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Hepatic Reticuloendothelial System Cell Iron Deposition is Associated with Increased Apoptosis in Nonalcoholic Fatty Liver Disease

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Abstract

The goal of this study was to examine the relationship between presence of hepatic iron deposition, apoptosis, histologic features and serum markers of oxidative stress and cell death in nonalcoholic fatty liver disease. Clinical, biochemical, metabolic and independent histopathologic assessment was conducted in 83 unselected patients with biopsy-proven nonalcoholic fatty liver disease (NAFLD) from a single center. Apoptosis and necrosis in serum was quantified using serum cytokeratin-18 (CK18) M30 and M65 ELISAs and in liver by TUNEL staining *in situ*. Serum malondialdehyde (MDA) and thioredoxin-1 (Trx-1) levels were measured to evaluate oxidative stress. Presence of reticuloendothelial system cell (RES) iron in the liver was associated with nonalcoholic steatohepatitis ($p < 0.05$) and increased hepatic TUNEL staining ($p = 0.02$), as well as increased serum levels of apoptosis-specific (M30, $p = 0.013$) and total (M65, $p = 0.006$) CK-18 fragments, higher MDA ($p = 0.002$) and lower antioxidant Trx-1 levels ($p = 0.012$) compared to patients without stainable hepatic iron. NAFLD patients with a hepatocellular iron staining pattern also had increased serum MDA ($p = 0.006$) but not M30 CK-18 levels or TUNEL staining compared to subjects without stainable hepatic iron. Patients with iron deposition limited to hepatocytes had a lower proportion of apoptosis-specific M30 fragments relative to total M65 CK-18 levels (37% vs. 25%, $p < 0.05$).

Conclusions—Presence of iron in liver RES cells is associated with NASH, increased apoptosis and increased oxidative stress. Hepatocellular iron deposition in NAFLD is also associated with oxidative stress and may promote hepatocyte necrosis in this disease.

Keywords

Nonalcoholic steatohepatitis; NAFLD; iron; cytokeratin-18; TUNEL; apoptosis; necrosis

Introduction

Nonalcoholic fatty liver disease (NAFLD) affects approximately 30% of adults in the United States, closely mirroring the obesity epidemic and prevalence of the metabolic syndrome¹. Nonalcoholic steatohepatitis (NASH), the severe form of NAFLD, is a multifactorial disease

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whereby the initial development of steatosis in the setting of insulin resistance is complicated by additional insults such as oxidative damage, mitochondrial dysfunction and ER stress¹. A potential contributing factor in many of these “second hits” is iron deposition². A recent study by our group showed that 35% of subjects enrolled in the NASH Clinical Research Network had stainable hepatic iron³. We also observed a relationship between the pattern of hepatic iron staining and disease severity in these patients; reticuloendothelial system cell (RES) iron staining alone was associated with advanced histologic features and a diagnosis of NASH, while iron staining exclusively in hepatocytes or a mixed hepatocellular/RES pattern was associated with comparatively less severe disease³.

Iron is known to increase cellular oxidative stress via production of reactive oxygen species (ROS) by catalyzing the Fenton reaction. ROS damage cell and organelle membranes via lipid peroxidation, causing altered membrane integrity and function⁴. ROS can also cause oxidative damage to nucleic acids (strand breaks, base adducts, molecular cross-links) and proteins (sulfhydryl oxidation, modification of prosthetic groups, fragmentation or structural changes) contributing to the cytotoxic effect of cellular iron accumulation^{5, 6}.

At different thresholds of oxidative damage the processes of reparative autophagy, apoptosis, or necrosis can be induced by the release of lysosomal enzymes⁷. Apoptosis can be induced by either extrinsic, death receptor-mediated pathways or intrinsic, intracellular pathways. Extrinsic pathways, such as FAS and TNFR, are thought to be dominant in NASH, but both extrinsic and intrinsic pathways are actuated by the mitochondrial release of cytochrome-c and initiation of apoptosis machinery by caspases-3 and -7^{8, 9}. Moderate lysosomal rupture has been shown to induce apoptosis, either through mitochondrial swelling and cytochrome-c release or direct caspase-3 activation and caspase-2 and 8 activation by lysosomal cathepsins¹⁰. In contrast, large-scale lysosomal breakage is associated with necrosis and limited caspase activation¹⁰. ROS- induced mitochondrial dysfunction initiates the intrinsic apoptosis cascade due to increased membrane permeability, release of cytochrome-c, and caspase 9/3 inhibition¹¹.

