### HEPCIDIN DEFICIENCY

Hepcidin deficiency is the key mechanism explaining iron overload in type *HFE*, *HJV*, *HAMP* and *TFR2* related hemochromatosis. The corresponding mutations lead, through disturbances of signal induction cascades, to decreased hepatic synthesis of hepcidin. In *HFE* hemochromatosis it was demonstrated in mice and confirmed in humans that correction of liver hepcidin secretion normalized iron metabolism<sup>45,46</sup>. Hepcidin deficiency leads to a sustained and unregulated activity of ferroportin with a double patho-physiological consequence<sup>5,47</sup>. On one hand, it leads to increased duodenal absorption of iron, and on the other hand, it enhances the release by macrophages into the blood of splenic iron originating from erythrophagocytosis. The overall result is increased plasma iron concentration associated to increased transferrin saturation. Beyond a certain level of transferrin saturation, peculiar biochemical iron species appear, named non-transferrin bound iron (NTBI)<sup>48,49</sup>. NTBI has the property to be very rapidly taken up by the liver<sup>50</sup>, pancreas and heart, and therefore produces parenchymal iron excess (namely hepatocytes for the liver). Moreover, an NTBI component, called labile plasma iron (LPI)<sup>51,52</sup>, which appears whenever plasma transferrin saturation is over 75%, corresponds to a potentially damaging iron species due to its high propensity for generating reactive oxygen species.

### IRON TRANSPORT ANOMALIES

The iron exporter Ferroportin can be involved in two types of diseases. In type A, mutations lead to a loss of activity of the protein<sup>53</sup>, and iron overload is related to decreased iron release from the macrophages as a consequence of functional deficiency. Conversely, in type B, the mutated ferroportin becomes resistant to hepcidin action<sup>54</sup>, thus despite an increased serum hepcidin level, the resulting “functional hepcidin deficiency” produces, through decreased ferroportin degradation, an increased ferroportin activity as in *HFE* hemochromatosis.

Mutations of the *ceruloplasmin* gene can hamper its ferroxidase activity (which transforms ferrous iron into ferric iron) which is mandatory for iron uptake by circulating transferrin after iron has been exported by ferroportin. As a consequence, excessive ferroportin degradation may occur, leading to decreased cellular export of iron<sup>55</sup>.

As one can see, in types HFE, HJV, TFR2, HAMP, and type B ferroportin hemochromatosis, cellular iron excess is due to an hepcidin deficiency with increased entry of excessive plasma iron into cells (predominantly parenchymal cells) whereas, in types A ferroportin disease and in hereditary aceruloplasminemia, cellular iron overload is related to decreased iron egress from cells with low circulating iron. These two different mechanisms have important implications both for the phenotypic expression of the diseases and for their therapeutic approaches.



## ETIOLOGY

Actors of iron metabolism can suffer genetic alterations leading to perturbations of its physiological equilibrium. According to the involved genes and subsequent affected mechanisms, the phenotypes differ, thus dividing hemochromatosis in different subtypes.

### HFE-RELATED (TYPE 1) HEMOCHROMATOSIS

*HFE* related hemochromatosis<sup>56</sup> is the classical, and first described form, of genetic iron overload. The *HFE* gene located on chromosome 6 codes for the membrane protein HFE, a MCH-Like protein whose definite role at the membrane remains unclear. Associated with  $\beta 2$ -globulin, TFR1 and potentially TFR2 and HJV, it plays a critical role in iron load sensing to regulate hepcidin secretion<sup>9,37-40</sup>. However, the definite subsequent signaling cascade, although interacting with the BMP/SMAD pathway, remains to be determined. Alterations in the protein can eventually lead to a decreased and unregulated hepcidin secretion, promoting iron absorption and iron overload<sup>57-61</sup>. The paramount role of liver in this alteration of iron metabolism has been proven by its evolution after liver transplantation in humans<sup>46</sup>.

