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REVIEW

Iron and non-alcoholic fatty liver disease

Laurence J Britton, V Nathan Subramaniam, Darrell HG Crawford

Laurence J Britton, Darrell HG Crawford, Gallipoli Medical Research Institute, The University of Queensland, Greenslopes Private Hospital, Brisbane, Queensland 4120, Australia

Laurence J Britton, The Princess Alexandra Hospital, Brisbane, Queensland 4120, Australia

V Nathan Subramaniam, QIMR Berghofer Medical Research Institute, Brisbane, Queensland 4120, Australia

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Correspondence to: Dr. Laurence J Britton, Gallipoli Medical Research Institute, The University of Queensland, Greenslopes Private Hospital, Newdegate Street, Greenslopes, Brisbane, Queensland 4120, Australia. l.britton@uq.edu.au
Telephone: +61-7-33947284
Fax: +61-7-33947767

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Abstract

The mechanisms that promote liver injury in non-alcoholic fatty liver disease (NAFLD) are yet to be thoroughly elucidated. As such, effective treatment strategies are lacking and novel therapeutic targets are required. Iron has been widely implicated in the pathogenesis of NAFLD and represents a potential target for treatment. Relationships between serum ferritin concentration and NAFLD are noted in a majority of studies, although serum ferritin is an imprecise measure of iron loading. Numerous mechanisms for a pathogenic role of hepatic iron in NAFLD have been demonstrated in animal and cell culture models. However, the human data linking hepatic iron to liver injury in NAFLD is less clear, with seemingly conflicting evidence, supporting either an effect of iron in hepatocytes or within reticulo-endothelial cells. Adipose tissue has emerged as a key site at which iron may have a pathogenic role in NAFLD. Evidence for this comes indirectly from studies that have evaluated the role of adipose tissue iron with respect to insulin resistance. Adding further complexity, multiple strands of evidence support an effect of NAFLD itself on iron metabolism. In this review, we summarise the human and basic science data that has evaluated the role of iron in NAFLD pathogenesis.

Key words: Iron; Fatty liver; Liver steatosis; Insulin resistance; Steatohepatitis; Diabetes mellitus; Adipose tissue

Core tip: Iron represents a potential therapeutic target for the treatment of non-alcoholic fatty liver disease (NAFLD). There are extensive data that link iron and disease pathogenesis in human studies as well as animal and cell culture models. Studies have predominantly focussed on the role of hepatic iron, although recently adipose tissue has emerged as a site at which iron may promote insulin resistance. In this review, we summarize the human and basic science data that have evaluated the role of iron in NAFLD pathogenesis.

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INTRODUCTION

The worldwide epidemic of obesity has led to a disturbing rise in the incidence of non-alcoholic fatty liver disease (NAFLD) and its complications[1,2]. NAFLD, regarded as the "hepatic manifestation of the metabolic syndrome", is now estimated to affect one billion individuals worldwide[1]. Non-alcoholic steatohepatitis (NASH), the aggressive form of the disease, can lead to cirrhosis and liver failure[3,4]. Indeed, NASH is predicted to soon become the predominant cause of advanced liver disease in the developed world[5] and the leading indication for liver transplantation[4]. NAFLD has also been increasingly recognised as an independent risk factor for the development of type Ⅱ diabetes mellitus, cardiovascular disease and hepatocellular carcinoma, the latter of which may occur even in non-cirrhotic individuals[3,6,7]. The factors that predispose patients to the development of steatohepatitis and fibrosis in NAFLD are not well understood and effective treatment strategies are lacking[8].

There is evidence that a modest degree of iron overload is associated with more advanced liver injury in NAFLD, although the mechanisms by which this might occur remain unclear[9,10]. A syndrome of increased hepatic iron in conjunction with the metabolic syndrome is commonly observed and has been termed dysmetabolic iron overload syndrome[9,11].

To date, the majority of studies have focussed mainly on the role of hepatic iron and mutations in the *HFE* gene, the gene mutated in type 1 hereditary hemochromatosis. Recently, however, it has become increasingly evident, that adipose tissue iron plays an important role in the pathogenesis of insulin resistance and therefore possibly NAFLD[12,13].

In this review, the potential involvement of iron in NAFLD pathogenesis is explored using the available data from human studies, as well as animal and cell culture models. In addition, the counterview that implicates NAFLD itself in the dysregulation of iron metabolism is outlined.

