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Altered expression of iron regulatory genes in cirrhotic human livers: clues to the cause of hemosiderosis?

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Hepatic iron deposition unrelated to hereditary hemochromatosis occurs commonly in cirrhosis but the pathogenesis of this condition is unknown. The aim of this study was to compare the expression of genes involved in the regulation of iron metabolism in cirrhotic ($n = 22$) and control human livers ($n = 5$). Transcripts were quantitated by real-time RT-PCR and protein levels were assessed by western blot. Hepatic iron concentrations (HICs) were measured by a spectrophotometric method. Levels of hepcidin mRNA did not differ between controls and cirrhotic livers; there was a highly significant correlation between hepcidin transcript levels and HIC in the latter group. Ferroportin, divalent metal transporter-1 (DMT1), and ferritin heavy chain mRNA levels were significantly higher in cirrhotic human livers than in controls ($P = 0.007$, 0.039, and 0.025, respectively). By western blot, ferroportin and DMT1 levels were generally diminished in the cirrhotic livers compared to controls; neither correlated with HIC. In contrast, the abundance of ferritin increased with increasing HIC in the cirrhotic livers, whereas transferrin receptor decreased, indicating physiologically appropriate regulation. In conclusion, hepcidin expression appears to be appropriately responsive to iron status in cirrhosis. However, there are complex alterations in DMT1 and ferroportin expression in cirrhotic liver, including decreases in ferroportin and DMT1 at the protein level that may play a role in aberrant regulation of iron metabolism in cirrhosis.
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Iron deposition unrelated to the *HFE* mutations of hereditary hemochromatosis is a common finding in cirrhotic livers, occurring in up to a third of explanted livers.[1] Hemosiderosis appears to be more common in the setting of advanced liver dysfunction and may be predictive of a poor prognosis. We recently reported that the presence of stainable iron on an index liver biopsy showing cirrhosis was associated with higher baseline MELD score.[2] Moreover, even when the more advanced degree of liver dysfunction in these patients was taken into account, they tended to decompensate more rapidly than patients without stainable iron. Ganne-Carrié *et al*[3] have made similar observations in patients with alcoholic cirrhosis. In addition, liver transplant patients whose explant shows heavy iron deposition are reported to be at increased risk of mortality from infection.[4] However, despite the prevalence and clinical significance of hemosiderosis, its cause remains unknown.

In the past several years, several important discoveries have provided new insights into the mechanisms governing iron metabolism. These include the identification of the iron transporters divalent metal transporter 1 (DMT1) and ferroportin (Fpn), a novel transferrin receptor 2 (TFR2), hemojuvelin (Hjv), and the iron regulatory peptide hepcidin.[5–11] All of these are expressed in liver, and mutations in the latter four are associated with rare forms of iron overload.[10,12–15] As the liver is important in the regulation of iron metabolism under normal circumstances, it is reasonable to surmise that hemosiderosis may result from dysregulated expression of one or more of iron-related proteins in cirrhosis.

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Studies examining the expression of some of these newly described proteins in patients with chronic liver disease indicate that this may be the case. Stuart *et al*[16] have reported that duodenal expression of DMT1 and Fpn, the iron transporters at the apical and basolateral surfaces of the enterocyte, respectively, are increased in cirrhotic patients. These alterations would be predicted to increase intestinal iron absorption, potentially contributing to hepatic iron deposition. These changes may reflect decreased levels of hepcidin, which downregulates intestinal iron absorption by causing internalization and degradation of Fpn. Consistent with this prediction, Détivaud *et al*[17] found that hepatic hepcidin expression in 16 patients with moderate to severe fibrosis correlated inversely with fibrosis stage. These intriguing findings may provide an explanation for the pathogenesis of hemosiderosis; however, they remain to be confirmed in a larger study. Furthermore, as a comprehensive assessment of the effects of cirrhosis on the expression of genes involved in hepatic iron metabolism is lacking, it is unknown whether there are additional defects in iron regulation that contribute to the development of hemosiderosis. Therefore, the aim of this study was to compare the expression of genes involved in the regulation of iron metabolism in cirrhotic and control human livers.

MATERIALS AND METHODS

Human Livers

Samples from cirrhotic livers removed at liver transplantation were collected at University of Iowa Hospitals and Clinics ('cirrhotics;' $n=22$). Clinical and laboratory data were abstracted from the medical record. The latest laboratory values before transplantation were recorded. Control livers were obtained from patients undergoing resection of liver lesions at the University of Iowa Hospitals and Clinics ($n=5$). Samples collected from surrounding macroscopically normal-appearing liver were evaluated histologically to confirm the absence of tumor. Complete blood counts and iron studies were obtained on the morning of surgery. Liver samples from both groups were snap-frozen in liquid nitrogen and stored at -80°C. Because of the potential variability of intraorgan iron concentrations and gene expression, iron quantitation and RNA isolation were performed on three separate specimens from each cirrhotic liver. Except where noted, results presented are those obtained from the sample with the median hepatic iron concentration (HIC). Owing to the small size of the control specimens, only one sample was available for each parameter. The study was approved by the Institutional Review Board of University of Iowa.

HIC

Quantitation of HIC was performed as previously described.[18] The upper limit of normal using this assay is 1500 μg/g dry wt.

Histochemical Staining for Iron

Perls' Prussian Blue staining was carried out on formalin-fixed, paraffin-embedded sections of livers using standard techniques. Iron deposition was assessed using the method of Edwards *et al*.[19]

Real-Time RT-PCR

Quantitation of hepcidin (HAMP), ferritin (FTH1, ferritin heavy polypeptide 1), transferrin (TF), and TFR2 gene expression were assessed using the TaqMan assay using the primers given in Table 1. PCR reagents for transferrin receptor (TFRC), Fpn (also known as Slc40a1), DMT1 (also known as Slc11a2), and Hjv(also known as hemochromatosis type 2) were purchased from Applied Biosystems (Foster City, CA, USA; TaqMan Assays on Demand, assays Hs99999911_m1, Hs00205888_m1, Hs00167206_m1, and Hs00377108_m1, respectively). Oligonucleotide probes for HAMP, FTH1, TF, and TFR2 were labeled with the fluorescent dye FAM on the 5′ end and with TAMRA or BHQ on the 3′ end as shown in Table 1.

