Stress Responses and Cellular Crosstalk in the Pathogenesis of Liver Disease Theme Issue

Liver Iron Loading in Alcohol-Associated Liver Disease

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Short Running Title: Liver iron in alcoholics

Number of Figures: 3  Number of Tables: 1

Funding: No funding was accessed/obtained for writing this review.

Conflicts of interest: The authors declare no conflict of interest.

Footnote: This article is part of a review series focused on the role of cellular stress in driving molecular crosstalk between hepatic cells that may contribute to the development, progression, or pathogenesis of liver diseases.
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Abstract

Alcohol-associated liver disease (ALD) is a common chronic liver disease with increasing incidence worldwide. Alcoholic liver steatosis/steatohepatitis can progress to liver fibrosis/cirrhosis, which can cause predisposition to hepatocellular carcinoma. Also, ALD diagnosis and management are confounded by several challenges. Iron loading is a feature of ALD. It can exacerbate alcohol-induced liver injury and promote ALD pathological progression. Knowledge of the mechanisms that mediate liver iron loading can help identify cellular/molecular targets and thereby aid in designing adjunct diagnostic, prognostic, and therapeutic approaches for ALD. Therefore, here, we reviewed the cellular mechanisms underlying alcohol-induced liver iron loading and discussed how excess iron in ALD patients can promote liver fibrosis and aggravate disease pathology. Essentially, alcohol-induced increase in hepatic transferrin receptor-1 (TfR1) expression and upregulation of high iron (HFE) protein in Kupffer cells (proposed) facilitate iron deposition and retention in the liver. Iron is loaded in both parenchymal and non-parenchymal liver cells. Iron-loaded liver can promote ferroptosis and thereby contribute to ALD pathology. Iron and alcohol can independently elevate oxidative stress. Therefore, a combination of excess iron and alcohol amplifies oxidative stress and accelerates liver injury. Also, via secretion of proinflammatory and profibrotic factors, excess-iron-stimulated hepatocytes directly or indirectly (through Kupffer cell activation) activate the hepatic stellate cells. Persistently activated hepatic stellate cells promote liver fibrosis, and thereby facilitate ALD progression.

Keywords: iron, liver, alcohol, transferrin receptor, TfR1
Introduction

Alcohol consumption is on the rise worldwide, and so is the incidence of alcohol-associated liver disease (ALD) \(^1\). With no standard laboratory diagnostic test to confirm ALD etiology, asymptomatic early stages, and high costs of disease management, ALD continues to pose challenges on all fronts. Abstinence is the only curative option \(^2\).

Iron loading is one of the characteristic features of ALD. Even mild to moderate alcohol consumption increases liver iron content \(^3\). This can aggravate alcohol-induced liver injury via various mechanisms and promote the pathological progression of the disease. Knowledge of these mechanisms that mediate liver iron increment in ALD and its consequences at cellular level may help identify cellular/molecular targets and thereby aid in designing better diagnostic, prognostic, and therapeutic approaches for ALD. Such investigations have proved useful in the past. For example, a study showed that liver iron content exhibited a negative correlation with the survival of ALD patients, and was thus predictive of mortality in alcoholic cirrhosis patients \(^4\).

Therefore, here, we reviewed the cellular mechanisms underlying alcohol-induced liver iron loading and discussed how excess iron in ALD patients can promote liver fibrosis and aggravate disease pathology.

High liver iron content in ALD

ALD patients/chronic alcohol consumers often show high hepatic iron levels \(^5-^9\). About 50% of ALD patients tend to show liver iron excess \(^10\). A study showed that the mean liver iron content (measured as µg/100 mg dry weight) in alcoholics was
156.4 ± 7.8, which was significantly higher than the controls (53±7)⁹. Alcoholic cirrhotic patients frequently show high liver iron content, which is associated with increased mortality⁴. Increment in liver iron occurs not only because of alcohol consumption but also due to additional factors and mechanisms involving the “second hit”, such as a high fat diet in combination with alcohol consumption. Regardless, high liver iron content can contribute to permanent liver injury and hepatocellular carcinoma¹¹. Indeed, with increased serum iron in alcohol consumers, there could be iron deposition in extra-hepatic organs such as the pancreas and heart, as seen in other iron loaded conditions¹². For example, an autopsy of a 54 year-old female with ALD showed iron overload in the liver as well as the pancreas, heart and stomach⁵.

**Pattern of iron deposition in hepatic cells in ALD**

There are two different proposals with regards to iron deposition in the different cell types of the liver. According to one proposal, in mild ALD, iron is preferably deposited in the hepatocytes (parenchymal cells of the liver). But as the condition progresses to severe ALD, iron loading is more obvious in the Kupffer cells (non-parenchymal cells in liver) compared to hepatocytes⁶. Pietrangelo A. has also supported the idea of non-parenchymal iron loading in the advanced stages of alcoholic liver fibrogenesis¹³. In contrast, another proposal suggests that in secondary iron overload syndromes such as ALD, iron accumulates in the reticuloendothelial system, which includes the Kupffer cells of the liver, and accumulates in the hepatocytes after the reticuloendothelial cells are saturated with iron¹⁴. Regardless, in ALD, iron deposition is observed in both hepatocytes and Kupffer cells, i.e., in parenchymal and non-parenchymal cells of the liver.
**Cellular mechanisms that increase liver iron in ALD**

Hepcidin, the liver secreted iron hormone is the regulator of systemic iron homeostasis\(^ {15}\). Alcohol-induced suppression of hepcidin expression is the main cause of systemic iron loading in alcohol consumers. Serum iron loading is further supported by alcohol-induced elevations in the expressions of iron transporters in the duodenum; duodenal divalent metal transporter 1 (DMT1) and ferroportin. These events enhance intestinal iron absorption i.e., increase iron entry into the circulation\(^ {16-19}\) and this forms the basis for liver iron loading in alcohol consumers.

