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The Interplay of Iron and Lipid Homeostasis in Non-Alcoholic Fatty Liver Disease

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Abstract

The liver is essential for numerous metabolic functions and is the primary site of iron storage and regulation in addition to maintaining critical functions in lipid metabolism. Both iron deficiency and overload have been demonstrated as being involved with metabolic dysfunction; hence, tight regulation of iron absorption is essential to maintain health. Approximately one-third of individuals suffering from non-alcoholic fatty liver disease have elevated hepatic iron concentrations, with increased iron associated with increased disease severity, suggesting a convergence in dysregulation between lipid and iron metabolism. Increasingly, the literature is demonstrating, using a myriad of model organisms and iron-loading methods, that iron loading induces dysregulation in multiple aspects of hepatic lipid metabolism. However, the molecular mechanisms involved, and their subsequent effects on human diseases, are unclear. As iron is a fundamental component of many enzymes and proteins involved in lipid metabolism and is involved in the production of free radicals and oxidative stress, the mechanisms are numerous. In this review, we examine and summarise the dysregulation that iron loading elicits on hepatic lipid availability, *de novo* synthesis, catabolism, and export. We propose that understanding the interplay between iron and lipid metabolism holds the key to unlocking the complexities of disease development and progression, ultimately leading to improved therapeutic avenues.

Keywords: cholesterol; iron metabolism; lipid metabolism; non-alcoholic fatty liver disease; triglycerides

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Introduction

Iron is an essential trace element and is a critical structural and functional component of many physiological systems. It exists in multiple forms in mammals, as labile iron, or as complexed in transferrin, ferritin, haemosiderin and haem-containing proteins, such as haemoglobin, myoglobin and haemo-enzymes (1, 2). Iron facilitates electron transfer (3), allowing it to catalyse enzymatic reactions and mediate oxygen transport, mitochondrial respiration, and DNA

biosynthesis (4). However, unregulated electron transfer may result in production of reactive oxygen and nitrogen species causing oxidative stress and cellular damage (5). The toxicity of iron is mitigated by regulation of iron metabolism, which starts with tight control of intestinal absorption (6). The average adult body contains 3–5 g of iron, with only 1–2 mg of iron being absorbed daily by the duodenum under healthy conditions so as to replace iron lost through mechanisms such as sweating, desquamation and menstruation (7). There is no controlled mechanism to eliminate excess iron once absorbed.

The liver is central to regulating whole body iron homeostasis, being the major location of iron storage and the production of the master iron regulating hormone, hepcidin (8, 9). Despite being tightly regulated, iron dysregulation, leading to deficiency or accumulation, can occur as the result of genetic (10) or nutritional factors (11). Mild to moderate iron accumulation is becoming increasingly frequent in the general population and is particularly associated with metabolic syndrome and its hepatic manifestation, non-alcoholic fatty liver disease (NAFLD), which has a global prevalence of approximately 30% (12–16).

The liver also plays a key role in maintaining lipid homeostasis and is a hub for fatty acid (FA) synthesis and circulating lipids through lipoprotein synthesis (17, 18). Under healthy conditions, a small fraction of the large quantity of fatty acids processed by the liver is stored within hepatocytes in the form of triglycerides (TG) (19, 20). Dysregulation of lipid metabolism is increasingly common among the global population (21, 22), leading to increased prevalence of hepatic lipid droplets and, eventually, NAFLD (23). The liver therefore represents a nexus for interaction between iron and lipid metabolic pathways.

Iron metabolism

Iron is a critical structural and functional component of multiple physiological systems, and these systems utilise iron's ability to switch between oxidation states to mediate electron transfer (24). However, uncontrolled electron transfer is toxic through the generation of free radicals, which can, in turn, induce oxidative stress (5). As mammals, including humans, lack the ability to excrete iron in a controlled manner, control of body iron is achieved through regulation of iron absorption. The liver plays a fundamental role in the regulation of iron, being the major site of iron storage and the production of hepcidin, a peptide hormone responsible for inhibiting iron's entry into the plasma from enterocytes (9).

Hepatocytes account for ~98% of hepatic iron storage under typical conditions (25). Most of the iron (~80%) is bound to ferritin, which acts as an iron reserve to maintain iron availability and to protect against oxidative damage. Transferrin and haem account for the majority of the remaining iron, ~5% and ~2–3%, respectively. Intracellular iron not specifically bound to proteins or other high molecular weight molecules is referred to as the labile iron pool, an ill-defined pool of chelatable and potentially redox-active iron bound to low molecular weight chelators (26). Despite the multiple levels of regulation, dysregulation of iron is associated with diseases such as diabetes and NAFLD (15, 27).

Iron in the context of NAFLD

Iron accumulation is inextricably linked with NAFLD, with ~30% of NAFLD individuals presenting with mild to moderate hepatic iron loading (28). The role of iron in NAFLD progression has been extensively studied through the lens of increased oxidative stress, which is a major contributing factor in the development of non-alcoholic steatohepatitis (NASH). Iron causes oxidative stress by catalysing the formation of free radicals (5), and markers of oxidative stress are known to increase with hepatic iron accumulation (29, 30). In NAFLD, oxidative stress can lead to depletion of adenosine triphosphate (ATP), oxidised nicotinamide adenine dinucleotide (NAD^+) and glutathione, lipid peroxidation, breaks in DNA strands, and damage to proteins, thereby altering their functions (31–33), leading to cell death.

The clinical significance of increased hepatic iron on progression of NAFLD is slowly becoming clearer, with modest increases in hepatic iron associated with more advanced NAFLD, liver injury and hepatocellular carcinoma (28, 34). However, it is also becoming clear that the role of iron is more complicated than just oxidative stress. Iron has been shown to have a role in altering hepatic equilibrium between lipid uptake, synthesis, oxidation, and export, leading to lipid accumulation due to impairment of mitochondrial β-oxidation gene and protein expression (35, 36), and dysregulated lipid synthesis (37–39). In mice, we demonstrated that iron loading increased the expression of genes involved in the biosynthesis of cholesterol (40) and increased lipid polyunsaturation in a hepatic cell line, potentially increasing susceptibility to oxidative stress (41). This review focuses on the role of iron loading in the dysregulation of lipid metabolism and the consequences for the development and progression of NAFLD.