Real-time RT-PCR was performed as previously described using TaqMan 18S Ribosomal RNA Control Reagents (Applied Biosystems) as an endogenous control.[20] Validation experiments showed similar amplification efficiencies for the target gene and the 18S PCRs. The comparative C_t method was used to calculate the amount of target mRNA, normalized to the amount of 18S RNA, and relative to an internal calibrator (control human liver), expressed as $2^{-\Delta\Delta C_t}$. Semi-quantitative RT-PCR for DMT1 isoforms was performed using primers described by Hubert and Hentze.[21]

Table 1 Primer and probe oligonucleotide sequences for HAMP, FTH1, TF, and TFR2

Gene	Sense	Antisense	Probe
HAMP	5′-CCACTTCCCCATCTGCATTT-3′	5′-GCAGCACATCCCACACTTTG-3′	5′-FAMCTGCTGCGGCTGCTGTCATCG-TAMRA-3′
FTH1	5′-TGGCTTGGCGGAATATCTCT-3′	5′-GCCCGAGGCTTAGCTTTCAT-3′	5′-FAMTGACAAGCACACCCTGGGAGACAGTGBHQ-3′
TF	5′-TCAGCAGAGACCGAAGA-3′	5′-CATCCAAGCTCATGGCATCA-3′	5′-TGCATCGCCAAGATCATGAATGGAGATAMRA-3′
TFR2	5′-CGGAGTGGCTAGAAGGCTACC-3′	5′-CTGCGTTGTCCAGGCTCAC-3′	5′-AGCGTGCTGCACCTCAAAGCCGTAMRA-3′

FTH1, ferritin heavy polypeptide 1; TF, transferrin; TFR2, transferrin receptor 2.

Table 2 HIC and clinical parameters related to iron metabolism of study subjects

	Controls ($n=5$)[a]	Cirrhotics ($n=22$)[b]	*P*
HIC (μg/g dry wt)[c]	401 ± 79	711 ± 193	0.23
Serum iron (μg/100 ml)[c]	61 ± 15	106 ± 15	0.03
TIBC (μg/100 ml)	263 ± 43	208 ± 11	0.16
Transferrin saturation (%)	24 ± 4	59 ± 7	0.01
Ferritin (ng/ml)	115 ± 160	332 ± 86	0.40
Hemoglobin (g/100 ml)	11.9 ± 0.9	11.4 ± 0.4	0.13
Hematocrit (%)	37 ± 2	38 ± 1	0.11

HIC, hepatic iron concentration; TIBC, total iron binding capacity.

[a]Underlying diseases: pancreatic cancer (2), metastatic colon cancer (1), metastatic renal cancer (1), symptomatic liver cyst (1).

[b]Etiologies of cirrhosis: hepatitis C (7), alcoholic liver disease (4), hepatitis C + alcohol (3), hepatitis B (3), autoimmune hepatitis (2), primary sclerosing cholangitis (1), primary biliary cirrhosis (1), nonalcoholic steatohepatitis (1).

[c]Data are expressed as median (±); all other data are means ± s.e.m.

Western Blots

Portions of frozen liver were homogenized and analyzed by western blot as previously described.[20] Primary antibodies include anti-TFRC (13-6800; Zymed Laboratories, San Francisco, CA, USA; dilution 1:500), anti-ferritin (F5012; Sigma, St Louis, MO, USA; dilution 1:1000), and anti-DMT1 (sc-30120; Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:200). The Fpn antibody has been previously described.[22] Peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Bound antibody was detected using chemiluminescence according to the manufacturer's instructions (ECL; GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The DMT1 and Fpn Westerns were stripped (Antibody Stripping Solution; ADI, San Antonio, TX, USA) and reprobed with an anti-β-actin antibody (A2066; Sigma; dilution 1:10 000). Bands were quantitated by densitometry using ImageJ software (NIH).

Statistical Analysis

Comparisons were carried out by Mann–Whitney *U*-statistics on medians when data were not normally distributed and unpaired two-tailed *t*-test when normally distributed. Spearman's rank order correlation test was used to determine nonparametric correlations between parameters and linear regression analysis to evaluate if linear correlation was present. *P*-values of <0.05 were considered significant.

RESULTS

Clinical Characteristics

The control group was comprised of 1 man and 4 women, with an average age of 63 (range 47–78). Two subjects had pancreatic cancer and liver nodules on imaging studies. They underwent laparotomy to exclude metastatic disease before pancreatectomy. Of the remaining patients, one had metastases from colon cancer, one had a metastasis from renal cancer, and one had a symptomatic liver cyst. Histologic evaluation of control livers revealed completely normal histology in two and mild, nonspecific changes in the remainder (two with mild portal inflammation without fibrosis and one with mild ductular proliferation). The cirrhotic group included 12 men and 10 women. Their average age was 50 years (range 24–67). Chronic hepatitis C was the most common underlying liver disease, followed by alcoholic liver disease and chronic hepatitis B.

Clinical parameters related to iron metabolism of the subjects are shown in Table 2. Transferrin saturations were significantly higher in the cirrhotics than in the controls (59 ± 7 *vs* $24 \pm 4\%$; $P<0.01$). This was a consequence of elevated serum iron levels in the cirrhotic group compared to the controls (106 ± 15 *vs* 61 ± 15; $P=0.03$), as well as a trend toward decreased total iron binding capacity. On average, serum ferritin levels were higher in the cirrhotics, but this difference was not significant. Hemoglobin and hematocrit levels were similar in the groups.

