

Pathways underlying iron accumulation in human nonalcoholic fatty liver disease^{1–3}

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ABSTRACT

Background: Mild iron overload is frequently observed in nonalcoholic fatty liver disease (NAFLD).

Objective: We aimed to study putative pathways underlying iron accumulation in NAFLD.

Design: Hepatic and duodenal expression of critical iron molecules in NAFLD patients with ($n = 32$) and without ($n = 29$) iron overload, hereditary hemochromatosis ($n = 10$), and controls ($n = 20$) were investigated. Phlebotomy treatment was performed in 14 NAFLD patients.

Results: The hepatic expressions of the iron-export protein ferroportin-1 (FP-1) and of the iron-sensing molecule hemojuvelin (HJV) were significantly lower in NAFLD patients. The mRNA expression of the iron-regulatory peptide hepcidin was increased in NAFLD patients with iron overload, which was paralleled by low duodenal FP-1 expression. Hepatic mRNA and serum protein concentrations of tumor necrosis factor- α (TNF- α) were increased in NAFLD patients and were inversely correlated with both liver FP-1 and HJV mRNA and positively associated with body mass index and hepatic hepcidin mRNA. Accordingly, TNF- α inhibited the FP-1 and HJV mRNA formation in HepG2 cells. Phlebotomy treatment of NAFLD patients reduced serum ferritin, transferrin saturation, and TNF- α concentrations and improved liver function tests.

Conclusions: Iron accumulation in NAFLD may result from an impaired iron export due to down-regulation of FP1 and ineffective hepatic iron sensing, as indicated by low HJV expression. TNF- α appears to play a role in exerting these regulatory changes. Increased hepcidin formation in iron-overloaded NAFLD patients, however, results in decreased duodenal FP-1 expression, whereas a reduction in liver FP-1 may perpetuate hepatic iron retention. Phlebotomy offers a safe and efficient therapy for these metabolic disturbances. *Am J Clin Nutr* 2008;87:1374–83.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and the most common cause of elevated liver enzymes in Western societies (1). Hyperferritinemia has been frequently observed in patients with the metabolic syndrome (2) and NAFLD (3). The term *insulin-resistance associated hepatic iron-overload syndrome* was coined to describe the frequent association between hepatic steatosis and body iron overload (4). Typically, in NAFLD, hepatic iron accumulation is mild and involves hepatocytes as well as sinusoidal Kupffer cells (5). Although

some investigators found an association of iron overload (6, 7) and presence of *HFE* mutations (8) with more progressed forms of NAFLD, this association was not confirmed by others (9–11). Nevertheless, iron reduction therapy was found to be beneficial with regard to NAFLD disease activity and insulin sensitivity (12, 13). Our knowledge of human iron metabolism has expanded over the past years because of the identification of new molecules (14). Although cells acquire iron via different pathways, which include the uptake of transferrin bound iron via transferrin receptors (TfRs) and of ferrous iron via the transmembrane protein divalent metal transporter-1 (DMT-1), only one iron exporter has been characterized—the transmembrane protein ferroportin-1 (FP-1) (15–17). After its transfer through the duodenal basolateral membrane, iron undergoes oxidation by the membrane-bound, copper-containing ferroxidase hephaestin before being incorporated into transferrin for further transport in the circulation (18). Hepcidin is a master iron-regulatory peptide (19), which is secreted mainly by hepatocytes in response to iron perturbations (20) and inflammation and hypoxia (21). Hepcidin exerts its regulatory functions on iron homeostasis via binding to FP-1, which thereby leads to FP-1 internalization, degradation, and thus to blockage of cellular iron export (22). Important up-stream regulators of hepcidin expression include hemojuvelin (HJV), a bone-morphogenetic protein coreceptor, (23) *HFE*, a nonclassic MHC class I molecule, and TfR-2 (24)—a liver-specific iron uptake molecule. Mutations of these genes are associated with low hepcidin formation and hereditary iron overload syndromes (25). Importantly, although in quantitative terms the liver is the major hepcidin-producing organ, macrophages (26, 27) and adipose

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² Supported by Austrian Research (FWF-P19664). FP-1 antibody was kindly provided by Andy McKie (Kings College, London).

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Received October 22, 2007.

Accepted for publication November 30, 2007.

tissue (28) can also excrete hepcidin. Elevated serum concentrations of proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), have been observed in NAFLD (29) and obesity (30). Moreover, TNF- α has been identified as an important regulator of iron homeostasis under inflammatory conditions, mainly via its stimulatory effects on ferritin formation (31, 32). The aim of this study was to elucidate putative mechanisms underlying iron accumulation in NAFLD by studying the expression of iron homeostasis molecules in the liver and duodenum.

SUBJECTS AND METHODS

Subjects

Sixty-one patients with a diagnosis of NAFLD were included. Ten patients with hereditary hemochromatosis (HH) homozygous for the C282Y mutation of the *HFE* gene were also studied. Accordingly, subjects with a history of relevant alcohol intake (>20g/d), viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, Wilson disease, or α_1 -antitrypsin deficiency or taking steatogenic medication were excluded.

None of the patients had signs of heart or renal insufficiency, cancer, autoimmune diseases, or systemic infections. Patients were considered to have NAFLD with iron overload when both

biochemical signs of iron overload—elevated serum ferritin concentrations and hepatic iron deposition as indicated by positive Perls stain (0–4)—were detected. Accordingly, patients with a histologic and clinical diagnosis of NAFLD and the absence of stainable iron and normal serum iron variables were identified as having NAFLD without iron overload. Because liver biopsy samples from truly healthy subjects could not be obtained, 20 subjects (13 women, 7 men) who underwent liver biopsy for unexplained elevation of liver enzymes were studied as control subjects. These patients had no serologic or clinical evidence of liver disease and normal liver histology. All control subjects had normal biochemical iron variables and no liver iron deposition. To screen for hemochromatosis-associated mutations in the *HFE* gene, C282Y, and H63D, genetic testing was performed in all patients and controls as described (33). None of the NAFLD patients studied were either homozygous for the C282Y or H63D mutations or compound heterozygous, respectively. Ten HH patients were homozygous for the C282Y mutation in the *HFE* gene. All patients underwent percutaneous liver biopsy, and the diagnosis of nonalcoholic steatohepatitis (NASH) or NAFLD was established as detailed below. Twenty-six NAFLD patients, 14 with and 12 without iron overload, and 10 control subjects underwent upper gastroduodenal endoscopy as a part of the diagnostic workup or for suspected peptic ulcer