Plasma lipid availability

Hepatic fatty acid is derived primarily from four sources: lipolysis of adipose tissue, *de novo* lipogenesis, clearance of chylomicron remnants and dietary lipids (18, 42–44). Under normal conditions, large quantities of fatty acid are processed by the liver. However, the rate of fatty acid acquisition is balanced by the rate of catabolism and secretion, and only a fraction (<5%) is stored within hepatocytes in the form of TG. Isotope studies have shown that the majority of hepatic

fatty acids (59%) under NAFLD conditions originate from adipose tissue, compared to 26% from hepatic *de novo* lipogenesis and 15% from diet (42). In the context of iron loading, it is frequently observed that iron increases plasma free fatty acids (FFA), TG and cholesterol, leading to hyperlipidaemia in rodents and humans (45–48), and suggesting a potentially significant role in the development of NAFLD. To date, iron has been demonstrated to affect mechanisms involved in increasing adipose tissue lipolysis and, thus FFA, and decreasing clearance of TG-rich lipoproteins (46), both of which may result in increased plasma lipids.

In both liver and adipose tissue, the process of hydrolysis of TG to FFA and glycerol requires three enzymes, the first of which, adipose triglyceride lipase (ATGL), also known as patatin-like phospholipase domain-containing protein 2, is the rate-limiting step and catalyses the hydrolysis of TG to diglycerides (DG) (49–51). The resultant DG are further hydrolysed by hormone sensitive lipase (LIPE), producing monoglycerides (MG). The final hydrolysis of MG to glycerol and FFA is catalysed by monoacylglycerol lipase (MGL) (49, 52). Increased expression of the *Lipe* gene was observed in the epididymal adipose tissue of mice with dietary haem-induced iron loading (53). Consistent with this finding, carbonyl iron-loaded mice have elevated Atgl and Lipe protein with a greater proportion of both phosphorylated in adipose tissue (54). The same mice exhibited increased glycerol and FFA concentrations in subcutaneous and visceral adipose tissue, strongly suggesting an increase in lipolysis (54). Surprisingly, mice injected with iron dextran were shown to have decreased *Lipe* gene expression with no effect on *Atgl* gene expression; nevertheless, Atgl protein and the ratio of phosphorylated to non-phosphorylated Lipe increased (55), consistent with observations in dietary iron loading (53, 54). Moreover, *in vitro* studies demonstrated that iron sulphate and transferrin increased the rate of lipolysis in primary rodent adipocytes in a dose-dependent manner (56, 57). Studies investigating the clinical relevance of iron loading reported an association between iron and lipolysis, with high plasma ferritin levels in obese women associated with high protein levels of both LIPE and ATGL (58). However, it must be recognised that serum ferritin is not a very specific marker of body iron and is also associated with other pathologies, including inflammatory conditions, of which NAFLD is one (59). Additionally, a study of 492 participants over the age of 40 years with a medical connection with type 2 diabetes mellitus showed an increase in fed and fasting plasma FFA concentration with multiple plasma markers of iron loading, including ferritin, transferrin, serum iron and non-transferrin-bound iron (NTBI). NTBI exhibited the strongest association, suggesting that the mechanism may involve lipid peroxidation (48).

The mechanism by which iron loading increases LIPE activity and lipolysis in adipocytes appears to differ between *in vitro* and *in vivo* models. *In vitro* iron loading of adipocytes indicates the mechanism as being independent of protein kinase A (PKA) activation via an unresolved pathway potentially involving lipid peroxidation (56). However, *in vivo* models suggested that the mechanism involved the upregulation of the β-adrenergic signalling pathway (55) and increased PKA activation (54). Consistent with the proposed mechanism *in vivo*, iron loading decreased *adiponectin* gene expression in adipose tissue and reduced plasma levels (60). Adiponectin is known to suppress Lipe activation (61, 62) and its gene expression is inhibited by β-adrenergic signalling via PKA (63).

Lipid uptake and trafficking

Notwithstanding the ability of FFA to diffuse across a lipid bilayer (64), a variety of proteins facilitate FFA transport across cellular membranes, including fatty acid transport proteins (solute carrier protein 27 [SLC27A1-6], previously referred to as FATP1-6), many of which exhibit acyl-CoA synthetase (ACS) activity (65), fatty acid translocase (FAT/CD36) and fatty acid binding proteins (FABPs). The scavenger receptor, CD36, and SLC27A2 and SLC27A5 are responsible for the majority of hepatic fatty acid import (66–68). *In vitro* models using primary mouse and human hepatocytes showed no change in *CD36* gene or protein expression with iron loading (45, 69). These studies, however, demonstrated that iron's effect on lipid uptake by hepatocytes was modulated by the availability of fatty acids; iron loading with a combination of oleic and palmitic acids (2:1) decreased lipid content and Cd36 protein expression in primary mouse hepatocytes (45). Interestingly, 24-h and 72-h iron-loaded primary human hepatocytes exhibited reduced expression of the *SLC27A5* gene, but the subsequent addition of palmitic acid increased both *SLC27A5* and *CD36* gene expression, compared to control and palmitic-only cells, indicating fatty acid saturation may alter iron's influence (69). In a model using *C. elegans*, iron loading increased lipid accumulation in both presence and absence of oleic acid through a mechanism dependent on ACS-20, an orthologue of mammalian SLC27A1/ SLC27A4 (70). In contrast, iron-loaded mouse models exhibited either no effect or reduced gene and protein expression of hepatic Slc27a2, Slc27a5 and Cd36 (71–73). Similarly, primary iron loading induced by hemojuvelin (Hjv) knockout (KO) did not change the hepatic protein expression of *Slc27a2* or *Slc27a5* (73) and no changes in gene expression were reported (38). Hence, iron loading did not appear to alter the expression of lipid import proteins in mouse models under either genetic (primary) or dietary iron loading. However, similar to *in vitro* observations, ApoE KO mice fed with a high fat diet with iron loading exhibited reduced *Cd36* and *Slc27a1* gene expression and Cd36 protein expression, while at the same time demonstrating increased

serum FFA and no change in hepatic FFA, suggesting either no change or a reduced hepatic lipid uptake (45). It therefore appeared that lipid availability and composition modulated the effect of iron loading on hepatic lipid import machinery.