Cytokeratin (or keratin)-18 (CK-18) is a cytoskeletal intermediate filament protein found in simple epithelial cells, especially of the digestive tract and together with keratin 8 are the only keratin intermediate filaments in hepatocytes and variants of these genes are associated with susceptibility to a variety of liver diseases¹². Circulating CK18 fragments generated by apoptosis and necrosis, though detectable in serum of non-liver disease controls^{13–17}, are elevated in a variety of liver diseases^{12–14}, but have been most widely studied in NAFLD; numerous studies have validated CK18 as a serum biomarker of NAFLD and/or to distinguish NASH from simple steatosis^{13–21}. The commonly used M30 antibody identifies an apoptosis-specific neo-epitope at the CK18 aspartic acid residue 396, generated by cleavage of early caspase-9 and caspase-3 and -7 during the execution phase of apoptosis. The use of two monoclonal antibodies, M5 and M6 (called M65), allows for measurement of all CK-18 fragments due to loss of cell membrane integrity from necrosis and/or apoptosis by caspase activity²². Thus, concurrent measurement of M30 and M65 assays permits quantification of the relative contributions of apoptosis and necrosis to cell death in a particular etiology²².

The goal of the present study was to determine the relationship between serum markers of oxidative stress and apoptosis/necrosis (fragmented M30 and total M65 CK-18 levels) and histologic measurement of NAFLD and apoptosis among NAFLD subjects with different hepatic iron pattern phenotypes. We aimed to test the hypothesis that increased NAFLD severity among patients with hepatic RES iron deposition is associated with increased

apoptosis and systemic oxidative stress, possibly as a consequence of iron loading in macrophages and other RES cells.

Patients and Methods

Patients

The study population comprised patients with NAFLD enrolled in a NAFLD registry and repository between 2003 and 2011. Liver biopsies were performed as part of routine clinical evaluation to confirm and stage the diagnosis of NAFLD and to exclude another cause for liver disease. All patients underwent standard clinical assessment with clinical, laboratory and serologic testing to exclude other causes of liver disease. Those who had previously undergone liver transplantation, had a diagnosis of concomitant viral hepatitis, hemochromatosis, or secondary iron overload or had any histopathologic diagnosis other than NAFLD were excluded from the study. Demographic data, standard laboratory tests, and serum were collected within 6 months of biopsy. Seventy nine consecutive patients who had given written informed consent to participate in the NAFLD registry were selected for inclusion into the study based on the availability of complete clinical and laboratory data, liver biopsy specimens for independent pathologic review and for TUNEL staining and stored serum for measurement of apoptosis and oxidative stress markers. Four patients with duplicate biopsies (mean time elapsed: 5 years 29 days) and complete associated data and specimens were included in the study as independent data points. All biopsies were stained for iron using Perls' stain and were classified as no iron, hepatocellular (HC) iron, or reticuloendothelial cell system (RES) iron [including patients with a mixed HC/RES pattern]. The study was approved by the Institutional Review Board of the Benaroya Research Institute.

Liver Histology

Histologic assessment for features of NAFLD using the NASH CRN scoring system including presence and grade of steatosis, lobular inflammation, fibrosis and ballooning was completed by a single hepatopathologist with expertise in NASH(MMY) who was blinded to all clinical and laboratory data²³. The NAFLD Activity Score (NAS), determined by the sum of the steatosis, lobular inflammation, and ballooning scores, was calculated. In addition, a diagnosis of definite NASH, or not NASH (including borderline/possible NASH) was rendered. Biopsies were scored for the presence and grade of HC and RES iron using Perls' staining as previously described³.

