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REVIEW ARTICLE

Iron Perturbations in Human Non-Alcoholic Fatty Liver Disease (NAFLD): Clinical Relevance and Molecular Mechanisms

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Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the insulin resistance syndrome and thus a frequent cause of elevated liver enzymes. The term "insulin-resistance associated hepatic iron overload syndrome (IR-HIOS)" has been coined to describe the frequent association of hepatic steatosis with increased levels of serum ferritin, normal or slightly elevated transferrin saturation and mild hepatic iron deposition. There is mounting evidence that increased iron stores in insulin resistance are associated with an unfavorable course of the disease and an increased prevalence of associated conditions such as diabetes, hypertension or cardiovascular disease. Iron depletion via phlebotomy has been demonstrated to improve several aspects of the insulin-resistance syndrome. Multiple interactions have been observed between molecules of iron and glucose metabolism. On a molecular level, impaired iron export has been demonstrated to be the principal mechanism of iron accumulation in fatty liver disease. Obesity-related inflammation, low ferroxidase activity associated with low copper bioavailability and decreased expression of the iron export molecule ferroportein have so far been identified as contributors to increased iron accumulation in human NAFLD.

Keywords: **Non-Alcoholic Fatty Liver Disease, Iron Overload, Insulin Resistance**

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the insulin-resistance syndrome (also known as the metabolic syndrome) and a frequent cause of elevated liver enzymes worldwide [1, 2]. The prevalence of NAFLD and other manifestations of insulin resistance are expected to rise further during the forthcoming decades as a consequence of an unhealthy and common coincidence of a sedentary lifestyle and a western diet. The histological spectrum of NAFLD ranges from benign steatosis without signs of inflammation to its severe manifestation, termed non-alcoholic steatohepatitis (NASH) [3, 4]. NASH can potentially progress to severe fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [5, 6]. NASH thus contributes significantly to the burden of liver disease in Western societies and is a recognized cause for liver transplantation [7].

It is generally accepted that changes occurring during the development of insulin resistance are

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also the pathophysiological basis of NAFLD. For insight into this subject reading of these reviews is recommended [8-10]. Besides these environmental and acquired contributors to the development of insulin resistance, diabetes or NAFLD, a genetic predisposition plays a role in the development of insulin resistance. Genetics have moreover been demonstrated to contribute significantly to impaired mitochondrial energy homeostasis and respiratory chain function which may be a key regulatory perturbation in insulin resistance and NAFLD [11, 12]. In addition, we have recently learned that adipose tissue is not only responsible for energy storage but is also to be considered an endocrine organ [10].

These endocrine properties become especially important as adipose tissue mass expands in response to excess calorie intake. Adipocytes secrete several hormone-like proteins modulating insulin sensitivity. The first of these adipocyte derived soluble factors linked to insulin resistance identified was tumor necrosis factor-α (TNF-α) [13]. Increased serum concentrations of pro-inflammatory cytokines such as TNF-α have been described in NAFLD and have been linked to activation of the crucial TNF-α transcription factor NF-kB by fatty acids [14]. Moreover, TNF-α activity induces oxidative stress which results in lipid peroxidation and cellular damage and has thus been implicated as an important factor in the progression of NAFLD to fibrosis and cirrhosis over time [15]. Several adipose tissue derived hormones, such as leptin, adiponectin, visfatin, RBP4 or resistin have been shown to facilitate changes in carbohydrate and lipid metabolism, thereby being involved in the pathogenesis of insulin resistance [9, 10]. Serum concentrations of leptin, RBP4 and resistin are increased in insulin resistant states but inadequately low concentrations of adiponectin is a characteristic of NAFLD. High circulating levels of leptin appear crucial in facilitating progression of NAFLD to NASH and consecutive liver cirrhosis [16]. Likewise, inappropriately low concentrations of adiponectin and its liver receptors are features of progressive forms of NAFLD [17]. Approximately 10 years have passed since hyperferritinemia has been recognized as a frequent and characteristic laboratory abnormality in patients with NAFLD [18], insulin resistance [19] and the metabolic syndrome [19, 20]. This review was designed to provide a brief overview of the relevance and pathomechanisms of increased iron stores frequently observed in patients with NAFLD.