HUMAN IRON HOMEOSTASIS

Iron is an essential nutrient required for erythropoiesis and multiple cellular metabolic functions[14,15]. An excess of iron is also, however, a potent cause of cellular injury from oxidative stress due to the generation of reactive oxygen species by the Fenton reaction[16]. Under usual conditions, intracellular protection from iron-induced oxidative stress is facilitated by sequestration of iron within ferritin[14].

Total body iron homeostasis is achieved predominantly by regulation of iron release from duodenal enterocytes and macrophages by the hormone hepcidin[15,17,18]. Predominantly produced by hepatocytes, hepcidin binds the enterocyte basal membrane iron transporter, ferroportin, causing its internalisation and eventual degradation, thus reducing iron release from duodenal enterocytes and other cells[15,18]. Ferroportin has been shown to be highly expressed in enterocytes, reticuloendothelial cells, and more recently, in adipocytes[15,19]. Thus, hepcidin regulates systemic iron balance by reducing intestinal iron absorption[15].

An understanding of the regulation of hepcidin (*HAMP*) gene expression has come about from studying human subjects with various forms of hereditary hemochromatosis, and by analysis of gene knockout rodent models. Hepcidin is regulated by many factors, including erythropoiesis, iron status, intracellular oxygen tension and inflammation[18].

Pathologic states of iron overload often lead to saturation of serum iron transporter, transferrin. As a result, serum levels of toxic non-transferrin bound iron (NTBI) rise. NTBI is readily absorbed by tissues such as the liver and cardiac muscle[18]. Tissue iron overload with NTBI results in increased oxidative stress and lipid peroxidation, leading to organ dysfunction. The common causes of iron overload include hereditary hemochromatosis, iron loading anemias (such as thalassemia) and parenteral iron overload from multiple blood transfusions[18].

INSULIN RESISTANCE AND THE PATHOGENESIS OF NAFLD

It has become evident that insulin resistance is associated with a more subtle degree of iron overload than is seen in hereditary hemochromatosis and thalassemia[9,10,12]. This is important as insulin resistance is central to the pathogenesis of NAFLD[3,20]. The presence of abdominal obesity and accompanying insulin resistance provide fertile conditions for the development of NAFLD. Indeed, NAFLD is often considered as the hepatic manifestation of insulin resistance and the metabolic syndrome[3]. Central obesity is associated with adipose tissue dysfunction, characterised by infiltration of adipose tissue with macrophages[21]. Dysfunctional adipose tissue produces adipokines that promote the development of insulin resistance[12]. The key sites of insulin action and resistance are the liver, skeletal muscle and adipose tissue[22]. In adipose tissue itself, insulin resistance potentiates lipolysis of triglycerides by hormone sensitive lipase[23]. This generates the majority of free fatty acid flux to the liver in NAFLD[24]. Insulin resistance in skeletal muscle leads to reduced uptake of glucose, whereas in the liver, insulin resistance enhances gluconeogenesis[25]. The

resultant compensatory hyperinsulinemia and relative hyperglycemia promote hepatic *de novo* lipogenesis and cholesterol synthesis and reduced catabolism of free fatty acid by oxidation[3].

Increased hepatic free fatty acid flux resulting from this dysregulation of hepatic lipid metabolism and more importantly by adipose tissue lipolysis, appears to be central to the pathogenesis of steatohepatitis *via* direct lipotoxicity[3,26,27]. A number of other mechanisms have been well demonstrated to be responsible for not only the development of steatohepatitis, but also steatosis itself. These mechanisms include dysregulated adipokine production[28,29], abnormal bile acid signalling[30], cytokine mediated effects[31], in particular as a result of increased gut cell permeability and TLR-4 receptor activation[32], endoplasmic reticulum stress[33,34] and oxidative stress[31,35]. Hepatocellular injury promotes cell death and steatohepatitis through a combination of apoptosis and cell necrosis[3]. These mechanisms also contribute to hepatic stellate cell activation and resultant development of hepatic fibrosis[36].