TABLE 1
Patients' characteristics at baseline¹

Group	NAFLD + Fe (n = 32)	NAFLD (n = 29)	HH (n = 10)	Control (n = 20)
Age (y)	55.6 ± 9.6 ^{2,3}	46.7 ± 14.6 ⁴	52.6 ± 11.2	45.6 ± 10.1
HFE C282 heterozygous [n (%)]	4 (12.5)	3 (10.3)	0	2 (10.0)
HFE H63D heterozygous [n (%)]	6 (18.7)	6 (20.7)	0	3 (15.0)
Patients with NASH [n (%)]	6 (18.7)	5 (17.2)	1 (10)	0
Steatosis grade (0–3)	1.4 ± 0.6	1.6 ± 0.6	0.5 ± 1.0 ⁵	0
Siderosis grade (0–4)	1.4 ± 0.7	0 ⁴	3.85 ± 0.3 ⁵	0
HIC ($\mu\text{g/g}$ dry wt)	1862.6 ± 948.5 ³	479.2 ± 231.8 ⁴	7922.1 ± 4831.0 ⁵	636.1 ± 203.4
HII ($\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{y}^{-1}$)	0.59 ± 0.2 ³	0.24 ± 0.12 ⁴	2.61 ± 1.33 ⁵	0.34 ± 0.12
Ferritin (mg/L)	796.3 ± 313.6 ³	196.1 ± 117.7 ⁴	2377.2 ± 1629.9 ⁵	134.4 ± 94.3
Tf saturation (%)	38.5 ± 10.1 ³	25.2 ± 5.6 ⁴	78.0 ± 12.0 ⁵	26.7 ± 9.4
AST (U/L)	37.8 ± 15.5	41.0 ± 19.6	34.0 ± 13.5	34.4 ± 11.7
ALT (U/L)	53.1 ± 36.8	59.8 ± 23.1	50.9 ± 22.6	50.9 ± 22.6
AP (U/L)	70.2 ± 16.6	76.2 ± 20.7	107.2 ± 50.0	64.2 ± 32.0
GGT (U/L)	79.5 ± 35.3 ³	85.5 ± 45.9	60.1 ± 87.2	51.2 ± 32.1
BMI (kg/m^2)	29.6 ± 3.3 ³	28.4 ± 4.6	25.6 ± 1.9 ⁵	26.8 ± 2.5
Fasting glucose (mg/dL)	115.6 ± 21.2 ³	102.9 ± 17.5 ⁴	104.8 ± 17.4 ⁵	94.7 ± 4.2
Cholesterol (mg/dL)	223.7 ± 44.3	234.0 ± 51.8	199.8 ± 30.7	225.9 ± 46.6
Triglycerides (mg/dL)	184.7 ± 108.8 ³	175.2 ± 90.0	141.3 ± 95.8	103.0 ± 52.6
HDL cholesterol (mg/dL)	52.7 ± 16.1 ³	54.6 ± 18.6	55.3 ± 13.2	70.6 ± 21.5
LDL cholesterol (mg/dL)	143.5 ± 43.5	147.8 ± 47.8	128.3 ± 32.3	141.0 ± 43.7
Hemoglobin (g/dL)	15.5 ± 1.3	16.1 ± 1.4	15.1 ± 0.6	15.0 ± 1.6
Leukocytes (cells/mL)	6468 ± 1832	7042 ± 1146	5524 ± 2435	5138 ± 2038
Platelets (cells/mL)	193 889 ± 33 648	203 000 ± 41 642	204 333 ± 53 972	214 700 ± 63 577
ESR (mm/h)	5.3 ± 5.0	4.8 ± 3.3	6.2 ± 4.6	5.1 ± 3.2

¹ NASH, nonalcoholic steatohepatitis; HIC, hepatic iron content; HII, hepatic iron index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; GGT, γ -glutamyltransferase; Tf, transferrin; ESR, erythrocyte sedimentation rate; NAFLD, nonalcoholic fatty liver disease; +Fe, with iron overload; HH, hereditary hemochromatosis.

² $\bar{x} \pm \text{SD}$ (all such values).

³ Significant difference between controls and NAFLD + Fe ($P < 0.05$).

⁴ Significant difference between NAFLD and NAFLD + Fe ($P < 0.05$).

⁵ Significant difference between HH and NAFLD + Fe ($P < 0.05$).

Calculations for statistical differences were carried out by Student's t test or Kruskal-Wallis test in the case of non-Gaussian distribution of variables and adjusted by Bonferroni correction for multiple testing.

disease. Written informed consent was obtained from all study participants to use one biopsy specimen for scientific purposes. The study was conducted in accordance with the ethical standards of the Helsinki Declaration of 1975 (revised in 1983).

Histologic examination of liver biopsy samples

The liver biopsy specimens were fixed in buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin, Mallory trichrome for morphologic evaluation, and Perls' stain was used to determine the liver iron load. All liver biopsy samples were at first analyzed independently by 2 pathologists unaware of the clinical data and the study objective according to the criteria detailed below. In the case of differing results from the histologic examination, samples were jointly reassessed by these pathologists and the result agreed on was used for the analysis. Liver biopsy samples were evaluated for the degree of steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2) according to Kleiner et al (34). NASH was diagnosed when the NASH Activity Score (NAS score) was ≥ 5 . A NAS score of 1–3 indicated NAFLD, and a score of 4 indicated borderline NASH. In the present study, patients with a NAS score of 4 were considered to have NASH.

Siderosis was determined semiquantitatively on histopathologic examination of Perls' stained liver biopsy samples. A score of 0 to 4 was determined: 0, granules absent or barely discernible at a magnification of 400 \times ; 1, barely discernible granules at a magnification of 200 \times but easily confirmed at 400 \times ; 2, discrete granules resolved at 100 \times magnification; 3, discrete granules

resolved at a magnification of 25 \times ; and 4, massive granules visible even at 10 \times magnification (35).

Laboratory evaluation

Venous blood was drawn after an overnight fast for determination of liver function, a full blood count, serum iron status (including ferritin, transferrin, transferrin saturation, and serum iron), C-reactive protein, erythrocyte sedimentation rate, and fasting glucose and lipid concentrations by standardized, automated laboratory methods. Serum TNF- α was measured with the use of Human TNF- α /TNFSF1A (Quantikine HS ELISA; R&D Systems, Minneapolis, MN), and the analysis was performed according to the manufacturers' instructions. Hepatic iron content (HIC) was measured by automated mass spectroscopy in patients and healthy control subjects and was expressed as $\mu\text{g/g}$ dry weight. The hepatic iron index was calculated as $\text{HIC}/56/\text{age}$ ($\mu\text{mol/g/y}$).