The primary function of FABPs is considered as being one of lipid chaperones; however, studies have also demonstrated a role in fatty acid import, storage and export (74). FABP1 is the most abundant FABP in hepatocytes and represents up to 5% of all cytosolic proteins (75, 76). FFAs are known as cytotoxic to cells (77, 78), and their binding to FABPs aids in decreasing their toxicity (79). Additionally, changes in FABP1 expression substantially affect the rate of fatty acid uptake, a property that other FABPs do not demonstrate (80, 81). Interestingly, the effect of iron loading on expression of FABPs is not uniform across all members in both *in vitro* and *in vivo* studies. Iron loading in primary mouse hepatocytes was demonstrated not to alter Fabp1 protein expression (45), while, in primary human hepatocytes, iron loading reduced FABP4 in an iron dose-dependent manner but increased *FABP5* expression (69, 82). Studies using dietary iron-loaded mice demonstrated reduced hepatic FABP1 protein (73), corroborated *in vitro* findings of reduced *FABP4* and increased *FABP5* gene expression, but exhibited no change in *FABP2* (71, 82). Although not reaching statistical significance, proteomic studies from iron-loaded mice were consistent with gene expression observations (73). Genetic models of iron loading showed the same relationship with hepatic FABPs as dietary models (73, 82, 83), although *Fabp5* gene expression was reduced in Hjv KO mice (82). Hepatic *Fabp2* gene and protein expression were similarly shown to be reduced in both Hfe and Hjv KO mouse models (82, 83). These dietary and genetic studies consistently demonstrated that iron loading reduced hepatic FABPs, consistent with reduced uptake and intracellular trafficking of fatty acids.

The introduction of a high lipid environment together with iron loading affects iron's relationship with FABPs. The addition of palmitic acid plus iron to primary human hepatocytes increased gene expression of both *FABP4* and *FABP5* (69), while primary mouse hepatocytes loaded with both oleic acid and palmitic acid plus iron exhibited reduced Fabp1 protein (45). Similarly, ApoE KO mice fed with a high iron and high fat diet also exhibited reduced hepatic *Fabp1*, *Fabp2*, *Fabp4* and *Fabp5* gene and protein expression (45). In contrast, Hfe KO mice fed with a high fat diet exhibited increased hepatic *Fabp1* gene expression, compared to both wild type and Hfe KO mice fed with a control diet (84). Together, these findings indicated that Fabp expression was sensitive to the composition of high fat treatment and genetic background of the model, demonstrating the importance of considering these factors when designing and interpreting such studies.

The liver is additionally able to import lipids through lipoprotein receptors (85–87). The low-density lipoprotein (LDL) receptor mediates the endocytosis of cholesterol-rich LDL from the plasma and recognises apoprotein B100 (86). Iron-loaded mice demonstrated no change in hepatic low density lipoprotein receptor (LDLR) mRNA or protein expression, suggesting that iron may not alter lipoprotein uptake (36, 46, 88). However, iron loading decreased the activity of lipoprotein lipase (LPL) (46), causing a reduction in clearance of lipoproteins from the plasma by multiple tissues, including adipose tissue, skeletal muscle, and the liver, and suggesting that reduced clearance of lipids could be partially or fully responsible for the elevated plasma lipids observed (89–91).

De novo lipid synthesis

Under normal circumstances, the hepatic *de novo* lipogenesis (DNL) pathway is rarely utilised (92). Nevertheless, just as enhanced fatty acid uptake can contribute to steatosis and NAFLD development, so does increased DNL (93, 94). DNL is the synthesis of fatty acid chains up to 16 carbons long (palmitate; C16:0) from acetyl-CoA subunits generated though glycolysis and other pathways (49, 95). The first step of DNL is the cleaving of citrate to form acetyl-CoA by ATP-citrate lyase (ACLY) (96, 97), followed by carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC/ACACA), the rate limiting step in DNL (98) (Figure 1). The final step in palmitate synthesis of adding acetyl-CoA to malonyl-CoA and subsequent cycles is carried out by fatty acid synthase (FAS) (99). In dietary iron-loaded mice, *Acaca* and *Fas* gene and protein expression were upregulated, and iron loading also increased their activity in rats (38, 100, 101). Thus, dietary iron loading upregulated the DNL pathway, leading to increased fatty acid synthesis. These observations were supported by a study reporting that iron loading of primary human umbilical vein endothelial cells (HUVECs) increased *FAS* gene expression and elevated the rate of 16:0, 16:1 and 18:1 fatty acid synthesis (37). Furthermore, iron loading increased expression of Acc2, the mitochondrial isoform of Acaca (45). Malonyl-CoA produced by Acc2 downregulated mitochondrial β-oxidation by inhibiting Cpt1 (102, 103), indicating iron loading may reduce Cpt1 activity. Although both hepatic ACACA and FAS expression increased with dietary iron loading, *Acly* gene and protein decreased, suggesting that increases in DNL may utilise a different source of acetyl-CoA (71, 73, 96). Rodent models in which iron loading was induced by bypassing duodenal regulation by injection of iron oxide nanoparticles (IONPs) or iron dextran showed decreased *Acly*, *Acaca* and *Fas* gene and protein expression, demonstrating differences between the two modes of iron loading (104, 105). In contrast, when mice fed with a high fat diet were iron-loaded by either dietary iron or injected IONPs, *Acaca* and *Fas* genes and proteins exhibited hepatic upregulation (45, 47).