IRON AND INSULIN RESISTANCE

The association between hyperferritinemia, insulin resistance and type Ⅱ diabetes is compelling. There is an increased prevalence of type Ⅱ diabetes associated with two common iron overload conditions, HFE-hereditary hemochromatosis (HH) and β-thalassemia major[12]. HH can lead to β-cell pancreatic loss and type Ⅰ diabetes, but whether HH causes type Ⅱ diabetes by unmasking insulin resistance through pancreatic β-cell loss or by causing insulin resistance itself remains controversial[12]. Animal data suggest that insulin sensitivity is enhanced in HH, but it has been difficult to tease out the relative contributions of β-cell loss and insulin resistance in human studies[12,37]. The case of β-thalassemia major is more clear, with evidence suggesting that both β-cell loss and insulin resistance are at play[12].

In those who have neither hereditary hemochromatosis nor another cause of overt iron overload such as thalassemia, the evidence for a pathogenic role of iron is also strong. In the National Health and Nutritional Education Survey (NHANES), 9486 US adults were studied[38]. The odds ratios for developing diabetes in those with elevated serum ferritin levels were high at 3.61 for women and 4.94 for men[38]. A further analysis of the NHANES cohort revealed that even after accounting for other factors such as age, race, alcohol consumption and C-reactive protein (CRP) levels, elevated serum ferritin concentration still accounted for a two-fold increase in the risk of the metabolic syndrome[38]. The risk of diabetes itself, has been shown to be strongly linked to serum ferritin concentration in healthy women, even within the normal range of ferritin[39]. In 2012, the European Prospective Investigation in Cancer and Nutrition (EPIC)-Potsdam study followed 27548 European adults for 7 years[40]. In this time, 849 subjects developed type Ⅱ diabetes. Serum ferritin concentration in the highest *vs* lowest quintile had a relative risk (RR) of 1.73 for the development of diabetes. This observation was made after adjusting for multiple variables including age, sex, body mass index, waist circumference, sports activity, education, occupational activity, alcohol, liver function test parameters, high sensitivity CRP (hsCRP), adiponectin, high density lipoprotein (HDL) and serum triglyceride concentration[40].

A recent review of 43 studies further supported these findings[41]. In this meta-analysis, the cohorts with the highest and lowest quartile of serum ferritin concentration were compared. The multivariable adjusted RR for the presence of diabetes was 1.91. This finding was consistent after including only studies that adjusted for inflammation (mostly hsCRP), RR 1.67. This related to a serum ferritin that was 43.54 ng/mL higher in type Ⅱ diabetics compared to controls. Studies assessing the relationship between type Ⅱ diabetes and transferrin saturation have yielded conflicting results[41-43].

The persistence of association between serum ferritin concentration and type Ⅱ diabetes after correction for hsCRP implies that inflammation alone does not entirely explain the association between hyperferritinemia and diabetes. However, it might be argued that even hsCRP may not reflect subtle degrees of inflammation as strongly as serum ferritin concentration.

SERUM FERRITIN CONCENTRATION AND NAFLD

The association between hyperferritinemia and histologic markers of liver injury in NAFLD is reasonably strong. In 2004, Bugianesi *et al*[44] found that serum ferritin concentration is not associated with hepatic iron concentration in NAFLD, but is a marker of severe histologic damage. Kowdley *et al*[45] demonstrated in the large NASH Clinical Research Network (CRN) cohort of 628 patients that a serum ferritin concentration greater than 1.5 times the upper limit of normal was independently associated with advanced fibrosis and increased NAFLD activity score. Sumida *et al*[46], have demonstrated the utility of incorporating serum ferritin into a clinical scoring system to predict steatohepatitis in Japanese patients with NAFLD.

However, other studies have not found such a clear association[47,48]. Notably, Valenti *et al*[47] showed in an Italian cohort of 587 patients with NAFLD that serum ferritin concentration did not predict fibrosis stage > 1, although the proportion of patients with fibrosis stage > 1 in this cohort was relatively small. As would be expected, serum ferritin concentration was higher in the patients who had hepatic iron staining than those who did not, but those with non-parenchymal iron had much higher ferritin values (606 μg/L) than those with hepatocellular iron (serum ferritin 354 μg/L) $P < 0.0001$.

This might suggest that macrophage iron can cause hyperferritinemia either by direct release of ferritin or cytokine-mediated stimulation of ferritin release by other cells. An earlier study by Chitturi *et al*[49] of 93 patients with NASH, 33% of whom had advanced fibrosis, found that serum ferritin concentration was not an independent predictor of advanced fibrosis.