There are several mechanisms/cellular events that facilitate liver iron loading in ALD. These have been depicted in Fig.1.

**Increased hepatic transferrin receptor-1 (TfR1)**

One mechanism involves increment in hepatic TfR1. Cellular TfR1 is the receptor for circulating iron-bound transferrin. It facilitates the entry of transferrin-bound iron into various cells. In a study, majority of habitual alcohol consumers/ALD patients showed increased expression of hepatic TfR1 (in hepatocytes), unlike healthy liver tissues\(^ {20}\). Alcohol-induced oxidative stress increases the activity of iron regulatory proteins (IRPs) and this is thought to be partly responsible for this increase in TfR1 expression\(^ {6,21}\). Kupffer cells of alcohol-fed rodents have also shown about 6-fold and 9-fold increases in TfR1 gene and protein expressions, respectively\(^ {22}\). This collectively indicates that alcohol-induced elevation in TfR1 expression promotes iron uptake in both parenchymal and non-parenchymal cells of the liver (Fig.1). Thus, TfR1 upregulation may partly explain the liver iron loading in ALD patients\(^ {20-22}\).

Interestingly, alcohol alone treatment to VL-17A cells neither altered the expressions
of TfR1 and IRP2 nor IRP1 RNA binding activity. However, a combination of alcohol and iron treatment to rat primary hepatocytes increased the expression of TfR1 (compared to iron alone treatment) partly through the increased activity of IRPs. Based on this, it can be extrapolated that the increased TfR1 expression observed in alcohol consumers is a result of combined effect of alcohol and iron.

Normally, the intracellularly operating IRP-Iron response element (IRE) system regulates cellular iron levels by acting on the transcripts for various iron-related genes including TfR1. Under cellular iron excess, the IRP-IRE system functions to reduce cellular TfR1 to reduce transferrin-bound iron entry into the cells. Alcohol-induced increment in hepatic TfR1 expression in the presence of hepatic iron loading suggests that alcohol can disturb the aforementioned TfR1-regulatory mechanism and cause or contribute to increased hepatocellular iron uptake.

Macrophages also show iron loading. These cells predominantly acquire iron due to phagocytosis of senescent RBCs. However, these cells do express DMT1, TfR1, hemoglobin scavenger receptor (CD163) and natural resistance associated macrophage protein1 (Nramp1). These proteins are involved in iron uptake and transport, and may contribute to the increment in liver iron levels.

**Putative role of high iron (HFE) protein**

The HFE protein may contribute to liver iron accumulation. HFE is a cell surface protein that appears to exhibit multiple functions. First, the HFE can bind to TfR2 to form an iron-sensing complex on the cell membrane. This complex regulates/induces hepcidin expression. Hereby, HFE functions as a regulator of hepcidin transcription. Second, HFE can affect the binding of iron-bound transferrin to TfR1.
When HFE binds to TfR1, the affinity of TfR1 to bind to iron-bound transferrin is reduced, and this reduces cellular iron uptake. Hereby, HFE functions as a regulator of cellular iron uptake. Third, HFE has shown to inhibit cellular iron efflux. Stable transfection-expression of HFE in human colonic carcinoma cell line increased cellular ferritin expression, indicating intracellular iron accumulation/elevation. However, this was not due to transferrin-dependent iron uptake. This suggested that the HFE expression prevented cellular iron efflux and facilitated intracellular iron retention, which resulted in the aforementioned intracellular ferritin elevation. Ferroportin is the sole known iron transporter (exporter) on the surfaces of various cell types including the hepatocytes and Kupffer cells. It has been postulated that HFE can interact with ferroportin and inhibit cellular iron release from macrophages (Fig.1). Alcohol activates HFE gene transcription in the Kupffer cells. Alcohol-exposed rat Kupffer cells showed increased Hfe mRNA levels. Based on the postulated function of HFE, this may reduce/inhibit cellular iron export and facilitate iron retention within the Kupffer cells. This may be an additional mechanism causing liver iron loading under the influence of alcohol (Fig.1). Interestingly, duodenal HFE mRNA expression in ALD patients with iron overload (defined as increased ferritin or transferrin saturation) was significantly higher than controls, unlike the expression levels in ALD patients without iron overload and ALD patients with anemia where levels were similar to controls. This suggested that increment in duodenal HFE expression was linked with systemic iron loading, and this could subsequently lead to iron deposition in the liver and other organs. Based on these data, it appears that HFE function may be cell-specific; mediating intracellular iron retention in one cell-
type, as postulated in case of Kupffer cells, while allowing systemic iron loading through duodenal cells. This hypothesis on the cell-specific nature of HFE needs to be confirmed.

**Enigma around ethanol-induced non-transferrin bound iron (NTBI) uptake**

Depending on the form of iron (transferrin-bound iron or non-transferrin-bound iron) cellular iron uptake can occur via two main mechanisms: transferrin-bound iron (TBI) uptake and non-transferrin-bound iron (NTBI) uptake. NTBI uptake occurs independent of TfR1 and contributes to cell toxicity when in excess. It involves NTBI transporters such as DMT1, zinc transporter ZIP14 (on hepatocytes), and ZIP8, and some L-type calcium channels in the cardiomyocytes that are believed to be involved in NTBI uptake. TBI uptake is regulated by the IRP-IRE system and functions by downregulating TfR1 expression under excess iron conditions. In contrast, NTBI uptake occurs despite iron loading. Hepatocytes and parenchymal cells of other tissues like pancreas and heart are prone to NTBI uptake. This explains iron loading in the liver and other organs.

