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Iron Levels in Hepatocytes and Portal Tract Cells Predict Progression and Outcome of Patients with Advanced Chronic Hepatitis C¹

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Abstract

Background & Aims—Iron might influence severity and progression of non-hemochromatotic liver diseases. We assessed the relationships between iron, variants in *HFE*, and progression and

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outcomes using data from the HALT-C Trial. We determined whether therapy with pegylated interferon (PegIFN) affects iron variables.

Methods—Participants were randomly assigned to groups given long-term therapy with PegIFN (n=400) or no therapy (n=413) for 3.5 y and followed for up to 8.7 y (median 6.0 y). Associations between patient characteristics and iron variables, at baseline and over time, were made using Kaplan-Meier analyses, Cox regression models, and repeated measures analysis of covariance. Iron was detected by Prussian blue staining.

Results—Patients with poor outcomes (increase in Child-Turcotte-Pugh score to ≥ 7 , development of ascites, encephalopathy, variceal bleeding, spontaneous bacterial peritonitis, hepatocellular carcinoma, death) had significantly higher baseline scores for stainable iron in hepatocytes and cells in portal tracts than those without outcomes. Staining for iron in portal triads correlated with lobular and total Ishak inflammatory and fibrosis scores ($P < 0.0001$). High baseline levels of iron in triads increased the risk for poor outcome (hazard ratio=1.35, $P=0.02$). Iron staining decreased in hepatocytes but increased in portal stromal cells over time ($P < 0.0001$). Serum levels of iron and total iron binding capacity decreased significantly over time ($P < 0.0001$), as did serum ferritin ($P=0.0003$). Long-term therapy with PegIFN did not affect levels of iron staining. Common variants in *HFE* did not correlate with outcomes, including development of hepatocellular carcinoma.

Conclusions—Degree of stainable iron in hepatocytes and portal tract cells predicts progression and clinical and histological outcomes of patients with advanced chronic hepatitis C. Long-term therapy with low-dose PegIFN did not improve outcomes or iron variables.

Keywords

Liver disease; hepatitis C virus; treatment; clinical trial; HCC

Introduction

Iron is a co-factor that influences the severity and progression of non-hemochromatotic liver diseases, especially steatohepatitis and viral hepatitis^{1–7}. The most common known genetic factor that leads to iron loading is genetic variation in the *HFE* gene. Normally, the protein product of this gene plays a key role in regulating the absorption of iron from the gut. Three genetic variations in *HFE*, namely, C282Y, H63D, and S65C, have been associated with hemochromatosis (HC). Adverse effects of hepatic iron (with or without *HFE* mutations) and *HFE* mutations (with or without elevated iron) on survival after liver transplantation have been described⁸. In order to produce an iron-overload phenotype in the absence of other genetic or environmental factors, both *HFE* alleles must be affected: e.g., homozygosity for C282Y (the major mutation); compound heterozygosity for C282Y and H63D; or compound heterozygosity for C282Y and S65C. Nonetheless, even in the presence of homozygosity for C282Y, most people, especially women, do not develop pathological iron overload, pointing to the importance of additional factors^{9–14}.

In an earlier report,¹⁵ we described significant positive associations among several measures of iron loading, *HFE* gene mutations, and severity of chronic hepatitis C (CHC) in the HALT-C cohort. Yet, the minor [H63D] *HFE* genetic variation led to improved virological responses to pegylated interferon [PegIFN] + ribavirin [RBV] lead-in therapy. Defects in immune functions have been described by others in persons carrying *HFE* genetic variations, and IFN therapy has been said to lead to decreases in hepatic iron¹⁶. The aim of this study was to assess the relationship of iron and *HFE* genetic variations to disease progression and outcomes in the HALT-C Trial and to assess whether long-term IFN therapy influenced iron variables. Our hypotheses were that greater amounts of hepatic and/

or total body iron would be associated with greater progression and adverse outcomes in participants with advanced chronic hepatitis C, and that long-term PegIFN therapy would be associated with decreased hepatic iron.

Patients and Methods

The HALT-C Trial

The HALT-C trial was a multicenter, prospective study of the safety and efficacy of PegIFN treatment in patients with advanced hepatitis C¹⁷. It was conducted at 10 clinical sites between August, 2000 and July, 2006 with extended observation through October, 2009. Inclusion criteria included age greater than 18 years, chronic hepatitis C without prior decompensation (i.e., no ascites, hepatic encephalopathy, bleeding varices), and non-response to prior treatment with interferon with or without ribavirin. Detailed criteria for inclusion and exclusion are listed in Supplemental Material.

