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Recent advances in genetic iron overload-related disorders

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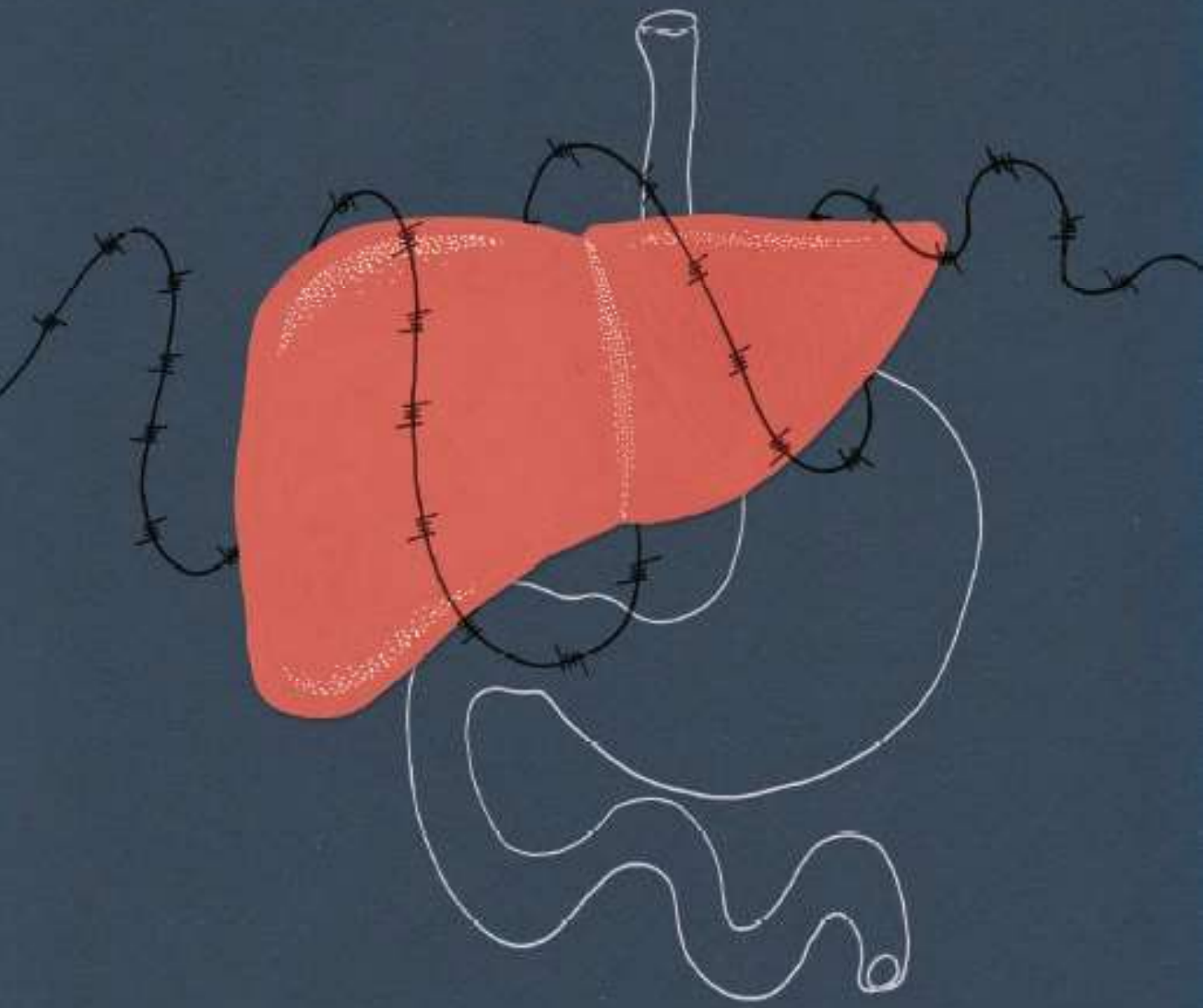
RECENT ADVANCES IN GENETIC IRON OVERLOAD-RELATED DISORDERS:

with special focus on ferritin and *HFE*-related hemochromatosis



Wenke Moris

Recent advances in genetic iron overload-related disorders: with special focus on ferritin and *HFE*-related hemochromatosis

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Recent advances in genetic iron overload-related disorders: with special focus on ferritin and *HFE*-related hemochromatosis

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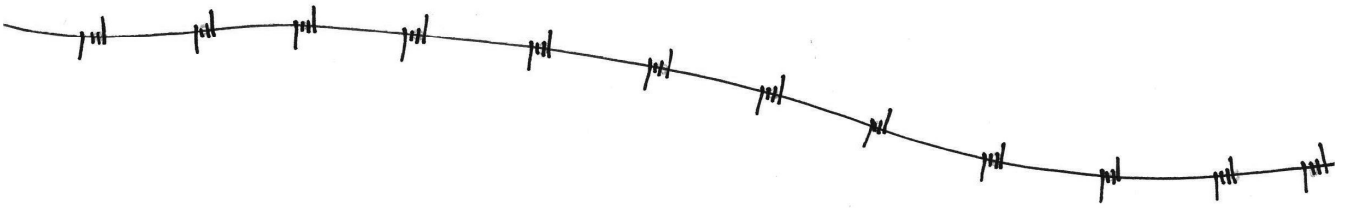
Table of contents

Chapter 1

General introduction



Body iron homeostasis

Iron

Iron is essential for life of all species. There is an abundance of this mineral on planet earth and the uptake and cellular processing was crucial in the process of evolution. In the human body, iron is found in every cell. It is required for numerous fundamental cellular processes including oxygen transport, enzymatic processes and host defense. The iron concentration is tightly regulated because an excess can lead to the generation of deleterious reactive oxygen species which can damage DNA, proteins and lipids, resulting in organ dysfunction.[1-3] Therefore, via different mechanisms and at various levels, iron homeostasis regulates the body iron balance.[4] In this introduction, firstly, we give a state of the art overview of iron homeostasis to get a better understanding of the disorders affecting this. Secondly, the pathophysiology of hyperferritinemia and its diagnostic challenges are discussed and finally we provide a comprehensive overview of the various aspects of *HFE*-related hemochromatosis.

Iron physiology

In a healthy adult, approximately 4 grams of iron are present in the body depending on gender and body weight.[5] Around 65% (2.5 grams) of the total iron content is found within erythropoietic cells and erythrocytes, 400 mg is found in iron containing proteins, 3-4 mg is found in the form of transferrin-bound iron and the remainder is stored in the storage pool, mainly in the liver, spleen, bone marrow and muscles as ferritin or hemosiderin.[6-8] Daily 20-25 mg of iron is used in erythropoiesis.[6]

In the body efficient iron recycling occurs: senescent erythrocytes are taken up by macrophages through phagocytosis mainly in the spleen but also in the liver. The average daily iron intake of 1-2 mg is only sufficient to compensate for the iron lost by sweat, menstrual blood and shedding of epithelial cells and hair.[9] Iron uptake is variable so that in periods of “iron hunger”, for example during menstruation, iron intake will increase via duodenal intestinal uptake and release.

Food is the most important external source of iron. Food iron can be divided into two forms: non-heme and heme-bound. Enterocytes of the duodenum and upper jejunum are capable of iron uptake. In persons who eat meat, heme-iron may contribute to 10-15% of the daily iron intake. Heme-iron is absorbed to a higher extent than non-heme iron since it is less influenced by dietary constituents and the higher pH of the small intestine.[10,11] In Figure 1.1 the different steps in iron uptake, cycling and distribution through the body are depicted schematically. In healthy individuals,

dietary, non-heme ferric iron (Fe^{3+}) is conversed by duodenal cytochrome-b (DCYTB) into the ferrous form (Fe^{2+}) enabling the absorption through the divalent metal transporter 1 (DMT1) on the enteric apical membrane.[4,12,13] Once in the cytosol of the enterocyte the imported Fe^{2+} enters the labile iron pool (LIP). From here it can be used for the cells own metabolic processes, it can be exported into the plasma or in case of low iron demand it will be stored into ferritin.[8,14]

The mechanism to absorb heme iron in the enterocyte seems to be facilitated through the involvement of heme carrier protein 1 (HCP1), however this mechanism is not fully elucidated. Inside the cell, iron is released from heme by heme oxygenase.[4]

Macrophages are another source of plasma iron, they acquire iron through recycling of iron after degrading erythrocytes by phagocytosis.

Iron is exported from the basolateral surface of the enterocyte into the circulation via ferroportin (FPN1), to date the only known iron export protein. FPN1 is expressed in a wide variety of cells, including macrophages, hepatocytes and enterocytes. Before iron can be loaded onto the iron transport protein transferrin (Tf), to be transported in the circulation, it needs to be oxidized to Fe^{3+} by a transmembrane bound copper ferroxidase.[12,15] In the enterocyte hephaestin is the ferroxidase needed for iron oxidation while in cells like hepatocytes, macrophages and astrocytes this is ceruloplasmin (CP).

Tf can bind to transferrin receptor 1 (TFR1) located on the surface of every cell for the uptake of iron. After binding, internalization by endocytosis takes place where it can be stored as an inert form into ferritin[12,16] (Figure 1.1).

The role of ferritin

Ferritin is the major iron storage protein and is present in every cell of the body, the highest concentrations of ferritin are found in the liver. Ferritin stores iron to maintain it in a bioavailable and non-toxic form and to have it as a reserve in case of iron depletion.

Under physiological conditions around 25% of the total body iron (0.8-1 g) is present in the storage pool, mostly as ferritin.[8] Ferritin is composed of 24 subunits of ferritin H-chains (heavy) and ferritin L-chains (light) and is capable of storing up to 4500 iron atoms.[4,17,18] The heavy chains are characterized by a ferroxidase site while the light chains are involved in the nucleation of the iron core of ferritin.[8] Both subunit types

play an important role in the iron-storage function. The ratio of heavy and light ferritin chains within ferritin varies per tissue type. In spleen and liver tissue more ferritin light chains are present while heavy chains are more present in heart and kidney.[8] In case of low iron demand, Fe^{2+} is stored into ferritin. Via channels Fe^{2+} will enter the apoferritin protein shell, there Fe^{2+} will be oxidated by ferroxidase sites within the heavy chain. Fe^{3+} will then migrate from the ferroxidase sites to the nucleation sites in the light chains of the protein shell, this facilitates the formation of small polynuclear iron clusters which act as a nucleation centre for mineral growth. One of these clusters will eventually become a dominant nucleation centre where the ferrihydrite mineral core will grow further through iron oxidation and mineralization.[8]

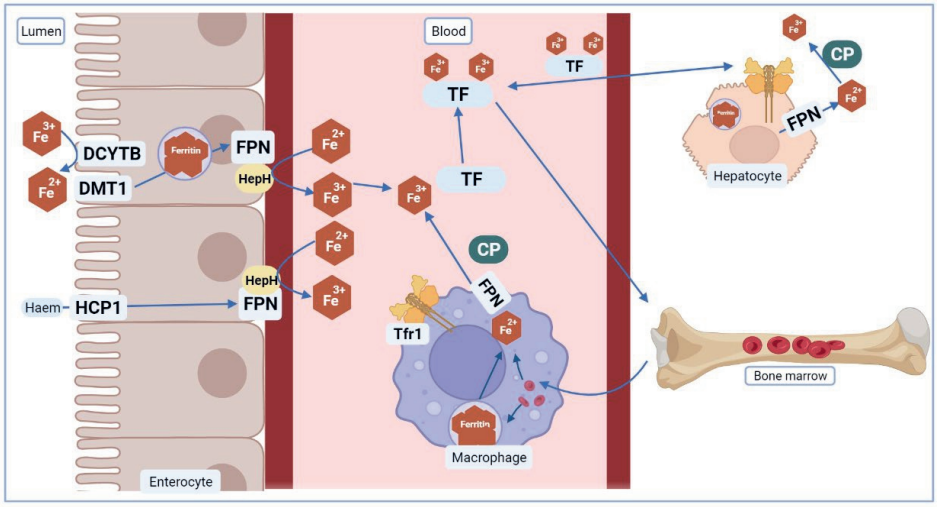


Figure 1.1 **Iron uptake, cycling and distribution through the body.** Non-heme ferric iron (Fe^{3+}) from the diet is conversed by duodenal cytochrome-b (DCYTB) into the ferrous form (Fe^{2+}) enabling the absorption through the divalent metal transporter 1 (DMT1) on the enteric apical membrane. Absorption of heme-iron from the diet in the enterocyte seems to be facilitated through the involvement of the heme carrier protein 1 (HCP1), however this mechanism is not fully elucidated. Another source of plasma iron are macrophages, they acquire iron from degrading erythrocytes after phagocytosis. Iron is released form the enterocyte and the macrophage via the ferroportin (FPN1) transporter, under the control of hepcidin. Fe^{3+} is transported into the blood compartment after oxidation by a transmembrane bound copper ferroxidase, hephaestin (HepH) (anchored to enterocytes). In other cells in the body like hepatocytes, macrophages or astrocytes the copper ferroxidase is ceruloplasmin (CP). In the normal physiological situation Fe^{3+} is transported on the carrier protein transferrin (TF) to cells that express transferrin receptor (TFR1).

In case of iron demand, stored iron can be mobilized to meet the body's needs. The molecular mechanisms to allow iron release are not completely understood however based on in-vivo experimentation there are two possible models.[8] The first model implies that in case of iron depletion the cytosolic ferritin can be taken up via autophagy by lysosomes where it can be degraded by cathepsins after which iron is transported back into the cytosol. Here iron can join the LIP for redistribution within the cell or it can be exported extracellularly.[8] The second model is that ferritin can release iron in the cytosol and that apoferritin can be degraded by the proteasome. It is hypothesized that both mechanisms are complementary. In that case iron-rich ferritin is degraded in the lysosome whereas iron-poor ferritin or apoferritin is degraded in the cytosol by the proteasome which results in a lower reincorporation of iron into ferritin.[8,19,20]

Apart from intracellular ferritin, ferritin is also found in the plasma. The plasma ferritin concentration consist principally of light chains and in contrast with intracellular ferritin its core contains relatively little iron.[4,18] Much about serum ferritin is still unclear for example its tissue of origin and its secretory pathways are still topics of discussion.[18] It has been shown that hepatocytes, macrophages and Kupffer cells secrete ferritin. [18,21]

Cellular iron homeostasis

Iron homeostasis is regulated by the balance of iron uptake, intracellular storage and iron loss. Regulating system proteins that play a role are iron-responsive elements (IRE) and iron regulatory proteins (IRP). The LIP acts as the trigger to activate the switch between the IRE/IRP system. When cells are iron depleted, the IRPs (IRP1 and IRP2) bind to the IREs. The binding to IREs in ferritin mRNA and FPN1 mRNA prevents its translation. The binding to IREs in TfR1 mRNA and DMT1 mRNA stabilizes the mRNA by preventing nuclease degradation. This will ensure iron uptake stimulation by preventing the degradation of TfR1 and DMT1 and it reduces iron storage by blocking ferritin and FPN1 translation which increases the LIP resulting in iron-repleted cells. As the LIP increases, this labile free iron contributes to assemble a [4Fe-4S] cluster which inactivates the RNA-binding of IRP1 and reverses the physiological effects. So IRP1 acts as a natural sensor of LIP and controls its activities by monitoring the IRE-IRP interaction.

In case of iron-replete cells IRPs lose their affinity for IREs which increases the translation of ferritin mRNA and FPN1 and mediates degradation of TfR1 mRNA and DMT1 mRNA. Through this mechanism iron toxicity is avoided by upregulation of ferritin as a storage protein and the unnecessary uptake of iron is prohibited.[8] In

enterocytes for example in case of iron demand this will result in an increased uptake of iron while the storage of iron is reduced. This IRE/IRP regulates iron on a cellular level, however it is also essential to regulate iron balance at the level of the whole organism.[8]

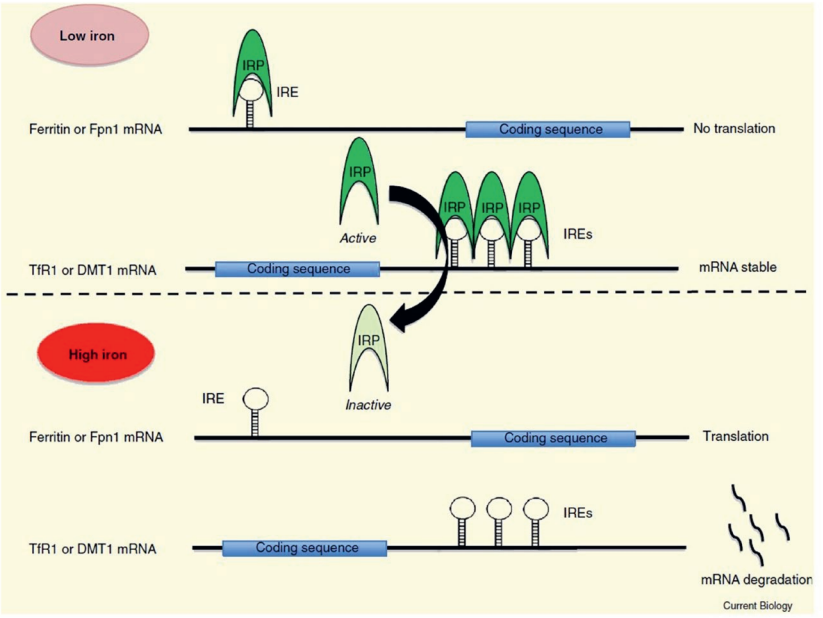


Figure 1.2 **Post-transcriptional regulation of the expression of required proteins for iron acquisition and storage.** In case of low cytosolic iron, the iron regulatory proteins (IRPs) bind to the 5' iron-responsive element (IRE) resulting in the prevention of the translation of ferritin or ferroportin (FPN1) mRNA or bind to the 3' IRE resulting in the stabilization of transferrin receptor 1 (TFR1) or divalent metal transporter 1 (DMT1) mRNA. In case of high cytosolic iron, IRPs are inactive and do not bind to IREs allowing the translation of ferritin and FPN1 mRNA and the degradation of TFR1 or DMT1 mRNA. *Reprinted from Current Biology, Vol 23/NO 15, J.Kaplan, D. M. Ward, The essential nature of iron usage and regulation, P642-646, © Elsevier Ltd. (2013), with permission from Elsevier.*

Systemic iron homeostasis

In iron balance and distribution, hepcidin is a key regulator. It is a hepatic peptide hormone that causes the internalization of FPN1, when sufficient iron has been absorbed.[4] Hepcidin secretion is regulated through the *HAMP* gene via pathways depicted in Figure 1.3.[12] The secretion of hepcidin is reduced by e.g. iron deficiency, increased erythropoiesis and hypoxia and is increased by iron overload and inflammation.[22]

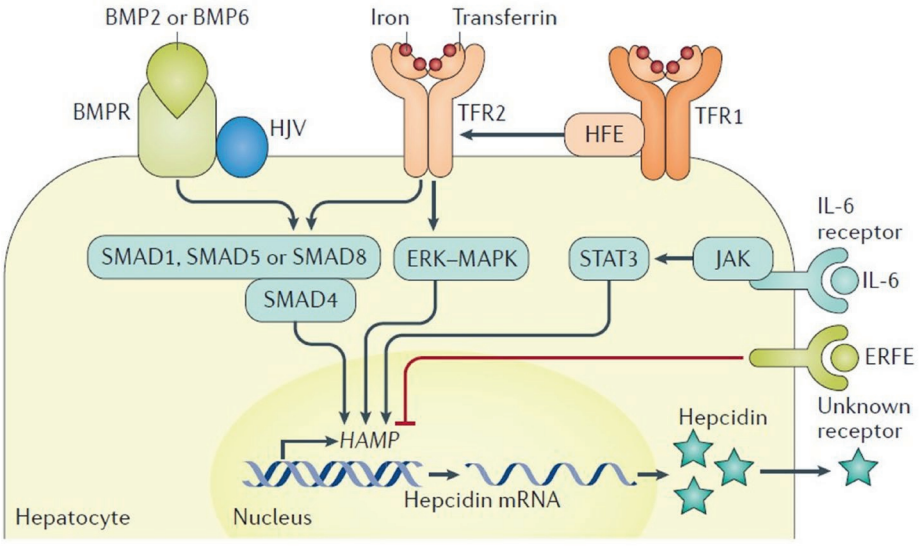


Figure 1.3 **Hepcidin regulation.** In case of increased cell iron stores, liver cells will produce bone morphogenetic protein 6 (BMP6) or BMP2 which will bind to the BMP receptors bound to hemojuvelin (HJV) on the hepatocytes. This will result in the phosphorylation of small mothers against decapentaplegic homologue 1 (SMAD1), SMAD5 or SMAD8 and these proteins will form a complex with SMAD4 eventually leading to HAMP transcription and hepcidin expression.[12,23,24] Transferrin saturation can also regulate hepcidin expression. Transferrin receptor 2 (TFR2) resembles TFR1 and can also bind iron-loaded transferrin however with lower affinity. In contrast to TFR1 which is expressed in all cells, TFR2 is mostly found in the liver. The expression of TFR2 is higher in case of excess iron and high transferrin saturation will result in a TFR2-HFE interaction. This interaction will lead to hepcidin induction through HAMP transcription, possibly through mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinase (ERK)[25] however this process is not completely understood.[12] In case of iron deficiency, HFE associates with TFR1 which leads to decreased SMAD signaling and decreased hepcidin expression.[4,12] Another regulator of hepcidin expression is chronic inflammation which is mediated by interleukin-6 (IL-6) production of inflammatory cells which induces janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT3).[12,26] Erythroferrone (ERFE) produced by erythroblasts during erythropoiesis can interact with unknown partners to decrease HAMP transcription.[12,27] *Reprinted with permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nat Rev Dis Primers (Haemochromatosis, Brissot P, Pietrangelo A, Adams PC, de Graaff B, McLaren CE, Loréal O.), © Macmillan Publishers Limited, part of Springer Nature. (2018).*

Pathophysiology of hyperferritinemia

Ferritin is a cellular iron storage protein. Serum ferritin is considered to be a reliable surrogate marker of body iron stores. Low serum ferritin levels provide absolute evidence of reduced iron stores. Hyperferritinemia is far less specific for systemic iron overload since ferritin is also an acute phase protein. Therefore serum ferritin levels will increase in case of infection, neoplasm and chronic or acute inflammation.

Hyperferritinemia is found in around 12% of subjects from the general population. It is defined as serum ferritin concentrations >200 µg/L in women and >300 µg/L in men. Determining the ferritin level is often executed as part of blood tests during an annual checkup, to check for iron storage or as a general routine bloodwork-up for patients with unexplained fatigue or liver test abnormalities. Hyperferritinemia is a frequent reason for referral to a medical specialist even in mild elevations.[28,29] It has a broad differential diagnosis and it is therefore challenging for physicians to determine the cause(s) of hyperferritinemia.

Within this variety of conditions there are multiple mechanisms underlying hyperferritinemia. It can be caused by increased ferritin synthesis as seen in patients with *HFE*-related hemochromatosis, non-*HFE* related hemochromatosis, hereditary aceruloplasminemia, secondary iron overload or in patients with anemia, associated with ineffective erythropoiesis. Another cause of hyperferritinemia is the increased synthesis or secretion of apoferritin (or light chain ferritin) seen in patients with chronic alcohol use, malignancies, reactive histiocytosis or the hereditary hyperferritinemia-cataract syndrome. Ferritin synthesis can be upregulated by inflammatory cytokines like tumor necrosis factor-α and interleukin-2.[17,30] Furthermore, hyperferritinemia is also caused by the increased release of ferritin from injured cells as seen in (non-) alcoholic fatty liver disease ((N)AFLD), chronic viral hepatitis, acute or chronic infections, auto-immune or rheumatologic conditions or massive liver necrosis.[7] The degree of ferritin elevation correlates with the degree of acute or chronic inflammation.

Diagnostic considerations and strategy for hyperferritinemia

In case of hyperferritinemia a smart diagnostic approach is required to unravel its cause. This is particularly relevant because treatment strategies vary depending on the cause of hyperferritinemia. In case of iron overload early detection is essential in order to start iron depletion therapy in time before iron accumulation results in organ damage.[31]

In certain cases, the degree of hyperferritinemia can help to distinguish its cause. Ferritin levels between 300-1000 µg/L are more often associated with NAFLD, metabolic syndrome, daily alcohol consumption, early stage of *HFE*-related hemochromatosis, malignancies or systemic inflammation. Ferritin levels between 1.000-5.000 µg/L are associated with *HFE*-related hemochromatosis, hereditary aceruloplasminemia, ferroportin mutations, the hereditary-hyperferritinemia cataract syndrome, alcoholic liver disease, viral hepatitis and secondary iron overload. Serum ferritin levels >10.000 µg/L are associated with Still`s disease, histiocytosis or fulminant hepatic failure.[7]

Elevated transferrin saturation

Serum ferritin should always be interpreted together with serum transferrin saturation (TSAT). TSAT levels ≥45% are defined as "increased" and in most cases this is an expression of iron overload. However, liver diseases can sometimes cause low transferrin levels which may lead to increased TSAT levels even in the absence of iron overload.[7] It is important to realize that TSAT is the result of the fraction of serum iron and transferrin and abnormalities of both components influence the outcome. This may not always be reflected in the resulting percentage. The accompanying clinical setting is always relevant for the interpretation of hyperferritinemia.

In hyperferritinemia patients with increased TSAT levels the next diagnostic step should be *HFE* genotyping, unless there are obvious other causes of the hyperferritinemia.[32,33] In case of *p.Cys282Tyr* homozygosity the diagnosis of *HFE*-related hemochromatosis is confirmed and iron depletion treatment should be started however there should also be attention for other explanations of hyperferritinemia like chronic alcohol consumption or obesity. In case of *p.His63Asp* homozygosity, *p.His63Asp* heterozygosity, *p.Cys282Tyr* heterozygosity or the absence of a mutation in the *HFE* gene there is no sufficient explanation for hyperferritinemia and there should be looked for other explanations. In case of persistent doubts about the presence of iron overload an additional investigation with for example MRI could be performed to rule out hepatic iron accumulation.[34,35] In case of *p.Cys282Tyr / p.His63Asp* compound heterozygosity there is no consensus, however in most cases it does not reflect iron overload.[36,37]

Normal transferrin saturation

In case of hyperferritinemia with a normal TSAT or with increased TSAT but in the absence of *p.Cys282Tyr* homozygosity a search for alternative explanations should be performed. In case of normal TSAT, hyperferritinemia is more often related to

inflammation, cell damage, metabolic abnormalities such as obesity or chronic alcohol consumption instead of iron overload. In this setting it is useful to explore the patients alcohol intake and to check for components of the metabolic syndrome (diabetes mellitus type 2, obesity, dyslipidemia, hypertension). Blood should be examined for inflammatory parameters (e.g. CRP, white blood cell count), abnormal liver tests (e.g. AST, ALT, alkaline phosphatase and gamma-glutamyl transpeptidase) and the presence of hepatitis B and C virus. Additionally performing a liver ultrasonography should be considered to check for other liver disease such as liver steatosis or the presence of cirrhosis.[33]

To date, non-alcoholic fatty liver disease (NAFLD) is a frequent cause for hyperferritinemia. In NAFLD patients in up to 30% patients elevated serum ferritin concentrations are found.[38] NAFLD is the most widespread liver disease in Western society, with a prevalence up to 25% in the general population. That means that hyperferritinemia is frequently found as a laboratory abnormality and that it has to be explored if iron overload and/or inflammation are present in patients. There is a need for better insight into the etiology of hyperferritinemia in this substantial group of patients.[39]

Additional diagnostic investigations in hyperferritinemia

Additional diagnostic work-up is indicated in the absence of a plausible explanation for hyperferritinemia. Previously, measurement of the liver iron concentration (LIC) by liver biopsy was considered as gold standard to detect iron accumulation. However this invasive diagnostical method with potentially serious complications and the risk for sample error is currently no longer essential to confirm the diagnosis of *HFE*-related hemochromatosis. It is only used in certain cases as explained in the hereditary hemochromatosis guidelines.[40-42] Newer non-invasive techniques are suggested and investigated such as LIC determination through MRI scanning.

The LIC currently is considered the best method to accurately assess body iron load, since the liver contains ≥70% of the body iron stores.[43] LIC determination by liver MRI is a good, non-invasive alternative to detect iron overload. The LIC measured by liver biopsy (LIC-b) and LIC measured by MRI (LIC-MRI) showed a good correlation ($r=0.87$).[44-48] Therefore, LIC-MRI quantification has been proposed as the new gold standard for diagnosing iron overload.[49] However, an important diagnostic difficulty is that the cut-off value of ≥36 µmol/g for LIC appears to be low since often the LIC is found to be mildly increased in hyperferritinemia associated with the dysmetabolic iron overload syndrome and/or alcohol (over)consumption in the absence of major

iron overload.[44] For that reason, many studies use different cut-off values, which hampers the interpretation of the LIC.[42,44,50,51] There is need for a well-defined, generally applicable cut-off value to differentiate between major iron accumulation and inflammation or mild iron overload.

Hyperferritinemia and elevated LIC: rare iron overload diseases

Evidently elevated LIC points to iron overload and the next step is to search for rare causes of hereditary hemochromatosis or acquired iron overload diseases. Within acquired iron overload disease, a distinction can be made between iatrogenic iron overload (e.g. frequent blood transfusions or iron infusions in case of chronic kidney diseases) and dyserythropoiesis (e.g. hematologic conditions such as thalassemia or syndromes with chronic compensated hemolysis).[52] In the absence of acquired iron overload disease DNA analysis should be performed to check for rare mutations causing non-*HFE*-related hemochromatosis: *HJV*-related, *HAMP*-related, *TFR2*-related and *SLC40A1*-related. Mutations in *SLC40A1* can lead to gain or loss of FPN1 function. Figure 1.4 provides an overview of the former classification of hereditary hemochromatosis and the newly proposed hereditary hemochromatosis nomenclature. The former type 4a hemochromatosis, now referred to as ferroportin disease, is excluded from the new classification due to its distinct phenotype. Another non-*HFE* related cause of hereditary hemochromatosis is hereditary aceruloplasminemia (HA). HA and ferroportin disease, are characterized by hyperferritinemia with a normal or low TSAT.[52,53]

Former hemochromatosis classification
Type 1 or *HFE* mutations
Type 2A or *HJV* mutations
Type 2B or *HAMP* mutations
Type 3 or *TFR2* mutations
Type 4A or *SLC40A1* (FPN1 loss of activity)
Type 4B or *SLC40A1* (FPN1 gain of activity)

New proposed hemochromatosis classification
HFE-related mutations
Non-*HFE* related mutations: • *HJV*-related • *HAMP*-related • *TFR2*-related • *SLC40A1*-related
Digenic mutations*
Molecularly undefined**

Figure 1.4 **Overview of the former and newly proposed classification of hereditary hemochromatosis disorders.** This new classification is developed by a working group of the International Society for the Study of Iron in Biology and Medicine (BIOIRON Society) * Double heterozygosity and/or double homozygosity/heterozygosity for mutations in two different genes involved in the iron metabolism (*HFE* and/or *non-HFE*) **When there is still no molecular characterization possible after sequencing known genes (provisional diagnosis) *Abbreviations: HFE: human homeostatic iron regulator; HJV: hemojuvelin; HAMP: hepcidin; TFR2: transferrin receptor 2; FPN1: ferroportin.*

HFE-related hemochromatosis

Hereditary hemochromatosis is the term for mutations in genes involving iron homeostasis which eventually result in iron accumulation. It can be divided into *HFE*-related hemochromatosis and non-*HFE*-related hemochromatosis (Figure 1.4). *HFE*-related hemochromatosis is caused by homozygous *p.Cys282Tyr* mutations in the *HFE* gene.[12,54] The HFE (human homeostatic iron regulator) protein is encoded by the *HFE* gene on chromosome 6. It is the most frequent form of hereditary iron overload.

Non-HFE-related hemochromatosis is caused by mutations which will all lead to insufficient hepcidin production, or resistance to hepcidin action. The loss of hepcidin function results in excessive iron absorption. This leads to iron accumulation since there is no active mechanism to excrete iron from the body.[55] When Tf becomes oversaturated the excess iron will be released into the circulation in the form of non-transferrin-bound iron which can result in cellar injury.[12,40] Another cause of iron overload is hereditary aceruloplasminemia (HA). This is a rare autosomal recessive disorder in which a mutation in the *CP* gene results in the absence of CP. The absence of CP largely inhibits the release of iron from the physiological storage cells resulting in their maximal saturation and contributing to iron accumulation in many organs like the liver, brain and pancreas (Figure 1.5).

Epidemiology

HFE-related hemochromatosis is the most prevalent genetic disorder in Northern European populations. The *p.Cys282Tyr* polymorphism in the *HFE* gene, an autosomal recessive mutation, is the most commonly occurring mutation resulting in hereditary hemochromatosis. Large population based studies found a prevalence of 0.3%-0.6%.[56-58] Overall, the genotype *p.Cys282Tyr* homozygosity is present in 1 in 250 Caucasians. In non-Caucasians the prevalence of *p.Cys282Tyr* homozygosity is much lower.[59] While the prevalence of *p.Cys282Tyr* homozygosity is high, the clinical penetrance is low and only a minority of these *p.Cys282Tyr* homozygous patients will eventually accumulate enough iron to result in organ damage. Regardless of the similar frequency of *p.Cys282Tyr* homozygosity in men and women the prevalence of clinical manifestations is very different.[12] Allen et al. found that only 1.2% of women and 28.4% of men with *p.Cys282Tyr* homozygosity matched the criteria consistent with a clinical diagnosis of *HFE*-related hemochromatosis while 55.4% of the women and 81.8% of the men with *p.Cys282Tyr* homozygosity had hyperferritinemia. There is a higher biochemical penetrance compared to the clinical penetrance.[60] Another study estimated the development of iron overload to occur in around 38-50% of

p.Cys282Tyr homozygotes and 10-25% eventually develop hemochromatosis-associated morbidity.[61] There is no *p.Cys282Tyr* homozygosity prevalence available in the Netherlands.

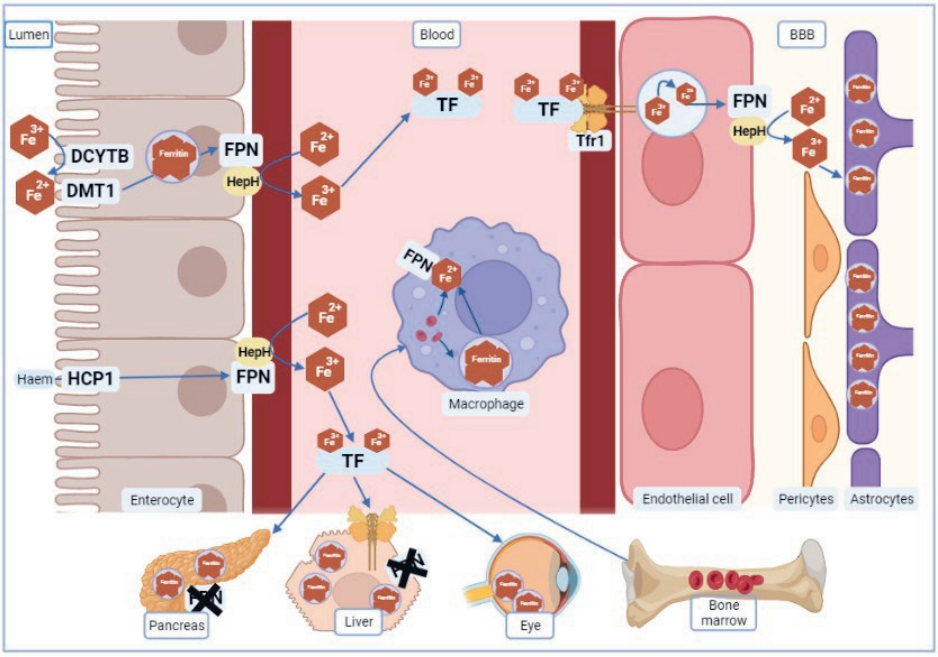


Figure 1.5 **The pathophysiology of hereditary aceruloplasminemia (HA)**. HA is characterized by cellular iron accumulation. Clinically expressions vary depending on the amount of accumulated iron. The most prominent clinical features are iron accumulation in the pancreas causing diabetes, in the liver resembling *HFE*-related hemochromatosis, in the eyes expressed as retina pigmentation and retinopathy and in the brain with different, variable, complaints. In the central nervous system iron is mainly accumulated in the basal ganglia and cerebellum, especially in the astrocytes and glia cells. Iron accumulation in HA is due to the inability to excrete cellular iron due to a mutation in the *ceruloplasmin (CP)* gene leading to the absence of CP. A ferroxidase (hephaestin (HepH) or CP) is necessary for a proper functioning of ferroportin (FPN). The mode of interaction between these two is not clear. The hypothesis is that due to absence of CP iron will accumulate in the cell resulting in low transferrin saturation (TSAT) and low hepcidin levels. This causes maximal iron absorption from the gut because enterocytic FPN is independent of CP as it uses hephaestin as a ferroxidase. This will lead to iron toxicity gradually causing the described complaints, mainly caused by iron driven oxidative stress and disturbance of cellular metabolism leading to cell death. *Abbreviations: Fe^{3}: ferric iron; Fe^{2+}: ferrous iron; DCYTB: duodenal cytochrome-b; HCP1: heme carrier protein 1; FPN: ferroportin; HepH: hephaestin; CP: ceruloplasmin; TFR1: transferrin receptor; TF: transferrin.*

Symptoms of *HFE*-related hemochromatosis

Around 75% of the patients diagnosed with *HFE*-related hemochromatosis are asymptomatic at the time of diagnosis, the diagnosis is often made during routine checks of blood samples.[57] Symptoms only develop in a later disease stage when enough iron is accumulated to result in organ involvement.[62] In men, symptoms most often develop after the age of 30 years and in women later, that is in the postmenopausal age.