RNA extraction from liver and duodenal biopsy samples and quantitative real-time polymerase chain reaction

A small tissue portion of the liver biopsy sample was separated and preserved for RNA and, when available, for protein extraction in Ambion's RNAlater solution (Applied Biosystems, Brunnam Gebirge, Austria). Total RNA was extracted from liver and duodenal tissue by using a guanidinium-isothiocyanate-phenol-chloroform-based procedure, as described previously (36). Reverse transcription was performed with 1 μg

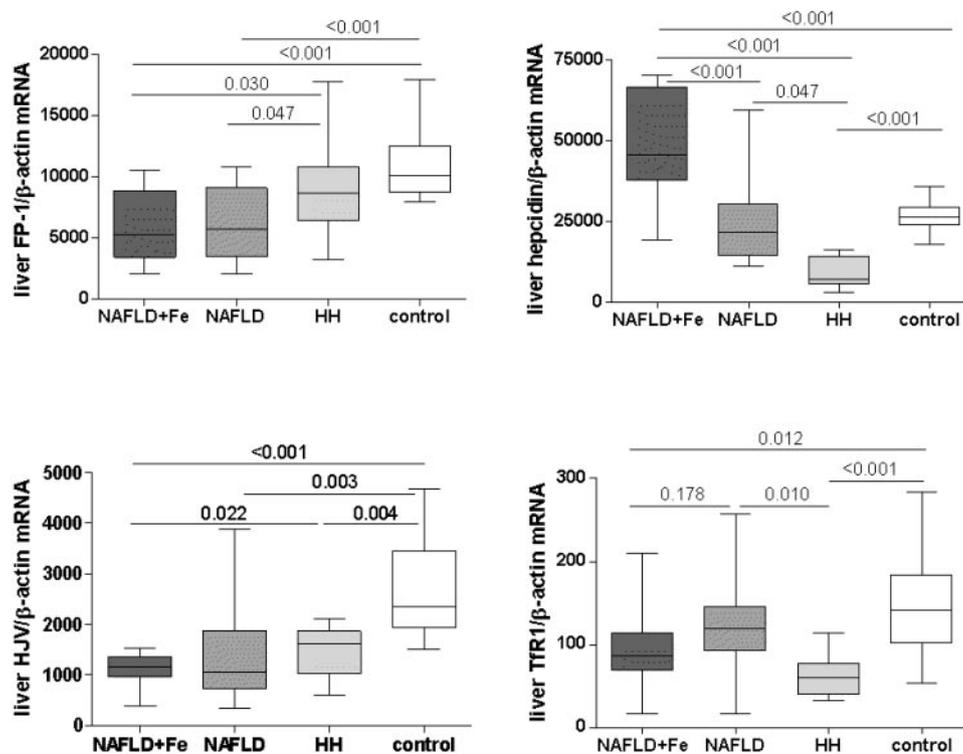


FIGURE 1. Hepatic mRNA expression of critical iron molecules in the control subjects ($n = 20$), the patients with nonalcoholic fatty liver disease with (NAFLD+Fe; $n = 29$) and without (NAFLD; $n = 32$) iron overload, and patients with hereditary hemochromatosis (HH; $n = 10$). mRNA from liver biopsy samples was extracted, and the expression of genes of interest was determined by quantitative real-time polymerase chain reaction analysis. Values are expressed as relative abundance normalized to β -actin cDNA concentrations and are presented as means (horizontal lines), 75th percentiles (boxes), and minimum to maximum ranges (vertical lines). Calculations for statistically significant differences between groups were performed by Student's t test and by nonparametric Kruskal-Wallis test in the case of non-Gaussian distribution of variables. The values above each horizontal line denote the P values for the comparison of the 2 groups at the end of the line.

total RNA, random hexamer primers (5 $\mu\text{mol/L}$), and dNTPs (62.5 $\mu\text{mol/L}$; Roche, Mannheim, Germany) and 200 U Molony murine leukemia virus reverse transcriptase (Gibco, Gaithersburg, MD). TaqMan real-time polymerase chain reaction (PCR) primers and probes were designed using Primer Express Software from Applied Biosystems (Vienna, Austria) and synthesized by Microsynth (Balgach, Switzerland). TaqMan probes were labeled with the reporter dye 6-carboxyfluorescein at the 5'-end and with 6-carboxy-tetramethyl-rhodamine at the 3'-end. For quantification of mRNA expression of genes of interest, the PCR reaction was carried on the MX4000 Multiplex Quantitative PCR System (Stratagene, Amsterdam, Netherlands) exactly as described (36). Specific cDNA concentrations were normalized to the amount of β -actin in liver biopsy samples.

The sense and anti-sense primers and TaqMan probes listed below were used (primer sense; primer anti-sense; probe). Commercially available probes for β -actin were obtained from Applied Biosystems and used according to the manufacturers' instructions.

- 1) TfR-1: 5'-TCCCAGCAGTTTCTTTCTGTTTT-3', 5'-CTCAATCAGTTCCTTATAGGTGTCCA-3', 5'-CGAGGACACAGATTATCCTTATTTGGGTACCACC-3'
- 2) ferroportin: 5'-TGACCAGGGCGGGAGA-3', 5'-GAGGTCAGGTAGTCGGCAA-3', 5'-CACAAACCGCCAGAGAGGATGCTGTG-3'
- 3) hepcidin: 5'-TTTCCCACAACAGACGGGAC-3', 5'-AGCTGGCCCTGGCTCC-3', 5'-CAGAGCTGCAACCCAGGACAGAGC-3'
- 4) hemojuvelin: 5'-CCCCATGGCGTTGG-3', 5'-GCATGTTCTTAAATATGATGGTGAGC-3', 5'-CAACGCTACCGCCACCCGGA-3'
- 5) DMT-1: 5'-CATCGTGGGAGCTGTCATCA-3', 5'-CCTGCTTATTGTTCCGGTTTACC-3', 5'-CAACATGTACTGCATTCTGCCTTAGTCAAGTCT-3'
- 6) TNF- α : 5'-GGTGCTTGTTCCCTCAGCCTC-3', 5'-CAGCAGAAGAGCGTGGTG -3', 5'-CTCCTTCCTGATCGTGGCAGGCG -3'

Protein extraction from liver and duodenal biopsy samples and Western blot analysis

Western blot analyses were carried out as described in detail previously (37). Briefly, protein extracts from duodenal and liver biopsy specimens were prepared by using radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mmol/L Tris HCl, pH 8.0, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 $\mu\text{g/mL}$ pepstatin, and 0.5 $\mu\text{g/mL}$ leupeptin), and 10 μg total protein was used for immunoblotting. Blots were incubated with 0.5 $\mu\text{g/mL}$ mouse anti-human FP-1 antibody or β -actin antibody for 1 h at room temperature and then stained with a horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (Dako, Copenhagen, Denmark).