Clinical background and relevance of hyperferritinemia

Since the first description of increased iron stores in NAFLD and/or insulin resistance, hyperferritinemia in NAFLD has been established as an important differential diagnosis of increased biochemical parameters of iron metabolism distinct from hereditary iron overload syndromes such as hereditary hemochromatosis or the sideroachrestic anemias [18]. The term insulin-resistance associated hepatic iron overload or dysmetabolic iron overload syndrome (IR-HIOS/DIOS) has been coined to describe the typical finding of hepatic steatosis along with mild to moderate iron deposition in liver biopsies and increased serum ferritin with normal or slightly elevated transferrin saturation (TfS) in patients with insulin resistance and features of the (dys-) metabolic syndrome [18, 21]. In contrast to hereditary iron overload syndromes, such as HFE-associated hemochromatosis, where massive iron accumulation is predominantly found in hepatocytes, iron deposition in NAFLD is generally mild and is found in hepatocytes as well as in macrophage derived Kupffer cells [22].

The relevance of increased iron stores for NAFLD disease severity and progression has been intensely investigated but has not yet been discussed conclusively. Several investigations found an association between iron [23-26] and HFE mutations [27, 28] and more progressed forms or the incidence of NAFLD; however, this association was not confirmed in subsequent studies [29-31]. Data concerning the relevance of excess iron in NAFLD disease progression is mainly hampered by the lack of an available prospective investigation with serial liver biopsies in a cohort of patients large enough to correct for established factors influencing disease progression such as obesity, female sex, and diabetes mellitus [29, 32]. However, such a study will be difficult to perform for practical reasons and due to ethical concerns. Notwithstanding, investigations examining iron depletion via phlebotomy in human NAFLD have unequivocally demonstrated beneficial effects of iron removal with regard to systemic or hepatic insulin resistance and to pancreatic insulin sensitivity [33-35]. In a parallel fashion, this association holds true for iron depletion and other cardiovascular risk factors [36]. These observations offer strong, though only indirect, evidence of the detrimental effects of excess iron stores in NAFLD. Moreover, a significant reduction in serum TNF-α concentration was found in response to

phlebotomy treatment in NAFLD patients, indicating amelioration of low-grade systemic inflammation in insulin resistant subjects upon iron removal [37].

Additional compelling support to the notion that increased iron stores may exert harmful effects on insulin resistance is derived from large epidemiologic studies. Patients with increased ferritin levels were subsequently found to develop a higher rate of diabetes and gestational diabetes [38-43]. Serum ferritin levels were found to be positively associated with BMI [44], visceral fat mass [45], serum glucose levels and insulin sensitivity [46], blood pressure [47], the metabolic syndrome [20, 48] and to be related to cholesterol levels [49]. Such a relationship between serum ferritin and metabolic markers was also found in a pediatric population [50]. These findings are clinically mainly important due to the high risk of developing cardiovascular or cerebrovascular diseases in a population with insulin resistance [51, 52]. In summary, these data reflect a close relationship between increased body iron stores, or at least biochemical evidence thereof, and several clinical manifestations of the insulin resistance syndrome.

Along the same line of evidence, a lower incidence of diabetes, lower postprandial serum insulin concentrations and higher pancreatic insulin sensitivity as reflected by improved beta cell function [53] was found in subjects who underwent previous phlebotomy treatment [54]. Iron depletion was also found to improve coronary vascular dysfunction in type 2 diabetics [55] and in patients with known coronary artery disease [56]. In addition to convincing clinical evidence of improved insulin sensitivity in response to iron depletion, iron chelation by desferoxamin improved insulin receptor signalling and glucose metabolism both in cell cultures and rat models [57]. Several investigations suggest, however, that serum ferritin overestimates the extent of iron overload in NAFLD. It is conceivable that hyperferritinemia is caused by both iron overload and adipose tissue associated low-grade inflammation [58, 59]. Accordingly, TNF-α and Interleukin-6, which are elevated in obesity and insulin resistance, are known to be important inductors of ferritin gene transcription leading to hyperferritinemia in inflammation even in the absence of iron overload [60].

