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Iron as a therapeutic target in chronic liver disease

Kouroumalis E *et al*. Iron as a therapeutic target in chronic liver disease

Abstract

It had been clearly realized more than fifty years ago, that iron deposition in the liver may be a critical factor for the development and progression of liver diseases. The recent clarification of ferroptosis as a specific form of regulated hepatocyte death different from apoptosis and the description of ferritinophagy as a specific variation of autophagy, prompted detailed investigations on the association of iron and the liver. In this review, we will present a brief review of iron absorption and handling by the liver with emphasis on the role of liver macrophages and the significance of the iron regulators hepcidin, transferrin and ferritin in iron homeostasis. The mechanistic and regulation of ferroptosis by endogenous and exogenous modulators will be examined. Furthermore, the involvement of iron and ferroptosis in various liver diseases including alcoholic and non-alcoholic liver disease, chronic hepatitis B and C, liver fibrosis and hepatocellular carcinoma (HCC) will be analyzed. Finally, experimental and clinical results on the results of interventions to reduce iron deposition and the promising manipulation of ferroptosis will be presented. Most liver diseases will be benefited by ferroptosis inhibition using exogenous inhibitors with the notable exception of HCC where induction of ferroptosis is the desired effect. Current evidence is mostly coming from *in vitro* and *in vivo* experimental studies and the need for well-designed future clinical trials is warranted.

Key Words: Iron overload; Liver diseases; Ferroptosis; Ferritinophagy; Ferroptosis modulators

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Core Tip: Iron overload may damage the liver in a variety of liver diseases such as cirrhosis and hepatocellular carcinoma affecting patient survival. In this review, we presented the evidence, both experimental and clinical, of the detrimental effects of iron

deposition in hepatocytes and other liver sinusoidal cells. Moreover, we examined the mechanism and implications of the recently described ferroptosis in the evolution of liver diseases. Ferroptosis, is a form of regulated hepatocyte death caused by excess iron and lipid peroxidation. Inhibition or induction of ferroptosis may profoundly improve the natural course of many liver diseases as demonstrated by a large number of experimental studies and few clinical data.

INTRODUCTION

The major suppliers of plasma iron are duodenal enterocytes and iron-recycling macrophages[1-3]. The duodenal cytochrome B reductase reduces inorganic trivalent iron reaching the duodenum into divalent iron and the surface divalent metal transporter 1 (DMT1) imports Fe^{+} into the cytoplasm. The gene SLC11A2 encoding DMT1 is activated in cases of iron deficiency or hypoxia because it bears a hypoxia-responsible element that interacts with the hypoxia-inducible factors (HIF1α and HIF2α) overexpressed in these situations[4-7]. The cytoplasmic iron sensors iron-responsive element (IRE) and the iron regulatory proteins (IRP1 and IRP2) also participate in absorption control as they stabilize the SLC11A transcript in iron deficiency or dissociate and degrade in iron overload[8]. Then the cytoplasmic divalent iron is transported to ferroportin, the only known iron exporter protein and then exported to the portal vein blood. Transportation is mediated by the chaperone protein poly (rC)-binding protein 2 encoded by the SLC40A gene[9]. The main regulator of ferroportin is hepcidin[10], but the IRP/IRE proteins and microRNAs are also involved[11]. Once in the portal vein the divalent iron is oxidized back to trivalent by the ferroxidases hephestin and ceruloplasmin and then carried in different cells bound to transferrin. Cells are importing iron by internalization of transferrin after binding to its receptor TRF1[12] and sorted into endosomes where iron is removed in the acidic environment, reduced again to $Fe2^{+}$ by the ferrireductase STEAP3 and released into the cytosol by DMT1[1,3]. Iron is then either exported by ferroportin or stored in ferritin or in the labile iron pool (LIP). On the other hand, heme

oxygenases (HOs) localized mainly in iron-recycling macrophages of liver and spleen, degrade heme to recover Fe^{2+}[2,13].

The regulation of hepcidin is critical in iron metabolism as binding of hepcidin to ferroportin in hepatocytes macrophages or enterocytes leads to internalization and degradation of ferroportin reducing thus iron export to the blood[2,3,10]. A decrease in hepcidin when iron is needed, leads to enhancement of ferroportin expression and increased iron absorption from the duodenum. In iron overload ferroportin is downregulated and iron absorption is reduced[14]. In addition to iron deficiency, inflammatory molecules like interleukin 6 also upregulate hepcidin expression[15]. HAMP is the gene encoding for hepcidin. Its promoter is activated by the complex of bone morphogenic proteins (BMP2, BMP4, BMP6) and their receptor. The complex phosphorylates the SMAD pathway which in turn activates HAMP[16,17]. Hemojuvelin (HJV) is a necessary co-factor for BMP-BMP receptor complex[18]. BMP6 is mainly expressed in the liver sinusoidal cells and induces hepcidin upregulation in a paracrine way during iron overload[19-21].

The second receptor of transferrin (TFR2), a low-affinity receptor of hepatocytes and erythroid precursors, is also an important inducer of hepcidin through the BMP/SMAD pathway[22-24] after forming a complex with HFE (the protein involved in Hereditary Hemochromatosis)[25]. Gene anomalies of either gene will lead to hepcidin downregulation[26-28].

HEPCIDIN INHIBITORS

In contrast to iron overload, hypoxia, anemia, and erythropoiesis reduce hepcidin expression[29,30]. The main inhibitor of hepcidin expression is erythroferrone (ERFE)[31], produced by erythroid cells in response to erythropoietic stimuli. ERFE downregulates hepcidin interfering with the BMP/SMAD pathway in hepatocytes[32-34]. Three other hepcidin inhibitors have been described. PIEZO1 and the immunophilin FKBP reduce HAMP expression by inhibiting the BMP/SMAD pathway[35,36]. The third hepcidin inhibitor is the ferritinophagy axis operating in both the enterocyte and the

macrophage. Ferritinophagy is a specialized form of autophagy resulting in the lysosomal breakdown of ferritin and iron release to increase the LIP. It is controlled by the nuclear receptor coactivator 4 (NCOA4)[37,38] during transportation of the absorbed iron to ferritin. On increased iron demand, NCOA4 functions as a cargo receptor for lysosomal degradation of ferritin. Excess iron leads to lipid peroxidation-mediated ferroptosis[38]. NCOA4 is similarly involved in macrophage ferritinophagy and iron release for erythropoiesis[39].

Iron ions are dangerous for cells. In iron overload redox-active iron increases and oxidative stress is induced through the formation of reactive oxygen species (ROS). Non-transferrin bound iron is mainly responsible for the redox-active iron when the capacity of iron binding proteins is not able to accommodate for the increased iron load. An additional dangerous form is the transit iron pool, which is the iron that does not bind to ferritin and other chelating proteins. This iron entity may also induce ROS[40]. Iron is a double-edged sword[41], which even under normal conditions may cause pathological damage. Iron induces hydroxyl radical production through the Fenton reaction[42]. The Fenton-Haber-Weiss reaction is caused by the free donation and acceptance of electrons during the transition between Fe^{2+} and Fe^{3+} states. Iron-catalyzed generation of hydroxide ions, hydroperoxyl and hydroxyl radicals is the result of this exchange. Under normal conditions free-radicals are quenched by the cellular antioxidant mechanisms[43]. When overproduced these free radicals promote the formation of other ROS such as thiyl and peroxyl radicals and a vicious circle is initiated leading to oxidation of lipids, proteins and nucleic acids[44]. Thus, in iron-loaded animals the products of lipid peroxidation such as malondialdehyde (MDA), isoprostanes and 4-hydroxynonenal (4-HNE) can be detected in the liver[45]. MDA and 4-HNE form mutagenic adducts, reacting with amino groups and DNA bases[46,47] that target the p53 tumor suppressor gene initiating apoptotic resistance to the cells[48]. 4-HNE correlates well with hepatic iron levels[49]. Iron metabolism was recently reviewed in detail[50-53].

FERROPTOSIS

The most important mechanism of iron-induced liver damage is the recently described ferroptosis, a name derived from the Greek word "ptosis", meaning a fall, and the Latin "Ferrum" or iron[54]. It is an iron-dependent regulated cell death characterized by iron accumulation, lipid peroxidation and the production of ROS that depends on the activity of NADPH oxidases[55,56]. The mitochondrial respiratory chain initiates lipid peroxidation by lipoxygenase (LOX) or cytochrome P450 reductase. The enzyme glutathione peroxidase 4 (GPX4), the antioxidant glutathione (GSH), the coenzyme Q10 (CoQ10), and the tetrahydrobiopterin (BH4) system are the defense mechanisms of the cell. They are further regulated by the nuclear factor erythroid 2-related factor (Nrf2)[57-59]. The process is controlled by multiple genes associated with iron uptake[60,61], lipotoxicity[62,63], and antioxidation responses[64,65].

Ferroptosis is regulated by several metabolic events such as lipogenesis and ferritinophagy. The mitochondrial tricarboxylic acid cycle fueled by glutaminolysis may promote ferroptosis induction. Phospholipid peroxidation is the critical event in ferroptosis. Production of ROS, iron, and phospholipids containing polyunsaturated fatty acids (PUFA-PLs) are the necessary requirements. The executioners of ferroptosis are phospholipid hydroperoxides (PLOOHs) synthesized from PUFAs, the precursor of PLOOHs[66].