Cell culture

HepG2 human hepatoma cells were cultured in RPMI 1640 medium supplemented with glutamine, penicillin, and streptomycin (100 U/mL) and 10% fetal calf serum. Cells were seeded in 6-well plates at passages 5 to 15 at a density of 5×10^5 cells per well in 3 mL medium. After being allowed to adhere for 12 h, the cells were either left untreated (control) or were treated with

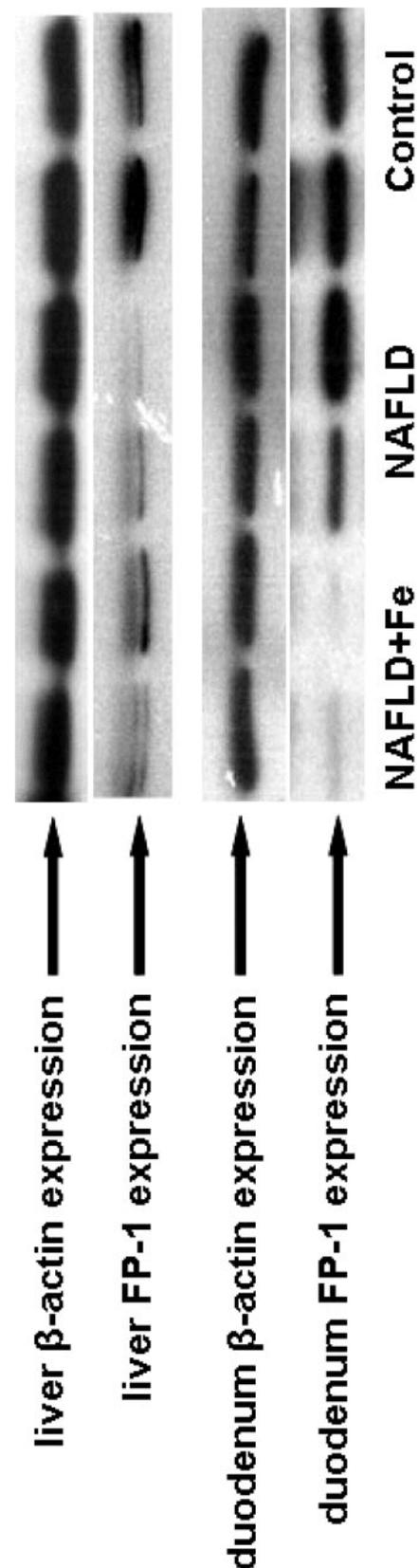


FIGURE 2. FP1 protein expression in liver and duodenal biopsy samples from control subjects and from the patients with nonalcoholic fatty liver disease with (NAFLD+Fe) and without (NAFLD) iron overload. Representative biopsy samples were used for the determination of FP1 protein expression by Western blot analysis. Expression of β -actin in duodenal and liver biopsy samples was analyzed and served as an internal loading control.

10 ng/mL of TNF- α (PeproTech EC, London, United Kingdom) for 24 h. Thereafter, cells were lysed with 1 mL RNA-Bee (Tel-Test, Friendswood, TX) per well, and RNA was extracted for quantitative PCR of critical iron transporters.

Statistical analysis

Statistical analyses were carried out with SPSS software (version 13.0; SPSS Inc, Chicago, IL). Calculations of statistical differences between the various groups were carried out by Student's *t* test or by nonparametric Kruskal-Wallis test in the case of non-Gaussian distribution of variables. Associations between the various variables in the different groups were calculated by using Spearman's rank correlation technique, and Bonferroni correction was used for multiple testing. The response to phlebotomy treatment was assessed by using paired *t* tests.

RESULTS

When investigating the NAFLD patients, we found that patients with iron overload were older than were patients without iron overload (Table 1). However, iron-overloaded NAFLD patients and NAFLD patients without liver iron accumulation were not different with regard to the frequency of *HFE* gene mutation carrier rates, the results of histologic grading, staging of fatty liver disease, and prevalence of NASH. Moreover, no differences in body mass index (BMI), liver transaminase, serum cholesterol, and triacylglycerol concentrations were observed between NAFLD patients with and without iron overload. However, NAFLD patients with iron overload had significantly higher concentrations of fasting glucose. Besides having a higher HIC and higher degree of siderosis, iron-overloaded NAFLD patients also had significantly higher serum ferritin concentrations and transferrin saturation than did NAFLD patients without iron overload. Importantly, patients with hereditary hemochromatosis had both, significantly higher serum and liver iron variables than NAFLD subjects with iron overload (Table 1), whereas none of the NAFLD patients with iron overload fulfilled the criteria for hemochromatosis (38). The prevalence of heterozygosity for the *HFE* C282Y and H63D mutations in NAFLD patients with and without iron overload and control subjects was comparable with that of the prevalence of the *HFE* gene mutation in the Central European white population studied (33).

Expression of critical iron metabolism genes in the liver and duodenum of NAFLD patients

We observed significantly lower FP-1 mRNA (Figure 1) and protein (Figure 2) expression in liver biopsy samples from NAFLD patients with or without iron overload than in control subjects. In comparison with controls, the expression of the iron-sensing molecule HJV was significantly lower in NAFLD patients (Figure 1), with a more prominent reduction in iron-overloaded NAFLD subjects. HJV mRNA expression in patients with *HFE*-associated hemochromatosis was similar to NAFLD patients without iron overload and was significantly lower than in controls. Hepatic TfR-1 mRNA expression was significantly lower in patient groups with iron overload, namely HH and NAFLD patients, than in healthy controls and NAFLD patients without hepatic iron accumulation (Figure 1). We found hepatic hepcidin mRNA expression in HH patients to be significantly lower than in controls and NAFLD patients. However, hepcidin mRNA expression was significantly higher in NAFLD patients with iron overload than in controls and NAFLD subjects without iron overload (Figure 1).

Iron absorption via enterocytes is pivotal for the regulation of body iron homeostasis. We determined the expression of key iron metabolism genes in the intestine. The expression of the apical iron transporter DMT-1 was not different between healthy control subjects and NAFLD patients with or without iron (Figure 3). As in the liver, we observed lower duodenal FP-1 mRNA expression in NAFLD patients than in control subjects (Figure 3). However, duodenal FP-1 protein expression was only significantly reduced in NAFLD patients with iron accumulation (Figure 2).

NAFLD patients have elevated TNF- α concentrations

Because the key proinflammatory cytokine TNF- α is known to be elevated in visceral obesity and NAFLD and because this cytokine has been shown to modulate body iron homeostasis, we studied TNF- α mRNA expression in the liver and measured serum TNF- α concentrations. Hepatic TNF- α expression was higher in NAFLD patients than in controls, with a further elevation in NAFLD subjects with iron overload (Figure 4). Likewise, we found the highest serum TNF- α concentrations in NAFLD patients with iron overload (Figure 4).

