NON-HFE IRON OVERLOAD: IS PHLEBOTOMY THE ANSWER?

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Abstract

Iron is an essential factor for life, however a physiologically optimal balance is critical. In this article we explore the role of iron as a co-factor in a range of chronic liver diseases and how it may contribute to the development of liver injury, fibrosis, cirrhosis and ultimately hepatocellular carcinoma. Whilst iron depletion therapy through phlebotomy is the most effective method of reducing iron stores, it is unclear whether this offers utility in the therapy of liver diseases in which iron is not the primary insult resulting in tissue injury. Here we examine the emerging evidence in the field of non-HFE hereditary haemochromatosis conditions associated with iron overload – is phlebotomy the answer?

Keywords ferritin; iron; phlebotomy; hepcidin; non-alcholic fatty liver disease; hepatitis C; porphyria cutnea tarda; alcoholic liver disease; metabolic syndrome.

Introduction

Iron (Fe) is an essential factor for life, however a physiologically optimal balance is critical. Too much or too little iron can have detrimental effects on human health. Evidence for the part that Fe plays in hepatic and extrahepatic diseases is fast gathering pace. In this article we explore the role of Fe as a co-factor in a range of chronic liver diseases and how it may contribute to the development of liver injury, fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC). Fe depletion through therapeutic phlebotomy is the most effective method of reducing body Fe stores, however it is unclear whether this offers utility in the therapy of liver diseases in which Fe is not the primary insult.

Regulation of iron metabolism in health and disease

In healthy individuals approximately 1-2 mg of Fe is absorbed from the diet per day to maintain Fe balance. Once absorbed, the Fe is bound by the plasma protein transferrin and is transported to the tissues where most of the Fe is taken up by the bone marrow for incorporation into haemoglobin for erythropoiesis, and to a lesser degree by the muscle for the synthesis of myoglobin and respiratory enzymes. The excess Fe is stored primarily in the liver. Macrophages degrade the erythrocyte-derived haemoglobin and release the Fe back into the plasma, so it can be re-utilised for erythropoiesis in the bone marrow. Too much Fe is detrimental to health as excess Fe generates free radicals causing oxidative stress and tissue damage primarily in the liver, heart and pancreas [1-3]. Providing further evidence as to the detrimental effects of Fe, it has now been shown that reduction of body Fe stores by phlebotomy therapy, even within the normal physiological range, is associated with reduced mortality from endpoints such as cancer [4].

Fe metabolism is tightly regulated by the Fe-regulatory hormone hepcidin, which is highly expressed by hepatocytes and at lower levels in other tissues including the kidneys. Hepcidin is a negative regulator of Fe absorption by the intestine, Fe release from macrophages and hepatic stores. It is secreted into the circulation and binds to the Fe exporter ferroportin, which is expressed on the surface of enterocytes, macrophages and hepatocytes, causing ferroportin internalisation and degradation. This limits the absorption and release of Fe and increases retention in the liver and macrophages [1, 5]. Hepcidin expression is influenced by the balance of a number of positive and negative regulators (Fig. 1). Excess Fe, inflammation and endoplasmic reticulum stress up-regulate hepcidin expression, which in turn, limits the availability of Fe for erythropoiesis and other Fe-dependent processes. Fe deficiency, erythropoiesis and reactive oxygen species (ROS) down-regulate hepcidin expression, which subsequently increases Fe bioavailability. Fe dependent regulation of hepcidin is controlled by bone morphogenetic protein /haemojuvelin/SMAD and HFE/transferrin receptor 2 (TFR2) signalling pathways. The pro-inflammatory cytokine interleukin-6 (IL-6) upregulates liver hepcidin via a STAT3 signalling pathway [1, 2]. During anaemia increased erythropoietic activity is required to down-regulate hepcidin expression and this is regulated by a number of factors including growth differentiation factor 15, while hypoxia and erythropoietin can also directly impair hepcidin expression. ROS reduces hepcidin via CCAAT/enhancer-binding protein α (C/EBPα) signalling pathway [1, 2].