Measurement of cell death and oxidative stress in the blood

Serum levels of malondialdehyde (MDA), a by-product of lipid peroxidation were determined using the Cayman TBARS Assay Kit (Ann Arbor, MI) following the manufacturer's instructions. Thioredoxin-1 (Trx1), a small protein with anti-oxidant and anti-apoptotic properties, was also measured in serum by ELISA following the manufacturer's instructions (Northwest Life Science Specialties' Thioredoxin-1 kit, Vancouver, WA). Serum levels of fragmented CK-18 from apoptosis (M30) and total CK-18 levels from the combination of apoptosis and necrosis (M65) were determined by ELISA following the manufacturer's instructions (Peviva, Stockholm, Sweden). Cell death due to apoptosis was calculated by the following equation: % Apoptosis = $[(M30 \text{ CK-18}/M65 \text{ CK-18}) * 100]$; likewise by definition, the remaining amount of cell death is considered to be due to necrosis and was calculated according to the formula, $100 - [(M30 \text{ CK-18}/M65 \text{ CK-18}) * 100]$.

TUNEL staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using ApopTagPlus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, Billerica, MA) following the manufacturer's instructions; DAB (Invitrogen, Carlsbad, CA), prepared just prior to use and 5 μ L of NiCl₂ (8% in distilled water) per mL of DAB was substituted for the Peroxidase Substrate to enhance visualization of dye. Specimens were counterstained using 2% methyl green for 4 min at room temperature followed by three washes each of distilled water, absolute ethanol and xylene. Quantification of apoptosis was determined by counting apoptotic cells in five random fields per patient at 400X magnification. Because the number of cells in each field varies widely between patients with ranging levels of steatosis, it was important to obtain a measure of cellularity for each patient. To estimate the total number of cells/field, two representative fields per patient were selected and overlaid with a 10x10 grid in ImageJ (Bethesda, MD). Ten squares within the grid were analyzed for the number of cells and this number was multiplied by 10 to approximate the total number of cells per field. Percent apoptosis was expressed as the average number of apoptotic cells/average total number of cells for each group. Scoring was performed in a blinded fashion through two independent assessments and the mean of these two scores were used for final analysis.

Statistical analysis

Demographic, clinical, and laboratory characteristics were recorded as number and percentage for categorical data and means and standard error or median and interquartile range for continuous data. Categorical data was analyzed using Fisher's exact test. Continuous variables including laboratory measures were not normally distributed and were analyzed using nonparametric statistics including Mann-Whitney rank sum test and Spearman rank correlation test or linear regression modeling adjusting for sex as a potential confounding variable. Differences in mean histological scores such as HC and RES iron grade, steatosis grade, fibrosis stage, lobular inflammation grade and NAS between groups were analyzed using ordinal logistic regression. Multivariate stepwise linear regression (cutoff $p < 0.20$) analysis was used to investigate the independent association of HC iron and % CK18 levels due to necrosis after adjustment for sex, age, BMI, ALT, and presence of diabetes. All statistical analysis was performed using STATA version 12.1 (College Station, TX).

Results

Patient Characteristics

Clinical and laboratory data of the study cohort is shown in Table 1. Forty seven patients (57%) were negative for hepatic iron staining and were categorized as "No Iron"; 27 patients each stained positive for HC iron or RES iron, including 18 patients positive for both HC and RES iron staining (i.e., a mixed HC/RES phenotype). Patients with either HC or RES iron deposits were more likely to be male and had a lower BMI compared to patients without iron. No significant differences were observed between either iron group and the iron negative group in age, proportion of Caucasians or presence of diabetes mellitus or obesity. Initially there were significant differences in several lab measurements (eg. ALT and AST), but most of these differences failed to remain significant after adjusting for sex in the analysis. Interestingly, even after adjusting for sex, serum ferritin values were still significantly higher in both iron groups compared to the no iron group (see Table 1). Subjects with either HC or RES iron had higher overall mean NAFLD histologic scores compared to those without iron; the NAS index and hepatocellular ballooning scores were significantly higher among patients with RES iron compared to those with no iron (Table 2). Subjects with RES iron staining were also significantly more likely to have a definitive diagnosis of NASH compared to subjects without iron (76% vs. 34%, $p < 0.05$).

