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REVIEW ARTICLE

# Non-alcoholic fatty liver disease: is iron relevant?

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**Abstract** Non-alcoholic fatty liver disease (NAFLD) is a common and ubiquitous disorder (Bedogni et al. in Hepatology 42:44–52, 2005; Bellentani et al. in Ann Intern Med 132:112–117, 2000) which in a proportion of subjects leads to non-alcoholic steatohepatitis (NASH), advanced liver disease and hepatocellular carcinoma. Although the factors responsible for progression of disease are still uncertain, there is evidence that insulin resistance (IR) is a key operative mechanism (Angulo et al. in Hepatology 30:1356–1362, 1999) and that two stages are involved. The first is the accumulation of triglycerides in hepatocytes followed by a "second hit" which promotes cellular oxidative stress. Several factors may be responsible for the induction of oxidative stress but hepatic iron has been implicated in various studies. The topic is controversial, however, with early studies showing an association between hepatic iron (with or without hemochromatosis gene mutations) and the progression to hepatic fibrosis. Subsequent studies, however, could not confirm an association between the presence of hepatic iron and any of the histological determinants of NAFLD or NASH. Recent studies have reactivated interest in this subject firstly, with the demonstration that hepatic iron loading increases liver cholesterol synthesis with increased lipid deposition in the liver increasing the cellular lipid burden and secondly, a large clinical study has concluded that hepatocellular iron deposition is associated with an increased risk of hepatic fibrosis, thus, strongly supporting the original observation made over a decade ago. An improvement in insulin sensitivity has been demonstrated following phlebotomy therapy but a suitably powered controlled clinical trial is required before this treatment can be implemented.

**Keywords** Fatty liver disease · Steatohepatitis · Iron overload

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## Introduction

It is widely acknowledged that obesity is a growing problem world-wide [1–6]. The prevalence of obesity (BMI >30) in the USA is reported as high as 34%, and it is increasingly common in the Asian Pacific region [7–11]. It is also widely accepted that the global obesity epidemic is linked to an increased incidence of a number of metabolic disorders especially type 2 diabetes mellitus and non-alcoholic fatty liver disease (NAFLD) [2]. The term NAFLD refers to a number of pathological conditions which include hepatic steatosis, non-alcoholic steatohepatitis (NASH), and cirrhosis [12–14] which may progress to hepatic failure [15–17] and hepatocellular carcinoma [18]. The occurrence of obesity together with impaired glucose tolerance, dyslipidemia, and hypertension has been termed the metabolic syndrome [19]. NAFLD is the hepatic manifestation of the metabolic syndrome [19–26]. NAFLD and NASH are increasingly recognized as part of the metabolic syndrome [27] and many cases that have been previously described as cryptogenic cirrhosis may in fact have been related to NASH [28, 29].

Only a proportion of patients with the metabolic syndrome have NAFLD and of these only a minority (but a significant minority) (~20%) will progress to steatohepatitis [30, 31]. The first step in this pathway is undisputedly lipid accumulation in the hepatocyte. However, what incites the progression from simple hepatic lipid deposition to hepatic inflammation remains unclear and controversial. This paper addresses the question of whether hepatic iron is relevant.

The development of NAFLD has been proposed to result from a two-stage process [27, 32]. Firstly, lipid accumulates in the liver, resulting primarily from dietary sources or from the flow of free fatty acids (FFAs) released from adipose tissue. This is frequently associated with IR (see below). The second stage is proposed to result from oxidative stress causing necro-inflammation and cytotoxicity [4] leading to NASH, fibrosis, and ultimately cirrhosis [33]. Many of the factors have been implicated in triggering this second stage in vivo and in vitro [34]. The factors that induce oxidative stress are multiple and are discussed below. Those include increased hepatic iron concentration.

As in other chronic liver diseases, for example, hepatitis C and alcoholic steatohepatitis, it has been observed that a significant proportion of patients with NAFLD have hyperferritinemia and elevated hepatic iron concentrations. This raises three questions: (1) what role does iron play in disease progression; (2) is there a direct correlation between iron indices and disease activity that could be used for screening or staging purposes; and (3) can we draw useful management conclusions from these observations?