Hepatic Iron Concentrations and Iron Staining

HICs tended to be higher in the cirrhotic livers, but this difference was not significant (Table 2). Notably, the range of HICs was much broader in the cirrhotic group (101–3268 μg/g) than among the controls (320–774 μg/g). Consistent with previous reports,[23,24] intraorgan HICs in some cirrhotic livers varied considerably (>5-fold).

There was a strong correlation between HIC and grade of iron staining ($r_s=0.89$; $P<0.0001$). A total of 13 had $\geq 1+$ stainable iron. Hepatocellular iron deposition predominated in the majority (11 of 13), with all but one also showing sinusoidal lining cell iron.

Hepatic Expression of Genes and Proteins Involved in Iron Metabolism

Levels of HAMP transcripts were similar in the control and cirrhotic groups ($P=0.976$; Figure 1). In contrast, levels of FPN, DMT1, and ferritin mRNA differed significantly between the groups (Figures 1 and 2). Fpn transcript levels were significantly higher in cirrhotics than in controls ($P=0.007$). In contrast, the 100 kDa band corresponding to Fpn was generally weaker in the cirrhotic livers than in the controls on western blot (Figure 3). Although there was considerable variability among the cirrhotic livers, Fpn levels did not appear to vary with iron content at the protein level nor did the abundance of its mRNA correlate with HIC (Figure 3; Table 3).

Like Fpn, levels of DMT1 mRNA were significantly higher in cirrhotic livers than in controls ($P=0.039$), whereas levels of DMT1 protein tended to be diminished in the cirrhotic livers. DMTh HIC or mRNA level (Figure 4; Table 3). Several DMT1 isoforms have been described, including an iron-response element (IRE)-containing form, a non-IRE form, and

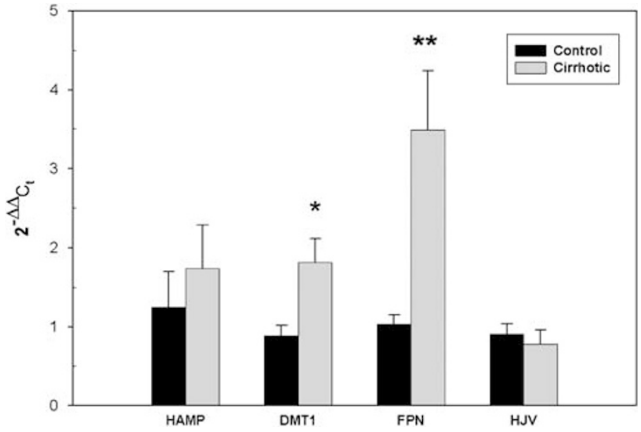

Figure 1 Relative mRNA expression of HAMP ($P = 0.976$) and hemojuvelin ($P = 0.864$) did not differ between the groups. DMT1 and FPN mRNA expression were significantly higher in the cirrhotics than in controls, $P = 0.039$ and 0.007, respectively. *$P = 0.039$; **$P = 0.007$.

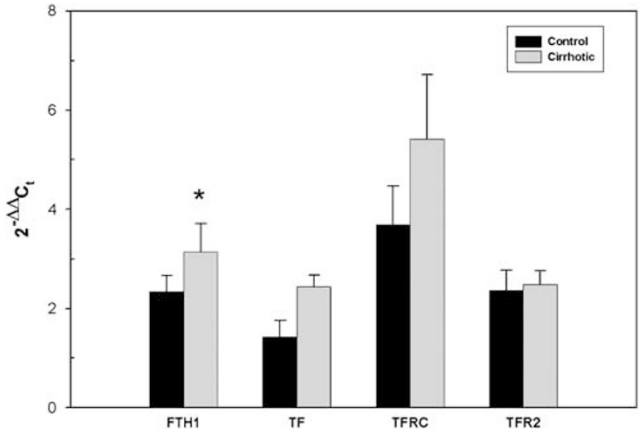

Figure 2 Relative mRNA expression of ferritin (FTH1) was significantly higher in the cirrhotics than in controls ($P = 0.025$); transferrin (TF) and transferrin receptor 1 (TFRC) mRNA levels also tended to be higher in the cirrhotics ($P = 0.055$ for both). Transferrin receptor 2 (TFR2) did not differ between the groups ($P = 0.99$). *$P = 0.025$.

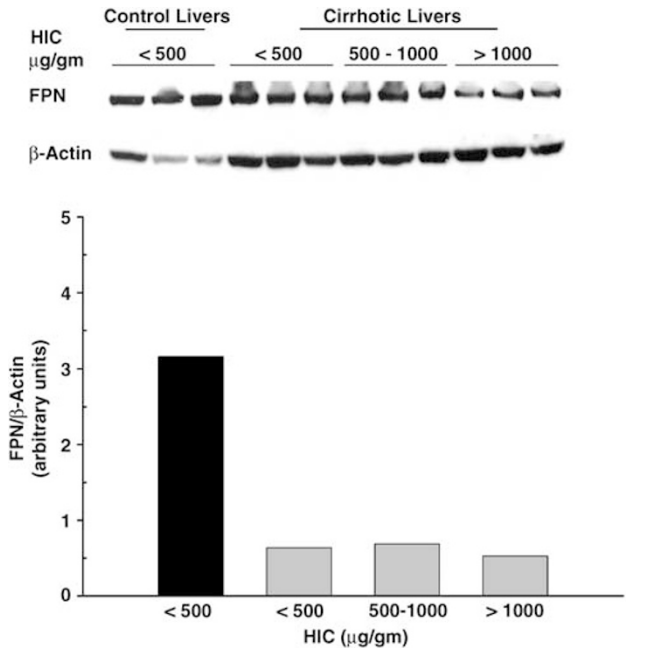

Figure 3 Western blot of ferroportin in control and cirrhotic human livers demonstrates that abundance of ferroportin in the cirrhotic livers is variable but generally lower than in the control livers and has no obvious relationship to hepatic iron concentration. Lysates from cirrhotic human livers are grouped according to iron concentration determined on samples from the same livers. Western blotting was performed as described in the 'Materials and methods'.

