

# Relative Contribution of Iron Burden, HFE Mutations, and Insulin Resistance to Fibrosis in Nonalcoholic Fatty Liver

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The mechanism(s) determining the progression from fatty liver to steatohepatitis is currently unknown. Our goal was to define the relative impact of iron overload, genetic mutations of HFE, and insulin resistance on the severity of liver fibrosis in a population of subjects with nonalcoholic fatty liver disease (NAFLD) who had low prevalence of obesity and no overt symptoms of diabetes. In a cohort of 263 prospectively enrolled patients with NAFLD, 7.4% of patients had signs of peripheral iron overload and 9% had signs of hepatic iron overload, but 21.1% had hyperferritinemia. The prevalence of C282Y and H63D HFE mutations was similar to the general population and mutations were not associated with iron overload. Although subjects were on average only moderately overweight, insulin sensitivity, measured both in the fasting state and in response to oral glucose, was lower. Univariate analysis demonstrated that the presence of severe fibrosis was independently associated with older age, female sex, overweight, aspartate/alanine aminotransferase ratio, serum ferritin level, fasting glucose and insulin levels, decreased insulin sensitivity, and with histologic features (degree of necroinflammation and steatosis). After adjustment for body mass index (BMI), age, sex, and degree of steatosis, ferritin levels (odds ratio [OR] = 1.77; 95% CI = 1.21–2.58;  $P = .0032$ ) and the oral glucose insulin sensitivity (OR = 0.53; CI = 0.33–0.87;  $P = .0113$ ) were independent predictors of severe fibrosis. In conclusion, the current study indicates that insulin resistance is a major, independent risk factor for advanced fibrosis in patients with NAFLD. Increased ferritin levels are markers of severe histologic damage, but not of iron overload. Iron burden and HFE mutations do not contribute significantly to hepatic fibrosis in the majority of patients with NAFLD. (HEPATOLOGY 2004;39:179–187.)

**N**onalcoholic fatty liver disease (NAFLD) is growing in importance, causing in a wide spectrum of hepatic injury, ranging from fatty liver to steatohepatitis and cirrhosis.<sup>1</sup> Whereas pure fatty liver

has a benign clinical course, nonalcoholic steatohepatitis (NASH) is a recognized cause of progressive liver fibrosis that eventually leads to cirrhosis, liver failure, and hepatocellular carcinoma.<sup>2–4</sup> NAFLD and NASH are the two most common chronic liver diseases in Western countries, with an estimated prevalence in the general population of 10% to 20% and 2% to 3%, respectively.<sup>2,5–7</sup> A two-hit theory<sup>8</sup> has been proposed to explain the progression from simple steatosis to NASH, fibrosis, or cirrhosis. The first hit is represented by excessive hepatic fat accumulation primarily owing to insulin resistance and is supported by the close relation observed between NAFLD and several features of the metabolic syndrome (visceral obesity, type II diabetes mellitus, and dyslipidemia).<sup>9–11</sup> The second hit is related mainly to increased intrahepatic oxidative stress, which promotes hepatic fibrosis through the production of reactive oxygen species and the activation of hepatic stellate cells.<sup>8</sup> Initially, hepatic iron was believed to be a determinant cofactor in the development of NASH, based on the observation of a close association with hepatic fibrosis and with heterozygosity for the

*Abbreviations:* NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; IRHIO, insulin-resistance-associated iron overload; HOMA-R, homeostasis model assessment parameter of insulin resistance; QUICKI, quantitative insulin sensitivity check index; FBG, fasting blood glucose; OGTT, oral glucose tolerance test; ISI, insulin sensitivity index; SI, sensitivity index; OGIS, oral glucose insulin sensitivity; HIC, hepatic iron concentration; HII hepatic iron index; SQUID, superconducting quantum interference device.

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C282Y mutation of the hemochromatosis gene (HFE).<sup>12,13</sup> However, although an increased prevalence of HFE mutations has been described for patients with NAFLD,<sup>14</sup> subsequent studies failed to confirm a significant increase in their hepatic iron burden and no association was found among hepatic iron, HFE mutations, and the severity of liver damage.<sup>14,15</sup> Conversely, data support the strong relation between liver fibrosis and components of the insulin resistance syndrome.<sup>9, 16–18</sup> Excess hepatic iron may cause insulin resistance and a specific syndrome was described (insulin resistance-associated hepatic iron overload [IRHIO]), characterized by hyperferritinemia with normal or high-normal transferrin saturation.<sup>19</sup> Although patients with IRHIO have a high prevalence of the features of the metabolic syndrome, the relation between IRHIO and NASH is unclear.

The purpose of the current study was to determine the amount of peripheral and hepatic iron burden, the presence and extent of insulin resistance, and the prevalence of HFE mutations in patients with NAFLD. In addition, we assessed the relation among iron overload, insulin resistance, and HFE mutation, as well as the relative contribution of these factors to the severity of hepatic damage.

## Materials and Methods

**Patients.** From January 2001 to October 2002, 263 consecutive newly diagnosed patients with NAFLD were enrolled prospectively in the study. The diagnosis of NAFLD in subjects with chronic hypertransaminasemia (>6 months) was based on the following criteria: exclusion of any other putative cause of chronic liver disease, evidence of bright liver at ultrasound scan, and liver biopsy.