## How does iron cause cellular and organ damage?

In contrast to alcoholic necroinflammation, the sources of oxidative stress in NAFLD are less clear [35–38]. However, excess FFAs in the hepatocyte lead to saturation of mitochondrial $\beta$-oxidation, peroxisomal $\beta$-oxidation which then results in hydrogen peroxide production [32, 39–41]. In the presence of free iron this will then generate hydroxyl radicals causing oxidative stress [31]. The end-product of peroxidation and malondialdehyde can activate hepatic stellate cells [42–44]. Reactive oxygen species (ROS) and lipid peroxidation may trigger the release of cytokines which may further cause liver damage [32, 45]. Early reports on the association of hepatic iron with NAFLD and NASH were controversial with two in particular not supporting a significant role for hepatic iron [46, 47]. However, more recent studies have strongly suggested a causative role for iron with several studies demonstrating that increased hepatic iron contributes to progression of NAFLD [47–52]. The recent very large study by Valenti et al. [41] has provided strong evidence that iron deposition predominantly in hepatocytes is associated with more severe liver damage in patients with NAFLD although HFE mutations cannot identify the patients with hepatocellular iron accumulation in NAFLD [41, 48, 52, 53].

Iron also appears to be implicated in a vicious cycle of interfering with insulin signalling leading to increased hepatic glucose production [53–55] and this aggravates IR and thus leads to more hepatic lipid deposition. Elevated TNF-alpha levels in the metabolic syndrome have also been linked to the net accumulation of fat within hepatocytes [56]. It is unclear whether iron has any effect on adipokine or cytokine levels.

Hepatic iron is increased in primary and secondary iron overload syndromes including hepatitis C, alcoholic hepatitis, and NASH. The pattern of iron deposition in patients with hemochromatosis was first described by Scheuer et al. [57]. Hemochromatosis associated iron deposition is predominantly hepatocellular in the periportal (zone 1) and decreasing towards the centrilobular areas (zone 2). A second pattern of iron deposition is predominantly reticuloendothelial without an obvious zonal gradient and associated with ferroportin disease and secondary forms of iron overload [58–60]. This pattern is more common in subjects with NAFLD and increased hepatic iron (Fig. 1).

## Insulin resistance, NAFLD, and iron

IR may be defined as a failure of target cells to respond to insulin and results from disordered insulin signalling downstream from the insulin receptor. Clinically, IR is characterized by normal or high blood glucose levels despite high serum insulin concentrations [61, 62]. In

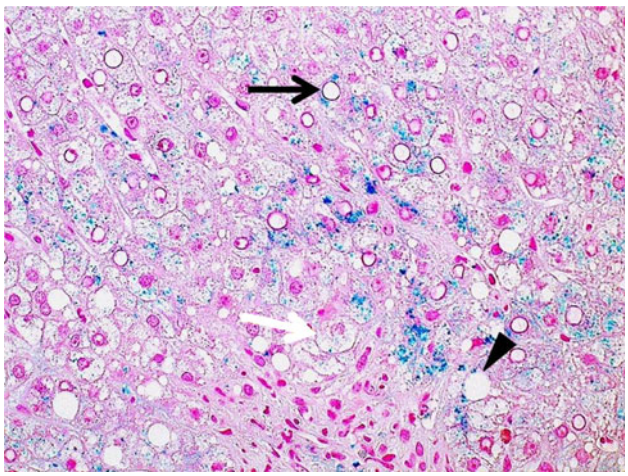

**Fig. 1** Liver biopsy from the patient with NASH and iron deposition. The arrows show the following: *black arrow* glycogen vacuolation of hepatocyte nucleus, *white arrow* ballooned hepatocyte, *Black arrowhead* steatotic hepatocyte (courtesy of Dr. Andrew Clouston)

NAFLD, IR results primarily in the disorders of carbohydrate and lipid metabolism which promote profibrogenic and proliferative events culminating in hepatic fibrosis and hepatocellular carcinoma. Several studies have supported a role for IR in the development of NASH [63] and these were reviewed in detail by Chitturi and George [64]. They propose that impaired insulin action prevents suppression of lipolysis thereby increasing serum FFA levels and FFA flux into the liver resulting in increased hepatic lipogenesis and the generation of hydrogen peroxide, a source of oxidative stress. In addition, several reports have suggested that type 2 diabetes is an independent risk factor for the development of hepatic fibrosis in NASH. Thus, Chitturi and George propose that IR is an important determinant for both hepatic steatosis and the progression of hepatic steatosis to steatohepatitis.