two additional splice variants.[21] Because the primers used in the quantitative PCR are expected to recognize all four isoforms, we performed semiquantitative RT-PCR using isoform-specific primers to determine whether expression of DMT1 isoforms is altered in cirrhotic liver. Both the IRE and non-IRE DMT1 isoforms were present in control and cirrhotic livers, and the IRE-containing transcript remained detectable in cirrhotic livers with elevated iron concentrations (Figure 5). The exon 1B variant was likewise present in all control and cirrhotic livers examined. In contrast, the exon 1A variant was detected in only a few cirrhotic livers; however, no relationship between the presence of this variant and increased hepatic iron was evident when additional livers were examined (data not shown). Thus, apart from the exon 1A variant, the repertoire of DMT1 expression is similar in control and cirrhotic liver, and is not clearly affected by iron content in the latter.

FTH1 mRNA levels were also significantly higher in the cirrhotic livers than in the controls (3.1 ± 0.6 *vs* 2.3 ± 0.3; $P < 0.03$), whereas TFRC and TF transcripts showed similar trends, which did not achieve statistical significance (Figure 2). Expression of TFRC and ferritin were assessed in cirrhotic livers by western blot (Figure 6). TFRC was readily apparent in livers with HICs $< 500\,\mu$g/g, but its abundance diminished progressively with increasing HIC, becoming undetectable in livers with HICs $> 1000\,\mu$g/g, whereas ferritin showed the inverse pattern. Thus, at the protein level, TFRC and ferritin are expressed in a physiologically appropriate manner relative to iron content in cirrhotic human livers, indicating that dysregulation of these proteins is unlikely to be causally related to hemosiderosis.

Previous studies indicate that hepatocytes are the predominant source of HJV and TFR2 transcripts in liver.[25,26] The finding that there were no significant differences in the level of HJV and TFR2 transcripts (Figures 1 and 2) among the groups suggests that, despite the variable content of inflammatory and fibrogenic cells in the cirrhotic livers, hepatocytes were the predominant source of RNA in those samples. These data are summarized in Table 3.

Table 3 Summary of alterations in iron regulatory genes and proteins in cirrhotic human livers compared to controls

	mRNA	Protein	Correlation with HIC
Hepcidin	↔	ND	+ mRNA
DMT1	↑	↓	− mRNA
			− Protein
Ferroportin	↑	↓	− mRNA
			− Protein
TFRC	↔	↔	− mRNA
			+ Protein
Ferritin	↑[a]	↔	− mRNA
			+ Protein
Transferrin	↔	ND	− mRNA
TFR2	↔	ND	− mRNA
Hemojuvelin	↔	ND	− mRNA

ND, not determined; DMT1, divalent metal transporter-1; TFRC, transferrin receptor; TFR2, transferrin receptor 2; HIC, hepatic iron concentration.

[a]Measured as FTH1 mRNA.

[b]Symbols in the last column indicate the presence (+) or absence (−) of correlation with the indicated parameter, not its direction.

Correlations Between Gene Expression, Hepatic Iron Concentrations, and Clinical Parameters

On the basis of analysis of the three separate samples from each liver, a highly significant correlation between HAMP mRNA levels and HIC was observed in the cirrhotic livers ($n = 66$, $r_s = 0.425$; $P < 0.0004$). A similar result was obtained when only data from a single sample from each cirrhotic liver was considered ($r_s = 0.508$; $P = 0.016$) (Table 4; Figure 7). Although no correlation was observed between HAMP transcript levels and HIC in the control group, this is likely a type II error due to the small number of samples in the control group, as previous work has shown a positive correlation between HIC and HAMP expression in human liver.[17]

DMT1 and Fpn transcripts were strongly correlated in cirrhotic livers ($r_s = 0.72$; $P = 0.0001$), suggesting that hepatic expression of DMT1 and Fpn may be coordinately regulated. There was no correlation with either of these transcripts and HAMP mRNA or HIC, suggesting that the expression of these iron transporters is not influenced by hepatic iron status, at least in cirrhotic livers (Table 4). Interestingly, a number of highly significant correlations were observed between HJV expression and that of other genes involved in iron metabolism including DMT1 ($r_s = 0.59$; $P = 0.0038$), Fpn ($r_s = 0.61$; $P = 0.0027$), TFRC ($r_s = 0.76$; $P < 0.0001$), and TF ($r_s = 0.48$; $P = 0.02$).

Correlations of HIC with clinical parameters of iron status were also assessed (Table 5). Serum iron and transferrin saturation both showed significant correlations with HIC in the cirrhotic livers ($r_s = 0.720$; $P < 0.0002$ and $r_s = 0.731$; $P < 0.0002$, respectively). A strong correlation between HIC and serum ferritin was observed in the cirrhotic livers ($r_s = 0.781$; $P < 0.0001$) with a similar trend seen in the control livers ($r_s = 0.90$; $P = 0.083$). These data suggest that serum iron studies provide a relatively good indirect assessment of HIC in cirrhosis, with ferritin being the single best predictor.

No relationships were identified between HAMP transcripts and serum markers of iron status in the control group. In the cirrhotic group there was a significant negative correlation between HAMP mRNA and total iron binding capacity ($r_s = -0.500$; $P = 0.021$), whereas positive correlations were observed between HAMP mRNA and transferrin saturation ($r_s = 0.428$; $P = 0.023$) and HAMP mRNA and serum ferritin ($r_s = 0.704$; $P = 0.0011$) (Table 5). The correlation of these parameters with hepcidin expression is consistent with the relationship of hepcidin and iron stores as reflected by the HIC and provides further evidence that hepcidin expression at the mRNA level is responsive to iron status in cirrhosis.

