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CELL DEATH REGULATION IN ASH AND ALCOHOLIC PANCREATITIS

Iron and steatohepatitis

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As the main iron storage site in the body and the main source of the iron-regulatory hormone, hepcidin, the liver plays a pivotal role in iron homeostasis. A variable degree of hepatic iron accumulation has long been recognized in a number of chronic liver diseases. Both alcoholic and non-alcoholic steatohepatitis display increased iron deposits in the liver, with an hepatocellular, mesenchymal, or mixed pattern, and recent reports have documented a concomitant aberrant hepcidin expression that could be linked to different coincidental pathogenic events (e.g. the etiological agent itself, necroinflammation, metabolic derangements, genetic predisposition). The present study reviews the pathogenic mechanisms of iron accumulation in steatohepatitis during alcoholic and non-alcoholic liver disease and the role of excess iron in chronic disease progression.

Key words

alcoholic liver disease, fatty liver disease, hepcidin, iron, steatohepatitis.

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Introduction

In spite of the diverse etiology, alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) share many epidemiological, clinical, and pathogenetic features. They both represent major causes of chronic liver disease worldwide,^{1,2} and both encompass a spectrum of disorders ranging from simple fatty liver (steatosis) to hepatocyte injury and inflammation (alcoholic steatohepatitis [ASH] and non-alcoholic steatohepatitis [NASH]), cirrhosis, and superimposed hepatocellular carcinoma (HCC). However, only a minority of individuals with steatosis of alcoholic or non-alcoholic origin progress to more severe forms of liver disease, indicating that factors other than the primary noxae (alcohol in ALD and aberrant hepatic free fatty acid [FA] flux in NAFLD) are involved in this transition.

Hepatocellular and/or mesenchymal iron deposition, usually slight or mild, might be found in chronic non-cirrhotic liver disease, regardless of its cause.³ Various non-specific factors (mainly inflammation and cell necrosis), together with polymorphisms in iron-related genes or pathogenic interactions between iron itself and the etiological agent (hepatotropic virus, alcohol, increased supply of free FA to the liver or insulin resistance), might be responsible. The clinical relevance of iron excess, in terms of fibrosis development and cancer risk, is still debated.

Regarding ALD and NAFLD, increasing data from experimental and clinical studies indicate that iron might sustain disease

activity and/or contribute to its progression. Furthermore, the coexistence of both ALD and NAFLD risk factors, and/or the presence of comorbidities (e.g. hepatitis C virus [HCV] and hepatitis B virus chronic viral hepatitis, variants of genes encoding proteins involved in iron homeostasis) might have additive or synergistic effects on both tissue injury and iron loading.⁴

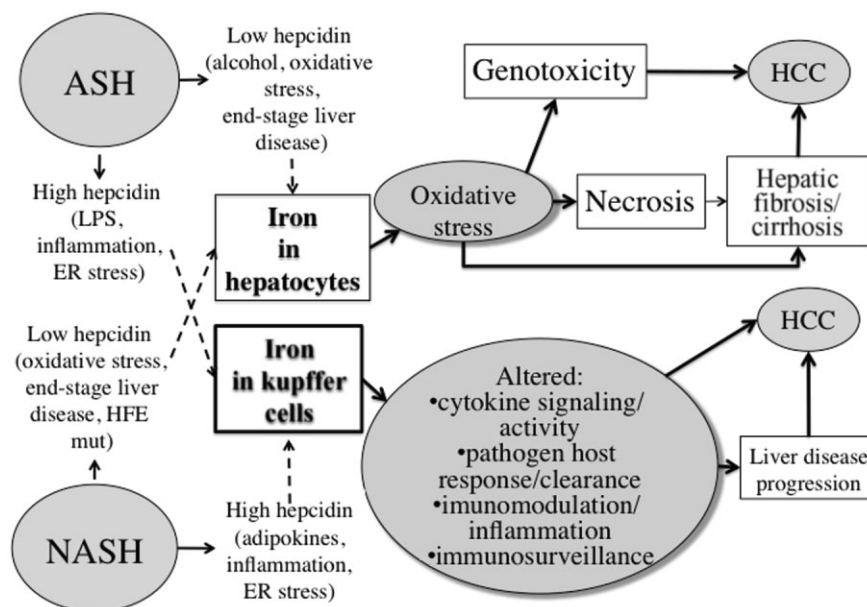
Although the pathogenic mechanisms underlying ALD and NAFLD are increasingly elucidated, therapeutic strategies are limited. Therefore, a further dissection of the role of iron in the pathogenesis of ALD and NAFLD could generate alternative and/or complementary therapeutic approaches for these common liver diseases.⁵

