Relative contribution of iron genes, dysmetabolism and hepatitis C virus (HCV) in the pathogenesis of altered iron regulation in HCV chronic hepatitis

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ABSTRACT

Background and Objectives

Hepatitis C virus (HCV) chronic hepatitis predisposes to iron overload, which negatively influences the prognosis of this infection. Since the underlying mechanisms of this iron overload are undefined, we analyzed the prevalence of altered iron parameters, and the relative contribution of viral, metabolic, and genetic factors in Italian patients.

Design and Methods

We studied the metabolic and biochemical characteristics of 143 previously untreated, biopsied patients with HCV who were not alcohol abusers. Hepatic iron was determined according to Deugnier, *HFE* genotype by restriction analysis, hepcidin, hemojuvelin, ferroportin-1, and transferrin receptor-2 mutations by denaturing high performance liquid chromatography and sequencing.

Results

Increased transferrin saturation was observed in 20%, hyperferritinemia in 22%, and histological iron deposition in 32% of patients. Ferritin was independently correlated with iron stores and host metabolic parameters, whereas hepatic iron deposition was correlated with ferritin and histological severity of hepatitis. Sinusoidal iron deposition was associated with metabolic alterations, including body mass index, insulin resistance, and LDL cholesterol. Conversely, the prevalence of *HFE* mutations and serum ferritin values increased with the severity of steatosis. The prevalence of *HFE* and β-globin mutations was not different from that of controls (31% and 2%, respectively). No *tranferrin receptor-2*, *hemojuvelin*, or *ferroportin-1* mutations were detected, but two patients carried the –72C>T *hepcidin* promoter mutation. The C282Y *HFE* mutation, *hepcidin* and *β-globin* mutations influenced iron stores. Both carriers of the –72C>T Hepcidin mutation had β-thalassemia trait, moderate iron overload, and liver cirrhosis.

Interpretation and Conclusions

Iron genes influence iron overload and steatosis development, but the major burden is related to HCV itself and host metabolic factors.

Key words: chronic HCV hepatitis, *HFE*, *hepcidin*, iron, liver steatosis.

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Chronic hepatitis due to hepatitis C virus (HCV) infection (CHC) is the leading cause of liver-related mortality in Western countries, due to progression to cirrhosis and hepatocellular carcinoma. A high prevalence of iron overload has been shown in patients with CHC characterized by different genetic backgrounds and exposure to environmental factors, suggesting that several mechanisms are involved, including inflammation, alteration of iron sensing, and deregulation of hepcidin release by hepatocytes.[1,2] Elevated iron stores have been reported to affect the outcome of antiviral therapy, and to promote fibrogenesis and the risk of hepatocellular carcinoma. Iron parameters are altered in only a proportion of patients,[1] so that understanding the pathogenesis of iron overload may have important clinical implications.[3-9] However, data on the relative role of acquired and viral factors, of mutations of the *HFE* gene responsible for hereditary hemochromatosis, and of the more recently identified genes responsible for rare cases of hereditary iron overload, are conflicting or still lacking.[10] The interpretation of altered iron parameters has recently become more complex, as there is now evidence indicating that hyperferritinemia often reflects metabolic alterations linked to insulin resistance and fatty liver in the general population.[11] It is noteworthy that steatosis and insulin resistance are detected in about 50% of CHC patients and are negative prognostic factors.[12-15] Based on experimental and clinical observations, it has been proposed that HCV plays a direct role in favoring the development of fatty liver,[14,16,17] in particular in subjects infected by genotype 3 viral strains.[14,18] Thus, it could be hypothesized that the pathogenesis of altered iron regulation and dysmetabolism of CHC are intertwined. In an attempt to shed light on the determinants of iron overload in CHC, we analyzed the prevalence of altered iron parameters and the relative contributions of viral and metabolic factors, and of genetic variations known to alter iron homeostasis.