Fatty acid elongation

Fatty acid elongation involves the addition of two-carbon units to a fatty acyl-CoA and is catalysed by four enzymatic reactions (106, 107) (Figure 1). In mammals, the initial step is a rate controlling condensation reaction, catalysed by enzymes referred to as elongation of very long-chain fatty acids (ELOVLs) (106). At present, seven ELOVL proteins have been identified: ELOVL1, ELOVL3, ELOVL6 and ELOVL7 prefer saturated and monounsaturated fatty acids as substrates whereas ELOVL2, ELOVL4 and ELOVL5 are selective for polyunsaturated fatty acids (108–110). ELOVL6 is involved in the elongation of C16:0 to C18:0 and C16:1 to C18:1 (111). Current literature reporting the effect of iron loading on hepatic ELOVL6 is contradictory, with both increased and decreased gene expression observed in C57BL/6 mice fed with similar concentrations of carbonyl iron for similar durations (38, 47, 71). ELOVL2 elongates C20:4 to C22:4 and C20:5 to C22:5 and protein levels were increased in rats fed with a high fat diet plus ferric citrate, although no change in activity was evident (112). Nevertheless, increases in the ELOVL family of proteins were consistent with increases in synthesis of longer-chain fatty acids and may explain the increase in fatty acid chain length observed in AML12 cells incubated with ferric ammonium citrate (41). *Elovl3* and *Elovl5*, which are believed to be involved in the elongation of multiple length fatty acids, exhibited reduced gene expression in mice fed with 2% carbonyl iron (38, 71).

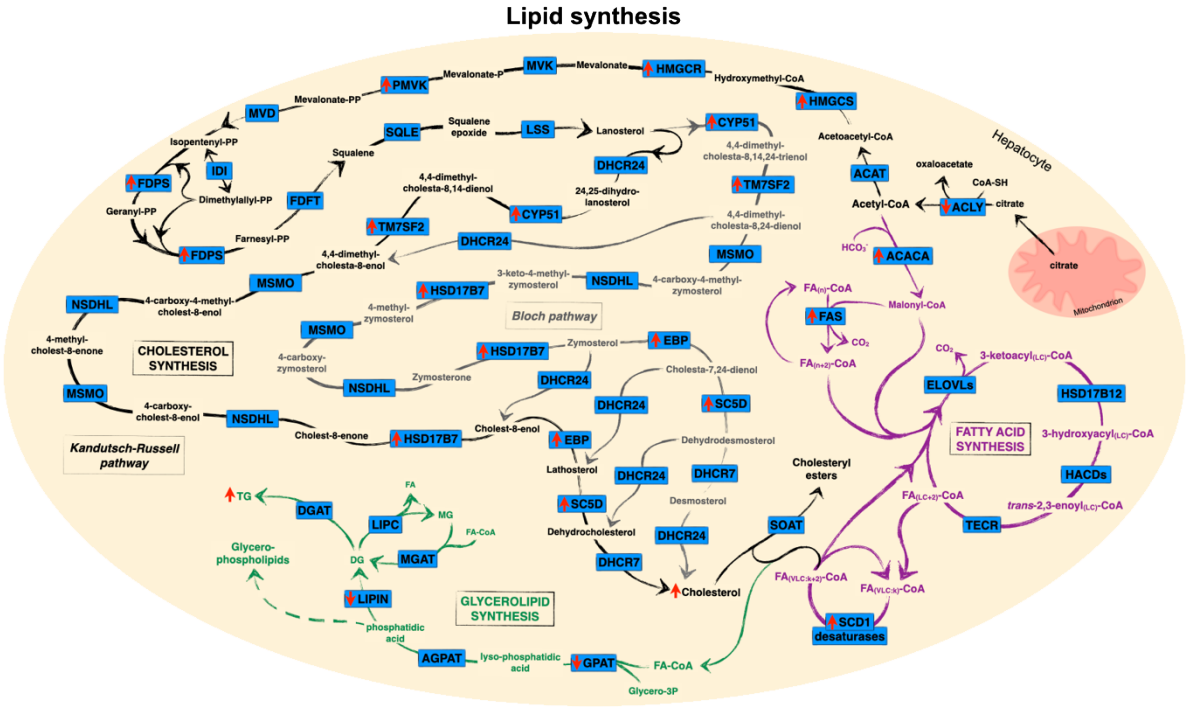

Figure 1: Association between hepatic iron accumulation and dysregulated lipid synthesis. Red arrows indicate direction of iron-associated regulation of gene, protein or metabolite expression as discussed in the text. The Bloch pathway of cholesterol synthesis is shown in black, with the branch to the Kandutsch–Russell pathway and its interconversions with the Bloch pathway shown in grey. Fatty acid synthesis, elongation and desaturation are shown in purple and glycerolipid synthesis is shown in green. In order to simplify the diagram, only some carbon numbers are shown and FA-CoAs destined for incorporation into glycerolipids are shown as being sourced from the pool of very long-chain FAs but may be sourced from any pool of cytosolic FA-CoA. $FA_{(n)}$: fatty acid containing n carbons; $FA_{(LC:k)}$: long-chain (or very long-chain [VLC]) fatty acid containing k double bonds. ATGL, adipose triglyceride lipase; DHCR24, dehydrocholesterol reductase; HACD, hydroxyacyl-CoA dehydratase; LIPC, hepatic lipase; LSS, lanosterol synthase; MGAT, monoglyceride acyltransferase; MSMO, methylsterol monooxygenase; MVD, mevalonate diphosphate decarboxylase; MVK, mevalonate kinase; NSDHL, NAD(P)-dependent steroid dehydrogenase-like; SOAT, sterol O-acyltransferase; SQLE, squalene epoxidase; TECR, *trans*-2,3-enoyl-CoA reductase. Other abbreviations are defined in the text. Pathways are based on data from KEGG (181) and Mazein et al. (182).

