

# Lack of evidence for the pathogenic role of iron and *HFE* gene mutations in Brazilian patients with nonalcoholic steatohepatitis

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## Abstract

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The hypothesis of the role of iron overload associated with *HFE* gene mutations in the pathogenesis of nonalcoholic steatohepatitis (NASH) has been raised in recent years. In the present study, biochemical and histopathological evidence of iron overload and *HFE* mutations was investigated in NASH patients. Thirty-two NASH patients, 19 females (59%), average 49.2 years, 72% Caucasians, 12% Mulattoes and 12% Asians, were submitted to serum aminotransferase and iron profile determinations. Liver biopsies were analyzed for necroinflammatory activity, architectural damage and iron deposition. In 31 of the patients, C282Y and H63D mutations were tested by PCR-RFLP. Alanine aminotransferase levels were increased in 30 patients,  $2.42 \pm 1.12$  times the upper normal limit on average. Serum iron concentration, transferrin saturation and ferritin averages were  $99.4 \pm 31.3$  g/dl,  $33.1 \pm 12.7\%$  and  $219.8 \pm 163.8$  µg/dl, respectively, corresponding to normal values in 93.5, 68.7 and 78.1% of the patients. Hepatic siderosis was observed in three patients and was not associated with architectural damage ( $P = 0.53$ ) or with necroinflammatory activity ( $P = 0.27$ ). The allelic frequencies ( $N = 31$ ) found were 1.6 and 14.1% for C282Y and H63D, respectively, which were compatible with those described for the local population. In conclusion, no evidence of an association of hepatic iron overload and *HFE* mutations with NASH was found. Brazilian NASH patients comprise a heterogeneous group with many associated conditions such as hyperinsulinism, environmental hepatotoxin exposure and drugs, but not hepatic iron overload, and their disease susceptibility could be related to genetic and environmental features other than *HFE* mutations.

### Key words

- Fatty liver
- Nonalcoholic steatohepatitis
- Iron overload
- *HFE* gene
- Alanine aminotransferase

## Introduction

Nonalcoholic steatohepatitis (NASH) was designated as such by Ludwig et al. in 1980 (1), and has been increasingly recognized as one of the most frequent diagnoses by hepatologists (2). Early studies (1-6) described NASH mainly in obese, middle-aged women, often associated with diabetes mellitus and hyperlipidemia. However, in recent years, other investigators (7-10) have found evidence of NASH association with male, nonobese, nondiabetic patients and with liver iron overload, which led to the hypothesis of iron playing a role in NASH pathogenesis.

The identification of the hereditary hemochromatosis *HFE* gene (11) on chromosome 6 and its 845 G→A (C282Y) and 187 C→G (H63D) mutations has enabled further investigation of this aspect based on the knowledge that compound heterozygotes (C282Y/H63D) and H63D homozygotes may have a mild or moderate iron overload (12). However, more recently, Gochee et al. (13) demonstrated that the presence of H63D does not result in significant iron overload, and that this mutation is not clinically significant in the absence of the C282Y mutation.

George et al. (14) found that the C282Y mutation of the *HFE* gene was significantly associated with the degree of iron staining, hepatic iron concentration and liver damage in Caucasian NASH patients from Australia. Bonkovsky et al. (15,16) expanded this concept by identifying both C282Y and H63D which were significantly related to NASH in North Americans. In view of these data, the aim of the present study was to investigate the evidence of iron overload and the prevalence of C282Y and H63D mutations among NASH patients from São Paulo, SP, Brazil.

## Patients and Methods

### Cohort of patients

Thirty-two patients who fulfilled the diag-

nostic criteria of NASH were enrolled in this study. NASH was defined histopathologically as macrovesicular steatosis, lobular inflammation and perivenular fibrosis morphologically indistinguishable from alcoholic disease in subjects whose alcohol consumption was less than 20 g/day. This study was approved by the local Ethics Committee and was performed according to the guidelines of the Helsinki declaration. Informed consent was obtained from each patient included in the study.

The initial database consisted of 112 patients suspected of having NASH who had been attended at the Gastroenterology Outpatient Clinic in the last 18 months. Suspicion of NASH was based on findings of a steatotic aspect of the liver by ultrasonography, serum aminotransferase elevation and negative serology for hepatitis B and C virus (HBsAg and anti-HCV antibody by the ELISA 3 test) in patients who denied alcohol consumption at hospital admission. All patients were evaluated by the same physician (M.M. Deguti) and received oral and written information about this protocol, with emphasis on the importance of providing reliable information. Patients with obesity (body mass index  $\geq 30$  kg/m<sup>2</sup>), diabetes mellitus type 2 or hyperlipidemia (cholesterolemia or triglyceridemia  $\geq 200$  mg/dl after a 12-h fast) were strongly urged to follow appropriate diets and physical exercise programs, and those who were using potentially hepatotoxic drugs were asked to discontinue them. The values of serum transaminases of 24 patients returned to normal levels within six months after taking these measures and these patients were not submitted to liver biopsies. Twenty-three subjects were excluded because they had not stated their actual alcohol consumption initially. Nine patients refused a liver biopsy and 24 had histopathological diagnoses other than steatohepatitis (non-specific changes with focal liver cell regeneration, steatosis, and cirrhosis without an etiological marker). The remaining 32 patients participated in the study.

## Methods

Serum alanine aminotransferase, iron, transferrin saturation and ferritin levels were obtained at the time of liver biopsy.