Design and Methods

Patients

Of 245 consecutive unrelated patients with CHC referred between January 2000 and January 2005, we studied 143 for whom serum and DNA samples, clinical data, and liver biopsy with evaluation of iron stores were available. The demographic and clinical features of the subjects considered in this study did not differ significantly from those of the subjects not included. All the patients lived in Northern Italy, although 28 (19%) had at least one ancestor from Central or Southern Italy. Patients with co-existent hepatitis B virus (HBV) or human immunodeficiency virus (HIV) infection, active alcohol abuse (>60 or 40 g/day for males or females, respectively, during the last 10 years), decompensated cirrhosis, hepatocarcinoma at diagnosis, or previously treated or under treatment with interferon were excluded. Part of this group has been previously described.[19] Data on age, sex, alcohol intake (g/day), body mass index (BMI), alanine transaminase (ALT), aspartate transaminase (AST), γ glutamyltransferase (GGT), serum ferritin, transferrin saturation, glucose, triglycerides, total, LDL and HDL cholesterol, and fasting serum insulin were available for each patient at diagnosis. Viral genotype and load, determined by standard methods, were available for 120 patients (85%). The demographic and clinical features of this cohort of patients are shown in Table 1. We used 291 healthy subjects (blood donors and relatives of patients of the same geographical origin), with normal liver tests and iron parameters and without diabetes, as controls for the studies of the prevalence of *HFE* and *β-globin* mutations. This group has been previously described.[20] Controls for the analysis of *hepcidin*, *hemojuvelin*, and *ferroportin-1* mutations have been previously described.[21,22]

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as revised in 2000, and was approved by the Institutional Review Board of the Ospedale Policlinico, Mangiagalli and Regina Elena Fondazione IRCCS, Milan, Italy. Informed written consent was obtained from each subject participating in the study.

Liver histology

Liver biopsy specimens were processed according to routine techniques. Tissue sections were stained with hematoxylin-eosin, silver, periodic acid Schiff, trichrome and Perls' stain for iron. Biopsies were reviewed and scored, according to Ishak, by a single expert pathologist, unaware of the patients' clinical status and genotypic analysis.[23] Iron deposits were assessed semi-quantitatively according to Deugnier.[24] Total iron scores, composed of parenchymal iron score, sinusoidal iron score and portal iron scores were determined for each case. Steatosis was identified when present and graded histologically according to the percentage of affected hepatocytes (<5%: grade 0, no significant steatosis, 5-33%: grade I, 34-66%: grade II, >66%: grade III steatosis).

Insulin resistance

Serum insulin levels were determined by radio-immuno assay (Biochem Immunosystems, Bologna, Italy), and insulin resistance was estimated by the homeostatic metabolic assessment insulin resistance index (HOMA-IR).[25]

Genotypic analysis

HFE mutations were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis. *Hepcidin* (all exons) and *hemojuvelin* (exons 3 and 4, which are hot spots for mutations) mutations, which when inherited in a homozygous state are responsible for juvenile hemochromatosis, were searched for in all patients and in controls by denaturing high performance liquid chromatography (dHPLC) and sequencing, as previously described.[21] In addition, *ferroportin-1* mutations (all exons and the promoter region), responsible for autosomal dominant iron overload disorders characterized by

Table 1. Demographic, clinical and genetic features of 143 Italian patients with HCV chronic hepatitis.

	Reference	*Value*	*Abnormal values*
Age (years)		49.8±13	
Sex: male/female		86/57 (60/40)	
Genotype 1/2/3/4*		71/32/13/4 (59/27/11/3)	
Viral load IU/mL 1000*		936 {316-3032}	
Alcohol g/day	<0	23±30	85 (59.5)
BMI Kg/m^2	<25	24.4±3.5	57 (40)
LDL cholesterol mg/dL	<130	112±32	
HDL cholesterol mg/dL	>40	44.3±12	
Triglycerides mg/dL	>160	104±48	
Uric acid mg/dL	<6M/5.6F	4.6±1.2	
AST IU/mL	<42	66±50	
ALT IU/mL	<42	102±77	
GGT IU/mL	<40	44 {27-79}	
Diabetes			17 (12)
HOMA-IR	<2.7	4.4±2.5	83 (58)
Liver histology			
Grade		6 {4-8}	
Stage		2 {1-4}	
Cirrhosis			24 (17)
Steatosis			
Grade I [5-33%]		59 (41)	
Grade II [34-66%]		12 (8.5)	
Grade III [>67%]		5 (3.5)	