Diagnosis of iron overload and tissue injury

Fe overload can be suspected from clinical information or elevated serum Fe biochemistry, but definitive diagnosis requires measurement of body Fe content (reviewed in [2]). This can be achieved by MRI-based R2 relaxometry, liver biopsy measurement of hepatic Fe concentration (HIC), or quantitative venesection [2, 6, 7]. Clinical features are nonspecific [2, 6, 7] whist a history of transfusion or administration of Fe (oral or intravenous) can be used to provide an estimate for the dose of administered Fe [8]

Serum measures of Fe biochemistry (transferrin saturation (TSAT) and serum ferritin) are well established in assessment of Fe overload in Hereditary Haemochromatosis (HH) and transfusion-related Fe overload. Unfortunately, measurements of serum ferritin may not accurately reflect the amount of Fe in the body because factors including body mass index, infection, inflammatory diseases or malignancy can also alter ferritin levels [1, 6, •9].

The most objective parameter of Fe overload is direct measurement of organ Fe concentration. Measurement of HIC accurately reflects total body Fe stores. Liver biopsy provides quantitative measurement of HIC and liver pathology, but it is invasive and subject to sampling error [10]. The most accurate non-invasive methods use magnetic resonance imaging [2, 11, 12]. A validated non-invasive MRI-based R2 relaxometry method has been described which accurately measures hepatic (HIC, FerriScan®) and cardiac (T2*/R2*) Fe concentration in a range of diseases [11, 13]. Once a certain threshold of HIC is exceeded, Fe can begin to accumulate in the heart and other organs [14]. HICs above 130 μmol/g dry weight are associated with increased risks of liver injury [15] whilst clinical cardiac toxicity occurs when HIC exceeds 270 μmol/g [16]. Changes in HIC generally precede changes in cardiac Fe

loading [14], acting as an early warning of possible future cardiac complications. Direct measurement of cardiac Fe deposition using the T2*/R2* MRI method enables assessment of the risk of Fe-induced arrhythmia and cardiac failure [13], and correlates with new echocardiographic measures using 2D speckle tracking [17].

Tissue injury can also be ascertained using non-invasive approaches. An increasing number of blood tests that estimate the likelihood of significant liver fibrosis or cirrhosis have been introduced. Most have been validated in chronic viral hepatitis and non-alcoholic fatty liver disease (NAFLD) [18, 19]. There have been various serum biomarkers suggested for use in Fe overload states, namely AST, platelets and ferritin [20, 21]. Transient elastography (FibroScan) is a non-invasive measure of liver 'stiffness' that gives an accurate indication of the presence of fibrosis [22]. It is a relatively simple, reproducible bedside test. Stebbing et al performed a meta-analysis and showed that a liver stiffness of 7.71 kPa for fibrosis (F2 or greater) had a sensitivity of 71.9% and specificity of 82.4% whereas for cirrhosis (F4) the results showed a cutoff of 15.08 kPa with a sensitivity of 84.45% and specificity of 94.69% [23]. Limitations include obesity and active inflammation, which can give falsely high results. Despite the good predictive values for cirrhosis, FibroScan scores do not correlate with serum ferritin levels in Hereditary Haemochromatosis (>7.1kPa) [24]. A longitudinal study in transfusion-independent β-thalassaemia patients showed correlation between serum ferritin levels and liver stiffness over 4 years [25].

Mechanism of iron-induced liver injury

An increase in hepatic Fe stores is linked to more severe fibrosis in alcoholic liver disease, non-alcoholic steatohepatitis (NASH) and viral hepatitis but not biliary causes of liver disease [26, 27]. In well-compensated cirrhosis, the presence of

stainable Fe on liver biopsy may be predictive of more rapid deterioration in liver function compared with patients without siderosis [28]. An excess of hepatocellular Fe generates ROS that cause lysosomal fragility [29], mitochondrial dysfunction [30] via lipid peroxidation of organelle membranes, resulting in the formation of protein and DNA adducts and mutation [31]. Usual cellular protective mechanisms are overwhelmed in Fe overload states leading to apoptosis and cell necrosis.