All subjects were tested for the presence of viral hepatitis (anti-hepatitis C virus, hepatitis B surface antigen and antibody), autoimmune disease (primary biliary cirrhosis, autoimmune hepatitis), sclerosing cholangitis, copper storage disease,  $\alpha_1$  antitrypsin deficiency, and drug-induced liver disease. Alcohol intake was assessed by interviews extended to family members and general practitioners. Patients with alcohol consumption greater than 20 g/d were excluded.

Bright liver was diagnosed on the basis of a hyperechoic liver parenchyma and posterior attenuation at ultrasounds. Liver biopsy was proposed to all patients, but it was not mandatory. Written consent was obtained from only 167 patients.

The study was approved by the senior staff committees of the university hospitals involved, as part of a larger observational study on NAFLD. Informed consent was

obtained from all subjects for data collection and investigations and for liver biopsy.

**Laboratory Investigations.** All subjects received a complete clinical, anthropometric, and laboratory investigation at the time of referral. Clinical data included age, sex, height, weight, and body mass index (BMI). BMI was calculated as weight (kg) divided by squared height ( $m^{-2}$ ). Obesity was defined as a BMI of at least  $30\text{ kg m}^{-2}$  and overweight as BMI in the range of 25 to  $29.9\text{ kg m}^{-2}$ . Patients had a routine liver biochemistry, insulin measurement, and iron status assessment (iron, transferrin saturation, and ferritin). Fasting glucose and insulin levels were used to calculate insulin sensitivity according to the homeostasis model assessment parameter of insulin resistance (HOMA-R)<sup>20</sup> and to the quantitative insulin sensitivity check index (QUICKI) and its reciprocal.<sup>21</sup> All patients with NAFLD were divided into three groups according to their fasting blood glucose (FBG) levels<sup>22</sup>: normal fasting glucose (FBG  $<110\text{ mg dL}^{-1}$ ), impaired fasting glucose (FBG  $110\text{--}125\text{ mg dL}^{-1}$ ), and diabetes mellitus (FBG  $>125\text{ mg dL}^{-1}$ ). A 120-minute oral glucose tolerance test (OGTT) with glucose and insulin determinations at 0, 30, 60, 90, and 120 minutes was performed in 206 patients. On the basis of a 120-minute blood glucose level after OGTT, these patients were further classified into normal glucose tolerance ( $<140\text{ mg dL}^{-1}$ ), impaired glucose tolerance ( $140\text{--}199\text{ mg dL}^{-1}$ ), and diabetes mellitus ( $\geq 200\text{ mg dL}^{-1}$ ).<sup>23</sup> Validated glucose-insulin models were used to derive the following OGTT-based insulin sensitivity indices: the insulin sensitivity index (ISI),<sup>24</sup> the sensitivity index (SI),<sup>25</sup> and the oral glucose insulin sensitivity index (OGIS).<sup>26</sup>

We tested 210 patients with NAFLD for the two most frequent mutations of the HFE gene, Cys282Tyr and His63Asp, using a single, multiplex amplification reaction (Nuclear Laser Medicine, Milan, Italy).<sup>27</sup> Data on the prevalence of HFE mutations in the Northeast Italian population were derived from 200 healthy blood donors.

Liver biopsy (167 patients) was performed within 1 month from the clinical and laboratory evaluation and was scored according to Brunt,<sup>1</sup> with minor modifications. Steatosis was present in all biopsies and was graded 1 to 3, according to the percentage of fatty infiltration (1, 0–33; 2, 34–66; 3, 67–100). Necroinflammation was graded 0 to 3 (0, absent; 1, occasional ballooned hepatocytes and no or very mild inflammation; 2, ballooning of the hepatocytes and mild-to-moderate portal inflammation; 3, intra-acinar inflammation and moderate portal inflammation). Fibrosis was graded 0 to 4 (0, absent; 1, perisinusoidal/pericellular fibrosis; 2, periportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis). NASH was diagnosed on the basis of the presence of fibrosis (grade 1 or

higher) or necroinflammation (grade 2 or higher). Staging and grading were performed by the same experienced pathologist who was blind to important risk factors.

A fresh specimen was available for 80 patients and was used for quantitative hepatic iron determinations. All liver tissue samples used for this purpose were of at least 0.5 mg dry weight and were frozen immediately at  $-20^{\circ}\text{C}$  after collecting. Hepatic iron concentration (HIC) was measured by atomic absorption spectrophotometry and expressed as  $\mu\text{g/g}$  dry weight. The hepatic iron index (HII) was calculated by dividing HIC ( $\mu\text{mol/g}$  dry weight) by the age (years) of the patient. The HIC and HII were not determined in the remaining patients because of insufficient available tissue specimens or occasional omissions at the time of liver biopsy.

In 51 subjects, the total hepatic iron content was also assessed by a noninvasive approach based on SQUID (a superconducting quantum interference device) biomagnetometry (model 5700; Tristan Technologies, San Diego, CA) as previously described.<sup>28,29</sup> In 32 patients, both HIC and SQUID determinations were available. SQUID measurements were performed randomly before or after liver biopsy, no longer than 3 months apart. The normal range for SQUID determination was set at 400  $\mu\text{g/g}$  wet weight on the basis of the average hepatic iron content in normal subjects from the same geographic area.