The most frequent and classical mutation of this gene is the p.Cys282Tyr (C282Y) mutation, which can lead to iron overload when present in the homozygous state<sup>62</sup>. The mutation prevalence is high in Caucasian populations<sup>63,64</sup> (10% of the subjects are heterozygous, 3 to 5 subjects per thousand are homozygous), but almost absent in the non Caucasian populations<sup>65</sup>. Other genotypes than C282Y homozygosity cannot explain overt hemochromatosis : C282Y heterozygosity, H63D heterozygosity or homozygosity and compound heterozygosity C282Y/H63D do not result in clinically significant iron overload in the absence of cofactors accounting for disturbed iron metabolism (alcoholism or metabolic syndrome)<sup>66,67</sup>. However some patients with compound heterozygosity C282Y/H63D may have, even without known cofactors, increased transferrin saturation and serum ferritin suggesting mild to moderate iron overload. Although they won't develop overt iron overload, and because many patients with C282Y homozygosity will also have only mild to moderate iron overload, the clinical relevance of compound heterozygosity is still debated. Moreover there is currently no data to define, biologically or clinically, what is a significant iron overload advocating for further diagnosis

workup. Thus the role of compound heterozygosity in the diagnosis remains elusive in clinical guidelines<sup>68,69</sup>.

Other rare (private) mutations of the *HFE* gene have been described associated to those frequent genotypes, thus explaining cases of iron overload<sup>70</sup>.

The phenotypic expression of C282Y homozygosity is quite variable, and the full-blown form of the disease (especially with cirrhosis) is rare<sup>71-74</sup>. Given that C282Y homozygosity is necessary but not sufficient for iron overload development, the role of modifying factors, impacting iron metabolism or hepcidin secretion, has been advocated. These factors can be acquired (diet<sup>75,76</sup>, alcohol<sup>77,78</sup>, hepatic dysfunction<sup>59</sup>, metabolic syndrome<sup>20,79,80</sup>), or genetic (gender-related<sup>63,64,71,81</sup> or iron genes-related factors: well documented in mice<sup>82-84</sup>, the modifying role of associated genes of iron metabolism appears more mitigated in humans<sup>85-89</sup>).

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## JUVENILE (TYPE 2) HEMOCHROMATOSIS

Described before the availability of genetic testing<sup>90</sup> this rare disease encompasses two entities characterized by the usual young age at diagnosis. Type 2A hemochromatosis is due to mutation of the hemojuvelin (*HJV*) gene<sup>91</sup> on chromosome 1 and type 2B is due to mutations of the hepcidin (*HAMP*) gene<sup>92</sup> itself, located on chromosome 19. Both are autosomal recessive diseases. It is a particularly severe form of hemochromatosis, usually affecting young patients (<30 years old), often associated with cardiac involvement and central endocrine impact (hypogonadotropic hypogonadism). Iron overload is massive and liver fibrosis is frequent although cardiac and endocrine manifestations are at the forefront. The major and early impact of *HJV* mutations on metabolism emphasizes its critical role in hepcidin secretion regulation. Hemojuvelin is expressed in muscle and liver, but iron metabolism is under the sole regulation of hepatocyte expressed hemojuvelin<sup>93</sup>. At the membrane of the hepatocyte, hemojuvelin act as a BMPs coreceptor modulating the BMP/SMAD pathway eventually enhancing hepcidin expression. Mutations of *HAMP* have obvious direct impact on hepcidin synthesis. Moreover, when associated with other form of hemochromatosis, mutations in the promoting region of *HAMP* have been described as a worsening factor of iron overload<sup>94</sup>.