Patients in whom serum HCV RNA persisted after 20 weeks of treatment with pegylated interferon alfa-2a, 180 µg weekly and ribavirin, 1000/1200 mg/day, were randomized at week 24 to either no treatment (control group) or to continue treatment with PegIFN, 90 µg subcutaneously every week (lead-in patients). The protocol was modified after two years to permit randomization of patients who had not responded to at least 24 weeks of combination treatment with pegylated interferon and ribavirin administered outside of the HALT-C trial, provided they met the same inclusion and exclusion criteria (express patients)^{17, 20, 21}. Because genetic testing, quantitative HIC, and other iron variables were not available on “express” patients, they were not included in this analysis. Furthermore 73 participants did not consent to genetic testing and were excluded from analysis of the genetic data.

During the randomized phase of the study, participants were seen every three months for interval history, physical examination, and laboratory testing to monitor the effects of medical therapy, and to assess for clinical endpoints and adverse events. The protocol called for repeat liver biopsy 18 months after randomization (24 months after enrollment for lead-in patients) and again 42 months after randomization (48 months after enrollment for lead-in patients).

The design of this trial (Fig. 1) and the major clinical, biochemical, and virologic responses to the lead-in phase of therapy have been reported^{20, 21}. Outcomes for the randomized trial included death from any cause, definite or presumed hepatocellular carcinoma, evidence of clinical decompensation [increase in CTP score to 7 or more, development of ascites, hepatic encephalopathy, variceal bleeding, spontaneous bacterial peritonitis], and/or a two point increase in Ishak fibrosis score. There was no benefit of therapy¹⁷. In fact, among those without cirrhosis at baseline, the risk of death during the randomized phase and during extended follow-up was higher in those treated with low-dose PegIFN²².

After completion of the randomized phase of the study, participants were invited to take part in a phase of extended observation, in which they were asked to return every six months for interval history, physical examination, lab studies, and hepatic/abdominal ultrasound examination until October, 2009. Thus, following the initial randomization to low-dose PegIFN or no treatment, the median duration of follow-up was 6.0 y (range= 0–8.7 y).

Two methods were used for histologic iron assessment performed on a 4 micron section from each biopsy specimen stained with Prussian blue at the central Armed Forces Institute of Pathology histopathology laboratory. The first, hereafter called the global hepatocytic iron score, assigned by consensus of the hepatic pathologists involved in the HALT-C Trial, employed the semiquantitative method described by Scheuer and Lefkowitz¹⁸ with a score

of 1 assigned to biopsies with hemosiderin in rare periportal hepatocytes or at the periphery of regenerative parenchymal nodules, a score of 2 to biopsies with hemosiderin in numerous periportal hepatocytes in zone 1 of hepatic acini or extensively around the periphery of regenerative parenchymal nodules, a score of 3 to biopsies with hemosiderin extensively in zones 1 and 2 of hepatic acini or more extensively in regenerative parenchymal nodules, and a score of 4 to biopsies with hemosiderin throughout all acinar zones or throughout regenerative parenchymal nodules.

In addition, a more detailed assessment of hepatic iron staining was performed by one of 3 hepatopathologists on the HALT-C pathology panel based on a semiquantitative scoring method used previously²³. Hemosiderin in hepatocytes was scored separately from that in reticuloendothelial (Kupffer) cells, and results were recorded as iron absent, iron present in <50%, or >50% of each cell type. For each biopsy specimen, the total numbers of portal tracts and proportion of portal tracts with any hemosiderin staining were recorded. Whether there was stainable iron in endothelial cells, portal stromal cells, or bile ducts was also recorded.

Hepatic iron concentrations (HIC) were measured on portions of 144 liver biopsy specimens from 3 of the clinical sites (University of California-Irvine Medical Center and Long Beach VAMC, University of Connecticut Health Center, and University of Massachusetts Medical Center) using a spectrophotometric procedure^{15, 24}. Hepatic iron indices (HII) were calculated as $\mu\text{mol Fe/g dry liver/age [y]}$.

Three variations in the nucleotide sequence of the *HFE* gene that cause changes in the resulting amino acid sequence of the HFE protein (C282Y, H63D, and S65C) were assessed (See Supplemental Material).