NAFLD patients with RES iron have increased TUNEL staining and oxidative stress

TUNEL staining was performed to investigate the relationship between hepatic iron and apoptosis (Figure 1). Patients with RES iron showed an increased mean percentage of TUNEL positive cells compared to no iron patients (6.9 vs. 4.8, $p=0.02$). However, no significant differences were observed between patients with HC iron compared to iron negative patients. There was a trend towards a positive association between %TUNEL stained cells and grade of RES iron ($r=0.33$, $p=0.01$) but not HC iron. There was also a trend towards a positive association between % TUNEL stained cells and total M65 CK-18 levels ($r=0.36$, $p=0.004$) but not M30 CK-18 levels.

To investigate the relationship between presence and pattern of hepatic iron stores and oxidative stress *in vivo*, levels of the lipid peroxidation product, malondialdehyde (MDA), and antioxidant/antiapoptotic protein, thioredoxin-1 (Trx1) were assayed in serum (Figure 2). MDA levels were increased in subjects with either HC ($p=0.006$) or RES iron deposits ($p=0.002$) compared to iron negative subjects. Both HC and RES iron positive patients also demonstrated lower Trx1 levels, and the differences were statistically significant in patients with RES iron ($p=0.012$). Overall, in both HC and RES iron positive subjects there was an inverse relationship between MDA and Trx1 ($r=-0.50$, $p<0.0001$), suggesting opposing effects on these markers by hepatic iron loading; iron positive subjects showed relatively increased MDA and decreased Trx1, while the opposite was true in patients without hepatic iron loading (Figure 2). There was also a trend for a positive association between MDA levels and both % TUNEL staining ($r=0.30$, $p=0.02$) and RES iron grade ($r=0.32$, $p=0.01$).

CK-18 levels suggest more cell death in patients with RES iron, but a higher proportion due to necrosis in patients with HC iron

A comparison of fragmented CK-18 levels from apoptosis alone (M30) and total CK-18 levels from the combination of apoptosis and necrosis (M65) for each group relative to the total M65 level in subjects without iron staining is shown in Figure 3. Patients with RES iron had the highest M30 and M65 levels which were more than twice the levels in patients without stainable iron ($p=0.013$ and 0.006 , respectively). The level of M65 ($p=0.043$) but not M30 was significantly different between patients with HC iron and no iron.

There was a significant difference in the calculated percentage of CK-18 levels due to apoptosis or necrosis ($\% \text{apoptosis} = [(M30 \text{ CK-18}/M65 \text{ CK-18}) \times 100]$) between the iron phenotype groups ($p=0.047$; Figure 4). Cell death estimated from CK-18 M30/M65 levels in patients without hepatic iron was almost exclusively due to apoptosis (97%). In contrast, the proportion of CK-18 due to apoptosis in patients with hepatic iron deposition ranged from 63–80%. Patients with HC-only iron had the highest proportion of CK-18 attributable to necrosis (37%). To determine if the presence of HC iron was independently associated with increased cell death due to necrosis, we performed stepwise multivariate linear regression analysis and adjusted for the following potential confounding variables known to be associated with NASH severity: age, sex, BMI, ALT and presence of diabetes. In this statistical model, HC-only iron was the only variable independently associated with necrosis-associated CK-18 ($p=0.011$).

Discussion

Previous studies have shown an association between the presence and pattern of hepatic iron deposition and disease severity in NAFLD^{2,3}. However, the mechanisms behind this association remain to be fully elucidated. The goal of the current study was to investigate the hypothesis that hepatic iron deposition in reticuloendothelial system cells was associated with increased apoptosis and serum markers of oxidative stress in NAFLD. As in our

previous multicenter study of 849 NAFLD patients (NASH Clinical Research Network), we found that RES iron was associated with NASH and more severe histologic features, while patients with HC iron deposition more frequently had milder pathology³. We found that the presence of both HC and RES hepatic iron in NAFLD patients was associated with increased oxidative stress (i. e., greater lipid peroxidation in the presence of decreased antioxidant capacity) as suggested by higher MDA levels and decreased Trx1 levels compared to NAFLD patients without iron staining. However, only RES iron was associated with significantly increased apoptosis as shown by greater numbers of TUNEL positive cells in the liver and higher serum levels of total and apoptosis specific CK18 fragments. We also found that a positive association between RES iron grade and both MDA levels and amount of TUNEL positive cells, providing indirect evidence that RES iron induced oxidative stress may mediate apoptosis. Subjects with HC iron had lower CK18 levels compared to subjects with RES iron, but with a higher proportion attributable to necrosis. This observation supports our previous hypothesis that HC iron contributes to NAFLD via a direct cytotoxic effect³.