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## TFR2 RELATED (TYPE 3) HEMOCHROMATOSIS

Type 3 related hemochromatosis is an autosomal recessive disease that can be considered as an “intermediate” disease between juvenile and *HFE* hemochromatosis. Caused by mutations of the transferrin receptor 2 gene (*TFR2*) located on chromosome 7, its clinical picture mimics *HFE* hemochromatosis although patients are usually younger and iron overload more severe<sup>95-101</sup>. Age of onset is usually described to be young adulthood (>30 years old) although several report of children with type 3 hemochromatosis do suggest that peculiar genotypes or cofactors could lead to more severe and earlier diseases<sup>100,102</sup>. Cardiac and endocrine dysfunctions are less frequent than in juvenile hemochromatosis. Arthropathy is not rare. *TFR2* is undoubtedly involved in hepcidin expression regulation, but its molecular mechanism remains unclear. *TFR2* is complementary to *HFE* for hepcidin regulation according to iron load sensing<sup>9,40</sup>. Moreover, *TFR2* is supposed to interact with *HFE* at the hepatocyte membrane and may modulate the BMP/SMAD pathway through a cross talk involving the MAP/Erk signaling pathway<sup>42</sup>.

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## FERROPORTIN DISEASE – TYPE 4 HEMOCHROMATOSIS

This disease is due to mutations of the ferroportin (*SLC40A1*) gene located on chromosome 2<sup>103,104</sup>. Unlike other types of hemochromatosis, inheritance is autosomal dominant. Although rare, it is more frequent than types 2 and 3 hemochromatosis and has been reported worldwide<sup>105-107</sup>. According to phenotypic expression it can be subdivided in two subtypes: i) Type A, the classical form, is characterized by normal or low transferrin saturation and liver biopsy shows macrophagic iron deposition ; ii) Type B variant which is more rare, is similar to types 1 and 3 hemochromatosis with elevated transferrin saturation and parenchymal iron deposition. Overall, the clinical manifestations of ferroportin disease are limited<sup>108</sup>, with only seldom cases of liver damage

reported which were frequently associated with cofactors. Liver damage may be more frequent in type B than in type A<sup>109,110</sup>.

As afore-mentioned, Ferroportin is the only known iron exporter at the cell membrane. In type A ferroportin disease, mutations lead to loss of iron-export function and cause iron accumulation within macrophages accounting for the predominant spleen iron overload seen by magnetic resonance imaging. Theoretically trapped in the macrophages, iron biological availability is low explaining the normal or low transferrin saturation and the potentially lower tolerance to venesections than in *HFE* hemochromatosis. In type B ferroportin disease, mutations lead to resistance of ferroportin to hepcidin activity, resulting in an excessive cellular iron efflux. Thus, the phenotypic picture mimicks that of type 1 Hemochromatosis with increased serum iron and transferrin saturation, and parenchymal iron deposition.

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## OTHER RARE IRON OVERLOAD DISEASES

Hereditary a(hypo)ceruloplasminemia is due to mutations of the ceruloplasmin gene<sup>111</sup>, which either totally inhibit protein production<sup>112</sup> or its ferroxidase activity<sup>113</sup>. Clinically, iron overload is associated with anemia and neurological symptoms. Other rare entities are presenting as anemia and iron overload syndromes: they are related to mutations of transferrin (atransferrinemia)<sup>114</sup>, DMT1 (Divalent Metal Transporter1)<sup>115-118</sup>, X linked sideroblastic anemia (ALAS2<sup>119</sup>, ABC7<sup>120</sup>), or glutaredoxin 5 (GRLX5) genes<sup>121</sup>.

## DIAGNOSIS WORK UP

The diagnosis work up of iron overload involves crucial steps to avoid misleading diagnosis due to confounding factors, and to optimize resource utilization. Many tools have been made available to help the physicians in this sequential strategy. Reference centers can ultimately be of primary importance to discuss difficult cases and assess the need for further specific explorations.