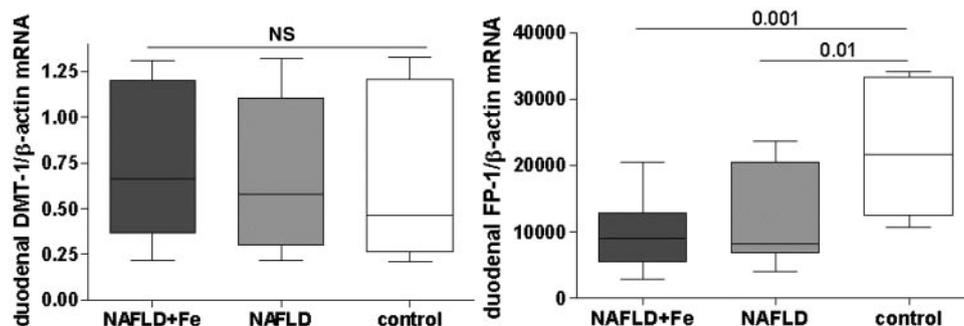


FIGURE 3. Expression of mRNA in duodenal biopsy samples from the control subjects ($n = 10$) and from the patients with nonalcoholic fatty liver disease with (NAFLD+Fe; $n = 14$) and without (NAFLD; $n = 12$) iron overload. mRNA from duodenal biopsy samples was extracted, and the expression of the genes of interest was determined by quantitative real-time polymerase chain reaction analysis. Values are expressed as relative abundance normalized to β -actin cDNA concentrations and are presented as means (horizontal lines), 75th percentiles (boxes), and minimum to maximum ranges (vertical lines). Calculations for statistically significant differences between groups were done by Student's *t* test and by non-parametric Kruskal-Wallis test in the case of non-Gaussian distribution of variables. The values above each horizontal line denote the *P* values for the comparison of the 2 groups at the end of the line.

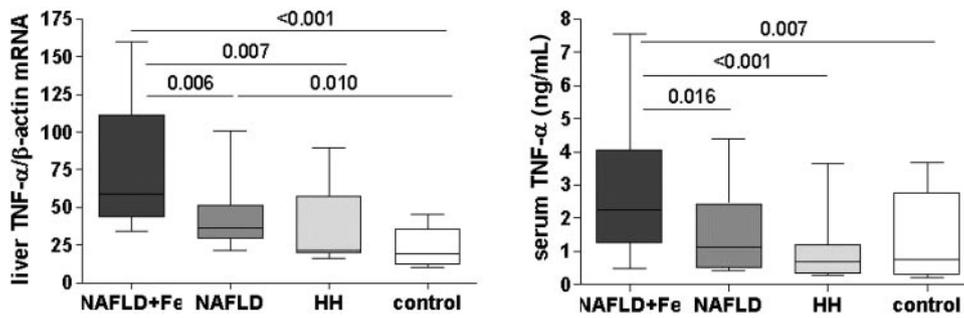


FIGURE 4. Expression of tumor necrosis factor- α (TNF- α) mRNA in the liver and serum concentrations of TNF- α for control subjects ($n = 20$), the patients with nonalcoholic fatty liver disease with (NAFLD+Fe; $n = 29$) and without (NAFLD; $n = 32$) iron overload, and patients with hereditary hemochromatosis (HH; $n = 10$). mRNA from liver biopsy samples was extracted, and TNF- α mRNA expression was determined by quantitative real-time polymerase chain reaction analysis, and values are expressed as the relative abundance of TNF- α normalized to β -actin cDNA concentrations. Concentrations of serum TNF- α were determined by enzyme-linked immunosorbent assay, and all data are presented as means (horizontal lines), 75th percentiles (boxes), and minimum to maximum ranges (vertical lines). Calculations for statistically significant differences between groups were performed by Student's t test and by non-parametric Kruskal-Wallis test in the case of non-Gaussian distribution of variables. The value above each horizontal line denotes the P value for the comparison of the 2 groups at the end of the line.

Correlation analyses

Due to known mutual interaction of key iron-regulatory molecules and regulation by cellular iron content we studied putative functional interactions by means of Spearman rank correlation analysis in controls and NAFLD patients, excluding HH patients (Table 2). This calculation provided evidence of a significant correlation between liver HJV, TfR, and FP-1 mRNA expression. In addition, serum ferritin concentrations correlated with the mRNA expression of hepatic iron-regulatory molecules investigated, namely FP-1, HJV, TfR-1, and hepcidin. Hepcidin mRNA expression was well correlated with the degree of siderosis and serum ferritin. BMI was negatively associated with hepatic and duodenal FP-1 and hepatic HJV mRNA expression but positively linked to hepatic TNF- α mRNA formation. Furthermore, we found that hepatic TNF- α mRNA was negatively associated with hepatic FP-1 and HJV mRNA expression and to be positively associated with serum ferritin, HIC, and liver hepcidin mRNA (Table 2).

Regulation of ferroportin and HJV expression by TNF- α in HepG2 cells

Hence, we set up in vitro experiments using HepG2 cells to identify a possible direct effect of TNF- α on the expression of HJV and FP-1. As shown in Figure 5, treatment of HepG2 cells with TNF- α resulted in a significant reduction in both FP-1 and HJV mRNA compared with untreated control cells, whereas TfR-1 expression remained unchanged.

Clinical effects of phlebotomy in iron-overloaded NAFLD patients

After the liver biopsies were performed, we conducted bi-weekly phlebotomies (500 mL blood) in 14 iron-overloaded NAFLD patients until serum ferritin was $\approx 100 \mu\text{g/L}$; these patients were followed-up 6 mo after the last phlebotomy. In response to phlebotomy treatment, we observed a fast decrease in transferrin saturation close to the lower limit of normal, even after the second phlebotomy ($21.1 \pm 4.5\%$), and a slower but

TABLE 2
Selected clinical and biochemical Spearman rank correlation coefficients (R) in the study cohort¹

	Liver TNF- α	Liver FP-1	Liver HJV	Liver TfR-1	Liver hep	dd DMT-1	dd FP-1	HIC	Sid	Ferritin	BMI
Serum TNF- α	-0.125	-0.273 ²	-0.309 ²	-0.119	0.031	0.038	0.037	0.191	0.246	0.386 ²	0.309 ²
Liver TNF- α		-0.532 ³	-0.332 ²	-0.142	0.533 ³	0.268	-0.085	0.343 ²	0.549 ³	0.599 ³	0.351 ²
Liver FP-1			0.554 ³	0.454 ³	-0.146	-0.238	0.253	0.078	-0.412 ²	-0.321 ²	-0.499 ³
Liver HJV				0.549 ³	0.078	-0.226	0.352	0.074	-0.353 ²	-0.531 ³	-0.271 ²
Liver TfR-1					0.152	-0.279	0.072	0.076	-0.360 ²	-0.394 ³	0.113
Liver hep						0.181	0.173	0.271	0.531 ³	0.409 ²	0.250
dd DMT-1							0.517 ³	0.001	0.227	0.207	-0.138
dd FP-1								0.273	-0.199	0.095	-0.491 ²
HIC									0.544 ³	0.398 ³	0.083
Sid										0.842 ³	0.212
Ferritin											0.278

¹ Correlation analysis included complete data from patients with nonalcoholic fatty liver disease with ($n = 21$) and without ($n = 22$) iron overload and control subjects ($n = 16$; all subjects combined, $n = 59$). FP1, ferroportin 1 mRNA expression; HJV, hemojuvelin mRNA expression; TfR-1, transferrin receptor 1 mRNA expression; hep, hepcidin mRNA expression; dd DMT-1, duodenal DMT-1 mRNA expression; dd FP1, duodenal ferroportin 1 mRNA expression; HIC, hepatic iron content; sid, siderosis on histologic examination; TNF- α , tumor necrosis factor- α .

² $P < 0.05$.