**Statistical Analysis.** All data were implemented on a personal computer and analyzed using StatView 5.0 (SAS Institute Inc., Cary, NC). Unpaired *t* test (two-tailed), the  $\chi^2$  contingency test, the Fisher exact test, and linear regression analysis were used, whenever appropriate. Nonparametric methods were also used for nonnormally distributed values (Kruskal-Wallis and Spearman rank correlation). Logistic regression analysis was used to identify the factors significantly associated with mild (stage 1-2) and severe fibrosis (stage 3-4) in patients with NAFLD, using two separate models. For practical purposes, parameters of insulin sensitivity were turned into parameters of insulin resistance by reciprocal transformation. Factors associated at univariate analysis were tested in multivariate analysis. The odds ratio (OR), the 95% confidence limits, and *P* values were calculated.

## Results

**Demographics and Laboratory Characteristics.** Most patients were males (83%; Table 1). They had a younger age and a lower BMI than females (age:  $39.4 \pm 11.2$  years vs.  $54.6 \pm 9.9$ ,  $P < .0001$ ; BMI:  $27.1 \pm 3.1$   $\text{kg m}^{-2}$  vs.  $28.4 \pm 4.6$ ,  $P = .044$ ). Obesity was diagnosed in 33% of the female patients and in 12% of the male patients. Low levels of high-density lipoprotein cholesterol

**Table 1. Anthropometric, Clinical, and Laboratory Data of Patients With NAFLD**

Characteristics	N = 263	Normal Range
Age (yr)	$42 \pm 12$	
BMI ( $\text{kg/m}^2$ )	$27.8 \pm 3.6$	
Normal weight/overweight/obese (%)	21/58/21	
Alanine aminotransaminases (U/L)	$82.4 \pm 50.1$	<45
Aspartate/alanine aminotransaminases	$0.54 \pm 0.20$	
Transferrin saturation (%)	$32.9 \pm 12.5$	20 to 55
Serum ferritin (ng/mL)	$239 \pm 235$	10 to 350
FBG (mg/dL)	$99.5 \pm 24.7$	70 to 110
120-m glucose (mg/dL)	$129 \pm 44$	<140
NFG/IFG/diabetes (%)	83/8/9	
NGT/IGT/diabetes (%)	68/25/7	
Fasting insulin ( $\mu\text{U/mL}$ )	$15.8 \pm 9.7$	4 to 25
HOMA-R (%)	$3.94 \pm 2.98$	<3.00
1/QUICKI	$3.11 \pm 0.29$	>2.60
SI	$40.3 \pm 12.9$	>40.0
ISI	$3.34 \pm 1.70$	>3.00
OGIS (mL/kg/min)	$8.84 \pm 1.77$	>11
HIC ( $\mu\text{g/g}$ dry weight) (N = 80)	$676 \pm 502$	400 to 1,000
HII (N = 80)	$0.33 \pm 0.27$	<1.1
SQUID HIC ( $\mu\text{g/g}$ wet weight) (N = 51)	$606 \pm 464$	<400

Abbreviations: NFG, normal fasting glucose; IFG, impaired fasting glucose; NGT, normal glucose tolerance; IGT, impaired glucose tolerance. Values expressed are  $M \pm \text{SD}$  or number of patients and prevalence.

were found in 10% and hypertriglyceridemia in 25% of the patients with NAFLD. There were no sex-related differences. The prevalence of impaired fasting glucose and diabetes mellitus was higher among females than among males (26% vs. 6% and 13% vs. 9%, respectively,  $P = .0013$ ). When grouped according to the 120-minute OGTT glucose values, the prevalence of impaired glucose tolerance or postload diabetes mellitus was also higher among females compared with males (56% vs. 27%,  $P = .002$ ; Fisher exact test). For most patients, diabetes mellitus was first diagnosed at the time of the study. Parameters of insulin resistance (HOMA-R, 1/QUICKI) were increased, whereas parameters of insulin sensitivity (SI, ISI, OGIS) were decreased in the majority of patients, independent of sex.

**Histological Findings.** Subjects who received a liver biopsy were younger ( $41 \pm 11$  years vs.  $45 \pm 13$ ;  $P = .008$ ) and had higher alanine aminotransaminase levels ( $92 \pm 56$  U/L vs.  $66 \pm 30$ ;  $P < .0001$ ) compared with subjects who refused liver biopsy. All other variables reported in Table 1 were not significantly different. No differences were observed for anthropometric and laboratory data between patients for whom HIC was/was not determined in liver biopsy specimens.

The amount of liver cells with fatty droplets ranged from 5% to 95% (median, 30%; Table 2). Necroinflammation was absent or mild (grade 1) in 54% of the subjects. Fibrosis was present in 62% of the biopsy

**Table 2. Histologic Data of Patients With NAFLD (N = 167)**

Score	No. of Patients (%)
Steatosis	
1	88 (52.7)
2	47 (28.1)
3	32 (19.2)
Necroinflammation	
0	16 (9.6)
1	74 (44.3)
2	55 (32.9)
3	22 (13.2)
Fibrosis	
0	63 (37.7)
1	38 (22.8)
2	30 (18.0)
3	27 (16.2)
4	9 (5.4)

specimens, with bridging fibrosis in 16% and cirrhosis in 5% of the patients. No patients with a histologic diagnosis of cirrhosis had clinical or laboratory signs of hepatocellular failure and/or portal hypertension.