In a large prospective population-based study from South Korea, 2410 healthy men aged 30 to 59 without sonographic evidence of steatosis were followed for 7545.9 person years[50]. Of these, 586 (24.3%) patients developed ultrasonographically detectable fatty liver. Baseline serum ferritin concentration was found to be a strong predictor of steatosis. This evidence is notable as it demonstrates an association early in the disease suggesting that the process that elevates serum ferritin concentration is contributing to NAFLD pathogenesis very early in the disease and pre-dates the development of steatosis. This implies that the ferritin association with NAFLD is not simply a result of NAFLD itself causing hyperferritinemia. Moreover, the results might tend to suggest that the link between hyperferritinemia and NAFLD could be explained by insulin resistance.

The strengths of these studies lie in the large numbers of individuals studied. However, serum ferritin concentration is an imprecise surrogate for body iron stores and its associations with both NAFLD and, type Ⅱ diabetes are clearly not enough to attribute causality with respect to iron in either of these conditions.

HEPATIC IRON AND NAFLD

The role of hepatic iron in NAFLD pathogenesis has largely focussed on the generation of oxidative stress by iron. Given that oxidative stress is an established key component of NASH pathogenesis[31], a role for iron mediating liver injury in NAFLD *via* this mechanism has been well studied. In NASH, oxidative stress leads to cell death *via* depletion of ATP, NAD and glutathione, and by direct damage to DNA, lipids and proteins within hepatocytes[31]. Furthermore, oxidative stress leads to an increase in the production of pro-inflammatory cytokines and a fibrogenic response[31]. Not only does oxidative stress potentiate steatohepatitis, characterised by inflammation and cell death, it can also increase steatosis by preventing the secretion of very low density lipoprotein (VLDL) by causing increased degradation of apolipoprotein B100 (ApoB100)[51]. In cultured primary rodent hepatocytes, the iron chelator desferrioxamine was able to restore ApoB100 and enhance VLDL export[51].

Reduced oxidative stress has been observed in the livers of rats fed an iron-deficient diet and after phlebotomy[52]. In a series of liver biopsies from patients with NAFLD, increased hepatic iron stores were found to be associated with increased lipid peroxidation[53]. In humans, iron overload has been shown to correlate with hepatic immunohistochemical staining for 7,8-dihydro-8-oxo-2′ deoxyguanosine (8-oxodG), a product of oxidative damage to DNA[54]. In this study, staining for 8-oxodG was significantly reduced with venesection[54]. Patients with NASH have been shown to have elevated levels of serum thioredoxin, a marker of oxidative stress, which declined following venesection[55]. In cultured AML-12 hepatocytes iron generated oxidative stress and led to impaired insulin signalling[56].

Iron also appears to have a direct role in the activation of hepatic macrophages and hepatic stellate cells. In humans with NAFLD, reticulo-endothelial iron has been shown to be associated with apoptosis, indicated by increased serum cytokeratin-18 (CK-18) fragments and increased hepatic TUNEL staining of liver sections[57]. *In vitro*, iron activates inflammatory signalling *via* hepatic macrophages[58]. Recently, dietary iron loading in leptin-receptor deficient mice was found to lead to inflammasome and immune cell activation with hepatocellular ballooning[59]. Furthermore, ferritin treatment of rat hepatic stellate cells has been shown to lead to a pro-inflammatory cascade by nuclear factor kappaB signalling[60].

Iron may also contribute to liver injury in NAFLD by generating endoplasmic reticulum stress[61]. In a mouse model of dietary iron overload and NAFLD, iron induced an unfolded protein response and endoplasmic reticulum stress[61]. Additionally, hepatic iron loading in mice up-regulates cholesterol biosynthesis pathways and this has been proposed as an additional mechanism of iron-induced liver injury in NASH[62]. The proposed mechanisms relating to hepatic iron in NAFLD pathogenesis are summarized in Table 1.