³ $P < 0.01$.

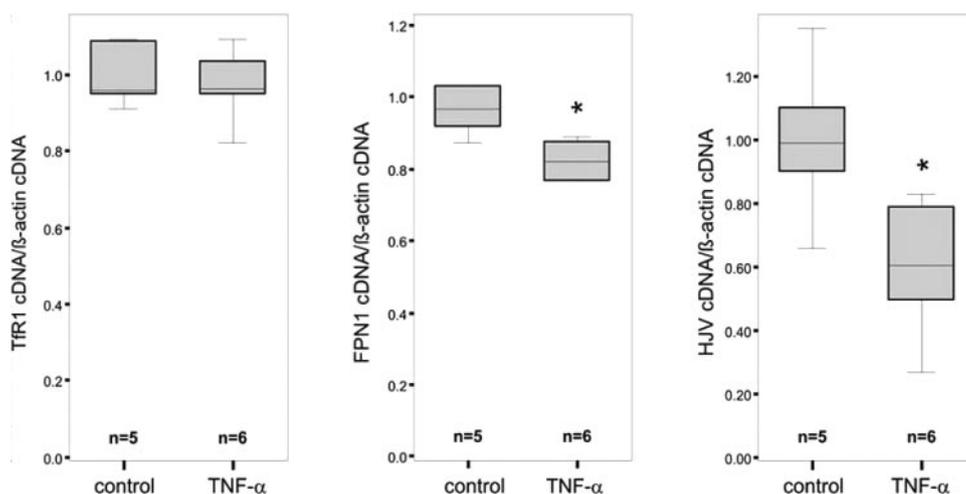


FIGURE 5. Modulation of mRNA expression of iron metabolism genes by tumor necrosis factor- α (TNF- α) in HepG2 cells. HepG2 human hepatoma cells were cultured and treated with TNF- α (10 ng/mL) for 24 h or left untreated (control). mRNA concentrations of the indicated iron metabolism genes were determined by quantitative real-time polymerase chain reaction and normalized to the mRNA expression levels of the housekeeping gene β -actin. Data are expressed as relative amounts of the unstimulated control. Data are presented as means (horizontal lines), 75th percentiles (Boxes), and minimum to maximum ranges (vertical lines). *Statistically significant difference between the 2 groups as determined by Student's *t* test ($P < 0.05$).

constant decline of serum ferritin concentrations. In parallel, we observed a significant reduction in serum TNF- α concentrations along with an improvement in liver function test results (Table 3). To estimate the amount of systemic iron overload, total iron removed during phlebotomy treatment was calculated as 0.5 mg Fe/mL blood removed. A total of 1880.4 ± 904.6 mg Fe was removed during phlebotomy treatment in these 14 patients. We found that the calculated amount of iron removed in these phlebotomized patients was significantly correlated with the HIC of liver biopsy samples ($R = 0.923$, $P < 0.001$) and with initial serum ferritin concentrations ($R = 0.570$, $P = 0.032$). Serum ferritin concentrations increased significantly within 6 mo after discontinuation of phlebotomy treatment (210.3 ± 92.6 ; $P = 0.002$), which indicated iron re-accumulation in these patients.

DISCUSSION

In this study we investigated putative molecular pathways underlying iron perturbations in patients with NAFLD. In line with previous investigations, we found the extent of iron overload in NAFLD patients to be mild compared with that in patients with hereditary hemochromatosis and to be overestimated by serum ferritin (4, 39).

TABLE 3

Changes in laboratory variables after phlebotomy treatment¹

	Before phlebotomy	After phlebotomy	P^2
Serum ferritin (mg/L)	918.9 ± 352.4	111.0 ± 74.1	0.001
Tf saturation (%)	42.9 ± 14.2	17.9 ± 4.3	0.001
Hemoglobin (g/dL)	15.6 ± 1.3	13.5 ± 1.1	0.001
AST (IU/L)	33.9 ± 22.7	28.6 ± 17.7	0.008
ALT (IU/L)	40.5 ± 20.2	29.0 ± 13.2	0.001
TNF- α (ng/mL)	2.7 ± 3.1	1.7 ± 2.0	0.036

¹ All values are $\bar{x} \pm SD$. Tf, transferrin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TNF- α , tumor necrosis factor- α .

² Calculations for statistical differences in response to phlebotomy treatment were performed by paired *t* tests.

Analysis of iron-regulatory molecules in liver tissue revealed a striking down-regulation of the liver iron exporter FP-1 and the iron sensing molecule HJV. Because FP-1 is the only known iron exporter in hepatocytes and in sinusoidal Kupffer cells, decreased FP-1 protein expression corresponds to the characteristic histologic pattern of iron deposition observed in NAFLD (5). The down-regulation of FP-1 mRNA in enterocytes and hepatocytes, both of which correlated with BMI and TNF- α concentrations, suggests that this occurs via a similar mechanism in the liver and the duodenum linked to visceral adipose tissue and associated inflammation. The assumption of a causative association between decreased hepatic FP-1 mRNA and protein expression in NAFLD patients and hepatic and systemic inflammation in vivo was supported further by decreased FP-1 formation in HepG2 cells on stimulation with TNF- α in vitro. Moreover, we found that the NAFLD patients responded to phlebotomy with a fast decrease in transferrin saturation and a gradual decline in serum ferritin, which indicates the slow mobilization of iron from storage sites, as was postulated previously (40). These changes in serum iron variables with phlebotomy treatment and down-regulation of the iron-export molecule FP-1 suggest that impaired release of iron from liver cells is a primary molecular mechanism underlying iron accumulation in patients with NAFLD. Moreover, we observed that the hepatic expression of the master iron-regulatory peptide hepcidin increased in NAFLD patients with progressive iron accumulation. Thus, the increased hepcidin concentrations in our patients most likely reflect the physiologic response to liver iron accumulation (20), because hepcidin concentrations in NAFLD patients without iron accumulation were similar to hepcidin mRNA concentrations in control subjects. Increased hepcidin formation in iron-overloaded NAFLD patients may thus be responsible for the observed down-regulation of FP-1 protein expression in the duodenum, which thereby limits iron absorption. However, the same mechanisms may facilitate iron retention within the liver, which contributes to iron-mediated radical formation and tissue damage in this organ as

reflected by increases in liver function indicators and their normalization with iron removal via phlebotomy in iron-overloaded NAFLD patients. In parallel, we found the hepatic HJV mRNA expression to be significantly lower in NAFLD patients than in control subjects. So far, HJV has been identified as a key up-stream regulator of hepcidin formation in response to iron accumulation, with a particular role in the sensing of dietary iron overload independently of inflammation (41). However, because hepcidin formation appears to be appropriate with regard to iron overload in NAFLD, the pathophysiological significance of markedly decreased HJV expression in such patients remains to be elucidated. This is important because impaired iron sensing due to down-regulation of hepatic HJV can lead to progressive iron accumulation as shown in juvenile hemochromatosis (42) and in HJV knockout mice (41). This goes along with the observations of reduced FP1 expression in HJV-deficient mice on induction of an inflammatory stage (41) and with the selective decrease of HJV mRNA concentrations in TNF- α -injected mice (43). Moreover, we found that TfR-1 mRNA decreased in NAFLD patients with iron accumulation and in patients with HH. This is likewise a reflection of liver iron overload and the consecutive metabolic down-regulation of TfR-1 mRNA via interactions between iron-regulatory proteins and iron-responsive elements (14).