Non-enzymatic/exogenous and endogenous/enzymatic pathways are implicated in lipid peroxidation. For the latter, LOXs and/or cytochrome P450 oxidoreductase mediate the induction of lipid peroxidation by dioxygenation of lipids. Exogenous/transporter mediated signaling pathways include the E cadherin-NF2-Hippo-YAP pathway, the glucose-regulated AMPK signaling pathway, and the p53 tumor suppressor pathway[67].

Mechanisms inhibiting ferroptosis are provided by three main biological pathways (Figure 1)[68,69]: (1) The GSH/GPX4 pathway, implicating the system Xc-, which is a membrane cystine/glutamate exchanger that imports cystine and exports glutamate. A critical role in this system has thecystine/glutamate antiporter SLC7A11. GPX4 is the

major protective system against lipid peroxidation[70]. In addition, the ferroptosis suppressor protein 1 acts mainly on the plasma membrane, and dihydroorotate dehydrogenase is an important defense molecule in mitochondria[71-75]; (2) Iron metabolism pathways, particularly the p62-Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 regulatory pathways[54]. Inhibition of ferritinophagy increases mitochondrial ferritin and protects from ferroptosis as evidenced in hypoxic macrophages. This is regulated by a hypoxia induced decrease of NCOA4 transcription, in combination with a microRNA 6862-5p-dependent degradation of NCOA4 mRNA[76]. The Nrf2 is a transcription factor that protects cells against oxidative and toxic damage and plays a significant role in regulating ferroptosis[77-79]. In hepatocellular carcinoma (HCC) and other tumors, activation of the p62-Keap1-Nrf2 pathway leads to reduced Nrf2 degradation, the protection of tumor cells against ferroptosis, and resistance to anticancer drugs[80]; and (3) The lipid metabolism pathways implicating p53 and various enzymes of lipid metabolism[54,66]. p53 is a tumor suppressor transcription factor that may prevent cancer by controlling the cell cycle, senescence and apoptosis. Ferroptosis is one of its antitumor mechanisms. p53 increased cell sensitivity to ferroptosis through repression of SLC7A11. The ferroptosis inhibitor fer-1 reversed this effect and induced SLC7A11 overexpression[62,81-83]. Additional biological factors inhibiting ferroptosis were recently identified. GTP Cyclohydrolase-1 is the rate-limiting enzyme for biosynthesis of tetrahydrobiopterin (BH4) which counteracts ferroptosis[84]. Transferrin and its cell surface TFR1 receptor have been implicated in the inhibition of ferroptosis[12]. CDGSH iron sulfur domain 1 negatively regulates ferroptosis protecting against lipid peroxidation in mitochondria[85].

EXOGENOUS FERROPTOSIS MODULATORS

Ferroptosis inhibitors are divided into two major groups: (1) Class I inhibitors, such as Deferoxamine (DFO) mesylate[86], suppress iron accumulation; and (2) Class II inhibitors, including ferrostatin-1, liproxstatin-1 and vitamin E, react with chain free radicals and can inhibit lipid peroxidation[87-91]. The activity of the first generation of

ferrostatin 1, specifically reduces the accumulation of lipid ROS. The second generation (SRS 11-92) and the third generation (SRS 16-86) of ferrostatins showed an increased metabolic stability[90] (Table 1).

The recently described inhibitor dynasore has characteristics of both classes, preventing both iron accumulation and lipid peroxidation[92]. Other inhibitors of ferroptosis have been identified. The cholesterol reducing drug probucol was found to suppress ferroptosis[93]. The RIPK1 inhibitor necrostatin-1, which suppresses necroptosis, has an additional effect in suppressing ferroptosis Selenium administration suppressed ferroptosis during stroke[94] while nitroxide XJB-5-131 targeted mitochondria and suppressed both apoptosis and ferroptosis[95]. However, it should be noted that these inhibitors have not been tested in the liver (Table 1).

Interestingly, a recent experimental finding showed that the mode of action of bicyclol, a common hepatoprotectant in China, was the prevention of ferroptosis. Furthermore, bicyclol attenuated cellular damage and lipid peroxidation induced by erastin. Additionally, Nrf2 inhibition and the subsequent reduction of GPX4 levels impeded the effects of bicyclol[96]. Finally, the anti-diabetic drug rosiglitazone inhibited ferroptosis and reduced hepatocyte death, acting as an ACSL4 inhibitor[97,98] (Table 1).

Ferroptosis inducers[99]

Class I inducers such as erastin, sorafenib, sulfasalazine and glutamate, deplete cellular cysteine by inhibiting system Xc- and the biosynthesis of GSH, resulting in the loss of GPX4 activity[81,100-102]. The low water solubility and metabolic instability of erastin has limited its clinical application[103], but a metabolically stable erastin derivative has been tested[104] (Table 1).

Class II inducers, including RSL3 and DPI compounds act by directly inhibiting GPX4[88,105-107], leading to the accumulation of lipid peroxides and eventual cell death. BSO and cisplatin also deplete GSH inducing ferroptosis. Cisplatin and erastin have a significant synergistic effect[108]. Interestingly, erastin promoted ferritinophagy and increased the free iron, lipid peroxidation, while RSL3 did not interfere with

ferritinophagy, suggesting that RSL3 induction of ferroptosis is not dependent on ferritin degradation[109] (Table 1).

Class III inducers such as FIN56 act by both direct degradation of GPX4 and indirect inactivation of GPX4 *via* the squalene synthase-mevalonate pathway of the mitochondrial electron transport chain[103,110]. FIN56 also acts by depleting GPX4 and CoQ10. In addition, statins such as simvastatin, enhance ferroptosis by inhibiting HMG-CoA reductase. It seems, that the lethality of FIN56 is increased when cells are co-treated with statins and FIN56[110] (Table 1).

In class IV inducers, ferroptosis is induced by excess iron, omega-3 PUFAs, or peroxides such as FINO2, that initiate lipid peroxidation and indirectly reduce GPX4 activity[111,112]. FINO2 is the only representative of class IV ferroptosis inducer tested so far, but several other inducers have been synthesized[103] (Table 1). PUFAs showed anticancer activity[113] but shortcomings like reduced bioavailability, limited resistance to oxidative degradation and lack of uptake specificity, impeded their use. The application of nanotechnology improved their therapeutic use[114]. Low density lipoproteins (LDL) are taken up by LDL receptor particularly expressed in tumor cells. LDL-based nanoparticles with docosahexaenoic acid (LDL-DHA NPs) were found to maintain their stability and specificity[115,116] (Table 1).

Experimental evidence suggested that there are additional biological inducers of ferroptosis, but their significance in human disease is still unknown. As mentioned above, ferritinophagy is a special recycling process of autophagy for the autophagic degradation of ferritin in lysosomes. It is mediated by the autophagic cargo receptor NCOA4, and leads to the initiation of ferroptosis[117]. Augmented ferritinophagy mediated by an increase of NCOA4 leads to induction of ferroptosis[64] (Table 1).

Reduction of iron-response element binding protein 2 significantly reduced erastin induced ferroptosis[55]. Increased activity of HO-1, the enzyme responsible for degradation of heme into ferrous iron, carbon monoxide, and biliverdin, increased LIP and initiated ferroptosis[118,119]. Artesunate as a derivative of artemisinin, is used in severe malaria[120]. Artesunate induces hematopoietic stem cell (HSC) ferroptosis but

chloroquine (a ferritinophagy inhibitor) reverses this effect, implying that artesunate induces HSC ferroptosis by activating ferritinophagy[121] (Table 1).

Magnesium isoglycyrrhizinate (MgIG) is a natural product with anticancer activity[122]. MgIG promotes HSC ferroptosis. Inhibition of HO-1 reduces MgIG-induced HSC ferroptosis implying that promotion of HSCs ferroptosis implying that the promotion of HSCs ferroptosis is mediated through upregulation of this enzyme[123] (Table 1).

LIVER MACROPHAGES IN IRON METABOLISM AND FERROPTOSIS

Kupffer cells and other liver and spleen macrophages take up heme from damaged or senescent erythrocytes and either export the extracted Fe^{2+} using ferroportin or store it in ferritin in the cytoplasm[124]. It has been shown that intracellular iron regulates the polarization of macrophages into M1 (pro-inflammatory) and M2 (anti-inflammatory) subtypes[125,126]. M1 macrophages have an iron storage capability with higher Hamp but lower FPN and IRP1/2 compared to M2 subtype[127]. M1 polarization is regulated by iron overload[128] but also by ROS production and p53 acetylation induced by iron overload[129]. Recently, experiments with cultured macrophages demonstrated that chronic iron overload may in fact downregulate M1 markers and show signs of M2[130].

During infection, hepcidin blocks this polarization to reduce iron export that could increase the growth of pathogens[131] which is reversed in case of intracellular pathogens. This is possibly achieved by an increased production of nitric oxide[132] and the expression of the phagolysosomal protein NRAMP1 both leading to induction of ferroportin and intracellular iron reduction[133].