**Variables Associated With Serum and Liver Iron Burden.** Serum ferritin levels were increased ( $>350$  ng/mL) in 21% of patients, whereas transferrin saturation was elevated only in 7.4% of patients. HIC ranged from 111 to 2458  $\mu\text{g/g}$  dry weight (median, 528) and HII ranged from 0.03 to 1.39  $\mu\text{mol/g/yr}$  (median, 0.26). HIC and HII were correlated significantly with transferrin saturation ( $r = 0.371$ ,  $P = .001$  and  $r = 0.270$ ,  $P = .022$ ), but not with ferritin levels ( $r = 0.050$  and  $r = 0.048$ , respectively). Seven male patients (9%) had an HIC greater than 1,500  $\mu\text{g/g}$  dry weight, but only one exhib-

ited signs of peripheral iron overload and carried an HFE mutation (H63D heterozygosity).

Sex distribution was not different according to quartiles of HIC. BMI was nonsignificantly lower in patients with increased HIC (Table 3). Of relevance, 35% of patients in the upper quartile had a normal BMI, compared with 20% of patients in the lower quartile. Higher HIC was associated with increased transferrin saturation but not with increased ferritin levels. FBG levels decreased with increasing quartiles, whereas basal insulin and insulin resistance indices were not different. A modest increase in OGIS was observed with increasing HIC. At liver biopsy, steatosis decreased without differences in necroinflammation and fibrosis.

These results were confirmed by SQUID biomagnetometry. SQUID determinations ranged from 59 to 2086  $\mu\text{g/g}$  wet weight (median, 443). HIC and SQUID values showed a linear correlation ( $r = 0.675$ ,  $P = .0021$ ). Transferrin saturation, but not ferritin levels, was correlated with SQUID ( $r = 0.333$ ,  $P = .022$  and  $r = 0.252$ ,  $P = .074$ , respectively). When data were grouped according to SQUID quartiles, values ranged from 0 to 278  $\mu\text{g/g}$  wet weight (first quartile;  $n = 13$ ), from 278 to 443 (second quartile;  $n = 12$ ), from 443 to 809 (third quartile;  $n = 13$ ), and greater than 809 (fourth quartile;  $n = 13$ ). No differences in age, sex distribution, and BMI were observed between the first and the fourth SQUID quartiles. The amount of fat in liver biopsy specimens tended to be lower in the subgroup with the highest SQUID value ( $29.4 \pm 20.8\%$  vs.  $36.2 \pm 25.2$  in the fourth and

**Table 3. Clinical and Laboratory Characteristics of Patients With NAFLD in Relation to HIC**

Characteristics	First Quartile (n = 20)	Second Quartile (n = 20)	Third Quartile (n = 20)	Fourth Quartile (n = 20)	P Value*
Age (yr)	44 $\pm$ 12	39 $\pm$ 14	40 $\pm$ 10	40 $\pm$ 9	.394
BMI ( $\text{kg/m}^2$ )	27.4 $\pm$ 3.3	28.1 $\pm$ 3.7	26.4 $\pm$ 2.5	25.9 $\pm$ 2.9	.083
Alanine aminotransaminases (U/L)	86.2 $\pm$ 52.6	98.9 $\pm$ 45.4	84.0 $\pm$ 34.9	65.3 $\pm$ 28.6	.111
Aspartate/alanine aminotransaminases	0.59 $\pm$ 0.32	0.53 $\pm$ 0.25	0.48 $\pm$ 0.18	0.53 $\pm$ 0.19	.342
Transferrin saturation (%)	28.3 $\pm$ 9.1	34.0 $\pm$ 11.1	33.0 $\pm$ 9.2	39.3 $\pm$ 16.1	.016
Serum ferritin (ng/mL) median (range)	144 (12-1,037)	156 (41-1,311)	181 (49-935)	181 (89-1,274)	.252
FBG (mg/dL)	112 $\pm$ 32	93 $\pm$ 17	92 $\pm$ 11	94 $\pm$ 14	.023
Fasting insulin ( $\mu\text{U/mL}$ )	14.6 $\pm$ 8.7	17.4 $\pm$ 9.9	11.2 $\pm$ 7.3	14.2 $\pm$ 6.1	.562
HOMA-R (%)	3.96 $\pm$ 2.49	3.88 $\pm$ 2.36	2.51 $\pm$ 1.59	3.35 $\pm$ 1.56	.327
1/QUICKI	3.11 $\pm$ 0.31	3.12 $\pm$ 0.27	2.93 $\pm$ 0.27	3.07 $\pm$ 0.25	.327
SI	40.3 $\pm$ 16.0	39.6 $\pm$ 11.7	42.9 $\pm$ 12.1	42.7 $\pm$ 14.1	.416
ISI	3.54 $\pm$ 2.33	2.90 $\pm$ 1.32	4.23 $\pm$ 1.30	3.92 $\pm$ 2.30	.189
OGIS ( $\text{mL/kg/min}$ )	8.47 $\pm$ 1.64	8.46 $\pm$ 1.75	9.40 $\pm$ 1.61	9.63 $\pm$ 1.52	.047
Steatosis degree (%)	42 $\pm$ 25	31 $\pm$ 22	28 $\pm$ 19	20 $\pm$ 16	.004
Inflammation score	1.9 $\pm$ 0.85	1.74 $\pm$ 0.81	1.90 $\pm$ 0.97	1.50 $\pm$ 0.51	.156
Fibrosis score	1.90 $\pm$ 1.33	1.47 $\pm$ 1.61	1.05 $\pm$ 1.23	1.40 $\pm$ 1.23	.166