*LDL: low density lipoprotein; HDL: high density lipoprotein; AST: aspartate transaminase; ALT: alanine transaminase; GGT: γglutamyltransferase. (): % values; median values and {}: interquartile range. M: males; F: females; BMI: body mass index; HOMA-R: homeostatic metabolic assessment insulin resistance index. *Available in 120 subjects.*

Table 2. Iron parameters, hepatic siderosis, and genetic background of 143 Italian patients with HCV chronic hepatitis.

	Value values	*Abnormal*
Ferritin ng/mL [>320/240 in M/F, respectively]	146 {69-283}	31 (22)
Transferrin saturation %	36.2±15.7	28 (19.6)
[> 45/40 in M/F, respectively]		
Total iron score [0]	0 <0-8>	46 (32.2)
Parenchymal iron score [0]	0 <0-3>	33 (23)
Sinusoidal iron score [0]	0 <0-3>	34 (23.7)
Portal iron score [0]	0 <0-0>	5 (3.5)
HFE status		
C282Y/H63D		2 (1.5)
C282Y/wt		3 (2)
H63D/H63D		3 (2)
H63D/wt		36 (25)
Wt/wt		99 (69.5)
β-thalassemia trait		3 (2)
Hepcidin exon 1-3		2 with –72C→T (1.5)
1 with IVS2 +7G→A (0.7)		
Hemojuvelin exons 3-4		none
Ferroportin-1 all exons and promoter		0/5 at risk
Transferrin receptor-2 all exons		0/2 at risk

*(): % values; []: reference values, median values and {}: interquartile range are shown; median and <>: 10th-90th centile range are shown. *Tissue iron score ≥6, unknown cause other than HCV, °parenchymal iron score ≥6, unknown cause other than HCV.*

non-parenchymal iron overload, were searched for by dHPLC and automated sequencing, as previously described,[22] in five patients with unexplained sinusoidal iron deposition. Given their known rarity outside specific ethnic settings, *transferrin receptor-2* mutations (all exons), which in a homozygous state are responsible for hereditary hemochromatosis, were searched for, by direct sequencing, in two subjects with unexplained severe iron overload. β-thalassemia trait was defined as the presence of mild anemia associated with decreased mean corpuscular volume (<75 fL), absence of iron deficiency, and increased HbA_2 (≥3.5%).

Statistical analysis

Results are expressed as means ± standard deviations, except for non-normally distributed variables which are presented as median values and interquartile ranges. Tissue iron scores were approximated to continuous variables for multivariate analysis. Mean values were compared by Student's t-test, ANOVA (for multiple comparisons), and Wilcoxon and Kruskal-Wallis tests (for non-normally distributed variables). Frequencies were compared by Fisher's exact test, and correlations were performed with Spearman's test. Variables selected by univariate analysis as significantly associated with iron indices were entered into linear regression models with the use of a forward stepwise elimination algorithm (variables with p>0.1 were eligible for removal). Results were considered statistically significant when the p value was less than 0.05 (two-tailed test). Analyses were carried out with JMP 6.0 statistical analysis software (SAS Institute Inc, Cary, NC, USA).

Results

Prevalence of altered iron metabolism and mutations of iron genes

The characteristics of the patients studied are shown in Table 1. Sixty per cent of the patients assumed alcohol (mean daily alcohol intake 23±30 g/day), 12% were diabetic, and 58 % had insulin resistance, as documented by increased HOMA-IR. Ninety-nine of the patients (69%) had histological evidence of steatosis, with the majority having grade I steatosis. Cirrhosis was present in 24 (17%) patients. Iron parameters and the prevalence of mutations / polymorphisms of genes involved in iron metabolism are reported in Table 2. Twenty-two percent of the patients had increased ferritin and 20% increased transferrin saturation. Liver siderosis was detected in 32 % of the patients, with equal prevalences of parenchymal and sinusoidal iron deposition.