Kupffer cells exhibit phagocytic dysfunction impairing iron homeostasis in the development of non-alcoholic fatty liver disease (NAFLD)[134-136]. In addition, they participate in the clearance of lipids in nonalcoholic steato hepatitis (NASH) through M1 polarization and the help of invariant natural killer T cells[137-139]. This composite role indicates that Kupffer cells can influence the development of ferroptosis, providing a new target for therapy in NAFLD.

Moreover, acute iron deprivation led to changes in metabolic and immunoregulatory genes in human macrophages resulting in impaired cell proliferation and reduced inflammation[140]. This is in contrast to the pro-inflammatory production of leukotrienes by the enzyme 5-LOX mediated by ferric iron in human macrophages[141]. As expected, ferroptosis has been the subject of detailed reviews[69,142-145] including description of ferroptosis regulators[146,147], ferroptosis in viral disease[148] and the role of macrophages in ferroptosis[149].

IRON IN LIVER DISEASE

Patients with chronic liver diseases may have hepatic and splenic iron loading usually inside Kupffer cells and splenic and bone marrow macrophages[150]. Sometimes, this is accompanied by low hemoglobin levels and indices that are suggestive of hemolysis indicating that hemolysis may have a role in the development of secondary iron overload[151]. However, a recent review emphasizes the role of low levels of hepcidin in various liver diseases as implicated in both iron deposition in hepatocytes and participation in stellate cell activation and liver fibrosis[152].

An excess of free iron exerts a toxic effect on the liver, favoring the progress of liver diseases[58,153] and abnormalities of iron regulation are reported in various liver diseases apart from inherited haemochromatosis[154]. Hyperferritinaemia has been the main manifestation of disturbed iron homeostasis in chronic liver diseases[155,156].

Opposite views have also been expressed. Data from cell culture experiments and animal models suggest that iron overload is only a weak fibrosis inducer and rarely causes serious liver damage not supporting the concept that iron overload is an important cause of liver toxicity. Iron may co-exist with other causes of inflammation and the resulting hepatocyte necrosis is the real driving force leading to fibrosis[157].

The role of iron overload and the significance of ferroptosis have been investigated in several liver diseases. The most common liver diseases will be discussed as well as the common end point of all, namely cirrhosis and HCC. The rather limited available information on other liver diseases will be presented.

NAFLD/NASH

The role of iron in liver damage has been extensively researched in the case of NAFLD. A new term was introduced, the dysmetabolic or insulin-resistance hepatic iron overload syndrome (DIOS or IR-HIO) which is characterized by high serum ferritin levels, unexplained iron overload and is associated with metabolic abnormalities[158-161]. IR-HIO is detected in one-third to half of patients with NAFLD[155,158,162,163]. The reason for the iron overload in NAFLD is still uncertain. A proposed mechanism was the redistribution of transferrin receptors (TfRs) to the cell surface caused by insulin[158,163,164]. Tfr1 was upregulated in mice on a high fat diet which may enhance iron hepatocellular uptake in NAFLD despite already increased hepatocellular iron[165]. The increase in serum ferritin may be due to the increased iron stores, the oxidative stress caused by lipid abnormalities, the systemic inflammation and a genetic background[166,167]. The implication of the presence of the Cys282Tyr HFE gene variant of hereditary hemochromatosis was also examined. A heterozygous mutation was associated with bridging fibrosis or cirrhosis in Caucasians[168-170]. By contrast, in knock out mouse models of hemochromatosis no progression to steatohepatitis or liver fibrosis was noted after a high-fat diet[171].

In addition, certain variants of ceruloplasmin are associated with increased liver iron stores and high ferritin in patients with NAFLD. They had also advanced liver fibrosis[172,173]. Ceruloplasmin mutations have been associated with iron deposition in the liver of other chronic liver diseases as well[174]. Excess dietary iron causes hepatic oxidative stress, inflammation and hepatocellular ballooning injury leading to NASH[175,176]. Oxidative stress interferes with mitochondrial function impairing fatty acid oxidation and production of different pro-inflammatory factors such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, MDA and nitric oxide[177-180] leading to NASH. Moreover, liver iron deposition increases liver cholesterol synthesis, lipid accumulation and impairs cellular stress responses, which further exacerbate NAFLD[181-184].

The pattern of hepatic iron deposition is important in NAFLD patients. Iron deposition in macrophages is associated with more advanced disease[185]. An important observation was recently reported emphasizing the role of liver macrophages in the pathogenesis of NASH. A histological structure, the crown-like structure, has been described in NASH. Iron-rich Kupffer cells surround dead hepatocytes, take up debris and induce inflammation and fibrosis. They have proinflammatory and profibrotic phenotypes, driving liver fibrosis[186]. The liver iron was significantly higher in patients with HCC associated with NASH and it was mostly localized in Kupffer cells[187]. Evidence suggested that iron may contribute to NAFLD pathogenesis and fuel the progression to NASH[178-180]. There are other factors associated with iron deposits in NAFLD.

Red cell fragility and erythrophagocytosis may also explain iron deposition in NAFLD. It could be the result of insulin resistance and membrane lipid abnormalities[188]. Recently, aristolochic acid-associated (atypical antipsychotic medications) were reported to induce NAFLD and link insulin resistance with iron metabolism dysregulation irrespective of drug-associated weight gain[189]. However, whatever the reason of iron deposition might be, the consequences are well documented.

Hyperferritinemia is frequent in patients with NAFLD. Sometimes, it is the first laboratory abnormality leading to further investigations[190]. In a large prospective population-based study from South Korea, serum ferritin was a strong early predictor of future development of steatosis indicating that the ferritin association with NAFLD is not a simple consequence of the disease itself[191]. Patients with high ferritin have more severe steatosis[192,193], presence of inflammation[194], advanced fibrosis[195] and increased mortality[196,197]. It was suggested that serum ferritin could be used as a marker to identify NAFLD patients likely to have NASH and fibrosis[166]. However, a clear association between serum ferritin and fibrosis could not be verified by others[198]. They reported that ferritin could not accurately predict advanced fibrosis in NAFLD[199,200]. The discrepancy may be explained by the findings of a recent investigation.

Hyperferritinemia was found in a quarter of NAFLD patients. Stainable iron was present in hepatocytes, Kupffer cells or more frequently in both. Importantly, serum ferritin was not related to the presence of NASH, but it increased with worsening of fibrosis and decreased in the cirrhotic stage[201].

Iron measurement by magnetic resonance imaging demonstrated that liver iron was the most important determinant of serum ferritin in NAFLD[202]. An important association of serum ferritin with the gut microbiome, was recently reported. Ferritin levels were associated with differences in gut microbial composition. Both negative and positive associations with particular microbial species were found and ferritin related bacterial species correlated with liver iron related genes. Moreover, the iron associated microbiome was also linked to liver fat load. Fecal transplantation from high ferritin mice to normal mice confirmed the human results and demonstrated an interplay among iron load, liver fat and gut microbiome, that could be exploited in future treatments[203].

Hepcidin in NAFLD

As in other liver diseases, extensive research was conducted on the possible role of hepcidin in NAFLD. Investigations tried to identify if the reported hepcidin abnormalities were the cause or the result of the iron overload observed in many cases of NAFLD. Hepcidin was demonstrated to be either increased or decreased in NAFLD. In obese individuals, adipose tissue expression of hepcidin was upregulated, irrespective of steatosis and NASH. The contribution of adipose tissue hepcidin to the serum hepcidin is not well studied, but it may potentially explain the increased serum hepcidin in NAFLD[182,204-207].

Furthermore, leptin was found to correlate with hepcidin levels in obese children. Leptin also upregulated hepcidin transcription in hepatocyte cultures indicating that hepcidin increase follows the leptin abnormalities in NAFLD[208,209]. Hepcidin downregulation, on the other hand, may be a consequence of oxidative stress secondary to iron overload[158-160,208,210]. Experimental evidence demonstrated that hepcidin down

regulation is a secondary phenomenon appearing after deposition of iron in the liver and the concomitant increase of oxidative stress[211]. Furthermore, an investigation on the relationship between iron stores and cardiovascular damage in patients with NAFLD, showed that ferritin was associated with the components of the metabolic syndrome but not with liver inflammation and damage. Hepcidin was increased due to the increased iron load[198]. Fat in the liver of mice increased the expression of the BMP binding endothelial regulator, which was produced in the sinusoidal endothelial cells and inhibited the BMP-SMAD pathway leading to a secondary inhibition of hepcidin. This is an additional explanation for the iron deposition in NAFLD[212].

Clinical data also indicate that hepcidin abnormalities are not the primary cause of the excess iron in the liver. HJV levels were low in NAFLD patients particularly in iron overloaded NAFLD and hepcidin levels were higher in high iron NAFLD. These findings reflect the effect of the results of iron accumulation as the primary event[213].

Individuals with the metabolic syndrome preserve the iron regulatory control of hepcidin and hepcidin progressively increased in response to the increase of iron stores[205,214-216]. In addition, serum hepcidin and HAMP mRNA in liver correlate to body iron stores irrespective of the degree of iron deposition. Thus, the DIOS syndrome seen in NAFLD is not related to an altered hepcidin synthesis[217].