The most frequent symptoms are fatigue/lethargy, arthropathies and decreased libido as depicted in Figure 1.6.[63] Liver test abnormalities can also be a reason to check for iron overload including serum ferritin and transferrin saturation. The first sign of liver dysfunction can be mildly to moderately increased liver enzymes (ALT/AST). When ferritin concentrations exceed 700-800 µg/L there is a risk of developing liver fibrosis and eventually liver cirrhosis which can lead to hepatocellular carcinoma (HCC) or liver insufficiency.[64] Liver cirrhosis is mainly seen in patients with ferritin concentrations >1000 µg/L.[65]

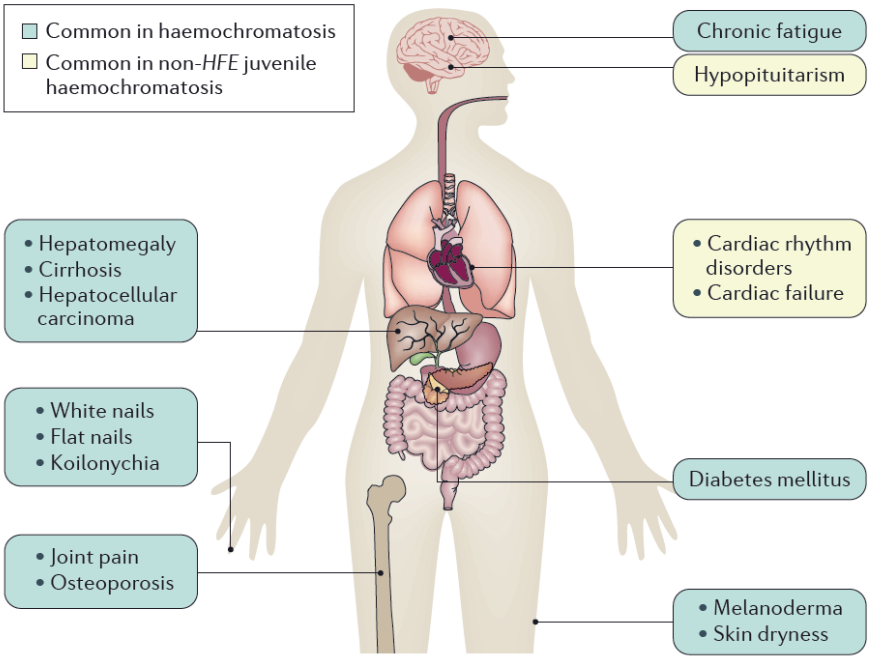


Figure 1.6 **Symptoms associated with *HFE*-related hereditary hemochromatosis.** *Reprinted with permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nat Rev Dis Primers (Haemochromatosis, Brissot P, Pietrangelo A, Adams PC, de Graaff B, McLaren CE, Loréal O.), ©Macmillan Publishers Limited, part of Springer Nature. (2018).*

It remains controversial whether the incidence of cancers other than HCC is increased in patients with *HFE*-related hemochromatosis. A Danish study found an association between *p.Cys282Tyr* homozygosity and the risk of cancer.[66] On the other hand, in a Swedish study no increased incidence of extrahepatic cancers was found in patients with *HFE*-related hemochromatosis.[67]

Iron overload can also result in cardiac disorders like cardiac arrhythmias and heart failure. The high iron content of mitochondria and a low content of antioxidants in the myocardium render the heart more prone to iron-induced oxidative stress[68] (Figure 1.6).
Many patients with *HFE*-related hemochromatosis experience arthropathy especially in the small finger joints but it is also seen in larger joints[63] (Figure 1.6). The pathogenesis of arthropathy is unclear. Despite the finding of iron deposits in the synovial membrane there is no association found between the extent of iron accumulation and the presence of arthropathy.
Diabetes mellitus can also be a complication of severe iron overload through iron-induced destruction of insulin-producing beta-cells, possibly combined with insulin resistance.[63] The accumulation of iron in pituitary cells can lead to secondary hypogonadism. In premenopausal women this will result in a decreased libido and amenorrhea while in men this will lead to a decreased testosterone production resulting in infertility through decreased spermatogenesis, reduced libido and erectile dysfunction.[69,70] In a very small subset of patients, clinically manifest and subclinical hypothyroidism is observed, presumably caused by iron accumulation in the thyroid[71] (Figure 1.6). In several published studies the prevalence of thyroid dysfunction in *HFE*-related hemochromatosis patients was found not to be increased compared to the general population.

A very tanned or greyish appearance of the skin can be another symptom of *HFE*-related hemochromatosis. This is probably caused by a combination of iron accumulation in the skin and stimulation of melanin production by melanocytes.[62] In the past this was seen more frequently. Therefore, in the 19th century hemochromatosis was described as “diabète bronze” because it frequently led to skin hyperpigmentation and diabetes mellitus[72] (Figure 1.6).

The diagnosis of *HFE*-related hemochromatosis has briefly been discussed in a previous paragraph of this chapter. In case of elevated TSAT combined with hyperferritinemia *HFE* genotyping should be performed.[32,33]

Treatment of *HFE*-related hemochromatosis

The treatment of iron overload in *HFE*-related hemochromatosis can be divided into two phases. The first phase is the depletion phase, which is based on the removal of excess body iron by lowering serum ferritin concentrations to 50-100 µg/L.[41,73] In case of TSAT >70% bloodletting needs to be continued until the serum ferritin level is <50 µg/L while in case of TSAT <70% serum ferritin levels of <100 µg/L are sufficient to continue with the second phase (Figure 1.7).

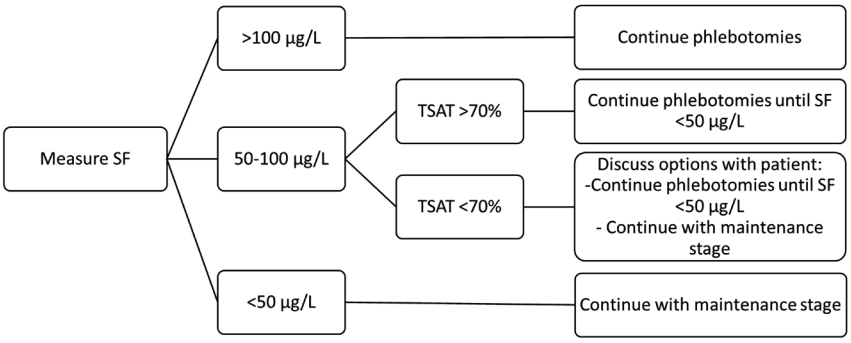


Figure 1.7 **Algorithm *HFE*-related hemochromatosis iron depletion treatment according to the Dutch guideline.** *Abbreviations: SF: serum ferritin; TSAT: transferrin saturation.*

The second phase is the maintenance phase with the aim of preventing iron re-accumulation. This is achieved by maintaining the ferritin concentrations below the upper range of normal in case of TSAT <70%. In case of TSAT >70% it is advised to maintain serum ferritin levels between 50-100 µg/L[42] (Figure 1.8).

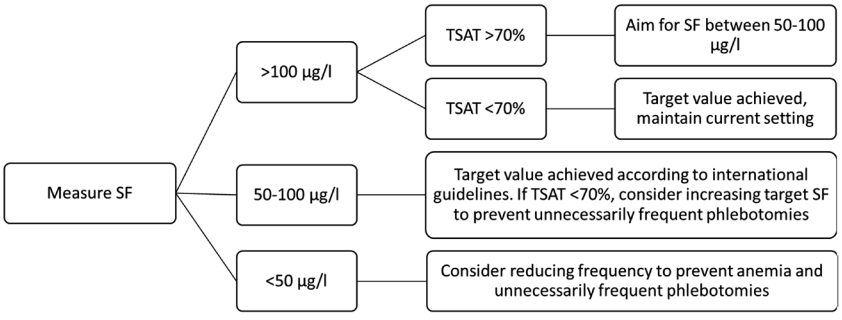


Figure 1.8 **Algorithm *HFE*-related hemochromatosis maintenance treatment according to the Dutch guideline**. *Abbreviations: SF: serum ferritin; TSAT: transferrin saturation.*

There are three different types of iron depletion treatments: phlebotomy, erythrocytapheresis and in some patients iron chelators can be used.

Despite the absence of scientific evidence from randomized clinical trials, phlebotomy treatment has proven to be very effective in the removal of accumulated iron. An extrapolation of clinical studies showed that iron depletion improves quantity and quality of life of all *HFE*-related hemochromatosis patients by improving chronic fatigue, cardiac function, reducing skin pigmentation, reversing hepatic fibrosis and stabilizing chronic liver disease. The benefits of therapy outweigh its potential risks or adverse effects.[12,74] Transient adverse effects are phlebitis, malaise and fatigue, occurring in 37-50% of patients.[75]

During phlebotomy treatment, in the depletion phase a volume of 500 ml blood is removed on a weekly basis, representing an iron loss of approximately 250 mg. During this treatment the hemoglobin levels are regularly checked and in case of evident decreases in hemoglobin the interval between treatments can be extended to every two weeks or longer. This frequency is continued until the target ferritin concentrations are reached and iron depletion has been attained. The duration of the induction therapy varies from months to years depending on the severity of iron overload.[75] The variability in the clinical phenotype makes it difficult to identify which patient needs maintenance treatment, and if so, how frequent phlebotomies should be performed. Usually, phlebotomies are performed between two to four times a year.

The other alternative iron depletion therapy is erythrocytapheresis. This technique selectively removes erythrocytes and returns valuable blood components such as platelets and clotting factors, etc. to the patient. With this technique it is possible to remove more erythrocytes and thus iron per single procedure. In patients with severe iron overload this is a good alternative treatment. It is also possible to substitute the removed erythrocytes with saline, albumin or other colloid solutions to prevent hemodynamic changes making it also suitable for patients with cardiovascular diseases.[76]

Chelation therapy is not standardly recommended as treatment for patients with *HFE*-related hemochromatosis. Patients with *HFE*-related hemochromatosis are advised to avoid oral iron therapy and alcohol abuse. Dietary restrictions have not been proven effectful.[77] Proton pump inhibitors (PPIs) have been suggested as an attractive additional therapy to reduce the need for phlebotomies by reducing gastric acid

secretion which results in decreased iron absorption.[78-80] Interestingly, studies about the occurrence of anemia during long-term use of PPIs in patients without *HFE*-related hemochromatosis are contradictory.[81,82] The study describing PPI associated anemia in patients without HH, did not rule out a pre-existent iron deficient state or possible upper gastrointestinal blood loss.[82] PPIs appear to have a different influence on iron absorption in patients with *HFE*-related hemochromatosis patients compared with healthy control subjects.

Aims and outline of this thesis

The current thesis is divided into two parts. The aim of the first part was to investigate the diagnostic challenges in hyperferritinemia. In **chapter 2**, we performed an extensive review of the available literature in which we aimed to investigate whether hyperferritinemia in NAFLD is associated predominantly with inflammation rather than iron overload. This is a relevant question since NAFLD is the most widespread liver disorder in Western society and hyperferritinemia was found in 30% of all NAFLD patients. The aim of **chapter 3** is to find an effective method to differentiate hyperferritinemia patients with none or only minor iron overload from patients with major iron overload. As previously explained the LIC is considered the best method to accurately assess body iron stores but there is a need for a clear-cut off value. In the past the liver iron index (LII) had been suggested to help interpretate, the LIC measured with liver biopsy, correctly. The LII is the LIC divided by the age of patients in years. The aim of **chapter 3** was to investigate if the LII has the same diagnostic value to interpretate the LIC-MRI.

In part 2 of this thesis, the focus is on hereditary hemochromatosis, mainly the *HFE*-related form. The aim of **chapter 4** was to provide an overview of the South Limburg population-based *HFE*-related hemochromatosis cohort with 360 patients, with respect to epidemiology, phenotype expression, disease manifestations, symptoms, efficacy of iron depletion therapy and long term follow up with morbidity and mortality.

In **chapter 5**, we aimed to develop a phenotypic predictor for the number of phlebotomies needed per year during maintenance treatment in *p.Cys282Tyr* homozygous patients. This tool is of great value to individualize therapy. Such a predictor or index might also help to select patients who will benefit most from a therapeutic alternative, e.g. erythrocytapheresis. In **chapter 6**, we focus on the

treatment of *HFE*-related hemochromatosis with respect to iron absorption in the small intestine. During PPI treatment a difference in iron absorption is observed between patients with *HFE*-related hemochromatosis and healthy subjects. Our aim was to explain the differences in iron absorption between patients with and without *HFE*-related hemochromatosis. In **chapter 7** a *p.Cys282Tyr* homozygous patient with upregulated hepcidin levels and lower iron concentration during a period of systemic inflammation is studied.

In **chapter 8** the use of erythrocytapheresis in patients with hereditary aceruloplasminemia to prevent disease progression is discussed. Finally, in **chapter 9** an integrative view on the key findings of all the studies presented in this thesis is discussed. The findings described in this thesis is are put in the perspective of the currently available literature and in terms of potential implications for future research.

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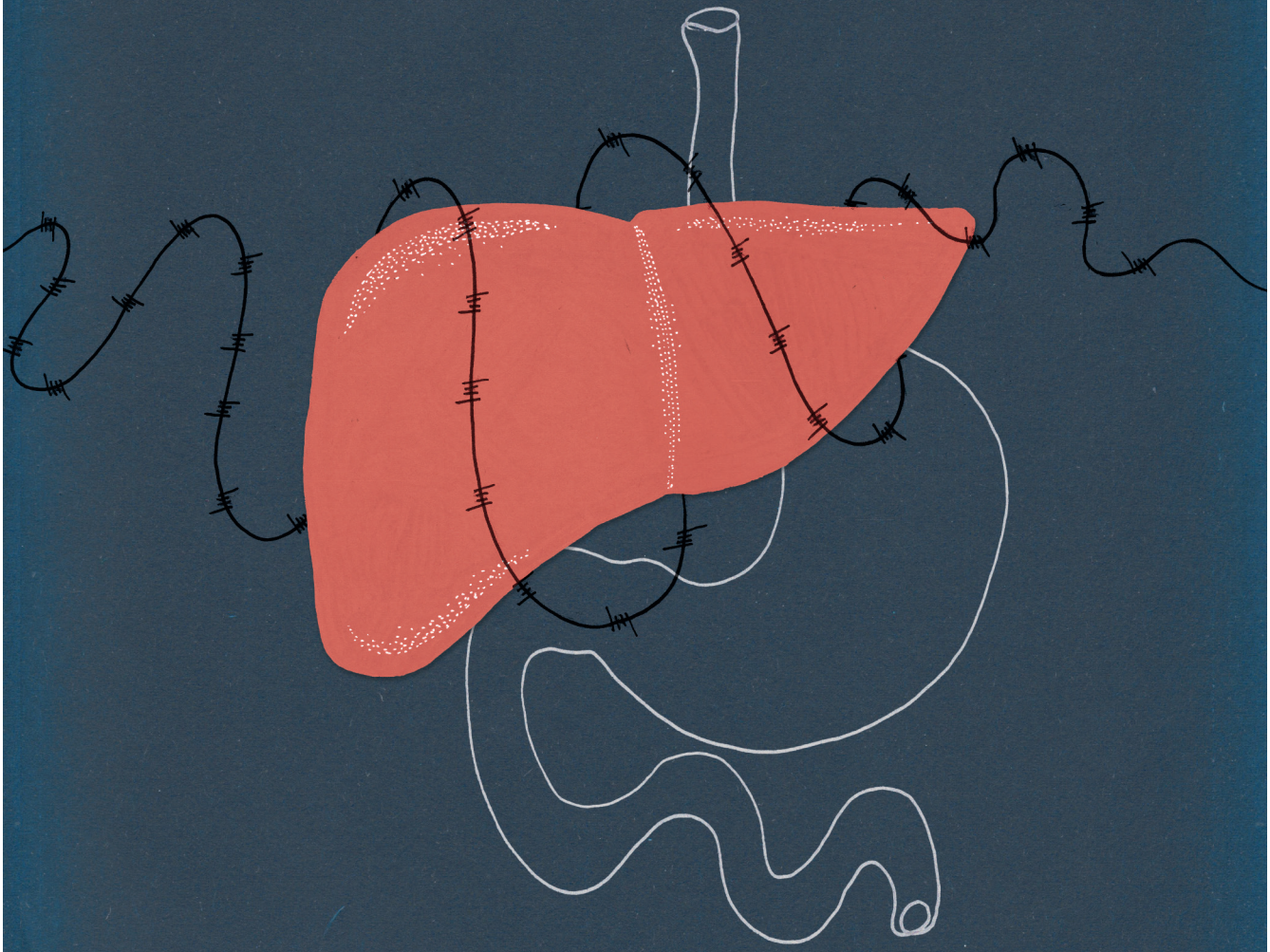
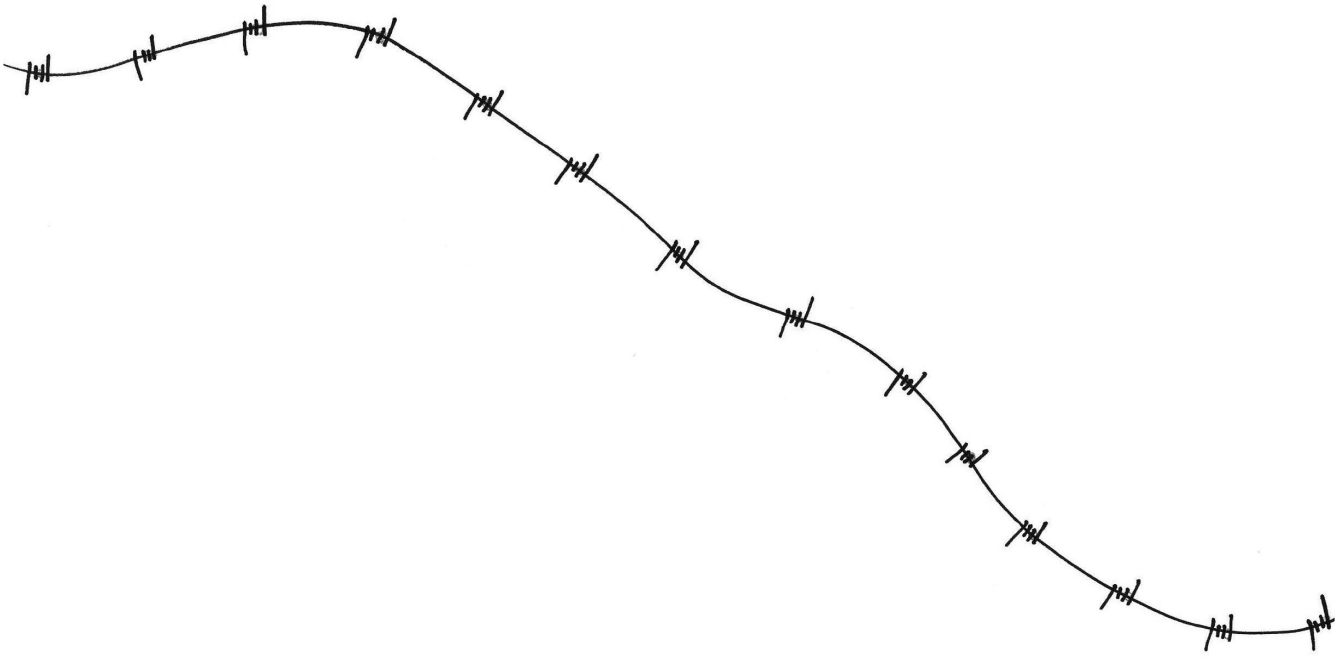
Part I

Understanding and interpreting hyperferritinemia



Chapter 2

Hyperferritinemia in Non-alcoholic Fatty Liver Disease: iron accumulation or inflammation?



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*These authors contributed equally to this work

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Abstract

Hyperferritinemia, observed in inflammation, in iron overload as well as in the combination of both, is found in about 30% of non-alcoholic fatty liver disease (NAFLD) patients. We summarised the evidence regarding the potential cause of hyperferritinemia in NAFLD, as this may affect the indicated therapy.

A systematic literature search was conducted in EMBASE, PubMed, MEDLINE and the Cochrane library.

In the majority of NAFLD patients, hyperferritinemia is due to inflammation without hepatic iron overload. In a smaller group, a dysmetabolic iron overload syndrome (DIOS) is found, showing hyperferritinemia in combination with mild iron accumulation in the reticuloendothelial cells. The smallest group consists of NAFLD patients with hemochromatosis. Phlebotomy is only effective with hepatocellular iron overload and should not be the treatment when hyperferritinemia is related to inflammation, whether or not combined with DIOS. Treatment with lifestyle changes is to date, probably the more effective way until new medication is becoming available.

Introduction

Non-alcoholic fatty liver disease (NAFLD), strongly associated with overweight and obesity, is the most common liver disorder in Western society, with a global prevalence of around 25%.[1] NAFLD ranges from hepatic steatosis (HS)[2] to non-alcoholic steatohepatitis (NASH), progressive liver fibrosis, cirrhosis and an increased risk to develop hepatocellular carcinoma.[3] It is considered the hepatic manifestation of the metabolic syndrome in which a generalised systemic inflammation plays an important role in the initiation and progression of diabetes mellitus and cardiovascular diseases.[4,5] The incidence of NASH is expected to increase even further over the next decades, which stresses the need to elucidate factors involved in disease progression. Diagnostic markers are urgently needed to identify NAFLD patients that progress to advanced liver- and systemic diseases. Serum ferritin is increased in about 30% of patients with NAFLD.[6,7] Ferritin is an acute phase protein but also a storage protein for iron within cells, being most pronounced in hepatocytes and macrophages.[8,9] Small amounts of ferritin are present in the circulation reflecting the body iron stores in healthy control subjects. Elevated serum ferritin levels can also occur in patients with mutations in genes involved in iron homeostasis, such as hereditary hemochromatosis (HH) (*e.g*. *HFE* mutations),[10,11] or in the case of the beta-thalassemia trait.[12] Transferrin saturation levels are usually increased (>45%) in hyperferritinemia caused by HH.[13]

In 34.5-51.5% of NAFLD patients dysmetabolic iron overload syndrome (DIOS) is present.[14,15] DIOS is characterised by hyperferritinemia with only a mild increase of both liver and body iron stores. It is associated with various compounds of the metabolic syndrome in the absence of any identifiable genetic mutation or other causes of iron excess.[16] As iron plays a role via the Fenton reaction in the generation of oxidative stress and thereby causes disease progression, it is important to distinguish if hyperferritinemia is due to classical hepatic iron overload or is the expression of inflammation. If hepatic iron overload in the classical pattern is the primary cause of hyperferritinemia, therapeutic bloodletting is the only therapeutic option. While in inflammation-related hyperferritinemia or in patients with DIOS, lifestyle changes such as a hypocaloric diet and physical activity are recommended instead of phlebotomies.

Therefore, the aim of this systematic literature search was to evaluate the role and value of hyperferritinemia as a marker of iron overload or inflammation in patients with NAFLD.

Methods

Literature search

A systematic literature search was performed in PubMed, Cochrane Library, MEDLINE and EMBASE up to 31st of October 2017 using the following (truncated) keywords: `Non*alcoholic fatty liver disease', `NAFLD', `Fatty liver`, `Hepatic steatosis` or `Liver steatosis` in any combination with `Hyperferritin*' and `Ferritin` and `Dysmetabolic iron overload syndrome`, `Dysmetabolic iron overload`, `Dysmetabolic hepatic iron overload`, `Dysmetabolic hepatic overload syndrome`, `DIOS', `DHIOS` or `iron overload`. This resulted in a total number of 530 hits (117 PubMed, 9 Cochrane Library, 252 EMBASE and 152 MEDLINE).

We excluded studies in which the population primarily consisted of patients with hyperferritinemia, obesity, diabetes and/or metabolic syndrome when they did not report on the role of hyperferritinemia in NAFLD. We also excluded studies performed *in vitro,* in animals, in minors, in languages other than English, case reports, conference abstracts, reviews, and duplicates.

Titles and abstracts of all 530 hits were screened, leaving 152 articles, of which the full text was checked. The initial screening was done by a single author (WM) and in case of doubt the reference was checked by a second author (PV). In case of disagreement on eligibility, the two reviewers came to consensus after discussing the article with a third reviewer (DJ or GK). Furthermore, eligible papers were cross-checked for references, which resulted in six additional papers. Finally, 42 papers were included in the current review (Figure 2.1). We assessed the quality of the included studies with regard to the risk of bias (Supplementary Table S2.1). Not all articles were described in detail also due to limited description of results applicable to the subject of the review.[17-19]

Results

HFE mutations and iron regulators in NAFLD

The frequency of mutations in genes involved in iron homeostasis has been studied in NAFLD patients with hyperferritinemia[12,15,20-34] (Supplementary Table S2.2). The overall prevalence of *HFE* mutations for *p.Cys282Tyr* ranged from 0-7.9% and 5-23.5% for homozygosity and heterozygosity, respectively. For *p.His63Asp* the prevalence was between 1-5.6% and 13-44.4% for homozygosity and heterozygosity, respectively. Some studies found a significantly higher prevalence of *HFE* mutations (*i.e.* for

homozygosity[20] or heterozygosity[20,28,29,30] of *p.Cys282Tyr* and/or heterozygosity of *p.His63Asp*[28]) in NAFLD patients versus control subjects. The majority of studies however, did not show significant differences in the presence of *HFE* mutations between NAFLD patients and control subjects.[12,15,24-27,31,33] Furthermore, no relation was observed between *HFE* mutations in NAFLD patients with high versus low serum ferritin[22] or with the presence or absence of iron overload.[23,32]

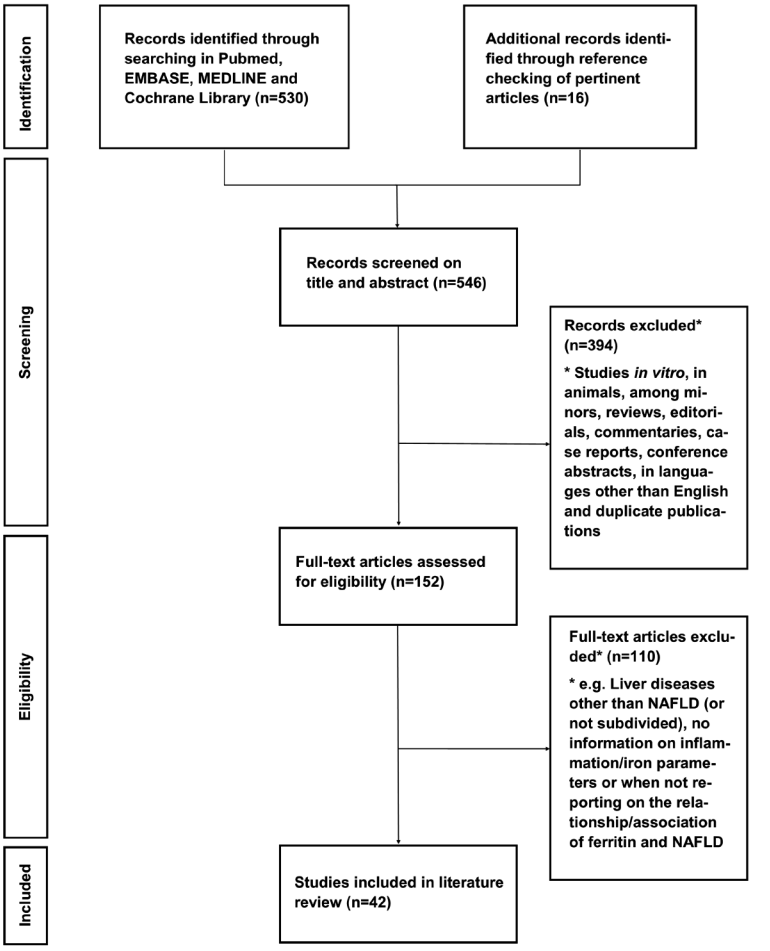

Figure 2.1 Flowchart study selection process. *Abbreviation: Non-alcoholic fatty liver disease (NAFLD).*

In addition to the *HFE* mutations, the beta-thalassemia trait, *alpha-1-antitripsin* mutation (AAT) and the *p.Ala736Val* variant of transmembrane protease serine 6 (TMPRSS6) were investigated in NAFLD. A significantly higher prevalence of the AAT mutation was found in NAFLD patients (±10%) compared to healthy control subjects (±3.5%).[12,21] The prevalence of beta-thalassemia was 9.1% in patients with NAFLD, there was no prevalence available in the control group.[12] One study assessing the prevalence of the *p.Ala736Val* variant of transmembrane protease serine 6, did not find a significant difference between NAFLD patients and healthy control subjects.[25]

The regulators of iron homeostasis focussing on serum transferrin receptor-1 (TfR1), TfR2, divalent metal transporter 1 (DMT1), ferroportin, hepcidin and hemojuvelin were also studied in NAFLD patients, but results were inconsistent.[12,21,23-25,34-41]

No significant correlations were found between NAFLD patients (NASH and HS) compared to control subjects in duodenal or serum DMT1 mRNA levels,[23,39,41] TfR1 and TfR2 mRNA levels[23,35,36,41], and serum, duodenal and hepatic ferroportin concentration.[23,24,35,38,39,41] Increased hepcidin levels were found in three studies[23,35,39] measuring hepcidin in NAFLD patients (NASH and HS) compared to control subjects. Furthermore, increased hepcidin levels were found to be associated with hepatic lobular inflammation and NAFLD activity scores (NAS)[40] and iron in reticuloendothelial cells[12] (Supplementary Table S2.2).

An important up-stream regulator of hepcidin expression is hemojuvelin. Two studies[23,37] investigating hemojuvelin found lower levels in NAFLD patients versus control subjects. Lower copper levels were associated with higher ferritin levels and increased prevalence of siderosis and hepatic iron in NAFLD patients.[24] Hepatic 8-oxodG levels, a product generated by hydroxyl radicals, was found to be positively correlated with body and hepatic iron deposition markers in NAFLD patients.[42]

Different patterns of hepatic iron deposition

The pattern of hepatic iron deposition can vary between iron deposition in the hepatocytes (classical iron overload), iron deposition in the reticuloendothelial cells or a combination of both cell types also called the mixed pattern. The histological NAS score,[43] assessing histological injury of NAFLD patients, is found to be significantly higher in patients with iron depositions in the reticuloendothelial cells,[14,22,44] when compared to iron depositions in hepatocytes (classical iron overload) or to iron deposition in the mixed pattern.[44] Patients with depositions in reticuloendothelial cells or mixed pattern were also found to have significantly higher serum ferritin than those with no iron overload or the hepatocyte pattern.[44] Furthermore, also a correlation was found between elevated serum ferritin levels and increased NAS.[22]

Hyperferritinemia related to inflammation

In addition to the standard inflammatory serum biomarkers measured in NAFLD patients, ferritin is often found to be significantly higher in NASH patients when compared to control subjects or patients with HS.[27,36,37,42,45-47] In several of these studies,[36,37,45] serum iron parameters were also investigated, however no significant difference in iron status was found between the groups.

A wide range of indirect and direct inflammatory parameters were found to be significantly higher in NAFLD or NASH patients when compared to control subjects (*e.g*. aspartate aminotransferase, thioredoxin, C-reactive protein, alpha-2-macroglobulin, ceruloplasmin, malondialdehyde, hepatic 8-oxodG, nitric oxide, interleukin-6, interleukin-8 and tumour necrosis factor alpha). Most studies also found ferritin levels to be significantly higher in NASH patients compared to controls and/or patients with HS and NAFLD patients compared to control subjects[27,36,37,42,45-49] (Supplementary Table 2.2). Most of the studies showed no signs of biochemical or histological iron overload to explain the higher ferritin levels.[2,27,36,37,45,48,49] Ferritin showed a significant positive correlation with lobular inflammation,[2,22,44] portal inflammation,[2,50] higher NAS,[22,44] severity of steatosis,[22,26,44,51] presence of fibrosis,[2,22,26,44,51] presence of NASH[48] and ballooning.[22,44]

Effect of interventions on hyperferritinemia in NAFLD

The proposed treatment of hyperferritinemia depends on its etiology. Moderate to vigorous physical exercise showed to decrease steatosis accompanied with significantly improved levels of serum ferritin as well as lipid peroxidation and adiponectine.[52] Eicosapentaenoic acid, an antilipidemic agent also showed to improve hepatic steatosis, fibrosis hepatic ballooning and lobular inflammation as well as ferritin levels in NASH patients.[53]

Phlebotomy therapy in NAFLD patients showed a significant decrease in ferritin levels and iron status[23,42,54,55] but also in parameters related to inflammation, *e.g*. alanine aminotransferase,[23,42,54] tumour necrosis factor-alfa[23] and hepatic 8-oxodG levels (a DNA base-modified product generated by hydroxy radicals).[42] In contrast, Adams *et al*. did not find improvement in liver enzymes, hepatic fat or insulin resistance during ferritin reduction by phlebotomy in patients with NAFLD in a randomised, controlled trial comparing phlebotomy and life style advices with life style advices only.[55]

Discussion

This literature review aimed to summarise the evidence for iron overload and/or inflammation as a possible cause of hyperferritinemia observed in patients with NAFLD. In more severe forms of NAFLD like NASH and advanced fibrosis, systemic inflammation is present in the majority of patients.[22] This low grade systemic inflammation has generally no characteristic laboratory abnormalities, but hyperferritinemia is frequently observed and this raises the question whether this is an expression of this low grade systemic inflammation in NAFLD.

With this extended literature review, three groups could be distinguished. First, the majority of NAFLD patients that have hyperferritinemia without evidence of hepatic iron overload or *HFE* mutations. These NAFLD patients are most likely to have hyperferritinemia related to ferritin release from damaged hepatocytes[56] and/or related to the systemic inflammatory status (Figure 2.2). Secondly, hyperferritinemia in NAFLD can also be explained as part of DIOS. In which inflammation up-regulates the hepcidin levels resulting in impaired iron export from the cells causing diminished iron availability[23] which can lead to mild hepatic iron overload in reticuloendothelial cells whether or not combined with iron in hepatocytes. About 30% of NAFLD patients have DIOS, which consists of hyperferritinemia in combination with only a mild increase of both liver and body iron stores associated with various compounds of the metabolic syndrome in the absence of any identifiable genetic mutation or other causes of iron excess.[16] The finding of a normal or moderately increased transferrin saturation supports DIOS. Iron overload in DIOS probably plays a prominent role in the pathophysiology of NAFLD, as it can generate reactive oxygen species and thereby lead to oxidative stress[57] causing severe cellular dysfunction, organ damage and promotes the development of insulin resistance and hepatocellular inflammation attributing to NAFLD progression.[58,59]

The third group is relatively small, consisting of NAFLD patients with identifiable *p.Cys282Tyr* homozygote *HFE* mutations (up to 7.9%). In the majority of these patients the increased serum ferritin levels are found in combination with elevated transferrin saturation (>45%). Therefore, screening for hemochromatosis mutations in NAFLD with hyperferritinemia should be considered in case transferrin saturation is also elevated.[60] The influence of the iron regulators (*i.e*. TfR1/2, ferroportin) was the subject of many studies however, no consistent results were found.

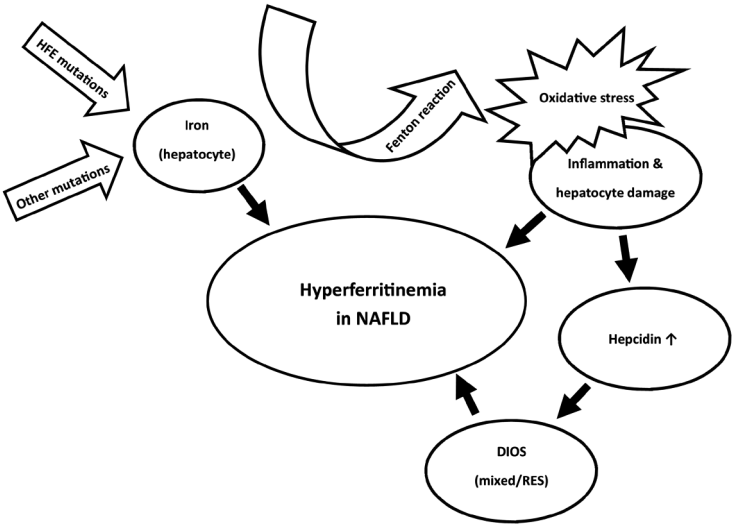

Figure 2.2 **Hyperferritinemia is observed in case of inflammation or in case of iron overload.** In non-alcoholic fatty liver disease (NAFLD) a minority of patients has significant hepatic iron overload, caused by mutations in the hemochromatosis gene or other mutations. However, in the majority of patients, there is no evidence of hepatic iron overload, in this case inflammation and hepatocyte damage can contribute to elevated serum ferritin levels. An up-regulation of hepcidin levels in inflammation can result in impaired iron export from the cells, which can lead to mild hepatic iron overload in hepatocytes, and/or reticuloendothelial cells (RES). Excess iron will contribute to the disease progression, since it will generate reactive oxygen species and lead to oxidative stress through the Fenton reaction.

Liver biopsy is still the gold standard as a diagnostic tool in NAFLD staging and is also useful to measure iron status. However, many patients are not willing to undergo a biopsy. MRI is a good alternative to measure the liver iron concentration (LIC) to determine iron accumulation in patients with hyperferritinemia. The reference value for the LIC is below 36 µmol/g dry weight but the LIC can also be elevated without liver iron excess (*e.g.* in patients with obesity or the metabolic syndrome). For this reason it is suggested to only diagnose iron overload in case the LIC exceeds 150 µmol/g dry weight.[16,61] It should be noted that others also use three times the upper limit of normal.[62] When increased iron accumulation is suspected, patients should be screened for hemochromatosis and therapeutic phlebotomies should be considered. In case of a hyperferritinemia accompanied by a normal transferrin saturation there is no need for *HFE* screening or therapeutic phlebotomy since the hyperferritinemia is most likely related to inflammation whether or not combined with DIOS. In these patients phlebotomy has not been proven more effective than

lifestyle changes.[59] In case of a clear inflammatory state, anti-inflammatory treatment and lifestyle changes should be considered.[16]

Based on the above, we suggest a diagnostic and therapeutic algorithm for the approach of NAFLD patients with hyperferritinemia (Figure 2.3).

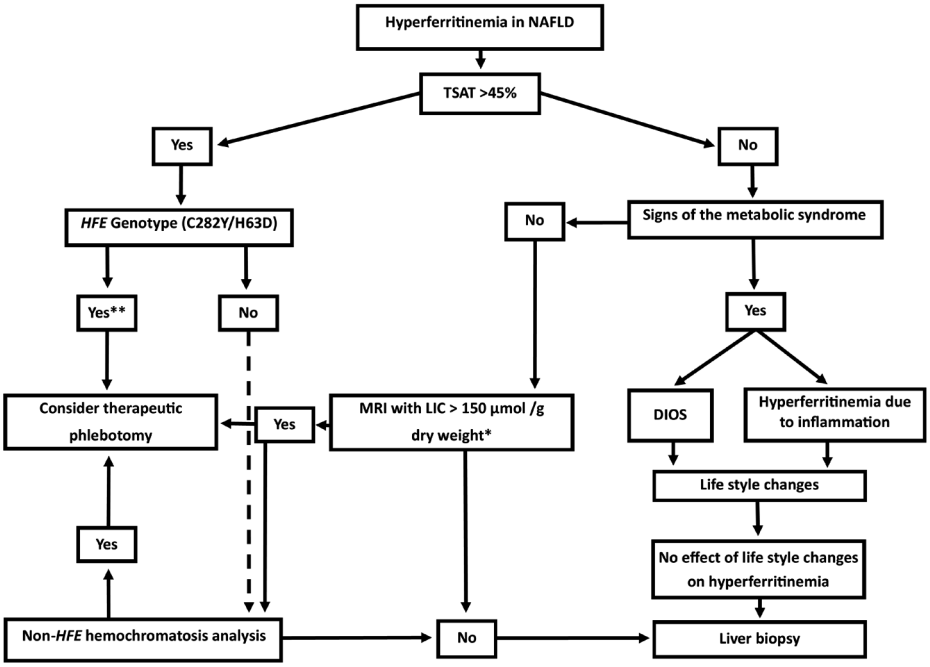

Figure 2.3 Diagnostic and therapeutic algorithm for the approach of hyperferritinemia in patients with NAFLD. *Advice based on Deugnier et al. 2017. **In case the serum ferritin is above 1000 µg/L, then assess for the presence of liver cirrhosis (e.g., liver biopsy, elastometry) Based on EASL Clinical Practice Guidelines for *HFE* hemochromatosis. J Hepatol 2010. *Abbreviations: Non-alcoholic fatty liver disease (NAFLD), Transferrin saturation (TSAT), dysmetabolic iron overload syndrome (DIOS), liver iron concentration (LIC).*

This review has several strengths, its focused research question, appropriate inclusion criteria and an extended search over a widespread period of years in four databases. We also assessed the quality of the included articles with regard to the risk of bias (Supplementary Table S2.1). A major limitation is the heterogeneity of the included studies with regard to their objectives, study population and methods. Furthermore, the studies are often based on relatively small retrospective and/or selective cohorts, which makes the results difficult to interpret. Besides, different methods were used to diagnose NAFLD and NASH, and liver biopsy was not always used as golden standard.[33,34,36] Another limitation is the possibility of selection bias in the studies, as

some included NAFLD/NASH patients referred for *HFE* mutation screening as a result of their hyperferritinemia enlarging the a priori chance for positive *HFE* mutations.[20,30] Also, multiple included studies were obducted by the same authors and the use of overlapping populations could not be ruled out.