The prevalence of *HFE* and *β-globin* mutations in this series of patients was not significantly different from that in the controls (*not shown*). No *hepcidin*, *hemojuvelin*, *ferroportin-1* or *transferrin receptor-2* mutations were observed in historical controls (*not shown in detail*). Thirty-one percent of patients carried either one of the two main *HFE* mutations. Three patients carried mutations of the *hepcidin* gene: two the –72C>T mutation in the promoter, one a variant in the second intronic sequence (IVS2+7G>A). No

Table 3. Factors associated with the presence of parenchymal and sinusoidal hepatic siderosis in 143 Italian patients with HCV chronic hepatitis.

	Parenchymal siderosis			*Sinusoidal siderosis*		
	Present n=30	*Absent n=103*	*p*	*Present n=30*	*Absent n=103*	*p*
Ferritin	264 {152-457}	130 {62-241}	0.0001	276 {146-450}	124 {62-240}	0.0002
BMI	25.2±1.8	24.1±3.8	0.02	25.3±2.2	24.1±3.7	0.02
LDL cholesterol	106±33	114±32	0.16	99±26	116±33	0.005
Uric acid	4.7±0.9	4.6±1.6	0.7	5.0±1.3	4.5±1.2	0.04

median and {}: interquartile range. BMI: body mass index; LDI: low density lipoprotein.

Table 4. Association between steatosis grade, viral and iron parameters in 143 patients with HCV chronic hepatitis.

	Steatosis grade			
	0	*I*	*II-III*	*p*
Number	67	59	17	
Steatosis %	0.6±1	8.8±5*	44±19*	<0.0001
LDL cholesterol	122±30	110±31	83±27*	0.0005
HCV genotype 3^	2/57 (3.5)	7/50 (14)	4/13 (31)	0.01
Ferritin ng/mL	92 {51-254}	156 {101-313}*	267 {88-430}	0.005
Transferrin saturation (%)	35.2±15	35.7±15	41.3±23	ns
HFE mutations	14 (21)	25 (42)	6 (35)	0.03
Total iron score	0 <0-5>	0 <0-9>	0 <0-11>	ns
Siderosis	19 (28)	21 (35)	2 (20)	ns
Sinusoidal siderosis	14 (21)	15 (25)	25(29)	ns

*(): % values, median and {}: interquartile range, <>: 10th-90th centile range are shown. Grade 0: 0-4%, I: 5-33%, II: 34-60%, III: ≥66%; * p<0.05 vs. absence of steatosis (grade 0), ^available in 120 cases.*

Table 5. Influence of iron genes on iron status in 143 patients with HCV chronic hepatitis.

	C282Y +	*H63D +*	*Hepcidin mutations Thal trait*	*negative*	*p*
n =	*5*	*40*	*4*	*94*	
Ferritin (ng/mL)	250 {28-567}	258 {91-307}	593* {14-1338}	193 {71-264}	0.007
Ferritin > 320 ng/mL	2 (40)	9 (22.5)	3 (75)	18 (19.8)	0.05
Transferrin saturation (%)	52.2±42*	37.9±14	50±27*	33.7±11	0.01
Siderosis present or iron reduction^	4#* (80)	13 (32)	3° (75)	32 (34)	0.066
Stage ≥3 (23)	2 (20)	15 (37.5)	2 (50)	37 (39.4)	ns

*(): % values, {}: interquartile range. ^presence of any degree of siderosis at biopsy or iron removed by phlebotomy or iron chelators > 3 g at iron depletion before liver biopsy. # 3 patients (phlebotomy) and ° 1 patient (desferrioxamine) were submitted to iron depletion before the liver biopsy corresponding to the clinical parameters collected, *p≤0.05 vs. patients negative for mutations.*

patient carried mutations of *hemojuvelin* nor, among those tested because potentially at risk (prevalent siderosis in Küpffer cells), of *ferroportin-1*, or of transferrin *receptor-2*.