Data Analysis

All data were analyzed by the data coordinating center [New England Research Institutes]. Most continuous variables were normally distributed, and means, standard deviations, and ranges were reported. Due to the left-skewed distribution of serum ferritin concentrations, for statistical analysis, we performed a $\log_{10}$ transformation on these results. [To clarify interpretation of results, values for serum ferritin in Figures and Tables were converted back to their antilogs.] For time to first clinical outcome, we used survival analysis methods, including Kaplan-Meier product limit analysis and Cox proportional hazards regression analysis. P values less than 0.05 were considered statistically significant. For comparison of the iron variables between the two treatment groups and for the outcome groups, over time, we used repeated measures analysis of covariance. These were done controlling for the baseline iron measures. The response variables were assessed at baseline, 24, and 48 months. Measures of iron status were not collected systematically beyond month 48, although clinical outcomes and other lab results were.

Results

Summary Overview of Participants Studied

Figure 1 provides a summary overview of the HALT-C Trial and the participants studied for this analysis. Among the 813 who agreed to participate in this study, 714 completed this phase. Most (659; 92%) of these also completed the extended follow-up phase. There were no significant differences in numbers of participants with outcomes or other events, comparing those treated with long-term low-dose PegIFN vs those not treated.

Selected Baseline Demographic, Laboratory, and Histological Features [Table 1]

As for the entire HALT-C cohort,¹⁷ none of the variables compared showed any statistically significant differences between the treatment vs control groups with the exception of age, which was slightly but significantly higher in the IFN-treated group (average age of 50.8 years versus 49.5 years for control group; $P=0.014$).

HFE Genotypes

Three single nucleotide polymorphisms (SNPs) in the *HFE* gene have been previously implicated in the development of hereditary hemochromatosis. These three SNPs are C282Y (which has been identified as the most severe mutation for causing hereditary hemochromatosis), H63D (which is the most frequent of these genetic variations) and S65C (which is rare in all populations studied to date). The frequencies of these SNPs are shown in Table 2. As expected, there were no significant differences between the control and PegIFN-treated groups (data not shown).

As described above, participants with a phenotype of iron overload at baseline were excluded from the Trial. Indeed, only one participant proved to be homozygous for the major mutation [C282Y^{+/+}] most often associated with hereditary hemochromatosis. 10 had compound heterozygosity for C282Y^{+/-} and H63D^{-/+}, a genotype that exhibits a lower penetrance for iron overload, which is usually mild^{1, 2, 4, 25}, and one each was compound heterozygous for H63D and S65C or for C282Y and S65C.

Measures of Iron Status at Baseline and Clinical Outcomes

We observed significant associations between the risk and rate of development of first clinical outcomes and the presence of higher grades of stainable iron in hepatocytes (Fig 2A) and higher percentages of portal triads with stainable iron (Fig 2B). The associations continued to be present also for the liver biopsies performed at 24 and 48 months: those who experienced clinical outcomes had significantly higher scores for hepatocellular stainable iron and percentages of portal triads with stainable iron and with progressive increases from 0 to 24 and from 24 to 48 months. In contrast, the likelihood of development of clinical outcomes was not significantly related to the presence of stainable iron in RE cells or portal stromal cells. By Kaplan-Meier analyses there were also trends (albeit not significant at the 5% level) for higher serum ferritin ($p=0.16$) or transferrin saturation ($p=0.11$) at baseline to be associated with development of outcomes. There were no associations among *HFE* genotypes and risks or rates of development of clinical outcomes. With use of Cox regression analysis of all time-varying iron variables, controlling for baseline levels and stratifying by treatment group, the baseline iron variables that were significant predictors of primary clinical outcomes were serum iron, TIBC, transferrin saturation (higher values directly correlated with outcomes; $p<0.0001$, $p<0.0001$, and $p=0.025$, respectively) and the percent of hepatocytes with stainable iron (higher values correlated with outcomes, $p=0.015$). The minor differences in significant results by the two methods of analyses are likely due to the use of tertiles for Kaplan-Meier vs continuous variables for Cox regression and the differing variables that were controlled for and correlated with baseline results. The hazard ratio (HR) for an outcome was significantly increased in subjects with stainable iron in portal triads at baseline ($HR=1.35$, $p=0.02$).