Several *in vitro* and *in vivo* studies provide evidence that exogenous iron induces apoptosis by increasing oxidative stress^{24–27}. Moreover, addition of the iron chelators baicalin²⁴, quercetin²⁴, deferasirox²⁵ or deferoxamine^{24, 25} all were shown to reduce various markers of oxidative stress and prevent apoptosis. Lin et. al., have recently provided further mechanistic insight into iron-mediated cell death; adiponectin (APN) gene therapy in C57BL/6J mice prior to intraperitoneal injection of iron dextran limited ROS production and protected animals from apoptosis²⁶. APN treatment resulted in phosphorylation of AMP-activated protein kinase (AMPK), PPAR α activation and finally induction of heme oxygenase-1 (HO-1) via binding of PPAR α to a PPAR α -responsive element in the promoter of HO-1²⁶. Interestingly, APN treatment also resulted in decrease hepatic iron staining compared to iron challenge alone which was reversed by inhibition of HO-1 with tin protoporphyrin, demonstrating the role of HO-1 in iron recycling²⁶. Iron exposure of isolated murine hepatocytes also led to NF- κ B activation and phosphorylation of p38 and ERK MAPKs concomitant with the ROS generation, reduced mitochondrial membrane potential, cytochrome c release, activation of the caspase cascade and cleavage of PARP protein²⁷.

A novel finding of this study was the greater degree of apoptosis with RES iron deposition, despite similar levels of serum indicators of oxidative stress in both HC and RES iron subjects. Apoptosis is usually observed in NASH patients concomitant with the onset of inflammation and is also thought to develop via several signaling mechanisms including oxidative stress, as well as increased pro-inflammatory cytokine production^{8, 9, 28}. Kupffer cells (KC), the main constituent of RES, are key effectors of the phenotype of surrounding cells including other KCs, hepatocytes, stellate cells, and neutrophils via the production of ROS, cytokines, chemokines, nitric oxide and prostaglandins^{29–31}. For example, KCs are a major source of TNF- α in the liver which promotes recruitment of neutrophils and other inflammatory cells, induces oxidative stress, cytotoxicity and ultimately apoptosis in hepatocyte and other cells^{29–30}. Ribera et al have shown in human NASH that TNF- α mediated induction of the caspase cascade via the death receptor TNF-R1 is a major apoptotic pathway and correlated with NF- κ B expression, and disease severity²⁸. In addition Peng et al have shown that FasL gene expression is mediated by NF- κ B and inhibition of NF- κ B attenuated apoptosis but not TNF- α expression³². In addition to expression of TNF- α and activation of the NF- κ B pathway, increased ROS and oxidative stress also promote apoptosis by activation of the JNK/AP1 serine kinase signaling cascade^{33–34}. Tsukamoto and others have shown that addition of iron activates KCs both *in vitro* and via erythrophagocytosis, inducing lipid peroxidation, NF- κ B activation and NF- κ B-mediated TNF- α expression and release which was abrogated by iron chelation treatment^{31, 35–39}.

Lastly, phagocytosis by KCs results in expression of TNF- α and death receptors FasL and TRAIL, suggesting a feed forward amplification of apoptosis⁴⁰. Taken together, these studies suggest a role of KC iron in apoptosis via the FAS, JNK and TNFR pathways through production of ROS, cytokines, NF- κ B and TNF- α , which could then be amplified through phagocytosis of erythrocytes and iron-containing apoptotic hepatocytes.