Clinical determinants of altered iron parameters and role of metabolic alterations

In the stepwise regression analysis considering, among the variables presented in Table 1, those significant at univariate analysis, ferritin correlated with transferrin saturation (p=0.003), HOMA-IR (p=0.05), uric acid (p=0.002), and parenchymal iron score (p=0.036), transferrin saturation correlated with age (p=0.048), ferritin (p=0.006) and alcohol intake (p=0.004), whereas siderosis, evaluated as total iron score, correlated with ferritin (p=0.03), and histological grade (p=0.02). To evaluate whether cellular iron distribution in the liver was related to different pathogenic mechanisms, we analyzed variables significantly associated with the presence of parenchymal and sinusoidal iron deposits (Table 3). Ferritin and BMI were associated with both parenchymal (p=0.0001 and p=0.02, respectively) and sinusoidal siderosis (p=0.0002 and p=0.02, respectively), whereas LDL cholesterol and uric acid were associated only with the presence of sinusoidal iron (p=0.005 and p=0.04, respectively). We then analyzed the relationship between different degrees of steatosis, and metabolic, viral, and iron parameters (Table 4). A significant decrease in LDL cholesterol was observed with increasing severity of steatosis (p=0.0005), and patients infected by genotype 3 had a higher prevalence of severe steatosis (p=0.01). Among iron parameters, ferritin, but not transferrin saturation, was significantly associated with steatosis (p=0.005). The prevalence of *HFE* mutations was higher in patients with steatosis than in those without (p=0.03).

Effect of mutations in iron genes on iron metabolism

An analysis of the effect of variants of iron-related genes on iron status, shown in Table 5, indicated that ferritin and transferrin saturation values were significantly influenced by the presence of mutations of the iron-related genes (p=0.007 and p=0.01, respectively), in particular by the C282Y *HFE* mutation and *β-globin/hepcidin* mutations (considered together because coexistent in two out of four patients). We also evaluated the relationship between the genetic variants and severe fibrosis (defined as Ishak stage ≥3) but did not find any significant association. However, two of the four patients with hepcidin mutations and/or β-thalassemia trait had cirrhosis, and three of the five carriers of the C282Y HFE mutations had undergone phlebotomy treatment prior to biopsy, potentially influencing the evolution of any liver damage. The two carriers of the –72C→T *hepcidin* promoter mutation were 60 and 73 years old, and did not have *HFE* mutations. Both had β-thalassemia trait, moderate iron overload (ferritin 1500 and 873 ng/mL, transferrin saturation 89% and 48%, presence of liver siderosis), and liver cirrhosis.

Discussion

In an attempt to shed light on the pathogenesis of iron overload in CHC, we characterized the iron status of a group of Italian patients with this condition and analyzed the relationship between iron status, metabolic and viral parameters, and mutations in iron related genes. Our results confirm that about 20% of such patients have increased ferritin and/or transferrin saturation and a higher number have evidence of liver iron deposits.[2] Mutations of iron-related genes were detected in about one third of patients with altered iron parameters, indicating that metabolic alterations and other, very likely HCV-related factors, played the major role in the alteration of iron parameters in the remaining patients.