Temporal Trends and Effects of Low-dose PegIFN Therapy on Findings at Liver Biopsy

Comparisons of the control and the PegIFN-treated groups for hepatic fibrosis score, necro-inflammatory score, global fat score and global hepatocytic iron score for the three liver biopsies (baseline, 24, and 48 months) are presented in Table 3. Table 3 also presents detailed iron scoring [hepatocytic iron (% positive), reticulo-endothelial (RE) iron (%

positive), stromal cell iron (% positive), the proportion of portal triads that were positive for iron (% positive)], and the hepatic iron concentration and the hepatic iron index. At baseline, there were no significant differences between the control and PegIFN-treated groups for any of these variables. However, as described previously for the entire HALT-C cohort,¹⁷ there were significant decreases in the necro-inflammatory scores at 24 months from 7.49 for control to 6.73 for the PegIFN-treated group ($P<0.0001$), and at 48 months from 7.14 for control to 6.33 for the PegIFN-treated group ($P<0.0001$).

For the entire cohort, the global hepatocytic iron scores for stainable hepatic iron showed a significant decrease with time ($P<0.0001$). The percent of biopsies with stainable iron in hepatocytes also decreased significantly ($P = 0.0005$) with time, but did not vary between the two treatment groups ($P = 0.66$). Conversely, the percent of biopsies with stainable iron in portal stromal cells increased significantly ($P < 0.0001$) with time, and indicated a strong trend towards increased frequency of stainable iron in these cells for the treated group ($P = 0.06$) compared to the control group (Fig 3).

The global hepatocytic iron score at 48 months was significantly lower ($P=0.024$) for the control group (score=0.34) than for the PegIFN-treated group (0.46). Both at 24 and 48 months the control group had a significantly lower frequency of RE cells that were positive for iron (28% vs 38% at 24 months; 28% versus 35% at 48 months).

By definition, none of the 813 participants in the original cohort had had any primary clinical outcomes at baseline. By 24 months, 60 participants had experienced a primary clinical outcome, whereas 715 participants had not. At 48 months, 84 additional participants (a total of 144) had had a primary clinical outcome and 586 had not. At 72 and 96 months 215 and 250 (38%) had experienced primary clinical outcomes. Global hepatocytic iron scores were higher at months 24 and 48, compared to baseline, for those participants with a clinical outcome compared to those without, ($P=0.032$). Participants who did not develop primary clinical outcomes showed no change in portal triads positive for iron, whereas participants who did develop primary clinical outcomes had significantly increased frequency of stainable iron in their portal triads ($P = 0.038$).

In liver biopsies from all three time-points, there were positive and highly significant correlations between the percent of portal triads that were positive for stainable iron and the Ishak inflammatory scores (both for lobular inflammation and the total Ishak score). In addition, there were significant associations ($p=0.0014-0.0184$) between steatosis scores and stainable iron at all biopsy time points, although the presence of stainable iron accounted for only a small percent of the variation in steatosis (0.6–1.5%). Both the percent of portal triads that were positive for iron and the global hepatocytic iron scores were also directly correlated with hepatic fibrosis scores, with significant time effects ($P= 0.047$ for the interaction term of the fibrosis and iron scores at baseline, 24 months and 48 months). Similarly, the percentage of portal triads that stained positive for iron produced an overall P -value for interaction of time and portal fibrosis of 0.020. In both cases, the relationship changed with time and became slightly weaker at 48 months (results not shown). In summary, therefore, higher degrees of stainable iron in hepatocytes and portal triads at baseline correlated with more fibrosis and with the occurrence of clinical outcomes, and those who experienced clinical outcomes had increases in stainable iron, compared to those without. There were no particular outcomes that were more frequent in those with stainable iron; rather, all outcomes occurred with greater frequencies and in about the same proportions (Supplemental Table 3).

HFE Genetic Variations and Clinical Course and Outcomes

HFE genetic variations (Table 2) did not correlate with primary clinical outcomes, including the development of hepatocellular carcinoma, nor did they correlate with greater increases in stainable iron in liver biopsies at 24 or 48 months (data not shown). There were no detectable effects of iron status or *HFE* genotypes on several markers of immunological functions previously described in this cohort^{26, 27} [intrahepatic cytotoxic T lymphocyte function or peripheral blood lymphocyte proliferation (data not shown)].