DISCUSSION

This work represents the first comprehensive assessment of iron-related gene expression in human livers. To our knowledge, it is also the only work concerning hepcidin expression in diseased human livers in which multiple samples from individual livers were assessed for both iron content and hepcidin expression, to take into account the variability of hepatic iron deposition within cirrhotic livers. Contrary to our expectations, we found that levels of HAMP mRNA are similar in control and cirrhotic human livers and correlate with HIC in the latter, suggesting that regulation of hepcidin gene expression in response to iron status is preserved in cirrhosis.

In assessing these results, it is important to consider the possibility that iron metabolism was abnormal in our control subjects, most of whom had malignancies. However, anemia of chronic disease or cytokine-driven acute phase physiology associated with malignancy would raise hepcidin expression,[27] thereby potentially increasing its levels in this group compared to the cirrhotics, which was not the case. Thus, although this factor seems unlikely to account for the lack of difference in HAMP mRNA levels between the groups, we cannot exclude the possibility that malignancy dampens hepcidin expression. In this context, it is worth pointing out that we originally intended to use donor livers as controls in this study, but abandoned this when our initial data showed a pattern of gene expression suggestive of an acute phase reaction (O Bergmann and K Brown, unpublished results). These observations underscore the challenges of working with scarce human tissues such as livers.

We must qualify our conclusion that hepcidin expression is regulated appropriately in cirrhotic livers by noting that we found no correlation between HAMP mRNA and HIC in control livers in this study, probably because of the small

Table 4 Spearman correlations between HIC and transcript levels for genes involved in iron metabolism in cirrhotic livers

	HIC	DMT1	FTH1	Fpn	HAMP	HJV	TF	TFRC	TFR2
HIC									
r_s	X	−0.002	−0.24	0.34	**0.508**	−0.069	−0.126	−0.419	−0.121
P	X	0.92	0.28	0.12	**0.016**	0.76	0.58	0.052	0.59
DMT1									
r_s	−0.002	X	0.320	**0.72**	−0.152	**0.59**	0.099	0.360	0.315
P	0.92	X	0.146	**0.0001**	0.50	**0.0038**	0.660	0.100	0.154
FTH1									
r_s	−0.24	0.320	X	0.139	−0.049	0.260	0.407	**0.724**	**0.485**
P	0.28	0.146	X	0.536	0.83	0.243	0.060	**0.0001**	**0.022**
Fpn									
r_s	0.34	**0.72**	0.139	X	0.014	**0.61**	0.065	0.080	0.291
P	0.12	**0.0001**	0.536	X	0.95	**0.0027**	0.774	0.724	0.189
HAMP									
r_s	**0.508**	−0.152	−0.049	0.014	X	−0.138	0.085	−0.282	0.034
P	**0.016**	0.50	0.83	0.95	X	0.54	0.71	0.20	0.88
HJV									
r_s	−0.069	**0.59**	0.260	**0.61**	−0.138	X	**0.483**	0.413	**0.761**
P	0.76	**0.0038**	0.243	**0.0027**	0.54	X	**0.023**	0.056	**<0.0001**

HIC, hepatic iron concentration; DMT1, divalent metal transporter-1; FTH1, ferritin heavy polypeptide 1; Fpn, ferroportin.
Parameters in bold indicate statistically significant correlations ($P<0.05$).

number of samples in this group. Given this limitation, it is possible that cirrhosis alters the relationship between HAMP mRNA and HIC in a quantitative manner, eg, hepcidin expression may be lower at any given HIC in cirrhotic liver than would be the case in normal liver. If so, the correlation between HAMP mRNA and hepatic iron in cirrhotic livers notwithstanding, hepcidin expression in cirrhosis may be deficient in absolute terms and thus contribute to inappropriate iron absorption. This issue may be resolved by examination of a larger number of bona fide normal human livers; however, such an undertaking will be difficult owing to ethical and logistic considerations.

The concept linking downregulated hepcidin expression with hemosiderosis is supported by recent studies showing decreased hepatic hepcidin expression after short-term alcohol administration to mice[28] and with increasing age in HCV polyprotein transgenic mice.[29] Although these data establish that hepcidin expression can be modulated by alcohol and HCV, two important causes of chronic liver disease in humans, the relevance of these observations to patients with cirrhosis is uncertain. On the one hand, there are no data to indicate that the alcohol-induced reduction in hepcidin expression results in hepatic iron accumulation, which would be unlikely in any case, given the brief (7 days) duration of the experiment.[28] Whether longer-term exposure to alcohol would result in persistent downregulation of hepcidin expression and/or dysregulation of iron metabolism is unknown at present, but it is worth noting that previous work in alcohol-treated rodents has failed to demonstrate spontaneous iron overload.[30,31] On the other hand, in contrast to HCV-infected humans, in whom hemosiderosis is uncommon before the development of advanced fibrosis,[32] HCV transgenic mice develop mild hepatic iron deposition in the absence of hepatic fibrosis. Thus, whether the causes and consequences of altered hepcidin expression in these animal models mimic those in human chronic liver disease remains to be determined.

In contrast to the lack of an effect of cirrhosis on hepcidin expression, we found that cirrhotic human livers demonstrate increased levels of Fpn and DMT1 mRNA, whereas the

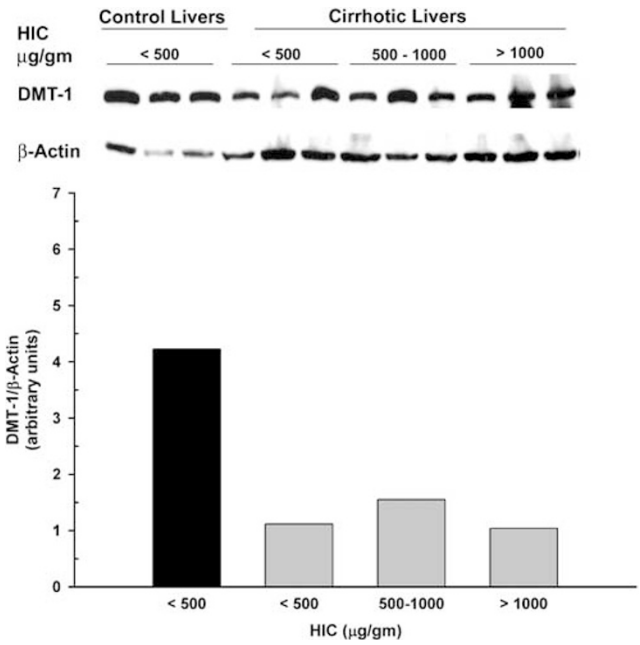

Figure 4 Western blot of DMT1 in control and cirrhotic human livers shows variable abundance of DMT1 irrespective of the presence of cirrhosis and of hepatic iron concentration. Western blotting was performed as described in the 'Materials and methods' using an antibody that detects all four DMT1 isoforms. The layout of the liver samples the same as in Figure 3.