ZIP14 and DMT1 can mediate NTBI uptake in hepatocytes (Fig.1). In the context of the effect of alcohol on these NTBI transporters and NTBI uptake, there have been some apparently differing observations. For example, in mice, chronic alcohol and/or iron feeding (15 weeks) caused significantly elevated levels of NTBI in serum and increased the expressions of hepatic DMT1 and ZIP14 at both mRNA and protein levels. This explained the observed increment in their liver iron content and indicated alcohol-induced elevation in NTBI and in NTBI uptake. Furthermore, in
human HepaRG cells (hepatic cell line), ethanol increased total iron content and this appeared to be mediated via elevations in the gene expression of DMT1 and Tfr1, indicating the utility of both NTBI and TBI uptake in the presence of alcohol.

However, in other studies, ethanol exposure dramatically reduced hepatic ZIP14 protein levels in mice and there was no major change in hepatic DMT1 in mice after 12 weeks of alcohol feeding. Since the data is variable, it would be interesting to further investigate and clarify the significance and role of NTBI uptake under the influence of alcohol.

**Alcohol and liver ferritin: some contradictions**

Ferritin (the iron storage protein present intracellularly and in the circulation) is elevated in response to elevation in iron and/or inflammation. It is composed of 2 types of chains- heavy (H) and light (L). Rats fed with alcohol for 7 weeks showed significantly increased levels of H-ferritin expression in the liver. Similarly, alcohol treatment to HepG2 cells increased the expressions of both H and L ferritin and alcohol increased L-ferritin synthesis in rat hepatocytes. Also, alcohol exposure to human hepatoma HepaRG cell line increased the expression of L-ferritin. Such an alcohol-induced increase in liver ferritin could be either a rescue mechanism to combat the alcohol-induced elevation in iron levels and store excess iron or it could be a response to alcohol-induced inflammation or both.

However, a study in mice fed with alcohol for 12 weeks showed decreased hepatic L-ferritin expression and there were no significant effects at the earlier time points. Likewise, in VL-17A cells, alcohol did not alter the expression of H-ferritin. These differential ferritin responses to alcohol require further investigation.
Combination of excess iron and alcohol enhances oxidative stress and aggravates ALD pathology

Under physiological conditions, normal levels of ROS produced by cellular mechanisms are utilized for cellular purposes and excess ROS are scavenged/tackled by the endogenous antioxidant mechanisms to prevent ROS-mediated injury. However, excess free iron can accelerate the Fenton reaction leading to the production of large amounts of ROS that saturate the endogenous antioxidant mechanisms. These free radicals increase oxidative stress and can cause immense cellular and tissue damage by acting on cellular organelles, DNA, proteins and lipids.

Both, iron-overload and alcohol can independently cause oxidative stress and lipid peroxidation. Thus, excess free iron and alcohol act in a synergistic manner to cause liver damage and the combined effect exacerbates liver injury. The fibrogenic potential of iron is enhanced when it acts with other hepatotoxins like alcohol. The catalytic ‘free’ iron could directly add to the hepatotoxicity of alcohol and/or amplify the generation of cytokines and fibrogenic mediators from the nearby Kupffer cells. This means that slight increase in tissue iron levels in the presence of alcohol (and other metabolites) can accelerate fibrogenesis and advance the liver disease. In the early stages of liver disease, iron-loaded hepatocytes release profibrogenic cytokines and sustain fibrogenesis, whereas at the advanced stages, fibrogenesis is primarily governed by iron-induced hepatocellular necrosis. Thus, in ALD, excess iron can enhance liver injury by acting as a co-factor for liver fibrogenesis. Also, the combined oxidative stress caused by alcohol and excess iron may cause DNA damage and mutations resulting in increased predisposition to liver cancer.
Ferroptosis in context

Ferroptosis: an iron-dependent cell death

Ferroptosis is a form of regulated cell death that is morphologically and biochemically distinct from other cell death patterns like apoptosis, autophagy, and pyroptosis. Its normal physiological function has not been established yet, but it has a role in pathology. Distinct from its role in hepatocellular carcinoma where it increases sensitivity to sorafenib (used for liver cancer treatment), in chronic liver diseases including ALD, ferroptosis aggravates hepatic damage. Generally, it has been implicated in the pathology of liver diseases via several signaling pathways.

Ferroptosis is iron-dependent cell death and is characterized by excessive iron accumulation and lipid peroxidation. Essentially, during ferroptosis, there is ample redox-active iron present, and glutathione peroxidase is unable to efficiently execute its antioxidant action and repair lipid peroxidation resulting in unrestricted lipid peroxidation and iron-dependent accumulation of high levels of lipid hydroperoxides.

Role of ferroptosis in ALD pathology

Alcohol metabolism generates a large amount of acetaldehyde, reduces the levels of the antioxidant glutathione in the mitochondria, and increases ROS production followed by elevated lipid peroxidation in liver cells. Studies confirm that alcohol treatment induces excessive accumulation of iron in the liver, and increases ROS accompanied by lipid peroxidation, thereby initiating ferroptosis. The key features of ferroptosis are iron and lipid peroxidation. Both liver iron loading and lipid
disorder are features of ALD, which creates a strong reason for ferroptosis initiation in the livers of ALD patients.

As previously discussed, excess iron generates free radicals and enhances oxidative stress/injury, and the liver is prone to oxidative injury in general. Thus, ferroptosis has a pathogenic role in excess-iron-induced hepatic damage and fibrosis, and excess iron is a risk factor for liver fibrosis and cirrhosis. This explains the role of iron overload in inducing ferroptosis and thereby contributing to ALD pathology.

**Effect of ferroptosis on hepatocytes**

Long-term alcohol consumption can cause liver iron loading and subsequently promote ferroptosis in the hepatocytes. Hepatocytes have myriads of functions including regulation of systemic levels of iron, glucose, and lipoproteins. So, regardless of the form of cell death (ferroptosis or otherwise) hepatocyte death or dysfunction is a critical factor for liver injury and failure. Hepatocytes that undergo ferroptosis burst and release damage-associated molecular patterns. These are proinflammatory in nature and activate NLRP3 (NOD-like receptor family pyrin domain containing 3) inflammasomes in the Kupffer cells leading to the release of a large volume of proinflammatory cytokines that aggravate disease pathology.