Iron homeostasis

Iron is a double-edged element. It is essential for growth and survival, due to its crucial role in many cellular functions, such as hemoglobin and DNA synthesis, mitochondrial respiration, oxidative phosphorylation, and other enzymatic functions. However, because of its ability to participate in the Fenton and Haber–Weiss chemistry, excess redox-active iron might lead to reactive oxygen species production, with consequent damage to membranes, proteins, and DNA.⁶

Given the essential need for iron, and the fact that in mammals there is no active mechanism for iron excretion, a tight regulation of iron absorption and recycling is required. The central regulator of iron homeostasis is hepcidin, a small peptide hormone

Figure 1 Molecular pathways leading to hepatic iron overload through hepcidin modulation in alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH). Putative pathways that lead to hepcidin stimulation or inhibition in ASH and NASH are shown, and the opposite effect and diverse iron deposition pattern that might arise during individual disease processes are emphasized. In addition, the role of iron excess into hepatocytes or Kupffer cells and relevant molecular pathways leading to disease progression and hepatocellular carcinoma are shown. ER, endoplasmic reticulum; HCC, hepatocellular carcinoma; HFE, human hemochromatosis gene *HFE*; LPS, lipopolysaccharide.



synthesized mainly by the liver and secreted in the bloodstream.⁷ It acts by binding ferroportin, the sole iron exporter present at the surface of duodenal enterocytes, macrophages, placental cells, and hepatocytes, leading to its degradation, with consequent reduced iron absorption from the gut, and increased iron retention in macrophages. Several stimuli have been shown to influence hepcidin expression. Both circulatory and tissue iron upregulate hepcidin transcription,⁸ via the Bone Morphogenetic Protein-Small Mothers Against Decapentaplegic (BMP-SMAD) signaling cascade, as a negative feedback mechanism that protects the body from excessive iron accumulation. Inflammation also stimulates hepcidin transcription, mainly via the Signal Transducer and Activator of Transcription 3 (STAT3) signaling pathway, as a protective innate immune defense to limit iron availability for invading pathogens or tumoral cells. However, if upregulation of hepcidin persists, iron-restricted erythropoiesis and anemia of chronic disease will follow. Recently, endoplasmic reticulum (ER) stress due to a variety of pathological signals has been shown to trigger hepcidin expression,⁹ indicating that not only extracellular, but also intracellular signals, might influence hepcidin levels and systemic iron homeostasis. Inhibitory stimuli for hepcidin also exist, such as iron depletion, hypoxia, and erythropoietic activity.¹⁰

In addition, it must be considered that in end-stage liver disease, regardless of its cause, a decreased hepcidin synthesis due to the reduced hepatocytic mass might also lead to excess iron deposition.

Iron in ALD and NAFLD

Alcoholic liver disease

Varying degrees of hepatic iron burden have been reported in patients with ALD. In alcohol users, serum iron markers have been shown to be raised even at early age.¹¹ With increasing and persistent alcohol abuse, iron accumulates in the liver, first in peri-