In our analysis of duodenal iron transporters, we found duodenal FP-1 mRNA to be lower in all NAFLD patients than in control subjects. However, FP-1 protein expression was only reduced in iron-overloaded NAFLD patients. In these patients

we also found hepatic hepcidin mRNA expression to be significantly increased. Because hepcidin has been established as the key regulator of mammalian iron homeostasis via posttranslational regulation of cellular FP-1 expression (22), our results suggest that hepatic iron accumulation induces hepcidin formation as an adequate metabolic response that consecutively leads to decreased duodenal iron absorption (44). It thus appears that hepatic iron overload in NAFLD develops despite low (and probably quantitatively not sufficiently decreased), intestinal iron absorption, whereas iron is retained in liver cells.

In response to phlebotomy treatment, we observed improvements in liver function tests and a reduction in serum TNF- α concentrations. Phlebotomy treatment was previously shown to be safe and beneficial with regard to insulin sensitivity in NAFLD patients (13, 39, 45). We suggest that these positive effects of iron reduction via phlebotomy might, at least in part, be related to lower TNF- α formation in response to iron depletion. Thus, removal of iron goes along with a subsequent reduction in oxidative stress via the Fenton reaction (46) and TNF- α formation, which highlights the usefulness of phlebotomy as a simple and effective add-on treatment option for patients with NAFLD. Hence, it appears that mutual interactions between iron perturbations and inflammation take place in human NAFLD, because inflammatory stimuli contribute to liver iron retention, and iron accumulation augments the inflammatory response.

Considering both the regulation of iron transporters in the liver and the duodenum, the origin of observed iron perturbations in NAFLD appears to be linked to visceral obesity and consecutive metabolic and inflammatory changes. We investigated TNF- α as

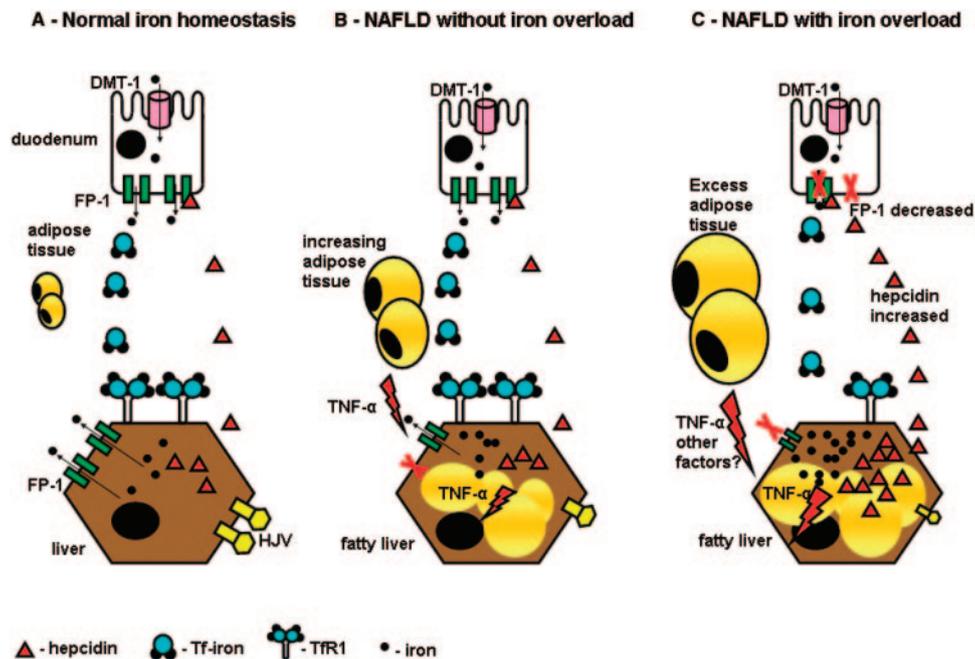


FIGURE 6. Proposed model of perturbations occurring in nonalcoholic fatty liver disease (NAFLD) iron homeostasis. A: Regulation of iron homeostasis under physiologic conditions. The amount of iron absorbed via duodenal enterocytes is tightly regulated by hepcidin according to body iron demands; hardly any iron deposits are found in the liver. B: Summary of changes in iron metabolism observed in NAFLD without iron overload. Low expressions of ferroportin-1 (FP-1) and hemojuvelin (HJV) are characteristic of NAFLD, even in the absence of iron accumulation; however, because no excess iron is deposited in the liver, hepcidin and consecutively duodenal FP-1 expressions are similar to those in control subjects. C: Changes in iron homeostasis in NAFLD with iron accumulation. Iron accumulates in NAFLD in association with significantly increased local and systemic mediators of inflammation, such as tumor necrosis factor- α (TNF- α), which most likely originates from expanding adipose tissues. Iron is progressively retained mainly because of impaired iron export from liver cells via cytokine-mediated down-regulation of ferroportin. Hepatic iron accumulation stimulates hepcidin formation, which results in the blockage of duodenal iron uptake to compensate for liver iron overload. DMT-1, divalent metal transporter-1.

a key cytokine known to be involved in the pathogenesis of NAFLD (29) and capable of regulating important molecules of iron homeostasis. The positive associations of TNF- α with BMI together with the well described formation of this cytokine by adipose tissue suggests that elevated TNF- α concentrations observed in NAFLD are at least in part a consequence of obesity (47). Nonetheless, because multiple cytokines and adipokines are crucial in the pathogenesis of NAFLD and insulin resistance, other adipose tissue-derived molecules besides TNF- α are likely to be involved in mediating changes in NAFLD iron homeostasis. A summary of perturbations of NAFLD iron homeostasis based on our findings is shown in **Figure 6**.

In conclusion, our findings suggest that perturbations of iron homeostasis observed in NAFLD patients are mainly a consequence of decreased iron mobilization from cells due to low expression of the iron exporter FP-1 and HJV. Progressive iron retention in the liver induces hepcidin formation to counterbalance hepatic iron accumulation by reducing duodenal iron absorption. Inflammatory cytokines of excess adipose tissue and the steatotic liver appear to be important mediators of NAFLD iron perturbations; however, phlebotomy treatment represents a safe and effective treatment option to ameliorate detrimental effects of iron.

The authors' responsibilities were as follows—EA: helped design and perform the research and wrote the manuscript; IT: performed the statistical analysis and helped perform the research; MT: helped perform the research; DL and MS: identified the patients and collected the biopsy specimens; HH and OD: performed the histopathologic analysis; CD: initiated the work, identified the patients, collected biopsy specimen, and wrote the manuscript; and GW: conceptualized the work and wrote the manuscript. All authors checked the final version of the manuscript. None of the authors of this study had any financial conflicts of interests to disclose.