Fatty acid desaturation

Upregulation of hepatic stearoyl CoA desaturase (Scd1) mRNA, protein and enzyme activity in rodents was reported in studies utilising both dietary and injection models of iron loading (47, 113, 114). SCD1 is the first enzyme involved in fatty acid desaturation and introduces a single double bond at Δ9 to produce monounsaturated fatty acids (C16:1 and C18:1) (115) (Figure 1). Scd1 protein increased proportionately with iron status in mice (113). Similarly, hepatic *Scd1* mRNA increased in ApoE KO mice fed with a high fat and high iron diet (45), suggesting dietary iron overload increased fatty acid desaturation. Interestingly, Hfe KO mice did not demonstrate altered hepatic *Scd1* gene expression compared to wild-type mice (116), potentially indicating a difference of iron-induced regulation between primary and secondary iron overload. In humans, elevated plasma ferritin was significantly associated with a higher Δ9 desaturase (SCD1) index (C16:1/C16:0) in a cross study of 447 female participants (117). In mice with iron loading, hepatic DG and TG species containing C18:1 represented the majority of the top 20 upregulated glycerolipids, indicating that increased Scd1 expression caused by iron may increase production of monounsaturated fatty acids (118). This was further supported by results from iron-loaded HUVECs, which exhibited increased synthesis of C16:1 and C18:1, compared to saturated counterparts C16:0 and C18:0 demonstrating greater SCD1 activity (37).

The relationship between fatty acid desaturase 1 (FADS1; Δ5 desaturase) and fatty acid desaturase 2 (FADS2; Δ6 desaturase) with iron loading was opposite to that observed for SCD1. Dietary iron loading increased both *FADS1* and *FADS2* mRNA expression but decreased protein activity (101, 119, 120). These enzymes introduce a double bond at position Δ6 on the acyl chain of linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) (121) and are essential for the synthesis of polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (122). Taken together, iron's effects on fatty acid desaturases lead to increased monounsaturated fatty acids and reduced polyunsaturated fatty acids, as observed in plasma and hepatic lipidomic studies (101, 119).

Fatty acid activation

Conversion of fatty acids to their active forms via thioesterification to add CoA to generate fatty acyl-CoA is a required step in fatty acid metabolism (49). For long-chain fatty acids, the reaction is catalysed by the ACS long chain family of enzymes. ACSL1 accounts for ~50% of total hepatic ACSL activity (123) and is known to physically interact with Cpt1 in rat hepatocytes with a potential role in targeting fatty acyl-CoA towards mitochondrial β-oxidation (124). *ACSL1* mRNA expression decreased with iron in a dose-dependent manner in HepG2 cells incubated with oleic acid (125) and, in iron-loaded mice, hepatic Acsl1 protein expression was reduced substantially (73). Links between elevated plasma ferritin and reduced *ACSL1* gene expression were reported in humans in visceral adipose tissue (126). *ACSL3* and *ACSL5* gene expression was reduced with iron loading in a dose-dependent manner in primary human hepatocytes (82) and was also decreased in Hjv KO mice (82). These studies indicated a reduction in fatty acid activation and mitochondrial β-oxidation with iron loading. The ACSL4 isozyme preferentially utilises arachidonic acid (C20:4) and overexpression promoted the conversion of arachidonic acid into phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and TG (127, 128). Protein expression of Acsl4 was upregulated in the liver of iron-loaded mice (129) and lipidomic investigation of iron-loaded mouse liver indicated increase in PI and TG containing arachidonic acid (118), suggesting increased activity.

Mitochondrial β-oxidation

Mitochondrial β-oxidation (Figure 2) is a process that shortens fatty acids into acetyl-CoA, which can be utilised for generating ketone bodies or fully oxidised for energy production via the tricarboxylic acid (TCA) cycle (130, 131). While β-oxidation can also occur in peroxisomes, only the mitochondria are capable of β-oxidation of short- and medium-chain fatty acids (49). Fatty acids must be activated and transported into the mitochondria via the carnitine shuttle (132). As discussed above, iron loading decreases fatty acid activation and significantly downregulates hepatic *Cpt1* mRNA and protein expression in genetic and dietary rodent models (35, 36, 53, 83, 105, 133). Mice fed with high fat plus iron also demonstrated decreased *Cpt1* mRNA and protein expression, suggesting decreased fatty acid transportation into the mitochondria (45, 105). The activity of rat CPT1 was also reduced with iron loading (101), and L-carnitine and L-acylcarnitine levels decreased in mice following 2 weeks of dietary iron loading (134). Iron loading therefore causes a reduction in mitochondrial fatty acid import, decreasing β-oxidation. While dietary iron loading did not alter *CPT2* gene expression (133), genetic iron loading through Hfe KO decreased *Cpt2* mRNA (83).

Once within the mitochondria, fatty acids undergo the four steps of β-oxidation (130). The first is dehydrogenation of acyl-CoA to trans-2-enoyl-CoA by one of the acyl-CoA dehydrogenases (ACADs). The last three steps are catalysed by mitochondrial trifunctional enzyme, which comprises two subunits, hydroxyacyl dehydrogenase alpha (HADHA), which completes the 2,3-enoyl-CoA hydratase and the 3-hydroxyacyl-CoA dehydrogenase processes, and hydroxyacyl dehydrogenase beta (HADHB), which completes the