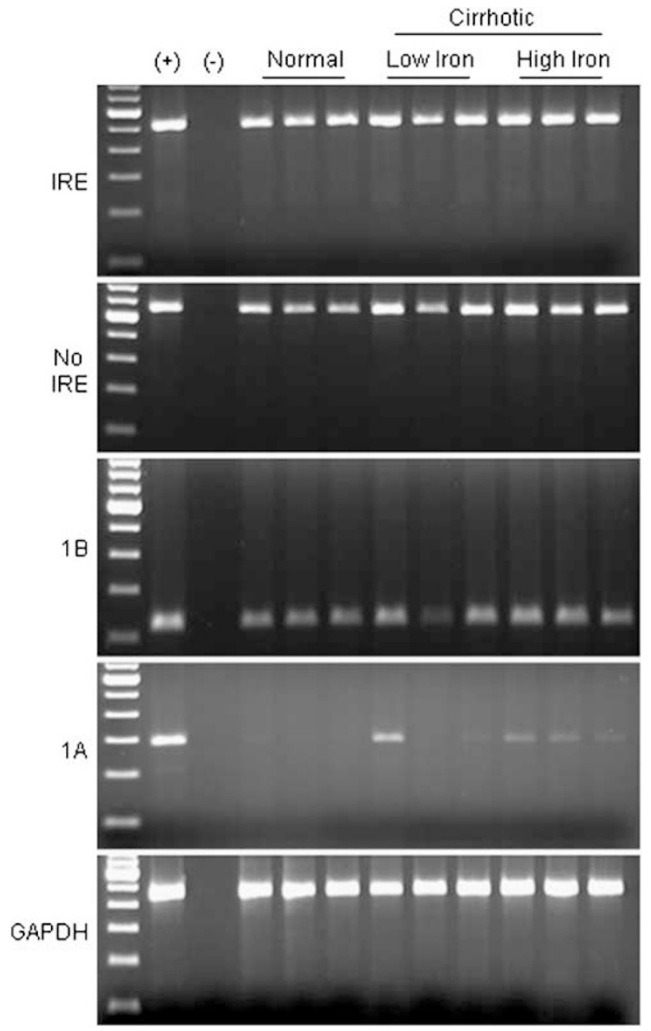

Figure 5 RT–PCR showing that the IRE, non-IRE, and exon 1B variants of DMT1 are expressed in control and cirrhotic human livers. A low level of expression of the exon 1A variant is detected only in some cirrhotic livers. Hepatic iron concentrations (HICs) were determined on samples taken from the same livers. 'Normal' indicates control human livers; 'low iron' and 'high iron' indicate HIC $<500\,\mu g/g$ and $>1500\,\mu g/g$, respectively. RT–PCR was performed as described in the 'Materials and methods'. The first lane is 100-bp ladder; the lane labeled '+' indicates the positive control (Caco-2 cells), '−' the negative control (no RT).

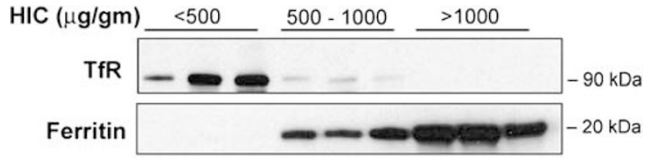

Figure 6 Western blot of transferrin receptor and ferritin in cirrhotic human livers demonstrates a reciprocal relationship between the abundance of transferrin receptor and ferritin that is physiologically appropriate vis-à-vis iron concentrations. Lysates from cirrhotic human livers are grouped according to hepatic iron concentration (HIC) as determined on samples taken from the same livers.

levels of the respective proteins tended to be lower in cirrhotic livers than in controls. Neither the mRNA nor the protein levels of the transporters correlated with hepatic iron concentrations, suggesting that the alterations in their expression were a consequence of cirrhosis and not a direct response to the presence of excess iron. The reason for the lack of correspondence between the mRNA and protein levels of the iron transporters is unclear. Little is known about the regulation of these genes in liver; however, the available data suggest that hepcidin and/or iron status has little, if any, influence on levels of Fpn mRNA. Yeh *et al* have reported that Fpn transcripts are unaltered in the livers of iron-deficient Belgrade rats (in which hepcidin expression is dramatically decreased); these investigators also observed that systemic administration of recombinant hepcidin to rats failed to modify hepatic Fpn mRNA levels.[33] We have found that both Fpn and DMT1 mRNA are increased in the livers of C57Bl6 mice fed a high fat diet or chronically administered 20% ethanol in the drinking water. In neither of these models of fatty liver does the expression of the iron transporters correlate with HAMP mRNA or HIC (O Bergmann and K Brown, in preparation). These data strongly suggest that hepatic levels of Fpn and DMT1 mRNA are not directly controlled by either HIC or HAMP expression, but may be influenced by inflammatory or stress-responsive pathways. Although examination of the promoter region of these genes will be helpful to confirm these speculations, it is noteworthy that these findings in murine models of liver injury are consistent with our observations in cirrhotic human livers.