Thus, IR is common in patients with obesity and a hallmark feature of the metabolic syndrome. Moirand et al. [65] first reported on a group of patients with metabolic disorders, who had normal transferrin saturations, hyperferritinemia, and mild to moderate iron overload on liver biopsy or MRI. An inflammatory state and other diseases associated with iron overload were excluded. In a follow-up article, these findings were termed insulin-resistance associated hepatic iron overload (IRHIO). It is questionable whether IRHIO really is a different entity from NAFLD and NASH as most of the patients were obese, had hyperlipidemia and an impaired glucose metabolism. Liver biopsies were performed in less than half of the study cohort. In this setting, patients have an increased risk of cardiovascular events and cancer development.

IR is almost universal in NAFLD and NASH [66]. It has been associated with increased hepatic iron levels [67, 68]. Improved glycemic control [69] as well as reduction of systemic iron by venesection [55] has been shown to reduce serum ferritin and hepatic iron concentrations. Several studies have emphasized the improvement of IR and glycemic control in patients with [55, 70] and without [71, 72] NAFLD by venesection.

Overall 96% of the patients with cirrhosis are glucose intolerant and about 30% are diabetic [73]. Diabetes that develops as a consequence of liver disease has been termed "hepatogenous diabetes". IR and diabetes are more prevalent in certain liver diseases, such as alcoholic liver disease, hepatitis C virus infection, non-alcoholic liver disease, and hemochromatosis, all of which are also associated with increased iron stores. This is significant as the influence of iron in the pathogenesis of IR and diabetes in the above mentioned diseases is attracting increasing attention. In a recent study from Korea [74] patients with hepatogenous diabetes were compared with patients with type 2 diabetes. HOMA-IR index, postprandial compared to fasting blood glucose levels and fasting insulin levels were all significantly higher in patients with hepatogenous diabetes.

Thus, it is of interest that the liver diseases which are most commonly associated with IR and diabetes are the ones that are also associated with iron overload.

## Oxidative stress and the role of iron

Oxidative stress represents an imbalance between the production of ROS and the body's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Cellular toxicity results from the disturbances of normal redox state by the production of peroxides and free radicals that damage cellular proteins, lipids, and deoxyribonucleic acid (DNA). Oxidative stress is the final common pathway leading to tissue injury in many diseases [75–78].

Iron has been implicated in the production of oxidative stress [79, 80]. Iron is a transition metal and readily engages in one-electron redox reactions between ferric (3+) and its ferrous (2+) form. It is essential in many critical cellular enzymes (such as ribonucleotide reductase) and in hemoproteins. On the other hand, its reactivity can cause cytotoxic tissue damage and lead to fibrosis in states of iron overload [81–83]. The capacity of labile cellular iron to catalyze the formation of highly ROS underlies its cellular toxicity as these radicals can damage a wide range of cellular macromolecules, including DNA [84], proteins, and lipids [50]. Although ROS are damaging, they are also generated during normal metabolism in organelles, such as mitochondria and peroxisomes. Under normal circumstances, the body uses a range of defence strategies to protect against excessive ROS accumulation and their effects, including various ROS-degrading enzymes, antioxidants, repair processes (e.g., DNA repair), and iron sequestration. Labile iron in the cell can be derived from the degradation of the iron storage protein ferritin, but the most damaging iron likely comes from extracellular sources. As body iron levels increase in hereditary hemochromatosis (HH) and other iron-loading states [85], the iron binding capacity of plasma transferrin may be exceeded and the proportion of free-iron in the plasma, or non-transferrin-bound iron (NTBI) can reach quite high concentrations (10–15 μM or more). NTBI is cleared very rapidly from the plasma by the liver and other organs [86–88]. It is thought that NTBI in the plasma per se is not unduly toxic, but rather the unstable component of NTBI that enters cells [89]. NTBI levels in HH are effectively reduced after phlebotomy. Hepatic iron overload has, thus, been shown to increase oxidative stress [49, 79, 90]. It is commonly found in patients with NAFLD and has been associated with IR [35].

A recent interesting study from Western Australia has provided important links between iron, lipid metabolism,

insulin resistance, and the pathophysiology of NAFLD and NASH [91]. Graham et al. [91] studied mice fed diets containing different amounts of iron to observe the effects of iron on hepatic cholesterol synthesis, specifically the effects of iron on the expression of enzymes coordinating the cholesterol biosynthetic pathway using microarray technology to identify the differences between groups of genes with related biological functions. They found that the expression of 3-hydroxy-3-methylglutarate-CoA reductase, a key rate-limiting enzyme in cholesterol synthesis as well as several other genes encoding enzymes in the cholesterol biosynthetic pathway, were positively regulated by hepatic non-hem iron content. In addition, hepatic cholesterol content was significantly correlated with hepatic non-hem iron levels. Graham et al. [91] concluded that iron-induced changes in hepatic enzyme expression resulted in functional increase in cholesterol production, thus providing a key link between hepatic iron and cholesterol synthesis.