Salient features of the eleven participants with *HFE* genotypes associated with hereditary hemochromatosis are summarized in Supplemental Table 1. Participant # 1 was C282Y^{+/+} homozygous and ten (participants # 2–11) were C282Y^{+/-} and H63D^{-/+} compound heterozygous. At baseline, although six had elevated serum ferritin levels and 5 had elevated serum transferrin saturations, only one (#5) had hepatic iron overload. Two (#4 and #10), one of whom at baseline had no stainable hepatic iron, experienced a clinical outcome; and another (#8) experienced a histopathological outcome (progression of fibrosis score from 3 to 6). There was no evidence of appreciable iron loading in these participants. Worthy of note is that four of ten participants carrying the H63D genetic variation experienced complete virological responses during lead-in therapy. Previously, we showed that participants with this genetic make-up are significantly more likely to achieve CVR and SVR than those without¹⁵.

Temporal trends in and effects of low-dose PegIFN therapy on serum measures of iron status

The effects of time and treatment group on serum iron levels, total iron binding capacity, the log of serum ferritin levels and the serum transferrin saturation scores are shown in Figure 4. Serum iron levels (Fig 4A) decreased significantly with time ($P < 0.0001$) and with the treatment group (the PegIFN-treated group had a larger decrease in serum iron levels than the control group; $P = 0.0304$). The P -value for the interaction of the time and treatment groups for serum iron levels was also significant ($P = 0.012$). Analysis of the serum iron binding capacity (Fig 4B) and ferritin values (Fig 4D) indicated similar, statistically significant decreases with time ($P < 0.0001$ and 0.0003 , respectively) and with treatment group ($P = 0.0004$ and 0.0475). The P -values for the interaction of time and treatment group were less than 0.0001 for both the iron binding capacity and serum ferritin values.

Prediction of disease progression in advanced chronic hepatitis C

Models to predict disease progression from baseline results of readily available lab tests (serum albumin, ALT, AST, total bilirubin, and platelet count), not including measures of iron status, were recently described by Ghany et al²⁸ for the HALT-C cohort. As described above, those more likely to experience outcomes also had higher serum irons and ferritins and lower total iron-binding capacities at baseline. Addition of the baseline measures of iron status to this model, revealed that lower TIBC at baseline was a significant additional predictor of outcomes ($p = 0.005$), whereas serum iron, ferritin, or global hepatocytic iron scores were not (details shown in Supplemental Table 2).

Discussion

In this paper, we provide long-term follow-up information on those participants in the HALT-C Trial who did not experience an SVR to lead-in therapy and who agreed to enter the randomized phase of the Trial. Our major findings in this large ($n = 813$) cohort, followed for up to 8.7 y (median of 6 y) are that, regardless of treatment group, (1) the presence and degree of stainable iron [global hepatocytic iron score; percent triads positive for iron] were predictive of adverse clinical outcomes; (2) stainable iron in hepatocytes decreased

significantly over time; (3) stainable iron in portal stromal cells increased significantly over time; (4) long-term low-dose IFN therapy was not associated with less stainable iron in the liver (in contrast to earlier findings with full-dose standard IFN)¹⁶; (5) there were no significant associations between *HFE* genetic variations and either clinical outcomes or measure of iron status; and (6) lower levels of TIBC contributed significantly to a model for predicting outcomes of CHC based on generally available liver tests (Suppl Table 2)²⁸.

Our results extend a number of previously published reports of the association of hepatic iron with adverse outcomes in CHC, as well as in other liver diseases, especially fatty liver/steatohepatitis^{1–6, 8, 15, 29–32}. The importance of iron as a co-morbid factor in CHC is emphasized by several recent reports of greater fibrosis, and greater risks of HCC development with more hepatic iron^{30, 32}. Conversely, iron reduction (usually by therapeutic phlebotomies) has consistently been associated with reductions in serum ALT levels, in severity of hepatic necro-inflammation, and risk of development of HCC^{1, 5, 31}. Iron reduction has also led to improved responses to IFN-based therapy of CHC²⁹. Among the possible reasons for improvement of CHC by standard IFN²⁹ is an effect to decrease hepatic iron¹⁶. This effect is diminished or abolished by ribavirin, which leads to iron loading due to the hemolytic anemia that attends its use. In this large study, we observed that long-term low-dose Peg-IFN did not lead to decreased hepatic iron, nor to any improvement in clinical outcomes or hepatic fibrosis (Table 3)¹⁷.