Hepatic stellate cells (HSCs) play a pivotal role in the production of extracellular collagen and connective tissue matrix resulting in hepatic fibrosis. When this occurs concurrently with dysregulated regeneration, cirrhosis and HCC may ensue [32]. HSCs are activated, in part, by oxidative stress due to Fe loading and a direct link between Fe and fibrogenesis via HSC activation has been demonstrated [33]. Liver progenitor cells (LPCs) are involved in hepatic regeneration during chronic liver injury [34], where their numbers are strongly correlated with disease severity [35]. LPCs are found in close proximity to HSCs and exhibit cellular cross-talk via a paracrine signalling mechanism [36]. LPCs are often observed in liver diseases associated with Fe overloading including HH, alcohol-related liver disease, chronic hepatitis B and C infection [35, 37, 38], non alcoholic fatty liver disease [39]. Dietary Fe loading of rats has also been shown to induce proliferation of LPCs [40].

Definition of Fe overload disorders

Fe overload exists when tissue or body Fe stores are elevated above the normal range. Such elevations can occur in a range of primary or secondary Fe overload disorders (table 1)**.** Primary (or genetic) aetiologies are reviewed extensively elsewhere [2]. For the remainder of this article we will focus on the role of iron as a secondary factor in a range of common liver diseases and malignancy.

Chronic Hepatitis C

Elevated serum iron parameters in patients with chronic hepatitis C are present in up to 36% of patients [41]. Patients with chronic hepatitis C have higher levels of steatosis, serum ferritin, TSAT and tissue Fe compared with other forms of chronic liver disease. The role of hepcidin in chronic hepatitis C remains unclear. Hepcidin levels may either be decreased in response to elevated levels of ROS or up-regulated due to inflammation and endoplasmic reticulum stress [42]. Fe contributes to the increased risk of hepatocellular carcinoma through DNA damage from Fe-induced adduct formation and chromosomal damage [43, 44]. In addition, hepatitis C virus may have a direct cytopathic effect on hepatocytes through the occurrence of Fe-dependent lipid peroxidation [45]. Individuals heterozygous for C282Y or H63D mutations in the *HFE* gene product in conjunction with chronic hepatitis C are at increased risk of developing hepatocyte damage and liver fibrosis [46]. Fe has been shown in many studies to impair immune responses and potentially lymphocyte-dependent clearance of the hepatitis C virus [47, 48]

Following the introduction of interferon monotherapy, lower levels of stainable Fe were shown to be associated with a better response to interferon alpha therapy [49] and treatment resulted in a reduction in HIC [50]. Later studies suggested that an HIC of greater than 1100 μg Fe /g was predictive of non-response in nearly 90% of patients [51, 52] and increased serum and/or hepatic Fe parameters were associated with a lower likelihood of response to interferon therapy [53]. However, following the introduction of combination interferon and ribavirin therapy, the presence of raised HIC no longer influenced the likelihood of a sustained virological response rate when treating patents [54]. Interestingly, elevated serum ferritin levels

remained a predictor of response to pegylated interferon and ribavirin and were also associated with more severe liver fibrosis [55]. A significant rise in serum ferritin levels during the initial weeks of treatment has been associated with a good treatment response; this is likely to reflect a higher level of inflammation during this phase [•56]. More recently, Ryan et al [57] showed that following initiation of pegylated interferon alpha and ribavirin therapy there was a significant increase in serum hepcidin levels and hypoferraemia (a reduced serum iron concentration and TSAT). The level of hypoferritnaemia was most marked in those with a most substantial decline in viral load, a well-known predictor of an increased likelihood of sustained viral response [58]. Thus changes in Fe metabolism, or effects of altered hepcidin levels directly, which is known to have an effect on the immune system, could play a significant role in chronic hepatitis C infection and possible success of therapy.

When therapeutic phlebotomy was trialed in patients with chronic hepatitis C it was initially shown to reduce serum alanine aminotransferase (ALT) [59], improvement in liver histology [60, 61] and lower the risk of HCC development [62]. Phlebotomy prior to therapy with interferon-α was variably shown to improve the likelihood of sustained virological response in some studies [59, 63] but not others[60, 61]. Fe depletion therapy has not been shown to effect hepatitis C virus levels [59]. More recently Gentile et al. showed that venesection to a serum ferritin <50ng/ml did not improve the outcome of pegylated interferon based therapy [•64]. Not only has phlebotomy therapy not been evaluated either prior to or during modern combination therapy containing ribavirin, it would be extremely difficult given the common complication of anaemia due to such therapy. At present Fe-depletion therapy cannot be recommended for treatment of chronic hepatitis C infection.