In an accompanying editorial, Sharp [92] expands on this story by adding the recent findings relating to the so-called unfolded protein response (UPR), which results from endoplasmic reticulum (ER) stress, and he proposes a model which comprehensively links iron, cholesterol, and IR in the development of NAFLD (Fig. 2). In this model, over-nutrition leads to IR, increased lipid deposition in the liver, and increased flux of FFA from adipose tissue to the liver. Associated inflammation leads to increased production of the adipokine, leptin, which in turn increases hepatic hepcidin production and thereby increased intracellular iron [93]. This increase in intracellular iron is positively associated with elevated hepatic cholesterol synthesis, further increasing the liver lipid burden. The combination of steatosis and cellular iron loading together with the increased FFA, could result in increased oxidative stress [49], which would exacerbate the progression from steatosis to NASH and its hepatic complications.

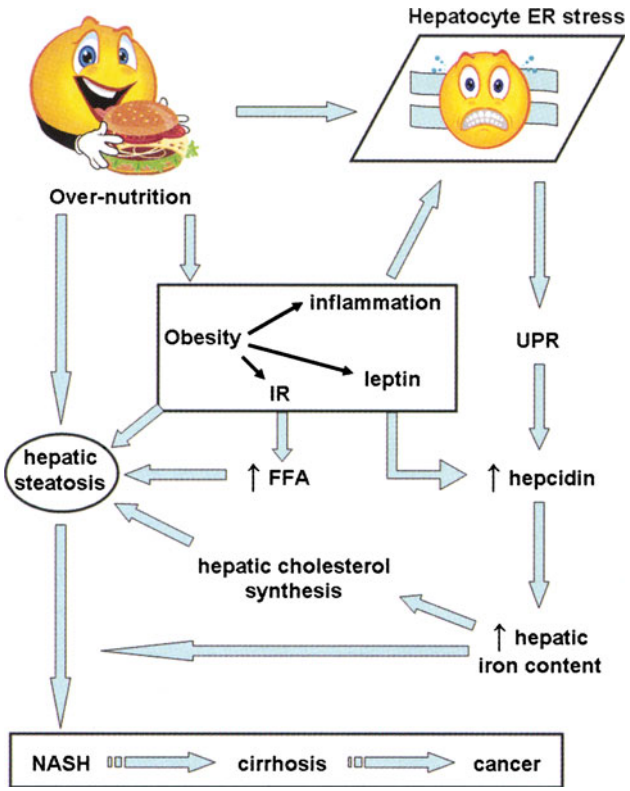


**Fig. 2** Hypothesized roles of cholesterol and iron in the development of NAFLD [92], with permission

## Why does iron accumulate in the liver?

It is not clear why some patients with NAFLD are prone to hepatic iron accumulation. Several mechanisms have been proposed. Aigner et al. [94] suggest an impaired release of iron from liver cells as an underlying mechanism for iron accumulation in NAFLD. They found down-regulation of ferroportin-1, the iron exporter in hepatocytes and sinusoidal Kupffer cells, and hemojuvelin gene expression. This also explains the characteristic pattern of iron deposition in NAFLD that is different to the pattern seen in hereditary hemochromatosis, i.e., hepatic and sinusoidal iron deposition without zonal gradient [59].

Iron accumulation may also be linked to copper homeostasis. One study [95] found low serum and liver copper concentrations as well as low serum ceruloplasmin levels, hepatic FP-1 mRNA and protein expression in patients with NAFLD, and iron accumulation. Low copper bioavailability may, therefore, lead to decreased ceruloplasmin ferroxidase activity and consequently impair FP-1 mediated cellular iron export. However, this is not necessarily by affecting ferroportin. Two recent interesting publications have demonstrated that oxidative stress/ROS generated by various ways in hepatic cells (a characteristic of NAFLD/NASH) can down-regulate ceruloplasmin by a novel mRNA decay mechanism that may contribute in hepatic iron accumulation by decreasing hepatic iron release [96, 97]. A dysregulated hepcidin production has also been suggested to contribute to iron accumulation [93].