A number of studies have looked at the relationship between hepatic iron concentration (HIC) and liver injury in NAFLD. George *et al*[63] showed that HIC was associated with increased fibrosis in 51 patients with NASH. Three subsequent and similar studies, however, have failed to reproduce these results[44,64,65]. Two much larger studies have looked at the association between hepatic iron (Perls') staining and liver histology in NAFLD with conflicting results. In a study of 587 Italian patients with NAFLD, Valenti *et al*[47] found that hepatocellular rather than reticulo-endothelial iron was associated with 1.7 fold increased risk of significant fibrosis compared to those without iron staining. Reticulo-endothelial iron was found to have a trend towards an association with a lower risk of significant fibrosis. Nelson *et al*[66], however, found seemingly contradictory results, with reticulo-endothelial iron being associated with greater risk of advanced fibrosis, lobular inflammation and hepatocellular ballooning in the US cohort of 849 patients enrolled in the NASH CRN database. In this study, the mean NAFLD Activity Score (NAS)[67] was 4.8 in the reticulo-endothelial iron staining group compared to 4.0 in the hepatocellular iron staining group. The exact reasons for this

Table 1 Proposed mechanisms for the involvement of iron in non-alcoholic fatty liver disease pathogenesis

Site	Mechanism
Hepatic iron	Oxidative Stress[31,53-57]
	Reduced VLDL export[51]
	Macrophage activation[57-59]
	Stellate cell activation[60]
	Endoplasmic reticulum stress[61]
	Increased cholesterol synthesis[62]
Adipose tissue iron	Reduced adiponectin[19,73,74]
	Reduced leptin[76]
	Increased resistin[75]
	Increased lipolysis[77,78]

discrepancy between these two large well-designed studies is unclear, although it is noted that there were some differences between the Italian and US cohorts including the frequency of steatohepatitis and beta-globin mutations[9].

One might argue, however, that the sum of the human data indicates that if hepatic iron does promote liver injury in NAFLD, then its effect is likely to be relatively small.

ADIPOSE TISSUE IRON AND INSULIN RESISTANCE

In recent years, there has been increasing recognition of the role of adipose tissue dysfunction in the development of insulin resistance and NAFLD[28]. Adipose tissue is undoubtedly a significant endocrine organ[68]. It is comprised of adipocytes (fat cells), a mixture of cells categorised as the stromal-vascular fraction including reticuloendothelial cells, predominantly macrophages[68]. Central obesity and the metabolic syndrome are characterised by infiltration of bone marrow-derived macrophages into adipose tissue[21,69]. Macrophage accumulation in adipose tissue is associated with obesity and the development of NAFLD[21,28]. A loss of regulatory T-cells and an increase in CD8+ effector T-cells characterises visceral adipose tissue in insulin resistance[28,70,71]. The net effect of this adipose tissue infiltration with immune cells is a state of systemic low grade inflammation that is mediated by a number of adipose tissue cytokines, termed adipokines[68]. Ectopic fat, such as omental (visceral) and epicardial or mediastinal fat, is dysfunctional tissue that is more likely to undergo inflammation[72]. In the case of visceral fat, this inflammation is particularly problematic with regards to liver physiology due to the direct transfer of adipokines to the liver *via* the portal vein[29].

Adipokines are polypeptides that are expressed significantly in adipose tissue in a regulated manner[29]. Of these, a number of important macrophage derived adipokines appear to play an important role in the development of NAFLD. Both tumour necrosis factor alpha and interleukin-6 have a pro-inflammatory role that may contribute directly to liver pathology in an endocrine fashion, and also *via* paracrine mechanisms that influence the production of other adipokines from adipocytes[28]. Adipokines produced by adipocytes which have been shown to influence NAFLD pathogenesis include adiponectin, leptin, resistin, suppressor of cytokine signalling-3 and secreted frizzled related protein 5[28,29].

Adipose tissue has been proposed as a site at which iron may have a major pathogenic role in NASH[9]. Unfortunately, to our knowledge, direct human data reporting iron concentrations in visceral adipose tissue and its significance in disease are lacking and this area represents both a target for future research and a technical challenge.