## CAUSES OF REFERRAL, CLINICAL PRESENTATION

Clinical features associated with iron overload are diverse and can be more or less associated: asthenia, impotence due to endocrinopathy, arthropathy and osteopenia, skin darkening, hepatomegaly and moderate transaminase increase, diabetes, cardiomyopathy (cardiac failure or rhythm disturbance). However, due to improved knowledge of the disease and more widespread screening, the currently major cause of referral is elevated serum ferritin level, detected in the context of suspected iron overload, or uncovered during routine biological check-up or work up for other suspected diseases.

## TO ASCERTAIN IRON OVERLOAD

The first step is to confirm that elevated serum ferritin is related to iron overload by assessing potential confounding factors, thus avoiding unnecessary explorations. This step is crucial and can be difficult as many frequent conditions can alter serum ferritin levels. Moreover, some of these causes can be associated with iron overload further increasing serum ferritin concentration.

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## CONFOUNDING CAUSES OF HIGH SERUM FERRITIN

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### ALCOHOL CONSUMPTION

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Alcohol can increase serum ferritin levels by different direct means: alcohol itself can induce ferritin synthesis<sup>122</sup>, and inhibit hepcidin synthesis which can lead to mild iron overload<sup>78</sup>. Moreover, alcohol can increase serum ferritin by indirect means: as hepatocytes are the main storage sites of ferritin, cell lysis related to alcoholic liver disease leads to release of ferritin in the bloodstream. Thus, serum ferritin should be interpreted with care in case of alcohol consumption and should be, if possible, controlled after a few months of abstinence. It should be kept in mind that marked fluctuations of serum ferritin levels are highly suggestive of intermittent phases of excessive alcohol consumption.

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### METABOLIC SYNDROME

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The definition of the metabolic syndrome initially suffered of controversy, but is now admitted to be<sup>123</sup> :

- Increased waist circumference (94cm in man and 80 in women, with population specific definitions)
- Increased triglycerides (or specific treatment): > 1.7 mmol/L
- Reduced HDL cholesterol (or specific treatment): < 1 mmol/L in men and 1.3 mmol/L in women
- Increased blood pressure (or specific treatment): Systolic  $\geq 130$  and/or diastolic  $\geq 85$  mm Hg
- Increased fasting glucose (or specific treatment) : >5.5 mmol/L

Metabolic syndrome is one of the most frequent causes of hyperferritinemia. Metabolic syndrome can be associated with hyperferritinemia (often comprised between 500 and 1200 µg/L) without or with mild iron overload <sup>124</sup> (insulin resistance associated iron overload or dysmetabolic hemosiderosis) and is usually associated with increased serum hepcidin levels <sup>125</sup>. However, iron burden remains of lower intensity as compared to the pronounced serum ferritin increase. Serum transferrin saturation is usually normal although it can sometimes be slightly increased. It leads frequently to an erroneous diagnosis of hemochromatosis.

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## INFLAMMATION

In the acute or chronic phase of inflammation, ferritin can be mobilized without iron excess, thus leading to high serum ferritin which can range from mild to very high levels. One should think of inflammation especially when serum iron is low. Therefore plasma C reactive protein (CRP) should always be part of the work up for hyperferritinemia.

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## LIVER DAMAGE

Acute or chronic liver injury resulting in hepatocyte damage can lead to increased serum ferritin regardless of the underlying cause. Actually, ferritin is mainly stored in hepatocytes and serum ferritin is a only minor part of total body ferritin stores and is used as a surrogate marker. Thus, similarly to aspartate amino transferase and alanine aminotransferase, intracellular ferritin can be released into the bloodstream secondary to hepatocyte injury. The determination of serum transaminase activities is therefore another important parameter to control for proper interpretation of hyperferritinemia.