Thus, excess iron, as found in ALD livers, can induce oxidative stress, cause iron-dependent cell death ferroptosis, promote inflammation, and thereby contribute to liver injury. Unsurprisingly, iron as an initiator of ferroptosis is linked with mortality related to ALD. Ferroptosis inhibitors like ferrostatin-1 can rescue the alcohol-induced hepatocyte death and limit alcohol-induced liver injury. Therefore, ferroptosis appears to be a promising target for ameliorating ALD pathology.
Cell-specific effect of ferroptosis

Unlike the aforementioned situation where ferroptosis in hepatocytes exerts a pathological effect and inhibition of ferroptosis in the hepatocytes is therapeutic, ferroptosis in HSCs show a completely opposite effect. Several studies in animal models have shown that ferroptosis in activated HSCs can reduce liver fibrosis and exert a curative effect. Also, blocking ferroptosis in the HSCs can promote liver fibrosis. Thus, the effect of ferroptosis appears to be cell-type specific. This presents challenges at the therapeutic front because selectively targeting ferroptosis in HSCs can be difficult. To enable this, specialized systems that exclusively target the HSCs will be required.

Links between alcohol, autophagy, ferritinophagy and ferroptosis

Autophagy: a cell survival mechanism that can also promote cell death

Autophagy is a conserved catabolic cellular process that is triggered following an insult or stress. It degrades damaged organelles and extra/unnecessary proteins, aiming to maintain a balance between protein degradation, synthesis, and recycling of cellular components. The process involves the formation of vesicles called autophagosomes, which deliver the cytosolic cargo to lysosomes for degradation, and then the material is recycled back to the cytosol. Dysregulation of autophagy has been implicated in metabolic and neurodegenerative diseases, inflammation, aging, and cancer. In the liver, autophagy maintains the cellular functionality of hepatocytes.
Autophagy degrades ferritin

Autophagy degrades ferritin, the iron-storage protein. This is called as ferritinophagy.

Ferritin degradation inside the autolysosomes leads to release of iron from ferritin.

This released free iron is likely to be transported back to cytosol leading to increment in ROS and oxidative stress, and this can trigger ferroptosis. Thus, ferritinophagy can play a role in triggering ferroptosis (Fig.2) \(^{49-53}\) and ferritin negatively regulates ferroptosis \(^{54}\). In HepG2 cells, autophagy inhibition increased ferritin heavy chain production \(^{55}\). In theory, this would aid in scavenging/accommodating free iron within ferritin leading to reduction in oxidative stress, and thereby, reduction in ferroptosis.

Collectively, data suggest that ferritinophagy can promote ALD pathology, in part via ferroptosis, because ferroptosis aggravates liver pathology (Fig.2).

Autophagy shows divergent relation with ALD- further clarity needed

Apparently, there is differing data in the context of the effect of alcohol of autophagy.

Studies indicate that alcohol exposure can increase autophagosome formation and trigger autophagy. This is a protective mechanism that selectively removes damaged mitochondria and hepatic lipids. However, alcohol can also impair lysosome function or lysosomal biogenesis leading to deficient autophagy in the hepatocytes, and contribute to ALD pathology (Fig.2) \(^{56}\). This apparently contrasting effects could be due to differential effects of acute and chronic alcohol on autophagy, differential effects of alcohol itself on autophagy or involve the fact that autophagy can mediate both cell survival and cell death; the latter may depend on cell-type and cell-context \(^{57}\). The reason/s for these differential effects need to be identified.

In addition, there can be apparently conflicting inferences involving autophagy, ferroptosis and ALD pathology (Fig.2). Inhibition of autophagy in alcohol-
fed mice increased hepatotoxicity, steatosis, oxidative stress, and hepatocyte apoptosis, and activation of autophagy blunted the alcohol-induced steatosis. This presents a protective role of autophagy under alcoholic conditions. However, experiments in HepG2 cells showed that inhibition/impairment of autophagy activates the p62-Keap1-Nrf2 pathway. This is protective against alcohol-induced ferroptosis, and thereby should reduce/decelerate ALD pathology. Unlike the previous scenario, this presents autophagy-impairment as having a protective role under alcoholic conditions (Fig.2).

These conflicting relationships which infer that autophagy can trigger ferroptosis but also decrease ALD pathology, and impaired autophagy can reduce ferroptosis but also accelerate ALD pathology require clarification.

**Intercellular events underlying iron-aggravated liver fibrosis in ALD**

Iron loading is one of the independent risk factors for fibrosis in ALD. Thus, it is important to review the intercellular events involved in the iron-facilitated progression to liver fibrosis.

Fig.3 summarizes the intercellular interactions, and the ways in which iron loading can exacerbate liver injury in ALD and promote liver fibrosis. Table 1 presents an overview of the effect of iron overload on some of the core cell types in the liver. Each cell-type of the hepatic lobule is actively involved in the fibrogenic process. The main cell types involved in this process are the hepatocytes, Kupffer cells and hepatic stellate cells (HSCs), while the liver endothelial cells (explained in Table 1) and fat storing cells (explained in the subsequent section) also play a role.
Interaction between hepatic stellate cells, hepatocytes and Kupffer cells

The hepatic stellate cells (HSCs) play a crucial role in liver fibrogenesis. Activation of HSCs is a normal phenomenon that mediates wound repair. Following repair, HSCs either revert to their quiescent state or undergo apoptosis. However, persistent liver insults keep the HSCs continuously activated. These HSCs secrete excessive amounts of profibrogenic factors and extracellular matrix that collectively induce a pathological state and form the basis of liver fibrosis. When liver iron exceeds 60 µmol/g, the HSCs get activated. Iron-induced promotion of fibrogenic mechanisms has been shown in murine HSCs, and the contribution of excess iron in enhancing liver fibrosis is well established 59,68,76.