Evidence for the role of adipose tissue iron in NAFLD pathogenesis mainly comes indirectly from the association between adipocyte iron and insulin resistance. In 2012, Gabrielsen *et al*[19] demonstrated that adipocyte iron reduced adiponectin gene expression, serum adiponectin levels and glucose tolerance in an adipocyte-specific *Ferroportin* knockout mouse model. Using the novel Ap2-Cre: $Fpn^{fl/fl}$ model they were able to selectively load iron into adipocytes. The model was developed following the observation that adipocytes are high expressers of ferroportin[19]. Using cultured pre-adipocytes (3T3-L1 cells) and chromatin immunoprecipitation analysis, iron was shown to alter acetylation and binding of the forkhead transcription factor Foxo1 to adiponectin gene promoter binding sites. In a human arm of the same study, they were able to demonstrate an inverse correlation between serum ferritin concentration and adiponectin that was independent of inflammation. This observation has subsequently been replicated in 492 Dutch individuals with risk factors for type Ⅱ diabetes[73]. Moreover, in obese patients undergoing bariatric surgery, two gene expression markers of increased adipocyte iron loading: increased hepcidin gene (*HAMP)* mRNA expression and decreased transferrin receptor 1 (*Tfr1*) mRNA expression were associated with reduced quantities of *Adipoq* (adiponectin gene) mRNA[74].

Iron-mediated dysregulation of two other adipokines has been demonstrated in rodent models. Dongiovanni *et al*[75] have shown that dietary iron loading in mice leads to increased expression of resistin *via* SOCS-3 which are mediators of insulin resistance. Recently, data from mouse and 3T3-L1 cell culture models found that iron down-regulates the expression of the appetite-suppressing adipokine, leptin - a hormone strongly implicated in NAFLD pathogenesis[29,76]. Intriguingly, this may help explain the symptom of anorexia in iron deficiency, although the significance of these findings in NAFLD is uncertain.

Adipose tissue iron has been shown to directly enhance lipolysis in isolated rat adipocytes and cultured 3T3-L1 cells[77,78]. As adipose tissue is the predominant source of free fatty acid flux to the liver[24], this is potentially a very important mechanism

of adipose tissue iron action in NAFLD, although these findings are yet to be demonstrated in animal models or humans. Potential mechanisms relating to adipose tissue iron in NAFLD pathogenesis are summarized in Table 1.

In summary, iron has been increasingly recognised as a regulator of adipose tissue function. Evidence supports a role for iron in the regulation of adipose tissue inflammation, adipokine regulation and adipose tissue lipolysis. At present, most of the evidence supports a role for adipose tissue iron in the pathogenesis of insulin resistance and type Ⅱ diabetes, although clearly these mechanisms may be highly relevant in NAFLD.

IRON-RELATED GENETIC POLYMORPHISMS IN NAFLD PATHOGENESIS

The most common inherited disorder affecting the hepcidin-ferroportin axis is type I hereditary hemochromatosis[16,18]. This usually results from homozygous p.C282Y mutation of *HFE* (HFE-hemochromatosis)[79]. The additional insult of NAFLD acts as a co-factor for the development of liver injury in C282Y homozygotes with hereditary hemochromatosis[80]. In non-hemochromatotics, the broader significance of *HFE* gene mutations as co-factors in the pathogenesis of NAFLD has received intense interest in recent years. The two most significant *HFE* mutations in Caucasian populations are the p.C282Y and p.H63D mutations[18].

Heterozygosity for the C282Y mutation is found in approximately 10%-11% of individuals in Caucasian populations[81,82]. C282Y heterozygosity is associated with a mild increase in serum iron markers, but not with overt hemochromatosis[82].

Many studies have looked at the association between *HFE* gene mutations and the incidence of NAFLD, but with conflicting results. These studies may have been limited by inadequate statistical power and heterogeneity of the cohorts. In 2011, Hernaez *et al*[83] published the results of a meta-analysis of 13 case-control studies specifically aimed at determining the association between *HFE* gene mutations and NAFLD. In contrast to a previous meta-analysis by Ellervik *et al*[84], they found no association between the C282Y/C282Y genotype and NAFLD. Similarly the presence of neither the C282Y mutation nor the H63D mutation resulted in an increased risk of NAFLD in Caucasians. In a sub-analysis of three studies of non-Caucasians, an association was found between the presence of the H63D mutation and the presence of NAFLD[83].

A limitation of the meta-analysis, as noted by its authors, is that it was not able to determine whether *HFE* gene mutations might have a disease modifying role in subjects after they have developed NAFLD[83]. This study appears to show that *HFE* gene mutations are generally no more common in subjects with NAFLD than in those without, however, the investigators were unable to determine whether those patients with NAFLD and *HFE* gene mutations are more likely to develop steatohepatitis and progressive liver injury than those without mutations.