portal hepatocytes, and later in Kupffer cells (KC).¹² Whole-body retention studies have demonstrated that alcoholics have a twofold increase in intestinal iron absorption.¹³ The degree of iron overload in ALD is usually relatively mild, but sometimes, particularly in the presence of cirrhosis, it is sufficiently severe to be mistakenly attributed to hereditary hemochromatosis (HH). There might be different reasons for iron accumulation in ALD and ASH. Both alcohol-induced ER stress¹⁴ and inflammation might lead to hepcidin upregulation and iron retention in liver macrophages (Fig. 1). However, alcohol seems also to suppress hepcidin expression in the liver after both acute¹⁵ and chronic^{16,17} exposure, *in vivo*^{15,17} and *in vitro*.¹⁵ In fact, ethanol downregulates the mRNA level¹⁶ and DNA binding activity¹⁵ of CCAAT/Enhancer Binding Protein-alpha (C/EBP α), a key transcription factor that regulates hepcidin expression, and these effects are abolished by inhibitors of alcohol-metabolizing enzymes and by antioxidants.¹⁵ This will eventually lead to increased iron absorption and consequent hepatocellular iron accumulation (Fig. 1). How do we reconcile these apparently contradictory data? Most likely, both mechanisms are operative in ALD, and it is the balance between these two opposed hepcidin-regulating stimuli and the modifying activity of cofactors (e.g. comorbidities, genetics, liver disease stage, and necroinflammatory activity) that will eventually determine the extent and distribution of hepatic iron deposits during the course of chronic ALD. Yet alcohol-induced hepcidin downregulation seems to play a dominant role in iron loading, particularly in hepatocytes, where iron can exacerbate cellular injury in a noxious vicious circle. In fact, patients with both acute and chronic ALD show decreased serum hepcidin levels, which progresses along with the progression of the underlying liver disease.¹⁸ When inflammatory and/or ER stress responses predominate, the superimposed upregulation of hepcidin might contribute to iron retention in KC, which will then result in perturbation of their function and release of pro-inflammatory cytokines^{19,20} (Fig. 1) (see also below). Yet the inhibitory effect of alcohol on hepcidin expression seems to be hierarchically dominant. In fact, experimental ethanol administra-

tion *in vitro*¹⁶ and *in vivo*¹⁷ blunts interleukin-6-¹⁶ or iron-mediated¹⁷ hepcidin upregulation in the liver.

As to the effect of excess iron on ALD progression, the presence of iron has been reported to be predictive of death²¹ and HCC²² development in alcoholic cirrhosis. Interestingly, in patients with HH, alcohol intake is a major determinant for phenotype severity and liver damage.^{23,24} Other sets of data show a correlation between body iron burden and the extent of fibrosis in ALD patients, as reviewed by Tavill and Qadri.²⁵ Therefore, clinical evidence exists that iron and alcohol synergistically contribute to exacerbate both hepatic iron burden and liver injury.

What about the underlying pathogenic mechanisms? Alcohol promotes the main pathological features of ASH (i.e. hepatocyte injury and death, inflammation, and fibrosis) by different mechanisms, including alcohol/driven oxidative stress, lipopolysaccharide toxicity, due to the increased gut permeability,¹ and ER stress.¹⁴ However, iron itself generates reactive oxygen species, inducing lipid peroxidation and oxidation of proteins and nucleic acids, leading to cell death.⁶ On the basis of the ability of both alcohol and iron to cause oxidative stress, stellate cell activation, and hepatic fibrogenesis, a synergistic effect on liver disease progression can be postulated (Fig. 1).²⁶ Another pathogenically relevant factor is in which hepatic cell type iron accumulates. While the pathogenic role of iron inside the hepatocytes on favoring cell damage and necrosis is obvious, excess iron accumulation in KC has recently emerged as a central event in hepatic toxicity in ALD (Fig. 1). In fact, the increased iron content of KC has been shown to induce the expression and release of the pro-inflammatory cytokine tumor necrosis factor- α and to activate the transcription factor nuclear factor- κ B in experimental animal models of ALD.^{19,20} These effects are abolished by treatment of KC *in vitro* and *ex vivo* with iron chelators.^{19,27}

NAFLD

Well-established risk factors for NAFLD are overweight and insulin resistance, the pathogenic cornerstones of the metabolic syndrome (MS).² The prevalence of NAFLD increases with the degree of obesity,²⁸ and is very high in type 2 diabetics.²⁹ In fact, NAFLD might be considered the hepatic manifestation of the MS.² Obesity plays a crucial role in the pathogenesis of NAFLD through the increased flux of free FA in the liver (from diet, adipose tissue, and de novo lipogenesis), which promotes hepatic steatosis.³⁰ The latter results from an imbalance between free FA acquisition and removal by oxidation or export as triglyceride into very low-density lipoproteins.^{28,30} However, while approximately 80% of obese individuals and 70% of those with type 2 diabetes present with hepatic steatosis, only a minority of them progress to NASH.³¹ Most likely, environmental and genetic factors influence the development and progression of NAFLD.^{2,32} Direct hepatocyte lipotoxicity, oxidative injury due to free radicals produced during free FA oxidation, cytokine-mediated cellular stress, and ER stress are the main mechanism leading to cell damage, stellate cells activation, collagen deposition, and progression to fibrosis in NAFLD.^{2,28}