Alcohol-related liver disease

Excessive alcohol consumption causes liver disease including steatosis, alcoholic hepatitis, cirrhosis and HCC. The amount of alcohol consumed does not have a direct correlation to the severity of the liver disease [65], suggesting that there are genetic and environmental factors influencing disease progression. It is possible that Fe could represent such an environmental factor. Alcoholic liver disease is often associated with elevated serum ferritin levels [66] and studies have suggested that it is the increased presence of Fe that acts synergistically with alcohol to cause increased rates of fibrosis [67, 68]. Alcohol induced hyperferritinaemia is most likely due to hepatocyte damage and induction of ferritin synthesis by pro-inflammatory cytokines [68]. Alcohol consumption leads to increased hepatocyte Fe uptake from desialylated transferrin in patients with ALD [69]. Alcohol and Fe induce oxidative stress which inhibits hepatic hepcidin production, resulting in increased Fe absorption and deposition in the liver [42].

A higher level of alcohol consumption in HH patients with HFE C282Y homozygosity leads to an increased rate of cirrhosis [70]. Animal studies have confirmed that in setting of alcohol excess, diet-induced Fe overload leads to an increase in oxidative stress in the liver and the development of liver fibrosis and cirrhosis [67, 68]. Conversely, Fe depleted diets lead to a reduction of alcohol induced liver disease [69]. It is the synergistic effects of alcohol and Fe on oxidative stress levels that accelerates an individual's progression to fibrosis and cirrhosis

To date, there have been no studies addressing the therapeutic utility of phlebotomy therapy in chronic alcoholic liver disease and thus no recommendation can be made regarding the use of phlebotomy treatment in this condition.

Non-alcoholic fatty liver disease (NAFLD)

NAFLD is a common cause liver disease and is becoming increasingly prevalent. Deposition of lipids causes the liver to be more vulnerable to damage from other stresses including hormones derived from adipose tissue (adipocytokines), oxidative stress and intestine-derived bacterial endotoxin [71]. Steatohepatitis is associated with nuclear factor kappa B and c-Jun N terminal kinase-mediated production of hepatic inflammatory cytokines such as tumour necrosis factor and interleukin (IL) 6, resulting in elevated serum aminotransferase levels and hepatomegaly [72, 73]. In NAFLD, hepcidin levels are affected by a variety of factors: increased iron stores, endoplasmic reticulum stress and inflammation via IL-6 increase hepcidin production, whereas oxidative stress leads to a decrease in hepcidin levels [74]. It is likely to be the combination of hepatic steatosis and an additional insult (Fe or alcohol) that initiates the oxidative stress cascade of lipid peroxidation, mitochondrial dysfunction, inflammation, apoptosis [71].

Ferritin levels are increased in 20-60% patients with NAFLD, but despite this only 5-10% patients have evidence of hepatic Fe overload [75]. Hyperferritinaemia in NAFLD usually reflects hepatic inflammation and fibrosis rather than true Fe overload. Kowdley et al. showed that a serum ferritin greater than 1.5 times upper limit upper limit of normal in patients with NAFLD is an independent predictor of advanced hepatic fibrosis [••76]. Hepatic Fe overload in patients with non-alcoholic steatohepatitis-related cirrhosis is observed more commonly in those patients that develop HCC than those who do not [77].

Fe-depletion therapy in patients with NAFLD, (even with normal body Fe stores) can result in the near normalisation of serum ALT levels and marked improvements in insulin sensitivity and improves insulin resistance [78]. Despite this,

it is generally thought that hepatic Fe overload does not play a major role in liver damage in those patients with NAFLD [79]. To date, phlebotomy has not been shown to have a significant impact in histological or clinical outcomes in NAFLD.