The issue concerning the effect of heterozygous mutations in progression to NASH was highlighted by an analysis of *HFE* mutations within the NASH CRN cohort[85]. This is a well-defined cohort of patients with biopsy proven NAFLD. Subjects with the H63D mutation had higher steatosis grades and NAS than their wild-type controls. However, those NAFLD patients with C282Y mutations had lower rates of hepatocyte ballooning and steatohepatitis.

Our group has previously shown that mice with homozygous knockout of the *Hfe* gene develop severe steatosis, steatohepatitis and early fibrosis when fed a high fat diet, whereas wild-type mice develop mild steatosis and no steatohepatitis or fibrosis when fed the same diet[86]. *Hfe* null mice had only modest increases in HIC, and it was proposed that the increased histologic injury seen in these animals may have been due to the lack of HFE protein rather than iron overload *per se*. *Hfe* null mice demonstrated dysregulated hepatic lipid metabolism with increased transcription of genes associated with *de novo* lipogenesis and reduced transcription of those associated with fatty acid oxidation[86].

A number of other non-*HFE* iron-loading polymorphisms have been proposed as modulators of NAFLD pathogenesis[9,87]. Of these, the A736V polymorphism of the *Trans-membrane protease serine-6 (TMPRSS6)* gene has been studied in patients with NAFLD. The *TMPRSS6* gene encodes for matriptase-2, an enzyme responsible for hemojuvelin cleavage that inhibits the bone morphogenetic protein-6 pathway, thus reducing hepcidin expression and increasing duodenal iron absorption[18,87]. Of 216 Italian patients with NAFLD, 38% had the AA genotype, 47% AV and, 15% VV[87]. The VV genotype is associated with increased hepcidin expression and reduced iron loading and in this study was associated with a trend (P = 0.05) towards a reduction in hepatocyte ballooning[87].

In summary, human and animal model data support a role for a co-toxic liver injury in the setting of hereditary hemochromatosis and NAFLD. Other more mild iron loading phenotypes such as heterozygous *HFE* gene mutations and polymorphisms of *TMPRSS6* may have disease modifying roles in NAFLD, although their effect is likely to be small.

CLINICAL TRIALS OF IRON REDUCTION THERAPY

Although associations of modest iron overload with NAFLD and diabetes appear reasonably well established, causality is difficult to determine using these studies alone. The most useful information with which

to more directly assess causality comes from human studies that have assessed the response to iron removal by venesection.

Venesection has been shown to improve glucose tolerance in healthy individuals and improve insulin sensitivity in type Ⅱ diabetics with a high serum ferritin concentration[88,89]. Moreover, in patients with the metabolic syndrome, venesection has been shown to improve metabolic syndrome parameters, including reduced blood pressure, blood glucose, glycosylated hemoglobin (HbA1C) and low-density lipoprotein/high density lipoprotein (LDL/HDL) ratio[90]. In patients with NAFLD and carbohydrate intolerance, venesection to near iron deficiency (decrease in serum ferritin from 299 ± 41 μg/L to 15 ± 1 μg/L) not only improved insulin sensitivity, as measured by fasting glucose, insulin and homeostatic model assessment-insulin resistance (HOMA-IR) score, but also improved serum alanine aminotransferase levels from 61 ± 5 U/L to 32 ± 2 U/L[91].

Two randomised controlled trials investigating venesection efficacy in NAFLD have recently been published. In a study of 38 Italian patients with NAFLD and hyperferritinemia, participants were randomised to venesection versus no venesection with liver biopsy before and after treatment[92]. Of the 38 enrolled participants, 21 underwent liver biopsy at the end of treatment. Despite the small numbers, histological improvement, defined by an improvement in NAS, was seen in 8 of 12 participants in the venesection group compared to 2 of 9 participants in the control group ($P = 0.04$)[92].

The largest randomised study of venesection in NAFLD to date involved 74 Australian participants with NAFLD[93]. These included patients with sonographically detected NAFLD and a wide range of serum ferritin concentration, including many within the normal range. Non-invasive assessment was performed to assess response to randomised therapy of either venesection with lifestyle advice versus lifestyle advice alone. There was no observed effect of venesection on hepatic steatosis determined by magnetic resonance imaging, serum ALT or CK-18 fragments. Somewhat surprisingly, there was also no effect on static and dynamic measures of glucose homeostasis including the HOMA-IR score and insulin sensitivity index[93].