However, despite the elevated serum hepcidin, duodenal iron absorption is increased because DMT1 is upregulated by the IRP1 activation probably due to as yet unidentified humoral factors in sera of NASH patients[218]. It seems therefore, that elevated hepcidin in NAFLD is either a reflection of hepatocellular inflammation in NASH, or that increased iron and the induction of hepcidin appears before the development of NAFLD or NASH[219].

So far, data suggests that the interplay between iron and lipid metabolism is multi-faceted in NAFLD. Moreover, it could be suggested that iron is directly implicated in NAFLD pathogenesis. The reports that increased dietary iron from red meat may predispose to type II diabetes and insulin resistance in humans, are supportive evidence for such an idea[220-222].

Contrasting results were also reported. An inadequate hepcidin production for a given level of iron load in NAFLD patients compared to controls has been reported[159]. An impairment in the ability of hepcidin to inhibit iron absorption was demonstrated in DIOS, suggesting hepcidin resistance in this condition[223]. The description of ferroptosis prompted new investigations on the effects of liver iron load in NAFLD although its exact role has not been clarified so far.

Ferroptosis was recently related to the induction of inflammation in the early stages of NASH being possibly the first hit[98]. Further studies indicated that ferroptosis play a critical role in the progression of NASH being a promising treatment target[224,225]. The enzyme arachidonate 12-LOX was found to promote the progression of NASH[226,227]. In that context it was demonstrated that arachidonic acid metabolism could trigger ferroptosis in a diet-induced NASH mice model[224]. Furthermore, the content of the central regulator of ferroptosis ACSL4 was increased in a rat NASH model and inhibition of the Mfn2/IRE1αACSL4 pathway could prevent occurrence and development of NASH[228] but the connection of NAFLD with ferroptosis is still debatable. Reviews on Iron and NAFLD pathogenesis have been presented[229-231].

ALCOHOLIC LIVER DISEASE

Early reports showed that stainable iron was present in the livers of alcoholics[232,233]. Hepatocyte iron deposition was considered as an important feature of alcoholic liver disease (ALD), although stainable iron in Kupffer cells was more prominent, particularly in the advanced stages of disease[234]. Ethanol consumption triggers iron overload[235]. It was shown in patients with ALD, that ethanol increased hepatocyte iron uptake by hepatocytes from circulating de-sialylated transferrin[236].

Almost half of the patients with ALD have HIO[237] with high values of plasma ferritin and transferrin saturation[238,239]. Drinkers from an early age have increased iron markers[208,240]. High liver iron was found to be predictive of HCC development or death in patients with alcoholic cirrhosis[241,242] sometimes acting synergistically with diabetes mellitus and viral hepatitis[243,244]. Ethanol is metabolized into acetaldehyde,

forming DNA and protein adducts that predispose to HCC[103]. Iron is directly implicated in HCC development since it accumulates in lysosomes through ferritinophagy and then into the cytoplasm as free iron[245]. The resultant production of free radicals through the Fenton reaction initially activates Kupffer and stellate cells ultimately leading to ferroptosis[246]. Additional significant production of ROS is mediated by CYPE1 which is directly induced by alcohol[247]. Alcohol consumption results in up to 20 folds increase of CYP2E1[248]. Mechanisms of alcohol induced HCC were reviewed[249].

The reason for the increased iron load in the liver of patients with ALD prompted the research on hepcidin regulation in ALD. Suppression of hepcidin transcription by ethanol was reported in cell cultures and experimental animals, possibly by inhibiting C/EBPa[250-253]. Iron induces activation of C/EBPa but ethanol inhibits this action leading to ineffective hepcidin[254]. Suppression of the BMP6/SMAD pathway by alcohol was also reported[255]. Hepcidin downregulation is also mediated by the induction of oxidative stress caused by either ethanol itself or free iron. Antioxidant treatment attenuates hepcidin downregulation. Ethanol may additionally increase hepatocyte iron uptake upregulating the expression of TfR[246] even in habitual drinkers[256]. Ethanol might also reduce hepcidin through proteins involved in liver regeneration but this should be further researched[257].

Ethanol exposure simultaneously increase the expression of DMT1 and FPN in the duodenum[254] and this has been linked to liver fibrosis[254,258]. Iron absorption was two-fold enhanced in chronic alcoholics[259]. Ethanol administration in mice model overexpressing adipose tissue lipin-1, accelerated iron accumulation followed by lipid peroxidation, reduction of GSH and induction of ferroptotic liver damage[260].

The effect of ethanol in hepcidin seems to be more complex than previously thought[261]. Ethanol was shown to increase transforming growth factor (TGF)-β expression and phosphorylation of SMAD2[262]. Increased activation of SMAD2/3 can abrogate the TGF-β-induced hepcidin upregulation[263]. Hepcidin is also suppressed by ethanol through the toll-like receptor 4 (TLR4) pathway. Ethanol did not suppress

hepcidin in TLR4 receptor mutant mice[264]. Interestingly, TLR4 deficiency protected animals from liver fibrosis[265,266]. Evidence suggests that ethanol action on TLR4 involves HSCs as both Kupffer cells and hepatocytes are unlikely candidates[267,268].

Both serum transferrin and serum hepcidin have been used as prognostic markers in ALD. Thus, low transferrin[269,270]were associated with worse prognosis[197,270-272]. Importantly, the prognostic value of serum transferrin was similar to other traditional prognostic scores like the model of end-stage liver disease (MELD) and the Glasgow alcoholic hepatitis score[273].

The recent identification of ferroptosis allowed for a better understanding of the connection between lipid and iron abnormalities observed in ALD[274]. Ferroptosis was downregulated during the repair of ethanol-induced liver damage, while a ferroptosis inhibitor or activation of Nfr2 pathway, reversed ROS accumulation and lipid peroxidation induced by ethanol[275,276]. Excessive ethanol activates genes like frataxin capable of liver injury induction[277]. More importantly, ferroptosis provided a strong link for the recently demonstrated crosstalk between the liver and the gut[278]. Lack of intestinal sirtuin 1 limited ferroptosis, normalized iron overload and ameliorated ethanol-induced liver damage[279]. Ferroptosis was also implicated in adipose-liver axis abnormalities observed in alcoholic steatohepatitis[260]. The overexpression of adipose specific lipin1 aggravated alcoholic steatohepatitis and iron deposition, increasing hepatic MDA levels[153,260].

Finally, an additional mechanism of ethanol induced liver damage was identified in severe alcoholic hepatitis patients. Iron overload triggers the activation of the metallopeptidase ADAM 17 which eventually increases TNF-α and soluble CD163, resulting in macrophage activation and promotion of inflammation of the liver[280]. Detailed reviews on iron and ALD were recently published[143,281,282].

CHRONIC HEPATITIS C

The effect of iron on hepatitis C virus (HCV) has been controversial. Inhibition of viral replication by iron due to the suppression of the nonstructural protein 5B has been

reported[283] as well as enhancement of replication[284]. HCV alters the expression of hepcidin and therefore the cellular iron metabolism[285,286]. Experimental evidence in the early phase of HCV infection showed an increased hepcidin expression followed by enhanced viral translation and replication. Interestingly, iron loading of macrophages accompanying hepcidin upregulation, resulted in induction of viral transmission to naïve cells[287]. Other experimental studies however, showed that hepcidin was low in HCV infected cell lines[288,289].

Inhibition of hepcidin expression was attributed to HCV-induced oxidative stress[290,291]. Experiments in chimpanzees on high iron diets demonstrated that liver damage was observed only in animals infected with HCV indicating a harmful effect of iron in HCV infection[292]. In the chronic phase of the infection, HCV interferes with the expression of the iron uptake receptor TfR1, a proven entry factor of HCV internalization[293,294]. The observed downregulation of hepcidin despite hepatic inflammation in chronic HCV[295] might be due to impairment of the BMP6/HJV pathway by TNF-α, that would suppress the transcription of HJV[296].

Clinical studies verified that HCV infection downregulates hepcidin[297-299] and serum hepcidin has been associated with the severity of liver disease[300]. More than 40% of patients have iron overload associated with a high rate of liver damage, inflammatory activity and an increased risk of hepatocarcinogenesis[301-303]. Hepatic iron and HCV proteins in combination, produce the toxic hydroxyl radical (·OH) that forms mutagenic bases such as 8-hydroxy-2-deoxyguanosine (8-oxodG)[304,305]. HCV patients had approximately a 10-fold increase of 8-oxodG in liver tissue compared to non-HCV control patients[306].

The hepatitis C antiviral long-term treatment against cirrhosis trial has convincingly demonstrated that iron in hepatocytes and portal tract cells predicts progression to decompensated cirrhosis, HCC and finally death[307]. Almost all liver tissues from HCV patients had some lysosomal iron deposits detected by electron microscopy and X-ray microanalysis, despite negative results on the classical Prussian Blue staining[308].

Increased serum aminotransferases were found only in HCV patients with stainable iron in the Kupffer cells but not in those with hepatocyte iron[309].