In conclusion, in the majority of cases, hyperferritinemia in NAFLD seems to be mainly related to inflammation. In some cases this can lead to mild hepatic iron accumulation in reticuloendothelial- or mixed pattern as an expression of DIOS, that is strongly related to the metabolic syndrome. Only in case of elevated transferrin levels, typically seen in classical iron overload (hepatocyte pattern) screening for *HFE* mutations should be performed. Phlebotomy is only effective when classical iron overload is proven. Lowering iron content probably decreases the generation of oxidative stress that plays a role in liver- and systemic inflammatory reactions. Large population studies will be necessary to evaluate the role of hyperferritinemia in the different stages of NAFLD.

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Table S2.1 The adjusted Quality Assessment scale with regards to risk of bias based on the NewCastle-Ottawa Quality Assessment scale

Author	Year	Selection				Comparability	Outcome		
		Is the study population defined adequately?	Is the comparative group defined adequately?	Representativeness of the study population?	Representativeness of the controls comparative group?	Comparability of groups on the basis of design or analysis	Assessment of the outcome iron	Assessment of outcome inflammation	In case of ≥2 groups that are compared: same method of ascertainment for groups
Adams *et al.*	2015	B*	B*	B	B	B*	B*	D	A*
Aigner *et al.* (Am J Clin Nutr.)	2008	A*	A*[1]	B	B	C	A* + B*	A* + B*	A*
Aigner *et al.* (gastroenterology)	2008	A*	A*	A*	B	C	C	C	A*
Akin *et al.*	2003	A*	NA	B	NA	C	D	D	NA
Barisani *et al.*	2008	A*	A*[2]	B	B	C	A*	D	A*
Beaton *et al.*	2014	A*	NA	B	NA	NA	A* + B*	A* + B*	NA
Boga *et al.*	2015	A*	B	A*	B	C	A* + B*	A* + B*	B
Bonkovsky *et al.*	1999	A*	D[4]	B	B	C	C	C	A [5]
Bugianesi *et al.*	2004	A*/B*[3]	NA	A*	NA	B*	A* + B*	A*	A*
Canbakan *et al.*	2007	A*	NA	B	NA	C	A* + B*	A* + B*	A*
Chitturi *et al.*	2002	A*	D[4]	A*	B	A*	A* + B*	A*	A*[5]
Deguti *et al.*	2003	A*	NA	A*	NA	C	A* + B*	A*	A*
Dongiovanni *et al.*	2015	A*	A*	B	B	C	A* + B*	A*	A*
Duseja *et al.*	2005	D[7]	NA	B	NA	NA	A* + B*	A*	NA
Fujita *et al.*	2009	A*	A*	A*	B	B*	C	C	A*
George *et al.*	1998	A*	D[4]	B	A	C	A* + B*	A*	A*[5]
Hagström *et al.*	2016	A*	NA	A*	NA	B*	A*	A* + B*	A*
Hoki *et al.*	2015	A*	D	A*	B	C	A* + B*	A*	B
Koruk *et al.*	2003	A*	B*	B	B	C	D	A* + B*	B
Kowdley *et al.*	2014	A*	NA	A*	NA	B*	A* + B*	A*	A*
Lin *et al.*	2005	B*	C	A*	B	C	B	D	B
Loguercio *et al.*	2004	A*	NA	A*	NA	B*	A*	A* + B*	A*
Manousou *et al.*	2011	A*	NA	A*	NA	B*	C	A*	A*
Nelson *et al.*	2011	A*	NA	A*	NA	B*	C	C	A*
Oh *et al.*	2015	A*	NA	B	NA	C	B*	B*	A*
Parikh *et al.*	2015	A*	B*	A*	B	A*	B*	A*	B
Rahka *et al.*	2010	A*	NA	A*	NA	NA	A*	A*	NA
Rametta *et al.*	2016	A*/B*[3]	A* (DIOS), B* (C), B* (HH)[5]	B	B[1]	B*	B*	D	A*

Table S2.1 (continued)

Author	Year	Selection				Comparability	Outcome		
		Is the study population defined adequately?	Is the comparative group defined adequately?	Representati-veness of the study population?	Representati-veness of the controls comparative group?	Comparability of groups on the basis of design or analysis	Assessment of the outcome iron	Assessment of outcome inflammation	In case of ≥2 groups that are compared: same method of ascertainment for groups
Sumida *et al.*	2003	A*	D[6]	B	B[6]	B*	B*	B*	A*
Tanaka *et al.*	2008	A*	NA	B	NA	NA	B*	A* + B*	NA
Tsuchiya *et al.*	2010	A*	A*	B	B	C	A* + B*	C	A*
Uraz *et al.*	2005	A*	NA	B	NA	D	A* + B*	A*	NA
Utzschneider *et al.*	2014	A*/B*[3]	C	B	B	A	B*	A* + B*	A*
Uysal *et al.*	2011	D[4]	B	B	B	C	B*	B*	A*
Valenti *et al.*	2003	A*/B*[3]	D[2]	A*	B	B*	A*	A*	A*[2]
Valenti *et al.*	2012	A*	C	B	B	B*	A*	A*	B
Valenti *et al.*	2006	A*	C	A*	B	B*	A*	A* + B*	B
Valenti *et al.* (J Hepatology)	2010	A*	C	A*	A*	A*	A*	C	B
Valenti *et al.* (Gastroenterology)	2010	A*	C	A*	A*	B*	A*	A*	B
Yoneda *et al.*	2010	A*	C	B	B	C	B*	A* + B*	B
Zamin *et al.*	2006	A*	C[6]	B	B[6]	C	A* + B*	A*	A*[2]
Zimmerman *et al.*	2011	A*	C	A*	B	A*	A* + B*	A* + B*	A*

[1] Answer applicable for all groups (since more than one comparative group) used in the study [2] Diagnosis NAFLD is not excluded (by biopsy or imaging) nevertheless a star was scored since this was not relevant for aim of the study and our review [3] Not all patients underwent a liver biopsy, some were diagnosed by imaging [4] Diagnosis of NASH by ultrasound examination [5] More than one comparative group with different answers [6] Not all comparative groups used since they were not relevant for the aim of the review

Quality assessment with regards to bias: Questions 1-4 relate to the selection process. Per question one star (*) can be scored. In case of 4, 3, 2-1 stars the risk of bias is ranked as low , intermediate or high , respectively. The comparative group could include non-NAFLD patients or subjects with NAFLD/ NASH. Due to the heterogeneity of the studies, question 2 was interpreted as follows: not applicable (in case of no comparative group or the comparative group being not relevant in context of this review (e.g. hepatitis C or alcoholic fatty liver disease patients); which was scored as high risk of bias. When studies lacked a control group and/ or when the diagnosis NASH was made by imaging, they were also scored as high risk of bias.

For question 6 and 7, more than one star could be scored. In case of 5, 4-3, 2-1 stars the risk of bias is ranked as low , intermediate or high , respectively. Some studies subdivide their patient group during the study, in this case only comparability and the method of ascertainment will be scored.

Table S2.2 All included studies and their results about inflammatory and iron parameters

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
George *et al.* 1998	51 NASH (yes) 2465 Controls	504 (62%)	homozygosity p.C282Y 8% vs. 1%* heterozygosity p.C282Y 24% vs. 12%* homozygosity p.H63D 4% vs. 2%¥ heterozygosity p.H63D 28% vs. 22%¥ (NASH vs. controls)	NP	NASH: TS (Elevated in 22%), HIC (Elevated in 23%) Sig. association between ferritin levels and HIC
Bonkovsky *et al.* 1999	57 NASH (yes) 348 Controls	447	homozygosity p.C282Y 3% vs. 0%* heterozygosity p.C282Y 17% vs. 11%¥ homozygosity p.H63D 6% vs. 3%¥ heterozygosity p.H63D 44% vs. 26%* (NASH vs. controls)	NP	NASH: Significant association between ferritin levels and the presence of iron deposits
Chitturi *et al.* 2002	93 NASH (yes) 206 Controls	387 (40%)	homozygosity p.C282Y 0% vs. 1%¥ heterozygosity p.C282Y 22% vs. 9%* homozygosity p.H63D 2% vs. 1%¥ heterozygosity p.H63D 17% vs. 25%¥ (NASH vs. controls)	NP	NASH: TS (elevated in 5%)
Deguti *et al.* 2003	3 NASH with hepatic siderosis 29 NASH without hepatic siderosis (yes)	♂ 764 *, ♀ NP ♂ 349*, ♀ 220	heterozygosity p.C282Y 0% vs. 4%¥ homozygosity p.H63D 0% vs. 4%¥ heterozygosity p.H63D 33% vs. 21%¥ (NASH with hepatic siderosis vs. without hepatic siderosis)	No significant difference in histological inflammation between groups	NP

Table S2.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Lin *et al.* 2004	33 NAFLD (no) 125 Controls	331	homozygosity p.C282Y 0% vs. 0%¥ heterozygosity p.C282Y 0% vs. 0%¥ homozygosity p.H63D 3% vs. 3%¥ heterozygosity p.H63D 3% vs. 3%¥ (NAFLD vs. controls)	NP	NP
Zamin *et al.* 2006[a]	29 NASH (yes) 20 controls	258 (69%)	homozygosity p.C282Y 0% vs. 0%¥ heterozygosity p.C282Y 34% vs. 25%¥ homozygosity p.H63D 3% vs. 0%¥ heterozygosity p.H63D 14% vs. 5%¥ (NASH vs. controls)	NP	NASH: No association between ferritin levels and the presence of iron deposits
Valenti *et al.* 2002	134 NAFLD (yes in 67 patients) 291 controls	45%	(homozygosity p.C282Y 1% vs. 0%¥ heterozygosity p.C282Y 17% vs. 2%* homozygosity p.H63D 2% vs. 3%¥ heterozygosity p.H63D 27% vs. 23%¥ (NAFLD vs. controls)	NP	NAFLD: TS (elevated in 12%) Sig. association between ferritin levels and the presence of iron deposits
Valenti *et al.* 2010 (Gastro-enterology)	587 NAFLD (yes) 179 controls	NP	heterozygosity p.C282Y 6% vs. 4%¥ homozygosity p.H63D 3% vs. 4%¥ heterozygosity p.H63D 26% vs. 29%¥ (NAFLD vs. controls)	NP	NAFLD: Significant association between ferritin levels and the presence of iron deposits

Table S2.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Nelson *et al.* 2011	556 NAFLD with negative iron stain (Fe -) (yes) 293 NAFLD with positive iron stain (Fe +) (yes)	106* 328 *	NP	NAFLD Fe-: NAS significantly higher among those with iron in RES	NP
Kowdley *et al.* 2012	500 NAFLD with SF > 1.5 x ULN 128 NAFLD with SF < 1.5 x ULN (yes)	547 * 121 *	heterozygosity p.C282Y 8% vs. 9%¥ homozygosity p.H63D 1% vs. 2%¥ heterozygosity p.H63D 20% vs. 24%¥ (NAFLD with SF 1.5x ULN vs. SF < 1.5 ULN)	NAFLD with SF > 1.5 x ULN : NAS* ↑ vs. NAFLD with SF < 1.5 x ULN	NAFLD with SF > 1.5 x ULN : Iron in RES and hepatocytes* ↑ vs. NAFLD with SF < 1.5 x ULN
Hagstrom *et al.* 2016	89 NAFLD with hyperferritinemia (♂ > 350 µg/ L, ♀ > 150 µg/ L) 133 NAFLD without hyperferritinemia (yes)	455 * (40%) 132*	NP	NAFLD with hyperferritinemia: sign. higher NAS and more fibrosis vs. NAFLD without hyperferritinemia No differences in CRP and WBC between groups	Total 204/ 222 patients with iron staining In NAFLD with hyperferritinemia sig. more positive iron staining 48% vs. 21% (NAFLD without hyperferritinemia)

Table S2.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Aigner *et al.* 2008 (Am J Clin Nutr.)	32 NAFLD biopsy proven Fe+	796	heterozygosity p.C282Y 12.5% vs. 10.3 ¥ heterozygosity p.H63D 18.7% vs. 20.7 ¥ (NAFLD Fe + vs NAFLD Fe -) Liver FP-1 mRNA/ protein expression, HJV mRNA: sign. Lower in NAFLD Fe+/ Fe- vs. 20 controls Hepatic TfR-1 mRNA, duodenal FP-1 protein expression sign. Lower in NAFLD	NAFLD Fe +: Serum TNFα sign. higher vs NAFLD Fe- No differences in NAS between groups	Siderosis grade sign. correlated with hepcidin in both groups
	29 NAFLD biopsy proven Fe- (yes)	196	Fe+ compared to controls and NAFLD Fe-) Hepcidin mRNA sign. higher in NAFLD Fe+ compared to controls/ NAFLD Fe- and correlates with ferritin in NAFLD Fe+/ Fe- No differences in DMT-1 expression between groups		
Tsuchiya *et al.* 2010	11 NASH	225 ¥	HJV mRNA , TfR2 mRNA/ protein, TfR1 mRNA, FP-1 Mrna/ protein sig. higher in	NP	NASH: Hepatic iron sign. elevated vs. controls
	17 HS (Yes)	179 ¥	NASH/ HS compared to controls NASH: Hepcidin mRNA*↑ vs. HS No differences in hepcidin protein		No differences in serum iron and TS between groups
	8 Controls	180 ¥	between groups		

Table S2.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Valenti *et al.* 2010 (J Hepatology)	274 NAFLD (yes)	46%	Heterozygosity p.C282Y 10% vs. 6%¥, Homozygosity p.H63D 5% vs. 3%¥ (NAFLD vs. controls) NAFLD: Beta-thalassemic trait *↑ vs controls and a sign. higher risk of hepatocellular siderosis No differences in prevalence of polymorphism FP-1 between groups NAFLD: (n57) Serum hepcidin sign. more frequently > 3 Nm in patients with non-parenchymal siderosis	NP	Liver siderosis in: Hepatocytes: 11.7% In non-parenchymal cells or Mixed pattern: 30.6% No hepatic iron: 57.7%
	179 Controls	NP			
Valenti *et al.* 2012	216 NAFLD (yes)	286	Heterozygosity p.C282Y 7% vs. 1%¥ Homozygosity p.H63D 4% vs. 3%¥ Heterozygosity p.H63D 25% vs. 28%¥	NP	NAFLD: Liver siderosis correlated with heterozygosity p.C282Y and homozygosity p.H63D
	271 Controls	37	Homozygosity TMPRSS6 p.Ala736Val ¥ (NAFLD vs controls)		
Hoki *et al.* 2015	25 NASH	182*	NASH: Duodenal DMT-1 mRNA *↑ vs controls	NASH: NAS*↑ vs HS	NASH: Iron absorption: TS, serum iron (After OIAT test) sig. higher vs. HS/ controls
	15 HS (Yes)	145	Duodenal FP- 1 mRNA ¥ not sig. different between groups Serum hepcidin-25 sig. Higher in NASH/ HS compared to controls		
	9 Controls	75*			

Table S2.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Oh *et al.* 2015	87 NAFLD with MPVA ≥250 min/ week Before/ after/ change 82 NAFLD with MPVA <250 min/ week Before/ after/ change (Yes)	198/ 171/ -27 *↓ NP* 	NP	NAFLD with MPVA ≥250 min/ week: TBARS *↓, Steatosis*↓ vs. NAFLD with MPVA <250 min/ week (more improvement) No significant differences in loghsCRP, logTNFα and IL-6 between groups	NP
Rametta *et al.* 2016	18 NAFLD (yes) 23 controls	140 63	Homozygosity p.C282Y 0% vs. 0%¥ Heterozygosity p.H63D 26% vs. 26%¥ Serum hepcidin ¥ (NAFLD vs controls)	NP	No differences in TS between groups
Aigner *et al.* 2008 (gastroenterology)	140 NAFLD (yes) 25 controls	NP	Heterozygosity p.C282Y 8% vs 12%¥ Heterozygosity p.H63D 14% vs 16%¥ (NAFLD vs controls)	NP	NP
Barisani *et al.* 2008	8 NAFLD Fe – 25 DIOS (yes)	266 1032	NAFLD Fe-: TfR1 mRNA *↑, DMT1 mRNA *↑, urinary hepcidin *↓ vs. DIOS No differences in TfR2 mRNA, FP-1 mRNA and hepcidin mRNA between groups	NP	NP

Table S2.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Boga *et al.* 2015	66 NAFLD (Yes) 35 Controls	140* 98*	NAFLD: HJV *↓ vs. controls No differences in serum hepcidin between groups	NAFLD: hsCRP *↑ vs controls	Positive iron staining in 18% in NAFLD patients no differences in TS between groups
Beaton *et al.* 2014	28 NAFLD (yes) pre phlebotomy (Baseline) 28 NAFLD Post phlebotomy	384 (61%) 88	NP	No differences in presence of NASH between groups No association found between ESR, CRP and SF	HIC elevated in 11% with sig. decrease post phlebotomy Sig. correlation between HIC and SF
Dongiovanni *et al.* 2015	23 NASH + 23 HS (yes) 10 Controls	689 240	Sig. higher FP-1 mRNA in NASH compared to HS and controls	NP	NP
Rahka *et al.* 2015	206 NAFLD (yes)	237	NP	Portal inflammation correlated with serum ferritin levels	NP
Manousou *et al.* 2011	65 NASH 47 HS (yes)	309 * 146 *	NP	No differences in CRP between groups In NASH + HS: Portal inflammation correlated with serum ferritin levels	No differences in serum iron and TIBC between groups
Yoneda *et al.* 2008	62 NASH 24 HS (yes)	279 * 165*	NP	NASH: CRP*↑ vs. HS	NP

Table S2.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Zimmerman *et al.* 2011	25 NASH (yes) 37 Controls	180 (sig. correlated with serum hepcidin)	Serum hepcidin not sig. different vs controls	NASH: CRP not sig. correlated with serum hepcidin NAS sig. correlated with serum hepcidin)	NASH: No iron deposition in liver biopsy
Utzschneider *et al.* 2013	15 NAFLD (yes) 15 Controls	362* 207*	NP	No differences in TNFα, hsCRP and L-6 between groups	NP
Parikh *et al.* 2015	55 NAFLD (yes)	51	NP	Presence of NASH correlated with SF	NP
Uysal *et al.* 2011	60 NASH (no) 28 Controls	118*↑ 27*	NASH: sTfR*↑ vs. controls No difference in hepcidin level between groups	NASH: TNFα, IL-6, IL-8 and MDA*↑ vs. controls	No sig. differences in serum iron and UIBC between groups
Bugianesi *et al.* 2004	263 NAFLD (yes) 200 controls	239 (21%)	Homozygosity p.C282Y 0% vs. 0% Heterozygosity p.C282Y 5% vs. 3% Homozygosity p.H63D 1% Heterozygosity p.H63D 24% vs. 19% (NAFLD vs controls)	In NAFLD: correlation between steatosis, fibrosis and serum ferritin levels	In NAFLD: TS (elevated in: 7.4%), no association found between hepatic iron and *HFE* mutations
Loguercio *et al.* 2004	305 NAFLD (yes)	NP (35%)	NP	Sign. correlation between: steatosis, fibrosis, lobular, portal inflammation and serum ferritin levels	Histological hepatic hemosiderosis (elevated in: 19%) (sign. correlated with SF)

Table S2.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Koruk *et al.* 2002	18 NASH (yes) 16 Controls	173 * 82*	NP	NASH: CRP*↑ vs. controls And hepatic inflammation, fibrosis, fatty infiltration not sig. correlated with serum ferritin levels	NP
Canbakan *et al.* 2007	38 HS 67 NASH (yes)	71* 110 *	NP	NASH: MDA and NO*↑ vs. HS	No hepatic iron deposition found in any group. No difference in serum iron, TS and TIBC between groups.
Tanaka *et al.* 2008	23 NASH pre EPA treatment 23 NASH post EPA treatment	228 * 124 *	NP	Pre EPA treatment TRX*↑ vs. post EPA treatment and no difference in TNFα	No difference in serum iron and TS between groups
Sumida *et al.* 2001	31 NAFLD (yes) 17 controls	170	NP	NAFLD: TRX*↑ vs. controls and sig. correlation between TRX and serum ferritin levels	NP
Adams *et al.* 2015	33 NAFLD with phlebotomy 41 NAFLD without phlebotomy (lifestyle advice)	Only in NAFLD with phlebotomy sign. decrease ferritin after intervention compared with before	NP	NP	NP

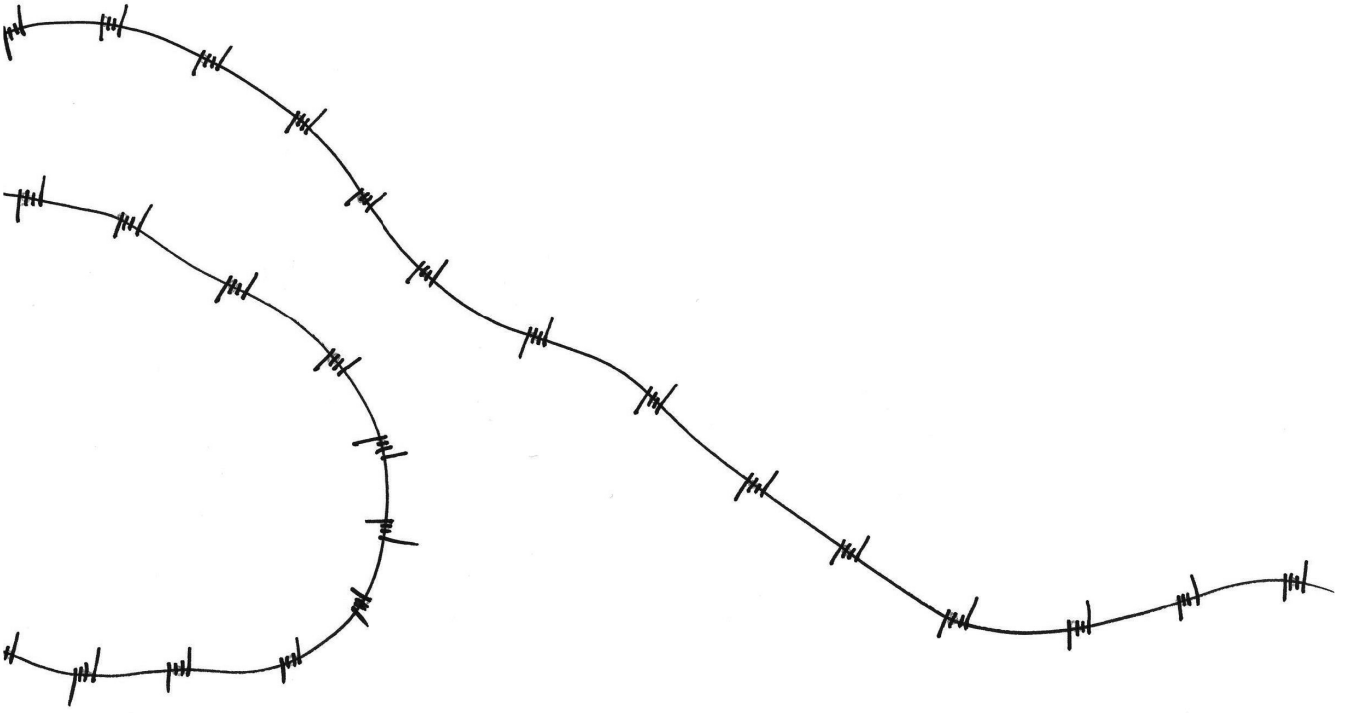
Table S2.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Fujita *et al.* 2009	38 NASH 24 HS (yes)	283 * 139 *	NP	NASH: Hepatic 8-oxodG vs. HS No difference in steatosis between groups HS: correlation between Hepatic 8-oxodG and serum ferritin levels	NASH: Sig. higher serum iron, TS and total iron score vs. HS
Akin *et al.* 2003	53 NASH (yes)	136	NP	NP	NP
Duseja *et al.* 2005	31 NASH (Yes)	NP	NP	NP	No association found between the presence of iron deposition and inflammation or fibrosis
Uraz *et al.* 2005	25 NASH (yes)	140	NP	NP	NP

*Abbreviations: not provided (NP), non-alcoholic steatohepatitis (NASH), hepatic iron concentration (HIC), transferrin saturation (TS), total iron-binding capacity (TIBC), NAFLD activity score (NAS), reticuloendothelial system (RES), iron in both hepatocytes and the reticuloendothelial system (mixed), serum ferritin (SF), upper limit of normal (ULN), moderate to vigorous intensity physical activity (MVPA), C-reactive protein (CRP), white blood count (WBC), patients with iron overload (Fe+), patients without iron overload (Fe-), tumour necrosis factor α (TNFα), ferroportin (FP-1), hemojuvelin (HJV), transferrin receptor (TfR 1), divalent metal transporter (DMT1), homozygosity for the alfa-1-antitrypsine mutation (non-MM genotypes AAT), Transmembrane protease serine 6 (TMPRSS6), oral iron absorption test (OIAT), Thiobarbituric acid reactive substances (TBARS), interleukin (IL), erythrocyte sedimentation rate (ESR), soluble transferrin receptor (sTfR), unsaturated iron binding capacity (UIBC), malondialdehyde (MDA), hepatic steatosis HS), purified eicosapentaenoic acid (EPA), nitric oxide (NO), thioredoxin (TXR). *significant difference between marked values in horizontal columns (p< 0.05), ¥ no significant difference between marked values in horizontal columns.*

Chapter 3

Hyperferritinemia and liver iron content determined with MRI: a new role for the liver iron index



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Submitted

Abstract

Goals

Hyperferritinemia is found in around 12% of the general population. Analyzing its cause can be difficult. In case of doubt about the presence of major iron overload most guidelines advice to perform an MRI as a reliable non-invasive marker to measure liver iron concentration (LIC). In general, a LIC of ≥36 µmol/g is considered to be elevated however in hyperferritinemia associated with for example obesity or alcohol (over)consumption the LIC can be ≥36 µmol/g in absence of major iron overload. So unfortunately, a clear cut-off value to differentiate iron overload from normal iron content is lacking. Previously the liver iron index (LII) (LIC measured in liver biopsy (LIC-b)/age (years)), was introduced to differentiate between patients with major (LII≥2) and minor or no iron overload (LII<2). Based on the good correlation between the LIC-b and LIC measured with MRI (LIC-MRI), our goal was to investigate whether a LII-MRI ≥2 is a good indicator of major iron overload, reflected by a significantly higher amount of iron needed to be mobilized to reach iron depletion.

Methods

We compared the amount of mobilized iron to reach depletion and inflammation-related characteristics in two groups (LII-MRI ≥2 versus LII-MRI <2) in 92 hyperferritinemia patients who underwent *HFE* genotyping and MRI-LIC determination.

Results

Significantly more iron needed to be mobilized to reach iron depletion in the LII-MRI≥2 group (mean 4741 SD±4135 mg) versus the LII-MRI<2 group (mean 1340 SD±533 mg), P<0.001. Furthermore, hyperferritinemia in LII-MRI<2 patients was more often related to components of the metabolic syndrome while hyperferritinemia in LII-MRI ≥2 patients was more often related to *HFE* mutations.

Conclusion

The LII-MRI seems with a cut-off value of 2 is an effective method to differentiate major from minor iron overload in patients with hyperferritinemia.

Introduction

Hyperferritinemia is common. It is found in around 12% of the general population and can be related to iron overload such as observed in *HFE*-related hemochromatosis (HH), non HFE-related hemochromatosis or secondary iron overload due to hematological conditions.[1,2] Other causes of increased serum ferritin levels include alcohol (over)consumption, metabolic syndrome, dysmetabolic iron overload syndrome (DIOS), chronic hepatic inflammatory diseases and non-hepatic inflammatory conditions.[3-5] Differentiation between hyperferritinemia resulting from iron overload or inflammation can be difficult. Nevertheless, early detection of hepatic iron overload is essential in order to start iron depletion therapy in time since accumulation of iron in the liver can lead to fibrosis, cirrhosis and hepatocellular carcinoma.[6] A consensus on a definition for "iron overload" is lacking.[7,8] Increased iron stores are defined as >2.0 g (2-4 g, mildly increased). While iron overload is defined as iron stores >4 g (4–10 g, moderately increased; 10–20 g, substantially increased; and >20 g, severely increased).[9-11] The amount of mobilized iron to reach iron depletion is the most objective and reliable indicator of the amount of iron accumulation. Since this can only be calculated in retrospect, there is need for a reliable diagnostic tool to predict the amount of iron overload.[7,12]

The liver iron concentration (LIC) currently is considered the best method to accurately assess body iron load, since the liver contains ≥70% of the body iron stores.[13] The LIC can be determined by liver biopsy (LIC-b) and by MRI (LIC-MRI). LIC-b and LIC-MRI show a good correlation (r=0.87).[14-18] Nowadays, LIC-MRI quantification has been proposed as the new gold standard for diagnosing iron overload[19], since liver biopsy is an invasive procedure with potentially serious complications and it carries the risk for sample error.[13] The reference value for the LIC is below 36 µmol Fe/g however in hyperferritinemia associated with obesity, metabolic syndrome, DIOS and/or alcohol (over)consumption the LIC can be ≥36 µmol/g in absence of major iron overload.[14] For this reason, different cut-off values were suggested[14,20-22] leaving a grey zone for LIC values between 36-150 Fel/g.

Bassett *et al*. introduced the liver iron index (LII), measured in liver biopsies, to differentiate between homozygous HH and other causes of iron accumulation like alcoholic liver disease or heterozygous hemochromatosis mutations. The LII is calculated as LIC (µmol/g dw) / age (years) and a LII-b ≥2 indicates iron overload in homozygous hemochromatosis patients in contrast to a LII-b <2 found in patients with alcoholic liver disease of heterozygous hemochromatosis mutations.[23] Later, there was implied that the LII can also be used to differentiate between all patients with major iron overload, so also the patients with secondary hemochromatosis or other

genetic hemochromatosis conditions and patients with only minor iron overload.[24] Suggesting that the LII can be a tool to overcome the grey zone in interpretating LIC values. We anticipate that, based on the correlation between LIC-b and LIC-MRI , the LII-MRI will be a good and reliable non-invasive method to diagnose patients with major iron overload. Since the MRI is now incorporated in guidelines and seen as the new non-invasive gold standard method to diagnose iron overload, there is a need to improve the interpretation of the LIC-MRI values and to overcome the grey zone of moderately elevated LIC-MRI values. Since the amount of mobilized iron to reach iron depletion is the most objective method to define iron overload, we will use this parameter as the gold standard to validate the LII-MRI.

We hypothesize that patients with a LII-MRI ≥2, based on the LIC measured with MRI, have major iron overload, which will be reflected by a significantly higher amount of iron that is mobilized to reach normal iron stores compared to patients with a LII-MRI <2. Secondly, we expect that patients with hyperferritinemia and a LII-MRI <2 have other causes for a hyperferritinemia such as metabolic syndrome, DIOS or alcohol (over)consumption but lack significant iron overload. Therefore, the aim of our study was to investigate whether the LII-MRI is able to discriminate major iron overload from minor increased iron stores in patients with hyperferritinemia.

Materials and methods

Study participants

We conducted a retrospective analysis of data obtained in patients with hyperferritinemia. We screened all patients referred to the outpatient hemochromatosis clinic of the Maastricht University Medical Centre (MUMC+), the Netherlands, between September 2016 and September 2018. Additionally we screened all patients who underwent a MRI according to the iron protocol, in the MUMC+, between June 2012 and October 2018. In the MUMC+ during that time period all patients with hyperferritinemia underwent a liver MRI and *HFE* gene analyses as part of the diagnostic process.

We included patients meeting all of the following criteria: a) serum ferritin levels above the upper limit of the reference range (>200 µg/L for women, and >300 µg/L for men) b) an MRI with LIC determination according to the Rennes University method[14] and c) *HFE* genotyping (Figure 3.1). There were no liver biopsies with LIC measurement available for these patients.

We included only patients in whom no iron had been mobilized before the first MRI. An exception was made for 11 patients in whom LII-MRI was well above 2, despite

iron mobilization before their first MRI. The Medical Ethics Committee of the MUMC+ had approved this study, waiving the requirement to obtain informed consent.

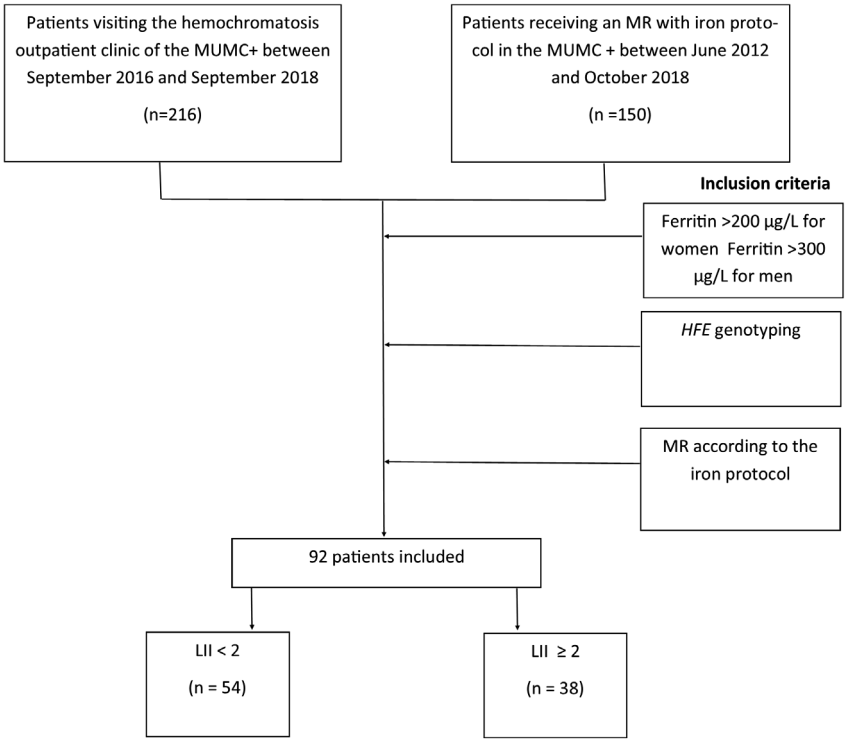

Figure 3.1 Study design and inclusion criteria.

The rationale behind introducing the factor age is that iron accumulation in a hereditary iron overload condition is a dynamic process in the course of life. On a yearly basis, patients with HH absorb about 1 gram more iron than the body requires.[25] A LII-b ≥2 was found to indicate major iron overload while a LII-b <2 is not indicative for iron overload syndromes but represents none or minor iron overload. For example an LIC of 75 µmol/g in a 35 year old patient means that after correction for age the LII =2.1. In a person aged 60, the same LIC corrected for age corresponds with a LII of 1.3. If this 60 years old person would have a hereditary condition resulting in iron overload the iron accumulation had continued for years and would have resulted in a significantly higher LIC.

MRI

The MRI equipment consisted of a Philips type Intera/Ingenia 1.5 T MRI. In all patients we used the signal intensity ratio method, which requires the calculation tool of the University of Rennes, France, to calculate the LIC.[13,14] Gandon et al. created this tool after correlating the liver-to-muscle signal intensity ratio with the LIC-b. The tool shows the gradient recalled echo T2 ++ sequence with the highest sensitivity (89%), specificity (80%), and a strong correlation (r=0.87) between the liver-to-muscle signal intensity ratio and LIC-b.[14] In most cases the MRI was performed within reasonable time after *HFE* gene analysis.

Study parameters

The following data were obtained from the medical files: age at time of diagnosis of hyperferritinemia, gender, dates of MRIs, alcohol usage, laboratory values including iron parameters, hemoglobin, liver enzymes and lipid parameters, relevant medication (proton pump inhibitor, H2 antagonists, antihypertensive and antihyperlipidemic drugs), presence of metabolic disorders (diabetes mellitus, obesity, hypertension and hypercholesterolemia) and relevant comorbidities (i.e. malignancies, chronic inflammatory disorders, rheumatic diseases, hematological diseases). All available data on mutations influencing the iron metabolism or the presence of blood transfusions to check for alternative explanations for iron accumulation were taken into account. The presence of the metabolic syndrome was defined when 3 out of the 5 following criteria were found. Hypertension (>130/85 mmHg), diabetes or fasting glucose levels above 5.6 mmol/L, elevated triglyceride levels (>1.7 mmol/L), decreased HDL levels (<1.29 mmol/L in women and <1.03 mmol/L in men) or elevated waist conferences (>88cm in women and >102 in men). When 2 out of 5 criteria where not known the presence of the metabolic syndrome was marked as a missing value. However since we have the waist conferences only sporadically available this will give an underestimation of the presence of the metabolic syndrome.

Mobilized iron

Since many patients had erythrocytapheresis and/or phlebotomies we decided to work with the amount of mobilized iron instead of the frequency of phlebotomy or erythrocytapheresis. We documented the amount of mobilized iron for all patients who reached iron depletion. For that, we used the dates the physicians had documented as the end of the initial therapy (serum ferritin concentration <50 µg/L). The amount of mobilized iron was calculated based on the number of phlebotomies

(i.e. 250 mg iron for each phlebotomy of 500 ml) and for erythrocytapheresis the the volume of erythrocytes (in ml) withdrawn by each erythrocytapheresis were multiplied by 0.80 based on the actual hematocrit of removed erythrocytes.[26] Patients treated with iron chelation therapy were excluded when calculating mobilized iron during the iron depletion stage. We defined iron overload as iron stores >4 grams; iron stores in the range of 2 grams to 3 grams are defined as mildly increased.

Statistical analysis

For the statistical calculations, we used *IBM SPSS* statistics version 23 for Windows. Continuous variables are expressed as mean with a standard deviation (SD) in case of non-skewed variables and as median with an interquartile range in case of skewed variables. The categorical variables are presented as absolute figures and percentages. Comparison of values between the groups in case of continuous variables was performed by using the independent T-Test in case of normally distributed variables and the Mann-Whitney U test in skewed distributed variables. Comparison of values between the groups in case of categorical variables was performed using the chi-square test or Fisher's exact test. Between patients there were differences in the amount of time between the diagnosis and the moment of iron depletion. To correct for these differences in time we used linear regression analysis. After correcting for the differences in time, the results were similar in all groups. We decided to show the non-corrected results in the text, in the table the corrected results are shown as well (see Table 3.2). Pearson correlation was used to assess the correlations between the parameters, in case of skewed distribution they were logarithmically transformed. Due to small subgroups no multivariate analysis was conducted to adjust for confounding factors. A two-sided P-value of $p<0.05$ was considered statistically significant.

Results

Patient characteristics

The population consisted of 92 hyperferritinemia patients of whom 72.8% was of male gender. The mean age at diagnosis of hyperferritinemia was 55 (13.5) years. Mean follow-up time was 5.4 (2.9) years. Only 49.4% (44/89) also had an increased transferrin saturation. Serum ferritin levels ranged from 245 to 10.888 µg/L. Thirty-eight of 92 patients (41.3%) had a LII-MRI ≥2 (Table 3.1). In 24.2% (16/66) of patients the metabolic syndrome was present. In 42.2% of the hyperferritinemia patients no symptoms associated with hemochromatosis were present and elevated ferritin levels

were often discovered during regular or sport checkups. The other patients presented with fatigue (23.3%), arthralgia (12.2%), liver test abnormalities (3.3%), excessive alcohol consumption (3.3%), a diagnosis of porphyria cutanea tarda (1.1%) and 14.4% patients were diagnosed following a family screening.