## RARE CAUSES OF ELEVATED SERUM FERRITIN

Some peculiar conditions can be associated with high serum ferritin without iron overload

- Hereditary Hyperferritinemia-Cataract Syndrome

Mutation in the Iron Responsive Element in the non-coding region of the messenger RNA of the L-Ferritin gene (*FTL*, coding for the light subunit of ferritin) causes hyperferritinemia (which can be very elevated, often above 1000 µg/L) associated, in the classical form, with an history of familial cataracts, often expressed in young subjects and leading to early surgical treatment<sup>126-128</sup>. Transmission is autosomal dominant. This condition is not associated with iron overload and thus there is no indication for venesection therapy. Although long term data are scarce, there is currently no data that advocate for a negative consequence of chronically elevated serum ferritin in this context. Beside this classical form, mutations in the coding region of the *FTL* gene have been recently described<sup>127</sup>. Those mutations, referred to as Hereditary L Ferritin syndrome in figure 2, lead to elevated serum ferritin without iron overload or cataract.

- Gaucher's disease

Gaucher's disease can be associated with high serum ferritin and normal transferrin saturation. It is an inherited metabolism anomaly (glucocerebrosidase deficiency) resulting in excessive storage of glucocerebroside in the liver, spleen, bone, and bone marrow. The clinical signs are anemia, thrombocytopenia, hepatosplenomegaly, and bone pain. Elevated serum ferritin is not at the forefront of the clinical picture and should not postpone referral to a referent center.



- Macrophage activation syndrome

Hyperferritinemia, which is a diagnosis criteria, is massively elevated ( $>5000 \mu\text{g/L}$ ) in the context of infectious (EBV), inflammatory (Still's syndrome) or hematological diseases. It is associated with general symptoms (fever, splenomegaly, cytopenia, high serum triglyceride levels) that need urgent referral for treatment.

## TO QUANTIFY IRON OVERLOAD

Once iron overload is suspected by elevated serum ferritin, and the potential confounding factors have been assessed, the next step is to assess body iron stores to quantify iron overload. Serum transferrin saturation should first be performed. If elevated *HFE* related hemochromatosis is the most likely diagnosis in the Caucasian population and should thus be confirmed by *HFE* C282Y testing before further exploration. Lack of C282Y homozygosity requires definite evaluation of iron overload.

Using the paramagnetic property of iron, Magnetic resonance imaging (MRI) is a fast and efficient non invasive technique to assess liver iron concentration<sup>129,130</sup>. It requires an 1,5 Tesla MRI device (which is the most frequent) and the algorithm for iron evaluation proposed by the Rennes University is freely accessible on the website [www.radio.univ-rennes1.fr](http://www.radio.univ-rennes1.fr).

A region of interest is drawn to compare the T2 signal between liver and paravertebral muscle, hyposignal (meaning dark liver as compared to paravertebral muscles), representing higher tissue iron concentrations. The very good correlation between hyposignal and hepatic iron overload allows to determine hepatic iron concentration with a satisfactory reliability. Moreover, it is highly relevant to evaluate hepatic versus an approximation of splenic iron load since a dominant splenic iron excess means preferential macrophagic iron deposition, therefore orientating the diagnosis towards transfusional iron excess or ferroportin disease<sup>131</sup>.

Alternative methods, using T2 relaxometry have been developed and are found to be more accurate to quantify liver iron content at all levels of iron overload<sup>130,132</sup>. However due to hardware requirement and lack of standardization they are not yet widely available.

If MRI is not available or contra-indicated, liver biopsy, using Perls staining, remains a reference method for diagnosing iron excess<sup>133</sup>. Biochemical determination of iron concentration remains the gold standard. Moreover, liver biopsy gives definite information regarding parenchymal or mesenchymal localization of iron overload which can be helpful in the diagnosis workup (review in Deugnier *et al.*<sup>134</sup>). However, due to its invasive nature, morbidity, cost and the increasing place of MRI, liver biopsy is rarely needed for diagnosing the type of iron overload. Indications today are mainly to evaluate iron overload consequences in terms of hepatic fibrosis and to search for possible co-factors such as steatohepatitis (alcoholic or not (NASH)). Regarding fibrosis evaluation, there is growing evidence that non invasive procedures such as serum markers and/or transient elastography, can give relevant information<sup>135,136</sup>.