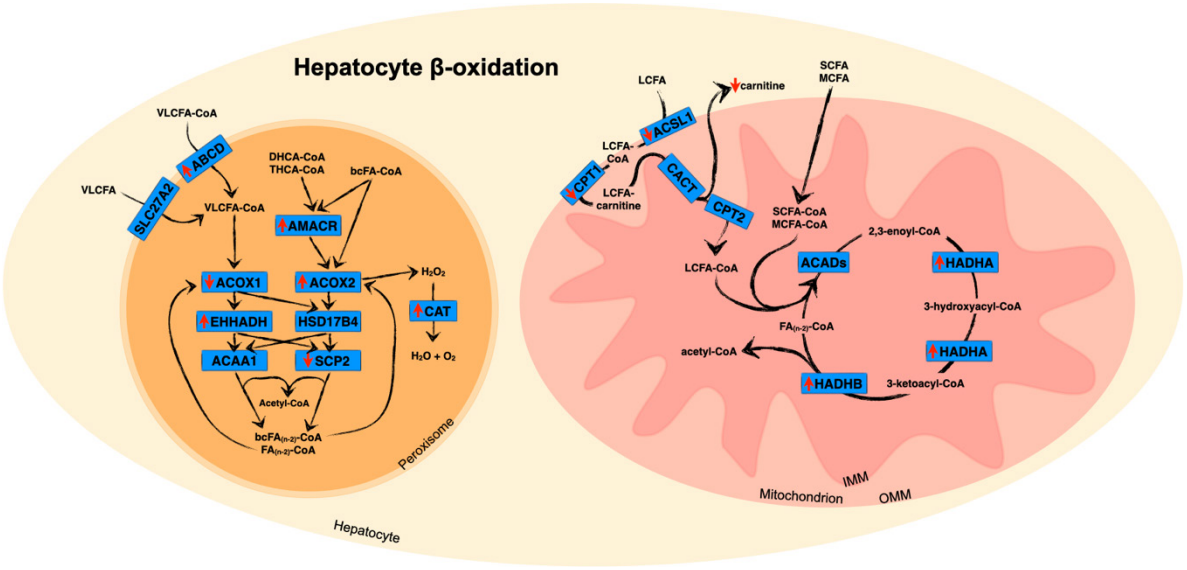

Figure 2: Association between hepatic iron accumulation and dysregulated β-oxidation. Red arrows indicate direction of iron-associated regulation of gene, protein or metabolite expression as discussed in the text. SCFA: short-chain fatty acid; MCFA: medium-chain fatty acid; LCFA: long-chain fatty acid; VLCFA: very long-chain fatty acid; bcAcyl-CoA: branched-chain acyl-CoA; CACT: carnitine acylcarnitine translocase. Other abbreviations are defined in the text. Pathways are based on data from KEGG (181).

3-ketoacyl-CoA thiolase reaction (130, 135). Iron loading of the human hepatocyte cell line HH4 reduced ACADVL protein, and in iron-loaded mice, *Acad10* gene expression was reduced (71), suggesting decreased β-oxidation of very long-chain fatty acids (136). Despite this, protein expression of multiple ACAD isoforms, including Acad10, was not significantly altered in dietary iron-loaded mice. In contrast, HADHA and HADHB proteins were increased, which would be consistent with an increase in mitochondrial β-oxidation (73, 137). The increase in the mitochondrial β-oxidation pathway, coupled with reduced CPT1, may indicate increased utilisation of short-chain fatty acids and decreased use of longer-chain fatty acids for mitochondrial β-oxidation. ApoE KO mice fed with a high fat and high iron diet exhibited reduced Acads and Hadha protein (45), suggesting decreased mitochondrial β-oxidation of short-chain fatty acids and a role for iron in decreasing mitochondrial β-oxidation in NAFLD.

Peroxisomal β-oxidation

While the majority of fatty acid β-oxidation is undertaken by the mitochondria, β-oxidation of very long-chain and branched chain fatty acids is dependent on peroxisomes (138). Fatty acids are imported across the peroxisomal membrane via members of the adrenoleukodystrophy (ALD) subfamily of the ATP-binding cassette (ABC) transporters (139, 140). The solute carrier, SLC27A2, is also associated with peroxisomal import (Figure 2) (141, 142). Analogous to mitochondrial β-oxidation, each cycle of peroxisomal β-oxidation requires four enzymatic steps of oxidation, hydration, dehydrogenation and thiolytic cleavage (143). The first reaction in rats and mice is catalysed by one of three isoforms of acyl-CoA oxidase (ACOX) (144). Palmitoyl-CoA oxidase (ACOX1) is specific for saturated and monounsaturated substrates, including very long-chain fatty acids and dicarboxylic fatty acids. ACOX2 specifically reacts with CoA esters of the bile acid intermediates 3α,7α-dihydroxycholestanoic acid (DHCA) and 3α,7α,12α-trihydroxycholestanoic acid (THCA) and branched chain fatty acids, and ACOX3 is active with branched chain fatty acids. The peroxisomal L-bifunctional enzyme (enoyl-CoA hydratase and hydroxyacyl-CoA dehydrogenase [EHHADH] or LBP) and D-bifunctional enzyme (hydroxysteroid-17-beta dehydrogenase [HSD17B4] or DBP) catalyse the second and third steps of peroxisomal β-oxidation. Both have enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities but with opposite chiral specificity (145). In rodents, the final step is completed by one of three different thiolases—3-ketoacyl-CoA thiolase A or B (Acaa1a or Acaa1b) or sterol carrier protein 2 (Scp2) (143).

There are indications that iron loading perturbs hepatic peroxisomal β-oxidation. Dietary iron-loaded mice exhibited upregulated hepatic gene expression of *Abcd2*, *Ehhadh,* and

Acot3 (38) as well as protein expression of Acox2, Ehhadh and 2,4-dienoyl-CoA reductase (DECR2), while *Scp2* gene and protein expression decreased and Acaa1a and Acaa1b did not change, consistent with an increase in peroxisomal fatty acid uptake and β-oxidation (38, 71, 73, 146). In IONP-injected iron-loaded mice, increased hepatic protein expression of Acox2 and alpha-methylacyl-CoA racemase (Amcr) was reported (104). Catalase (Cat) gene and protein expression was also increased in these mice, suggesting increased generation of hydrogen peroxide, a product of peroxisomal β-oxidation (104). These studies suggest that iron loading is associated with increased hepatic peroxisomal β-oxidation of branched-chain fatty acids and bile acid intermediates. Interestingly, hepatic *Acox1* gene expression was decreased in dietary, iron-dextran injected and Hfe KO mice (83, 84), suggesting decreased peroxisomal β-oxidation of straight and monounsaturated fatty acids in both primary and secondary iron loading.