Table 3.1 Patient characteristics of LII-MRI ≥2 group vs the LII-MRI <2 group.

	Liver iron index ≥2 N=38	Liver iron index <2 N=54	p-value
Age at diagnosis (years)	52 (15)	58 (13)	0.294
Gender (♂)	76.35% ♂	70.4% ♂	0.528
BMI kg/m^2	25.5 (3.2)	27.4 (4.2)	0.026
Abnormal liver tests	5.3% (2/38)	38.9% (21/54)	<0.001
Presence of Diabetes Mellitus	13.2% (5/38)	13.0% (7/54)	0.978
Presence of hypertension	23.7% (9/38)	57.4% (31/54)	0.001
LDL cholesterol (mmol/L)	2.72 (±1.42)	3.46 (±1.88)	0.041
Alcohol usage (U/day)	1.0 (1.0)	1.7 (2.6)	0.449
Proton pump inhibitor usage	23.7% (9/38)	25.9% (14/54)	0.807
MCV (fl)	95.87 (7.04) (30/38)	92.31 (5.07) (35/54)	0.021
Serum iron (µmol/l)	80.5 (219.7)	39.0 (133.3)	<0.001
Transferrin Saturation (%)	64.7 (27)	36.7 (13.4)	<0.001
Serum ferritin (µg/L)	2032 (2091)	683 (260)	<0.001
Metabolic syndrome	16% (4/25)	29.3% (12/41)	0.222
Presence of liver steatosis	29.0% (9/31)	58.5% (24/41)	0.013
Presence of liver fibrosis	11.8% (4/34)	14% (6/44)	0.808
LIC-MRI (µmol/g)	243.2 (95.8)	57.4 (19.0)	<0.001
LII-MRI	4.66 (1.82)	1.02 (0.36)	<0.001
Presence *p.Cys282Tyr* homozygotes	60.5% (23/38)	3.7% (2/54)	<0.001

Results are presented as mean (SD) or as a percentage with the exact numbers. *Abbreviations: LDL, low-density lipoprotein cholesterol; PPI, proton pump inhibitor; BMI, body mass index; LIC, liver iron concentration; LII, liver iron index.*

MRI measurements

Patients were subdivided into two groups based on the LII outcome of their MRI. Patient characteristics for each group are summarized in Table 3.1. In 38 patients the LII-MRI was ≥2 (mean 4.66 (1.82)) and in 54 patients the LII-MRI was <2 (mean 1.02 (0.36)). There were significantly more components of the metabolic syndrome (higher BMI, hypertension, liver test abnormalities, LDL cholesterol and steatosis) present in patients with a LII-MRI <2 (Table 3.1). There is a positive correlation between the LII-MRI and the ferritin levels at the time of the MRI (r 0.596, p<0.001).

Genetic mutations in relation to the liver iron index

Twenty-three of 38 LII-MRI ≥2 patients were homozygous for the *p.Cys282Tyr* mutation. In two of 54 LII-MRI <2 patients, homozygosity for the *p.Cys282Tyr*

mutation was found with ferritin levels of 655 µg/L and 561 µg/L. Half of the *p.Cys282Tyr*/ *p.His63Asp* compound heterozygosity patients (5/10) had a LII-MRI ≥2. Only seven patients with hyperferritinemia in combination with a normal transferrin saturation had a LII-MRI ≥2, four had rare non-*HFE* mutations, two had a mutation of the ceruloplasmin gene resulting in aceruloplasminemia, and one had secondary hemochromatosis resulting from frequent blood transfusions due to hereditary spherocytosis. In all LII-MRI ≥2 patients a genetic mutation affecting the iron metabolism was found (Supplementary Table S3.1). In three LII-MRI ≥2 patients these mutations were not known to result in iron accumulation. One patient being *p.Cys282Tyr* heterozygous also fit the criteria for the metabolic syndrome and frequently consumed alcohol. The two others were *p.His63Asp* homozygous patients, without documented components of the metabolic syndrome, however the presence of non-*HFE* mutations was never excluded. In Supplementary Table S3.1 we provide a detailed overview of the mutations affecting iron metabolism found in both groups.

Mobilization of iron in relation to liver iron index results

Not all patients received iron depletion therapy. In 20.7% (19/92) no iron depletion therapy was initiated, in 16 LII-MRI <2 patients this was due to the absence of iron overload and only lifestyle changes were given. In three LII-MRI ≥2 patients iron depletion was not possible due to a progressive anemia. In 8.7% (8/92), all with a LII-MRI <2, only a very small number of phlebotomies or erythrocytapheresis were started as a trial, they were stopped before the iron depletion stage was reached and most patients were switched on lifestyle changes. In 5.4% (5/92) patients treatment was started, but the iron depletion stage was not reached yet before the end of follow-up and 3.3% (3/92) received iron chelators. Iron depletion was reached in 57 patients: 29 patients in the LII-MRI ≥2 group and 28 in the LII-MRI <2 group. There was a strong positive correlation between the initial LII-MRI and the amount of mobilized iron during iron depletion ($r=0.737$, $P<0.001$). The amount of iron mobilized to reach iron depletion was significantly higher in the LII-MRI ≥2 group versus the LII-MRI <2 group: 4741 (4135) versus 1340 (553) mg iron (mean difference between groups 3401 mg, 95% CI 1817,4985 $p< 0.001$; see Table 3.2). In the LII-MRI ≥2 group, men needed to mobilize significantly more iron in order to reach iron depletion compared to women; 5351 mg (4373) versus 2401 mg (1819) respectively ($p=0.046$). The decrease in serum ferritin values at diagnosis to iron depletion was significantly higher in the LII-MRI ≥2 group (1565 (1459) µg/L) versus the LII-MRI <2 group (589 (282) µg/L) (mean difference between groups 976, 95% CI 412,1540, $p=0.001$; see Table 3.2). The mean hemoglobin after reaching iron depletion was 8.5 mmol/l in both groups.

Table 3.2 **Group comparison for the decrease in ferritin levels and the total amount of mobilized iron between the moment of diagnosis and iron depletion.**

	Amount of mobilized iron between diagnosis and iron depletion		Decrease in ferritin between diagnosis and iron depletion	
	LII-MRI ≥2	**LII-MRI <2**	**LII-MRI ≥2**	**LII-MRI <2**
Number of patients	29	28	29	28
Mean (SD)	4741 mg (4135)	1340 mg (533)	1565 µg/L (1459)	589 µg/L (282)
Mean difference between both groups (95%CI), p-value	3401*(1817,4985) p<0,001		976**(412,1540) p=0.001	

*If corrected for the differences in time within patients, between the diagnosis and iron depletion, the amount of mobilized iron was similar (3256 (95%CI 1652, 4859), p=0.000) ** If corrected for the differences in time within patients, between the diagnosis and iron depletion, the amount of mobilized iron was similar (991 (95%CI 414,1567), p=0.001)). *Abbreviations: N, patient population; SD, standard deviation; CI, confidence interval; LII, liver iron index.*

Discussion

In individual cases of hyperferritinemia it is difficult to differentiate between iron overload and inflammation as a cause of hyperferritinemia. Therefore a reliable non-invasive method is needed to diagnose iron overload because iron overload can result in organ damage.

In this study we have shown that patients with hyperferritinemia and a LII-MRI ≥2 have significantly higher iron stores. This was based on the finding that in patients with a LII-MRI ≥2 a significantly higher amount of iron had to be mobilized to reach iron depletion (4.7 grams versus 1.3 grams in the LII-MRI <2 patients). In neither the LII-MRI ≥2 nor the LII-MRI <2 group the treatment to reach the iron depletion stage resulted in anemia.

The patients with LII-MRI <2 had a significantly higher prevalence of components of the metabolic syndrome (Table 3.1) and had to mobilize a significantly lower amount of iron to reach iron depletion. These observations point to other explanations for hyperferritinemia and for moderately increased LIC's with none or only minor iron overload such as obesity, DIOS or NAFLD.[5]

We further investigated whether genetic mutations in the *HFE* gene were more frequently associated with LII-MRI ≥2. Homozygosity of the *p.Cys282Tyr* mutation in the *HFE* gene is the most common genetic mutation resulting in major iron overload. However, not all patients with *p.Cys282Tyr* homozygosity develop iron accumulation due to a low penetrance.[27] (Supplementary Table 3.1) Other contributing reasons are menstrual blood loss, occult blood loss from the digestive tract or medication which,

in combination with relatively low ferritin levels, can explain the LII-MRI <2 in the two *p.Cys282Tyr* homozygous patients.[28] In all our *p.Cys282Tyr* homozygous patients an increased transferrin saturation was found. Other *HFE* genotypes, like *p.Cys282Tyr* heterozygosity, *p.His63Asp* heterozygosity, and *p.His63Asp* homozygosity do in general not lead to major iron overload.[29-33] The effect of *p.Cys282Tyr*/ *p.His63Asp* compound heterozygosity on iron accumulation is controversial however in most cases is not associated with major iron overload.[30,34] To cause iron overload these mutations probably need to be accompanied by co-factors such as alcohol (over)consumption or metabolic syndrome.[35] All patients in our study with possible other explanations of major iron overload such as secondary iron overload, non-*HFE* mutations and mutations in the ceruloplasmin gene resulting in aceruloplasminemia were found in the LII-MRI ≥2 group (Supplementary Table S3.1). We deliberately not excluded patients with other causes of iron overload like aceruloplasminemia or secondary hemochromatosis. The LII-MRI can help to detect which mutations carry a low risk of developing iron overload, and can help to identify *p.Cys282Tyr* homozygous patients with a low phenotypic penetrance. On the other hand the LII-MRI helps to differentiate which patients should be checked for *HFE* and non-*HFE* mutations.

The LII-MRI is of clinical relevance to identify hyperferritinemia patients in definite need for iron depletion therapy. There are no strict criteria to indicate when iron depletion therapy should be started, not even in patients with *HFE*-related hemochromatosis.[7,35-38] A recent study showed beneficial evidence for iron depletion therapy in *HFE*-related hemochromatosis patients with ferritin levels between 300 µg/L and 1000 µg/L.[39] In DIOS and patients with NAFLD, iron depletion therapy is not advised as the hyperferritinemia is not related to significant iron accumulation.[40] Our results support these findings since in the LII-MRI <2 patients (e.g. DIOS and NAFLD patients) a significantly lower amount of iron needed to be mobilized to reach iron depletion compared to the LII-MRI ≥2 group (1340 (553) mg iron versus 4741 (4135)). Life style changes can beneficially affect metabolic syndrome, NAFLD and inflammation. However, in our retrospective analysis no data were available on the effect of life style changes on serum ferritin levels and iron stores.

In case of persistent iron overload when *HFE* gene analysis is negative and alternative explanations for hyperferritinemia (chronic alcohol consumption, inflammation, cell necrosis, tumors, NAFLD, metabolic syndrome) are lacking, the Dutch hemochromatosis guideline and the AASLD guideline advise to perform respectively a LIC-MRI or a LIC-b as the next step.[30,41] The EASL guideline advise the assessment of liver iron stores by MRI or liver biopsy in hyperferritinemia patients with normal or

low (≤45%) transferrin saturation in the absence of more common causes of hyperferritinemia.[7]

We introduced the role of the LII-MRI to indicate major iron overload. We suggest that in case of a LII-MRI ≥2 typical *HFE* and if negative additional non-*HFE* analyses should be performed and iron depletion should be initiated timely. In case of LII-MRI <2 there is no major iron overload present and other causes of hyperferritinemia should be considered such as the metabolic syndrome.

A strength of our study is that all patients with hyperferritinemia underwent *HFE* genotyping in combination with LIC-MRI. Many patients with a LIC-MRI >36μmol/g were diagnosed as having hepatic iron overload and were treated with phlebotomies or erythrocytaphereses until they reached iron depletion. This gave the opportunity to directly relate the amount of mobilized iron to reach iron depletion to the genetic mutations and the height of hyperferritinemia in these patients. There is no referral bias since the MUMC+ performed MRIs and *HFE* DNA analysis in all patients referred with hyperferritinemia, independent of the transferrin saturation. In addition the MUMC+ is, next to their specific hemochromatosis outpatient clinic also a city hospital and receives referrals of hyperferritinemia of all general practitioners in the environment.

A limitation of this study is its retrospective design. First, not all physicians documented iron depletion precisely at the time point when ferritin levels had decreased to below 50 μg/L. Second, the study population is relatively small and not all patients reached the phase of iron depletion during the follow-up period. Another limitation is that in some patients there was significant time between *HFE* gene analysis and the MRI. We cannot rule out that in this time period patients changed their lifestyle like decrease their alcohol intake or lose weight.

In conclusion, the LII-MRI is an effective method to help the differentiation between major and minor iron overload in patients being analyzed for hyperferritinemia. Hyperferritinemia in patients with LII-MRI <2 is more often related to components of the metabolic syndrome. The LII-MRI is not only suitable for patients with *HFE* hemochromatosis but also with non-*HFE* hemochromatosis and secondary causes of iron overload. The study topic is of interest since the use of MRI has now overtaken liver biopsy in most cases of differential diagnosis of hyperferritinemia. The study findings could potentially help address the diagnostic process and treatment decisions.

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Supplementary data

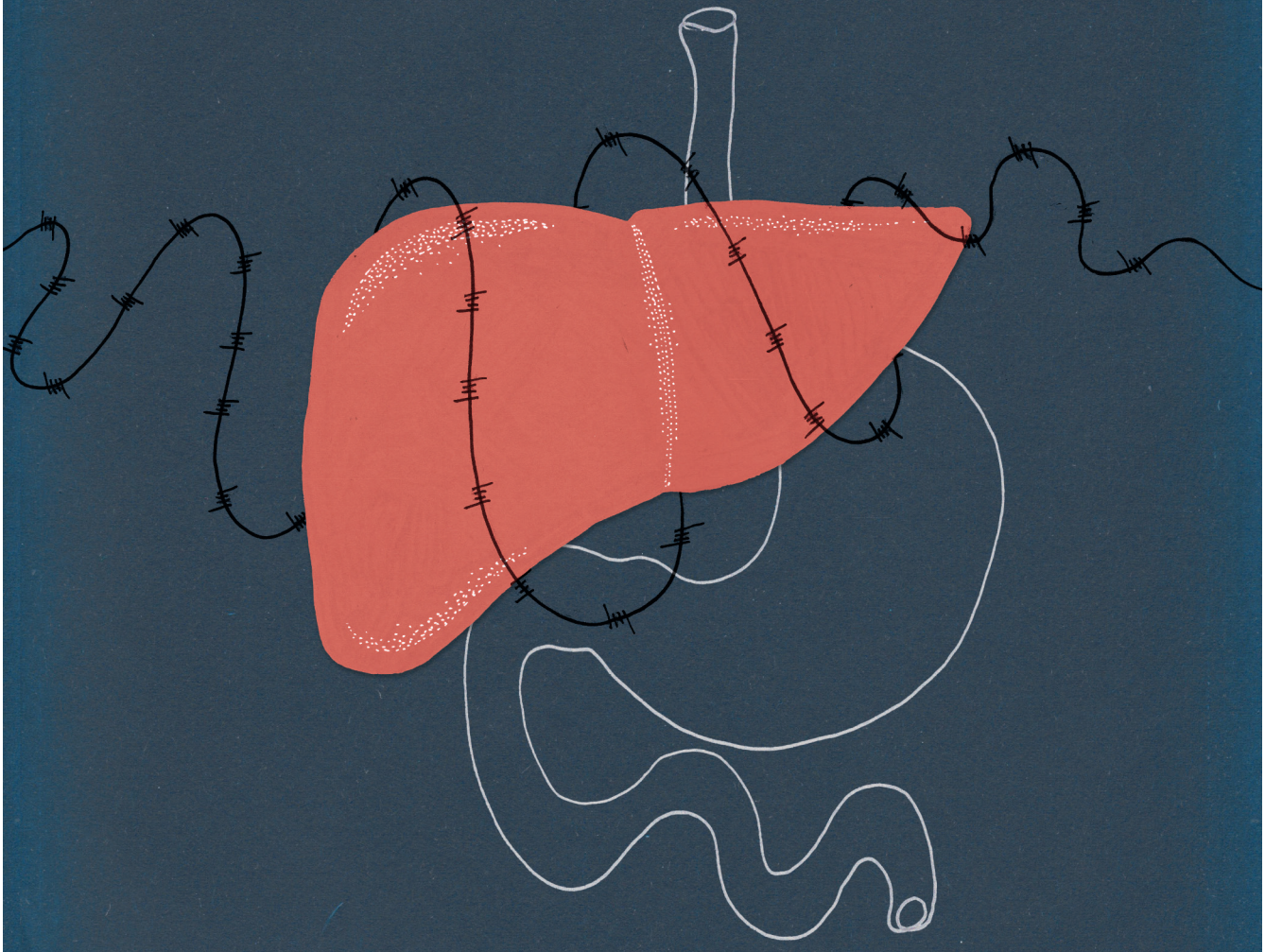
Table S3.1 Overview of the different mutations involving the iron metabolism, within both groups.

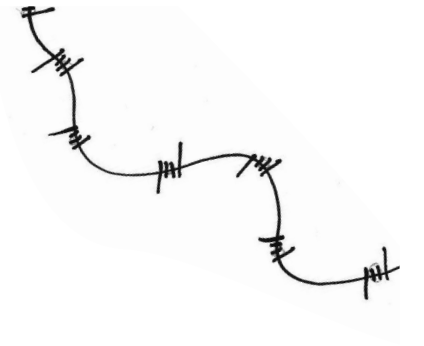
	MRI-LII ≥2 (n=38)	MRI-LII <2 (n=54)
p.Cys282Tyr homozygosity	23/38	2/54 *
p.Cys282Tyr/ *p.His63Asp* compound heterozygosity	5/38	5/54
p.Cys282Tyr heterozygosity	1/38 **	8/54
p.His63Asp homozygosity	2/38 ***	3/54
p.His63Asp heterozygosity	1/38 ****	8/54
Heterozygosity mutation in TRF1 gene[1]	0/38	2/54
p.His63Asp heterozygosity & Heterozygosity mutation in TRF1 gene[1**]	0/38	1/54
p.Ser65Cys heterozygosity	0/38	1/54
p.His63Asp /p.Ser65Cys Compound heterozygosity	0/38	1/54
Mutation ceruloplasmin gene	2/38	0/54
Mutation HAMP gene[2]	1/38	0/54
p.H63D heterozygosity & mutation in SLC40A1 Gene (type 4) [3]	1/38	0/54
p.H63D heterozygosity & mutation in SLC40A1 Gene (type 4) & mutation in HJV gene[4]	1/38	0/54
p.Cys282Tyr / *p.His63Asp* heterozygosity & and mutation in SLC40A1 Gene (type 4)[5]	1/38	0/54
Absence of gene mutation affecting iron metabolism	0/38	23/54

* Ferritin levels were 655 µg/L and 561 µg/L, there is probably no phenotypic expression. ** This patient also fit the criteria for the metabolic syndrome and frequently consumed alcohol. *** There were no documented components of the metabolic syndrome, however the presence of non-HFE mutations was not excluded. **** Hereditary spherocytosis requiring frequent blood transfusions resulting in secondary hemochromatosis. 1. These patients are heterozygous carrier of the unclassified variant c.1473G>A in exon 11 of the TFR2 gene. Genetic heterozygosity of this variant is not known to cause relevant iron accumulation. **The additional effect of the heterozygosity for the *p.His63Asp* mutation in the *HFE* gene is unknown. 2. This patient is a heterozygous carrier of the unclassified variant p.Gly71Ser in the Hepcidine (HAMP) gene. This variant has never been previously found so there is no knowledge if this is a pathogenic mutation or a rare polymorphism. 3. This patient is heterozygous carrier of the pathogen p.Val162del mutation in the SLC40A1 gene. This deletion is described as a pathogenic mutation resulting in hemochromatosis type 4, an autosomal dominant form of hemochromatosis. The additional effect of the heterozygosity for the *p.His63Asp* mutation in the *HFE* gene is unknown. 4. Triple mutation: this patient is a heterozygous carrier of the pathogen p.(Val162del) mutation in the SLC40A1 gene. Resulting in hemochromatosis type 4. The patient is also compound heterozygous for the *p.His63Asp* mutation and *p.Cys282Tyr* mutation in the *HFE* gene. And the patient is heterozygous carrier of the unclassified variant p.(Arg288Gln) in the HJV gene. This variant has never been found previously so there is no knowledge if this is a pathogenic mutation or a rare polymorphism. 5. Triple mutation: this patient is heterozygous carrier of the pathogen p.(Val162del) mutation in the SLC40A1 gene. Resulting in hemochromatosis type 4. The patient is also compound heterozygous for the *p.His63Asp* mutation and *p.Cys282Tyr* mutation in the *HFE* gene. *Abbreviations: NA; Not applicable.*

Part II

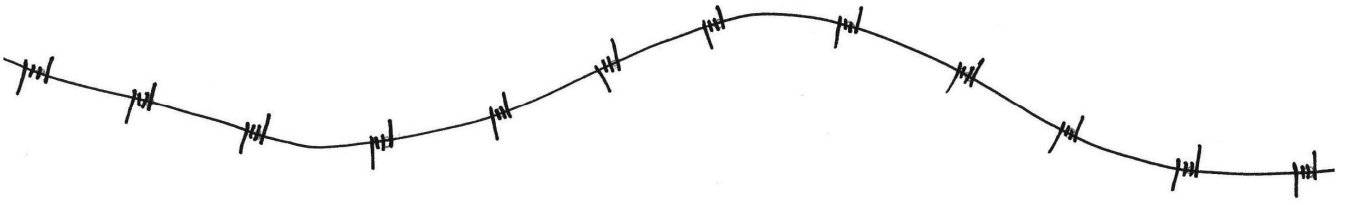
Current clinical aspects of *HFE*-related hemochromatosis and iron homeostasis





Chapter 4

Cohort profile: the *HFE*-related hemochromatosis South Limburg cohort



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To be submitted

Introduction

HFE-related hemochromatosis (HH) is the most common autosomal recessive, genetic disorder in the Caucasian population. The most prevalent form is homozygosity for the *p.Cys282Tyr* variant in the *HFE* gene (type 1 hemochromatosis), on chromosome 6, first discovered by Feder et al. in 1996.[1,2] HH is most commonly seen in populations of Northern European origin, particularly of Celtic or Nordic ancestry, in which the prevalence is close to 1 per 200-250.[3-5] It is characterized by parenchymal iron overload due to increased iron absorption.[1] Although the prevalence of *p.Cys282Tyr* homozygosity is high, phenotypic expression is low and can be classified in four stages (see Table 4.1).[6-9]

Table 4.1 Classification of disease severity for *HFE*-related hemochromatosis.

Stage	Mutation	TSAT (%)	SF (µg/L)	Clinical manifestations
0	*p.Cys282Tyr*	<45	<200 women, <300 men	-
1	*p.Cys282Tyr*	≥45	<200 women, <300 men	-
2	*p.Cys282Tyr*	≥45	≥200 women, ≥300 men	-
3	*p.Cys282Tyr*	≥45	≥200 women, ≥300 men	+ (Without impact on survival: fatigue, arthropathies, impotence, liver test abnormalities etc.)
4	*p.Cys282Tyr*	≥45	≥200 women, ≥300 men	+ (With impact on survival: liver disease (Fibrosis, cirrhosis, HCC), cardiomyopathy, diabetes mellitus)

This five-grade scale was described by Brissot et al. in the French National Health Authority (Haute Autorité de Santé) guidelines for the management of *HFE* hemochromatosis.[10] *Abbreviations: TSAT: Transferrin saturation; SF: serum ferritin, HCC: hepatic cellular carcinoma.*

Biochemical penetrance (abnormal iron status) is higher than clinical penetrance. Clinical manifestations of iron overload (stage 3-4) are less frequently seen.[6,11] Patients without evidence of iron overload (stage 0-1) are usually found through screening of first-degree family members of *p.Cys282Tyr* homozygotes. Clinical features related to iron overload include arthropathies, liver-related abnormalities varying from asymptomatic hypertransaminasemia to fibrosis, cirrhosis and eventually even hepatocellular carcinoma (HCC).[12] Associated but much less evident are impotence[13,14], chronic fatigue[14], hyperpigmentation, diabetes[15], cardiomyopathy and cardiac arrythmias.[1] As treatment, phlebotomies are most often used. With each 500 ml of full blood around 250 mg or iron can be removed from the body. An alternative treatment is erythrocytapheresis in which more erythrocytes can be removed per procedure compared to a phlebotomy. Erythrocytapheresis is less frequently used and is currently in the Netherlands only performed in specialized centers.[16] Iron depletion treatment may prevent the disease onset and minimize its severity especially if

started before the presence of end-organ damage. This emphasizes the importance of early diagnosis to prevent progression to iron overload and organ damage. It is essential that information on HH is based not only on data from expert centers but also on population-based data revealing the true epidemiology, phenotypic expression with disease course and disease behavior of HH.

Here we describe the first Dutch hemochromatosis cohort, containing all identified *p.Cys282Tyr* hemochromatosis patients from the South Limburg region in the Netherlands. Patients from this cohort have been described in publications or have participated in studies on hemochromatosis performed by our group in the past three decades.

Methods

Study design and data collection

Setting

South Limburg is a geographical area in the southeast region of The Netherlands, enclosed by Belgium and Germany. In January 2021, South Limburg had 594.212 inhabitants.[17] The medical care of its entire population is provided by two large hospitals. The Zuyderland Medical Centre is a large general hospital, created in 2015 after the merger of two regional hospitals in Heerlen and Sittard-Geleen (258 patients). The second hospital is the Maastricht University Medical Center+ (102 patients). South Limburg provides an ideal setting for population-based research due to limited cross-border search for health care and low migration rates.[17]

Design

The cohort was set up by searching in databases of each hospital in the region: (1) the regional clinical genetics registry with *HFE* analysis, using all *p.Cys282Tyr* homozygotes; (2) hospital billing system using the International Classification of Disease, Tenth Revision Code E83.1); (3) presence of hyperferritinemia and hemochromatosis in medical history of (electronic) patients record forms and (4) databases of the multidisciplinary hemochromatosis team or its members.

The data collection of this cohort was started in the late eighties of the past century and follow-up was performed until July 2021. Data collection started from the date of diagnosis, which could be the moment of inclusion or a date in the past. The end of follow-up was defined as death or the time point of the last data collection and was

documented for each patient separately. The end of the follow-up period was defined as the last visit with available information. Reasons for end of follow-up (see Figure 4.1) were: a) death b) migration to another province or country c) discharge from hospital care with referral to their general practitioner because of persistently low serum ferritin (SF) levels without need for maintenance therapy (phlebotomies or erythrocytaphereses) d) lost to follow-up for unclear reasons. In most of the lost to follow-up cases regularly planned checks of SF levels had ceased without further explanation. In case of death of a patient the date and reason of death were retrieved from the patients' medical files. All medical electronic and paper records were screened on-site by two investigators. (WM and CvD)

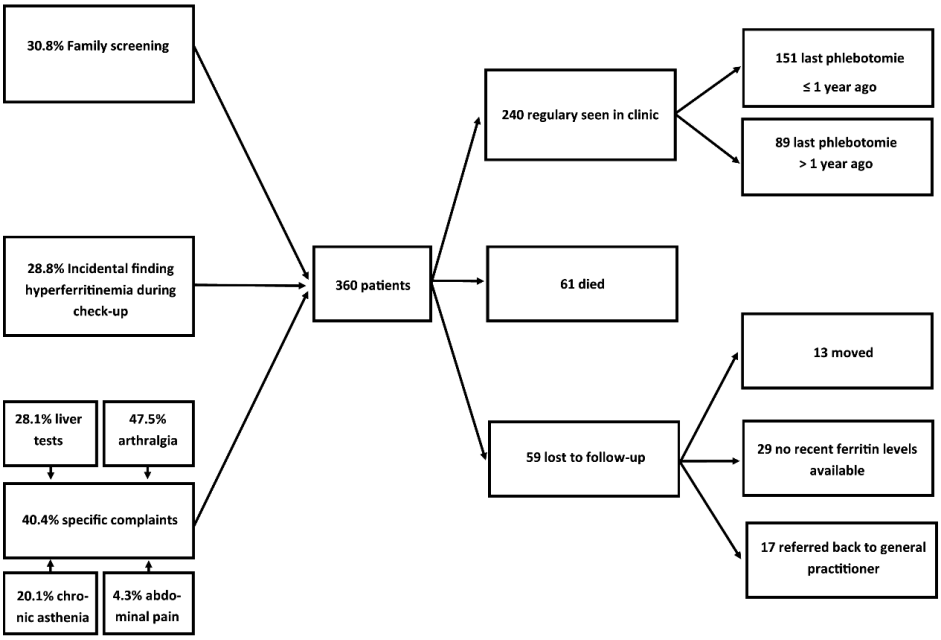

Figure 4.1 **An overview of all the reasons of referral to the outpatient clinic and the reasons for the end of follow-up**. In case patients were still regularly seen in the outpatient clinic at the end of follow-up we showed if their last treatment was ≤ of > one year ago.

Definitions

The main inclusion criterium was *p.Cys282Tyr* homozygosity. For patients diagnosed before 1996 we also included HLA typing suggesting homozygous HH, in most of these cases *HFE* analysis was eventually performed and the diagnosis was confirmed. Disease severity of HH was defined according to stages 0-4 (see Table 4.1) as

described by Brissot et al. in the French National Health Authority (Haute Autorité de Santé) guidelines for the management of *HFE* hemochromatosis.[18] Phenotypic expression was defined as ≥ stage 2. We classified each patient according to her/his highest stage of disease severity in the course of the disease. Therefore at least SF level and transferrin saturation (TSAT) at time of diagnosis had to be known. To classify patients into stage 2, 3 or 4 we needed eight variables (fatigue, arthralgia, hypogonadism, cirrhosis, fibrosis, HCC, diabetes and cardiomegaly). Patients were classified into one of these stages when at least six of the eight variables were known, otherwise classification was considered not reliable (see Table 4.1). Information whether patients with diabetes mellitus were insulin-dependent or on oral medication was not available, all patients with diabetes were assigned to stage 4. In addition, it should be noted that when using this scale there is only checked for the presence of these variables and not for a causal link between these variables and HH.

Hemochromatosis-related death was defined as death due to a hemochromatosis-related decompensated liver cirrhosis or HCC. Liver disease was defined as the presence of liver test abnormalities, liver steatosis (diagnosed by liver ultrasound and/or liver biopsy), fibrosis (diagnosed by liver biopsy or FibroScan®, (F1-F3) or cirrhosis (diagnosed by liver imaging, FibroScan® (F4) or liver biopsy). Alcohol consumption was documented as absent, average, moderate or excessive. For males excessive drinking was scored in case of intake of ≥ three alcoholic beverages a day. For females excessive drinking was scored in case of intake of ≥ two alcoholic beverages a day. Arthropathy was defined as patient-reported joint pain and as a surrogate marker, knee or hip replacements below 60 years of age. We defined chronic fatigue as patient-reported chronic tiredness. We defined endocrinological comorbidities as diagnosis of osteoporosis and/or osteopenia, hypogonadism, impotence, hypothyroidism or hyperthyroidism, diabetes mellitus. Cardiac comorbidities were documented when patients were known with any cardiologic diagnosis or condition. The presence of cardiomyopathy was documented separately. To determine the difference in the disease course before and after the diagnostic developments (discovery of the *HFE* gene), we chose the year 2000, when the EASL International Consensus Conference on hemochromatosis was published, as the turning point.[19]

Treatment

Phlebotomy remains the mainstay in the treatment of hemochromatosis. The depletion stage is the first phase of the treatment in which phlebotomies are performed at high frequency to induce iron depletion. In this stage phlebotomies are

usually carried out weekly with regular measurement of SF and hemoglobin levels. In our cohort iron depletion was pragmatically defined by the treating physician, based on SF levels, usually ≤100 µg/L. Although previously, SF levels ≤50 µg/L were pursued, this strategy was not based on scientific evidence.[7,20] The 2018 Dutch guideline advises a target SF level of ≤50 µg/L or 50 µg/L-100µg/L in combination with a TSAT <70%.[21] The depletion phase is followed by the maintenance phase to avoid iron re-accumulation and to keep SF levels ≤100 µg/L. In this phase phlebotomies are performed less frequently compared to the depletion phase. A substantial number of patients in our cohort received erythrocytapheresis as alternative therapy for phlebotomy. With erythrocytapheresis, in the depletion phase per single treatment procedure 350-800 ml of erythrocytes were withdrawn once every 2-3 weeks. In the maintenance phase erythrocytaphereses are performed depending on the serum ferritin levels.

Data collection

The following minimal data set was obtained from the medical files: reason for referral, age at time of diagnosis, results of *HFE* analysis, gender, when applicable date and cause of death, intoxications, medication (in particular use of proton pump inhibitors), comorbidities (i.e. malignancies, chronic inflammatory disorders, rheumatic diseases, hematological diseases), family history, previous blood donations, postmenopausal state in women. We also included BMI, presence of metabolic syndrome and of its individual components (e.g. diabetes mellitus, obesity, hypertension and hyperlipidemia), results of laboratory tests (iron parameters, liver enzyme tests, inflammatory parameters), results of diagnostic tests (MRI with iron protocol, liver ultrasound, FibroScan®, liver biopsy), clinical data related to hemochromatosis (presence of arthralgia, consultation of rheumatologist, knee/hip prosthesis performed, endocrine disorders, cardiomyopathy and fatigue). With respect to treatment the following data were collected: a) amount of iron needed to remove in order to reach the iron depletion stage b) the date at which iron depletion was reached and c) the mean amount of phlebotomies or erythrocytaphereses per year in the maintenance stage.

Statistical analyses

Data from hemochromatosis patients living in the South Limburg region between the seventies of the past century until July 2021 were obtained during regular care and were collected from various sources. Due to the retrospective data collection there are multiple missing values. For the statistical calculations, we used IBM SPSS statistics version 23 for Windows. Since all of the continuous variables are skewed, they are

expressed as median with an interquartile range. The categorical variables are presented qualitatively, as events or percentages. Comparison of values between groups was performed by using Mann-Whitney U, Chi-square or Fisher's exact testing. A two-sided p-value of $p<0.05$ was considered statistically significant.
The Ethics Committees of the Maastricht University Medical Centre + (MUMC+) and the Ethics Committee of the Zuyderland Medical Centre had approved the study protocol and waived the need for informed consent.

Results

Cohort characteristics

The case finding strategy resulted in 360 individuals diagnosed with HH between 1971 and 2021 in the South Limburg region. Baseline patient characteristics are shown in Table 4.2.

The median follow-up period from diagnosis until end of study or lost to follow-up was 9.9 years [13.4]. Of the 360 diagnosed patients, 66.7% is still regularly seen at the outpatient clinics of the two hospitals. Of the 120 patients in whom follow-up ended (see Figure 4.1), 61 patients had died during the follow-up period, 13 had moved outside the region, 17 were referred back to their general practitioner due to persistently low SF levels without the need maintenance therapy (phlebotomies or erythrocytaphereses) and 29 patients were lost to follow-up: regularly planned checks of SF levels had ceased without further explanation (see Figure 4.1).
At time of diagnosis the median age was 53 years, 60.3% of the patients were male. Of the women, 85.7% were postmenopausal at time of diagnosis. We found an elevated SF in 91.9% of patients (median value 845 µg/L [1162]) and in 95% of patients an elevated TSAT (TSAT ≥45%; median 82.4% [26.8]). Of all patients with both SF and TSAT values available at diagnosis both were elevated in 97.7%. In the patients without hyperferritinemia the *HFE* analysis was part of a family screening. In women the median age at diagnosis was 56 [15] years and in men 52 [16] years (p=0.004). The median SF at diagnosis was 551 µg/L [680] in women and 1149 µg/L [1430] in men (p=0.000). The median TSAT at diagnosis was 75% [29] in women and 85% [21.8] in men (p=0.000). A BMI in the normal range was found in 33.3% of patients, 43.8% was overweighted, 16% had obesity and 6.9% had morbid obesity. Information whether patients had been blood donor was available in 118 patients. Before the HH diagnosis, 8.3% had been blood donor.

Table 4.2 Baseline characteristics

Baseline characteristics		Reference value	Missing values
Demographic factors			
Male, n(%)	360 (60.3)		*0*
Age at diagnosis(years), median [IQR]	53 [16]		*0*
Death during follow-up period, n(%)	61 (17.9)		*19*
Metabolic factors			
BMI (kg/m^2), median [IQR]	27 [6.2]	*18.5 - 25*	*72*
Hypertension, n(%)	102 (30.1)		*21*
Hyperlipidemia, n(%)	72 (27.3)		*96*
Metabolic syndrome, n(%)	73 (21.5)		*20*
Diabetes mellitus (type 1 or 2), n(%)	61 (17.5)		*12*
Serum biochemistry			
Hb (mmol/l), median [IQR]	9.4 [1.1]	*7.5 - 10*	*55*
MCV (fl), median [IQR]	95 [6]	*80 - 100*	*103*
Serum iron (µmol/L), median [IQR]	35 [12.1]	*11 - 30*	*35*
Serum transferrin (g/L), median [IQR]	1.9 [0.3]	*1.9 - 3.2*	*51*
Transferrin saturation (%), median [IQR]	78 [28.9]	*<45*	*40*
Serum Ferritin (µg/L), median [IQR]	845 [1162]	*<200 women, <300 men*	*15*
CRP (mg/L), median [IQR]	2 [2]	*<10*	*186*
Ferritin above 1000, n(%)	150 (43.5)		15
Liver test abnormalities, n(%)	148 (43.9)		23
Hyperferritinemia at diagnosis, n(%)	319 (91.9		13
Possible disease manifestations			
Presence of malignancy, n(%)	85 (24.5)		15
Presence of HCC, n(%)	20 (5.8)		15
Steatosis, n(%)	83 (41.5)		160
Fibrosis, n(%)	12 (14.3)		276
Cirrhosis, n(%)	26 (12.1)		145
Visit rheumatology, n(%)	75 (21.1		5
Protheses, n(%)	52 (14.9)		10
Arthralgia, n(%)	197 (55.6)		6
Cardiac comorbidities, n(%)	58 (16.3)		4
Fatigue, n(%)	144 (42.9)		24
Endocrine disease, n(%)	101 (28.5)		5
Hypogonadism, n(%)	10 (2.8)		5
Relevant clinical information			
Classification alcohol usage, (%)			
average	52.3		
moderate	20.3		
excessive	27.4		
PPI use at diagnosis, n(%)	86 (25.4)		21
Premenopausal women at diagnosis, n(%)	18 (14.3)		234
Blood donor at diagnosis, n(%)	30 (25.2)		241
Mobilised iron to reach iron depletion (g), median [IQR]	3.75[4.25]		152

Reasons of referral

Patients were referred to the hemochromatosis outpatient clinics for several reasons: a) in 28.8% for incidental finding of hyperferritinemia during regular check-up, sport

screening or as part of a screening blood test for unspecific complaints b) in 30.8% as part of a family screening for hemochromatosis and c) in 40.4% SF levels were checked because of specific complaints such as unexplained liver test abnormalities in 28.1%, arthralgia in 47.5%, chronic fatigue in 20.1% and abdominal pain in 4.3% (Figure 4.1).