NOTE. HIC was divided into quartiles, according to the following ranges: first, 0 to 325  $\mu\text{g/g}$  dry weight; second, 325 to 528; third, 528 to 855; fourth,  $>855$ . Values expressed are M  $\pm$  SD or median (range).

\*Spearman rank correlation.

**Table 4. Anthropometric, Clinical, and Laboratory Data of Patients With NAFLD According to HFE Genotype**

Characteristics	No Mutation (n = 150)	H63D (n = 50)	C282Y (n = 10)
Age (yr)	42 ± 13	41 ± 11	48 ± 10
Body mass index (kg/m <sup>2</sup> )	27.7 ± 3.6	27.9 ± 3.8	26.6 ± 5.2
Alanine aminotransaminases (U/L)	80.9 ± 48.3	82.2 ± 49.4	92.9 ± 90.2
Aspartate/alanine aminotransaminases	0.55 ± 0.21	0.55 ± 0.20	0.63 ± 0.20
γ-glutamyl transferase (U/L)	76.5 ± 99.8	98.7 ± 98.9	178.7 ± 190.7*
Transferrin saturation (%)	32.2 ± 11.7	35.8 ± 13.8	40.1 ± 16.7*
Serum ferritin (ng/mL)	153 (12-1,581)	175 (8-1,274)	190 (97-935)*
HIC (μg/g dry weight)	495 (111-2,458)	556 (141-1,898)	795 (761-1,136)
SQUID (μg/g wet weight)	378 (82-1,871)	667 (144-2,086)	430 (230-949)
FBG (mg/dL)	98.1 ± 23.8	99.7 ± 17.9	108.6 ± 20.4
Fasting insulin (μU/mL)	16.7 ± 11.6	16.0 ± 9.3	19.3 ± 14.2
HOMA-R (%)	3.97 ± 3.14	3.91 ± 2.46	5.87 ± 4.07
1/QUICKI	3.12 ± 0.29	3.11 ± 0.30	3.25 ± 0.38
SI (N = 206)	40.8 ± 12.7	38.3 ± 14.3	31.2 ± 14.6
ISI (N = 206)	3.28 ± 1.64	3.18 ± 1.77	3.02 ± 2.09
OGIS (mL/kg/min) (N = 206)	8.89 ± 1.85	8.79 ± 1.92	8.07 ± 2.28
Steatosis score (1/2/3%) (N = 167)	53/25/22	50/36/14	67/17/17
Inflammation score (0/1/2/3%) (N = 167)	10/43/33/14	6/44/36/14	17/33/33/17
Fibrosis score (0/1/2/3/4%) (N = 167)	36/25/16/18/5	36/17/25/17/6	33/17/33/0/17

\*Unpaired *t* test,  $\chi^2$ , or Fisher exact test: *P* < .05. Values expressed are M ± SD or median (range).

first quartiles, respectively, *P* = .563), whereas there was no difference in the degree of inflammation or fibrosis.

When the HIC and SQUID data were pooled (*n* = 99) and expressed in quartiles, it was further confirmed that transferrin saturation was significantly higher, whereas glucose levels, the amount of fat infiltration, and the degree of inflammation were significantly lower in the subgroup with the highest HIC (data not shown).

**Variables Associated With HFE (C282Y and H63D) Mutations.** The prevalence of the C282Y and H63D mutations in patients with NAFLD was not increased significantly when compared with 200 blood donors with the same ethnic background. No C282Y homozygosity was found both in patients with NAFLD and in controls. C282Y/H63D compound heterozygosity was present in 1.2% of patients with NAFLD and in 1.0% of controls. H63D mutations (96% heterozygosity and 4% homozygosity) were present in 24% of patients and C282Y heterozygosity was present in 4.8% of patients. Similar values were found in the control population, where the prevalence of H63D heterozygosity was 19% and C282Y heterozygosity was 2.8% (*P* = ns). The overall presence of an HFE mutation was not different in relation to sex (34% in females and 30% in males).

Values of patients carrying the H63D mutation (both homozygotes and heterozygotes grouped together) and the C282Y mutation (heterozygosity only) were compared with patients with NAFLD without mutations. C282Y/H63D compound heterozygosity was excluded (Table 4).

Only the presence of the C282Y mutation was associated with a significant increase in serum iron and trans-

ferrin saturation and, to a lesser extent, with higher ferritin levels. However, liver iron concentration, measured by either HIC or SQUID, was not significantly increased in this subset of patients. Among liver function tests, only γ-glutamyl transferase levels were higher in patients with the C282Y, but not the H63D, mutation. Liver histology was not different in relation to HFE genotype.

