Several non-hereditary liver diseases are associated with mild hepatic iron overload such as viral hepatitis, excessive alcohol consumption, non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH) [1–7]. In fact, the term insulin resistance or dysmetabolic iron overload syndrome was introduced to describe the association of body iron overload and NAFLD [8]. The contribution of HFE-mutations to the development of iron overload in these diseases remains debated [9,10] (NAFLD/NASH [8,11,12] (HCV [13–15]) (alcohol [16]), but is probably slight.

The underlying molecular mechanism centers on dysregulation of the iron regulatory hormone hepcidin. Hepcidin is primarily produced by hepatocytes, but there is also evidence for production by macrophages [17] and fat cells [18]. Hepcidin internalizes and subsequently degrades the cellular iron exporter ferroportin, present on the cell surface of macrophages and enterocytes, resulting in hypoferremia. Hepcidin is upregulated by inflammation, by the presence of increased body iron levels and inhibited by increased erythropoietic activity. These respective regulatory pathways are thought to protect the body from (i) extracellular proliferating pathogens, (ii) the harmful effects of iron overload and to (iii) ensure that body iron availability matches iron needs.

Recent evidence has demonstrated that perturbation of hepcidin underlies non-hereditary (or non-hemochromatotic or acquired) mild iron overloading hepatic diseases (NHIOD). Hepcidin expression is affected by the upstream stimulatory factor 1 (USF1), a well established susceptibility gene for familial combined hyperlipidemia, a complex disorder of lipid metabolism [19]. USF1 together with USF2 controls cis and trans regulation of hepcidin expression [20]. Other circumstantial evidence of a link between hepcidin, glucose and lipid metabolism comes from hepcidin producing hepatic adenomas in severe anemic type 1a glycogen storage disease (GSD1a) patients [21], and hepcidin production by fat cells [18]. Interestingly, many of the diseases that are part of the metabolic syndrome have a disrupted iron homeostasis [3,4] (e.g. diabetes [1,2,8], hypertension [22] and elevated body mass index [23]). Thus, we are only starting to discover the pleiotropic role of hepcidin.

Alcoholic and hepatic viral infections are the most typical examples of acquired mild forms of iron overload. Liver biopsies in these disorders consistently show iron deposits in hepatocytes and Kupffer cells [5,24]. Elevated liver iron deposits are also commonly observed in NAFLD [5,24].

Iron can induce oxidative stress, which can cause tissue injury. Indeed, hepatic iron deposits have been associated with more advanced forms of NHIOD, including liver fibrosis, cirrhosis and hepatocellular cancer in some reports (NAFLD/NASH [9,11,25,26] , HCV [27–29] , alcohol [30,31]), but this was not confirmed in others (NAFLD/NASH [12,32,33] , HCV [16,30]).

In NAFLD, there is increased urinary hepcidin excretion [34] and elevated liver hepcidin expression [35] but hepcidin levels are relatively low for the level
of iron overload [34], suggesting ineffective liver iron sensing.

In this issue of the Journal of Hepatology, Fujita et al. [36] confirm that in chronic hepatitis C virus (HCV), hepcidin expression is inadequate for the body iron stores, but can be restored upon viral eradication. In addition, they found that hepatic hepcidin expression reflects circulating hepcidin levels. This finding suggests that serum hepcidin measurements might become a proxy parameter for hepatic hepcidin and aid in the management of patients with acquired forms of mild hepatic iron overload such as in HCV.

### 1. Hepcidin peptide

Human hepcidin is mainly produced by hepatocytes as an 84 amino-acid (aa) pre-prohepcidin [37]. Subsequent post translational processing results in the biologically active 25 aa form (hepcidin-25, molecular mass is 2789 Da) that is secreted in the plasma. Additional amino-acid degradation results in the production of two smaller isoforms (hepcidin-20 and -22) [38,39], that have unknown biological significance.