Even minor increases in iron load in heterozygous carriers of C282Y or H63D gene mutations for hemochromatosis were found to induce more fibrosis in chronic HCV infection[310,311]. Genotype 3-infected patients had more frequently elevated liver iron which was associated with hepatic steatosis in genotype 3 infection[312]. Thalassemia patients further indicate that iron is adversely implicated in the progress of HCV increasing morbidity and mortality due to more severe liver disease[313].

Liver iron adversely affected the response to interferon (IFN)-based treatments[314]. Thus, in IFN-treatment, ferritin levels increased regardless of sustained virologic response (SVR) and decreased at about 3 years post-treatment. This is not the case with direct-acting antivirals (DAAs)[315,316] where SVR is achieved irrespective of iron status[317-320]. A recent study demonstrated that elevated serum ferritin and ERFE levels before DAAs treatment were restored after treatment and correlated with changes of LDL cholesterol levels, but only in men[321].

Plasma ferritin, liver iron and transferrin saturation are also increased[322] and elevated serum ferritin has been related to liver fibrosis[323]. An additional reason for increased liver iron in HCV patients is the reported increased hemolysis particularly in advanced stages of the disease[151]. Despite the evidence presented above, different results in relation to the role of HCV-induced iron overload were presented[324,325]. Elevated serum ferritin and iron within the liver played no significant role in the progression of liver damage[326,327]. Moreover, the significance of hemochromatosis mutations has been questioned as a risk of HCV disease[328].

Recently, it was suggested that ferroptosis may be implicated in the natural course of HCV[58]. Importantly, HCV replication was inhibited by an iron-dependent mechanism like ferroptosis, which was mediated by the desaturation of oleate to highly unsaturated fatty acids by the enzyme fatty acid desaturase 2 (FADS2.) This is a key determinant of cellular sensitivity to ferroptosis. FADS2 suppression significantly enhanced HCV

replication, whereas the ferroptosis inducer erastin sensitized HCV to DAAs altering the conformation of the HCV replicase[329].

CHRONIC HEPATITIS B

Iron favors hepatitis B virus (HBV) mRNA expression in HepG2 cells[330]. Increased serum and cellular iron uptake and decreased hepcidin expression have been reported in HBV infection[297,331]. Hepatitis B infected patients frequently show iron deposition in hepatocytes and elevated liver iron concentration (LIC) leading to increased disease severity[332,333]. Serum ferritin levels are also increased in patients with chronic HBV[332].

Levels of hepcidin are increased in early stages of HBV and reduced in the cirrhotic stage[334,335]. Co-infection with hepatitis D increases the iron load[332]. However, results on serum hepcidin are not uniform. Reduced levels were reported in patients with or without cirrhosis[336] while another report found that hepcidin was slightly increased in patients without cirrhosis and those with HCC[335]. The reason for the discrepancy is not clear. Nonetheless, decreased hepcidin levels and elevated transferrin saturation and ferritin levels were associated with fibrosis severity in patients with chronic HBV[337].

It should be noted that iron deposition in the liver was considered a secondary phenomenon. Damaged hepatocytes during viral hepatitis undergo necrosis and the released iron is scavenged by Kupffer cells[240,338]. However, this proposition cannot account for the deposition of iron in the hepatocytes. The implication of HBV in iron deposition is exemplified in a case report. A female patient with symptoms of iron overload had highly increased serum ferritin and transferrin saturation. All symptoms disappeared and all iron abnormalities normalized after HBV antiviral treatment as the sole therapy[339].

LIVER FIBROSIS AND CIRRHOSIS

Nearly 6 decades ago, it was shown that iron on liver biopsies was associated with manifestations of advanced disease compared to non-iron loaded cirrhotics[340], an observation later confirmed. Cirrhotics with hemosiderosis are more likely to be

classified as Child class B or C with higher MELD scores than cirrhotics without stainable iron[341,342].

As mentioned before, hyperferritinemia and high liver iron predict the risk of advanced liver fibrosis in NAFLD[166,179,343]. A recent study of a large number of NAFLD patients and a long follow-up of a mean of 8.4 years emphasized the fact that it is the non- parenchymal iron deposition that leads to serious liver disease[344].

Fibrosis is increased by iron through increased HSC-cell proliferation and selectively increased collagen synthesis without interference with non-collagen proteins[345,346]. Experiments with cultured HSCs showed that incubation with either ferritin or transferrin increased nuclear factor kappa-B translocation and HSCs activation[347,348] and enhancement of α-smooth muscle actin, collagen and vimentin synthesis[349]. Isoprostanes, the peroxidation products of arachidonic acid produced during the iron-induced oxidative stress, increased HSC-collagen-production and TGF-β release from the Kupffer cells[350]. Furthermore, 4-HNE upregulated the expressions of collagen and the TIMP-1 inhibitor of metalloproteases in HSCs[351].

Elastin, another component of extracellular matrix is affected by iron. Elastogenesis is modulated in cultured human skin fibroblasts by iron as the levels of both elastin protein and elastin mRNA levels are increased 3-fold[194]. Liver iron load induces both, TGF-β[352] and BMP-6[353,354]. The connection between the fibrosis and hepcidin pathways and the significance of SMAD4 as the common link between the two pathways have been demonstrated[353]. Other signaling pathways related to fibrosis are modulated by iron. Iron deficiency stimulated Notch signaling[355]. Recently, iron-loading revealed a protective role of β-catenin (component of cadherin complex that stimulates Wnt signaling) against liver fibrosis[356]. Hepcidin has also a protective role in liver fibrosis suppressing HSCs activation[357]. BMP6, the main hepcidin inducer, has a similar protective role in fibrosis inhibiting HSCs activation[358]. Evidence on the role of ferroportin in liver fibrosis is limited. Ferroportin is increased in activated HSC and the anti-fibrotic action of hepcidin in HSC mentioned before may be mediated by degradation of ferroportin[357].

Clinical evidence confirms the importance of iron metabolism in the development of fibrosis. Ferritin levels were associated with progress to decompensation and increased mortality in cirrhosis[359]. However, ferritin concentration has a poor sensitivity as a marker of liver fibrosis, since it also increases as a result of inflammation[360]. Transferrin has also a clinical significance in HCV- and HBV-related cirrhosis. Transferrin was associated with advanced fibrosis and as a predictor of survival in cirrhotic patients[269,301,338]. Low hepcidin can cause iron overload and increased oxidative stress in liver[361] which in combination with other factors such as genetic, viruses and alcohol can eventually lead to liver fibrosis[362].

Low values of hepcidin are a predictor of mortality and development of HCC in alcoholic cirrhosis[258,363]. Similarly, in HBV cirrhosis hepcidin is low compared to patients without cirrhosis[335,364] where values where similar to healthy controls[335,365]. In HCV-related cirrhosis and alcoholic cirrhosis hepcidin was significantly lower than in HBV cirrhosis[365-367].

Hepcidin is not reduced in the early stages of NAFLD, but eventually drops in advanced fibrosis, similar to other liver diseases[368]. Unlike ferritin[369], serum hepcidin is a reliable marker of fibrosis stage and severity of fibrosis in NAFLD[368,370]. A low hepcidin/ferritin ratio can differentiate between cirrhosis and non-cirrhosis in patients with HBV, HCV and NAFLD[367] but not in ALD patients possibly because ethanol directly inhibits hepcidin expression as mentioned above.

Ferroptosis

Inevitably, the role of ferroptosis in liver fibrosis was recently investigated. Its role is debatable as both induction and attenuation of liver fibrosis has been reported. Ferroptosis increased the susceptibility to fibrosis in mice on a high-iron diet. The effect was reversed by a ferroptosis inhibitor[12]. However, other studies showed that ferroptosis attenuated HSC activation and reduced liver fibrosis. The ferroptosis inducers erastin and sorafenib reduced liver fibrosis increasing ferritinophagy[371]. A natural product, MgIG, increased ferroptosis leading to reversion of fibrosis[123]. The

anti-malarial agent Artemether increased the p53-dependent ferroptosis and inhibited HSC activation[372]. In this context, artesunate, a derivative of artemisinin with immunomodulating properties, induced ferroptosis of activated HSCs possibly triggering ferritinophagy[121]. The iron implication in liver fibrosis has been recently reviewed[152,373].

HCC

Liver iron overload has long been linked to HCC tumorigenesis and tumor growth[147,374-376]. Iron incubation of an HCC cell line increased mesenchymal and metastatic markers, a fundamental defect in cancer development[377]. Patients with hereditary haemochromatosis showed a 20-200-fold increased risk of HCC development[378,379]. An iron score was significantly higher in HCC-NASH patients than in NASH controls. In HCC patients iron localization was mainly sinusoidal[187]. Importantly, iron deposition in the portal tract was associated with poor survival of HCC patients after curative resection[380]. Similar findings were reported in a prospective study of HCC in alcoholic cirrhosis[241] and in HCV-associated cirrhosis[381].