Porphyria cutanea tarda (PCT)

PCT is due to an acquired deficiency of hepatic uroporphyrinogen decarboxylase (UROD) and characterised by skin and liver involvement caused by porphyrin accumulation. Many factors contribute to the susceptibility of an individual acquiring clinically detectable PCT including hepatitis C virus infection, alcohol consumption, smoking, Human Immunodeficiency Virus infection, oestrogens and end stage renal failure. The above factors are capable of causing oxidative stress within the liver, potentially reducing hepcidin production thus increasing Fe absorption from the intestine and Fe release from macrophage stores, increasing serum iron levels and HIC [80]. Although hepatic Fe deposition in PCT is usually mild to moderate, it may promote the oxidisation of porphyrinogens, leading to the crystalisation of porphyrins [81].

Fe deficiency protects individuals from reduced UROD activity [82] and it has been long established that venesection, to decrease the Fe load in the liver, in almost all cases leads to remission [83]. The therapeutic aim is to reduce ferritin to below <25ng/mL [84, 85]. Maintenance of low serum ferritin levels will prevent recurrence of the condition [86]

Metabolic syndrome, cardiovascular disease and cancer

The metabolic syndrome (METS) includes abdominal obesity, dyslipidaemia, insulin resistance, glucose intolerance and hypertension. Increased Fe stores have

been postulated to play a role in pathogenesis of insulin resistance by increasing oxidative stress [87]. Patients with type 2 diabetes often have hyperferritinaemia due to the inflammation, insulin resistance and steatosis often associated with type 2 diabetes [88]. A reduction of ferritin levels in rats with type 2 diabetes via either phlebotomy or dietary Fe-restriction leads to an improvement in HbA1c levels [89]. In a study in which patients with METS were randomly assigned to Fe reduction therapy with phlebotomy, reduction of ferritin by two sessions of phlebotomy resulted in a reduction of systolic blood pressure, improvements in markers of cardiovascular risk and glycemic control [90]. Multiple studies now demonstrate that Fe depletion therapy can reduce HbA1c levels, increase insulin release and sensitivity. A recent review article has addressed the issue of Fe and metabolic syndrome in depth [91].

In patients with steatosis, increased Fe stores have been linked to a faster progression to cardiovascular disease [92]. Increased serum ferritin has been shown to be an independent risk factor for the presence of coronary artery calcium [93] although there is no evidence from population studies that ferritin is a risk factor for cardiovascular disease [94]. There is also no evidence that HFE gene mutations are associated with an increased risk of cardiovascular disease [95]. Moreover, mortality from cardiovascular disease is not associated with raised serum ferritin levels [96]. A large randomised clinical study of phlebotomy therapy in US veterans failed to demonstrate any risk reduction of cardiovascular events through phlebotomy therapy [4].

Fe has also been implicated as a factor which may increase the risk of cancer. The role of Fe in HCC development is well-documented and hepatic Fe overload, whether due to primary or secondary disorders of iron overload, is associated with an increased risk of HCC. The development of HCC is due to DNA damage related to Fe

toxicity, impaired liver regeneration and the wound-healing response, LPC proliferation and the evolution of cancer stem cells [97, 98]. It has also been demonstrated that patients with HFE C282Y homozygosity have a three-fold increase in the risk of colorectal cancer and breast cancer [99] and blood donation has been shown to be associated with lower cancer risk [100]. Zarcharski et al showed that in a predominantly older male population with peripheral vascular disease that Fe reduction therapy with venesection to a ferritin target range from 12-60 ng/ml resulted in a significant reduction of new visceral malignancies over a follow up period of 4.5 years [4]. Whilst these studies are encouraging, there is currently no consensus supporting a recommendation that blood donation should be advocated to reduce the risk of cancer *per se*.

Conclusion

Hepatic Fe is an important cofactor producing liver injury through the induction of oxidative stress, cellular damage and altered hepcidin production. Hepatic Fe deposition may contribute to increased fibrosis, cirrhosis and HCC. Phlebotomy therapy to reduce hepatic Fe stores and serum ferritin has only been shown to be of benefit in Hereditary Haemochromatosis and PCT. Hepatic Fe clearly has a synergistic affect on fibrosis and cirrhosis when due to alcohol, chronic hepatitis C or NAFLD. Evidence for a role of Fe in hepatic and extrahepatic diseases continues to accumulate and approaches to modulate Fe bioavailability or reduce body stores through phlebotomy may eventually become attractive therapeutic options.

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