Overall, although there are promising results from small studies, venesection cannot currently be recommended as a suitable therapy for the majority of patients with NAFLD[94]. However, whether there are sub-groups of non-hemochromatotic NAFLD patients with increased iron that would benefit from venesection, remains to be determined by further studies.

IRON METABOLISM IN NAFLD

So far, we have discussed the effect of iron on the pathogenesis of NAFLD and insulin resistance. It is also necessary to consider to what extent NAFLD and associated conditions, such as insulin resistance and obesity, might themselves mediate iron metabolism.

Serum hepcidin levels are typically elevated in individuals with NASH[95]. As this in itself fails to explain iron loading in NASH, one might consider that dysregulated iron metabolism occurs in NASH independently of hepcidin. In this regard, *Transferrin receptor-1* (*Tfr1*) has been shown to be upregulated as a consequence of a high fat diet in mice which may lead to hepatocellular uptake in NAFLD despite already increased hepatocellular iron[96]. Also, divalent metal transporter 1, which is responsible for import of iron from the duodenal lumen into enterocytes is up-regulated in patients with NASH, despite increased serum hepcidin[97]. Another intriguing finding is that increased red cell fragility in response to a high fat diet in rabbits leads to increased erythrophagocytosis[98]. This may explain increases in hepatic reticuloendothelial iron that have been observed in some NASH cohorts[66].

It seems likely that elevated hepcidin in NASH is either a reflection of hepatocellular inflammation or simply that increased iron, which induces hepcidin, pre-dates the development of NASH. Indeed, hepcidin expression appears to be directly enhanced by insulin and down-regulated in the setting in insulin resistance, thus indicting a possible mechanism for iron loading as an early event in the pathogenesis of NAFLD and type Ⅱ diabetes[99]. Furthermore, it has been observed that hepcidin is expressed in white adipose tissue and is increased in obesity[100]. Although the contribution of adipose tissue-derived hepcidin to the serum hepcidin pool is uncertain, this is another potential factor that may explain increased serum hepcidin in NASH. Further complexity in these relationships arises when one considers that iron deficiency has been shown to be associated with obesity and in women with obesity and NAFLD[101,102]. Together, these findings suggest that the interaction between iron and lipid metabolism is multi-faceted. It seems that "just enough" but "not too much" iron may be critical in preventing dysfunctional lipid metabolism.

If one accepts a causal role for iron in NASH pathogenesis, then variations in dietary iron may explain much of the spectrum of iron loading in NASH. Although there is no specific evidence relating iron intake to NASH pathogenesis in humans, increased dietary iron, particularly from red meat, seems to predispose individuals to the development of insulin resistance and type Ⅱ diabetes[103-105].

CONCLUSION

In summary, there is considerable evidence that links increased iron stores with insulin resistance and NAFLD. This includes a number of studies that have identified serum ferritin concentration as a predictor of liver injury. Hepatic iron itself is attractive culprit for liver injury, although the cellular location of iron within the liver may vary between genetically distinct

populations. Increasingly, adipose tissue iron has been linked with adipose tissue dysfunction, including the dysregulation of adipokines, enhanced adipose tissue lipolysis and adipose tissue inflammation. These are plausible candidate mechanisms that may link adipose tissue iron to liver injury. However, assessment of adipose tissue iron concentrations in individuals with well characterised NAFLD remains a goal for future studies.

Iron-related genetic polymorphisms, such as those of the *HFE* gene, may contribute to NAFLD pathogenesis, although it would appear that, other than for individuals with hereditary hemochromatosis, the effect of these polymorphisms, is likely to be small. The complexity of these relationships between iron and NAFLD is further increased when one considers the possibility that NAFLD itself is likely to have a number of effects on iron metabolism.

Finally, venesection studies have offered a unique opportunity with which to assess causality of iron loading in the pathogenesis of NAFLD. The available data suggest that venesection is unsuitable as a general treatment for all patients with NAFLD. Therefore, the key for future human studies will be to determine whether a subset of patients with NAFLD can be identified that might still benefit from therapeutic manipulation of iron homeostasis.

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