### 2. Hepcidin assay

Progress in this field has been hampered by the difficulties many researchers face by measuring hepcidin. Until recently, only few tools were available to measure hepcidin. Largely because antibodies are hard to raise. Hepcidin has a small and compact structure and antigenic epitopes are scarce. In addition, hepcidin is conserved among a wide range of species which complicates the induction of antibodies in hosts such as rabbit. As a consequence, immunochemical methods based on specific anti-hepcidin antibodies are largely unavailable. Lastly, quantitation of hepcidin is complicated by its tendency to aggregate [40] and stick to laboratory plastics, necessitating implementation of robust laboratory procedures.

Currently, many studies use the serum-based commercial immunoassay that measures the hepcidin precursor prohepcidin rather than the bioactive peptide. The relevance of these data is controversial because prohepcidin levels fail to correlate with urinary and serum hepcidin [41,42]. Furthermore, when measured in healthy controls, serum prohepcidin levels are around 40–50 times higher than those observed for bioactive hepcidin (≈180 ng/ml versus ≈4 ng/ml) (unpublished observations). This suggests that prohepcidin and hepcidin have different biological profiles [41–44].

Ganz and Nemeth et al. have developed an immuno-dot blot assay using non-commercially available antibodies for the semi-quantitative measurement of the sum of all urinary hepcidin isoforms [45]. The results of studies that employ this method have greatly improved our understanding of the regulation of hepcidin in patients with distorted iron homeostasis [46].

More recently, new assays for the various hepcidin isoforms: hepcidin-25, -22 and -20 have been developed on a platform of novel technologies such as surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) and liquid chromatography tandem MS (LC/MS–MS) [38,47–49]. With the use of internal standards these MS techniques enable reliable quantitative hepcidin analysis in urine and serum (Fig. 1) [50,51]. In fact, MS optimally uses an internal standard to correct for extraction, variable ionization and suppression of the analyte. Internal standards are spiked to the clinical samples and co-analysed. The ratio between the synthetic internal standard and endogenous peptide is determined by MS, allowing calculation of the absolute amount of hepcidin.

In the current issue Fujita et al. exploit SELDI-TOF MS to (semi)quantify circulating hepcidin-25 levels [36,47]. This method shares the drawback of most currently available hepcidin analysis since it lacks the use of an internal standard. This precludes value assignment, necessitating expression in arbitrary units, and resulting in somewhat imprecise measurements. Nevertheless, their results provide important proof of principle insights in biologically relevant circulating hepcidin levels in HCV before and after treatment.

### 3. Clinical utility of the hepcidin assay

Before measurement of hepcidin in urine and serum can be introduced into routine clinical practice, several issues need to be addressed. This starts with establishment of reference values among a wide variation of controls, and the assessment of a diurnal variation or fluctuation in relationship to meals.

Proof of principle findings of a circadian (either intrinsic or diet related) variation with generally lower values in the fasted morning samples than in the afternoon [38], may necessitate standardized sampling in the morning after an overnight fast (as was nicely done by Fujita et al.).

Another relevant question is whether urine is a reliable alternative for serum to assess biologically relevant hepcidin levels. Urine hepcidin levels correlate with hepcidin expression in liver biopsies [52], but discrepancies have been reported [34]. Urine levels also correspond to circulating levels [38] and nicely fit in the concept of hepcidin as the iron regulatory peptide [46,53]. Still, we caution against the uncritical use of urine concentrations as a universal surrogate for circulating levels since urinary secretion of hepcidin will depend on glomerular filtration and tubular re-absorption. In addition, hepcidin mRNA has been detected in the kidney, which suggests the potential for local production and release into the urine [54].
4. Hepcidin resistance