There are a number of cellular conditions that are thought to favor either necrosis or apoptosis, which potentially could explain our observations that HC iron may promote greater necrosis compared to the other iron phenotypes. Apoptosis is a deliberate, ATP-dependent process that usually occurs gradually while necrosis is a rapid event involving plasma membrane rupture subsequent to ATP depletion, thus availability of ATP is recognized as a key determinant for which mode of cell death predominates¹⁰. Iron-mediated mitochondrial lipid peroxidation contributes to pore formation in mitochondrial membranes or “mitochondria permeability transition” (MPT) and subsequent release of mitochondrial ROS^{41, 42}. Both necrosis through ATP depletion, or caspase-dependent apoptosis induced by cytochrome c release are consequences of MPT, but the degree of mitochondria involvement may determine the extent of ATP depletion and hence the development of necrosis or apoptosis²⁹. Depletion of the antioxidant glutathione in both the mitochondrial and cytosolic compartments has been shown to promote oxidative stress-induced necrosis, whereas selective cytosolic glutathione depletion sensitizes hepatocytes to TNF- α -induced apoptosis independent of oxidative stress^{43, 44}. Several studies have investigated the origins of cell death via necrosis or apoptosis in cultured hepatocytes or using *in vivo* animal models following chemically induced superoxide formation using menadione or diquat^{33, 45}. Evidence from these studies suggests that when extensive oxidant damage overwhelms the cellular antioxidant capacity, necrosis may result, whereas with moderate oxidative stress apoptotic pathways predominate.

There are some limitations of our study worth noting, such as the possible effect of elevated MDA levels after prolonged serum storage⁴⁶; potentially explaining higher levels in the subjects with hepatic iron. However, using linear regression modeling including MDA level, duration of serum storage and presence of iron, only the presence of iron (Beta 0.45, $p < 0.001$) and not serum storage duration (Beta 0.03, $p = 0.786$) was significantly associated with MDA levels. We also recognize that this cross-sectional study cannot imply causation and that additional mechanistic studies will be required to definitively establish the role of RES cell iron in the induction of various apoptotic pathways.

In conclusion, we found that both HC and RES iron deposition are associated with increased oxidative stress compared to patients without stainable iron. However, RES but not HC iron deposition in this disease is characterized by increased apoptosis in the liver as shown by both *in situ* TUNEL staining in the liver and circulating CK-18 levels and suggests a mechanism for increased disease severity. By contrast, our data suggest that selective hepatocellular iron deposition in NAFLD may preferentially promote cell necrosis versus apoptosis. Longitudinal studies will also help delineate the dynamic relationship of iron and oxidative stress and the role of these in NAFLD progression.

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List of abbreviations

NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NAS	NAFLD activity score
HC	hepatocellular
RES	reticuloendothelial system
CK18	cytokeratin-18
MDA	malondialdehyde
Trx-1	thioredoxin-1
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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Fig. 1. Quantification of TUNEL staining in patients with hepatic iron deposition

TUNEL staining was performed and positive nuclei were counted and normalized to the number of cells per field by two blinded investigators in liver tissue of NAFLD patients with or without iron staining. Values are presented as mean $\pm$ SEM. P values are shown and significant differences are indicated by the arrow (Mann-Whitney test).

Fig. 2. Differences in oxidative stress markers based on presence of iron deposition

Serum levels of the lipid peroxidation byproduct, malondialdehyde (MDA, white bars, left axis), and antioxidant enzyme thiolredoxin-1 (Trx1, black bars, right axis) are shown together. Values are presented as mean $\pm$ SEM. P values are shown and significant differences are indicated by the arrow (Mann-Whitney test).

Fig. 3. The effect of hepatic iron deposition on serum CK-18 levels

Serum levels of fragmented (M30, white bars) and total (M65, black bars) CK-18 were determined by ELISA and are shown according to the presence of either HC or RES iron staining. Levels are shown relative to the level of total M65 CK18 in patients without iron staining. P values are shown and significant differences are indicated by the arrow (Mann-Whitney test).