Triglyceride synthesis

Iron overload is frequently associated with increased hepatic TG, leading to elevated hepatocyte lipid droplet formation in both *in vitro* and *in vivo* studies (69, 118, 119, 147). Lipid droplets primarily comprise TG and cholesteryl esters, and protect hepatocytes from lipotoxicity (148). Lipidomic investigation of homogenised livers of mice injected with iron dextran demonstrated that iron does not cause the accumulation of only a single type of glycerolipid but instead results in a broader increase in MG, DG, TG and sterol lipids (118). Thus, iron appears to induce lipid accumulation and storage phenotype not only by increasing the synthesis of fatty acids but also by increasing their incorporation into glycerolipids. Glycerol-3-phosphate acyltransferase (GPAT) is the first enzyme of the TG synthesis pathway, producing lysophosphatidic acid (149) (Figure 1). Acylation of lysophosphatidic acid to phosphatidic acid by acylglycerol-3-phosphate O-acyltransferase (AGPAT) is completed in the endoplasmic reticulum (ER) membrane. Subsequent dephosphorylation by phosphohydrate phosphohydrolase (LIPIN) generates DG (149). The final reaction is acylation of DG by diglyceride acyltransferase (DGAT) to form TG. Interestingly, microarray and proteomic studies did not pick up any changes in the expression of genes or proteins associated with the TG synthesis pathway in the presence of iron loading (45). However, activity of GPAT was reduced when incubated with iron sulphate (Fe^{2+}) or iron chloride (Fe^{3+}) (150), raising the question of what caused the TG to accumulate? ApoE KO mice fed with a high fat and high iron diet exhibited no change in *Gpat1*, *Agpat2* or *Dgat2* gene and protein expression, potentially due to iron loading decreasing TG synthesis in animals fed with a high fat diet (45). Dietary iron loading similarly reduced hepatic gene expression of *Lipin1* in mice (38). HepG2 cells incubated with IONP showed no change in *LIPIN1* gene expression; however, iron loading in combination with oleic acid substantially increased *LIPIN1* gene expression (47) and this was similarly observed in high fat-fed mice with IONP iron loading. These studies suggest that further investigations are required into the effect of iron on TG synthesis to fully unravel the role of iron.

Cholesterol synthesis

Dysregulated cholesterol metabolism, including increased availability of free cholesterol, is associated with NAFLD progression (151–153). The liver has a fundamental role in *de novo* cholesterol synthesis, which is highly regulated throughout a multistep process (154) (Figure 1). In brief, synthesis begins with the generation of mevalonate from acetate, itself a multistep process utilising acetyl-CoA acetyltransferase (ACAT) to form acetoacetyl-CoA, which subsequently has an additional acetyl-CoA added by HMG-CoA synthase, generating HMG-CoA (155). The final reaction, catalysed by HMG-CoA reductase (HMGCR), is both rate limiting and the committed step in the process (156, 157). Mevalonate is subsequently converted by several reactions into two activated isoprenes (155). The condensation of multiple activated isoprenes by farnesyl-PP synthase (FDPS) and farnesyl-pyrophosphate farnesyltransferase (FDFT) generates squalene, the precursor of all steroids, a 30-carbon polyunsaturated linear molecule. Squalene undergoes a series of oxidation and reduction reactions to produce the tetracyclic compound, lanosterol, from which cholesterol can be generated via two different, albeit interconnected, routes, termed the Bloch and Kandutsch–Russell pathways (158, 159).

Dietary iron loading was found to upregulate *Hmgcr*, phosphomevalonate kinase (*Pmvk*) and *Fdps* in mice, which may increase production of mevalonate (40, 160). Furthermore, gene expression of *Cyp51*, delta-14-sterol reductase (*Tm7sf2*), *Hsd17b7*, cholestenol delta-isomerase (*Ebp*) and *Sc5d* was shown to increase hepatic cholesterol accumulation. Incubation of HUVECs with iron upregulated *Hmgcr* and *Pmvk* gene expression and cholesterol biosynthesis (37). Supporting these findings, hydroxymethylglutarate-CoA synthase 2 (Hmgcs2) and Hmgcr protein expression is also increased in dietary iron-loaded mice and rats (73, 88), and hepatic *Hmgcr* gene expression is upregulated in mice fed with a high fat and high iron diet, demonstrating that iron induced increases in hepatic and plasma cholesterol and cholesteryl esters through increased *de novo* lipogenesis (118, 161), supporting a role for iron in further elevating cholesterol in NAFLD. While iron loading has been associated with reduced activity of the *Hmgcr* gene in rats, hepatic cholesterol concentration was not impacted and, as stated by the authors, was consistent with increased

oxidative stress, indicative of oxidative damage to the membranes in which the enzymes reside (162).

Approximately 90% of actively metabolised cholesterol is utilised for bile acid synthesis (163). The first reaction is rate-limiting and is catalysed by members of the cytochrome P450 (CYP) family (164). *Cyp7a1* gene and protein expression was reduced in the liver of iron-loaded mice and rats (88). It was demonstrated that iron loading reduced the activity of *Cyp7a1* in rat hepatic microsomes (162). Iron loading subsequently decreased the synthesis and removal of bile acids by the liver and could be an alternative cause of plasma and hepatic cholesterol accumulation.

Lipid metabolism regulation

Multiple transcription factors are involved in regulating lipid metabolism, of note are sterol regulator element binding proteins (SREBPs) and peroxisome proliferator-activated receptors (PPARs) (165–167). SREBP1c is the primary isoform in the liver and is involved in regulating genes involved in fatty acid synthesis, while SREBP2 activates the cholesterol uptake and synthesis pathway (165). Iron loading was demonstrated to increase the hepatic expression of Srebp1 protein in mice and rats fed with a high iron diet (100, 101, 168). However, rats injected with gleptoferron exhibited no change in gene or hepatic protein expression (88), despite an increase in Srebp2 protein expression. *In vitro* HUVECs iron loaded with ferric ammonium citrate were reported to elevate *SREBP2* gene expression (37). PPARα is highly expressed in the liver and has a fundamental role in upregulating mitochondrial and peroxisomal β-oxidation, ketogenesis and lipoprotein assembly (166–167). Iron loading in rodents, induced either by diet or dextran injection, was shown to reduce hepatic *Pparα* and *Pparγ* gene and protein expression (36, 100, 101, 105, 161). These studies support the observed iron-associated alterations to hepatic lipid metabolism, with increased SREBP1 and SREBP2 increasing the expression of lipid biosynthetic genes, and decreased PPARs decreasing the expression of lipid catabolic pathways (35, 100, 101, 133, 169).