Iron-loaded hepatocytes release profibrogenic factors and can directly activate the HSCs (Fig.3). In addition, these hepatocytes can release profibrotic/proinflammatory factors and stimulate the Kupffer cells 77. Alcohol increases the translocation of lipopolysaccharide (LPS) from the intestine to the liver, which additionally stimulates the Kupffer cells. Once activated, the Kupffer cells release proinflammatory and profibrotic factors, such as tumor necrosis factor (TNF)-α, interleukins (ILs)-1,6,8,10, interferon (IFN)-γ, transforming growth factor (TGF)-β1, platelet derived growth factor (PDGF), β-fibroblast growth factor (FGF), monocyte chemoattractant protein (MCP)-1, and reactive oxygen species (ROS). These cytokines in turn activate the HSCs (Fig.3) 77–80. Essentially, injured hepatocytes can directly activate the HSCs or indirectly activate HSCs by stimulating the Kupffer cells to secrete profibrotic factors that in turn activate HSCs. Regardless, upon activation, HSCs differentiate into myofibroblasts and synthesize and release excessive amounts of extracellular matrix
comprised of elastin, collagen, and other matrix proteins, thereby exhibiting liver fibrosis (Fig.3) \(^{59,77}\).

Activation of nuclear factor-κB (NF-κB) correlates with liver inflammation and fibrosis in ALD \(^{81}\). Alcohol-induced accumulation of iron in Kupffer cells can activate nuclear factor-κB (NF-κB) and worsen experimental ALD/alcoholic steatohepatitis \(^{22,65,82}\).

Alcoholics show increased levels of LPS in the circulation. Iron and LPS are believed to activate NF-κB in the Kupffer cells and induce the synthesis of proinflammatory cytokines like TNF-α \(^{19}\). TNF-α plays an important role in liver injury. Normally hepatocytes are not negatively affected by TNF-α. However, alcohol sensitizes the hepatocytes to injury by TNF-α and causes hepatocyte cell death via apoptosis \(^{80,83}\).

These dead cells would be engulfed by the Kupffer cells (Fig.3). In animal models, upon Kupffer cell depletion or inactivation, alcohol-induced effects such as inflammation, fatty liver and necrosis were dampened. Thus, Kupffer cells play an important role in the pathological progression of ALD \(^{19}\).

**The adipocyte context**

In addition to Kupffer cells and HSCs, other cells in the surrounding such as the adipocytes from adipose tissue are involved in ALD pathogenesis. Independent of the effect of alcohol, it has been speculated that lipid peroxidation by-products released from iron-overloaded hepatocytes are able to stimulate collagen gene transcription in the neighboring fat-storing cells directly or via activation of Kupffer cells \(^{84}\). This may further aggravate ALD pathogenesis in cases with iron overload.

Notably, excess-iron-generated ROS and lipid-peroxidation by-products can activate both Kupffer cells and HSCs (Fig.3) \(^{13}\).
Also, alcohol induces inflammation in the adipose tissue. Alcohol-induced lipolysis in the adipocytes (which promotes hepatic steatosis) together with inflammatory responses in the macrophages release increased levels of free fatty acids, adipokines (such as leptin) and cytokines (such as TNF-α and IL-6) into the portal circulation. These adipokines like leptin have proinflammatory effects on the liver. Leptin (along with other endocrine factors) activates the HSCs and Kupffer cells (that produce increased TNF-α), and thereby promotes hepatic inflammation and fibrosis (Fig.3). High levels of TNF-α can damage the liver hepatocytes, as discussed previously. Also, leptin and acetaldehyde together can enhance the production of IL-6 in the HSCs (Fig.3). Interestingly, leptin levels correlate with liver disease severity in patients with alcoholic cirrhosis. In addition, iron loading in the adipocytes reduces the production of the anti-inflammatory adipokine adiponectin. This can further promote inflammation and contribute to liver injury.

Note that while the aforementioned cellular interactions showcase an iron perspective, ALD pathology is additionally driven by both adaptive and innate immune systems and involves the recruitment of various immune cells that create a pro-inflammatory environment in the liver. As such, the liver has abundant lymphocytes scattered through its parenchyma, and it is also rich in cells of the innate immune system such as the natural killer cells. Iron does play a role in liver pathology via the immune cells. For example, iron deficiency dampened concanavalin A-induced intra-hepatic inflammation in mice. It also reduced intrahepatic lymphocyte infiltration.
Low liver iron content- a phenomenon to be investigated

In a study by Varghese et al., mice models showed gradual elevation of serum iron levels during 12 weeks of alcohol feeding. Elevations in duodenal ferroportin (gradually increased at 8 weeks and further at 12 weeks) and duodenal DMT1 (significantly increased at 8 weeks but dropped to control levels at 12 weeks) supported this increment in serum iron. In contrast to these elevations, hepatic and serum hepcidin expression gradually decreased during the 12-weeks of alcohol exposure.11 This alcohol-induced decrement in hepcidin is an expected response and is also seen in ALD patients16–19. Here, the lack of hepcidin upregulation despite elevation in serum iron levels reiterates the insensitivity of hepcidin to increasing systemic iron levels in the presence of alcohol.

Distinct from the frequently observed hepatic iron elevation in alcoholics, here, in mice models, hepatic iron levels were seen to drop after 12 weeks of alcohol feeding.11 The pattern of liver iron decrement matched fully with the patterns of decrements of hepatic TfR1 hepatic ferritin expressions through the 12 weeks of alcohol exposure. This drop in liver iron content is an unexpected response because several studies in humans have shown increased liver iron content in alcohol-consumers/ALD patients5–7.