**Variables Associated With the Severity of Fibrosis.** Using univariate analysis, the presence of mild (stage 1-2) fibrosis was independently associated with increased BMI and serum ferritin levels, whereas severe (stage 3-4) fibrosis was associated with older age, female sex, increased BMI, increased aspartate/alanine aminotransferase ratio, higher serum ferritin levels, increased fasting glucose and insulin levels, and increased insulin resistance and decreased insulin sensitivity (as expressed by HOMA-R and OGIS, respectively; Table 5). HIC and the prevalence of HFE mutations were not related to the severity of fibrosis. Among the histologic features, severe fibrosis was associated with the degree of necroinflammation and, less significantly, with the score of steatosis.

Using multivariate analysis, after adjustment for age, sex, and BMI, only the ferritin level was independently associated with mild fibrosis, whereas increased ferritin levels and OGIS were associated with severe fibrosis (Table 6, Fig. 1). Similar associations were observed when OGIS was replaced by ISI or SI (not reported in details). Multivariate analysis confirmed the association between severe fibrosis and both serum ferritin levels (OR = 1.77; CI = 1.21-2.60) and OGIS (OR = 1.56; CI = 1.11-

**Table 5. Factors Associated With Mild (stage 1–2) and Severe (stage 3–4) Liver Fibrosis in Patients With NAFLD (N = 167) at Univariate Analysis**

Characteristics	Mild Fibrosis (Stage 1–2)		Severe Fibrosis (Stage 3–4)	
	Odds Ratio (95% CI)	P Value	Odds Ratio (95% CI)	P Value
Female sex	1.97 (0.56–6.88)	.290	4.21 (1.17–15.19)	.028
Age (yr/10)	1.04 (0.74–1.48)	.811	2.17 (1.43–3.31)	.0003
BMI (kg/m <sup>2</sup> /5)	2.01 (1.15–3.52)	.014	2.12 (1.06–4.27)	.035
Alanine aminotransferase (mU per mL/10)	0.95 (0.89–1.03)	.220	1.05 (0.98–1.12)	.149
Aspartate/alanine aminotransferase	1.60 (0.19–13.17)	.662	27.4 (2.3–329.8)	.009
FBG (mg/dL/10)	0.90 (0.73–1.12)	.361	1.27 (1.03–1.57)	.029
Serum insulin ( $\mu$ U/mL/5)	1.15 (0.93–1.43)	.207	1.35 (1.06–1.73)	.014
HOMA-R (%)	1.10 (0.91–1.32)	.319	1.36 (1.11–1.67)	.003
1/QUICKI	3.23 (0.82–13.41)	.092	35.92 (5.41–238.76)	.0002
100/OGIS (mL/kg/min)	1.27 (1.02–1.58)	.036	1.62 (1.26–2.08)	.0002
100/ISI	2.37 (1.23–4.54)	.010	2.90 (1.45–5.80)	.0025
10/SI	1.41 (1.06–1.86)	.018	1.77 (1.30–2.40)	.0003
Transferrin saturation (%)	0.99 (0.95–1.02)	.391	1.02 (0.98–1.05)	.346
Serum ferritin (ng/mL/100)	1.32 (1.06–1.67)	.017	1.49 (1.18–1.88)	.001
Liver iron (HIC- $\mu$ g/g dry weight) (N = 80)	1.03 (0.97–1.09)	.355	0.96 (0.89–1.03)	.261
Iron SQUID ( $\mu$ g/g wet weight) (N = 51)	1.02 (0.88–1.14)	.977	1.07 (0.95–1.20)	.257
Steatosis score	1.11 (0.71–1.76)	.644	1.66 (0.99–2.80)	.0556
Inflammation score	1.07 (0.70–1.64)	.742	2.15 (1.28–3.61)	.0037

NOTE. Data were tested using two separate models, using fibrosis score 0 for comparison.

2.20) after additional adjustment for the degree of steatosis.

OGIS was inversely correlated with the degree of steatosis ( $r_s = -0.297$ ;  $P = .0025$ ) and fibrosis ( $r_s = -0.390$ ;  $P < .001$ ), but not with the necroinflammatory score ( $r_s = -0.030$ ;  $P = .762$ ). Likewise, serum ferritin levels and the degree of both steatosis ( $r_s = 0.309$ ) and fibrosis ( $r_s = 0.311$ ;  $P < .0001$  for both), but not of inflammation ( $r_s = -0.041$ ;  $P = .601$ ), were significantly correlated. At linear regression analysis, increased ferritin levels were correlated with decreased insulin sensitivity, as assessed by OGIS ( $r^2 = 0.035$ ;  $P = .020$ ).

No significant association was found among hepatic iron stores, prevalence of HFE mutation, and degree of fibrosis.

## Discussion

The aim of the current study was to dissect the contribution of iron and insulin resistance by measuring several parameters of iron burden and insulin sensitivity in a large

NAFLD series with a low prevalence of metabolic abnormalities, possibly obscuring the results (obesity, diabetes mellitus). This is particularly relevant considering that iron overload has been observed more frequently among male, nonobese, nondiabetic patients with NAFLD. The data indicate that iron burden and HFE mutations are not relevant risk factors for fibrosis in patients with NAFLD. In contrast, insulin resistance plays a major role not only as a pathogenic factor for the onset of disease, but also in determining the severity of liver involvement. In these patients, increased ferritin levels are markers of the severity of liver fibrosis, more likely expression of their metabolic state than of peripheral or hepatic iron overload.