Strengths of this study include a large, well-characterized cohort with prospective and extended follow-up (Fig. 1). There was little drop-out of participants during the lead-in and randomized phases, and, even during the extended follow-up phase, continued patient follow-up was at least 70 percent for at least 6.5 years and 55% for at least 8.7y after randomization²².

Our study, although the largest undertaken to date and with the longest and best follow-up thus far achieved among longitudinal studies of CHC¹⁷, has limitations. First, enrollment was limited to persons with advanced CHC who had failed at least one (often more than one) course of adequate IFN ± ribavirin therapy. Second, we took pains to exclude participants who, at baseline, had iron overload. The reason was that we did not want to confound results by introducing the variable of pre-existent iron loading. We were highly successful in excluding participants with hemochromatosis from the Trial. Indeed, only 1/1050 (Participant #1 of Supplemental Table 1) who later had *HFE* genetic testing carried the genotype (C282Y^{+/+}) strongly associated with hemochromatosis, and this participant did not express an iron-overload phenotype (Supplemental Table 1)¹⁵. In long-term follow-up, this participant did not develop clinical or histopathological outcomes and did not develop HCC. Another limitation is that we do not have full long-term follow-up data on participants who experienced the more severe outcomes (liver decompensation, liver transplantation) because they did not continue in the study and because their iron status at the time of these serious outcomes was usually unknown.

We speculate that this drop-out of subjects with more advanced and/or rapidly progressing liver disease is the main reason why the frequency of stainable iron in hepatocytes decreased over time in this study (Fig 3A&B). The rise in stainable iron in portal stromal cells suggests an intra-hepatic redistribution of iron from hepatocytes, which, when iron-loaded, may be more susceptible to oxidative damage and death, with the iron being taken up by macrophages and eventually moving into the portal tract stromal cells (Fig 3C).

Iron, the most abundant element of the whole of earth, and fourth most abundant element of the earth's crust, is an essential element for virtually all forms of life on our terrestrial home. Despite its abundance, during the course of evolution and throughout most of human history,

organisms, including humans, have had difficulties in absorbing sufficient amounts of iron to meet their needs for formation of heme, hemoproteins, and other iron-containing proteins. Thus, several proteins have developed, which can help to increase the absorption of iron from the gastrointestinal tract, whereas there are no well-developed physiological processes for the shedding of excess iron. The liver is the primary site for iron storage, and genetic or acquired defects leading to excessive iron uptake and storage are characterized by gradual hepatic iron deposition, development of hepatic fibrosis and cirrhosis, and, at times, of hepatocellular carcinoma (HCC). Indeed, among subjects with cirrhosis due to hemochromatosis, there is a life-time risk of HCC of ~40%.

The iron-loading that may complicate chronic viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease (including non-alcoholic steatohepatitis), and other hepatic disorders is of multi-factorial origin. However, one key mechanism is an inappropriately low level of hepcidin production and/or secretion by the liver. Hepcidin has emerged as the primary hormone that regulates iron metabolism. Hepcidin is made by and released from many cell types, but especially by hepatocytes. Its release is increased by pro-inflammatory cytokines, such as IL-1, IL-6, TNF α (hepcidin is an acute phase reactant), and by *HFE*, *HFE2* (hemojuvelin), transferrin receptor-2, bone morphogenetic proteins, and other factors that respond to hepatic iron status, to hypoxia, and to the degree of adequacy of tissue oxygen delivery. Hepcidin production is suppressed by the transmembrane serine protease TMPRSS6 (also known as matriptase-2), which is required to sense iron deficiency^{33–35}[see reviews^{36, 37}]. In the case of CHC, serum hepcidin levels have been found to be inappropriately low for the degree of iron loading³⁸, and expression of HCV genes has led to decreased hepcidin gene expression due to HCV-mediated defects in histone deacetylase activity in infected hepatocytes³⁹.

The complexity of the interplay between iron and CHC is underscored by our recent finding that mutations in *HFE*, particularly the common H63D variation, while being associated with higher hepatic and total body iron, nonetheless is also associated with significantly higher likelihood of complete and sustained responses to IFN + ribavirin therapy¹⁵.