Epidemiology: incidence, prevalence and penetrance

The mean annual rate of newly diagnosed HH patients between 2016 and 2020 in South Limburg was 3.7 per 100.000 inhabitants. Based on our data, at January 2021 the point prevalence of *p.Cys282Tyr* homozygosity found in the South Limburg region was 50 per 100.000 inhabitants (0.05%). The point prevalence of the patients with phenotypic expression (stage ≥2) was 40.4 per 100.000 inhabitants (0.04%) and of patients with clinical manifestations (stage 3-4) was 29.3 per 100.000 inhabitants (0.03%). Indication for *HFE* gen analysis was based on the presence of clinical symptoms or lab abnormalities (together 69%) or on family screening of first degree relatives with HH (31%).

Liver disease

In 43.9% of patients liver test abnormalities were found at diagnosis. Liver cirrhosis was found in 12.1% of the patients and liver fibrosis in 14.3%. In 82 of the 200 patients (41.2%) examined by liver ultrasound, liver steatosis was present. In 56 patients a liver biopsy was performed, in most patients before the year 2000 when liver biopsy had a more prominent role in the diagnosis of hemochromatosis. In the 26 patients in whom a liver cirrhosis was found 11 were diagnosed before the year 2000 while the other 15 were diagnosed between 2000-2021.
Of all patients with liver cirrhosis, 63.6% had BMI ≥25, 27.3% fulfilled the criteria for the metabolic syndrome and 73.1% had a SF ≥1000 µg/L at diagnosis. Of all patients with liver cirrhosis 30% had excessive and 25% moderate alcohol intake and 73.1% of them were males.

In 20 of 345 patients (5.8%) a HCC was found, nine were diagnosed with HH before 2000. In 14 patients HCC occurred in a non-cirrhotic liver compared to six of the patients in whom HCC occurred in a cirrhotic liver. It should be noted that the absence or presence of liver cirrhosis was mostly based on liver imaging. HCC and a cirrhotic liver was found in six patients; two did have a cirrhotic liver at HH diagnosis, in one patient no liver imaging was performed at HH diagnosis, in one the diagnosis of HH, HCC and liver cirrhosis were simultaneously and in the two remaining liver cirrhosis was already found at HH diagnosis.

The patients with HCC had significantly higher SF levels (3002 µg/L [2758]) compared to the patients without a HCC (815 µg/L [975], p=0.000), at the time of HH diagnosis. In all but one of the patients diagnosed with HCC the SF level at diagnosis of hemochromatosis was ≥1000 µg/L. Of the patients with HCC, 90% were males.

Arthropathy

During the follow-up period 55.6% of the patients complained of arthralgia, 21.1% consulted a rheumatologist, and 14.8% had a knee or hip replacement. In 12 patients the knee or hip replacement was performed before the age of 60 years. There is no association between the gender and the presence of arthralgia. (p=0.449)

Malignancies other than HCC

In total, in the 360 patients, at the end of follow-up 103 cancers were registered during the life time of the patients. See Figure 4.2 for an overview of the different malignancies recorded.

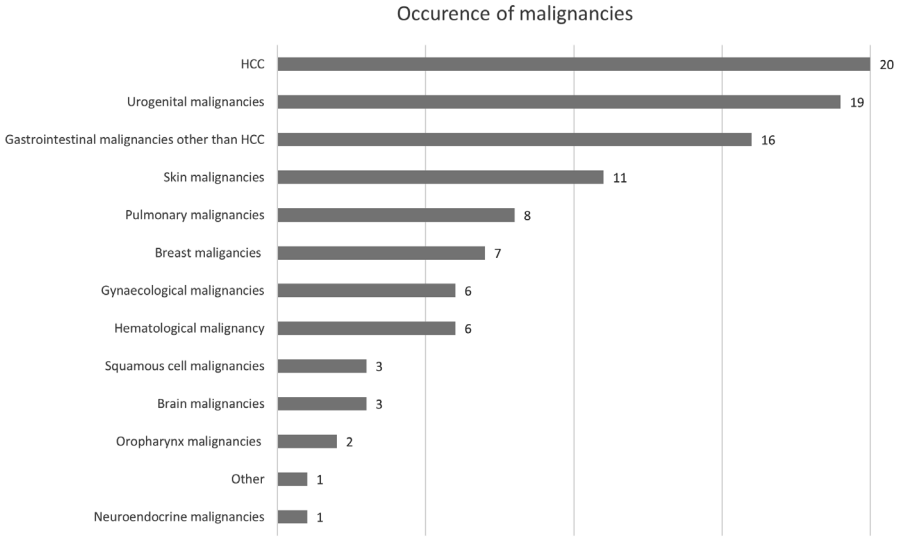

Figure 4.2 Overview of all the different categories of malignancies observed during the follow-up period of the HH patients.

Other disease manifestations

In 16.3% (58/356) of the patients cardiac comorbidities were found, in eight of these patients evidence for a cardiomyopathy was found. A substantial percentage of 42.9% of the patients complained of fatigue. In 30.1% of the patients one or more endocrine

disorders were diagnosed: in 19% diabetes mellitus, in 6.4% hypothyroidism or hyperthyroidism, in 3.9% osteopenia or osteoporosis and in 2.8% hypogonadism.

Mortality

Sixty-one patients (17.9%) died during the follow-up period, the median age at time of death was 73 years [15]. In 25% of patients who died during the follow-up period, HCC was the cause of death. In 5% a decompensated liver cirrhosis was the cause of death. The patients with a decompensated liver cirrhosis were also excessive alcohol drinkers and/or had evidence of the metabolic syndrome and/or liver steatosis. In Figure 4.3 the various causes of death are shown.

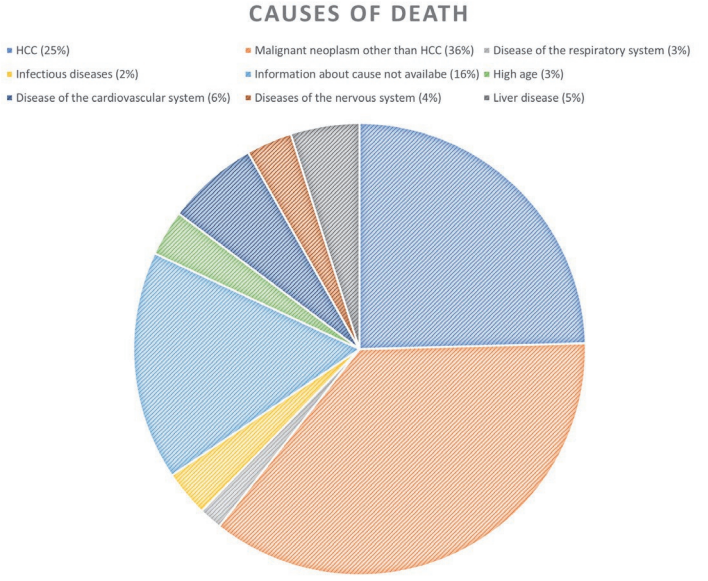

Figure 4.3 Overview of all the causes of death of the 61 patients who died during the follow-up period.

Hemochromatosis classification by stages

In Table 4.3 an overview is given about the classification of patients in the five-grade scale. The median TSAT and SF for each group is included and when available the median amount of mobilized iron to reach the iron depletion stage. In our cohort 90% had phenotypic expression (stage ≥2). 18.3% were in stage 2, 48.3% were in stage 3 and 23.4% were in stage 4 (Table 4.3).

Table 4.3 Classification of all patients for each stage with biochemical parameters and mobilized iron.

Stage	N (294)	TSAT (%)	SF (µg/L)	Mobilized iron to reach depletion stage (grams)
0	3	26	40	0
1	25	64 (33)	150 (133)	0
2	54	84 (23)	791 (849)	2.95 (3.38) (n=32)
3	142	83 (21)	897 (928)	3.85 (4.35) (n=110)
4	70	85 (20)	1633 (2951)	4.28 (4.45) (n=42)

Abbreviations: TSAT: Transferrin saturation; SF: serum ferritin. Median values (IQR) are shown.

Iron depletion therapy

In 89.2% of all patients at one point during follow-up, phlebotomies or erythrocytaphereses, have been performed to induce iron depletion. In 78.9% of our patients the depletion stage was reached during the follow-up period.
The median amount of iron mobilized to reach iron depletion was 3.75 grams [4.25], for women this was 2.28 grams [2.50] and for men 4.5 grams [4.75] (p=0.000). At the time of diagnosis 25.4% of the hemochromatosis patients was on proton pump inhibitor prescription. Of all the patients in the maintenance stage, 30.9% (56/181) was no longer in need for treatment (phlebotomies or erythrocytaphereses) during the follow-up period. During the maintenance stage approximately one, two, three, four and five yearly phlebotomies were seen in 19.9%, 22.7%, 20.4%, 5%, 1.1%, respectively. Of all the patients who were still regularly seen at the clinic, their last phlebotomy was more than one year ago in 35.7%.

Discussion

We have presented data of the first Dutch cohort of homozygote *p.Cys282Tyr* hemochromatosis patients from the South Limburg region.

Epidemiology

From our cohort we report evidently lower prevalence rates (0.05%) of homozygote *p.Cys282Tyr* hemochromatosis compared to previous studies (0.4-0.68%) that were based on population-based genetic screening. While in studies based on genetic screening the reported clinical penetrance is very low (varying from <1% to 13.9%)[20,22], in our cohort over 90% of patients had phenotypic HH expression stage ≥2 and 73% stage ≥3.[20,22] The differences in epidemiological data on *p.Cys282Tyr* homozygosity between large population studies based on genetic screenings and cohort studies that are based on clinical and laboratory abnormalities reflect the

much lower clinical penetrance and variable phenotypic expression of *p.Cys282Tyr* homozygosity.

Referral

In our cohort the reasons for referral for HH analyses were laboratory test abnormalities (30%), presence of symptoms (40%) or family screening (30%). Symptoms involve various organ systems and vary from chronic fatigue/lethargy to arthropathies, liver disease, endocrine disturbances and cardiac arrythmias. Physicians from various background/specialty can become involved such as general practitioners, rheumatologists, internists, gastroenterologists, hepatologists, cardiologists, etc. For that reason it is important to create multidisciplinary awareness for hemochromatosis in order to optimize more early diagnosis and initiation of iron depletion treatment to prevent or reduce iron-related organ damage.

Symptoms and disease manifestations

We report on the prevalence of various symptoms and disease manifestations in our cohort of hemochromatosis patients. It should be noticed that data for comparison, that is from a control group or from the general population are lacking here. While the prevalence of joint complaints and joint disorders in our cohort was high,[23-26] this holds also true for the general population. To date, in some studies the prevalence of arthropathy in HH patients when compared with controls was found not to be increased.[5,11] We found a high prevalence of fatigue/lethargy but this complaint is also frequently reported in the general population and previously performed case-control studies did not find an increased prevalence of fatigue in HH patients.[11,27-29] The same goes for endocrine disorders such as diabetes mellitus, hypogonadism, impotence and thyroid disorders.[30] Cardiac arrythmias were not seen more frequently in HH patients compared to controls.[11,31] The prevalence of various symptoms and disease manifestations of HH appear to have decreased in the past decades due to an earlier diagnosis and initiation of treatment facilitated by the introduction and implementation of the *HFE* analysis since 1996.[32] Future studies on prevalence of symptoms and organ manifestation of HH should include appropriate data from control groups or from the general population. Despite the finding of symptoms and organ manifestations known to be related to HH, we did not investigate a causal relationship due to the lack of a control population.

Liver disease in hemochromatosis

At the time of diagnosis increased liver enzymes were present in 43.9% of the HH patients in our cohort. During follow-up liver fibrosis, cirrhosis and HCC were seen in

13.1%, 12.7% and 5.8% of our patients respectively. Our findings are in line with data from previous studies pointing to a lower prevalence of liver-related morbidity in HH as previously assumed.[11,33-35] Proportionally, most of the patients diagnosed with liver cirrhosis, fibrosis or a HCC in our cohort were diagnosed with HH before the year 2000, when the EASL International Consensus Conference was published and only a few years after the discovery of the *HFE* gene. Of all patients with cirrhosis or HCC seen at liver units of university hospitals respectively only 0.5-1% and 1-2% were diagnosed with hemochromatosis.[9,21-23] Despite the presence of p.Cys282Tyr homozygosity, physicians must remain alert for the presence of liver steatosis, viral or alcoholic liver disease.

While in our cohort at the time of diagnosis 60% were males, male predominance was even more pronounced in the HH patients with liver cirrhosis (75%) and HCC (90%). These findings are in agreement with a previously observed male predominance in HH patients with advanced liver fibrosis.[6,22,33,34] Female HH patients in our cohort were diagnosed at a significant later age and with significantly lower SF and TSAT levels compared to male HH patients. The gender difference can be explained by the protective effect of menstrual blood loss and the antioxidant effect of oestrogen.[12,36,37] Probably because of a more restrictive policy in performing liver biopsies and liver imaging the prevalence of liver-related morbidity may have been underestimated in our cohort. The role of the liver biopsy in HH has become less important after implementation of *HFE* analysis. And starting from 2015 the non-invasive FibroScan® has been used on a regular basis in our centers. Current guidelines advice to perform a liver biopsy and/or FibroScan® in patients with SF levels ≥1000 µg/L[7,20,21] because these patients have an increased risk of developing cirrhosis with a prevalence of 20-45%.[38,39] In contrast, in patients with SF level <1000 µg/L at diagnosis less than 2% had cirrhosis or fibrosis, in the absence of other risk factors such as fatty liver disease, viral hepatitis or excessive alcohol use.[38-41] In our cohort we found liver cirrhosis in 7.6% and 17% of patients with SF <1000 µg/L and patients with SF levels >1000 µg/L respectively.

Hepatocellular carcinoma

The risk for HCC is known to occur predominantly in HH patients with liver cirrhosis.[7,42] A remarkable finding in our cohort is that of the 20 HH patients with HCC, 14 had no evidence at all of liver cirrhosis at the time of their HCC diagnosis. Up to now HCC screening, as advised by guidelines, is limited to hemochromatosis patients with liver cirrhosis.[7] Previous reports on analyses of patients with non-cirrhotic HCCs showed a high prevalence (in more than 50%) of stainable iron while the prevalence of

p.Cys282Tyr mutations in these patients was very low (<2%).[43-45] The hypothesis is that iron overload in the liver may promote hepatic carcinogenesis by oxyradicals resulting in lipid peroxidation and DNA damage.[42] This observation should be interpreted with caution since we lack information about other risk factors for the development of a HCC like steatosis, alcohol or viral liver disease. In addition, not in all patients a FibroScan® or liver biopsy was available and the presence of liver cirrhosis by liver imaging only can be missed. However the observation of HCC development in hemochromatosis patients in the absence of liver cirrhosis deserves further evaluation.

Treatment

In our cohort all diagnosed HH patients with iron overload were offered iron depletion therapy. The standard of care consists of phlebotomies but substantial experience has been gained in our region with erythrocytapheresis. In controlled trials and follow-up studies we have repeatedly reported on efficacy of erythrocytapheresis.[16,46] There is substantial evidence that iron depletion, started before the presence of cirrhosis and/or diabetes significantly reduce morbidity and mortality of HH.[7,47,48] Therefore, it is crucial to start iron depletion therapy as early as possible in the disease course to reduce iron accumulation and prevent further organ damage.[1]

Mortality

During follow-up 61 patients with HH died. We consider that in at least 18 patients death was related to iron accumulation: HCC in n=15 and decompensated liver cirrhosis in n=3. It should be noted that factors as excessive alcohol usage, the metabolic syndrome and liver steatosis were also present in many of these patients.

Strengths and weaknesses

A major strength lies in the population-based character of the cohort. Population-based cohorts comprise the full spectrum of disease phenotypes from mild to a more severe disease course. Interest in hemochromatosis in the South Limburg region started in the eighties of the past century. Since then, 53 scientific papers on HH have been published by the South Limburg multidisciplinary hemochromatosis team.

To build this cohort we have performed extensive searches in four databases of South Limburg hospitals. Since we included the database of the Clinical Genetics department we are ensured to have included all diagnosed HH patients of the South Limburg region. Whether completeness is (near) 100% needs to be checked against a representative sample of HH registries by general practitioners in our region. Our

follow-up time is long with a median duration of 9.9 years. This allows us to report in detail on disease course, manifestations and development of complications.
A major limitation is the retrospective collection of patient data from electronic and paper hospital records which has resulted in missing data with respect to patient histories, disease manifestations, laboratory and imaging results. Liver biopsies were not routinely performed and FibroScan® was readily available starting from 2015. These factors may have led to an underestimation of liver disease manifestations. Based on guidelines, patients were offered iron depletion therapy. This will have affected the disease course, the presence and severity of organ manifestations in our HH patients. Due to the absence of a control population, a causal relationship could not be investigated.

Conclusions

We reported on the first Dutch clinical, population based cohort of 360 HH patients followed for a median period of 9.9 years after their diagnosis. Inclusion in this cohort was based on symptoms, laboratory abnormalities or family screening of first degree relatives, not on population-based genetic screening for pC282Y homozygosity.
What essentially new information has been obtained from this cohort:

- A regional incidence of 3.7 per 100.000 inhabitants and prevalence of 50 per 100.000 inhabitants of pC282Y homozygosity was observed.
- According to the five-grade classification scale of phenotypic expression 90% of our patients had phenotypic penetrance (stage ≥ 2).
- Organ involvement and symptomatology were much lower than previously reported, possibly due active iron depletion therapy.
- During median follow-up of 9.9 years hepatocellular carcinoma was diagnosed in 20 HH patients (5.8%).
- In contrast to previous publications, in 14 of these 20 patients with HH and HCC, the HCC developed in a non-cirrhotic liver.
- Further analyses of risk factors for development of HCC in non-cirrhotic hemochromatosis are warranted.

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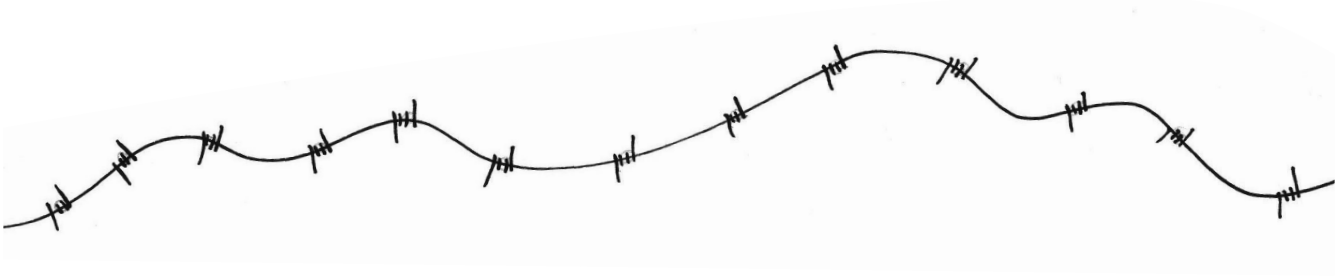
Chapter 5

The modified iron avidity index: a promising phenotypic predictor in *HFE* hemochromatosis



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Abstract

Objective

Phenotypes of the *HFE*-related hemochromatosis vary considerably, making it hard to predict the course of iron accumulation. The aim of this retrospective study was to determine if the Iron Avidity Index (IAI) is a good phenotypic predictor of the number of phlebotomies needed per year during maintenance treatment (NPDMT) in patients with homozygous *p.Cys282Tyr* hereditary hemochromatosis (HH).

Methods

Patients with HH homozygous for *p.Cys282Tyr* where included when on maintenance treatment for at least 1 year. The IAI (ferritin level at diagnosis/age at diagnosis) was calculated.

Results

Ninetyfive patients were included in the analysis. Linear regression analysis showed the confounding effect of sex on the relationship between IAI and NPDMT. A modified IAI, adjusted for sex, was calculated. As proton pump inhibitor (PPI) use was independently associated with NPDMT, the group was split in PPI users and non-PPI users. A positive correlation between the modified IAI and the NPDMT was shown in both groups (PPI $r=0.367$, $p=0.023$; non-PPI $r=0.453$, $p<0.001$). An ROC was computed to measure the accuracy of the modified IAI to predict who needed 0–2 vs. ≥3 maintenance treatments per year. The AUROC in the PPI and non-PPI group were respectively 0.576 (0.368–0.784) and 0.752 (0.606–0.899).

Conclusion

The modified IAI is a fairly good predictor in non-PPI-using homozygous *p.Cys282Tyr* HH patients, to differentiate who needs ≥3 maintenance phlebotomies per year. Therefore, this index might help to select patients that benefit from an alternative less frequent therapy, e.g. erythrocytapheresis.

Introduction

Hereditary hemochromatosis (HH) is an autosomal recessive disease, which leads to accumulation of body iron in mainly parenchymal organs of the body. When untreated, this disease can result in cirrhosis, severe arthropathy, cardiac diseases and diabetes.[1,2] To prevent this from happening, treatment is essential and phlebotomy is currently the standard therapy, which consists of the removal of 500 ml whole blood, representing an iron loss of approximately 250 mg. The initial treatment goal is to lower the ferritin level to around 50 µg/L, and thereafter in the maintenance treatment to keep it between 50-100 µg/L.[3] The most common hemochromatosis genotype is homozygosity for *p.Cys282Tyr*; a single-base change mutation on chromosome 6, short arm, that results in the substitution of cysteine for tyrosine at position 282 of the HFE protein. The variability in the phenotype makes it hard to identify which patient needs maintenance phlebotomy therapy, and if so, how frequent. Phlebotomy therapy is not without side effects[4] and compliance with maintenance therapy declines over the years.[5] Therefore, it would be of great value to have a tool to predict phenotype; thereby individualizing therapy since alternative therapies are available, such as erythrocytapheresis. Erythrocytapheresis removes more erythrocytes, and therefore iron, per session compared to phlebotomy (427 mg iron vs. 205 mg[6]), while sparing plasma proteins, coagulation factors and platelets. However, the costs of a single erythrocytapheresis session are greater compared to a phlebotomy and more expertise is necessary. To pinpoint the group who needs frequent maintenance phlebotomies, we aimed to determine if the iron avidity index (IAI) is a good phenotypic predictor of the number of maintenance phlebotomies per year in homozygous *p.Cys282Tyr* HH patients in a proof of concept study. We hypothesize that the higher the IAI (calculated by serum ferritin levels at diagnosis divided by age at diagnosis), the more phlebotomies per year are required for patients on maintenance treatment.

Patients and methods

Study population

This retrospective study was conducted among homozygous *p.Cys282Tyr* HH patients, visiting the outpatient clinic of the Zuyderland medical centre, Heerlen/Brunssum. All patients had to be in maintenance treatment for at least 1 year. Furthermore, the ferritin level at diagnosis and the age at diagnosis had to be known. Patients who were still in the initial phase of treatment (de-ironing) and those who were treated

with erythrocytapheresis during maintenance treatment were excluded. Patients treated with erythrocytapheresis during the initial phase only and with phlebotomy during maintenance treatment were not excluded. A total of 127 homozygous *p.Cys282Tyr* HH patients were evaluated, 95 patients were included; 17 patients were excluded because they were still in the initial phase of treatment or on maintenance treatment for <1 year, three patients because they were on erythrocytapheresis during maintenance treatment and for another 12 patients the data on ferritin and/or age at diagnosis were missing. The Medical Ethics Committee Atrium-Orbis-Zuyd approved this study, waiving the requirement to obtain informed consent. This study protocol conformed to the provisions of the declaration of Helsinki. This study was registered at trialregister.nl (NTR4684).

Study parameters

The following data, summarized in Table 5.1, were obtained from the medical file: age, sex, age at diagnosis, results for genetic testing on hemochromatosis mutations, serum ferritin at diagnosis, number of phlebotomies during initial and maintenance treatment, mobilized iron (the number of phlebotomies during initial treatment multiplied by 0.25 g [the amount of iron removed per treatment]) and the use of a proton pump inhibitor (PPI).

Table 5.1 Baseline characteristics homozygous *p.Cys282Tyr* hemochromatosis patients Zuyderland Medical Center.

	Female (n=38)	Missing values (n)	Male (n=57)	Missing values (n)	p-value
Age (in years) (Mean [SD])	60.8 [12.8]	0	62.8 [9.1]	0	0.811*
Age at diagnosis (in years) (Mean [SD])	50.9 [12.0]	0	51.8 [8.6]	0	0.814*
Ferritine (µg/L) at diagnosis (Mean [SD])	949.4 [1176.6]	0	1742.5 [1330.6]	0	<0.001*
Number of phlebotomies in de-ironing phase (Mean [SD])	12.5 [7.4]	15	32.3 [17.0]	26	<0.001*
Number of phlebotomies per year during maintenance treatment (Mean [SD])	2.0 [1.5]	0	3.1 [1.7]	0	0.007*
Mobilised iron (g) (Mean [SD])	3.0 [2.6]	14	7.7 [4.4]	24	<0.001*
Use of protonpump inhibitor (n [%])	13 [34.2]	0	25 [43.9]	0	0.347**

Continuous variables are expressed as mean ± standard deviation (SD), and categorical variables as absolute figures and percentages. *Mann-Whitney U /**Chi-square test.

Statistical analysis

The data were analyzed using IBM SPSS statistics version 22.0 (IBM Statistics for Macintosh, Chicago, IL, USA). In descriptive analyses, continuous variables were expressed as mean ± standard deviation (SD), and categorical variables as absolute figures and percentages.

Linear regression analysis was performed to assess the association between the number of phlebotomies during maintenance treatment (NPDMT) and IAI (Model 1). Furthermore, the analysis was adjusted for demographic characteristics (age, age at diagnosis and sex) (Model 2) and the use of PPI (Model 3).

To investigate which covariate had the strongest confounding effect on the association between NPDMT per year and IAI, the crude analysis (Model 1) was adjusted for all factors separately. The factors with the strongest significant effects where thereafter omitted separately from the fully adjusted model to determine if the factors were indeed confounders of the association between NPDMT per year and IAI. Furthermore, it tested whether or not the factors were independently associated with the NPDMT.

Missing values were excluded list wise. As a result of skewed distribution, IAI was logarithmically (Log) transformed. The B of the linear regression analyses signifies the change in number of phlebotomies per year during maintenance treatment resulting from one (Log) change in IAI.

Pearson correlation was used to assess the correlation between the index and the NPDMT. An ROC analysis was performed to measure the accuracy of the index to predict who needed 0–2 vs. ≥3 maintenance treatments per year. $p<0.05$ were considered to denote significance.

Results

Sixty per cent of the patients were male and 40% were using PPIs. The male patients showed significantly higher levels of ferritin at diagnosis, more phlebotomies both during the initial and maintenance phase, and a higher level of mobilized iron compared to female patients. Patient characteristics are summarized in Table 5.1.

The association between NPDMT and IAI was assessed by linear regression analysis. After adjustment for sex, age and age at diagnosis (model 2), the association between NPDMT and IAI was no longer significant (B 0.744, 95% CI-0.090; 1.577, p=0.080). The association became significant again after adjustment for PPI use (B 0.772, 95% CI 0.048; 1.496, p=0.037) (model 3) (Table 5.2).

Table 5.2 Linear regression analysis of NPDMT per year vs. the logarithmic transformed IAI.

NPDMT	IAI		
Model	**B**	**95% CI**	**p-value**
1	1.182	0.405; 1.959	0.003
2	0.744	-0.090; 1.577	0.080
3	0.772	0.048; 1.496	0.037

Model 1 is the crude analysis. Model 2 is adjusted for demographical characteristics (sex, age, age at diagnosis). Model 3 is Model 2 plus use of proton pump inhibitors.

When separately adjusting the crude analysis for the possible confounders, several factors were associated with NPDMT. Female sex (B -0.746, 95%CI -1.476; -0.015, p=0.045) was associated with a lower NPDMT, as was the use of PPI (B -1.611, 95% CI -2.204; -1.019, p<0.001).

Age and age at diagnosis did not reach statistical significance. Thereafter, we omitted sex and PPI use separately from the fully adjusted model. When omitting sex, the association between NPDMT and IAI became stronger again, demonstrating the confounding effect of this factor. PPI use did not affect the analysis and is therefore not a confounder in this analysis, but independently associated with NPDMT.

Therefore, a modified IAI was calculated to adjust for sex. This was based on the assumption that in the young *p.Cys282Tyr* homozygous patients, body iron stores remain normal or increase only slightly until the end of growth; meaning iron only accumulates from the age of 20 onwards. Therefore, 20 years were subtracted from "age at diagnosis" in males. Although, iron accumulation in women is also less distinct during growth and development, the physiological blood loss during menstruation in the premenopausal phase should be taken into account. Therefore, 20 years were added to the "age at diagnosis" for women, to correct for the childbearing years in which iron accumulation per year is less pronounced. This led to the following formula: ferritin at diagnosis / (age at diagnosis minus 20) when male, and ferritin at diagnosis / (age at diagnosis plus 20) when female.
Pearson correlation showed a positive relationship between the NPDMT and the modified IAI (r 0.343, p=0.001), (because of skewed distribution, modified IAI was

logarithmically transformed), compared to a Pearson correlation coefficient of r=0.299, p=0.003 for the association between the non-adjusted IAI and the NPDMT (Table 5.3).

Table 5.3 **Correlation (r) of respectively the IAI and the modified IAI vs. the number of phlebotomies during maintenance treatment per year for the entire study population, with corresponding p-value.**

	Pearson correlation (r)	p-value
IAI	0.299	0.003
Modified IAI	0.343	0.001

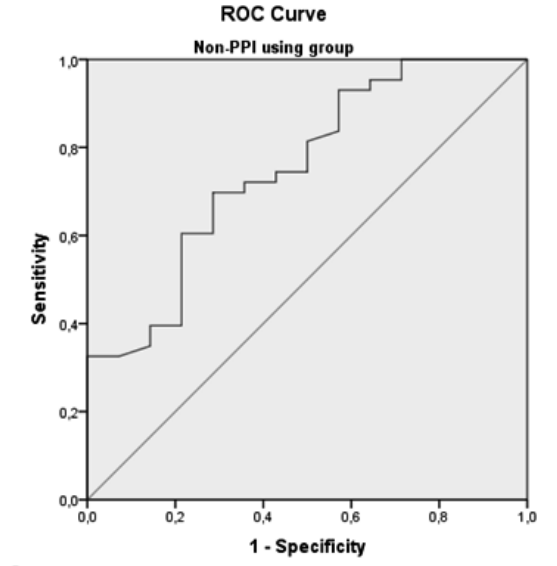
Since PPI use was independently associated with NPDMT, separate analyses for the modified IAI in PPI users and non-PPI-users were conducted; showing a Pearson correlation coefficient of 0.367, p=0.023 for PPI-users and 0.453, p<0.001 for non-PPI-users (Table 5.4). To measure the accuracy of the modified IAI to predict who needs 0-2 vs. ≥3 maintenance treatments per year an ROC was computed. The AUROC in the PPI group and non-PPI group were respectively 0.576 (0.368-0.784) and 0.752 (0.606-0.899) (Figure 5.1). The AUROC in the PPI group and non-PPI group were respectively 0.576 (0.368-0.784) and 0.752 (0.606-0.899) (Figure 5.1). The cut-off values for optimal sensitivity and specificity were calculated using the Youden index (Table 5.5).

Table 5.4 **Correlation (r) of the modified IAI vs. the number of phlebotomies during maintenance treatment per year for respectively non-PPI users and PPI-users, with corresponding p-value.**

Modified IAI	Pearson correlation (r)	p-value
Non-PPI using groups	0.453	<0.001
PPI using groups	0.367	0.023

Table 5.5 **Cut-off values, sensitivity and specificity of the mIAI for the overall cohort, the non-PPI-using group and the PPI-using group.**

	Cut-off mIAI (log transformed)	Sensitivity (%)	Specificity (%)	AUROC
Overall group	≥1.455	71.4	61.2	0.671
Non-PPI-using group	≥1.102	69.8	71.4	0.752
PPI-using group	≥1.31	72.7	48.1	0.576



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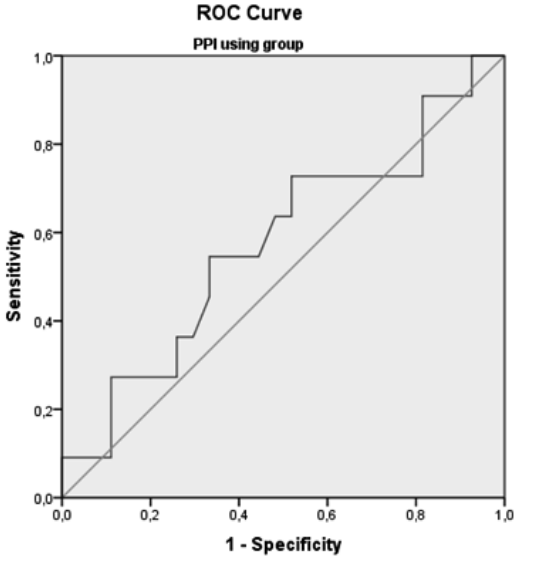

B

Figure 5.1 **The receiver-operating characteristic (ROC) curves of the modified IAI for the prediction of ≥3 maintenance treatments per year in hemochromatosis patients, for the non-PPI using group (A) and the PPI using group (B).**

Discussion

As a result of the variability in the phenotype in HH, it would be of great value to have a tool to predict a patient's phenotype and thereby individualize therapy.

To our knowledge, this is the first study that proposed a prediction model to calculate how many phlebotomies per year are needed in the maintenance phase for homozygous *p.Cys282Tyr* HH patients. Our retrospective study showed a positive correlation between the modified IAI (dividing ferritin at diagnosis by age at diagnosis minus 20 when male, and ferritin at diagnosis divided by age at diagnosis plus 20 when female) and the NPDMT per year.

An AUROC was calculated to measure the accuracy of the index to predict who needed ≥3 maintenance treatments per year. A cut-off value of ≥3 phlebotomy treatments per year was chosen, since it has been shown that with an increasing frequency of phlebotomies the compliance will decrease.[5] An AUROC of 0.752 was found in the group not using PPI, indicating that the modified IAI is a fairly good predictor to indicate who need ≥3 maintenance phlebotomies per year. However, the predictive properties were less distinctive in homozygous *p.Cys282Tyr* HH patients using PPI (AUROC 0.576).

The relationship between PPI use and phlebotomy frequency in patients with hemochromatosis has been shown previously. Hutchinson *et al*.[7] showed that PPI use resulted in a significant reduction in the maximum increase in serum iron concentration following ingestion of a test meal, containing highly bioavailable iron. Furthermore, van Aerts *et al*.[8] showed that PPI use significantly reduces the amount of blood removed annually. The IAI can be used to indicate the need of ≥3 phlebotomies per year as maintenance treatment with a cut-off value of 1.455, or more specifically 1.1 when the patient is not taking a PPI and 1.3 when taking a PPI.

Other predictive models have been developed, such as the iron reabsorption index (IRI)[9] and the hepatic iron index.[10] However, these indicators have some limitations, as they can only be calculated after at least a year of maintenance therapy or are invasive. Manet *et al*. calculated the IRI in a prospective cohort of 316 hemochromatosis patients, 25% were treated over 10 years.[9] This index was calculated taking into account the entire time of maintenance therapy and was expressed as milligrams of iron removed per day of treatment. IRI corresponds to the rate at which iron must be removed to maintain stable low serum ferritin levels,

therefore, it reflects the expressivity of phenotype in a given patient in its environmental background. However, because of the retrospective nature of this index, determination of IRI is of no practical help for predicting maintenance therapy at its start and is no substitute for serum ferritin as a tool for treatment control.[9]

Bassett *et al.*[10] introduced the 'hepatic iron index' (liver iron index: LII), i.e. the liver iron content in µmol/g dry weight divided by the age in years. However, this index was used more as a genotypic predictor, where it seemed of importance for the differential diagnosis and for younger subjects who were screened as members of families of patients identified with HH, before genetic testing was available. They found homozygous HH patients to have values >2, whereas heterozygotes and patients with liver disease had values <2.[10] If the liver iron content is expressed as lg/mg protein, this discriminatory value would be 0.224.[11] The disadvantage of this index is the invasive character, because a liver biopsy is needed.

Our study also has several limitations. First, it has a retrospective design, this carries the risk of selection bias, since only patients with a complete follow-up/data were included. Furthermore, adjusting for other possible factors such as weight, BMI, menopause (in women) and diet was not possible because this information could not be retrieved. In addition, it should also be noted that the study population belongs to the group of patients that expressed a biochemical and/or clinical penetrance of the homozygous *p.Cys282Tyr* HH. Longitudinal prognostic studies showed that 38–76% of homozygous individuals developed raised iron parameters (biochemical penetrance). The clinical penetrance however was lower: up to 38% in men and up to 10% in women.[12] Approximately 50% of female and 20% of male adult *p.Cys282Tyr* homozygotes have normal serum ferritin levels and may never require phlebotomy therapy.[13] This latter group might be seen in clinic more often, due to the progress in genetic testing. Whether or not our index could be used is this group of patients should be studied first.

Validating this index in a prospective study might point out other factors, which can help to optimize the index. Therefore, further studies are warranted before it can be introduced in clinical practice.

In conclusion, the modified IAI is a fairly good predictor for homozygous *p.Cys282Tyr* HH patients not using PPI to differentiate who needs ≥3 maintenance treatments per year. It will be very helpful to predict phenotype and select a specific group that needs to undergo frequent phlebotomies that could be selected for other forms of therapy,

e.g. erythrocytapheresis, as shown by this proof of concept study. Future prospective studies are needed to optimize the modified IAI, taking into account other factors that could influence the phenotype.

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Response to can modifier gene mutations improve the predictive value of the modified Iron Avidity Index in Type 1 Hereditary Hemochromatosis?

Pauline L. M. Verhaegh, Wenke Moris, Ger H. Koek and Cees Th. B. M van Deursen
Liver International 2016;36(11):1714.

To the Editor

We thank Drs Zanella, Di Lorenzo and Biasiotto for the interest in our recent publication.[1,2] In our study, we aimed to investigate if the modified Iron Avidity Index (mIAI) is a good phenotypic predictor for maintenance phlebotomies per year in homozygous *p.Cys282Tyr* hereditary hemochromatosis (HH) patients. We showed that the mIAI is a fairly good predictor to indicate who needs ≥3 maintenance phlebotomies per year in patients not using proton pump inhibitors (PPIs) (AUROC 0.752). However, the predictive properties were less distinctive in homozygous *p.Cys282Tyr* HH patients using PPI (AUROC 0.576). The cut-off values for optimal sensitivity and specificity were calculated using the Youden index (Table 5.5).

The mIAI value for the patient presented by Dr Zanella et al. is 1.9 (when log transformed), which is indeed higher than the cut-off (for all groups), indicating that the patient needs ≥3 maintenance phlebotomies per year.

It should be noted that our study population consists of patients who expressed biochemical and/or clinical features of the homozygous *p.Cys282Tyr* HH. Longitudinal prognostic studies showed that 38-76% of homozygous individuals developed raised iron parameters (biochemical penetrance). The clinical penetrance, however, was lower: up to 38% in men and up to 10% in women.[3] Approximately 50% of female and 20% of male adult *p.Cys282Tyr* homozygotes have normal serum ferritin levels and may never require phlebotomy therapy.[4] This latter group might be seen in clinic more often, due to genetic testing of first degree relatives of HH patients.

Whether the mIAI could predict the iron burden in a group of patients showing no clinical or biochemical penetrance or whether it could identify the presence of modifier gene mutations should be studied in larger cohorts of patients first.

We calculate the mIAI for all hemochromatosis patients in our hospital to examine whether mIAI scores can be correlated with different genetic backgrounds for hemochromatosis and different clinical manifestations. We expect that by conducting larger HH cohort studies it will be possible to adjust the mIAI score taking modifier gene mutations and other factors, such as serum iron and transferrin saturation, into account as has been suggested by our colleagues Drs Zanella, Di Lorenzo and Biasiotto.