The role of intrahepatic iron deposition as a source of oxidative stress in patients with NAFLD initially attracted much interest,<sup>12–15</sup> but conclusions were conflicting, in part due to the different techniques used to determine liver iron concentration. When hepatic iron stores were assessed by both HIC and Perls' stain, the results were poorly correlated<sup>13,15</sup> and the strength of association between fibrosis and hepatic iron store was stronger for Perls' stain.<sup>14</sup> As the determination of HIC provides a measure of iron in dry liver tissue specimens, it can be influenced by the fat content of hepatocytes and by the way samples are preserved. A post-hoc analysis of our data shows a fairly good correlation between HIC and Perls' stain ( $r_s = 0.330$ ;  $P = .0043$ ), which was not significantly influenced by the degree of steatosis (test for negative interaction;  $P = .853$ ).

**Table 6. Independent Predictors of Liver Fibrosis in Patients With NAFLD at Multivariate Logistic Regression Analysis**

Characteristics	Odds Ratio (95% CI)	P Value
Mild fibrosis (grade 1–2)		
Serum ferritin (ng/mL/100)	1.52 (1.08–2.13)	.016
Severe fibrosis (grade 3–4)		
Serum ferritin (ng/mL/100)	1.69 (1.18–2.43)	.0045
100/OGIS (mL/kg/min)	1.56 (1.12–2.18)	.009

NOTE. Data in multivariate analysis were corrected for age, sex, and BMI.

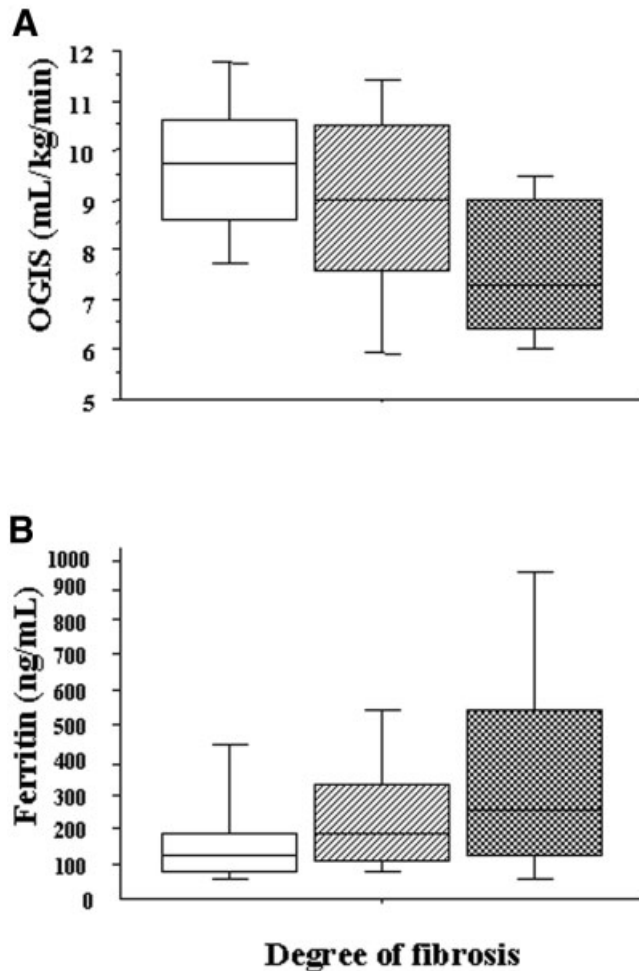


Fig. 1. Distribution of oral glucose insulin sensitivity (OGIS) (A) and serum ferritin levels (B) according to the degree of liver fibrosis. The values within the box range from the 25th to the 75th percentiles. The median is shown by the horizontal bar. The vertical bars represent the values between the 10th and the 90th percentiles. (Open bars), absent; (lightly shaded bars), stage 1 to 2; (heavily shaded bars), stage 3 to 4.

Biomagnetic liver susceptometry (SQUID) is a noninvasive method to determine *in vivo* the iron content of the liver. It has been validated extensively in patients with iron storage diseases or hematologic disorders.<sup>28,29</sup> Theoretically, the sensitivity of SQUID should not be affected by the fat content of hepatocytes, but determinations might be biased by the subcutaneous fat of the abdominal wall. This limitation does not apply to our study because most patients were lean. SQUID measurements showed a good correlation with transferrin saturation and HIC determination. When analyzed separately, both SQUID and HIC demonstrated that iron content was not systematically increased and no relation was present between liver iron levels and fibrosis. This conclusion supports the finding that the iron burden of our patients was largely below the fibrogenic threshold.<sup>30</sup> It is noteworthy that the higher the hepatic iron accumulation, the lower the fat

content and BMI when measured either by SQUID or by HIC. Previous studies also reported that patients with increased steatosis and/or type II diabetes mellitus had a lower hepatic iron burden.<sup>13,15</sup> Provided that the amount of liver fat does not interfere with the determination of iron content, the relative contribution of iron to NAFLD and its proinflammatory role might be higher in lean patients, in whom the lower amount of steatosis may not be sufficient to trigger the inflammatory response.