The role of HFE gene mutations in iron overload related to NAFLD is controversial with some studies showing an increased incidence of homozygous or heterozygous (C282Y) mutations [98] and others finding no association [35, 46, 48, 49, 99]. However, the recent work of Valenti et al. [41] strongly suggests that it is iron deposition rather than HFE mutation that is the key factor in the pathogenesis of NAFLD and NASH. A significant correlation between steatosis and fibrosis was seen in a study of 214 C282Y homozygotes and this remained significant even

after the adjustment for other factors, such as the degree of iron loading and alcohol intake [100].

## Serum ferritin

Ferritin is an ubiquitous 450 kD protein. Its 24 subunits form a hollow sphere that can store up to 4,000 iron atoms. Tissue ferritin and hemosiderin form the iron storage of each cell, but in iron overload it is primarily the hepatocytes and reticuloendothelial cells that are involved [101]. Ferritin has an extraordinary capacity for uptake, storage, and release of iron. It, therefore, stores iron in its bioavailable form and protects cells from the cytotoxic effect of ionized iron.

The origin and function of serum ferritin are still unknown. It is more heavily glycosylated and carries comparatively little iron. In the steady state, serum ferritin levels reflect total body iron stores. In hereditary hemochromatosis, elevated serum ferritin levels have also been associated with advanced fibrosis [102]. However, levels vary with age and gender and are altered by inflammation and parenchymal damage. The work of Ruddell et al. [103] from Brisbane strongly indicates that ferritin is a cytokine which acts on the ferritin and transferrin receptors to further release tissue cytokines and activate Kupffer and stellate cells exacerbating fibrosis. Serum ferritin best reflects body iron stores and raised levels indicate iron overload in the absence of inflammation. Thus, the level may be elevated in steatohepatitis out of proportion to liver and body iron stores. In contrast to hereditary hemochromatosis, however, the transferrin saturation is not elevated in this situation [54].

## Non-alcoholic fatty liver disease, serum ferritin, and iron

As discussed above, iron may be involved in the production of oxidative stress as a "second hit". Thus, iron indices may not be reliable markers for hepatic iron overload or severity of disease in NAFLD as both conditions lead to an increase in serum ferritin level. Serum ferritin was positively associated with mild and moderate iron overload in univariate and multivariate analysis in a study from Bugianesi et al. [48], however, there was no correlation between serum ferritin and hepatic iron content. This suggests that serum ferritin is a marker of IR rather than hepatic iron overload in NAFLD and NASH [104, 105]. Several other studies have shown similar correlations between serum ferritin and NASH [49, 102, 106, 107]. An important issue with respect to serum ferritin as a marker for iron overload and inflammatory activity is its role as an acute phase reactant protein. It has been suggested that the metabolic syndrome, in particular T2DM is a proinflammatory condition as evidenced by elevation of C-reactive protein (CRP) and TNF-alpha levels. In this setting, plasma soluble transferrin receptor levels may correlate more closely with iron stores [108].

Yoneda et al. [109] from Japan have recently investigated the serum ferritin concentrations, HFE gene mutations and IR in 86 Japanese NASH patients (24 with steatosis and 62 with NASH verified by liver biopsy as well as 20 control subjects). They observed specifically the diagnostic utility of serum ferritin levels as a means of distinguishing NASH. They found that the serum ferritin concentration was significantly higher in NASH patients than in patients with simple steatosis ($P = 0.006$). Notably, there was no significant difference between the groups with respect to HFE gene mutations but the serum ferritin level was related to insulin resistance. They concluded that high serum ferritin concentrations are distinguishing features of Japanese NASH patients independent of HFE gene mutations.

## The effect of phlebotomy

If iron overload does indeed lead to the "second hit" and, therefore, significantly influences the progression of hepatic fibrosis, depletion of iron should be able to at least attenuate this. A recent study indeed found that phlebotomy is an effective and safe treatment option to ameliorate the detrimental effects of iron in patients with NAFLD [94]. In this study, iron indices as well as serum transaminase levels significantly improved in 32 patients with NAFLD and iron overload. Phlebotomy has also been associated with improved IR [55, 70–72, 110].