Varghese et al. attributed the decrement in hepcidin expression partly to decreased hepatic iron levels. The authors proposed that this could be either due to alcohol-induced hepatomegaly and alcoholic steatosis and/or mobilization of iron to other tissues. The concept of mobilization of iron from liver to other tissues was supported by their observation that hepatic ferroportin expression showed a tendency to increase after 4 and 12 weeks of alcohol exposure, which would facilitate cellular iron egress.11 The reason for decrement in liver iron content needs
to be fully understood, particularly because it involves the function of ferroportin, the sole known unidirectional cellular iron transporter.

Liver iron loading in ALD: diagnostic, prognostic, and therapeutic perspectives

Liver iron and ALD diagnosis and prognosis
Currently, there is no single diagnostic test to confirm ALD. One of the challenges at the diagnostic front is that the symptoms of ALD are not obvious at the early stages. Suspected cases are often tackled based on patient-derived information about their alcohol intake (patient history) supported by laboratory tests. Crabb et.al have reviewed this topic in detail. Note that liver iron loading by itself cannot be used for the diagnosis of ALD or any chronic liver disease because there are several other liver conditions such as haemochromatosis and non-alcoholic fatty liver disease that show high liver iron content. An old study indicated that liver iron in ALD has a prognostic value. It showed that alcoholic cirrhosis patients with detectable liver iron had a lower survival rate than those without. However, other authors suggested that hepatic iron overload is a poor prognostic factor in ALD.

Liver iron and ALD therapeutics: alcohol abstinence
Although there are FDA-approved therapies for alcohol use disorders that help reduce cravings for alcohol, there is no FDA-approved drug to treat ALD. Alcohol abstinence is the only curative option and liver transplantation is the definitive treatment for liver diseases (including ALD) in the end stage.
Cessation of alcohol has shown to reduce liver iron deposits. For example, a study found that ALD patients who abstained for more than 3 months had reduced liver iron content compared to ALD patients with active alcoholism (average intake of 164.4 g/day). Also, drinking lesser amount of alcohol has shown to cause lesser liver iron deposition. For example, in a study, mean liver iron concentrations were significantly higher in alcoholic patients (drank more than 80 g/day for 3 or more years prior to and inclusive of the study period) compared to controls who did not drink excessive amounts of alcohol (i.e. did not drink more than 20 g/day).

**Liver iron and ALD therapeutics: discussing phlebotomy**

Hemochromatosis is an iron-overload disease in which patients show high systemic and liver iron loading, in addition to iron deposition in other organs. For hemochromatosis patients that show high iron loading, life-long periodic phlebotomy is the mainstay of therapy where the aim is to reduce the level of iron and thereby limit excess-iron induced organ damage. In a patient with ferroprotein disease (hereditary iron loading disorder), long term phlebotomy decreased hepatic iron accumulation.

This questions whether phlebotomy could be used for ALD patients that show liver iron overload. Firstly, just like in case of hemochromatosis where not all patients demonstrate enough iron overload to cause organ damage, not all ALD patients show liver iron loading. Some ALD patients may be anemic. Secondly, in ALD patients that show liver iron loading, the levels hardly ever surpass 2-3 times the upper limit of the norm. Thirdly, phlebotomy has several limitations, one of which is the possibility of developing anemia. Therefore, while phlebotomy is a
suitable option for iron-overloaded hemochromatosis patients, it is not a suitable therapeutic option for ALD patients.

**Liver iron and ALD therapeutics: iron chelation**

In general, apart from phlebotomy, another therapeutic approach for reducing liver iron content is iron chelation by using iron chelators like deferoxamine, deferiprone and deferasirox \(^{102-104}\). Deferiprone decreased hepatocyte iron overload in chronically ethanol-fed rats \(^{105}\). A novel iron chelator M30 reduced alcohol-induced injury in rat hepatocytes. There was a significant attenuation of ethanol-induced apoptosis, oxidative stress and secretion of inflammatory cytokines \(^{106}\). Thus, these chelators could be tried for ALD cases that show liver iron overload.

Naturally occurring compounds, namely flavonoids are also potential therapeutic agents. These have shown to impair ALD pathological progression by maintaining iron balance. For example, quercetin, which exhibits iron-chelating and anti-oxidant properties, dampened alcohol-induced liver damage in mice \(^{44}\). Such natural compounds could be tested in alcohol-treated animal models and then relevant clinical trials could be established.

**Liver iron and ALD therapeutics: synthetic hepcidin**

Hepcidin deficiency is the main cause of iron loading in ALD patients \(^{16,23}\). Therefore, hepcidin treatment is a promising therapeutic approach. Natural hepcidin is expensive and has undesirable pharmacological properties such as having a short half-life. In contrast, minihepcidins are synthetic in nature. These mimic the action of hepcidin and are pharmacologically more favorable \(^{99}\). Intraperitoneal injections of minihepcidin to mice models of haemochromatosis showed significant reductions in
liver iron loading. This experiment could be repeated in alcohol-fed animal models to extrapolate whether the approach would be successful in reducing liver iron loading in ALD patients.

**Liver iron and ALD therapeutics: targeting ferroptosis**

Interestingly, not the liver iron loading itself, but ferroptosis, the iron-dependent process that contributes to liver damage in ALD, has been targeted for therapy. Ferroptosis inhibitors and repressors have shown protective effects against alcohol-induced liver damage. For example, the ferroptosis inhibitor ferrostatin-1 reduced lipid peroxidation and alcohol-induced liver injury in vivo. Similarly, another ferroptosis inhibitor dimethyl fumarate significantly improved alcohol-induced liver injury in ethanol-fed mice. Also, deficiency of intestinal SIRT1 in mice has shown protection from alcohol-induced hepatic injury via mitigation of ferroptosis.