A second question raised by our findings is whether the abundance of Fpn in the liver is regulated directly by hepcidin, as is the case in the intestine.[34] Our data showing

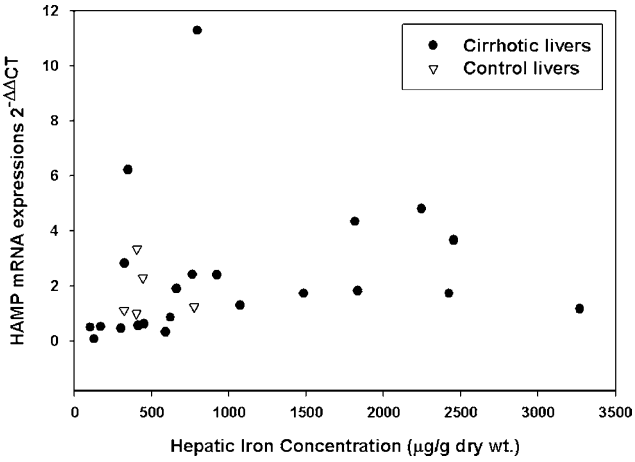

Figure 7 Relationship between HAMP mRNA and hepatic iron concentration in control and cirrhotic human livers. Circles represent cirrhotic livers, and inverted triangles indicate control livers.

decreased levels of Fpn in the cirrhotic livers with no increase in HAMP mRNA do not strongly support such a mechanism; however, it is important to note that we did not measure circulating or hepatic levels of hepcidin. Assuming that local hepcidin production is preserved in diseased liver and that it normally mediates Fpn degradation and internalization in hepatocytes and/or Kupffer cells in an autocrine or paracrine manner, an alternative mechanism, such as impaired synthesis or localization of Fpn, may lead to increased turnover of the protein and decreased levels in cirrhosis.

A further obstacle in assessing the potential role of the transporters in the pathogenesis of hemosiderosis is the lack of information concerning their function in the liver. In the intestine, DMT1 and Fpn constitute the 'mucosal block' to iron uptake, respectively, regulating the entry and egress of iron from enterocytes. Consistent with this role, the abundance of both proteins increases in the gut in response to iron deprivation and decreases with iron repletion. In contrast, Trinder *et al*[35] have reported that DMT1 behaves in an inverse manner in the liver, with hepatocyte DMT1 immunoreactivity increased in iron overload and decreased in iron deficiency. These investigators have proposed that hepatocyte DMT1 may be involved in the clearance of non-transferrin bound iron (NTBI) from portal blood, preventing the entry of low molecular weight iron into the systemic circulation and diverting it into hepatocytes, which have a robust capacity for iron storage. If so, hepatocyte DMT1 may be particularly important in cirrhosis, as duodenal expression of iron transporters is increased in cirrhotics, thus creating the potential for iron uptake to exceed the binding capacity of transferrin, leading to the presence of NTBI in the portal circulation.[16] The decrease in DMT1 protein in the cirrhotic livers appears unlikely to play a primary role in the development of hemosiderosis, but may contribute to the increase in circulating levels of NTBI reported in cirrhotic patients.[36]

Table 5 Spearman's correlations between serum iron parameters and HICs and transcript levels of genes involved in iron metabolism

Comparison	Control livers		Cirrhotic livers	
	r_s	*P*	r_s	*P*
HIC vs				
Serum iron	**−1.00**	**0.017**	**0.720**	**<0.0002**
TIBC	−0.700	0.233	−0.258	0.258
Transferrin saturation	−0.667	0.233	**0.731**	**<0.0002**
Serum ferritin	0.900	0.083	**0.781**	**<0.0001**
Fpn vs				
Serum iron	0.400	0.750	0.305	0.179
TIBC	0.200	0.917	**−0.549**	**0.0099**
Transferrin saturation	0.211	0.917	**0.441**	**0.0452**
Serum ferritin	−0.400	0.750	0.154	0.542
DMT1 vs				
Serum iron	−0.400	0.750	−0.060	0.797
TIBC	−0.800	0.333	−0.427	0.053
Transferrin saturation	−0.316	0.750	0.0033	0.989
Serum ferritin	0.400	0.750	−0.195	0.438
HAMP vs				
Serum iron	−0.500	0.450	0.347	0.123
TIBC	−0.300	0.683	**−0.500**	**0.021**
Transferrin saturation	−0.154	0.783	**0.428**	**0.023**
Serum ferritin	0.600	0.350	**0.704**	**0.0011**

TIBC, total iron binding capacity; HIC, hepatic iron concentration; DMT1, divalent metal transporter-1; Fpn, ferroportin.
Parameters in bold indicate statistically significant correlations ($P<0.05$).

In contrast to DMT1, we speculate that the decrease in Fpn in the cirrhotic livers may be mechanistically linked to the pathogenesis of hemosiderosis. Although Kupffer cells are the major cellular source of Fpn in liver, hepatocytes also express Fpn.[6,37] Decreased Fpn in either or both of these cell types would be expected to result in intracellular iron retention, which is consistent with the variable iron deposition in parenchymal and nonparenchymal cells in siderotic livers.[38] However, if decreased Fpn were the sole abnormality, the resulting increase in hepatic iron content would be expected to elicit a physiological response leading to decreased intestinal iron absorption that would prevent progressive iron loading. The fact that this does not occur indicates that there are likely to be additional abnormalities, perhaps involving the sensing of iron content or biosynthesis or secretion of hepcidin, that participate in the pathophysiology of secondary iron overload.

Despite the recent advances in this area, much remains to be learned regarding regulation of hepatic iron metabolism.