## PRIMARY OR SECONDARY IRON OVERLOAD

Once iron overload has been identified and quantified, its primary or secondary nature must be determined.

Oral supplementation, although rarely, can lead to iron overload<sup>137</sup>. It is thus necessary, through careful questioning, to ensure that the patient has not undergone prolonged iron supplementation. It is of major importance to assess this point in patient seeking sportive performance for professional<sup>138</sup> or non-professional reasons, as it has been considered that iron supplementation could increase hemoglobin status.

The main cause of secondary iron overload is represented by hematological conditions. Chronic or rare anemias such as thalassemia major, sickle cell disease, myelodysplastic syndromes, and congenital anemias, can be associated with iron overload.

Two, more or less associated, mechanisms can be involved:

- Increased iron load through repeated transfusions represents, a major cause of iron overload (each transfused unit provides 200–250 mg of iron so that significant iron excess develops after 10–20 units). This

mechanism leads to an increased recycling of red blood cells process with enhanced iron deposition within macrophages, mainly in the spleen but also in hepatic Kupffer cells.

- Hepcidin deficiency. Ineffective erythropoiesis leads, through a yet not clearly defined pathway, to inhibition of hepcidin expression. The proposed role of Growth Differentiation Factor 15<sup>139</sup> is now questioned since a more direct link between erythropoiesis and hepcidin secretion has been recently reported. Indeed, a major breakthrough is represented by the discovery of the hormone named erythroferrone which corresponds very likely to the long-sought “erythropoietic factor” (Kautz et al, 2013, Biolron, London). This could explain why iron overload can develop in chronic anemia, like thalassaemia, even in the absence of transfusions<sup>140</sup>, and why hepcidin expression is relatively low in those diseases despite transfusional iron excess (which should lead to increased hepcidin expression).

Past history of chemotherapy treatment should also be sought. Growth factor or sometimes multiple transfusions used in this context can also lead to iron overload. The long term outcome of “transitional” secondary iron overload is not known, but due to the absence of natural and effective iron elimination route, it is likely that iron overload can persist for years.

## TO IDENTIFY THE GENETIC ORIGIN OF IRON OVERLOAD

Identification of the genetic cause of primary iron overload is driven by the combination of patient’s clinical and biological data that suggest the possible underlying physiological mechanism.

A precise determination of the suspected disease is highly recommended as most of the genetic studies are not performed in routine and are both expensive and time consuming. Thus, a multidisciplinary approach is often required in complex cases and the help of referral centers should be sought.

As an example, in France, a national reference center, working in a network with several regional competence centers, has been established (<http://www.centre-reference-fer-rennes.org/>) and proposes, on a weekly basis, multidisciplinary meetings where difficult cases are discussed in order to support physicians in their diagnosis work-up.

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## PATIENT AND FAMILY DATA

Patient's clinical and biological data have to be gathered through the initial phases of work-up. It should be emphasized that, due to important between and within-day variations of iron biological parameters (especially serum iron and transferrin saturation<sup>141,142</sup>), repeated measurements should be performed especially to avoid false positive results.

Family history is a major point to assess. Careful search for putative diagnosis of iron overload in relatives can suggest the presence of a dominant or recessive disease and strengthen the need for genetic exploration.

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## DECISION TREE

The decision tree is summarized in Figure 2.

Serum transferrin saturation is the initial key point.

- Increased transferrin saturation

The most likely diagnosis in Caucasians is *HFE* related (or type 1) hemochromatosis as confirmed by C282Y homozygosity. If there is a family history of dominant transmission the type B ferroportin disease (hemochromatosis 4B) should be sought.