Several studies suggested an association between HCC and dietary iron overload from beer fermented in steel drums in black Africans[382-385]. Experimental evidence identified several mechanisms of iron involvement in HCC development. HCC cells like many other cancer cells upregulate iron uptake and intracellular iron accumulation since cancer cells are dependent on iron[386,387]. The generation of ROS by iron favors carcinogenesis through genomic instability, and DNA repair defects[388,389]. ROS maintain the oncogenic phenotype of cancer cells[390,391]. The direct hepatocarcinogenic effect of free iron in the pathogenesis of HCC was demonstrated in an animal model on iron-rich diet where the tumor developed without fibrosis or cirrhosis[392,393]. Iron deposition directly decreased p53 protein level and its activity in the liver, facilitating thus the development of HCC[394]. An important mediator of intracellular iron is the protein leucine-rich repeat protein 5 (FBXL5). Exposure of FBXL5 knockout animals to chemical or viral carcinogens resulted in an increased liver tumor formation. More

importantly, low levels of FBXL5 in HCC patients were associated with a poor prognosis[395]. Ferritin heavy chain (FTH) acts as a protector of HCC cells increasing their cellular resistance to ferroptosis. FTH therefore, acts as an oncogene in the genesis and progression of HCC[396].

HCC patients in contrast to other cancers have low hepcidin levels[397-399]. Many mechanisms lead to the final decrease of hepcidin in HCC, including downregulation of inducers such as HAMP, TfR and HJV and upregulation of suppressors such as matriptase 2 and GDF15[400]. Hepcidin downregulation increases cellular proliferation and HCC risk due to reduction of the hepcidin protection against HSC activation. The downregulation of hepcidin in HCC is attributed to the effects of cirrhosis rather than to HCC itself. Cirrhotic patients also show decreased hepcidin expression irrespective of disease etiology[152,336,399], while the hepcidin:ferritin ratio has been reported to decrease with fibrosis progression[373].

Ferroptosis and its inducers have been extensively investigated in HCC as it is considered an effective tumor suppression mechanism[81,401-403]. On the other hand, genes negatively regulating ferroptosis increase HCC drug-resistance[404]. Sorafenib, a drug used for treatment of advanced HCC is an example. The drug can induce the expression of metallothionein-1G (MT-1G). Upregulation of MT-1G is a negative regulator of ferroptosis and confers resistance to sorafenib[405]. Some studies found that haloperidol can facilitate the cascade of ferroptosis induced by sorafenib in HCC[406].

In contrast to the negative regulators of ferroptosis, ACSL4 can positively regulate ferroptosis in HCC[97]. Inhibition of ACSL4 protects sorafenib-induced ferroptosis in HCC cells. A human study demonstrated an upregulation of the ACSL4 protein in HCC tissue from surgical specimens with a good response to sorafenib as a postsurgical adjunct treatment[407]. ACSL4 can therefore serve as a prognostic factor for survival and disease-free survival time[407,408]. Natural omega-3 PUFAs are the main peroxide substrates in ferroptosis and have anti-tumor activity[409], a fact that has been therapeutically exploited[410]. PUFAs consumed in the form of fish can reduce the risk of HCC development[411]. Ceruloplasmin inhibited ferroptosis in HCC cells interfering

with iron metabolism. Inhibition of ceruloplasmin increased the accumulation of iron and ROS production facilitating erastin-induced ferroptosis in HCC cells[412].

Additional regulators of ferroptosis in HCC are the long non coding RNA molecules (lncRNAs) but their role has not been elucidated[413]. Erastin-induced ferroptosis upregulated the lncRNA GABPB1-AS1 in HepG2 cells, silencing the gene encoding peroxiredoxin-5 peroxidase eventually leading to a reduction of the cellular antioxidant capacity[414]. The predictive value of lncRNAs associated with ferroptosis in HCC was recently addressed. A nine and a five ferroptosis signature models were established which identified two groups of patients. The high-risk group had enhanced tumorigenesis and worse prognosis[415,416].

Equally, the non-coding circular RNAs (circRNAs) seem to play a role in the development of HCC through ferroptosis. The circ0097009 endogenous RNA regulates the expression of SLC7A11, a key regulator of cancer cell ferroptosis, in HCC. Circ0097009 therefore, may be used as a potential target for HCC treatment[417].

Furthermore, ferroptosis-related genes (FRGs) were identified and found upregulated in HCC tissues. Three clusters were determined. A high expression of cluster 3 was associated with the worse prognosis and a higher histological stage[418]. A different approach on the use of ferroptosis as a prognostic marker in HCC was recently presented. A novel ferroptosis-related ten gene signature stratified HCC patients into two risk groups[419]. Those in the high-risk group had significantly reduced survival. The role of ferroptosis in HCC generation and progress has been recently reviewed[420].

CHOLESTATIC DISEASES

Hepcidin was significantly lower in patients with primary biliary cholangitis and primary sclerosing cholangitis compared to patients with other chronic viral and metabolic liver diseases. Low hepcidin was maintained even after two years of treatment[421]. The reason for low hepcidin is probably the suppression of STAT3 phosphorylation by accumulated bile acids. Hepcidin remains lower in cholestatic

cirrhosis compared to non-cholestatic cirrhosis, indicating a critical role of cholestasis in low values of hepcidin[422].

AUTOIMMUNE HEPATITIS

There is experimental evidence suggesting that iron is implicated in autoimmune hepatitis (AIH) through ferroptosis involvement. The classical AIH-inducer, Concanavalin A (ConA), showed an overproduction of reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite in a mouse model of AIH. This effect was attenuated by Fer-1, indicating that ConA induced ferroptosis in the liver. Moreover, gadolinium chloride (a Kupffer cell depleting agent) inhibited RNS and hepatocyte ferroptosis[423]. Indoleamine 2,3-dioxygenase 1 (IDO1) is an intracellular heme enzyme involved in autoimmune diseases[424]. IDO1 upregulation is also involved in ConA-induced hepatocyte ferroptosis through RNS accumulation and hepatocyte ferroptosis. An IDO1 inhibitor and an IDO1 knockout suppressed this effect indicating that that IDO1 promotes hepatocyte ferroptosis by triggering nitrative stress[425].

Clinical evidence also supports the detrimental effect of iron in AIH. Ferritin and iron are increased in serum of 65% and 58% of naïve patients with AIH which is resolved after successful treatment[426]. Increased serum ferritin was independently associated with advanced fibrosis in patients with untreated AIH[427]. Moreover, serum hepcidin was low in patients with liver autoimmune disease[367,421]. Interestingly, in AIH, low serum hepcidin levels remain after 2-year treatment, similar to observations in autoimmune cholestasis. A plausible explanation could be that hepcidin is involved in the liver autoimmune process[428].

ISCHEMIA-REPERFUSION INJURY

Although ischemia-reperfusion injury (IRI) is not strictly a liver disease as it occurs in other organ transplantations, iron is clearly involved in the pathogenesis of hepatic abnormalities. Ferroptosis is implicated in the pathogenesis of IRI through GPX4 inactivation[59,429]. Iron overload and upregulation of the ferroptosis indicator PTGS2 are

prominent characteristics of IRI in the liver[59]. An analysis of the clinical data of 202 live-donor liver transplantations, showed a high serum ferritin level indicating iron overload[430]. Use of ferroptosis inhibitors such as Fer-1, α-tocopherol, and DFO prevented hepatic IRI.

ACUTE LIVER FAILURE

Ferroptosis is also involved in the development of acute liver failure (ALF). In sepsis-induced ALF, analysis of the liver infiltrate revealed that FRGs may be responsible for the development of liver failure through B cells and natural killer cells[431]. The commonest reason for ALF however, is acetaminophen (APAP) toxicity where lipid peroxidation leads to hepatocyte ferroptosis and ALF[432].

GSH is important for the inactivation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) implicated in APAP. GSH reduction and GPX4 inhibition are common in APAP-induced cell death[433]. The viability of mouse hepatocytes in the presence of APAP was improved by fer-1 without restoring the cellular GSH level, an indication that suppression of conversion from APAP to NAPQI was not the reason for the protective effect of fer-1[434]. Consistently, other experiments confirmed the implication of ferroptosis in APAP-induced hepatocyte cell death[432,435-437]. An additional mechanism of APAP-induced ferroptosis is the significant hepcidin reduction probably through activation of HIF1a[434,438-440].

However, the ferroptosis participation in APAP and other drug-induced liver injury is disputed. An earlier report showed that α-tocopherol did not improve APAP-induced liver injury and lipid peroxidation was not involved in APAP hepatotoxicity[441]. A recent review suggested that APAP-induced hepatotoxicity should be identified as programmed necrosis and not ferroptosis or other types of cell death[442]. Therefore, more research is required before ferroptosis inhibitors are recommended as treatment of APAP toxicity.

SICKLE CELL LIVER DISEASE

Sickle cell liver disease (SCD) is an inherited disease caused by the presence of hemoglobin S. Under hypoxic conditions, red blood cells are dehydrated and form the characteristic sickle cells[443,444]. The formation of hemoglobin S is due to a single substitution of an amino (glutamic acid to valine) in the beta-globin chain[444]. Viral hepatitis and iron overload are the two reasons for liver disease development in SCD, both related to multiple transfusions[445]. The source of liver iron in SCD is mainly blood transfusions and intravascular hemolysis[446]. Liver iron deposition occurs mainly in the Kupffer cells[447]. Liver iron deposition can occur in non-transfusion dependent patients[448], and there is a single case of a patient who never received blood transfusions[449]. Hemosiderosis may lead to fibrosis, and overt cirrhosis[445,448,449].