An important and still unresolved question is whether long-term, sustained iron reduction to near iron depletion can help to prevent or forestall disease progression and/or development of HCC in CHC. This has been reported in a long-term non-randomized prospective study from Japan³¹. In view of the relative ease and safety of iron reduction and adherence to a low iron diet, a prospective, randomized, controlled trial of iron reduction, in persons who cannot tolerate, or have not responded to, PegIFN + ribavirin therapy would be welcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CHC	chronic hepatitis C
CVR	complete virological response
HALT-C	hepatitis C anti-viral long-term treatment to prevent cirrhosis
HC	hemochromatosis
HCC	hepatocellular carcinoma

HCV	hepatitis C virus
HFE	the gene that is mutated in classical hereditary hemochromatosis
HIC	hepatic iron concentration
HII	hepatic iron index
PegIFN	pegylated interferon alpha-2a
RE	reticulo-endothelial
RNA	ribose nucleic acid
SNP	single nucleotide polymorphism
SVR	sustained viral response
TIBC	total iron binding capacity
TNF	tumor necrosis factor

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Figure 1. Schematic of the HALT-C Trial and participants studied

**Each patient counted more than once.

Figure 2. Measures of iron status at baseline and development of clinical outcomes

A. Time to first clinical outcome by global hepatocytic iron score

B. Time to first clinical outcome by percentage of portal triads that contained stainable iron. Clinical outcomes were defined as described previously¹⁷. Stainable iron was assessed by a panel of hepatopathologists, as described in Materials and Methods, who had no knowledge of clinical features or outcomes.

Figure 3. Effect of time and treatment on hepatic stainable iron

Results are mean + SE. The global hepatocytic iron score fell significantly over time ($P < 0.0001$). There was significant decrease in the percent of biopsies with stainable iron in hepatocytes ($P = 0.0005$) and increases in the percent of biopsies with stainable iron in portal stromal cells ($P < 0.0001$), with a strong trend to be greater in PegIFN group ($P = 0.06$).

Figure 4. Effect of time and treatment on serum measures of iron status

Results are mean + SE. Serum iron and TIBC fell significantly, and progressively over time ($p < 0.0001$) and significantly more in the PegIFN group ($p = 0.03$ and $p = 0.0004$). There were no significant changes in transferrin saturation. Serum ferritin also fell significantly over time ($p = 0.0003$), but less so in the PegIFN group ($p = 0.048$).

Table 1

Selected Demographic, Lab, and Histological Features at Baseline

Variables	Control Group	PegIFN- Treated Group	P-values Control vs PegIFN- treated
N in this study	413	400	
Gender			0.63
Male	71%	72%	
Female	30%	28%	
Race			0.16
Non-Hispanic White	70%	72%	
Black	16%	20%	
Hispanic (White)	10%	7%	
Other	3%	2%	
Age [y]	49.5	50.8	0.014
Body Mass Index [kg/m²]	30.0	30.0	0.65
<i>Results of Blood tests</i>			
Mean Serum AST [U/L]	89.8	87.6	0.60
Mean Serum AST [ratio to ULN]	2.14	2.11	0.80
Mean Serum ALT [U/L]	115	106	0.11
Mean Serum ALT [ratio to ULN]	2.3	2.15	0.21
Mean Serum Alkaline P'ase [ratio to ULN]	0.84	0.83	0.85
Mean Serum Albumin [g/dL]	3.86	3.89	0.30
Mean Serum Globulin [g/dL]	3.64	3.61	0.54
Mean Serum total bilirubin [mg/dL]	0.78	0.81	0.34
Mean INR	1.04	1.04	0.93
Mean Serum Iron [mcg/dL]	132	134	0.63
Mean Total Iron Binding Capacity [mcg/dL]	372	370	0.61
Mean Serum Iron Saturation [%]	37%	38%	0.53
Mean Serum Ferritin [ng/mL]	366	350	0.56
<i>Findings at Baseline Liver Biopsy</i>			
Cirrhosis (Ishak fibrosis 5–6) [%]	40%	40%	0.90
Positive for Iron in Hepatocytes [%]	37%	36%	0.81
Positive for Iron in R-E Kupffer Cells [%]	26%	29%	0.40
Positive for Iron in Stromal Cells [%]	36%	31%	0.19
Positive for Iron in Triads [%]	59%	61%	0.62
Ave Number of Triads per Biopsy	14.7	15.1	0.54
<i>HCV variables</i>			
Mean Log ₁₀ Serum HCV RNA Levels [IU/mL]	6.46	6.47	0.60
HCV Genotype			0.46
Genotype 1	93%	95%	
Genotype 2	3%	1%	