Furthermore, it is well known that classical iron overload syndromes such as hemochromatosis or transfusional iron overload lead to hepatic and peripheral insulin resistance as body iron accumulation increases [61, 62]. Thus, the spectrum of the relationship between iron and glucose metabolism ranges from iron-overload associated insulin resistance in hemochromatosis or transfusional iron overload to insulin-resistance associated iron overload observed along with various features of the metabolic syndrome.

Molecular mechanisms contributing to perturbations of iron homeostasis in insulin resistance

Derived from the above mentioned clinical observations, it is clear that iron and glucose metabolisms are mutually influencing each other. This review will first briefly give an overview of physiological regulation of iron metabolism and then outline what is known about molecular mechanisms underlying iron perturbations in insulin resistant states (Fig. 1). Our knowledge of physiological regulation of human iron metabolism has expanded over the past years due to the identification of several new key molecules. While cells acquire iron via different pathways which include the uptake of transferrin bound iron by transferrin-receptors (TfR-1 and TfR-2) and the uptake of ferrous iron via a transmembrane protein named divalent metal transporter-1 (DMT-1) [63], so far only one iron exporter has been characterized, comprising the transmembrane protein ferroportein (FP-1) [64]. Following its transfer through the duodenal baso-lateral membrane, iron undergoes oxidation by the membrane bound copper containing ferroxidase hephaestin before being incorporated into transferrin for further transport in the circulation [65]. Iron is then mainly required for heme biosynthesis in erythropoiesis and other heme containing enzymes, whereas excess iron is mainly stored in the liver, which is the central organ in the regulation of body iron homeostasis [66].

Hepcidin is a master iron regulatory peptide, which is secreted mainly by hepatocytes in response to iron perturbations, inflammation and hypoxia [67, 68]. Hepcidin exerts its regulatory functions on iron homeostasis via binding to FP-1 thereby leading to FP-1 phosphorylation, internalisation, degradation and thus to blockage of cellular iron export [69]. Important up-stream regulators of hepcidin expression include hemojuvelin (HJV), a bone-morphogenetic protein co-receptor [70], HFE, a non-classical MHC class-1 molecule [71] and TfR-2, a liver specific iron uptake molecule [72]. Mutations of these genes are associated with inappropriately low hepcidin formation and hereditary iron overload syndromes. Importantly, although the liver is the major hepcidin producing organ in quantitative terms, macrophages