CORONAVIRUS DISEASE 2019

There is considerable evidence to suggest an association between ferroptosis and coronavirus disease 2019. Cytokines produced during the infection, upregulate hepcidin expression and subsequently ferroportin suppression leading to iron accumulation. In addition, severe acute respiratory disease coronavirus 2 downregulates the expression of GPX4, contributing further to the initiation of Fenton reaction and production of massive amounts of ROS and ferroptosis[450].

TARGETING IRON

There have been many attempts to reduce iron overload, which is uniformly considered harmful for liver diseases irrespective of etiology. However, it should be remembered that iron loading is not similar between patients and between stages of various diseases[152]. Dietary iron restriction was effective in reducing liver fibrosis and steatosis in diet-induced NAFLD animal models[451,452].

Phlebotomy was the traditional treatment in hereditary haemochromatosis. It was shown to increase erythropoiesis, reverse liver fibrosis to some extent and restore life expectancy[453,454]. Phlebotomy was used to treat NASH patients but the results and the clinical benefit are contradictory[455]. Phlebotomy improved liver enzymes, insulin

resistance and liver histology in the majority of NAFLD patients, but it was not fully successful in DIOS insulin resistant patients with slight ferritin increase[182,456-458]. Insulin sensitivity was improved by phlebotomy in type II diabetics with a high serum ferritin[459]. Moreover, in patients with the metabolic syndrome, phlebotomy improved metabolic parameters, including glycosylated hemoglobin A1c and LDL/high-density lipoprotein ratio[460]. Four interventional studies with more than 400 patients were analyzed in a meta-analysis. It was shown that phlebotomy improved liver enzymes, insulin resistance and the lipid abnormalities[461].

In contrast, no effect was reported in two prospective randomized controlled trials. The first, which is the largest series so far, was conducted in NAFLD patients[462], and the second in DIOS patients with insulin resistance[463]. Thus, it is probably illogical to use phlebotomy in patients with NASH until more extensive studies are available[464].

Phlebotomy reduced the marker of oxidative stress 8-hydroxy-2'-deoxyguanosine in HCV patients who had failed IFN therapy. Fibrosis and inflammation were also reduced but HCV titers were unaffected. None of the patients developed HCC after six years of follow-up[306,465]. Reduction of HCC development in HCV patients after phlebotomy was verified in additional studies[466,467]. Phlebotomy was also reported to improve the response to IFN in chronic HCV[468].

Iron chelation is an additional intervention to reduce liver iron. DFO was successfully used to control fibrosis in hemochromatosis[469]. Studies in several animal models revealed that iron chelation decreased the stability of procollagen mRNA[470], and reduced elastin mRNA[194]. DFO also reversed HSC activation, and induced apoptosis of activated murine HSCs[471]. More recently, a combination of DFO with pegylated IFN-α showed a synergistic anti-fibrotic effect in rats[472]. ROS degrade the apolipoprotein B100 (apoB100) component of VLDL enhancing thus hepatocyte steatosis in rodents. DFO restored apoB100 and increased VLDL secretion[473]. No firm conclusions can be drawn however, without the results of clinical trials. It should be noted that inhibition of hemoxygenase-1 decreased hepatic iron deposition and attenuated liver fibrosis in rats[474].

Interestingly, commonly used drugs like the calcium channel blockers were found to induce HSCs apoptosis and reduce DMT1 expression, hepatic iron deposition and liver collagen in mice and cellular experiments[475]. Hepcidin may be a promising agent for the treatment of liver iron overload. Hepcidin administration attenuated iron deposition in mice models of hemochromatosis[476-478] while its expression ameliorated fibrosis severity. This is due to the inhibition by hepcidin of the TGFβ1-induced SMAD3 phosphorylation in HSCs, a pathway that requires the presence of ferroportin in stellate cells[357]. Similar reduction of liver fibrosis was observed with BMP6 overexpression in murine and human NAFLD[358].

Hepcidin responds to iron conditions in HCV patients, but the response is impaired. Response is relatively limited. Thus, correction of hepcidin regulation may improve the clinical progress in iron-overloaded HCV patients[479]. Hepcidin manipulation may be beneficial in the management of HCC as well. The iron chelator deferasirox induced apoptosis in hepatoma cells lines and decreased liver tumor development in mice increasing HAMP mRNA expression. However, toxicity and the lack of response in some patients may be a problem in human trials[480]. Additionally, some HCC patients have increased hepcidin expression and downregulation of hepcidin may be required. In a murine HCC model with high liver hepcidin, the traditional Chinese medicinal herb dandelion polysaccharide reduced hepcidin expression. It also arrested cell cycle and suppressed the proliferation of HCC cell line[481]. It is therefore, a logical candidate for clinical trials in HCC.

Hepcidin agonists or inhibitors have been tested *in vitro* and in laboratory animals that could be potential treatment for hepcidin dysregulation in liver disease[482]. It should be noted that synthetic mini-hepcidins were designed and already tested in Hamp-/- mice. Serum iron was reduced after chronic administration of this mini-hepcidin[476].

Ferroptosis is the current therapeutic target in the treatment of iron overload diseases. It should be stressed however, that the effects of ferroptosis in chronic liver diseases depends on the cell type and the specific environment. In liver fibrosis for example,

ferroptosis has different effects on hepatocytes and HSCs as will be detailed later[483]. A future challenge is to develop drug delivery systems targeting ferroptosis in specific cell types. In ALD and in NAFLD, ferroptosis is implicated in liver damage, and ferroptosis inhibition would be beneficial[225,276,432]. Ferroptosis-induced liver injury could be reversed by sestrin 2, an antioxidant protein increased by ferroptosis inducers[484].

Ferroptosis inducers

In contrast to other liver diseases where ferroptosis is detrimental and therapies are directed towards inhibition of ferroptosis, HCC is benefited by enhancement of ferroptosis. Thus, ferroptosis inducers are used in advanced HCC. Sorafenib, a multi-kinase inhibitor, is the ferroptosis inducer most extensively studied[103,485]. In HCC acts by inhibiting cellular proliferation and neo-angiogenesis. Additionally, it induces ferroptosis in HCC cells[486]. It has been reported that sorafenib decreases the uptake of cystine in the Xc- system and starts the chain of events leading to the ferroptosis induction through the accumulation of ROS which is the result of GSH depletion and loss of GXP4 activity[487]. Excessive ROS production also results in the inhibition of the retinoblastoma protein Rb, an important negative regulator of cell proliferation[488].

Prolonged administration increases the resistance of HCC cells to sorafenib. ABCC5, a recently described regulator of ferroptosis, increased the generation of GSH and reduced the production of ROS through stabilization of SLCA11 and subsequent inhibition of ferroptosis. Downregulation of ACCC5 reduced the resistance to sorafenib[489]. Other proteins reducing the sorafenib-induced ferroptosis through stabilization of SLCA11 have been recently described[490,491].

Haloperidol, promoted erastin and sorafenib-induced ferroptosis, indicating that haloperidol can be used in combination with sorafenib to achieve either dosage or resistance reduction[404,406,492]. An upregulation of Nrf2 through activation of the p62-Keap1-Nrf2 pathway inhibited sorafenib-induced ferroptosis in HCC cell lines[63,493]. Interestingly, trigonelline, the active ingredient of the traditional Chinese medicine fenugreek can increase ferroptosis by acting on Nrf2, therefore reducing sorafenib

resistance[494]. The leukemia inhibitory factor receptor (LIFR) overexpression increased sorafenib induced ferroptosis of HCC cell lines whereas reduced LIFR expression increased resistance to ferroptosis[495].

A recent observation reported an additional target for HCC treatment. Lactate-rich hepatoma cells exhibit increased resistance to the ferroptosis induced by common ferroptosis inducers. Moreover, lactate uptake mediated by the monocarboxylate transporter 1 (MCT1) enhances the production of monounsaturated fatty acids blocking ferroptosis. Inhibition of the MCT1-mediated lactate uptake enhanced ferroptosis[496]. In contrast to the presented evidence, a recent report indicated that sorafenib may not be an inducer of ferroptosis at least in many cancer cell lines[497]. Other drugs that could be used in the treatment of HCC based on increased ferroptosis were recently described[498,499]. Heteronemin, a marine terpenoid induced ferroptosis in HCC cells by reducing GPX4[500]. IFN-γ was also confirmed to inhibit system Xc- activity and increase ferroptosis[501]. Lenvatinib, another kinase inhibitor used in advanced HCC treatment, also acts through the inhibition of the system Xc-. Fibroblast growth factor receptor-4 (FGFR4) increased the activity of the system Xc- and lenvatinib inhibited FGFR4 increasing ferroptosis. Interestingly, patients with HCC positive for FGFR4 had a longer progression-free survival compared to those with FGFR4-negative HCC. Nrf2 upregulation also decreased the sensitivity of HCC to lenvatinib[502]. Moreover, low-density lipoprotein nanoparticles (LDL-DHA NPs), selectively induced HCC cell death in mouse models. LDL-DHA NPs enhanced lipid peroxidation due to both GSH depletion leading to GPX4 inactivation and direct degradation[410].