Variables	Control Group	PegIFN- Treated Group	P-values Control vs PegIFN- treated
Genotype 3	3%	2%	
Others (4, 5 or 6)	1%	2%	

Table 2*HFE* Genotypes of Participants Who Consented to Genetic Testing (N=740) [N (%)]

Genotype	Total
WT ($^{-/-}$) at all 3 loci	510 (69%)
H63D $^{+/-}$ only	146 (20%)
H63D $^{+/+}$ only	16 (2%)
C282Y $^{+/-}$ only	44 (6%)
C282Y $^{+/+}$ only	1 (<1%)
S65C $^{+/-}$ only	9 (1%)
S65C $^{+/+}$ only	1 (<1%)
Compound Heterozygotes	
C282Y $^{+/-}$, H63D $^{-/+}$, S65C $^{-/-}$	10 (1%)
C282Y $^{-/-}$, H63D $^{-/+}$, S65C $^{+/-}$	1 (<1%)
C282Y $^{+/-}$, H63D $^{-/-}$, S65C $^{-/+}$	1 (<1%)
Total	739* (100%)

* One participant who provided consent for genetic testing had missing genotype data.

Table 3

Hepatic Histopathology in Participants Studied [Mean (SD) or N (%)]

	Control	IFN – Treated	P-values
Biopsy at Baseline			
N	413	400	
Fibrosis score	4.13 (1.27)	4.08 (1.24)	0.56
Necro-inflammatory score	7.39 (2.01)	7.50 (2.09)	0.43
Global fat score	1.33 (0.89)	1.37 (0.95)	0.58
Global hepatocytic iron score	0.51 (0.72)	0.49 (0.69)	0.72
Detailed iron scoring			
- Hepatocytic iron [% +]	149 (37%)	141 (36%)	0.81
- R-E iron [% +]	143 (36%)	121 (31%)	0.19
- Stromal cell iron [%+]	105 (26%)	112 (29%)	0.40
- Triads with iron [%+]	237 (59%)	236 (61%)	0.62
Hepatic iron concn (ug/g dry wt)	1479 (763)	1344 (794)	0.44
Hepatic iron index	0.53 (0.28)	0.48 (0.31)	0.46
Biopsy at 24 Months			
N	340	336	
Fibrosis score	3.92 (1.47)	3.92 (1.47)	0.99
Necro-inflammatory score	7.49 (2.03)	6.73 (2.33)	<0.0001
Global fat score	1.31 (0.83)	1.33 (0.80)	0.77
Global hepatocytic iron score	0.36 (0.63)	0.38 (0.63)	0.72
Detailed iron scoring			
- Hepatocytic iron [% +]	101 (30%)	99 (29%)	0.87
- R-E iron [% +]	95 (28%)	127 (38%)	0.0087
- Stromal cell iron [%+]	156 (46%)	169 (50%)	0.32
- Triads with iron [%+]	191 (57%)	194 (58%)	0.82
Hepatic iron concn (ug/g dry wt)	1368(605)	1334 (748)	0.82
Hepatic iron index	0.51 (0.25)	0.48 (0.27)	0.57
Biopsy at 48 Months			
N	284	282	
Fibrosis score	4.00 (1.60)	4.07 (1.60)	0.61
Necro-inflammatory score	7.14 (2.19)	6.33 (2.30)	<0.0001
Global fat score	1.14 (0.69)	1.23 (0.76)	0.15
Global hepatocytic iron score	0.34 (0.59)	0.46 (0.70)	0.0237
Detailed iron scoring			
- Hepatocytic iron [% +]	71 (25%)	85 (30%)	0.17
- R-E iron [% +]	79 (28%)	100 (35%)	0.0505
- Stromal cell iron [%+]	142 (50%)	158 (56%)	0.15
- Triads with iron [%+]	166 (59%)	172 (61%)	0.57

	Control	IFN – Treated	P-values
Hepatic iron concn (ug/g dry wt)	1521 (809)	1360 (650)	0.36
Hepatic iron index	0.61 (0.63)	0.49 (0.25)	0.28

Fewer participants underwent liver biopsies at 24 & 48 months because of a variety of reasons. The major reasons included participant declined, participant had died, participant had undergone liver transplantation, participant had withdrawn from the study, and investigators felt biopsy should not be performed.