If the C282Y *HFE* mutation is absent, the next relevant information is the age of presentation: in young patients (<30 years old) either type A (hemojuvelin mutations) or type B (hepcidin mutation) juvenile hemochromatosis (type 2 hemochromatosis) should be looked for. In older patients, transferrin receptor 2 mutation (or type 3 hemochromatosis), type B ferroportin disease (hemochromatosis 4B) or private mutations of the *HFE* gene (which require complete sequencing of the gene instead of routine *HFE* C282Y test) can be evoked. However

patient with very early presentation of type 3 hemochromatosis have been reported, and conversely patient with type 2 hemochromatosis and late presentation have also been reported. Thus if age is a clue for deciding which genetic test should be performed at first, if negative an unusual age of presentation must be considered.

- Normal or low transferrin saturation

In this case, the most likely diagnosis is the classical form of ferroportin disease (type A) which can be confirmed by sequencing. However, given its simplicity, plasma ceruloplasmin levels should also be determined despite the rarity of hereditary aceruloplasminemia. The latter diagnosis will of course be more likely in cases of anemia and/or neurological symptoms. Ceruloplasmin levels are typically not detectable but, in some cases, ceruloplasminemia is only significantly decreased.

## MANAGEMENT OF NON HFE HEMOCHROMATOSIS

### FAMILY SCREENING

Family screening is very important in the management of patients with genetic iron overload. It can provide precious clues in the diagnosis work-up of the patient but also help to determine if a genetic anomaly is a pathogenic mutation or a simple polymorphism by studying the genotype / phenotype correlations within the family. Mostly, family screening, following the diagnosis of a specific mutation in a given patient, allows earlier diagnosis thus preventing the development of iron-related organ damage related .

### PHLEBOTOMY

The mainstay of treatment in genetic iron overload is removal of iron burden. Phlebotomies (venesections) remain the most efficient and convenient way to remove iron by forcing the bone marrow to use stored iron for intense erythropoiesis.

Treatment is performed in non-HFE hemochromatosis in a similar way to that of the type 1 form. The initial induction phase will remove the excessive iron and the maintenance phase will prevent its recurrence<sup>143</sup>.

During induction phase, phlebotomy is performed on a weekly basis using a weight based volume of 7ml/Kg up to 550ml. Hemoglobin should be monitored on a monthly basis. In case of anemia treatment should be postponed until resolution ; if necessary volume and / or frequency of phlebotomy should be reduced. Serum ferritin is monitored to assess treatment efficiency. Monitoring frequency relies upon serum ferritin value: monthly as long as ferritin remains above the normal range, then fortnightly until the goal of 50µg/L is reached.

Once iron depletion has been achieved, the aim of maintenance treatment is to prevent recurrence of iron overload. Venesection is thus performed every 2-4 months to maintain a serum ferritin value close to 50µg/L.

Special attention should be given to iron overload related to iron transport anomalies, like ferroportin disease, as anemia could occur more frequently. Therefore, hemoglobin levels should be closely monitored and phlebotomies should be performed initially less frequently to test for hematological tolerance.

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## OTHER THERAPEUTIC ASPECTS

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### DIET

Although very commonly questioned by patients, and probably partially involved in the variable expression of the disease, no studies showed beneficial effect of dietary modification or alimentary iron avoidance in patient undergoing phlebotomy treatment. Thus, it is advised to maintain a healthy diet without stringent restrictions regarding iron. However, iron supplemented food should be avoided and it is usually recommended to limit vitamin C intake due to its possible toxic effect.

Special emphasis should be given to alcohol consumption. It has been clearly shown that, like in many liver diseases, excessive alcohol consumption increases liver damage <sup>77</sup>. Moreover, a direct inhibition effect of alcohol on hepcidin secretion favors iron overload.

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## ORAL CHELATION

Deferasirox (Exjade®) is an oral iron chelator, which, taken once daily, is used in post-transfusional iron overload. An international study in *HFE* hemochromatosis showed satisfactory results regarding safety and efficiency <sup>144</sup>. Although off label, this treatment could be helpful in case of contraindication (anemia) or poor tolerance to phlebotomy. Moreover, it could be useful, in addition to venesections, in patients with massive iron overload and organ damage requiring very rapid removal of iron burden.