Fig. 4. Proportion of CK18 fragments due to apoptosis or necrosis according to hepatic iron pattern

The percentage of CK-18 fragments due to apoptosis and necrosis, calculated using the following equation: [%apoptosis = (M30/M65)*100] or %necrosis=100-[(M30 CK-18/M65 CK-18)*100] were significantly different among groups ($p=0.047$). Values are presented as mean $\pm$ SEM. Statistical analysis was performed using the Kruskal-Wallis test.

Table 1

Clinical and serologic differences of patients according to hepatic iron stain status.

Characteristic	No Iron	HC Iron	RES Iron	Total
Number	47	27	27	83
Age, (yrs)	48.8 ± 12.0	50.9 ± 11.9	48.9 ± 12.1	49.4 ± 12.0
Male sex, n (%)	20 (42)	22 (81) [†]	21 (78) [†]	49 (59)
Caucasian, n (%)	39 (83)	17 (63)	20 (74)	65 (78)
BMI (kg/m ²)	34.6 ± 6.8	30.1 ± 3.7 [†]	30.5 ± 4.1 [†]	32.8 ± 6.0
Obese (≥ 30 BMI)	29 (66)	14 (56)	16 (62)	48 (62)
Diabetes mellitus	17 (36)	7 (26)	6 (22)	26 (31)
AST (U/L)	34 (27–48)	45 (32–64)	52 (32–71)	40 (27–57)
ALT (U/L)	55 (36–90)	82 (52–139)	103 (57–164)	60 (42–118)
Alkaline phosphatase	88 (67–113)	65 (59–78) [#]	71 (58–84)	76 (64–101)
Total bilirubin (mg/dL)	0.6 (0.4–0.7)	1.1 (0.7–1.6) [#]	1.1 (0.7–1.7) [†]	0.7 (0.4–1.25)
HDL (mg/dL)	42 (36–47)	37 (31–41)	35 (29–40)	39 (33–45)
Fasting glucose (mg/dL)	103 (94–118)	99 (95–116)	104 (96–127)	103 (95–116)
Fasting insulin (IU/mL)	12 (9–19)	17 (12–20)	14.5 (12–16.5)	15 (11–21)
HOMA-IR	3.3 (1.9–4.9)	4.3 (3.3–6.0)	6.0 (4.4–8.6)	4.0 (2.8–6.7)
Hemoglobin (g/dL)	14.0 (12.9–15.1)	15.4 (14.7–16.2)	15.3 (14.6–16)	14.7 (13.6–15.8)
Ferritin (ng/mL)	122 (81–231)	368 (237–757) [#]	360 (236–800) [#]	225 (105–344)
C-reactive protein	3.8 (2.6–6.6)	2.5 (1.2–3.2)	2.6 (1.2–4.5)	2.9 (1.5–5.6)

Values are presented as numbers and percentages or medians and interquartile ranges, except for BMI and age (means and standard deviations).

[#]P<0.05;

[†]P<0.005 compared to No Iron group using Fisher's exact test for categorical variables and linear regression modeling adjusting for sex for continuous variables.

Table 2

Histological differences between patients according to hepatic iron stain status.

Characteristic	No Iron	HC Iron	RES Iron
Steatosis, (0–3)	1.8 ± 0.10	1.9 ± 0.15	2.0 ± 0.14
Lobular Inflammation, (0–3)	1.5 ± 0.09	1.6 ± 0.11	1.7 ± 0.10
Ballooning, (0–2)	0.6 ± 0.11	0.8 ± 0.14	1.1 ± 0.14 [†]
Fibrosis Stage, (0–4)	1.3 ± 0.16	1.5 ± 0.22	1.7 ± 0.37
NAS, (1–8)	4.0 ± 0.18	4.2 ± 0.33	4.8 ± 0.27 [#]
HC Grade, (0–3)	NA	1.6 ± 0.14	1.1 ± 0.20
RES Grade, (0–2)	NA	1.1 ± 0.17	1.5 ± 0.10
Definite NASH diagnosis, n (%)	16 (34)	12 (44)	18 (67) [#]

Values are presented as mean ± SEM, except for presence of NASH diagnosis (numbers and percentages). All p-values were determined by ordinal regression except presence of NASH diagnosis (Fisher's exact test).

[#] P<0.05;

[†] P<0.005 compared to No Iron group