First, we confirmed that ferritin levels, associated in previous studies with the severity of liver disease and therapeutic failure,[3,6] reflect iron overload. Indeed, ferritin was independently associated with transferrin saturation and hepatic iron deposition, in particular in parenchymal cells. However, HOMA-IR, the insulin resistance index, and uric acid also proved to be independent predictors of ferritin, and alcohol intake of transferrin saturation. These data indicate that, as observed in the general population,[11] metabolic and acquired factors play a major role in determining the increase of iron parameters in CHC. Conversely, the independent association between tissue iron score and histological activity, as well as the negative correlation between ferritin and LDL cholesterol, point to the strong influence of the host-virus interaction on iron metabolism and liver damage. Given the cross-sectional design of the study, cause and effect relationships could not be ascertained. Interestingly, iron supplementation has previously been demonstrated to enhance HCV replication in hepatocytes, but evidence is controversial, since an opposite effect has recently been reported.[26,27] Thus, additional studies are still required to clarify this important issue.

Next, considering the high prevalence of both iron overload and steatosis in patients with CHC, we analyzed their inter-relationship. It is known that ferritin is increased in about 30% of patients with non-alcoholic fatty liver disease and metabolic syndrome, the leading cause of hyperferritinemia in the general population,[20] and results of this study indicate that also in CHC the presence of steatosis markedly influenced serum ferritin values, which were higher in patients with more severe steatosis. An opposite trend was observed for LDL cholesterol, confirming a direct role of HCV in the pathogenesis of fatty liver by inhibiting VLDL secretion.[28] As previously shown, we did not observe a higher prevalence of *HFE* gene mutations in patients with CHC, but interestingly, as recently reported by our group in patients with non-alcoholic fatty liver disease,[20] we did find an increased prevalence of *HFE* mutations in patients with steatosis. It could be hypothesized that iron excess facilitates fatty liver pathogenesis because of the interaction of ferritin with ApoB secretion by hepatocytes.[29] It is noteworthy that iron overload has recently been found to facilitate oxidative stress, steatosis development, and hepatocarcinogenesis in a mouse model transgenic for HCV.[7] Thus, these data indicate that altered iron metabolism and steatosis are intertwined, and that the interaction between the HCV core protein, ferritin and nascent lipoproteins in the endoplasmic reticulum may play an important role in the pathogenesis of CHC. The few subjects positive for the C282Y *HFE* mutation and those carrying *hepcidin* mutations and/or heterozygous for *β-globin* mutations, characterized by ineffective erythropoiesis and increased iron absorption, had more severe iron overload. Very recently, Sartori *et al.*[30] reported that β-thalassemia trait was significantly associated with iron stores and stage of fibrosis. In the present study we did not find a relationship between iron and fibrosis, possibly because patients with more severe iron overload underwent phlebotomy prior to biopsy, and because of the low prevalence of mutations of iron-related genes with consequent lack of statistical power of the analyses. However, we did detect a significant effect of genetic background on the presence of iron overload. Interestingly, we showed that the –72 C>T *hepcidin* mutation, present in two patients, was associated with iron overload and cirrhosis. This mutation, so far not observed in healthy subjects with normal iron parameters,[21] has previously been detected in a heterozygous state, and shown to aggravate the clinical phenotype and biochemical indices of iron overload in *HFE* compound heterozygous individuals.[21] The characterization of the functional significance and prevalence of this inherited factor needs to be established in future studies. Previously, another *hepcidin* promoter variation (-25G>A) has been linked to iron overload and altered regulation of hepcidin release after iron challenge,[31] and it is therefore interesting to note that the –72C>T mutation is very close to the TATA box and the transcription start of *hepcidin*.[32]

In conclusion, our data indicate that iron genes influence iron overload in CHC patients, likely by influencing hepcidin release, but suggest that the major burden is related to HCV hepatitis by direct mechanisms, and indirectly by inducing steatosis in co-operation with host metabolic factors. Conversely, iron genes may in turn predispose to steatosis.

Authors' Contributions

LV conceived the study, collected, analyzed and interpreted the data, and drafted the manuscript; SF and ALF conceived the study, analyzed and interpreted the data, and drafted the manuscript; EP collected the data, contributed to data analysis and interpretation, and revised the manuscript; PD, GB, LC and PA performed biochemical and genetic tests, contributed to data analysis and interpretation, and revised the manuscript; MM reviewed liver histologies, contributed to data analysis and interpretation and reviewed the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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