The prevalence of the two major mutations of the HFE gene of hemochromatosis (C282Y and H63D) was not significantly increased in our patients and matched those of the general population.<sup>31</sup> In addition, mutations were not associated with an increased hepatic iron burden or with more severe fibrosis. Only the presence of the C282Y mutation in its homozygous form is associated with increased serum transferrin saturation.<sup>32</sup> Most current data suggest that an increased level of liver iron simply acts as a cofactor in the progression from fatty liver to NASH only in geographic areas with a large prevalence of mutations of the HFE gene of hemochromatosis. However, a recent study,<sup>14</sup> although confirming the increased prevalence of C282Y heterozygosity among Australian patients with NAFLD compared with ethnically matched blood donors, failed to demonstrate increased iron stores and any association among iron, HFE mutation, and liver fibrosis.

It is noteworthy that an increased level of serum ferritin, although not correlated with transferrin saturation and hepatic iron content, was one of the most relevant independent predictors of severe fibrosis in our patients with NAFLD. Increased ferritin levels were previously reported to identify patients at risk for NASH among patients with normal transferrin saturation.<sup>33</sup> Hyperferritinemia with normal to mildly increased transferrin saturation may occur in IRHIO, which can represent up to 70% of non-HFE-related nonalcoholic hepatic iron overloads.<sup>19</sup> Patients with IRHIO are predominantly male and middle-aged, and most share one or more features of the metabolic syndrome and have histologic evidence of NAFLD. Conversely, increased ferritin levels have been observed in several features of the metabolic syndrome, like type II diabetes mellitus, where it is associated with increased steatosis and inflammation,<sup>34</sup> and in male patients with hypertension.<sup>35</sup> In epidemiologic studies, serum ferritin concentration was the second strongest determinant of blood glucose in regression models and the third strongest determinant of serum insulin. Its concentration also correlated positively with plasma triglycerides, high-density lipoprotein, and OGTT-derived index of insulin resistance, suggesting that serum ferritin could be a marker of the insulin resistance syndrome.<sup>36</sup> Transferrin receptors, glucose transporters, and insulin-

like growth factor II receptors colocalize in microsomal membranes in cultured adipocytes. Insulin causes the simultaneous translocation of all three proteins to the cell membrane.<sup>37</sup> However, substantial iron overload is not a typical feature of type II diabetes mellitus. Whether high ferritin levels in the metabolic syndrome are an expression of true iron overload is a matter of debate.

The metabolic syndrome is highly prevalent among patients with NAFLD, and particularly among patients with NASH,<sup>9</sup> and the metabolic disease might be amplified by iron accumulation. In our patients, intrahepatic or peripheral iron burden was not related to insulin resistance parameters. Possibly, increased ferritin levels in NAFLD are simply an expression of a metabolic derangement and/or of hepatic damage because widespread activation of inflammatory cytokines would increase transcription of ferritin messenger RNA in macrophages that may subsequently transfer ferritin to hepatocytes.<sup>38</sup> It is likely that the metabolic derangement of our patients with NAFLD has a larger effect on ferritin levels than hepatic iron stores, mostly in the normal range.

All parameters of insulin resistance were significantly related to the severity of liver damage, but OGIS gave the strongest independent association. An index derived from a single basal glucose and insulin determination, like HOMA and QUICKI, may be biased by a high variability due to the relative weight of small changes in fasting insulin determination. This problem is overcome in OGTT-derived formulas, which also include several post-load insulin measurements. OGIS is based on a well-validated physiologic glucose-insulin model and its performance was superior to other OGTT-based indices.<sup>26</sup>

The current study strengthens the role of insulin resistance as the main determinant for the severity of the disease, but does not answer the question whether this is directly related to insulin resistance or indirectly through steatosis. Insulin itself may have profibrogenic properties. For example, incubation of hepatic stellate cells with glucose or insulin leads to overexpression of connective tissue growth factor,<sup>39</sup> which is involved in liver fibrosis both in human chronic liver disease and in experimental models. Subclinical inflammation has been suggested to be part of the metabolic syndrome.<sup>40</sup> A direct link among insulin, inflammation, and oxidative stress<sup>41</sup> has been suggested by the observation that chronic activation of IKK- $\beta$  in ob/ob mice, triggered by cytokines involved in oxidant and inflammatory stresses, is associated with insulin resistance. In the current study, the strong association between reduced insulin sensitivity and severe fibrosis, independent of the degree of hepatic steatosis, would suggest a primary role of insulin resistance in the severity of the

disease. The possible bias of a "burn out" fat content due to excessive fibrosis is ruled out by the small number of patients with histologic (not clinical) cirrhosis.

In conclusion, risk factors for NASH in patients with NAFLD can be the same as those directly involved in the onset of fatty liver, with insulin resistance acting both as the first and second hits. The different outcome of the disease might be related to the relative impact of metabolic derangements, environmental conditions, and host factors, like the genetic and hormonal milieu, as in other conditions associated with insulin-resistance, such as diabetes mellitus, hypertension, and cardiovascular disease.

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