Ferroptosis can be used for stratification of HCC patients to predict both prognosis and suitability for immunotherapy. For that purpose, a ferroptosis-related prognosis risk score model has stratified patients into two subgroups based on six FRGs (FRGs)[503].

Ferroptosis inhibitors are promising drugs in the treatment of various liver disease, although evidence is mainly based on laboratory data. NAFLD and NASH progress is worsened by induction of ferroptosis[98,224,504]. Alleviation of NASH can be achieved by

ferroptosis inhibitors, such as liproxstatin-1 or ferrostatin-1[225,505]. Administration of the ferroptosis inducer RSL3 aggravated hepatic steatosis, and inflammation) in diet-induced NASH mice, while administration of liproxstatin-1 ameliorated NASH severity and rescued animals from cell death[225].

Other drugs, such as Ginkgolide B and dehydroabietic acid, alleviated NASH severity inhibiting ferroptosis through upregulation of the p62-Keap1-Nrf2 pathway[506-508]. Thymosin β4 (Tβ4) improved liver lipid metabolism markers in NAFLD rat models and inhibited the palmitic acid-induced hepatocyte death in the LO2 cell line. Ferrostatin-1 increased the effect of Tβ4, which is attenuated by erastin indicating that the protection of hepatocytes is mediated by ferroptosis reduction[509]. The enzyme, enoyl coenzyme A hydratase 1 (ECH1) is an important component of mitochondrial fatty acid β-oxidation. ECH1 knockdown aggravated liver inflammation, and fibrosis in mice NAFLD models, while fer-1 administration alleviated liver damage again suggesting that the beneficial effect of ECH1 may be due to inhibition of ferroptosis[505].

Liver fibrosis is another disease that may be treated by ferroptosis regulators[510]. Inhibition of ferroptosis by ferrostatin 1 reversed liver fibrosis induced by a high-iron diet, and carbon tetrachloride[511], while induction of ferroptosis by liver iron overload aggravated APAP-induced fibrosis in mice[483]. However, ferroptosis is a double-edged sword in liver fibrosis. When ferroptosis is targeting the activated HSCs, the induction of ferroptosis is beneficial. The cystine/glutamate antiporter SLCA11 was shown to increase ferroptosis as mentioned before[55]. Inhibition of SLC7A11 enhanced ferroptosis in HSCs and attenuated liver fibrosis[512]. Thus, erastin and sorafenib induced ferroptosis in HSCs, and reduced liver fibrosis in mice[371,513].

There is growing evidence that natural products may effectively be used in the treatment of liver fibrosis. Artesunate can attenuate liver fibrosis by triggering ferritinophagy-mediated ferroptosis in HSCs[121]. Artemether can also induce ferroptosis in HSCs by increasing iron and ROS in HSCs[514] and promoting p53-dependent ferroptosis[372]. MgIG can induce ferroptosis in HSCs by increasing the activity of the enzyme HO-1[123].

Chrysophanol isolated from the rhizome of rhubarb can inhibit the HBV x protein-induced activation of HSCs through ferroptosis and alleviate HBV related fibrosis[515]. Finally, wild bitter melon extracts can downregulate GPX4 and SLC7A11 in activated HSCs, by inducing ferroptosis[516]. Two proteins regulating ferroptosis in HSCs could be the future targets in the treatment of liver fibrosis. The ZFP36/TTP and ELAVL1/HuR are critical regulators of HSCs ferroptosis[371,513]. ZFP36 protects against ferroptosis and ELAVL1 contributes to ferroptotic cell death.

Three more diseases may be benefited from ferroptosis inhibitors. Fer-1 improves I/R-mediated liver[59,224,276]. ALF is also a candidate for similar treatment based on experimental data. Glycyrrhizin, an active constituent of the liquorice root, reduces the ferroptosis during ALF, inhibiting oxidative stress through the Nrf2/HO-1/high mobility group box 1 pathway[517]. Finally, reduction of liver iron load will most certainly benefit ALD. Phlebotomy however, is not recommended in patients with ALD.

An interesting approach to reduce iron load in ALD is the stabilization of erythrocytes and reduction of hemolysis. Administration of N-acetylcysteine or protective heme carriers like haptoglobin and hemopexin have been tested. Erythrocyte stabilizers include vitamins such as B12 or folate[281]. Ferrostatin-1 can also reduce alcoholic liver damage[276], indicating participation of ferroptosis in ALD progress. Dimethylfumarate reduced lipid peroxidation and alleviated liver cell ferroptosis leading to ALD improvement in a murine model[275]. Currently, no effective treatment can be recommended for ethanol-induced iron overload. Modulation of ferroptosis for the treatment of chronic liver diseases has been recently reviewed[282].

NUTRIENTS AS TREATMENT OPTIONS OF LIVER IRON OVERLOAD

Vitamin A

Retinoid signals are reduced in NAFLD livers of humans and mice[518,519] and are epigenetically silenced in HCC[520]. Administration of the synthetic retinoid, tamibarotene improved oxidative stress and iron deposition in iron-fed mice. Retinoids downregulate the hepatic expression of HJV, leading to liver hepcidin downregulation

and ferroportin upregulation[521,522]. Retinoids also attenuated insulin resistance and hepatic steatosis in a murine model of NAFLD[523,524]. Attenuation of hyperinsulinemia may prevent the development of HCC in NAFLD[525].

Vitamin C

A very large observational study with more than 8000 participants demonstrated that dietary vitamin C supplementation reduced plasma ferritin levels[526] indicating that vitamin C reduces iron deposition increasing iron mobilization. In a murine model of ALD, vitamin C administration restored the decreased hepatic hepcidin and downregulated intestinal ferroportin leading to HIO amelioration[527]. Therefore, it is reasonable to supplement vitamin C in ALD and chronic HCV patient with liver iron deposition.

Vitamin D

Evidence from thalassemia major and hereditary hemochromatosis indicated that iron overload suppressed vitamin D, as there was a negative correlation between liver iron and 25-hydroxyvitamin D levels[528-530]. In hereditary hemochromatosis levels of vitamin D were partially restored after phlebotomy[531]. Moreover, vitamin D depletion exacerbated HIO in HJV knockout mice, corrected by the administration of the calcium channel blocker verapamil and not by vitamin D supplementation[475,532]. These results indicate the presence of a link between iron and calcium and justify the use of calcium channel blockers as a treatment modality of iron deposition in patients with reduced levels of vitamin D as frequently observed in ALD, NAFLD and chronic HCV[533-536].

Zinc

Zinc-deficient diet led to increased plasma ferritin and development of HIO in rats, while zinc supplementation returned liver iron to normal[537]. Clinical studies also indicated that iron deficiency anemia was frequently associated with zinc deficiency[538,539] implying that a physiological crosstalk between iron and zinc seems to

exist. Zinc plus iron administration in rats, ameliorated anemia more efficiently than iron alone[540]. The therapeutic effects of zinc on chronic liver diseases have been reviewed[541].

Folate

The Solute Carrier Family 46 Member 1 (SLC46A1) is the major importer of heme-iron in the duodenum but it is also present in the liver. In murine liver specific SLC46A1 knockdowns its role in liver iron deposition was investigated. SLC46A1 was found to take up heme in the liver and contribute to hepatic iron deposition. Interestingly, heme inhibited folate uptake after downregulation of SLC46A1 expression, but folate supplementation had no effect in heme uptake and SLC46A1 expression indicating that folate deficiency was the result of secondary liver heme uptake excess[542]. A combined administration of iron and folate in rats, significantly reduced liver iron compared with iron alone[543].

Riboflavin

Contrary to the agonistic use of the previous nutrients in the treatment of liver iron deposition, riboflavin antagonists, such as galactoflavin, may be used in HIO[544]. This is because riboflavin deficiency led to reduction of iron absorption[544,545]. A detailed discussion on the role of nutrients in chronic liver diseases was recently published[546].

CONCLUSION

It is well documented, that iron in the liver is a double-edged sword. It is a necessary element in a large number of metabolic pathways, but it is equally harmful if either the amount or its cellular localization is impaired. Increased iron deposition negatively affects most chronic liver diseases. The interplay with the lipid metabolism prompted an extensive investigation for the role of iron in NAFLD/NASH. Moreover, the description of ferroptosis as a discrete form of regulated hepatocyte death, opened the way for the therapeutic modulation of iron overload in many diseases. Interestingly,

most liver disease are benefited by ferroptosis inhibition. A notable exception is HCC where the therapeutic target is ferroptosis induction.

The current evidence involves the integration of information from experimental models and less so, from patient findings. Further experimental *in vitro* and *in vivo* investigations are warranted to find more suitable molecules, with wider availability and better specificity that could regulate ferroptosis. In this context, it is interesting to note that many natural products may influence iron metabolism and ferroptosis. Furthermore, it should be stressed that clinical trials involving ferroptosis regulation are scarce and sometimes inconclusive. Therefore, to draw validated conclusions, further well-designed randomized trials in humans are urgently required.

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