The presence of iron further complicates the complex pathogenic picture of NAFLD. Variable degrees of hepatic iron accumulation, usually mild or moderate, are common in NAFLD.³ The term "dysmetabolic iron overload syndrome" (DIOS),³³ more

recently also referred to as "insulin resistance-associated hepatic iron overload",³⁴ was first introduced to define cases of unexplained hepatic iron excess characterized by high serum ferritin levels, normal serum iron, and associated metabolic abnormalities. The histological pattern of DIOS was described as mixed parenchymal and mesenchymal in 85% of patients.³ In two large, recent series of 480 Italian³⁵ and 849 American³⁶ NAFLD patients, hepatic iron deposition was found in approximately 50% and 35% of cases, respectively, with an hepatocytic, mesenchymal, or mixed pattern. Serum ferritin levels are commonly raised in NAFLD, due to hepatic iron overload, hepatic or systemic inflammation, oxidative stress, and likely insulin resistance.³⁷ As DIOS and hyperferritinemia associated with the MS or NAFLD share many clinical and epidemiological features, they could be considered two different faces of the same health problem.³⁸

Although hepatic iron excess is common in NAFLD patients, its cause and clinical significance are still being debated. Early *in vitro* studies have suggested that insulin, due to its ability to stimulate cellular uptake of nutrients, might also cause increased iron uptake.³⁷ However, more recent reports have suggested that hepcidin might be directly responsible for iron disturbances during NAFLD. Bekry *et al.*³⁹ have shown the ectopic expression of hepcidin in white adipose tissue in obese individuals; hepcidin mRNA levels were increased and correlated with the inflammatory state, independently of steatosis and NASH. Moreover, leptin, which is commonly increased in obesity, was demonstrated to enhance hepcidin mRNA expression *in vitro* through the Janus Kinase 2 (JAK2)/STAT3 signaling pathway,⁴⁰ and a significant positive correlation between serum leptin and hepcidin has been reported in obese children.⁴¹ Additional factors that might contribute to hepatic iron excess in NAFLD are necrosis,⁴² which might lead to iron leaking from dying hepatocytes and subsequent phagocytosis by liver macrophages, or the induction of hepcidin by inflammatory cytokines,⁷ causing iron accumulation in KC (Fig. 1). In agreement with this hypothesis, a recent study on MS patients, with and without NASH, reported increased hepcidin levels compared to matched controls.⁴³ Interestingly, hepcidin was positively correlated with hepatic lobular inflammation in NASH, non-NASH patients, and in the whole MS group, while in the NASH group, it was also positively correlated with the NAFLD Activity Score (NAS) score (which accounts for steatosis, inflammation, and hepatocyte ballooning). Interestingly, none of the MS patients had increased iron load at the liver biopsy, indicating that high hepcidin was not due to tissue iron excess, but most likely to necroinflammation per se. An additional trigger for hepcidin activation in these patients is likely ER stress (a known activator of hepcidin expression), which has been associated with NAFLD and its main risk factors, namely obesity and type 2 diabetes²⁸ (Fig. 1). Conversely, as oxidative stress has been shown to down-regulate hepcidin expression in ALD¹⁵ or HCV-related⁴⁴ liver disease, the same phenomenon might well occur in NASH, where oxidative stress is well documented. Controversy surrounds the relationship between the human hemochromatosis gene *HFE* polymorphisms with both liver iron accumulation and the risk of fibrosis in NAFLD.³⁸ The discrepancy among different studies might be due to different ethnicities, patient inclusion criteria, and referral bias.