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## LEGENDS TO FIGURE

Figure 1: **Iron metabolism regulation.** HCP : Heme carrier protein. DMT1 : Divalent Metal Transporter 1. HJV : Hemojuvelin. TFR1 : Transferrin Receptor 1. TFR2 : Transferrin Receptor 2. BMP Receptor : Bone Morphogenic Protein receptor.

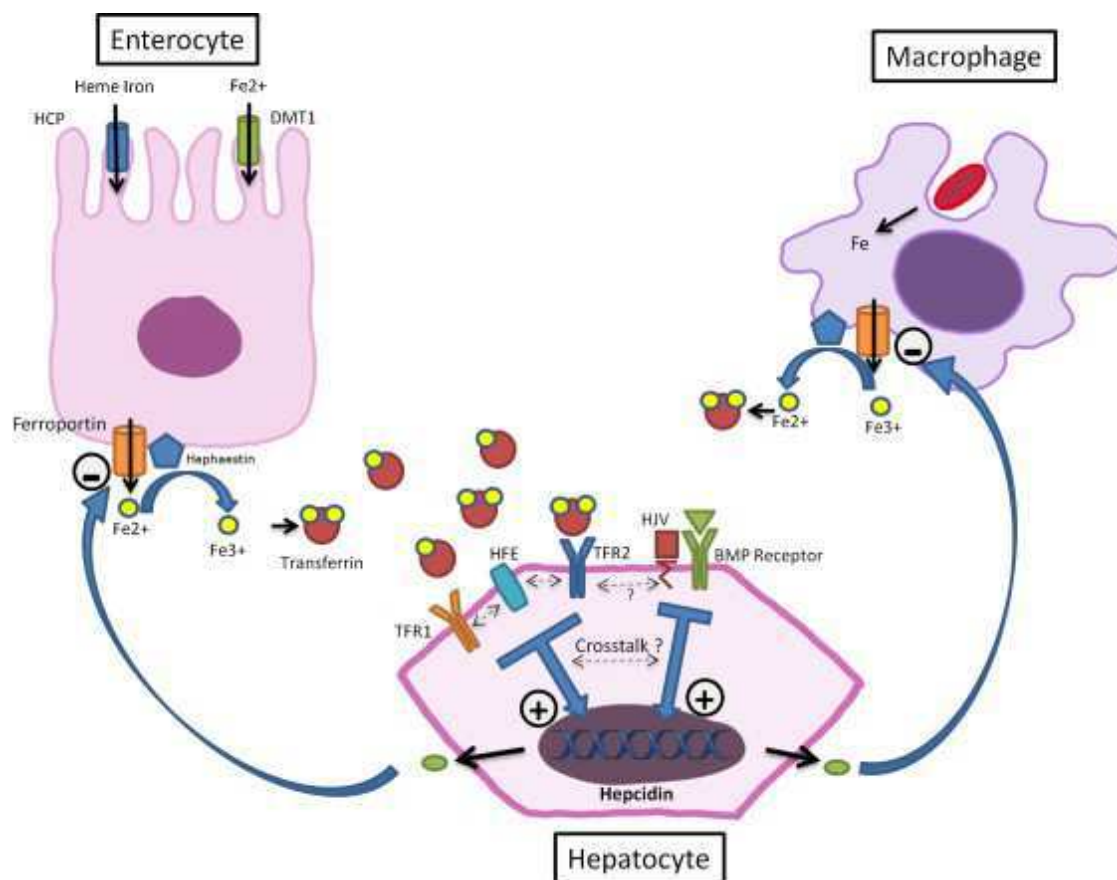


Figure 2: **Decision tree for diagnosis of genetic iron overload.** HC : Hemochromatosis