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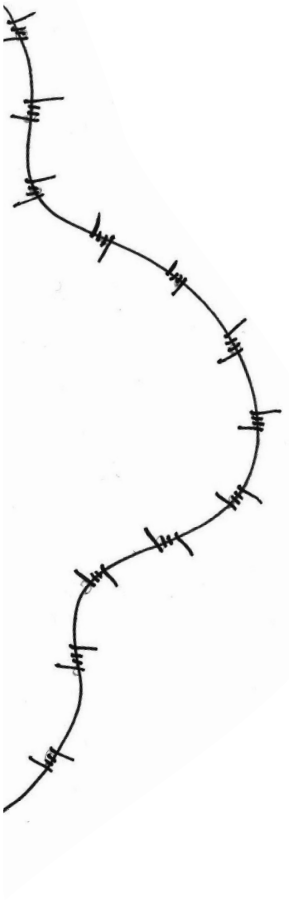
Chapter 6

Absorption of non-heme iron during gastric acid suppression in patients with hereditary hemochromatosis and healthy controls



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Am J Physiol Gastrointest Liver Physiol 2021;320(6):G1105-G1110

Abstract

Introduction

Phlebotomies are performed in hereditary hemochromatosis (HH) to maintain normal iron concentrations. Proton pump inhibitors (PPIs) can reduce the number of phlebotomies in HH patients. However, in patients without HH, the iron concentrations do not appear to be compromised when using PPIs. Therefore, we aim to explain the differences in iron absorption between patients with and without HH.

Methods

In ten *p.Cys282Tyr* homozygous HH patients with normalized iron stores and ten healthy control subjects (HCs), the iron parameters and hepcidin concentrations were determined before ingestion of a pharmacological dose of 50 mg iron (Fe^{3+}) polymaltose, and hourly for four hours afterwards. This was repeated after seven days' treatment with pantoprazole 40 mg once daily.

Results

Serum iron concentrations and transferrin saturation percentages dropped significantly during PPI use in the HH patients, while no changes were observed in the HCs. Hepcidin concentrations were lower in the HH patients compared with the HCs both before and during PPI use. In both groups hepcidin levels did not significantly decrease during the treatment.

Conclusions

Seven day PPI use significantly reduces iron absorption in HH patients but not in HCs. Changes in hepcidin concentrations could not explain these different PPI effects on iron absorption probably due to a small sample size.

Introduction

The most prevalent form of hereditary hemochromatosis (HH) is homozygosity for the p.Cys282Tyr variant in the *HFE* gene.[1] This condition is characterized by an ineffective regulatory feedback mechanism in which circulating hepcidin concentrations are disproportionally low for body iron stores. Since hepcidin is the key regulator of systemic iron metabolism, persistently low hepcidin concentrations will result in excessive iron absorption leading to iron accumulation.[1-3] Iron overload causes damage to parenchymal tissues and can lead to liver cirrhosis, severe arthropathy, diabetes mellitus, cardiac disease, and premature death.[4] The standard therapy for iron overload in HH is phlebotomy. However, patients can experience side effects with significant burden, reducing quality of life and requiring additional hospital visits.[5,6]

Proton pump inhibitors (PPIs) have been suggested as an attractive additional therapy to reduce the need for phlebotomies by reducing gastric acid secretion which results in decreased iron absorption.[7,8] Studies about the occurrence of anemia during long-term use of PPIs in patients without HH are contradictory.[9,10] Furthermore, the study describing PPI associated anemia in patients without HH, did not rule out a pre-existent iron deficient state or possible upper gastrointestinal blood loss.[10] To date, it is unclear via which mechanism PPIs appear to have a different influence on iron absorption in patients with HH and without HH. We hypothesized that HH patients benefit from a reduction in bioavailable iron via gastric acid inhibition, because their hepcidin levels are already disproportionally low. While in patients without HH the use of PPI will not result in a reduction in iron absorption because their hepcidin concentrations will decrease in response to gastric acid inhibition and the accompanying reduction in bioavailable iron.

Methods

Patients

We conducted a non-randomized proof of concept study, between January 2015 and January 2016, with ten *p.Cys282Tyr* homozygous HH patients with normalized iron stores and ten gender-matched healthy control subjects (HCs). The HH patients were recruited from the outpatient department of the Zuyderland medical centre in Heerlen/Brunssum, The Netherlands. The HCs were recruited among personnel of the medical centre and their acquaintances. All participants gave written informed consent, in keeping with the Declaration of Helsinki. The regional ethics committee

Atrium-Orbis-Zuyd approved the study. In both groups, men and menopausal women between the age of 18 and 65 years with ferritin concentrations <400 µg/L for at least three months were included. HH patients needed to be homozygous for *p.Cys282Tyr*, on maintenance treatment for at least one year and had their last phlebotomy ≥6 weeks before entering the study. HCs did not have *HFE* mutations ([compound] heterozygosity or homozygosity for *p.Cys282Tyr* or *p.His63Asp*). Exclusion criteria for both groups were coexistence of acute or chronic inflammatory disorders, such as inflammatory bowel disease or rheumatoid arthritis, hepatitis B, hepatitis C or HIV infection. Also, anemia, an active malignancy, alcohol intake of >21 units a week for men and >14 units a week for women, and present PPI treatment or other gastric acid-suppressing medication were criteria to exclude patients from the study. The same goes for the use of medication that interfered with PPIs e.g. vitamin C supplements, the use of iron supplements and previous side effects of PPIs.

Study design

On the first test day, after an overnight fast, baseline blood samples were drawn between 7.30 and 8.00 a.m. (T0) after which the participants ingested iron polymaltose (Ferrum Hausman, Vifor, Germany) containing a pharmacological dose of 50 mg of Fe^{3+} iron on a small piece of white bread. After ingesting the iron polymaltose, blood samples were drawn hourly for four hours (T1-T4). The participants did not receive breakfast until the 3rd blood sample (T2) was drawn, to reduce the chance of an interference with the outcome (Figure 6.1).

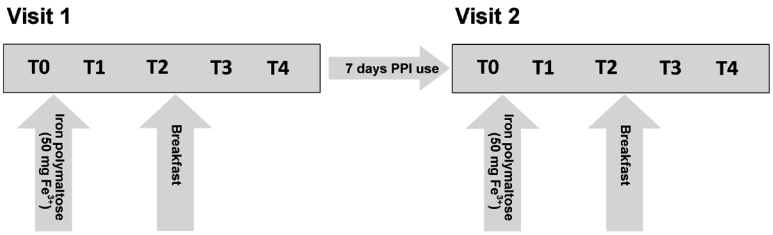

Figure 6.1 **Study design.** The baseline blood samples were drawn between 7.30 and 8.00 a.m. (T0) after which the participants ingested iron polymaltose (Ferrum Hausman, Vifor, Germany) containing a pharmacological dose of 50 mg of Fe^{3+} iron on a small piece of white bread. After ingesting the iron polymaltose, blood samples were drawn hourly for four hours (T1-T4). All patients consumed the same breakfast of +/- 300 kcal consisting of bread, coffee and water after the third blood sample (T2). The following day the participants started using 40 mg pantoprazole orally once daily, before breakfast, for seven days. On the seventh day, we repeated the oral iron challenge and blood sample collection. In all blood samples serum iron, transferrin, transferrin saturation and hepcidin concentrations were measured.

They all consumed the same breakfast of +/- 300 kcal consisting of bread, coffee and water. The following day the participants started using 40 mg pantoprazole orally once daily, before breakfast, for seven days. On the seventh day, we repeated the oral iron challenge and blood sample collection.

Laboratory analyses

Blood samples for hepcidin analysis were stored at -80°C. Serum iron and transferrin were determined on the days of the test by standard laboratory analysis. Transferrin saturation was calculated from serum iron and transferrin: iron (µmol/L) x 4.5) / transferrin (g/L).

Hepcidin measurements were performed in freshly thawed serum samples by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry using an internal standard for quantification.[11,12] Hepcidin-25 concentrations were expressed as nmol/L (nM). The median reference concentrations for serum/plasma hepcidin-25 (Dutch population) are 4.5 nM for men, 2.0 for premenopausal women and 4.9 nM for postmenopausal women.[13] The hepcidin test lower limit of detection was 0.5, in case of a hepcidin concentration below 0.5, the result is shown as <0.5.

Statistical analysis

Statistical analyses were carried out using SPSS, version 23 for Windows (IBM Statistics for Macintosh, Chicago, IL, USA). A power calculation was not possible since this is an exploratory study and no previous data regarding hepcidin concentrations in hemochromatosis were available. Data are expressed as mean (SD) for continuous variables and frequency (%) for categorical variables. Comparison of baseline values between the HH group and the HC group were performed using the independent T Test in case of continuous variables and using the Fisher's exact test for categorical variables.

The comparison of repeated measurements, before and during PPIs, within groups and between groups (HH patients and HCs), was performed using a linear mixed model with a first order autoregressive (AR1) covariate type for the repeated measurements. Fixed factors included the following variables: group (HH patient vs. HC), PPI use (before vs. during PPI use), and the time points of the blood sample collection (T0, T1, T2, T3 and T4). The random factor was the participant ID. The repeated variables included the test days (before vs. during PPI use) and the time points of the blood sample collection. Furthermore, EM means were calculated. In

case of hepcidin concentrations < 0.5, we performed statistical analyses with hepcidin concentrations of 0.5, 0.25 and 0.01 to test if this would lead to different outcomes. This was not the case, therefore in this paper we reported the value 0.25 when hepcidin analysis showed <0.5. A Bonferroni correction was used to correct for multiple testing. P-values were considered significant when ≤0.05.

Results

Patient baseline characteristics

Of the 126 *p.Cys282Tyr* homozygous HH patients regularly visiting the outpatient clinic, 24 matched the inclusion criteria. Of these 24 HH patients, ten agreed to participate in the study.

Twenty-three HCs were screened for the *HFE* mutations *p.Cys282Tyr* and *p.His63Asp* and their ferritin concentrations were checked. In 13% (3/23) hyperferritinemia was observed and in 43.5% (10/23) heterozygosity for one of the *HFE* mutations was found (2/10 *p.Cys282Tyr* and 8/10 *p.His63Asp*). These HCs were then excluded leaving ten subjects who fulfilled the inclusion criteria. Baseline characteristics are summarized in Table 6.1.

In this gender-matched study the majority of participants was male (70%). Age and BMI were not significantly different between groups.

Table 6.1 Baseline characteristics of the study population.

Baseline features	Hemochromatosis patients (n=10)	Healthy control subjects (n=10)	P-value
Age (Year)	55.3 ± 8.2	50.1 ± 9.4	0.205
Gender (male)	7 (70%)	7 (70%)	>0.999
BMI (kg/m^2)	26.8 ± 2.8	24.5 ± 3.0	0.100
Smoking (yes)	2 (20%)	1 (10%)	>0.999
Alcohol (yes)	10 (100%)	7 (70%)	0.211
CRP (mg/l) (0-10)	1.8 ± 2.6	0.8 ± 1.1	0.282
ALT (U/L) (0-40)	25.8 ± 8.8	26.7 ± 8.8	0.821
GGT (U/L) (0-40)	52 ± 29.0	47 ± 42.2	0.761
Hb (mmol/l) (8.5-11♂; 7.5 -10 ♀)	9.7 ± 0.8	9.3 ± 0.7	0.258
Ht (L/L) (0.41-0.51 ♂ 0.36-0.47 ♀)	0.45 ± 0.04	0.44 ± 0.03	0.500
Serum ferritin (µg/L) (30-400)	98.3 ± 110.0	153.1 ± 91.6	0.242
Transferrin saturation (%) (16-45)	61.2 ± 18.2	26.7 ± 10.6	0.000
Transferrin (g/L) (2-4.1)	2.0 ± 0.2	2.5 ± 0.3	0.000

Data are expressed as absolute numbers (percentage), mean ± SD. *Abbreviations: CRP, C-reactive protein; ALAT, alanine aminotransferase; GGT, gamma-glutamyl transferase; Hb, hemoglobin; HT, hematocrit.*

Serum iron and transferrin saturation

In HH patients both serum iron concentrations before and during PPI treatment were significantly higher than those obtained from HCs at all-time points, with a mean difference between the two groups of 14.2 µmol/l (p=0.001) before PPI use and 9.9 µmol/l (p=0.013) during PPI use (Figure 6.2a).

In the HC group serum iron concentrations before and during PPI use showed no significant difference (mean difference of 0.02 µmol/l, p=0.985). In the HH patients, lower serum iron concentrations were found during PPI use compared with before PPI use with a mean difference of 4.35 µmol/l (p<0.001) (Figure 6.2a).

Similar results were obtained for transferrin saturation (TSAT) (Figure 6.2b). HH patients had higher TSAT percentages at all-time points, both before (mean difference 39.18%, p<0.001) and during PPI use (mean difference 28.25%, p=0.001) compared with HCs. In the HH patients, significantly lower TSAT percentages were shown during PPI use compared with before PPI use (mean difference 10.33%, p<0.001). In the HC group no significant difference was found in TSAT comparing before and during PPI use (mean difference 0.60, p=0.760).

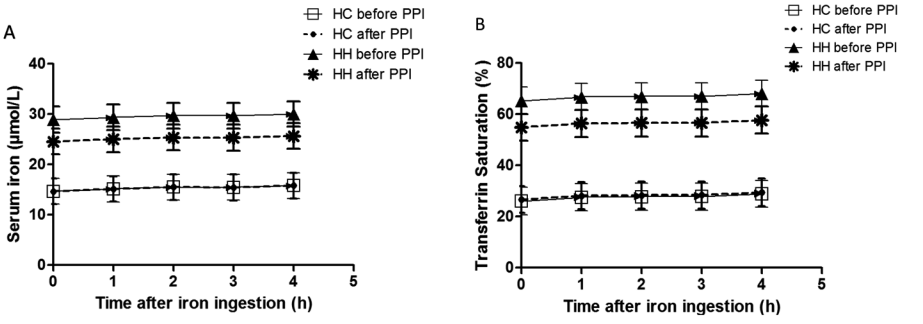

Figure 6.2 **Effect of PPI on serum iron concentrations and transferrin saturation over time in patients with HH (n=10) and HC subjects (n=10) after oral iron supplementation the day before and on day 7 of PPI administration.** (a) Serum iron concentrations (b) transferrin saturation. *Values represent estimated means with a standard error of the mean calculated by mixed model analysis. *Abbreviations: HC, healthy control subjects; PPI, proton pump inhibitor; HH, hereditary hemochromatosis patients.*

Hepcidin

HH patients had significantly lower hepcidin concentrations at all-time points compared with HCs, both before (mean difference 2.46 nmol/L, p=0.002) and during

PPI use (mean difference 1.69 nmol/L, p=0.029) (Figure 6.3a-b). The hepcidin concentration in the HC group showed lower concentrations during PPI use compared to before PPI use, however this difference was not statistically significant (mean difference 0.54 nmol/L, p=0.166). Also in the HH patients no statistically significant difference in hepcidin concentrations was found during PPI use compared with before PPI use (mean difference 0.23 nmol/L, p=0.549) (Figure 6.4).

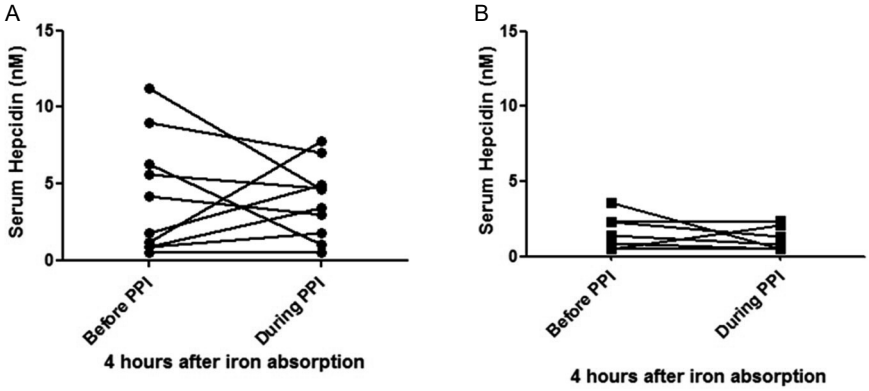

Figure 6.3 **Serum hepcidin concentrations, measured four hours (T4) after the intake of iron polymaltose, displayed for each individual patient/healthy control subject before and during PPI.** (a) The serum hepcidin concentrations for each healthy control subject (n=10), in five subjects the hepcidin level decreased (n=4) or stayed unchanged (n=1) comparing before vs during PPI (b) The serum hepcidin concentrations for each hemochromatosis patient (n=10) in seven patients the hepcidin level decreased (n=5) or stayed unchanged (n=2) comparing before vs during PPI use, in two other patients the measured increase was not more than 0,1nM.

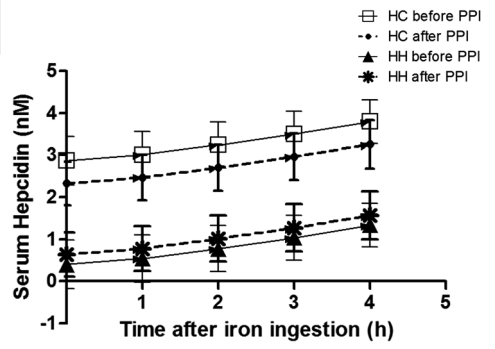

Figure 6.4 **Hepcidine course after iron ingestion before and after PPI use in HH and HCs.** No significant differences were seen between the hepcidin concentrations before and during PPI use within HCs (n=10) and within HH patients (n=10) (HC group mean difference of 0.54, p=0.166; HH group mean difference 0.23, p=0.549). Between group analysis showed a statistically significantly lower hepcidin concentration in HH patients for all time points (before PPI use mean difference 2.46, p=0.002; during PPI use mean difference 1.69, p=0.029). *Values are estimated means with a standard error (SE) of the mean calculated by mixed model analysis. *Abbreviations: HCs, healthy control subjects; PPI, proton pump inhibitor; HH, hereditary hemochromatosis.*

Discussion

This proof of concept study shows that short term use of PPIs leads to significantly lower circulating iron concentrations in iron-depleted HH patients, whereas it has no effect on serum iron concentrations in HCs. These results support the finding that reducing the acidity of the gastric content with PPIs leads to a decrease in iron absorption in HH patients.[7,8]

Iron in the food can be present in the non-heme and/or heme-bound form. Iron is absorbed mainly in the duodenum and the upper jejunum. In persons who eat meat, heme-iron may contribute to 10–15% of the daily iron intake. Heme-iron is absorbed to a higher extent than non-heme iron. In contrast to non-heme iron, heme-iron is less influenced by dietary constituents and the higher pH of the small intestine.[14,15] Heme-iron is absorbed into the enterocyte through the heme carrier protein 1. Inside the cell iron is released from heme by heme oxygenase.[3] Non-heme iron absorption takes place mainly on the apical membrane of the enterocyte via the divalent-metal transporter-1 (DMT1).[3,16] This transporter is selective for ferrous iron (Fe^{2+}). Since ferric iron (Fe^{3+}), is the predominant form present in the diet a reduction step of ferric iron to ferrous iron is necessary for absorption. The reduction is catalysed by duodenal cytochrome-b, a major intestinal ferrireductase.[3,17,18] DMT1 is a H+-coupled cotransporter and it functions optimally at acidic pH.[19] PPIs reduce the acid content of gastric secretions resulting in higher pH up to 6. As a result the DMT1 function will decrease and the reduction of ferric iron will be diminished, with less ferrous iron available for absorption via the DMT1.[20] In patients with HH, intestinal DMT1 is upregulated which may result in increased iron absorption into the enterocyte.[20,21] Results of a mice study suggested that in case of a more acidic pH, the ferrireductase activity is lower.[22] This mechanism suggests that HH patients on PPIs would have increased iron absorption through upregulated DMT1 and increased ferrireductase activity. However, in patients with HH treated with PPI, far less phlebotomies were needed to maintain a stable serum ferritin concentration. This indicates that the inhibitory effects of PPI use, lowering gastric acid secretion, on the uptake of iron, overruled the promoting effects (upregulated DMT1 and increased ferrireductase activity) seen in the intestinal cells in hemochromatosis.[7,8,23] Our results also show significantly lower circulating iron concentrations in HH patients after PPI use. These results are in line with Hutchinson *et al*., who previously reported a significant reduction in increase of iron concentrations following an iron challenge, after seven days of PPIs, in HH patients.[23] Furthermore, our study showed no decrease in circulating iron concentrations after PPI use in HCs. An explanation for this might be the fact that iron, whether originating from heme or from non-heme sources, can only

leave the enterocyte via ferroportin, a process that is regulated by hepcidin.[24] This protein is disproportionally low in hereditary hemochromatosis but not in HCs, suggesting that in HCs hepcidin concentrations could be lowered to ensure enough iron uptake from the enterocyte, whereas HH patient cannot use hepcidin to regulate their iron concentrations.

We did not observe a significant decrease in serum hepcidin concentrations after one week of PPIs in HCs. As shown in Figure 6.3 there was a wide variation in hepcidin concentrations in HCs both before and after PPI administration. Because of these wide variations and our small sample size in this study it is not possible to determine the possible effect of hepcidin on the differences in iron absorption.

It should be noted that an increase in iron concentrations after the iron challenge was not seen in our study. Previous studies measuring iron concentrations following an oral iron challenge used ferrous sulphate. These studies did observe an increase in iron parameters.[25,26] We aimed to measure the effect of gastric acid inhibition so in our study the use of a ferric preparation was essential. Furthermore, most of the iron in our food is in the ferric form. However, the lack of rise in iron concentrations after iron administration cannot solely be related to the administration of ferric iron compared to ferrous iron.[27] Hutchinson *et al.* also used ferric chloride and showed an increase in iron concentrations.[23] A possible explanation for the lack of iron increase in our study could be that absorption of ferric preparations is up to seven times better when taken with food and our patients were fasted while the patients of Hutchison *et al*. received their ferric chloride combined with an iron enriched meal.[28]

Strengths of this study include the accurate study protocol, including an hourly monitoring of serum iron parameters and the accurate selection process of the HCs to exclude acquired or genetic confounding factors, including *HFE* genotypes susceptible for iron overload. Moreover, using the stable portion of iron polymaltose instead of an iron enriched meal allowed an exact amount of Fe^{3+} to be ingested. Furthermore, the effect of PPI use on hepcidin concentrations after an oral iron challenge has never been studied before. We recognize that the current study has limitations. First, the period of PPI use was short and no gastric pH measurements were done to check the effect of PPI. However, continually measuring gastric pH can be experienced as invasive and the decrease in acidity following use of PPI has already been clearly documented, even after seven days of PPI.[29]

Furthermore, the study population was small. The sample size was based on previous studies researching serum ferritin concentrations. Because of the lack of data on hepcidin concentrations in hemochromatosis patients using PPI, a power calculation for hepcidin as outcome measure could not be performed. Therefore, the results on hepcidin should be interpreted with caution and it is necessary for future studies on hepcidin to include a larger study population.

In conclusion, our proof of concept study has shown that PPI use significantly reduces serum iron concentrations in HH patients but not in HCs, indicating that PPI use reduces iron absorption only in HH patients. The presupposition that PPI use in HCs will not result in a reduction in iron absorption because their hepcidin concentrations will decrease in response to lowering gastric acidity could not be confirmed. However, it should be noted that the sample size was small. Future studies should include a larger study population and preferably also different doses of PPIs to unravel the pathophysiological mechanisms.

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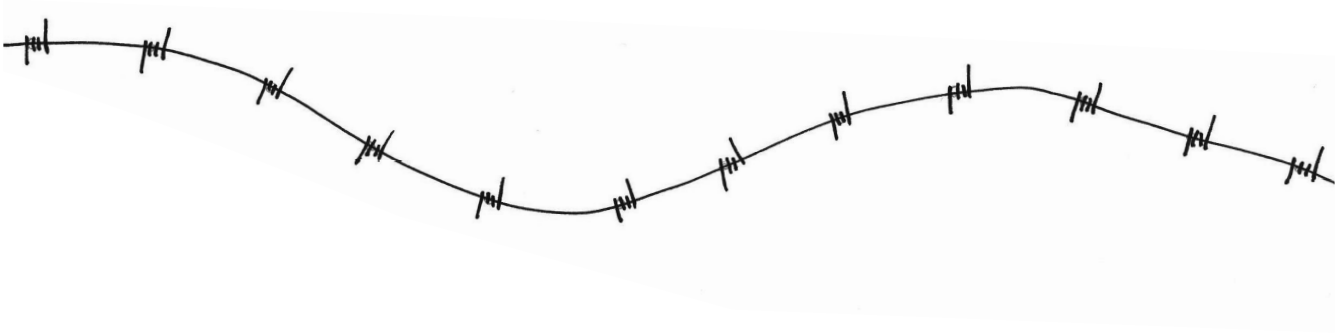
Chapter 7

Inflammation can increase hepcidin in *HFE*-hereditary hemochromatosis



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Clinical Case Reports 2021;9(5):e04114

Abstract

We present a *p.Cys282Tyr* homozygous patient with high hepcidin levels and normal iron parameters during systemic inflammation. This suggests that in the absence of a proper functioning HFE, resulting in blockage of the BMP/SMAD pathway, the innate low hepcidin concentration can be upregulated by inflammation, probably via the JAK/STAT3 pathway.

Case report

The production of hepcidin, a peptide hormone synthesized by the hepatocyte, is stimulated by iron overload and inflammation through largely distinct pathways.[1] In *HFE*-hereditary hemochromatosis (HH) the hepcidin level is inadequately low for body iron stores, resulting in continuous iron absorption in spite of elevated body iron stores.[1] Less is known about hepcidin levels in *HFE*-HH in times of inflammation. We report the case of a *p.Cys282Tyr* homozygous patient with elevated hepcidin levels and correspondingly lower iron parameters at a time of systemic inflammation. The patient, a Caucasian man, was diagnosed in November 2011 with *p.Cys282Tyr* homozygosity in the *HFE* gene, at the age of 51. At that time, he had a serum ferritin level of 1319 µg/L, a serum iron of 36 µmol/L, and a transferrin saturation of 73%. He was treated with weekly phlebotomies and after 17 phlebotomies his ferritin level became 126 µg/L, whereafter maintenance treatment was started. Three years after reaching the depletion stage, the patient participated in a proof of concept study to investigate the role of acid suppression on the course of serum iron and hepcidin concentrations after a single oral ingestion of 50 mg iron polymaltose (Fe^{3+}). The experiment consisted of two test days; test day 1 before proton pump inhibitor (PPI) use and test day 2 after seven days of PPI use. Just before the start of the study, the patient's serum ferritin level was 89 µg/L, serum iron 27.8 µmol/L, and transferrin saturation 46%. His last phlebotomy was almost 4 months before. The results of the first test day showed a high hepcidin and a low serum iron level (Figure 7.1).

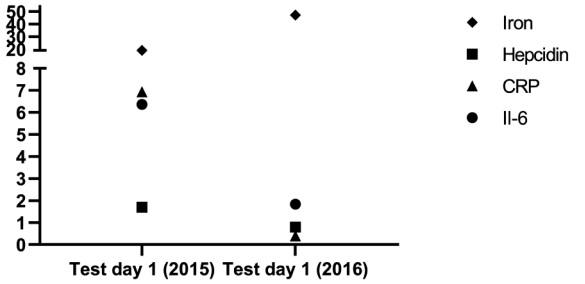

Figure 7.1 **Overview of iron and inflammation parameters measured during the first and second experiment.** This figure shows the values of iron, hepcidin, CRP and Il-6 values of a patient with homozygous *HFE*-HH. During the first test day in 2015 the patient was recovering from a cold. One year after full recovery, without signs of systemic inflammation, the study was repeated. All presented results are of the first test day so before the use of pantoprazole. Reference values: Iron values: 11-30 µmol/L , Hepcidin values: <0.5-14.7 nM for men with a median of 4.5nM, CRP: 0-10 mg/L, IL-6: 0.447-9.96 pg/mL.

The serum ferritin level was 164 µg/L with a transferrin saturation of 32%. On the second test day, after 1 week of pantoprazole 40 mg daily, the hepcidin level decreased and the iron parameters were higher, but still lower than usual for this patient; serum iron level 25.7 µmol/L, transferrin saturation 44%, and serum ferritin 137 µg/L (Table 7.1).

Table 7.1 The iron and inflammation parameters measured during the first and second experiment.

	First experiment (2015)		Second experiment (2016)	
	Test day 1 (Before PPI use)	Test day 2 (After PPI use for 7 days)	Test day 1 (Before PPI use)	Test day 1 (After PPI use for 7 days)
Cold symptoms	yes	no	no	no
Serum iron (µmol/L)	19	25.7	47.3	51.5
Hepcidin (nM)	1.7	0.5	0.8	0.5
CRP (mg/L)	6.93	1.25	0.4	0.27
IL-6 (pg/mL)	6.36	2.08	1.84	1.29

Results of both test days of the first and second experiment are shown. During the first test day in 2015 the patient was recovering from a cold. The second test day seven days later was after seven days of PPI use, the patient already was recovered at that time. One year later without signs of a cold or systemic inflammation, the study was repeated. Again the first test day was before PPI use and the second test day was after seven days of PPI use. Reference values: serum: Iron 11-30 µmol/L , Hepcidin: <0.5-14.7 nM for men with a median of 4.5nM, CRP: 0-10 mg/L, IL-6: 0.447-9.96 pg/mL Abbreviations: Proton pump inhibitor (PPI), C-reactive protein (CRP), interleukin (IL).

This was unexpected since in *p.Cys282Tyr* homozygous patients, PPI administration has been shown to reduce the need for phlebotomy by reducing the iron absorption.[2] Searching for the explanation, the patient was contacted and he reported that during the first test day he was recovering from a cold but did not want to cancel his participation. During this cold, he felt ill and had a runny nose, a sore throat, and a cough. He did not have a fever and his symptoms were self-limiting within a couple of days, without the need for medication. C-reactive protein (CRP) and interleukin-6 (IL-6) levels were measured and both were increased suggesting a systemic-inflammatory response. One year later, after a full recovery, the experiment was repeated, following the same protocol. The results of the first test day, before the use of PPIs, showed a low hepcidin level and increased serum iron levels, in the absence of elevated CRP and IL-6 values (Figure 7.1 and Table 7.1). Body iron excess and inflammation are both known to stimulate hepcidin production.[3] Studies in *HFE*-knockout mice report conflicting results concerning hepcidin values after exposure to an inflammatory stimulus.[4-7] The only human study so far on the subject comprises a case report of an iron-depleted *p.Cys282Tyr* homozygous patient with a variant Schnitzler's syndrome, an autoinflammatory condition. The patient had periods of fever with peaking IL-6 followed by increased hepcidin concentrations and hypoferremia. After treatment

with an anti-inflammatory cytokine interleukin-1 receptor antagonist, IL-6 levels normalized and hepcidin levels reduced and became undetectable, in agreement with what is expected for iron-depleted *HFE*-HH.[8] Upregulation of hepcidin is explained by the fact that *Hamp* expression, the gene encoding for hepcidin, is regulated by several pathways (Figure 7.2). On the one hand, hepcidin expression is regulated by the Bone Morphogenetic Protein/Small Mothers Against Decapentaplegic (BMP/SMAD) pathway in response to body iron levels. On the other hand, proinflammatory factors can increase hepcidin expression mostly through the janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) signaling pathway.[9,10] It has been suggested that HFE plays a role in transducing an iron-induced signal through the BMP/SMAD signaling pathway to stimulate *Hamp* transcription while the inflammatory pathway (STAT3), activated by IL-6, does not require HFE.[11,12]

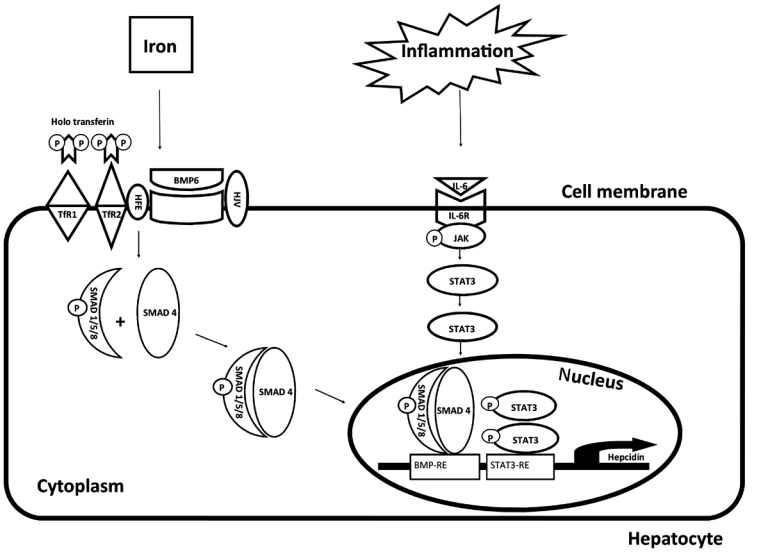

Figure 7.2 **Pathways involved in the regulation of Hepcidin**. Iron transported by transferrin, together with a number of co-factors such as Hemojuvelin, BMP6 and HFE, can activate the SMAD complex. In the nucleus the SMAD complex together with the STAT3 complex induces the production of Hepcidin. Inflammation activates the JAK/STAT3 complex via IL-6. In the nucleus the JAK/STAT3 complex induces the production of Hepcidin in cooperation with the BMP-SMAD complex. This figure was adapted after Muckenthaler et al. 2017 and Silvestri et al. 2019. *Abbreviations: TfR1, Transferrin receptor; BMP, Bone morphogenetic protein; HJV, Hemjuvelin; IL-6, interleukin 6; IL-6R, Interleukin 6 receptor; JAK, Janus kinase; STAT, Signal Transducer and Activator of Transcription; SMAD, Small Mothers Against Decapentaplegic*.

Our case of a *p.Cys282Tyr* homozygous patient confirms that hepcidin levels can increase in hemochromatosis, with consequently also lower serum iron levels, due to an inflammatory state. It suggests that the JAK/STAT3 pathway can still induce hepcidin production in spite of attenuation of the BMP/SMAD pathway in patients with HFE-HH and in this way decrease iron absorption.[11,13] Based on these observations, intervention via the JAK/STAT3 pathway could reduce excess absorption and accumulation of iron in patients with HH and deserves further exploration.

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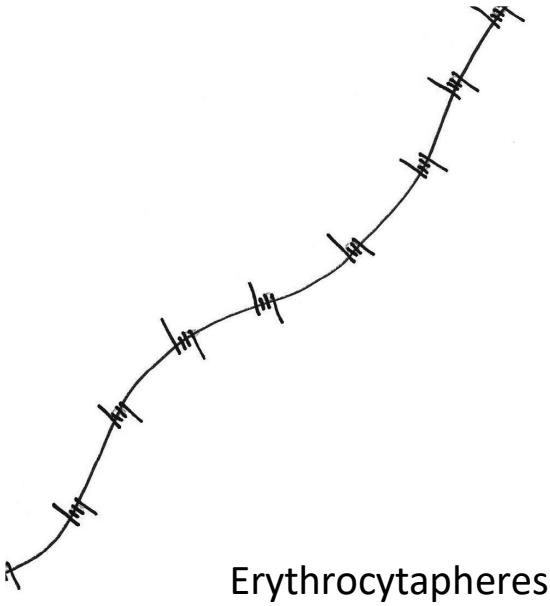

Chapter 8

Erythrocytapheresis in aceruloplasminemia prevents progression of cerebral iron accumulation after chelator-induced normalization of iron stores: report of two cases

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*Shared last authorship

Submitted

Abstract

Aceruloplasminemia is a rare disorder characterized by the absence of ceruloplasmin due to mutations in the ceruloplasmin genes. This results in iron accumulation leading to diabetes, anemia, retinal degradation, and neurological disorders. We describe two cases with iron accumulation in the brain causing neurological symptoms in one. Initial treatment with iron chelators removed much or all of the excessive peripheral iron evidenced by decreased ferritin levels and decreased or normalized liver iron content on MRI. Because of the intolerable side effects of the chelation therapy, including anemia, therapeutic erythrocytapheresis was initiated under the hypothesis that further iron accumulation would be prevented by engendering erythropoietic activity in the bone marrow resulting in increased iron demand. Repetitive therapeutic erythrocytapheresis turned out to be very successful in preventing further iron accumulation. Anemia did not occur because of the adjustment of the removed erythrocyte volume based on hemoglobin values. The patient without neurological symptoms remained neurologically symptom-free during the nine years of repetitive therapeutic erythrocytapheresis. The severe neurologic symptoms in the other patient did not improve, despite normalization of the peripheral iron content. To our knowledge, this is the first report on therapeutic erythrocytapheresis in aceruloplasminemia. It prevented the progression of cerebral and peripheral iron accumulation without causing symptomatic anemia.

Introduction

Hereditary aceruloplasminemia (HA) is a rare autosomal recessive disorder usually resulting from bi-allelic mutations in the *ceruloplasmin* (*CP*) gene on chromosome 3.[1] CP is a multicopper containing ferroxidase that plays a role in cellular iron export. It catalyzes the oxidation of intracellular ferrous iron (Fe^{2+}) that is transported by ferroportin (FPN) into ferric (Fe^{3+}) iron enabling its binding to transferrin, the carrier protein for iron in the blood[2-4] (Figure 8.1).

HA is characterized by progressive iron overload especially within the liver and brain. The mechanistic explanation of HA is not fully understood and under debate. CP is expressed by a large number of cells including macrophages, astrocytes and hepatocytes.[5-7] It is suggested that, in the absence of CP, iron is not exported from these cells since a ferroxidase (hephaestin or ceruloplasmin) is necessary for a proper functioning of the basolateral transporter FPN. This results in a low transferrin saturation and low hepcidin synthesis.[8] The low hepcidin concentration induces an increased intestinal iron absorption since enterocytic FPN relies on the ferroxidase hephaestin.[8] This leads to progressive iron accumulation that can cause organ dysfunction through the formation of free radicals.[9] Recent data remarkably show that iron deposits are predominantly located in the hepatocytes, whereas the resident macrophages in the liver, the Kupffer cells, and spleen macrophages do not have increased iron deposits debating the previously suggested mechanism of decreased iron export by decreased CP function.[10] This indicates that the iron metabolism in HA is still not fully elucidated and will be a reason for further research.