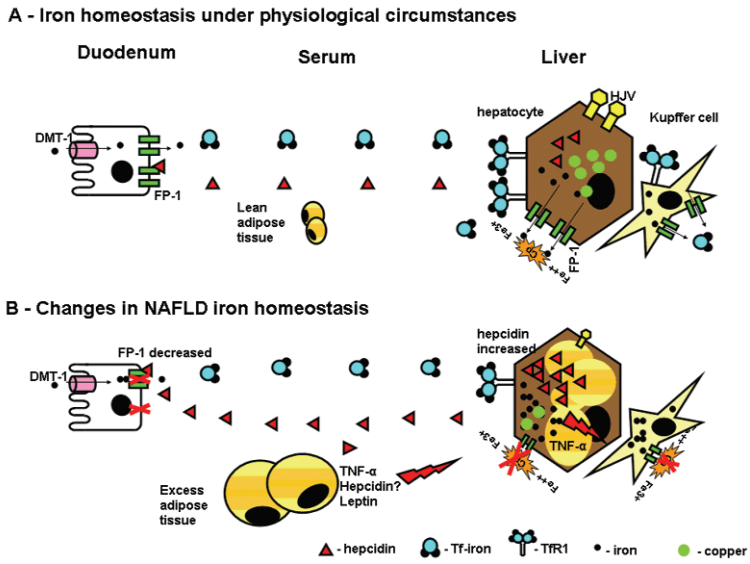

Figure 1. Current knowledge of molecular links between obesity or insulin resistance and iron homeostasis. Figure 1A depicts regulation of iron homeostasis under physiological circumstances. Iron is taken up in duodenal enterocytes via DMT-1 and FP-1 before being incorporated into transferrin. Iron requiring tissues such as the erythron (not shown) or the liver take up iron mainly via endocytosis of Tf-loaded transferrin receptors. Iron homeostasis is tightly regulated via hepcidin, which inhibits excess iron absorption from the duodenum as well as iron recycling from macrophages. Figure 1B summarizes changes known to occur in human NAFLD. Iron is primarily retained in hepatocytes and Kupffer cells due to low expression of the only known iron export molecule ferroportein (FP-1). This down-regulation may occur as a consequence of systemic and hepatic inflammation (TNF-α), as well as increased adipose tissue expression of hepcidin or leptin. Iron mobilisation from liver cells is additionally impaired due to low copper bioavailability in NAFLD which leads to low ceruloplasmin ferroxidase activity and a further decrease in FP-1 expression. Iron accumulation in liver cells induces hepcidin expression which in turn down-regulates duodenal iron absorption to counterbalance hepatic iron deposition.

and adipose tissue can also excrete hepcidin [73, 74]. TMPRSS6 comprises a recently identified serin-protease which is required for adequate hepatic hepcidin down-regulation in response to loss of iron or anemia, thus mutations of TMPRSS6 cause iron deficiency due to inappropriately high levels of hepcidin [75, 76].

As clinical evidence of iron perturbations induced by mechanisms associated with insulin resistance increases, several investigations have aimed to elucidate underlying mechanisms. Hence, the master iron regulatory peptide hepcidin was demonstrated to be synthesized in excess adipose tissue in morbidly obese individuals [74], corresponding to previously observed increased urinary hepcidin concentrations in insulin resistant subjects [77, 78]. It is, however, not clear to what extent adipose tissue derived hepcidin is pathophysiologically important; as in quantitative terms, the main source of hepcidin is the liver. Obese adipose tissue is heavily infiltrated with bone-marrow derived macrophages and displays histological features of inflammation. One can assume that inflamed adipose tissue macrophages are the main source of hepcidin rather than adipocytes [79, 80]. We and others have recently demonstrated that increased hepcidin synthesis occurs in the liver of NAFLD patients with iron deposition and hepcidin mRNA levels directly correlate with the extent of NAFLD iron overload [37, 81]. As iron accumulation is a key stimulus of hepcidin production, increased systemic hepcidin levels appear to be a consequence rather than the cause of hepatic iron accumulation. In particular, persistently high hepcidin levels would induce iron deficiency as opposed to iron accumulation which is observed in biopsies of patients with NAFLD. In a similar fashion, it has recently been demonstrated that pancreatic beta-cells express hepcidin which thus may be involved in iron and glucose metabolism at the same time [82].

As insulin is a key anabolic regulator of human body homeostasis associated with increased uptake and storage of nutrients, it is noteworthy that insulin has been able to increase both the TfR1 cell surface expression and ferritin synthesis in cell culture experiments indicating the potential of insulin to increase iron uptake and storage [83, 84]. Several links were found between adipocytokines and parameters of iron metabolism. A positive association has been found between increased iron stores and circulating RBP4 (retinol-binding protein 4), as both decrease in response to iron depletion [85]. A similar relationship has been observed between serum visfatin concentrations and biochemical parameters of iron metabolism [86]. However, these observations may simply reflect the co-incidence of increased iron stores and other markers of insulin resistance and does not yet molecularly link these adipocytokines to the homeostasis of iron metabolism. In contrast, the adipocytokine leptin which is increased in obesity and insulin resistance, has been found to upregulate hepcidin transcription via JAK2/STAT3 dependent signalling pathways [87]. Thus, leptin

induced hepcidin synthesis may directly contribute to iron perturbations in obesity, diabetes or NAFLD.