Increased hepatic, urinary and serum hepcidin levels are found in obesity [18], dysmetabolic iron overload syndrome [34,35] and the metabolic syndrome (our unpublished observations). This (moderate) increase is likely due to a combination of (i) (low grade) inflammation and cytokine induced hepcidin production in hepatocytes [46,53,55] and (ii) hepcidin production by fat cells [18] especially in severe obese. At first sight, these increased absolute levels contrast to the mild parenchymal overload as observed in NHIOD, because hepcidin inactivates ferroportin which, in theory, should decrease iron uptake by the intestine. However, the precise functional effect of circulating hepcidin levels on iron release of various cell-types, among them enterocytes, is unknown yet. There is some evidence of hepcidin-independent regulation of iron absorption (reviewed in [56]). Other data suggest that hepcidin effects might be cell specific [57]. It is therefore plausible to postulate that in obesity related NHIOD (and possibly also in HCV and alcoholic liver disease) enterocytes are relatively resistant to circulating hepcidin, resulting in a relatively high intestinal iron absorption for the circulating hepcidin levels, while macrophages are more sensitive, leading to RES iron sequestration.

5. Hepcidin/ferritin ratio and common mixed pattern of iron deposits

Key in the report of Fujita et al. is their novel findings on reduced liver iron sensing in chronic HCV and especially its reversibility upon viral eradication. Similar to earlier studies in HH [51,58], they assessed inadequate iron sensing by calculation of the hepcidin/ferritin ratio. In our view the suitability of this ratio as a measure of inadequately increased duodenal iron uptake in NHIOD is uncertain. There might be unknown molecular players that regulate iron stores but are not taken into account.

The pattern of iron deposits in NHIOD are typically mixed: i.e. found in both Kupffer cells and the hepatocytes [5,24,59]. Regulation of hepatocyte hepcidin expression through the iron-sensing pathway appears to be independent of Kupffer cells [60,61]. These observations suggest that the hepcidin/ferritin ratio is only suitable as a measure to assess the adequacy of hepcidin regulation by the iron stores of parenchymal cells. This would implicate that a low ratio primarily inadequately predicts increased intestinal iron uptake when ferritin in the denominator reflects the iron levels in these parenchymal cells, such as occurs in various forms of HH. Hence, in NHIOD the decrease in hepcidin/ferritin ratio is likely to overestimate the reduction of iron sensing of parenchymal iron levels and the subsequent inadequate...
increase of intestinal iron absorption. It moreover fails to explain the observed Kupffer cell iron loading. It is therefore tempting to speculate that in HCV and other NHIOD local RES produced small amounts of inflammatory cytokines and hepcidin are involved in RES iron sequestration by decreasing ferroportin activity [62,63].

Clearly, more work is warranted to unravel the biological mechanism explaining the distorted iron homeostasis in these patients to assess the suitability of the hepcidin/ferritin ratio or its alternative log transformations as a marker for inadequately elevated intestinal iron uptake.

6. Hepcidin in the management of patients with NHIOD

By assessing serum hepcidin levels in HCV Fujita et al. [36] corroborate previous findings on a decreased liver iron sensitivity as a uniform mechanism underlying mild hepatic iron overload. Down regulation of liver hepcidin expression by increased hepatic reactive oxygen species (ROS) by inhibition of the DNA-binding of C/EBPα might be an important causal factor in this apparent decrease in hepatocyte iron sensing (Fig. 1).

Based on this role of hepcidin in NHIOD in general and chronic HCV in particular we propose that hepcidin may contribute to predicting and monitoring of iron overload in these patients. Relatively low hepcidin levels may indicate an increased risk of iron overload and normalization possibly points to the successful HCV eradication by antiviral regimes. Normalization of hepcidin levels by iron reduction (by e.g. dietary restriction, phlebotomy, iron chelation), antioxidants or hepcidin inducers or supplements, may perhaps be suitable as add-on treatment options for patients with NHIOD to improve the detrimental effects of iron on liver function. It is conceivable that in the case of HCV, hepcidin elevating therapies lead to an improved response to antiviral treatment. However, before we can translate these newly acquired insights into clinical practice, additional studies will be needed to further unravel critical cellular iron transporters involved in iron homeostasis in NHIOD.

Finally, if hepcidin proves to be a useful marker in the management of non-hemochromatotic iron overload reliable, accessible, high throughput, affordable and reproducible methods will be needed to facilitate the measurements that hepatologists need.

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References