Clinically, HA can result in a triad of adult-onset neurodegenerative symptoms, insulin-dependent diabetes mellitus, and retinal degradation however they do not need to be all present together.[9] Neurodegeneration in HA is mainly seen in the dentate nuclei of the cerebellum, the thalamus, and the basal ganglia. It may become progressively symptomatic later in adult life with a poor prognosis.[11] The clinical expression, however, is not uniform. The most frequent neurological manifestations in Japanese patients were movement disorders like cerebellar ataxia, chorea, tremor, dystonia, and eventually cognitive decline and dementia. The Caucasian phenotype seems to be characterized more frequently by parkinsonism and psychiatric symptoms.[11] No clear genotype-phenotype association was found.[1] In HA patients erythropoiesis mainly depends on the direct utilization of transferrin-bound absorbed iron as the release of iron from macrophages is hampered resulting in slight to moderate microcytic anemia.[2,12,13]

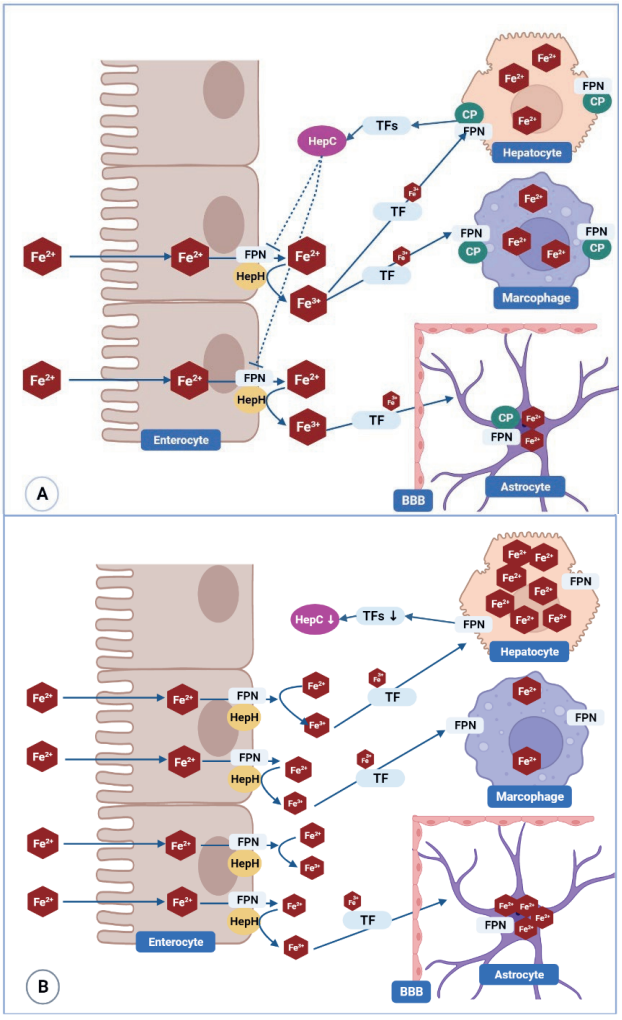

Figure 8.1 **Cellular iron efflux in normal individuals (a) and patients with HA (b).** (a) Effective cellular iron efflux requires the oxidation of iron after transportation by ferroportin (FPN). In small intestinal epithelial cells, the ferroxidase is hephaestin (Heph), and in other cells like astrocytes, hepatocytes and macrophages, it is ceruloplasmin (CP). The iron saturation of the iron-transporting blood protein transferrin (TF) regulates intestinal iron absorption via hepcidin. This protein causes the internalization of FPN, thereby preventing cellular iron efflux. (b) The permanent absence of CP in patients with HA results in progressive iron accumulation in non-intestinal cells because of the blocked iron efflux. The resulting decreased transferrin saturation leads to a reduced hepcidin (HepC) production, thereby increasing the iron efflux by small intestinal epithelial cells. There is still debate about the mechanism and location of iron accumulation since a recent study did not show iron accumulation in liver (Kupffer cells) and spleen macrophages.

The only available information on therapeutic strategies is presented in case reports. Herein mainly iron chelating drugs are used. Iron-chelating agents can gain excess to intracellular iron pools were they can diminish the iron excess in peripheral tissues but unfortunately not in the brain. An important problem with these drugs is that they are often not tolerated because of side effects, including worsening anemia.[14] An alternative, phlebotomy, did not reduce peripheral and cerebral iron overload, and in some cases, neurological symptoms even developed during this therapy.[6,15,16] It also aggravated pre-existing anemia.[16,17]

Therefore there is need for a more effective and tolerable treatment that reduces, or at least prevents, further iron overload without causing symptomatic anemia, especially in the hope of preventing (deterioration of) neurological symptoms. We hypothesized that therapeutic erythrocytapheresis (TE) could accomplish this and investigated this treatment in two cases that had been treated before by chelators to normalize peripheral iron concentration. TE allows a more precise and selective reduction in iron-containing erythrocytes preventing the development of symptomatic anemia. The resulting increased iron demand of the enhanced erythropoietic activity in the bone marrow might then prevent further iron accumulation.

Case report

Case 1

A 46-year-old woman, known with insulin-dependent diabetes mellitus, was admitted to our hospital in 2009 for diabetes regulation after a severe episode of ketoacidosis. Four years earlier, progressive neurological symptoms like forgetfulness, cognitive deterioration, and gloomy mood leading to frequent disagreements and altercations had started. She had been admitted multiple times to psychiatric units and had undergone numerous tests, including neurological examination, cerebral MRI, EEG, and brain perfusion scanning (HMPAO SPECT). Neither tests had shown any explanatory anomalies.

Eventually, during her submission in the hospital in 2009 they considered the diagnosis of HA for the first time when encompassing all her medical problems; normocytic anemia, hyperferritinemia, low transferrin saturation, and an extremely low CP (0.04 g/L) (Table 8.1). A renewed brain MRI showed global atrophy and iron accumulation in the cortex and basal ganglia. Severe cognitive impairment was evident by a low Cambridge Cognitive Examination (CAMCOG) score of 64/105

(scores below 84 are suspect for a cognitive disorder). Her Mini-Mental State Examination (MMSE) test had decreased to 22/30 compared to 29/30 in 2005 (below 24 is abnormal, lower scores suggest more significant impairment). A liver MRI revealed an iron deposit of 350 µmol/g dry weight (ref.: <36). The diagnosis of HA was confirmed with the finding of two *CP* mutations p.C2701C>T and p.C.2227T>G on chromosomes 3. In retrospect, there was already mild iron excess present on the MRI scan in 2005. However, the scan technique at that time was not optimal for the detection of iron accumulation. Additional DNA analysis also showed compound heterozygosity (*p.Cys282Tyr*/*p.His63Asp*) of the *HFE* gene. Inspired by therapy described in a case report, fresh frozen plasma twice a week combined with an iron chelator (deferiprone) was started. The rationale to add fresh frozen plasma is that it contains ceruloplasmin.[18] This therapy was switched to deferasirox, after one year of treatment because her neurological symptoms did not improve, and the hyperferritinemia persisted (Table 8.1). No amelioration in the iron excess in neither the liver nor the brain was seen on MRI scans. At the beginning of 2013, deferasirox was discontinued because of renal toxicity (Table 8.1). At that time, the cognitive impairment had deteriorated (04-2013: CAMCOG 50/104 and MMSE 16/30). In February of 2013, TE was started. The volume of removed erythrocytes was adjusted based on values of hemoglobin and hematocrit measured prior to each procedure. After 12 months of treatment with seven TEs and a total iron removal of 1763 mg, her serum ferritin had decreased from 796 µg/L to 552 µg/L. Her impaired cognitive functioning and venous access problems hindered compliance with TE, and we did not manage to remove the optimal amount of iron. Following one year of therapy, her MMSE score ameliorated slightly to 18/30. Since the beginning of 2014, TE was performed twice a year, keeping ferritin levels stable around 400-500 µg/L. In 2017 TE failed two times due to increased venous access problems and agitation. This and her severely decreased quality of life from progressive cognitive impairment made us discontinue TE with her legal representative's consent. She passed away in April of 2018 at the age of 55.

Table 8.1 Laboratory findings of the two cases at repeated moments in treatment period.

	At diagnosis		After using deferiprone		During treatment with deferasirox		Before start TE		4 years after start TE	9 years after start TE
	Case 1	**Case 2**	**Case 1**	**Case 2**	**Case 1**	**Case 2**	**Case 1**	**Case 2**	**Case 1**	**Case 2**
Serum ferritin (µ/L)	2310	2329	1573	2900	NA	53	667	203	1043	295
Serum iron (µmol/L)	4.9	8.1	NA	NA	NA	NA	4.7	NA	4.5	5.4
Transferrin (g/L)	1.09	2.08	NA	1.46	NA	NA	2.09	NA	1.78	2.95
Transferrin saturation (%)	20	17.3	NA	NA	NA	NA	9	NA	NA	7.3
Hemoglobin (g/dL)	12.57	13.54	13.21	13.37	12.73	8.54	11.44	12.41	13.54	12.89
Creatinine (µmol/L)	62	NA	65	80	136	129	94	83	70	96

Case 2

In 2009 her 45-year-old brother, also known with insulin-dependent diabetes mellitus since the age of 38, was screened for HA. At that time, he did not experience cognitive impairment. A complete blood count showed a normal hemoglobin and a hyperferritinemia of 2329 µg/L (Table 8.1). DNA diagnostics confirmed the same two *CP* mutations and heterozygosity for the *p.Cys282Tyr HFE* mutation. The liver MRI showed iron accumulation of 350 µmol/g dry weight (Figure 8.2), and the cerebral MRI showed impressive iron accumulation in the basal ganglia and the cerebral cortex and mild iron accumulation in the cerebellar cortex without signs of atrophy (Figure 8.3). Treatment was started with the administration of zinc in an attempt to neutralize the oxidative effect of intracellular iron[19], soon followed by deferiprone. Because of progressive hyperferritinemia, the treatment was altered to deferasirox (Table 8.1). The ferritin level normalized (53 µg/L); however, deferasirox was discontinued due to severe anemia (hemoglobin 8.54 g/dL), renal toxicity, and stomach pain (Table 8.1). A new MRI of the liver showed a normalization of the iron content from 350 to 40 µmol/g dry weight (Figure 8.2), but the cerebral MRI showed no decrease in iron excess and new signs of discrete atrophy. TE was then initiated with a frequency of 3-4 treatments yearly to keep the ferritin level below the upper limit of normal (<300 µg/L). Each session, an average of 500ml erythrocytes and thus 430mg of iron has been effectively removed. Over the years, the mean hemoglobin pre-treatment was 12.5 g/dl and post-treatment 9.4 g/dl. To date, December 2021, after more than nine years of repetitive TE therapy, there was no evidence of further iron accumulation. Ferritin levels remained within the lower normal range, and the MRI of

the liver did not show any iron re-accumulation. The increased iron content in the brain had remained stable on MRI without causing cognitive impairment (his MMSE score remained maximal).

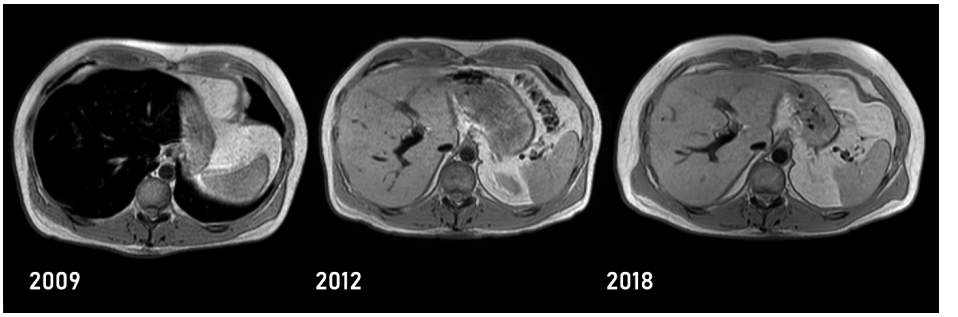

Figure 8.2 **Liver MRI of case 2.** The MRI in 2009 shows an important reduction in signal intensity in the liver consistent with iron accumulation. The signal intensity of the liver improves of time consistent with a significant decrease in iron deposition.

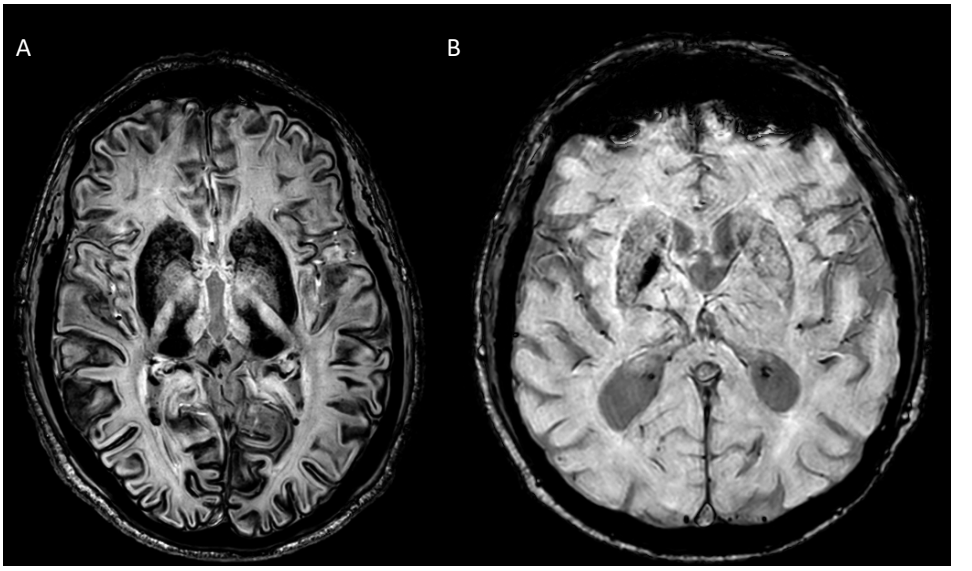

Figure 8.3 **Brain MRI** (a) Brain MRI of case 2 shows susceptibility artifact, consistent with iron overload seen in the thalamus, putamen, dentate nucleus both sides and to a lesser extent in the globus pallidus. The cerebral cortex is better aligned on the gradient sequence than normal, also consistent for iron overload. There is very minimal susceptibility artifact/iron overload seen high in the cerebellar cortex. (b) a brain MRI of a random person without iron deposition.

Discussion

To our knowledge, this is the first report describing TE as a successful therapy for HA by preventing increased iron accumulation peripherally and in the brain after normalization of iron stores with chelating agents, without side effects including anemia.

In the first case, TE was started before resolving peripheral iron accumulation due to an intolerance for deferasirox treatment, and it diminished liver iron overload. In the second case, deferasirox had already resolved peripheral iron accumulation, and TE was used to prevent iron re-accumulation. In both cases, TE could prevent further increase in iron accumulation, even in the brain. Despite persistent but stable iron accumulation in his brain, case two did not develop cognitive impairment. The cognition and physical situation of case one deteriorated, which prevented further treatment since good compliance and venous access are necessary for a successful TE treatment. In contrast to iron chelation and phlebotomies, no side effects of TE were reported. However, the treatment was less effective in case one, likely due to the advanced stage of neurological symptoms before start of the treatment and the inability to remove brain iron. This confirms that early diagnosis is vital since, most likely, initiation of treatment in a pre-symptomatic stage will delay the onset of neurological symptoms drastically.[14]

The only available information about treatment in HA is based on expert opinions and sporadic case reports. Therapies described for HA to prevent disease progression are phlebotomies, zinc sulfate, fresh frozen plasma, and iron chelators like deferoxamine[2,20-24], deferasirox[12,24-27], and deferiprone[26,28]. Vroegindeweij et al. published in 2020 a detailed overview of iron chelation treatments in 48 HA patients.[14] Only 20 of these 48 patients were treated for a longer period of time (≥4 years) . Eleven of these 20 patients had progressive neurological symptoms during the treatment period.[11,14-17,23,29-31] In only four of these progressive patients, the serum ferritin levels were normalized during the follow-up period[14,30], in four patients the ferritin levels did not normalize[15,16,23,24,31], and in three patients no follow-up ferritin levels were mentioned.[11,17,29] Of these eleven patients two were treated with deferoxamine[23,29], two with deferiprone combined with vitamin E and C[30], three with phlebotomies[15,16,31] and four with consecutive treatment with two types of iron chelators (deferoxamine/deferiprone) in two cases even temporarily combined with phlebotomies.[10,14,17,30] Only in the patients on deferoxamine monotherapy and in two patients with phlebotomies no side effects were reported while in all other patients

side effects were seen and often treatment needed to be temporary stopped or a dose reduction was required.[31] A normalisation of ferritin levels was only seen in deferiprone monotherapy and in two patients with duotherapy.[14,30] Nine of the 20 patients had remained asymptomatic during the follow-up period. In eight of these, serum ferritin levels normalized. These patients were treated with deferasirox monotherapy[32,33], deferasirox combined with phlebotomies[11,30] or two consecutive iron chelators (deferoxamine/deferiprone)[28,30,34]. In four of the nine patients, side effects of their treatment were experienced, and a treatment interruption or lowering of the dose was required.[11,28,30]

A notable finding was that in only a few patients it was stated for how long it took to achieve normal ferritin levels and for how long this was maintained if at all ferritin levels were mentioned. In none of the available brain MRIs an improvement was seen after treatment. Despite the promising results of iron chelators to reduce serum ferritin and iron accumulation in the liver[6,12,35] and to stabilize brain iron accumulation, their effect on neurological symptoms is variable.[6,30] Deferasirox seems to be the most promising iron chelator in HA. It has been described as being able to lower ferritin levels, hepatic iron and stabilize or decrease neurological symptoms. None of the asymptomatic patients treated with deferasirox for four years or longer developed symptoms during the follow-up period.

In many patients treated with deferoxamine or deferiprone, the maximum amount of iron excess could not be mobilized. Due to side effects like creatinine rise, skin rash and aggravation of anemia long-term use of these chelators is limited.[6,26,30,36] The follow-up ranges from one week to 18 months with the exception of one patient with a therapy duration of 16 years, nevertheless with periodic breaks due to complications of iron deficiency anemia.[17]

There are differences between these previous case reports and our second case, who did not develop neurological symptoms and did not experience significant side effects. Our patient had documented progressive brain iron accumulation during chelating therapy before starting TE, which was only halted after onset of TE. Second, our follow-up period of nine years was rather long.

A limitation of this study is that we cannot state a definite causality between the treatment and the absence of neurological symptoms in the second case. It could be the natural disease course that varies between patients, probably due to genetic and environmental factors involved in the phenotypic expression of the disease in this

patient.[27] Neurological manifestations were found to be present in 50.0-73.6%[2,37] of patients with HA with a median age of onset of 51.[37]

It cannot be excluded that the presence of compound heterozygosity of the *HFE* mutation contributed to the rapid disease progression in case one. The effect of compound heterozygosity of the *HFE* mutation or other *HFE* mutations on the disease course of HA should be investigated further in the future. To our knowledge, this combination of HA and compound heterozygosity has not been previously described.

We suggest TE as a viable option in patients with HA according to the following method. First, normalization of iron stores by the removal of peripheral iron excess by iron chelation therapy. Afterwards, start TE treatment to prevent re-accumulation of iron by keeping the ferritin levels within the reference range. We succeeded in maintaining low serum ferritin levels by decreasing the hemoglobin to an average of 9.7 g/dL after every TE. As most HA patients present with mild anemia, TE may be preferred because of the selective removal by way of apheresis and the individualized approach that takes the patient's total blood volume and hemoglobin into account. TE is a more effective method than phlebotomy as it can remove at least double the amount of iron during a single procedure. At the same time, less volume is removed and hypovolemia and anemia can be prevented. [38]

Conclusion

TE appears to be a promising therapy to prevent the progression of peripheral and cerebral iron accumulation in HA patients with good compliance and tolerance without causing severe anemia. Since iron accumulation in the brain does not seem to decrease with any therapy, early identification seems vital to timely start the appropriate treatment to prevent the onset of neurological symptoms. Further research is needed to explain the mechanisms involved in the disturbances in iron homeostasis and the inability to remove brain iron in HA and investigate whether TE monotherapy could be a promising treatment in HA.

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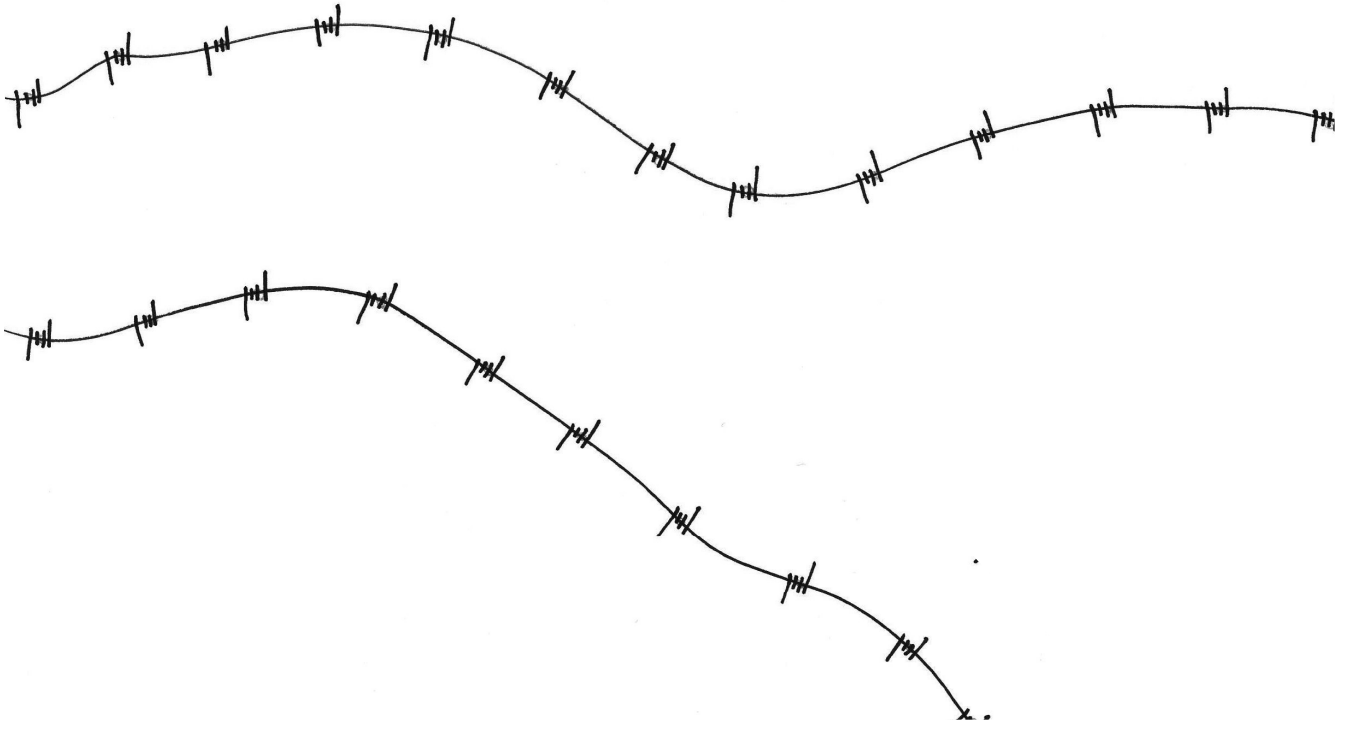
Chapter 9

General discussion



General discussion

Main findings

Serum ferritin is frequently determined in clinical practice and abnormal results are often encountered. Low values of serum ferritin indicate iron deficiency, but what about the interpretation of elevated results? Hyperferritinemia can reflect increased body iron stores, however, since ferritin is also an acute phase reactant, increased levels of this protein can be caused by inflammatory processes. Differentiation between iron overload and inflammation as a cause of hyperferritinemia is essential, nevertheless this often proves to be complex.

This thesis comprised two parts. The focus of part one was on hyperferritinemia. The evidence regarding the role of hyperferritinemia in non-alcoholic fatty liver disease (NAFLD) was summarized. It was suggested that liver MRI iron measurement could be a useful tool to help in differentiating hyperferritinemia patients with major vs. none or only minor iron overload. In the second part *HFE*-related hemochromatosis was elaborated on, a frequent cause of iron overload and hyperferritinemia. The disease course in the South Limburg cohort in daily practice and a tool to predict the phenotypic expression of *HFE*-related hemochromatosis was presented. As for the treatment of iron overload a possible mechanism explaining why proton pump inhibitors (PPIs) reduce iron absorption in patients with *HFE*-related hemochromatosis and not in healthy control subjects was investigated. The case of a patient with *HFE*-related hemochromatosis showing upregulation of hepcidin in the course of an inflammation was presented and there was elaborated on the possible underlying, nearly unexplored mechanism. Lastly in part two, erythrocytapheresis was introduced as a new treatment strategy to reduce the body iron stores in hereditary aceruloplasminemia, a rare cause of iron overload.

Below, the main findings are discussed and the current knowledge about the presence and absence of iron overload in hyperferritinemia and *HFE*-related hemochromatosis is put into perspective. Furthermore, future perspectives within this research field will be addressed.

Hyperferritinemia in NAFLD

With a prevalence up to 25%, NAFLD is the most widespread liver disease in Western society.[1,2] The term NAFLD comprises simple steatosis, a more benign form of fat accumulation and non-alcoholic steatohepatitis (NASH), a chronic inflammatory state which can eventually progress to fibrosis, cirrhosis and hepatocellular carcinoma

(HCC).[3,4] NAFLD is considered the hepatic manifestation of the metabolic syndrome in which a generalized systemic inflammation plays an important role in the initiation and progression of diabetes and cardiovascular diseases.[5,6]

Since hyperferritinemia is found in 30% of NAFLD patients it is important to gain further insight in the underlying cause of hyperferritinemia.[7] In **chapter 2** a systemic literature review was conducted summarizing the available evidence for iron overload and/or inflammation as a possible cause of hyperferritinemia in NAFLD patients. There were three groups of patients distinguished. The first group comprises the majority of NAFLD patients, in these patients hyperferritinemia is due to inflammation without hepatic iron overload. The second, smaller, group included patients with the dysmetabolic iron overload syndrome (DIOS), showing hyperferritinemia in combination with mild iron accumulation in the reticuloendothelial cells. DIOS is associated with various components of the metabolic syndrome but DIOS patients lack other identifiable genetic mutation or other causes of iron excess.[8] The third group was relatively small and consisted of NAFLD patients with *HFE*-related mutations.

Not only chronic inflammation leads to disease progression but also mild hepatic iron overload can play a prominent role in the pathophysiology of NAFLD. Iron overload can generate reactive oxygen species through the Fenton reaction and thereby lead to oxidative stress.[9] This can cause severe cellular dysfunction, organ damage and promotes the development of insulin resistance and hepatocellular inflammation attributing to NAFLD progression.[10,11] To prevent disease progression it is important to start treatment in time. The treatment choice depends on the underlying cause of hyperferritinemia since in case of hyperferritinemia related to inflammation whether or not combined with DIOS, phlebotomy is not more effective than lifestyle changes.[11-13]

The review presented in **chapter 2** contributes to the awareness that there are three different causes of hyperferritinemia in NAFLD and to the frequency of their occurrence. Since treatment strategies differ according to the cause of hyperferritinemia, it is important to make a correct diagnosis. To simplify the process of determining the underlying cause of hyperferritinemia, a diagnostic and therapeutic algorithm for the approach of hyperferritinemia in NAFLD was developed (Chapter 2, Figure 2.3). Herein there is recommended to perform *HFE* analysis when transferrin saturation (TSAT) is elevated (>45%) and to search for components of the metabolic syndrome in case of normal TSAT levels. Phlebotomies are only

recommended in case of genetic mutations known to result in iron accumulation. In the absence of signs of the metabolic syndrome it is advised to perform MRI with determination of liver iron concentration (LIC).

Interpreting hyperferritinemia

Most guidelines advice to use the MRI as a good, non-invasive marker to measure the LIC as part of the analysis of hyperferritinemia.[14,15] The reference value for the LIC is below 36 µmol Fe/g. Since the LIC can also be elevated without liver iron excess (e.g. in patients with obesity or the metabolic syndrome) it is suggested to test for iron overload only in case the LIC exceeds 150 µmol Fe/g.[16,17] In addition, the LIC estimated by MRI with the signal intensity ratio (SIR) method has a tendency to overestimate LIC values leaving a grey zone with diagnostic uncertainty between levels of 60-170 µmol Fe/g.[18,19] This often leads to an incorrect interpretation of results ensuing in an incorrect diagnosis of `hemochromatosis` and unnecessary phlebotomies.

To resolve this issue the liver iron index (LII) was proposed in **chapter 3**. The LII-MRI is calculated by dividing the LIC (measured with MRI) by the age of the patient. Values ≥2 identify patients with major iron overload while values <2 are an indication of none to only mild iron overload. This is substantiated by a significantly higher amount of iron needed to be mobilized to reach the iron depletion stage in hyperferritinemia patients with a LII-MRI ≥2 versus a LII-MRI <2. The amount of iron mobilized to reach iron depletion is seen as the most objective reflection of iron overload. In addition the presence of *HFE* mutations were more often found in LII-MRI ≥2 patients while components of the metabolic syndrome were more often found in the LII-MRI <2 group which also supports the hypothesis that values ≥2 identify patients with major iron overload.

The factor age was introduced since iron accumulation is a dynamic process in the course of life effected by many different factors, like age. For that reason, age should be taken into account when interpretating iron overload. Patients with mutations leading to major iron overload absorb about 1g more than the body's requirement each year this will result in a higher LIC at a relatively lower age.[20] While patients with conditions associated with minor iron accumulation will need more years to accumulation enough iron to result in a small increase in LIC. For example, the LII helps to prevent overinterpretation of iron overload in situations where no significant accumulation has taken place as for example the 80 year old DIOS patient with a LIC of 150 µmol Fe/g. More importantly, it triggers practitioners to expand DNA analysis in young patients with a LIC not yet exceeding 150 µmol Fe/g.

A major strength of this study is that all hyperferritinemia patients at the outpatient clinic received MRI and *HFE* genetic testing independent of their TSAT, ruling out selection bias. A limitation of the study is that there was not correcteded for the factor gender. Gender can affect the LIC since it is known that male *HFE*-hemochromatosis patients display biochemical and symptomatic hemochromatosis sooner and to a greater degree than women.[21] The protective effect in women can be explained by the physiological iron loss during menstruation, the antioxidant effect of oestrogen[22] and by sex-specific *HFE* and non-*HFE* genetic modifiers.[17,23]

The LII-MRI facilitates the interpretation of the intermediate LIC values and helps the clinicians to choose a direction in which additional diagnostic work-up and research needs to be done: search for inflammatory conditions or perform non-*HFE* gene analysis.

Hyperferritinemia accompanied with an elevated TSAT is most often associated with iron overload. The most common genetic iron overload condition is *HFE*-related hemochromatosis on which will be elaborated on in part two.

The medical history of HFE-related hemochromatosis

Through the past 150 years the knowledge about *HFE*-related hemochromatosis has developed enormously. In 1865 Trousseau was the first to postulate a new syndrome involving diabetes, pigmented liver cirrhosis and bronze-coloured skin, later referred to as 'bronze diabetes'.[24] The term `hemochromatosis` was introduced by the German pathologist von Recklinghausen in 1889 after staining massive iron deposition during liver autopsy from a patient with `bronze diabetes`.[25] He hypothesized that something circulating in the blood ('hemo') was responsible for skin and organ damage and pigmentation ('chromatosis').[26] It was only in the 20th century in 1935 that the English doctor Joseph Sheldon concluded that in hemochromatosis, excess iron was the cause of organ toxicity and that it was a hereditary metabolic disease. A turning point was when in 1976, hemochromatosis was shown to be an inherited autosomal recessive HLA-linked disease, which led to population studies with HLA assessment and family screening contributing to an increasing number of detected cases.

The biggest milestone in the history of hemochromatosis was when in 1996 a candidate gene for hereditary hemochromatosis, termed *HFE* (human homeostatic iron regulator), was identified on chromosome 6. Two common mutations were identified: *p.Cys282Tyr* and *p.His63Asp*, and it was possible to detect whether patients were heterozygous, homozygous or wild-type for these mutations.[27-29]

Changing disease course

With the discovery of the *HFE* gene and due to increased awareness, the majority of cases are nowadays diagnosed at an earlier disease stage, often even before the development of clinical and biochemical manifestations of the disease.[28] It also led to the recognition that the phenotypic expression of *HFE*-related hemochromatosis is rather low. There is a five-grade scale of disease severity available to classify patients. It ranges from the presence of *p.Cys282Tyr* homozygosity in the absence of any biochemical or clinical symptoms (stage 0) to increased biochemical parameters and clinical symptoms with manifestations of organ damage, predisposing to early mortality (stage 4)[30] (Chapter 4, Table 4.1).

The disease `hemochromatosis` has changed in the course of time and the patients with `bronze diabetes`, the classical hallmark of severe advanced disease or hemochromatosis-related mortality are now seen less frequently.[26] In **chapter 4**, the first Dutch hemochromatosis cohort was described, containing all identified *p.Cys282Tyr* hemochromatosis patients from the South Limburg region in the Netherlands. The cohort consists of 360 patients which were followed for a median period of 9.9 years after their diagnosis. A *p.Cys282Tyr* homozygosity prevalence of 0.05% among the population was observed, which is much lower compared to prevalence rates (0.4-0.68%) based on population based genetic screening.[31,32] This was expected as it is known that phenotypic expression of *p.Cys282Tyr* is low. Only a smaller proportion of the patients have been diagnosed by family screening resulting in a higher phenotypic penetrance in this specific population (stage ≥2 in over 90% and stage ≥3 in 73%). Despite a high prevalence of phenotypic penetrance remarkably fewer organ involvement and symptomatology were reported compared to what was reported before the implementation of *HFE* analysis.[33-35] These findings are in line with more recent studies probably due to active iron depletion therapy and early diagnosis.[36-38] In addition, there was also reported on the prevalence of HCCs in our *HFE*-related hemochromatosis population. Remarkably 14 of the 20 patients with a HCC were found in a non-cirrhotic liver. This finding is new and clinically relevant. It is in absolute contrast with findings from previous publications[39] and warrants the need for future research in *HFE*-related hemochromatosis with appropriate control groups to investigate the current disease course, its related symptoms and possible genetic or environmental factors affecting the occurrence of a HCC in *HFE*-related hemochromatosis.

The treatment of HFE-related hemochromatosis

While in the past decades big steps have been made in the diagnosis of *HFE*-related hemochromatosis, treatment of *HFE*-related hemochromatosis has remained unchanged. The most effective treatment, bloodletting, was already introduced in 1952 by Davis and Arrowsmith.[2] The goal of the treatment is the repeated withdrawal of erythrocytes, the most easiest way to reduce the iron concentration because hemoglobin contains an iron core. This stimulates erythropoiesis and thereby the mobilization of (excess) iron stored in organs like the liver. A more recently introduced alternative treatment is erythrocytapheresis, a technique using an apheresis machine that selectively removes erythrocytes and returns valuable blood components such as thrombocytes, clotting factors, plasma proteins, etc. back to the patient. In the last 25 years an increasing number of studies on erythrocytapheresis were published pointing to a higher efficacy with a significant decrease in the number of required procedures compared to phlebotomies. Compared to phlebotomies, less adverse events occur during erythrocytapheresis, quality of life is better, costs are less and compliance is better.[40,41] The costs for a single erythrocytapheresis procedure are three times higher compared to a phlebotomy procedure. By decreasing the amounts of procedures needed erythrocytapheresis can become cost effective. Therefore it is necessary to select patients with high amounts of excess iron. Due to the difference in phenotypic expression it is not always easy to predict which patient are good candidates for erytrocytapheresis. In **chapter 5**, the modified iron avidity index (mIAI) calculated by serum ferritin levels at diagnosis divided by age at diagnosis minus 20 when male, and ferritin at diagnosis divided by age at diagnosis plus 20 when female was proposed. The mIAI appears to be a fairly good predictor in *HFE*-related hemochromatosis patients not taking PPIs, to differentiate the patients needing ≥3 maintenance phlebotomies per year from the rest. Therefore, this index has the potential to help in selecting patients who may benefit from erythrocytapheresis in the maintenance stage.

Various factors such as genetic modifiers (non-*HFE* mutations or digenic mutations), environmental factors (blood donations, physiological blood loss) and lifestyle factors (alcohol, diet) influence the phenotypic expression in *HFE*-related hemochromatosis.[42] In the mIAI only a small proportion of these factors were taken into account, due to the retrospective collection of available patient data. It is essential to validate the mIAI in a separate, preferably prospective study and thereby take into account other factors influencing the phenotype, before the mIAI can be implemented in clinical practice.[43-45]

PPIs have been proposed as a treatment option in *HFE*-related hemochromatosis. During PPI use the amount of phlebotomies needed per year was significantly reduced. PPIs were effective by reducing the amount of iron absorbed in the digestive tract.[46,47] It is quite remarkable that in patients without *HFE*-related hemochromatosis, iron deficiency rarely is a consequence of chronic PPI use. In **chapter 5** there was confirmed that lowering gastric acidity by PPIs resulted in a reduction in iron absorption in *HFE*-related hemochromatosis patients and not in healthy control subjects. There was investigated if a decrease in hepcidin concentration in healthy controls in response to lower gastric acidity could explain for the differences in iron absorption between these groups, since this feedback mechanism of hepcidin is lacking in *HFE*-related hemochromatosis. However, with this proof of concept study the hypotheses could not be confirmed, probably due to a small sample size. Future studies should include a larger study population and preferably also different doses of PPIs to further unravel the pathophysiological mechanisms.

While conducting the studies presented in this thesis, an interesting finding was observed which is described in **chapter 6.** Here, a *p.Cys282Tyr* homozygous patient with high hepcidin levels and normal iron parameters during systemic inflammation was presented. More in line with expected parameters in *HFE*-related hemochromatosis, low hepcidin and higher iron levels were found when repeating the measurements in the same patient, but in the absence of systemic inflammation. The increase in hepcidin levels observed during inflammation has previously only been described in one *HFE*-related hemochromatosis patient.[48] It was assumed that patients with *HFE*-related hemochromatosis can still induce hepcidin production through the JAK/STAT3 pathway in spite of attenuation of the BMP/SMAD pathway and in this way decrease iron absorption.[49,50] Based on these observations, interventions via the JAK/STAT3 pathway could potentially reduce excess absorption and accumulation of iron in patients with *HFE*-related hemochromatosis and deserve further exploration. Apart from providing insight into an until now unexplored mechanism in *HFE*-related hemochromatosis, it seems unlikely that medication interfering the JAK/STAT3 pathway will be considered as a therapeutic option of *HFE*-related hemochromatosis. Since medication interfering with the JAK/STAT3 pathway is associated with several potentially serious side-effects while the currently available treatment options (phlebotomies and erythrocytapheresis) are known to be safe.

Recommendations for a new classification of hemochromatosis

Over time, it became clear that the genetic basis of *HFE*-related hemochromatosis was more heterogeneous than previously assumed and that several variants in other iron-controlling genes (non-*HFE* genes) were progressively associated with iron overload. In most cases gene defects causing insufficient hepcidin production were found.[26] The working group of the International Society for the Study of Iron in Biology and Medicine (BIOIRON Society) proposed to solely use the term 'hemochromatosis' for a unique genetic clinical-pathological condition characterized by increased TSAT, iron overload in the liver and not in the spleen, involvement of peri-portal hepatocytes with iron-spared Kupffer cells and symptoms and/or signs associated with iron overload. The new proposed classification of *HFE*-related and non-*HFE*-related hemochromatosis disorders is given in the introduction in Figure 1.4. Hereditary aceruloplasminemia (HA), a genetic condition resulting in systemic iron overload, is not included within the term hemochromatosis.

Hereditary aceruloplasminemia

HA is a rare autosomal recessive disorder usually resulting from bi-allelic mutations in the *ceruloplasmin (CP)* gene on chromosome 3.[51] Its prevalence was estimated to be approximately one per two million people.[52] There is still much unknown about the disease pathophysiology and the workings mechanism of treatment possibilities. The only available information of therapeutic strategies has been presented in case reports and current treatment strategies are often associated with progressive anemia. In **chapter 7**, erythrocytapheresis was introduced for the first time. It seems to prevent progression of cerebral iron accumulation after chelator-induced normalization of iron stores. There was also discussed what is known about the pathophysiology of HA. CP is a multi-copper-containing ferroxidase that plays a role in cellular iron export. It catalyzes the oxidation of intracellular ferrous iron (Fe^{2+}) that is transported by ferroportin into ferric (Fe^{3+}) iron to enable its binding to transferrin, the carrier protein for iron in the blood[53-55] (Chapter 7, Figure 7.1). CP is expressed by a large number of cells including macrophages, astrocytes and hepatocytes.[56-58] There is hypothesed that in the absence of CP, iron is not mobilized from these cells resulting in low transferrin saturation and low hepcidin synthesis. This causes increased intestinal iron absorption since enterocytic ferroportin relies on the ferroxidase hephaestin.[59]

With the mechanism of HA pathophysiology in mind it does not seem beneficial to start the treatment with phlebotomies or erythrocytapheresis since the export of iron from the cells is limited. For that reason there was suggested to normalize iron stores

with iron chelators and then switch to erythrocytapheresis to prevent re-accumulation. This to reduce long term use of iron chelators and their associated risk of aggravating anemia. Erythrocytapheresis keeps serum ferritin levels within the reference range to effectuate the utilization of absorbed iron for erythropoiesis only, instead of further deposition in the parenchymal cells. None of the case reports with phlebotomy monotherapy in HA induced the normalization of hepatic iron stores. So despite the fact that it would be interesting to investigate if erythrocytapheresis monotherapy could also be a good alternative treatment in HA, this does not seem likely. Further research is needed to explain the inability to remove iron from the brain in HA and also to investigate the ability of erythrocytapheresis to stabilize already present neurological symptoms.