Assembly and secretion of very low-density lipoproteins

The final method by which hepatic lipid loading may be decreased is through export. Fatty acid export from the liver is primarily in the form of TG and cholesteryl ester-rich lipoproteins, including very low-density lipoproteins (VLDL) and high-density lipoproteins (HDL), for delivery to muscles for oxidation and to adipose tissue for storage (170, 171). VLDL production is a two-step process beginning in the ER lumen (172) incorporating small quantities of TG on to apoB100 facilitated by microsomal triglyceride transfer protein (MTP) (173). *In vitro* studies have repeatedly demonstrated iron loading and ferritin post-translationally inhibiting the secretion of ApoB100, leading to increased ER-associated degradation in hepatic cell lines (174–176). Interestingly, while iron dextran-injected mice showed increased *Mtp* and *ApoB100* gene expression (36), rats injected with iron dextran exhibited no change in *Mtp* gene expression when fed with a control diet or high fat diet (177). Furthermore, dietary iron-loaded mice showed no change in *ApoB100* gene expression (40). A recent proteomic study supported no change in hepatic Mtp protein levels in either dietary iron-loaded or Hjv KO mice, suggesting iron did not alter Mtp expression. Similarly, no changes were reported in hepatic ApoB100 with dietary iron-loading in microarray or proteomic studies. Despite this, animal models and humans exhibit an association between iron parameters and plasma VLDL and LDL (69, 178, 179).

Interestingly, multiple studies have reported that iron loading upregulated hepatic expression of other lipoproteins, primarily ApoAIV and ApoCIII (38, 40, 45). Increased hepatic ApoAIV increased TG export by promotion of ApoB100 lipoprotein assembly, and increased VLDL secretion, resulting in raised plasma TG concentrations (180). Similarly, ApoCIII was shown to upregulate VLDL production, suggesting iron loading may increase hepatic lipoprotein production and secretion through increased ApoAIV and ApoCIII expression.

Conclusion

The liver is central to maintaining whole body lipid homeostasis. The role of overnutrition in dysregulating the activity of metabolic pathways leading to common metabolic conditions, including NAFLD, is well understood. It is increasingly clear from the literature that iron loading induces substantial and complex alterations in hepatic lipid metabolism and appears to play a role in both initial development and subsequent progression of NAFLD through increased lipid availability, *de novo* synthesis, altered lipid composition and dysregulated hepatic lipid catabolism and export. The exact molecular mechanisms by which iron alters hepatic lipid homeostasis are yet to be fully elucidated. Further investigation and understanding of iron, lipids and their interactions are ultimately required to further our understanding of metabolic diseases such as NAFLD.

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Author contributions (CRediT)

Clinton J Kidman: conceptualisation, visualisation, writing of original draft, reviewing and editing of manuscript. Cyril DS Mamotte: supervision, writing, reviewing and editing of manuscript. Keea R Inder-Smith: writing, reviewing and editing of manuscript. Mark J Tobin: supervision, reviewing and editing of manuscript. Mark J Hackett: supervision, reviewing and editing of manuscript. Ross M Graham: conceptualisation, supervision, project administration, reviewing and editing of manuscript.

Conflicts of interest

The authors declared no potential conflict of interest with respect to research, authorship and/or publication of this article.

Abbreviations

ABC, ATP binding cassette; ACAA, ketoacyl-CoA thiolase; ACAD, acyl-CoA dehydrogenase; ACC/ACACA, acetyl-CoA carboxylase; ACAT, acetyl-CoA acetyltransferase; ACLY, ATP-citrate lyase; ACOX, acyl-CoA oxidase; ACSL, acyl-CoA synthetase; AGPAT, acylglycerol-3-phosphate-O-acyltransferase; AMACR, alpha-methylacyl-CoA racemase; CAT, catalase; CYP, cytochrome P450; DECR2, dienoyl-CoA reductase; DG, diglyceride; DGAT, diglyceride acyltransferase; DHA, docosahexanoic acid; DNL, *de novo* lipogenesis; EBP, cholestenol delta-isomerase; EHHADH, enoyl-CoA hydratase and hydroxyacyl-CoA dehydrogenase; ELOVL, elongation of very long chain fatty acids; EPA, eicosapentanoic acid; ER, endoplasmic reticulum; FABP, fatty acid binding proteins; FADS, fatty acid desaturase; FAS, fatty acid synthase; FAT/CD36, fatty acid translocase; FATP, fatty acid transport protein; FDFT, farnesyl-pyrophosphate farnesyltransferase; FDPS, farnesyl-pyrophosphate synthase; FFA, free fatty acids; GPAT, glycerol-3-phosphate acyltransferase; HADHA, hydroxyacyl dehydrogenase alpha; HADHB, hydroxyacyl dehydrogenase beta; HDL, high density lipoprotein; HJV, haemojuvelin; HMGCR, hydroxymethylglutarate-CoA reductase; HMGCS, hydroxymethylglutarate-CoA synthase; HSD17B, hydroxysteroid-17-beta dehydrogenase; HUVEC, human umbilical vein endothelial cells; IONP, iron oxide nanoparticle; KO, knockout; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LIPE, hormone sensitive lipase; LIPIN, phosphohydrate phosphohydratase; MG, monoglyceride; MGL, monoglyceride lipase; NAFLD, non-alcoholic fatty liver disease; NTBI, non-transferrin bound iron; PE, phosphoethanolamine; PI, phosphoinositol; PKA, protein kinase A; PMVK, phosphomevalonate kinase; SCD1, stearoyl-CoA desaturase; SCP2, sterol carrier protein 2; SLC27, solute carrier protein 27; TG, triglyceride; TM7SF2, delta-14-sterol reductase; VLDL, very low density lipoprotein.

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