In summary, in analogy with ALD, during the dynamic evolution of NAFLD, diverse signals might arise that modulate hepcidin

expression in the opposite direction, depending on patient genetic determinants, comorbidities, timing and entity of the primary liver injury, and liver disease stage or inflammatory activity. So far, available data seem to indicate that in NAFLD hepcidin induction by systemic inflammation (related to obesity), intrahepatic inflammation, and/or intrahepatic cytokine-related stress and ER stress (secondary to excessive free FA influx) has a central role in iron retention in the liver, and likely in white adipose tissue (Fig. 1).

As to the effect of hepatic iron accumulation on disease progression, a recent study, while failing to detect a correlation between the presence of *HFE* mutations and the severity of hepatic fibrosis, showed that hepatocellular iron accumulation was associated with more severe liver fibrosis.³⁵ In contrast, another study reported that reticuloendothelial iron accumulation is correlated with the histological features of NASH (inflammation, hepatocyte ballooning, and fibrosis) and with advanced fibrosis.³⁶ As it is known that iron load in KC could impair their function^{19,20,27} (Fig. 1), this event might also contribute to disease progression.⁴ Interestingly, heterozygosity for the C282Y mutation of *HFE* gene has been associated with lower insulin release and the development of NAFLD,⁴⁵ suggesting that *HFE* mutations and/or the consequent, even mild, iron overload might act as facilitator of the disease. Finally, a significant relationship between hepatic iron content and HCC progression has been reported, suggesting a carcinogenic or cocarcinogenic role for iron in NASH.⁴²

What about the pathogenic mechanisms underlying the effect of iron load on the progression of NAFLD? Both free FA^{2,28} and iron⁶ excess in the liver have been demonstrated to induce oxidative injury, indicating that when hitting the liver together, they might exert a synergistic hepatotoxic effect. In the same vein, accumulating evidence suggests that hepatic mitochondrial dysfunction might contribute to NAFLD development and severity,⁴⁶ as occurs in experimental iron overload.⁶ A number of studies suggest that hyperglycemia, hyperinsulinemia, and adipokines secreted by the adipose tissue might have a direct fibrogenic role, independently of hepatocyte injury and stellate cell activation.³² Hepatic iron excess might contribute to the impairment of glucose homeostasis by influencing insulin signaling. In fact, iron removal by phlebotomy or iron chelators has been shown to improve insulin sensitivity and/or metabolic control in healthy individuals, type 2 diabetics, and NAFLD patients,^{37,47,48} reinforcing the idea of a possible role of iron in favoring insulin resistance, which is a main factor involved in NAFLD progression and severity.

Ferritin levels also have been shown to correlate with the severity of NAFLD, both in the absence⁴⁹ or presence⁵⁰ of hepatic iron load, and independently of type 2 diabetes, body mass index, age, sex, and hepatic iron deposition.⁵⁰ This suggests that ferritin per se might exert a role on disease progression in NASH through still undefined mechanisms.

Conclusions and perspectives

Both ALD and NAFLD are commonly associated with varying degrees and diverse patterns of hepatic iron deposition, whose cause and significance are being progressively elucidated.

In both conditions, a direct modulation of hepcidin expression by the causative agent (alcohol or fat) might play a role in the modification of systemic and/or intrahepatic iron traffic, leading to tissue iron load. While during the long-lasting course of ALD and

NAFLD opposite regulatory forces on hepcidin expression might cause diverse patterns of hepatic iron accumulation, alcohol-induced hepcidin downregulation seems to play a dominant role in hepatocytic iron loading in ALD, while necroinflammation favors preferential iron deposits in KC in NAFLD. Once liver iron burden is established, it might act at both the hepatocellular and mesenchymal levels to maintain and enhance liver injury and disease progression, regardless of its cause (Fig. 1).

Further studies are needed to elucidate the molecular events that link iron excess and the clinical features of ALD and NAFLD, or the consequence of different patterns of tissue iron accumulation on disease severity. Nevertheless, in view of the available experimental and clinical data, it is time to consider the use of iron-removal strategies as adjuvant therapy in patients with ASH and NASH. Ideally, iron removal should be achieved by using permeable, hepatotropic "weak" iron chelators able to target the redox-active intracellular iron pool in hepatocytes and KC without affecting the circulatory iron pool or systemic iron stores (the drawback of phlebotomy).

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