Valenti et al. [41] recently reported a study of 587 Italian patients with NAFLD and 187 control subjects. They found that the presence of predominantly hepatocellular iron deposition was associated with increased risk of hepatic fibrosis compared with the absence of detectable siderosis. This strongly supports the original observation by George et al. from Brisbane who found on multivariate analysis in a series of 51 patients with NASH that HFE mutations were associated with increased hepatic iron, acinar inflammation, and steatosis [49]. This study was further confirmed by a similar report from the US by Bonkovsky et al. However, the study of Valenti supports the findings reported from Sydney by Chitturi et al. that HFE mutations were not associated with increased risk of fibrosis. Kowdley in an accompanying editorial proposed that two important factors may explain the inconsistent findings, the prevalence of HFE mutations in the different populations at risk [111] and the proportion of patients with advanced disease, both of which could influence the power of the study. Nelson et al. [51] reported that the presence of the C282Y mutation was associated with advanced hepatic

fibrosis in a cohort of 126 patients with NASH ascertained from several centres in the US and Canada. The conclusion from these studies is that HFE and increased hepatic iron are probably relevant to the development of NAFLD and NASH when they are present [101, 112, 113] but in some communities iron is not a prominent feature of the disease [114]. Of the two factors, increased hepatic iron would seem to be more relevant.

### NAFLD, hepatocellular cancer, and iron

Primary hepatocellular cancer has an increasing incidence in developed countries [115, 116]. It is now the fifth most common cancer worldwide and because of its overall poor prognosis it is the third most common cause of cancer death. Half of this increase is due to hepatitis C virus-related cirrhosis. As the incidence of the metabolic syndrome, including NAFLD is also rising, there is an increasing number of patients with progressive liver disease [117], cirrhosis, and hepatocellular cancer in this patient group [118–122]. Thus, patients with NASH and cirrhosis should be screened at regular 6-monthly intervals with serum alpha fetoprotein and ultrasound examination.

Iron appears to be a risk factor for development of HCC in these conditions [48, 83, 123]. Sorrentino et al. [123] found in a recent retrospective study that iron overload in patients with NASH-related cirrhosis may be associated with development of HCC. The group enrolled 51 patients with NASH-related cirrhosis and HCC and compared them to a control group of 102 patients with NASH-related cirrhosis but without HCC. The group of patients who had developed HCC on a background of NASH-cirrhosis was found to have larger iron deposits on histologic examination of liver biopsy specimen. The multivariate analysis showed that the sinusoidal iron deposits were more frequent and larger in the HCC/NASH group than in the control group.

Iron overload, even if mild, has also been associated with cancers other than HCC [124]. Osborne et al. [125] found in a large prospective study of 30,000 subjects who were followed for 14 years, that male patients with C282Y hemochromatosis were at increased risk for developing colon cancer (RR 2.28) and female C282Y hemochromatosis patients were at an increased risk of developing breast cancer (RR 2.39). Heterozygotes and compound heterozygotes were not at an increased risk. This has important implications for screening and surveillance.

## Summary, conclusions and clinical relevance

The increasing incidence of obesity and the metabolic syndrome and the related disorders of hepatic steatosis (NAFLD) and steatohepatitis (NASH) have important implications for the diagnosis and management of liver disease. There is increasing evidence for a significant relationship between iron, insulin, steatosis, and hepatic injury. It has been proposed that the mechanism of hepatic injury is mediated by cumulative effects of oxidative cellular injury which may be aggravated by tissue iron. There is also increasing evidence that venesection or phlebotomy therapy leads to improved plasma insulin levels and IR in subjects with NAFLD [70, 110] but without hemochromatosis [55, 70]. What is now required is a suitably powered controlled trial of phlebotomy therapy in NAFLD before this therapy can be recommended in clinical practice.

Although the role of hepatic iron in NAFLD and NASH has been controversial, the weight of evidence from very recent studies suggests that when increased hepatic iron is present it is relevant to the progression of the disease. Unanswered questions include the following: (1) why do only 20–60% of NAFLD patients have abnormal iron indices? The presence of one or two copies of the C282Y mutation for hemochromatosis is relevant in some patients [49, 98]; (2) are there different clinical characteristics of NAFLD patients with or without abnormal iron indices? This seems unlikely on the available evidence; (3) among persons with iron deficiency states, does NAFLD present as less severe disease? There are no firm data to suggest this is so; and (4) do obese persons with primary or secondary iron overload states have an increased prevalence of NAFLD/NASH? As mentioned earlier a large study of 214 C282Y homozygotes showed a significant correlation between hepatic steatosis and fibrosis which remained significant even after the adjustment for other factors, such as alcohol intake.

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