Frataxin is a mitochondrial protein that predominantly participates in iron homeostasis and oxidative stress. A study showed that alcohol reduced the expression of frataxin and the deficiency of frataxin increased sensitivity to alcohol-induced ferroptosis (Fig.2). Restoration of frataxin reversed this effect. Thus, frataxin can be an additional therapeutic target to tackle ALD.

**Summary**

Increased serum iron due to chronic alcohol consumption increases iron uptake in the hepatocytes and Kupffer cells facilitating both parenchymal and non-parenchymal iron loading in the liver, and in parenchymal cells of other organs. Hepatic iron deposition is mediated via upregulation of TfR1 and HFE (proposed). Both iron and alcohol can independently induce oxidative stress, so the combined
effect accelerates hepatic injury. Excess iron-simulated hepatocytes and Kupffer cells secrete inflammatory and profibrogenic factors that activate the hepatic stellate cells. Chronic activation of hepatic stellate cells mediates the development of liver fibrosis. Iron loading promotes ALD progression via induction of oxidative stress and the activation of HSCs and Kupffer cells. Other cells such as the liver sinusoidal endothelial cells, the liver immune cells (from both adaptive and innate immune systems) and the adipocytes also contribute to the iron-mediated liver injury in ALD.

Authors' contributions: Primary investigation, Writing-original draft: Kevin Ferrao and Najma Ali; Conceptualization, Supervision, Writing and Editing: Kosha J. Mehta

Acknowledgements

This article is made open access with the financial support of King’s College London, UK.


doi:10.3389/fmolb.2022.928321


doi:10.1101/cshperspect.a008813


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Figure Legends

Figure 1. Cellular events underlying alcohol-induced iron-loading in different cell types
Alcohol consumption decreases hepcidin levels in the circulation. In turn, this increases intestinal absorption of iron. Elevated serum iron levels cause iron deposition in various cell types including the hepatocytes and Kupffer cells in the liver via elevation in TfR1 and HFE (proposed). Also, alcohol-induced elevation of NTBI transporters ZIP and DMT1 on hepatocytes, as observed in some studies, aid in hepatocyte iron loading. Green arrows with yellow stars indicate variability in results with regards to alcohol-induced elevation of these NTBI transporters.

Figure 2. Putative associations between autophagy, ferritinophagy and ferroptosis under the influence of alcohol
Alcohol seems to have a dual effect of autophagy i.e., it can both stimulate and impair autophagy. This differential effect of alcohol on autophagy and the consequent ambiguity is indicated through an asterisk in the figure. Degradation of ferritin via autophagy is ferritinophagy. Ferritinophagy can trigger ferroptosis whereas increment in ferritin can increase the probability of accommodating free iron, thereby reducing excess-iron-induced oxidative stress, and consequently reducing ferroptosis. Autophagy can also trigger ferroptosis through ferritinophagy-independent routes such as those involving frataxin deficiency, and degradation of damaged or excess cellular components that eventually increases free iron levels and/or lipid peroxidation. Interestingly, while autophagy can trigger ferroptosis, which can exacerbate ALD pathology, autophagy appears to also impart a protective
effect and decrease or blunt ALD pathology. This apparently opposing concepts have been indicated by questions marks in the figure and need further clarification.

**Figure 3. Intercellular events depicting the role of iron in enhancing alcohol-induced liver fibrosis**

Alcohol can cause iron-loading in the hepatocytes and Kupffer cells. Oxidative injury to hepatocytes due to excess iron and alcohol can lead to hepatocyte death. Kupffer cells phagocytose dead/damaged hepatocytes and get activated. Activated Kupffer cells release profibrotic cytokines and activate the hepatic stellate cells (HSCs). Additionally, profibrotic/inflammatory cytokines released from injured hepatocytes together with ROS and acetaldehyde produced from alcohol metabolism in the hepatocytes activate the HSCs. Following activation, HSCs secrete profibrotic factors and excessive extracellular matrix that collectively form the basis for liver fibrosis. Adipocytes also play a role in promoting alcohol-induced liver fibrosis, and together with excess iron, the pathology may be aggravated.
Table 1 Overview of the most prominent effects of iron overload on the core liver cells and the associated underlying mechanisms