Recently, we aimed to analyse potential pathways underlying iron accumulation in human NAFLD by studying the expression of key iron regulatory molecules in human liver and duodenal biopsies [37]. Several lines of evidence suggest that iron deposition in human NAFLD is primarily a perturbation of iron export. This has already been suggested from a clinical study examining the response of iron overloaded NAFLD patients [34]. First, we found a striking down-regulation of the only iron export molecule FP-1 in human NAFLD compared to unaffected liver tissue, suggesting a decreased rate of iron mobilisation from liver cells. Additionally, we found that iron overloaded NAFLD subjects respond to phlebotomy treatment with a fast decrease in TfS and are prone to develop anemia in response to phlebotomy, which also corresponds to slow mobilisation of iron from storage sites such as the liver. Interestingly, low FP-1 expression was found in NAFLD patients independent of iron deposition which indicates that contributing factors are required besides low FP-1 expression for iron deposition in NAFLD to develop. NAFLD patients with iron accumulation present with significantly increased hepatic hepcidin mRNA levels, whereas NAFLD patients without signs of excess body iron have normal hepatic hepcidin mRNA expression. Increased hepcidin production correlates directly with hepatic iron concentration indicating an intact physiological response to full iron stores in the liver. TfR1-mediated iron uptake does not seem to be involved in NAFLD iron accumulation. NAFLD patients with low hepatic iron present with higher TfR1 levels compared to patients with NAFLD and iron overload or patients with HFE-associated hemochromatosis. This pattern suggests physiologically reduced expression of TfR1 in response to iron accumulation in order to limit Tf/TfR1-mediated iron uptake in liver cells where iron stores are full.

As copper is an important modulator of iron homeostasis, we examined if copper status was linked to iron perturbations in NAFLD. Copper is the key molecule in hephaestin ferroxidase activity in the duodenal enterocytes where it facilitates loading of iron to apotransferrin. In a similar manner, copper is required for ceruloplasmin ferroxidase activity to mobilize iron from storage sites such as the liver or the reticuloendothelial system. In NAFLD, iron and copper stores are inversely related [88]. Significantly lower liver and serum copper concentrations were found in NAFLD patients with iron accumulation. Low serum or liver copper concentrations were found to be associated with low serum activity of the ferroxidase ceruloplasmin. In addition, lower hepatic expression of FP-1 was detected in rats on a copper deficient diet. These observations demonstrate that besides low FP-1 expression associated with low-grade systemic inflammation; inadequate copper bioavailability further impairs iron export from liver cells. Low copper bioavailability impairs iron transport across the cell membrane via decreased ceruloplasmin-dependent oxidation of Fe^{2+} to Fe^{3+} and consecutive loading of Fe^{3+} to apo-transferrin. Moreover, adequate copper supply has been demonstrated to induce FP-1 expression [89] and copper-dependent membrane-bound ceruloplasmin expression is necessary for FP-1 protein expression and cell surface stability [90].

Conclusions

In summary, hyperferritinemia is a clinically relevant finding in NAFLD and other insulin resistant conditions, since excess iron appears to be associated with adverse outcomes, increased insulin resistance and thereby accelerated disease progression. Moreover, removal of excess iron via phlebotomy is a safe and beneficial additive treatment which can easily be offered to these patients and is linked to favorable effects on parameters of insulin resistance and inflammation. Iron deposition occurs mainly due to impaired iron export from liver cells in response to low expression of the iron export molecule FP-1 and decreased ferroxidase activity associated with NAFLD copper deficiency.

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