Implications for clinical care and future research

Elevated serum ferritin levels create a great challenge for physicians to determine whether hyperferritinemia truly represents iron overload. Wrongly interpreting hyperferritinemia can lead on the one hand to the missing of a diagnosis, for example a malignancy, and on the other hand it can result in a huge number of consultations, the overuse of *HFE*-gene analysis, and incorrectly performing phlebotomies. With the studies presented in this thesis there was aimed to create awareness for the different possible causes of hyperferritinemia in NAFLD, in addition, there was a tool proposed to help interpret intermediate MRI results. This is an important step forward in the analysis of hyperferritinemia to prevent patients from being misdiagnosed with hemochromatosis and to guide clinicians when there is an indication to check for non-*HFE* mutations. There is a need for future extensive research to optimize the LII and to systematically search for potential factors influencing the LII.

A known cause of hyperferritinemia is *HFE*-related hemochromatosis which has been revolutionized since it was first described. Due to early diagnosis its previously classic manifestations has become more rare instead of characterizing the condition, as was previously stated. In addition, it became clear that phenotypic expression in *HFE*-related hemochromatosis is rather low. This is influenced by genetic, environmental and lifestyle factors, by a more early diagnosis and more early onset of therapy. More than 20 years after the discovery of *HFE* gene analysis, the manifestations of hemochromatosis have changed completely. This thesis showed that severe organ involvement is less frequent nowadays, and that symptomatology differs from what was observed previously. As long term follow up is available, the cohort study pointed to a remarkable finding that 75% of diagnosed HCCs during follow-up were being present in non-cirrhotic livers. Nowadays HCC surveillance is only advised in cirrhotic

livers. Therefore there is a need for future large longitudinal case-control studies. It is important to investigate symptomatology in *HFE*-related hemochromatosis over time and also to relate other contributing factors in patient with hemochromatosis developing other diseases like NAFLD and alcoholic liver diseases. As iron is essential for life it will effect cellular metabolic processes and generation of radical oxygen species in overload not only found in genetically susceptible patients but also strongly related to the Western lifestyle. This brings a new spot on the element iron that was symbol of the international Expo in 1958, Brussels Belgium.

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Addendum

Summary

Serum ferritin is one of the most frequently requested laboratory tests in both primary care and referral settings. Ferritin is a cellular iron storage protein and for that reason serum ferritin is a reliable surrogate marker of body iron stores. Low serum ferritin levels provide absolute evidence of reduced iron stores. However, high serum ferritin levels (hyperferritinemia) are far less specific for systemic iron overload since ferritin is also an acute phase protein and will increase in case of infection, neoplasm and acute or chronic inflammation. Hyperferritinemia is defined as serum ferritin concentrations >200 µg/L in women and >300 µg/L in men, and is found in around 12% of the general population. Since hyperferritinemia is common and often does not reflect iron overload it is a great challenge for physicians to determine its exact cause.

This thesis consists of two parts. In the first part the focus is on the diagnostic difficulties in patients with hyperferritinemia, in the second part on *HFE*-related hemochromatosis.

HFE-related hemochromatosis is a frequent cause of hyperferritinemia, and is associated with iron overload. The most prevalent form is homozygosity for the p.Cys282Tyr variant in the *HFE* gene and this is the most common autosomal recessive, genetic disorder found in Caucasians. It is most commonly seen in populations of Northern European origin, in which the prevalence is close to 1 per 200-250 persons. *HFE*-related hemochromatosis is characterized by low hepcidin levels which result in a persistent iron absorption leading to iron accumulation in the body`s tissues and organs, particularly the liver, pancreas, joints, heart and the skin. Iron accumulation will eventually lead to organ damage resulting in hepatic cirrhosis, primary liver cancer, arthropathy, cardiomyopathy and diabetes mellitus. To maintain a normal life expectancy iron depletion therapy should be started in time in order to prevent iron accumulation and its complications.

Part 1- Understanding and interpreting hyperferritinemia

A frequent cause of hyperferritinemia is non-alcoholic liver disease (NAFLD), the most widespread liver disorder in Western society. In 30% of patients with NAFLD, hyperferritinemia is found, however its origin is a subject of discussion. Prior to starting therapy the etiology of hyperferritinemia should be investigated since iron depletion therapy is not advised in inflammation-related hyperferritinemia nor in patients with the dysmetabolic iron overload syndrome. In **chapter 2** an extensive

literature search was performed to investigate whether hyperferritinemia in NAFLD is an expression of iron overload or inflammation. It was shown that in the majority of cases hyperferritinemia in NAFLD is related to inflammation. In a smaller group, dysmetabolic iron overload syndrome is found, showing inflammation related hyperferritinemia in combination with mild iron accumulation in the reticuloendothelial cells. In the smallest group, hyperferritinemia in NAFLD is related to genetic disturbances of iron homeostasis such as *HFE*-related hemochromatosis.

Because of the broad etiological spectrum of hyperferritinemia it is a challenge to determine its cause. When clinical examination and additional laboratory tests do not provide a certain diagnosis, the liver iron concentration (LIC) can be used. Previously, the LIC was determined in a liver tissue sample collected via liver biopsy. Liver biopsy however, is an invasive procedure with potentially serious complications and the risk of sample error. For that reason, nowadays, the LIC measured by MRI is used (using a specific iron protocol). The LIC is considered the best method to accurately assess body iron load, since the liver contains ≥70% of the body iron stores. However, there are difficulties with interpreting the LIC with the generally used cut-off value of ≥36 µmol/g. This value appears to be low since often the LIC is found to be increased in hyperferritinemia associated with the dysmetabolic iron overload syndrome and/or alcohol (over)consumption in the absence of major iron overload. Previously, the liver iron index (LII) was introduced to differentiate patients with *HFE*-related hemochromatosis from patients with alcoholic liver disease or heterozygous hemochromatosis mutations. The LII is calculated by dividing the LIC measured with liver biopsy by the age of the patient in years. The rationale behind introducing the factor age is that iron accumulation in *HFE*-related hemochromatosis is a dynamic process gradually increasing in the course of life. On a yearly basis, patients with *HFE*-related hemochromatosis absorb about 1g more iron than the body requires. Since a good correlation was found between the LIC measured by biopsy and by MRI scan, the aim of the study was to investigate if the LII derived from the LIC measured by MRI could also be used to interpret hepatic iron presence (**chapter 3**). A retrospective cohort study was conducted involving hyperferritinemia patients who underwent a MRI according to the iron protocol and *HFE* gene analyses as part of the diagnostic process. Patients with hyperferritinemia and a LII-MRI ≥2 have significantly larger iron stores. This was based on the finding that patients with LII-MRI ≥2 had to mobilize a significantly higher amount of iron to reach iron depletion while patients with LII-MRI <2 had a significantly higher prevalence of components of the metabolic syndrome and had to mobilize a significantly lower amount of iron to reach iron depletion. It was concluded that LII-MRI is an effective method to help differentiating between major

and minor iron overload in patients being analyzed for hyperferritinemia. The LII-MRI it is not only suitable to find patients with *HFE*-related hemochromatosis but also to detect major iron overload caused by non-*HFE* hemochromatosis or secondary causes.

Part 2 - Current clinical aspects of HFE-related hemochromatosis and iron homeostasis

Although the prevalence of *p.Cys282Tyr* homozygosity is high, its phenotypic expression is low. In some patients there is no iron overload and depletion therapy is not indicated. In **chapter 4**, an overview of the population based *HFE*-related hemochromatosis South Limburg cohort is given. Data from all subjects with identified *p.Cys282Tyr* hemochromatosis in this geographical area enclosed by Belgium and Germany, with low migration rates, were collected. The aim of the study was to get a better insight in the epidemiology, phenotypic expression, disease manifestations and complications of *HFE*-related hemochromatosis and to create awareness to achieve an early diagnosis to prevent irreversible organ damage. The cohort contains 360 patients, followed for a median period of 9.9 years after their diagnosis. Remarkably fewer organ involvement and symptomatology were reported compared to what was previously published, possibly due to active iron depletion therapy and early diagnosis. The prevalence of hepatocellular carcinomas (HCCs) in our hemochromatosis population was reported and remarkably only 20 patients with a HCC were found of which 14 were found in a non-cirrhotic liver which is in contrast with previous publications were HCCs are almost exclusively found in cirrhotic livers.

The treatment of *HFE*-related hemochromatosis consists of two phases. The first one is the depletion phase in which the excess iron is removed while in the maintenance phase the re-accumulation of iron is prevented. The most frequently used treatment modality is bloodletting (phlebotomies). During each session 500ml of blood is removed, containing approximately 250 mg of iron. This is comparable with the amount taken for blood donation. In the iron depletion phase, phlebotomies are performed weekly until serum ferritin levels are below 100 µg/L or below 50 µg/L in case the transferrin saturation levels is >70%. The number of phlebotomies needed in the depletion phase is quite variable and depends on the amount of iron accumulated in the body. The number of phlebotomies in the maintenance phase varies between two and six per year. An alternative treatment is erythrocytapheresis, in which more erythrocytes can be removed per procedure and valuable blood components such as platelets and clotting factors can be returned to the patients. With this technique it is possible to remove more erythrocytes and thus iron per single procedure, nevertheless it is used less frequently due to costs and is only performed in specialized

centers. However, in patients with severe iron overload this is a good alternative treatment.

Because of the variability in phenotypic expression it is difficult to identify patients in need for maintenance treatment, and when identified, how frequently treatment sessions should be applied. In **chapter 5**, retrospectively analyses of *HFE*-related hemochromatosis patients in the maintenance phase were investigated to research how to predict the patient's phenotype and thereby individualize treatment. The modified iron availability index (mIAI) was developed, calculated by serum ferritin levels at diagnosis divided by age at diagnosis minus 20 when male, and ferritin at diagnosis divided by age at diagnosis plus 20 when female. The mIAI seems a fairly good predictor in *HFE*-related hemochromatosis patients not taking proton pump inhibitors (PPIs) to differentiate patients needing ≥3 maintenance phlebotomies per year. Therefore, this index might help to select patients who benefit from an alternative less frequent therapy such as erythrocytapheresis.

Patients using PPIs were excluded since PPIs are known to have a significant effect on the amount of phlebotomies needed per year by lowering the amount of iron absorbed in the digestive tract. It is remarkable that in patients without *HFE*-related hemochromatosis iron deficiency is not a frequent side effect of chronic PPI use. In **chapter 6**, a proof of concept study was conducted in which serum iron and hepcidin levels after a pharmacological dose of 50 mg iron (Fe^{3+}) polymaltose were measured in patients with *HFE*-related hemochromatosis and in healthy controls. These measurements were repeated after seven days' treatment with PPIs. With this study, the reduction in iron absorption in *HFE*-related hemochromatosis patients in contrast to healthy control subjects was confirmed after lowering gastric acidity by PPIs. The assumption that a decrease in hepcidin concentration in healthy control subjects, in response to the reduced availability of Fe^{2+} for absorption, can explain the difference in iron absorption between these groups, could not be confirmed probably due to a small sample size.

HFE-related hemochromatosis is characterized by low hepcidin levels leading to continuous iron absorption and thus elevated serum ferritin and serum iron levels. In **chapter 7**, the case of a patient with *HFE*-related hemochromatosis with elevated hepcidin and normal iron levels during an episode of systemic inflammation was presented. When iron parameters and hepcidin levels were measured one year after his full recovery low hepcidin levels and higher iron levels were found as expected in a patient with *HFE*-related hemochromatosis. It was suggested that in the absence of a

proper functioning HFE, resulting in blockage of the BMP/SMAD pathway, the innate low hepcidin concentration can be upregulated by inflammation, probably via the JAK/STAT3 pathway. This is a rather unexplored area within *HFE*-related hemochromatosis research and could lead to new treatment possibilities.

Chapter 8 focused on hereditary aceruloplasminemia, a rare genetic condition in which iron gradually accumulates in the brain and various internal organs. This can lead to diabetes, retinal degradation and neurological disorders. It can also result in anemia since iron sequesters in the cells and is not available for the production of erythrocytes. The current treatment strategies are associated with many side effects as for example an aggravation of the already present anemia. Two hereditary aceruloplasminemia patients were treated with erythrocytapheresis in order to prevent iron re-accumulation after peripheral iron normalization by chelators. Erythrocytapheresis seems a good treatment possibility because it allows a more precise and selective reduction in erythrocytes preventing the development of symptomatic anemia. This is the first report on therapeutic erythrocytapheresis in hereditary aceruloplasminemia, that prevented progression of cerebral and peripheral iron accumulation without causing symptomatic anemia.

Finally, in **chapter 9**, an overview of the main findings is given and discussed. The thesis ends with a discussion on new insights and future perspectives in the field of interpreting hyperferritinemia and *HFE*-related hemochromatosis.

Addendum

Nederlandse samenvatting

Ferritine is een van de meest verrichte testen in het bloed in zowel de eerstelijns- als de tweedelijnsgezondheidszorg. Ferritine is een eiwit waarin ijzer in de cel wordt opgeslagen en om die reden is het een betrouwbare marker om te bepalen wat de ijzervoorraad van het lichaam is. Een verlaagd ferritine in het bloed is een bewijs voor een ijzertekort echter een verhoogd ferritine (hyperferritinemie) in het bloed is niet erg specifiek voor een overschot aan ijzer. Dit komt omdat ferritine ook zal stijgen in de aanwezigheid van een infectie, kanker of ontsteking. Ferritine spiegels worden gedefinieerd als verhoogd wanneer ze bij vrouwen boven de 200 µg/L zijn en bij mannen boven de 300 µg/L.

In 12% van de algemene bevolking wordt een verhoogd ferritine aangetroffen. Omdat deze verhoogde waarde meerdere oorzaken kan hebben en niet altijd op een overschot aan ijzer duid, is het vaak een uitdaging voor artsen om de juiste verklaring te vinden.

Dit proefschrift is opgedeeld in twee delen. In het eerste deel wordt de nadruk gelegd op de moeilijkheden bij de diagnostiek van een verhoogd ferritine. In het tweede deel wordt gefocust op een erfelijke ijzerstapelingsziekte genaamd hemochromatose, een vaak voorkomende verklaring van een verhoogd ferritine en gekarakteriseerd door ijzeroverschot.

Dit proefschrift focust op de meest voorkomende ijzerstapelingsziekte genaamd `*HFE*-gerelateerde hemochromatose`, veroorzaakt door een mutatie (genaamd p.Cys282Tyr) in het *HFE*-gen. Dit is de meest voorkomende autosomaal, recessief genetische aandoeningen binnen de Kaukasische populatie. In de Noord-Europese populatie komt de aandoening het meest voor met een prevalentie van 1 per 200-250 mensen. Hemochromatose wordt gekenmerkt door lage waarden van het hormoon hepcidine, de belangrijkste regulator van het ijzerhuishouding in het lichaam. Hierdoor zal de opname van ijzer in de darm niet meer worden verminderd wanneer er reeds genoeg ijzer in het lichaam aanwezig is. Uiteindelijk resulteert dit in een ophoping van ijzer in het lichaam, met name in de lever, alvleesklier, gewrichten, het hart en de huid. Uiteindelijk kan dit resulteren in beschadiging van deze organen en kan het zorgen voor een verlittekening van de lever (levercirrose), leverkanker, gewrichtsklachten, hartfalen en suikerziekte. Om deze complicaties te voorkomen en te zorgen dat patiënten een normale levensverwachting behouden is het nodig om deze aandoening tijdig op te sporen en te behandelen.

Deel 1- Het begrijpen en interpreteren van hyperperferritinemie

Hyperferritinemie wordt vaak aangetroffen bij patiënten met een niet-alcoholische leververvetting (NAFLD), de meest voorkomende leveraandoening in de westerse samenleving. In ongeveer 30% van patiënten met NAFLD wordt een hyperferritinemie gevonden, toch is de onderliggende oorzaak van het verhoogd ferritine vaak een onderwerp van discussie. De aard van de behandeling is sterk afhankelijk van de oorzaak van de hyperferritinemie dus voor een behandeling gestart kan worden is het belangrijk om de oorzaak duidelijk te krijgen.

In **hoofdstuk 2** werd een uitgebreid literatuuronderzoek verricht om te onderzoeken of hyperferritinemie bij NAFLD een uiting is van ijzeroverschot of van ontsteking. Hieruit bleek dat in de meerderheid van de gevallen hyperferritinemie bij NAFLD gerelateerd is aan ontsteking. In een kleinere groep wordt het eveneens aan ontsteking gerelateerd maar gecombineerd met een mild ijzeroverschot in de afwezigheid van genetische mutaties die dit ijzeroverschot kunnen verklaren. In de kleinste groep is hyperferritinemie bij NAFLD gerelateerd aan genetische mutaties binnen de ijzerhuishouding zoals *HFE*-gerelateerde hemochromatose. Er werden alleen aderlatingen geadviseerd in de laatste groep, de genetische mutaties in het ijzerhuishouding. In de eerste twee groepen, beide gerelateerd aan ontsteking worden geen aderlatingen maar leefstijladviezen zoals gewichtsreductie geadviseerd.

Vanwege de vele verschillende oorzaken van hyperferritinemie is het een uitdaging om de juiste verklaring vast te stellen. Wanneer een algemene klinische analyse en aanvullend laboratorium onderzoek geen uitsluitsel kunnen geven over de diagnose, kan de lever ijzer concentratie (LIC) worden gemeten. Vroeger werd de LIC bepaald aan de hand van een weefselmonster van de lever verkregen via een leverbiopsie. Een leverbiopsie is echter een invasieve procedure met een risico op ernstige complicaties. Daarom wordt tegenwoordig de LIC gemeten met behulp van een MRI scan waarbij een specifiek ijzerprotocol wordt gebruikt. De LIC wordt beschouwd als de beste methode om de ijzerbelasting van het lichaam nauwkeurig te bepalen, aangezien de lever ≥70% van de ijzerreserves van het lichaam bevat.

De interpretatie van de LIC kan echter moeizaam zijn. De afkapwaarde van ≥36 µmol/g is laag en blijkt vaak verhoogd bij patiënten met bijvoorbeeld overmatig alcoholgebruik of obesitas zonder dat er een evident ijzeroverschot aanwezig is. Eerder werd de lever ijzer index (LII) geïntroduceerd, berekend door de LIC (gemeten via een leverbiopt), te delen door de leeftijd van de patiënt (in jaren). De factor leeftijd wordt ingevoerd omdat ijzerstapeling bij hemochromatose een dynamisch

proces is waardoor in de loop van het leven het overschot aan ijzer zal toenemen. Patiënten met *HFE*-gerelateerde hemochromatose nemen jaarlijks ongeveer 1 gram meer ijzer op dan het lichaam nodig heeft. In dit kader zal dus in patiënten met hemochromatose, vergeleken met andere oorzaken voor milde ijzerstapeling, ook op vroegere leeftijd een groter ijzeroverschot gezien worden. Er wordt een goede correlatie gevonden tussen de LIC gemeten via leverbiopt en via een MRI-scan. Om die reden was het doel van de studie om te onderzoeken of de LII afgeleid van de LIC gemeten middels MRI ook betrouwbaar is om te differentiëren tussen evident ijzeroverschot en geen of mild ijzeroverschot.

In **hoofdstuk 3** wordt een retrospectieve cohortstudie beschreven bij hyperferritinemie patiënten die zowel *HFE*-gen analyse als een MRI volgens het ijzerprotocol ondergingen. Middels deze studie werd bevestigd dat patiënten met hyperferritinemie en een LII-MRI ≥2 significant meer ijzer overschot hebben, dit werd gebaseerd op de bevinding dat bij patiënten met een LII-MRI ≥2 significant meer ijzer verwijderd moest worden om een normale ijzervoorraad te bereiken. Daarnaast werden bij patiënten met een LII-MRI <2 significant vaker componenten van het metabool syndroom gevonden. Het metabool syndroom is een stofwisselingsstoornis waarbij minimaal drie van de volgende kenmerken aanwezig zijn: een te hoog cholesterolgehalte, een hoge bloeddruk, overgewicht, een grote buikomtrek of een hoge bloedsuikerspiegel. Er werd geconcludeerd dat de LII-MRI een effectieve methode is om te helpen bij het differentiëren tussen evident en geen of mild ijzeroverschot bij patiënten met een hyperferritinemie.

Deel 2 – De huidige klinische aspecten van *HFE*-gerelateerde hemochromatose en het ijzer metabolisme

Hoewel *HFE*-gerelateerde hemochromatose vaak voorkomt, is de fenotypische expressie laag. Dit wilt zeggen dat niet alle patiënten ijzeroverschot hebben en dus niet allen een behandeling nodig hebben. In **hoofdstuk 4** wordt een overzicht gegeven van alle patiënten met *HFE*-gerelateerde hemochromatose in Zuid-Limburg. Gegevens van alle geïdentificeerde personen met deze aandoening werden verzameld. Het voordeel van Zuid-Limburg is dat dit geografische gebied ingesloten wordt door België en Duitsland en een lage migratiegraad heeft. Het doel van de studie was een beter inzicht te krijgen in de epidemiologie, fenotypische expressie, ziekte manifestaties en complicaties van *HFE*-gerelateerde hemochromatose en om bewustwording te creëren zodat de aandoening tijdig wordt erkent en onomkeerbare orgaanschade voorkomen kan worden. Het cohort bevat 360 patiënten, die gedurende een mediane periode van 9.9 jaar na de diagnose werden gevolgd. Een opvallende bevinding was

dat er minder orgaanbetrokkenheid werd gerapporteerd in vergelijking met wat eerder werd gerapporteerd, waarschijnlijk als gevolg van vroegtijdige diagnose en behandeling. Bovendien werd het voorkomen van leverkanker in onze hemochromatose populatie gerapporteerd, er werden 20 patiënten met leverkanker gediagnosticeerd waarvan 14 in een lever zonder cirrosekenmerken. Dit is in tegenstelling met eerdere publicaties waarin leverkanker bijna uitsluitend in patiënten met levercirrose werd gevonden.

De behandeling van hemochromatose bestaat uit twee fasen. De eerste fase wordt de depletiefase genoemd, waarin het ijzer overschot uit het lichaam wordt verwijderd. Tijdens de tweede fase, de onderhoudsfase wordt het opnieuw opbouwen van een ijzeroverschot voorkomen. De meest gebruikte behandelmethode zijn aderlatingen. Tijdens elke sessie wordt 500 ml bloed afgenomen, vergelijkbaar met de hoeveelheid die voor bloeddonatie wordt afgenomen. In de depletiefase worden wekelijks aderlatingen uitgevoerd totdat het ferritine gehalte onder de 100 µg/L ligt of onder de 50 µg/L in geval van een transferrine verzadiging boven de 70%. Het aantal aderlatingen dat in de depletiefase nodig is, varieert erg en is afhankelijk van de grootte van het ijzer overschot. Het aantal aderlatingen in de onderhoudsfase kan wisselen van nul tot zes per jaar.

Een alternatieve behandeling is erytrocytaferese, hierbij worden per procedure meer rode bloedcellen verwijderd en kunnen belangrijke bloedbestanddelen zoals bloedplaatjes en stollingsfactoren aan de patiënten worden teruggegeven. Met deze techniek is het mogelijk per procedure meer rode bloedcellen en dus ijzer te verwijderen. Wel wordt deze procedure minder vaak toegepast en kan het alleen in gespecialiseerde centra uitgevoerd worden. Bij patiënten met een ernstig overschot zou dit echter een goed alternatief kunnen zijn.

Vanwege de variabiliteit in fenotypische expressie is het moeilijk om patiënten te identificeren die een onderhoudsbehandeling nodig hebben, en hoe vaak de behandeling toegepast zal moeten worden. In **hoofdstuk 5** zijn retrospectief *HFE*-gerelateerde hemochromatose patiënten in de onderhoudsfase onderzocht om te onderzoeken hoe het fenotype van de patiënt voorspeld kan worden en of daarmee de behandeling geïndividualiseerd kan worden. De gemodificeerde ijzer aviditeit index (mIAI) werd ontwikkeld, deze wordt berekend door de ferritine spiegels bij diagnose te delen door de leeftijd bij diagnose min 20 als het om mannen gaat, en plus 20 als het om vrouwen gaat. Bij *HFE*-gerelateerde hemochromatose patiënten die geen protonpompremmers (PPI's) gebruiken, lijkt de mIAI een vrij goede voorspeller, om de

patiënten te onderscheiden die ≥3 onderhoud aderlatingen per jaar nodig hebben. Om die reden zou deze index kunnen helpen om patiënten te selecteren die baat hebben bij een alternatieve, minder frequente therapie zoals erytrocytaferese.

Deze index is niet betrouwbaar bij patienten die PPI's gebruiken, omdat PPI's het aantal benodigde aderlatingen per jaar verminderden, door het verlagen van de ijzeropname in het spijsverteringskanaal. Het is opmerkelijk dat patiënten zonder *HFE*-gerelateerde hemochromatose die chronisch PPI's gebruiken niet vaak een ijzertekort hebben. In **hoofdstuk 6** werd een proof of concept studie uitgevoerd waarin serum ijzer en hepcidine spiegels werden gemeten na een farmacologische dosis van 50 mg ijzer polymaltose (Fe^{3+}) bij patiënten met *HFE*-gerelateerde hemochromatose en bij gezonde personen. Deze metingen werden herhaald na een behandeling met PPI's gedurende zeven dagen. Met deze studie werd de vermindering van de ijzeropname gedurende het gebruik van PPI's bij *HFE*-gerelateerde hemochromatose patiënten in tegenstelling tot gezonde personen bevestigd. De veronderstelling dat een daling van de hepcidine spiegels bij gezonde personen in reactie op verlaging van de maagzuurgraad door de PPI, het verschil in ijzerabsorptie tussen deze groepen kan verklaren, kon niet worden bevestigd, waarschijnlijk vanwege een te kleine onderzoekspopulatie.

HFE-gerelateerde hemochromatose wordt gekarakteriseerd door lage hepcidine spiegels die resulteren in aanhoudende opname van ijzer in de darm onafhankelijk van de ijzervoorraad in het bloed. Dit resulteert in hoge ferritine en ijzer spiegels in het bloed. In **hoofdstuk 7** werd een casus gepresenteerd van een patiënt met *HFE*-gerelateerde hemochromatose met onverwacht verhoogde hepcidine en normale ijzer spiegels tijdens een periode waarin hij een infectie/ `griep` doormaakte. Bij het overmeten van de spiegels, een jaar na zijn herstel, werden lage hepcidine spiegels en hoge ijzerspiegels gevonden, meer naar verwachting bij een patiënt met *HFE*-gerelateerde hemochromatose. Er werd gesuggereerd dat bij een slecht functionerend HFE eiwit, tijdens een periode van ijzeroverschot de BMP/SMAD-route wordt geblokkeerd resulterend in een verlaagd hepcidine en aanhoudende ijzeropname. Maar dat ondanks een slecht functionerend HFE eiwit bij hemochromatose, tijdens een periode van ontsteking/infectie, hepcidine productie wel kan worden gestimuleerd, waarschijnlijk via de JAK/STAT3-route. Dit is vooralsnog een relatief onbekend gebied binnen het *HFE*-gerelateerde hemochromatose onderzoek en zou kunnen leiden tot nieuwe behandelingsmogelijkheden.

In **hoofdstuk 8** werd aandacht besteed aan erfelijke aceruloplasminemie, een zeldzame genetische aandoening waarbij zich geleidelijk ijzer ophoopt in verschillende inwendige organen inclusief de hersenen. Dit kan leiden tot suikerziekte, aantasting van het netvlies van het oog en neurologische aandoeningen maar ook door bloedarmoede omdat ijzer zich ophoopt in de cellen en hierdoor niet beschikbaar is voor transport naar het beenmerg om daar gebruikt te worden voor de aanmaak van rode bloedcellen. De beschikbare behandelingsstrategieën gaan gepaard met vele bijwerkingen zoals bijvoorbeeld een verergering van de reeds aanwezige bloedarmoede.

Er worden twee patiënten met erfelijke aceruloplasminemie gepresenteerd welke werden behandeld met erytrocytaferese om te voorkomen dat er opnieuw ijzer overschot wordt opgebouwd nadat het ijzeroverschot in het bloed en de perifere organen eerst middels ijzer chelatoren werd bereikt. Ertrocytaferese lijkt een goede behandelingsmogelijkheid omdat het een meer selectiever en gepersonaliseerd het ijzer kan verwijderen zonder dat er een bloedarmoede ontstaat. Dit is het eerste keer dat erytrocytaferese bij erfelijke aceruloplasminemie wordt gebruikt, het lijkt een toename van ijzer in de hersenen en verder perifere ijzeroverschot te voorkomen zonder een anemie te veroorzaken.

Tenslotte wordt in **hoofdstuk 9** een overzicht van de belangrijkste bevindingen gegeven en besproken. Het proefschrift eindigt met een discussie over nieuwe inzichten en toekomstperspectieven op het gebied van de interpretatie van hyperferritinemie en *HFE*-gerelateerde hemochromatose.

Addendum

Impact paragraph

Serum ferritin is one of the most frequently requested laboratory tests in both primary and secondary care. In 12% of the general population an increased serum ferritin level (hyperferritinemia) is found.[1] Hyperferritinemia can be caused by various conditions and for that reason, its interpretation is challenging for physicians and can lead to misdiagnoses. Therefore, it is important to raise awareness for hyperferritinemia and its different causes and to have diagnostic tools available which are easy to interpret.

In this thesis, an overview of the etiology of hyperferritinemia in non-alcoholic fatty liver disease (NAFLD) was given. NAFLD is associated with an unhealthy lifestyle and has a prevalence of 25%. Due to the ongoing obesity epidemic it is the main cause of chronic liver diseases in the Western world.[2,3] Hyperferritinemia is found in 30% of NAFLD patients[4], and is rarely caused by iron overload but mainly due to the chronic low grade systemic inflammation. The prevalence of both NAFLD and hyperferritinemia is expected to rise due to the growing global burden of diseases related to an unhealthy lifestyle.

Within NAFLD, there are several possible causes explaining hyperferritinemia with varying treatment options. With this thesis, clinicians' attention is drawn to the different causes of hyperferritinemia.

Measuring the liver iron concentration (LIC) with MRI, has an important role in the diagnostic work-up of hyperferritinemia. However, LIC values, and especially intermediate values, are difficult to interpret and do not always give a definite answer to the presence or absence of iron overload. The suggested liver iron index contributes to the correct interpretation of the MRI results and avoids misinterpretation of the results.

Less than half of hyperferritinemia cases referred to the outpatient clinic account for *HFE*-related hemochromatosis.[5] *HFE*-related hemochromatosis is the most common autosomal recessive disorder in the Northern European population. Due to the discovery of the *HFE* gene in 1996[6] the awareness of this condition improved and patients can be identified in an earlier state. The patients with `bronze diabetes`, the classical hallmark of severe advanced disease, or hemochromatosis-related mortality are seen less frequently.

To date, besides diabetes mellitus, liver cirrhosis and bronze skin, symptoms such as arthralgia, fatigue, cardiac complaints and impotence haven been linked to *HFE*-related hemochromatosis. However, not all of these associations were confirmed in case-control studies. Nevertheless these symptoms result in frequent testing of iron parameters (serum ferritin, serum iron, transferrin, transferrin saturation) by different types of physicians like rheumatologists, orthopedic surgeons, urologists, cardiologists or endocrinologists. And through the accessibility of *HFE* gene analysis, the diagnosis sometimes will be made in subjects with only very limited elevations of serum ferritin and transferrin saturation (biochemical iron overload) in the absence of clinical disease symptoms. To date, it is known that phenotypic expression of the disease is low and that not all patients require treatment at the time of diagnosis. With data from the Dutch South Limburg population-based cohort study an overview was given of the recent epidemiology, phenotypic expression and disease course bringing awareness to the disease and its course, not only for gastroenterologist and internist/hematologist but all physicians confronted with *HFE*-related hemochromatosis. In addition, an unexpected finding of a high number of non-cirrhotic hepatocellular carcinomas rises the need for future research.

The treatment of *HFE*-related hemochromatosis has remained the same for many decades. Phlebotomy is still the corner stone in the treatment but more recently erythrocytapheresis is becoming an attractive alternative. Through the more selective removal of erythrocytes, more iron can be removed with less procedures. Erythrocytapheresis is however less frequently used due to higher costs and is currently only performed in specialized centers.[7] With the modified iron avidity index patients who will benefit from a less frequent therapy like erythrocytapheresis can be selected to provide the optimal treatment for the patient.

This thesis has also given insight in still relatively unexplored pathways within iron homeostasis. Proton pump inhibitors (PPIs) are among the most frequently used drugs worldwide.[8] PPIs are also suggested as a treatment for *HFE*-related hemochromatosis since they can decrease iron absorption.[9] It is unclear why PPI use does not initiate an iron deficiency in the large population of chronic users without *HFE*-related hemochromatosis while they do decrease ferritin levels in *HFE*-related hemochromatosis. In this thesis the precise mechanism explaining this difference could not be established however the proof of concept study on iron absorption paved the way for future research requiring larger study populations.
Another relatively unexplored path analyzed in this thesis was the ability of hepcidin production, as a result of inflammation, in patients with *HFE*-related

hemochromatosis, through the JAK/STAT3 route. Based on the observations in this thesis, intervention via the JAK/STAT3 pathway deserves further exploration since it could reduce excess absorption and accumulation of iron in patients with *HFE*-related hemochromatosis and will lead to new treatment possibilities.

Lastly, a new treatment for hereditary aceruloplasminemia was introduced. Despite the fact that hereditary aceruloplasminemia is a very rare condition and therefore societal impact will be low, there is a need for a good treatment. Current therapeutic regimens are often associated with side-effects like aggravating anemia. Erythrocytapheresis has the ability to selectively remove erythrocytes, to return valuable blood components to the patient and closely monitor patients hemoglobin levels, thus preventing the aggravation of anemia.

In conclusion, this thesis will contribute to a better understanding of hyperferritinemia in NAFLD and to a better interpretation of liver iron concentrations measured by MRI used in the diagnostic work-up of hyperferritinemia and hemochromatosis. In addition, it provides insight in the current disease course of *HFE*-related hemochromatosis and relatively unexplored pathways within the iron homeostasis.

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A

Addendum

List of publications

Verhaegh PL, **Moris W**, Koek GH, van Deursen CT. The modified iron avidity index: a promising phenotypic predictor in HFE-related haemochromatosis. Liver Int. 2016 Oct;36(10):1535-9. doi: 10.1111/liv.13121. Epub 2016 Apr 6. PMID: 26992127.

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Submitted

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To be Submitted

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Addendum

Dankwoord

Toen ik eind 2014, in mijn 5de jaar van de opleiding geneeskunde, aan mijn wetenschapsstage begon had ik nooit gedacht dat dit zou resulteren in een proefschrift. Geboeid door het onderwerp besloot ik om tijdens de laatste twee jaren van mijn opleiding, naast mijn coschappen, het onderzoek dat ik was begonnen verder af te ronden. In de loop van de tijd ontstonden er nieuwe ideeën en toen ik in 2017 een opleidingsplek tot MDL-arts bemachtigde, werd een jaar onderzoek ingepland om zoveel mogelijk af te ronden en om nieuwe projecten op te starten.

Na een jaar onderzoek, pakte ik mijn opleiding tot MDL-arts weer op. Daarnaast bleef ik verder werken aan mijn promotieonderzoek. Door de combinatie met mijn fulltime opleiding ging dit minder snel dan gehoopt, maar des te meer trots ben ik dat het gelukt is en dat het nu eindelijk klaar is! Het is me gelukt door de steun en hulp van veel mensen en in dit hoofdstuk wil ik een aantal mensen persoonlijk bedanken.

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Mijn beoordelingscommissie

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Zuyderland Medisch Centrum

In 2016, mocht ik, meteen na de opleiding geneeskunde, jullie team komen versterken. Mijn eerste baan als arts en wat was dat spannend. Gelukkig werd ik met open armen ontvangen en al snel mocht ik een heel aantal collega`s ook vrienden noemen. Bedankt Aimée, Eline, Ellen, Maaike, Limmie, Renee en Sharona voor alle gezellige feestjes en etentjes samen. Door jullie had ik niet alleen op het werk maar ook na de werkuren een geweldige tijd. Daarnaast ook dank aan Chantal & Michèle voor onze vriendschap en geweldige reis naar Sri Lanka. Ik denk nog vaak terug aan deze onvergetelijke reis gevuld met geweldige feestjes, wandelingen, bergbeklimmingen maar ook slechte beslissingen resulterend in een bezoek aan de hondsdolheid poli of een massage in een sketchy achterbuurt. Bedankt lieve Floor voor alle geweldige momenten samen, met jou zijn er geen saaie momenten. Auwiejee, onze kennismaking was redelijk onconventioneel maar ik had het niet willen missen om jou als vriendin te hebben!

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Addendum

Curriculum vitae

Wenke Moris was born on the 27th of January 1992 in Hasselt, Belgium. She graduated from secondary school at the Heilig-Grafinstituut in Bilzen, Belgium. After graduating in 2010 she started medical school at Maastricht University. During medical school she did several internships abroad, including an internship Internal Medicine at Kasturba Medical College in Manipal, India and an internship Hepatology at Sheila Sherlock Liver Centre in the Royal Free Hospital in London, UK. During her master, she started on a research project regarding hyperferritinemia and hemochromatosis under direct supervision of dr. G. Koek, as part of a scientific internship at the Gastroenterology department of Maastricht University Medical Center, The Netherlands. After graduating medical school in 2016, she started working as a resident Internal Medicine (ANIOS) at Zuyderland Medical Center in Sittard-Geleen and Heerlen under supervision of dr. J. Buijs. In 2017, she started her residency Internal Medicine (AIOS) in the Zuyderland Medical Center under supervision of dr. J. Buijs, as first part of her residency program in Gastroenterology and Hepatology under supervision of Prof. dr. A. Masclee. Wenke interrupted her residency for a year in 2018 to work on the department of Gastroenterology, Geriatrics, Internal and Intensive Care Medicine at Zuyderland Medical Center as a PhD student also affiliated to the NUTRIM, School of Nutrition, Toxicology and Metabolism and the Department of Internal Medicine, Division of Gastroenterology and Hepatology, of Maastricht University Medical Center to continue the research project she was working on since her research internship in 2014. From 2019, she continued her residency Internal Medicine in Zuyderland Medical Center simultaneously with her PhD research. In 2020, she started with the Gastroenterology and Hepatology part of her residency program in Zuyderland Medical Center in Sittard-Geleen and Heerlen under the supervision of dr. J. Keulemans and dr. A. van Nunen. In 2022, she started the 5th year of the residency program in Maastricht University Medical Center under the supervision of dr. J. Kruimel and dr. J. Haans.