<table>
<thead>
<tr>
<th>Liver cell type and its generic function</th>
<th>Prominent effects of iron overload</th>
<th>Underlying cellular mechanisms in context of iron loading</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hepatocytes</strong> (Hepatic parenchymal cells, make majority of liver parenchyma, exhibit various functions including sensing iron in the circulation and secreting the iron-regulating hormone hepcidin) ¹⁵</td>
<td>Increased oxidative stress resulting in damage to cellular organelles, lipids, proteins and DNA ⁴⁴,⁵⁹. Cell death</td>
<td>Excess-iron-induced elevation in ROS production is via the Fenton reaction ⁴⁴,⁵⁹. Excess ROS causes lipid peroxidation which contributes to different types of cell deaths including ferroptosis ⁵⁹–⁶¹. Increased synthesis and secretion of hepcidin ¹⁵. Hepcidin is induced via the BMP-SMAD pathway ¹⁵. [Notably in ALD, hepcidin synthesis and secretion is reduced due to alcohol-induced inhibition of the BMP-SMAD pathway ⁶², attenuation of JAK/STAT signaling ¹⁶ and oxidative stress ¹⁹,⁴⁴]</td>
</tr>
<tr>
<td><strong>Kupffer cells</strong></td>
<td>Increased production of inflammatory cytokines ⁶⁴</td>
<td>Iron-loading can activate nuclear factor-κB (NF-κB) ⁴⁴,⁶⁵, which can stimulate the production of pro-</td>
</tr>
<tr>
<td>(Hepatic non-parenchymal cells, clear microorganisms, dead cells, debris and circulating endotoxin)</td>
<td>Enhanced inflammatory response to LPS $^{22,67}$</td>
<td>Disruption of mitochondrial homeostasis and increased generation of mitochondrial superoxide partly promote inflammatory response to LPS $^{67}$</td>
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<tr>
<td><strong>Hepatic stellate cells (HSCs)</strong> (Hepatic non-parenchymal cells, generally quiescent, mediate wound healing following an injury)</td>
<td>Persistent cell activation and proliferation leading to promotion of fibrosis $^{59}$</td>
<td>Stimulation of the expressions of type I collagen and α-SMA (markers of fibrosis), increased production of TGF-β1, and activation of TGF-β pathway $^{68,69}$</td>
</tr>
<tr>
<td>Extracellular ferritin stimulates inflammatory pathway in HSCs $^{70}$.</td>
<td>Activated HSCs exhibit a receptor for H-ferritin. Binding of ferritin (H-ferritin) activates NF-κB through PI3 kinase, PKCζ, MEK1/2, MAPK, and IKKα/β. Thereby, extracellular ferritin acts as a proinflammatory mediator $^{70}$.</td>
<td></td>
</tr>
<tr>
<td><strong>Liver sinusoidal endothelial cells (LSECs)</strong> (Hepatic non-parenchymal cells, form liver sinusoids)</td>
<td>Induce hepcidin production in the hepatocytes $^{72}$</td>
<td>LSECs can sense iron and produce BMPs in response. BMPs 2 and 6 can induce hepcidin synthesis in hepatocytes via BMP-SMAD pathway $^{15,72}$. [Note that in ALD, hepcidin synthesis and secretion is impaired].</td>
</tr>
<tr>
<td>a fenestrated endothelium that allow movement of selective molecules, play a role in role in clearance of macromolecules from blood(^{63}), differentiated LSECs maintain HSC quiescence and help prevent fibrosis (^{71})</td>
<td>reduced due to the previously explained reasons</td>
<td>Following chronic liver injury (including persistent iron overload), LSECs can de-differentiate, activate the HSCs, which leads to increased production of extracellular matrix, LSECs lose their fenestrations (defenestration) and function (^{63,71,73})</td>
</tr>
</tbody>
</table>
Iron

Hepcidin
gene

Normal/high hepcidin levels

HFE gene

HFE interacts with ferroportin and inhibits iron export (proposed)

Alcohol

Increased serum iron

Increase iron saturation of transferrin

Hepatocyte

Cell death

Iron and cell debris phagocytosed by Kupffer cell

Key:

- Upregulation
- Downregulation
- Ferroportin
- Hepcidin
- Non-transferrin bound iron
- Transferrin-bound iron

HFE

HFE gene

Reduced ferroportin degradation

Increased iron saturation of transferrin

Increased iron saturation of transferrin

Reduced transferrin receptor 1 (TfR1)

Upregulation

Reduced levels of hepcidin lead to increased transcription of DMT1 gene

Entry of iron into cells

Intracellular iron trapping

Iron loading & oxidative stress

ZIP14

Non-transferrin bound iron

DMT1

Reduced levels of hepcidin lead to increased transcription of DMT1 gene

Apical region

Basolateral region

Enterocyte

Ferroportin degradation

Hepcidin

Transferrin (Tf) - Iron (Fe)

Hepcidin gene

Reduced hepcidin levels

Hepcidin

Iron saturation of transferrin

TfR1

Increased hepcidin in circulation

HFE

Iron uptake

Transferrin (Tf) - Iron (Fe)

TfR1

Increased hepcidin in circulation

HFE

Increased iron saturation of transferrin

TfR1

Increased iron saturation of transferrin

HFE

Increased iron saturation of transferrin

HFE

Increased iron saturation of transferrin

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Increased iron saturation of transferrin

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Increased ir
Autophagy triggered Ferritin degradation (ferritinophagy) Release of free iron Increased oxidative stress Ferroptosis triggered

Frataxin deficiency Excess free iron Lipid peroxidation

Degradation of damaged mitochondria, protein aggregates & excess peroxisomes

Autophagy impaired

Ferritin heavy chain increment Increased accommodation of free iron Reduced oxidative stress Ferroptosis reduced or inhibited

Activation of p62−Keap1−Nrf2 pathway Reduced lipid peroxidation

ALD pathogenesis decreased ALD pathogenesis exacerbated

Increased oxidative stress

Alcohol*
Excessive extracellular matrix secreted

Activated hepatic stellate cells
Increased α-SMA, TGF-β, Collagen

Activated Kupffer cells
Profibrogenic factors and inflammatory cytokines released (examples TNF-α, ILs-1,6,8,10, IFN-γ, TGF-β1, PDGF, β-FGF, MCP-1) & ROS

Hepatocyte death
Iron overload
Increased oxidative stress
Acetaldehyde
Alcohol
Phagocytosis by Kupffer cells
Hepatocyte injury
Activated Kupffer cells
Activation of Kupffer cells
Iron accumulation
Lipopolysaccharide
Lipid peroxidation by-products
Increased oxidative stress
Activated collagen gene transcription in neighboring fat-storing cells
Hepatocyte
Lipid peroxidation
Increased lipid peroxidation
Acetaldehyde
Sensitization to produce more inflammatory mediators
Alcohol
Liver Fibrosis
Activated collagen gene transcription in neighboring fat-storing cells
Increased leptin (in chronic consumers)
Leptin
Collagen release
Adipocyte
Increased leptin (in chronic consumers)
Alcohol
Increased lipid peroxidation
Alcohol metabolism
Liver
Increased lipid peroxidation
Acetaldehyde
Alcohol sensitis hepatocytes to TNF-α injury
Phagocytosis by Kupffer cells
Activation of Kupffer cells