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1. Ludwig J, Hashimoto E, Porayko MK, *et al.* Hemosiderosis in cirrhosis: a study of 447 native livers. Gastroenterology 1997;112:882–888.
2. Kayali Z, Ranguelov R, Mitros F, *et al.* Hemosiderosis is associated with accelerated decompensation and decreased survival in patients with cirrhosis. Liver Int 2005;25:41–48.
3. Ganne-Carrié N, Christidis C, Chastang C, *et al.* Liver iron is predictive of death in alcoholic cirrhosis: a multivariate study of 229 consecutive patients with alcoholic and/or hepatitis C virus cirrhosis: a prospective follow up study. Gut 2000;46:277–282.
4. Brandhagen DJ, Alvarez W, Therneau TM, *et al.* Iron overload in cirrhosis—HFE genotypes and outcome after liver transplantation. Hepatology 2000;31:456–460.
5. Gunshin H, Mackenzie B, Berger UV, *et al.* Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 1997;388:482–488.
6. Abboud S, Haile DJ. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. J Biol Chem 2000;275: 19906–19912.
7. Donovan A, Brownlie A, Zhou Y, *et al.* Positional cloning of zebrafish *ferroportin1* identifies a conserved vertebrate iron exporter. Nature 2000;403:776–781.
8. McKie AT, Marciani P, Rolfs A, *et al.* A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell 2000;5:299–309.
9. Kawabata H, Yang R, Hirama T, *et al.* Molecular cloning of transferrin receptor 2. J Biol Chem 1999;274:20826–20832.
10. Papanikolaou G, Samuels ME, Ludwig EH, *et al.* Mutations in *HFE2* cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet 2004;36:77–82.
11. Pigeon C, Ilyin G, Courselaud B, *et al.* A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 2001;276:7811–7819.
12. Montosi G, Donovan A, Totaro A, *et al.* Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. J Clin Invest 2001;108:619–623.
13. Njajou OT, Vaessen N, Joosse M, *et al.* A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. Nat Genet 2001;28:213–214.
14. Camaschella C, Roetto A, Calí A, *et al.* The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. Nat Genet 2000;25:14–15.
15. Roetto A, Papanikolaou G, Politou M, *et al.* Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet 2003;33:21–22.
16. Stuart KA, Anderson GJ, Frazer DM, *et al.* Increased duodenal expression of divalent metal transporter 1 and iron-regulated gene 1 in cirrhosis. Hepatology 2004;39:492–499.
17. Détivaud L, Nemeth E, Boudjema K, *et al.* Hepcidin levels in humans are correlated with hepatic iron stores, hemoglobin levels, and hepatic function. Blood 2005;106:746–748.
18. Brown KE, Khan CM, Zimmerman MB, *et al.* Hepatic iron overload in blacks and whites: a comparative autopsy study. Am J Gastroenterol 2003;98:1594–1598.
19. Edwards CQ, Carroll M, Bray P, *et al.* Hereditary hemochromatosis. Diagnosis in siblings and children. New Engl J Med 1977;297:7–13.
20. Brown KE, Broadhurst KA, Mathahs MM, *et al.* Expression of HSP47, a collagen-specific chaperone, in normal and diseased human liver. Lab Invest 2005;85:789–797.
21. Hubert N, Hentze MW. Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: implications for regulation and cellular function. Proc Natl Acad Sci USA 2002;99:12345–12350.
22. Han O, Kim EY. Colocalization of ferroportin-1 with hephaestin on the basolateral membrane of human intestinal absorptive cells. J Cell Biochem 2007;101:1001–1010.
23. Villeneuve JP, Bilodeau M, Lepage R, *et al.* Variability in hepatic iron concentration measurement from needle-biopsy specimens. J Hepatol 1996;25:172–177.
24. Emond MJ, Bronner MP, Carlson TH, *et al.* Quantitative study of the variability of hepatic iron concentration. Clin Chem 1999;45: 340–346.
25. Niederkofler V, Salie R, Arber S. Hemojuvelin is essential for dietary iron sensing, and its mutation leads to severe iron overload. J Clin Invest 2005;115:2180–2186.
26. Fleming RE, Migas MC, Holden CC, *et al.* Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. Proc Natl Acad Sci USA 2000;97: 2214–2219.
27. Nemeth E, Valore EV, Territo M, *et al.* Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2002;101:2461–2463.
28. Harrison-Findik DD, Schafer D, Klein E, *et al.* Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription and leads to increased duodenal iron transporter expression. J Biol Chem 2006;281:22974–22982.
29. Nishina S, Hino K, Korenaga M, *et al.* Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription. Gastroenterology 2008;134:226–238.
30. Batey RG, Johnston R. Effects of alcohol, carbon tetrachloride, and choline deficiency on iron metabolism in the rat. Alcohol Clin Exp Res 1993;17:931–934.
31. Gentry-Nielsen MJ, Preheim LC, Lyman KN, *et al.* Use of rat models to mimic alterations in iron homeostasis during human alcohol abuse and cirrhosis. Alcohol 2001;23:71–81.
32. Guyader D, Thirouard AS, Erdtmann L, *et al.* Liver iron is a surrogate marker of severe fibrosis in chronic hepatitis C. J Hepatol 2007;46: 587–595.
33. Yeh KY, Yeh M, Glass J. Hepcidin regulation of ferroportin expression in the liver and intestine of the rat. Am J Physiol Gastrointest Liver Physiol 2004;286:G385–G394.
34. Nemeth E, Tuttle MS, Powelson J, *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306:2090–2093.
35. Trinder D, Oates PS, Thomas C, *et al.* Localisation of divalent metal transporter 1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. Gut 2000;46:270–276.
36. Fiorelli G, De Feo TM, Duca L, *et al.* Red blood cell antioxidant and iron status in alcoholic and nonalcoholic cirrhosis. Eur J Clin Invest 2002;325:21–27.
37. Zhang AS, Xiong S, Tsukamoto H, *et al.* Localization of iron metabolism-related mRNAs in rat liver indicates that HFE is expressed predominantly in hepatocytes. Blood 2004;103: 1509–1514.
38. Brunt EM, Olynyk JK, Britton RS, *et al.* Histological evaluation of iron in liver biopsies: relationship to HFE mutations. Am J Gastroenterol 2000;95:1788–1793.