REFERENCES

- Neuschwander-Tetri BA. Fatty liver and the metabolic syndrome. *Curr Opin Gastroenterol* 2007;23:193–8.
- Wrede CE, Buettner R, Bollheimer LC, et al. Association between serum ferritin and the insulin resistance syndrome in a representative population. *Eur J Endocrinol* 2006;154:333–40.
- Zelber-Sagi S, Nitzan-Kaluski D, Halpern Z, et al. NAFLD and hyperinsulinemia are major determinants of serum ferritin levels. *J Hepatol* 2007;46:700–7.
- Mendler MH, Turlin B, Moirand R, et al. Insulin resistance-associated hepatic iron overload. *Gastroenterology* 1999;117:1155–63.
- Turlin B, Mendler MH, Moirand R, et al. Histologic features of the liver in insulin resistance-associated iron overload. A study of 139 patients. *Am J Clin Pathol* 2001;116:263–70.
- Fargion S, Mattioli M, Fracanzani AL, et al. Hyperferritinemia, iron overload, and multiple metabolic alterations identify patients at risk for nonalcoholic steatohepatitis. *Am J Gastroenterol* 2001;96:2448–55.
- George DK, Goldwurm S, MacDonald GA, et al. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. *Gastroenterology* 1998;114:311–8.
- Valenti L, Dongiovanni P, Fracanzani AL, et al. Increased susceptibility to nonalcoholic fatty liver disease in heterozygotes for the mutation responsible for hereditary hemochromatosis. *Dig Liver Dis* 2003;35:172–8.
- Bugianesi E, Manzini P, D'Antico S, et al. Relative contribution of iron burden, HFE mutations, and insulin resistance to fibrosis in nonalcoholic fatty liver. *Hepatology* 2004;39:179–87.
- Chitturi S, Weltman M, Farrell GC, et al. HFE mutations, hepatic iron, and fibrosis: ethnic-specific association of NASH with C282Y but not with fibrotic severity. *Hepatology* 2002;36:142–9.
- Younossi ZM, Gramlich T, Bacon BR, et al. Hepatic iron and nonalcoholic fatty liver disease. *Hepatology* 1999;30:847–50.
- Fargion S, Dongiovanni P, Guzzo A, et al. Iron and insulin resistance. *Aliment Pharmacol Ther* 2005;22(suppl 2):61–3.
- Valenti L, Fracanzani AL, Dongiovanni P, et al. Iron depletion by phlebotomy improves insulin resistance in patients with nonalcoholic fatty liver disease and hyperferritinemia: evidence from a case-control study. *Am J Gastroenterol* 2007;102:1251–8.
- Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. *Cell* 2004;117:285–97.
- Abboud S, Haile DJ. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J Biol Chem* 2000;275:19906–12.
- Donovan A, Brownlie A, Zhou Y, et al. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* 2000;403:776–81.
- 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.
- Vulpe CD, Kuo YM, Murphy TL, et al. Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nat Genet* 1999;21:195–9.
- Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* 2003;102:783–8.
- 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;276:7811–9.
- Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest* 2002;110:1037–44.
- Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004;306:2090–3.
- Babitt JL, Huang FW, Wrighting DM, et al. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet* 2006;38:531–9.
- Kawabata H, Fleming RE, Gui D, et al. Expression of hepcidin is down-regulated in TFR2 mutant mice manifesting a phenotype of hereditary hemochromatosis. *Blood* 2005;105:376–81.
- Pietrangelo A. Hereditary hemochromatosis—a new look at an old disease. *N Engl J Med* 2004;350:2383–97.
- Nguyen NB, Callaghan KD, Ghio AJ, et al. Hepcidin expression and iron transport in alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L417–25.
- Peyssonnaud C, Zinkernagel AS, Datta V, et al. TLR4-dependent hepcidin expression by myeloid cells in response to bacterial pathogens. *Blood* 2006;107:3727–32.
- Bekri S, Gual P, Anty R, et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. *Gastroenterology* 2006;131:788–96.
- Crespo J, Cayon A, Fernandez-Gil P, et al. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* 2001;34:1158–63.
- Weisberg SP, McCann D, Desai M, et al. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;112:1796–808.
- Torti FM, Torti SV. Regulation of ferritin genes and protein. *Blood* 2002;99:3505–16.
- Weiss G. Modification of iron regulation by the inflammatory response. *Best Pract Res Clin Haematol* 2005;18:183–201.
- Datz C, Lalloz MR, Vogel W, et al. Predominance of the HLA-H Cys282Tyr mutation in Austrian patients with genetic haemochromatosis. *J Hepatol* 1997;27:773–9.
- Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313–21.
- Searle J LB, Crawford DHG, Powell LW. Iron storage disease. In: MacSween RNM, Burt AD, eds. *Pathology of the liver*. 4th ed. New York, NY: Churchill Livingstone, 2002.
- Theurl I, Ludwiczek S, Eller P, et al. Pathways for the regulation of body iron homeostasis in response to experimental iron overload. *J Hepatol* 2005;43:711–9.
- Theurl I, Mattle V, Seifert M, et al. Dysregulated monocyte iron homeostasis and erythropoietin formation in patients with anemia of chronic disease. *Blood* 2006;107:4142–8.
- Kowdley KV, Trainer TD, Saltzman JR, et al. Utility of hepatic iron

- index in American patients with hereditary hemochromatosis: a multicenter study. *Gastroenterology* 1997;113:1270–7.
39. Guillygomarc'h A, Mendler MH, Moirand R, et al. Venesection therapy of insulin resistance-associated hepatic iron overload. *J Hepatol* 2001;35:344–9.
 40. Piperno A, Vergani A, Salvioni A, et al. Effects of venesections and restricted diet in patients with the insulin-resistance hepatic iron overload syndrome. *Liver Int* 2004;24:471–6.
 41. 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;115:2180–6.
 42. 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.
 43. Constante M, Wang D, Raymond VA, et al. Repression of repulsive guidance molecule C during inflammation is independent of Hfe and involves tumor necrosis factor- α . *Am J Pathol* 2007;170:497–504.
 44. Ludwiczek S, Theurl I, Muckenthaler MU, et al. Ca(2+) channel blockers reverse iron overload by a new mechanism via divalent metal transporter-1. *Nat Med* 2007;13:448–54.
 45. Valenti L, Fracanzani AL, Fargion S. Effect of iron depletion in patients with nonalcoholic fatty liver disease without carbohydrate intolerance. *Gastroenterology* 2003;124: 866–7.
 46. Rosen GM, Pou S, Ramos CL, et al. Free radicals and phagocytic cells. *FASEB J* 1995;9:200–9.
 47. Day CP. From fat to inflammation. *Gastroenterology* 2006;130:207–10.