Liver biopsy specimens were fixed in buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin, Masson's trichrome, reticulin and Perls' Prussian blue. Each biopsy was read blindly by one experienced pathologist (L.C.C. Gayotto). NASH was graded 1 to 4 according to steatosis intensity (the percentage of hepatocytes with fat vacuoles), necro-inflammatory injury and architectural damage. Architectural damage grade 4 was assigned to cirrhosis. The presence of iron pigments was assessed in Kupffer cells, lobular hepatocytes and periportal regions, and graded from 0 to 4 on a semiquantitative basis for each deposition site as follows: 0, absence of iron; 1, presence of iron at minimum intensity; 2 and 3, presence of iron at intermediate intensity, and 4, presence of iron at maximum intensity.

All patients but one were tested for both C282Y and H63D mutations. Genomic DNA was extracted from peripheral blood leukocytes by standard techniques, and mutation analyses were carried out as described (17). DNA amplification was performed by the polymerase chain reaction with the primers 5'-CTC.AGG.CAC.TCC.TCT.CAA.CC-3' and 5'-TGG.CAA.GGG.TAA.ACA.GAT.CC-3' for the G845A locus codon 282 and 5'-GCC.ACA.TCT.GGC.TTG.AAA.TT-3' and 5'-ACA.TGG.TTA.AGG.CCT.GTT.GC-3' for the C187G locus codon 63, which was followed by restriction fragment length polymorphism analysis with the *Sna*BI and *Bcl*II enzymes, respectively.

## Statistical analysis

Statistical analysis was performed using the unpaired Student *t*-test or the Fisher exact test when appropriate. Continuous vari-

ables are reported as means  $\pm$  SD and categorical variables as frequency or percentage.

## Results

As shown in Table 1, in this cohort of 32 patients, age ranged from 32 to 76 years, average 49.2 years. Ethnic origin was as follows: 72% Caucasians, 12% Mulattoes and 12% Asians. Fifty-nine percent of patients were females, 50% obese, 53% hyperlipidemic and 31% diabetic type 2. Other clinical features were the continuous use of amiodarone and the daily inhalation of industrial substances for at least six months.

Serum alanine aminotransferase was increased in 30 patients (93.5%), and the highest value was 4.8 times the upper normal limit (average  $2.42 \pm 1.12$ ). Serum iron concentration was normal (50-150  $\mu$ g/dl) in 93.5%, average  $99.4 \pm 31.3$  g/dl. Transferrin iron saturation was within the normal range (20-40%) in 68.7%, average  $33.1 \pm 12.7\%$ . Ferritin level was normal (females: 25-250  $\mu$ g/dl; males: 50-500  $\mu$ g/dl) in 78.1% of women, average  $219.8 \pm 163.8$   $\mu$ g/dl and in 76.9% of men, average  $444.8 \pm 305.9$   $\mu$ g/dl.

Table 1. Clinical and demographic characteristics of the 32 patients in the present study.

Variable	Characteristics
Age	49.2 $\pm$ 17.2
Females	19 (59%)
Ethnic origin	
Caucasian	23 (72%)
Mulatto	4 (12%)
Asian	4 (12%)
Others	1 (3%)
Clinical features	
Obesity	16 (50%)
Hyperlipidemia	17 (53%)
Diabetes mellitus type 2	10 (31%)
Nonobese, nonhyperlipidemic and nondiabetic	7 (22%)
Amiodarone use	2 (6%)
Industrial substance inhalation	3 (9%)

Data are reported as number of patients with percent in parentheses, except for age which is reported as mean  $\pm$  SD in years.

The histopathological study revealed steatosis grade 2 in 5 cases (15.6%), grade 3 in 20 (62.5%) and grade 4 in 7 (21.9%). Necroinflammatory injury was grade 1 in 13 cases (40.6%), grade 2 in 5 (6.3%), grade 3 in 12 (37.5%), and grade 4 in 2 (15.6%). Ten patients (31.2%) had architectural damage grades 1, 2 and 3; the remaining two patients were already cirrhotic, with grade 4 damage (6.3%).

Liver biopsies submitted to Perls' staining revealed siderosis in only three patients, all of whom were males. The first one, an obese and hyperlipidemic Caucasian, had siderosis grade 2 in both lobular hepatocytes and Kupffer cells. The second patient was a diabetic Asian and presented siderosis grade 1 in lobular hepatocytes and grade 3 in Kupffer cells. The third one, a Caucasian without any associated condition, presented grade 1 siderosis homogeneously distributed in the periportal region, lobular hepatocytes and

Kupffer cells.

Of the 31 patients whose DNA was obtained, one was C282Y +/-, seven H63D +/- and one H63D +/+. There were no C282Y ++ or compound C282Y/H63D heterozygotes. The allelic frequencies found in this cohort of patients for C282Y and H63D were 1.6 and 14.1%, respectively. Among the three patients with liver siderosis, only one (an obese and hyperlipidemic Caucasian) had an *HFE* mutation, H63D +/- . The patient who had the H63D +/+ mutation was an obese, hyperlipidemic female with hyperferritinemia, but with no hepatic iron deposition.

The biochemical, histopathological and genetic profile of NASH patients was compared according to the presence or absence of siderosis in liver tissue (Table 2). Patients with hepatic iron overload had significantly higher levels of serum ferritin ( $P < 0.05$ ); however, there was no statistically significant correlation of hepatic iron overload with necroinflammatory injury or with *HFE* mutation frequencies.

Table 2. Biochemical, histopathological and *HFE* mutational results of patients with and without hepatic siderosis.

	With hepatic siderosis (N = 3)	Without hepatic siderosis (N = 29)
Female:male	0:3	19:10
Biochemical profile		
Alanine aminotransferase (xNL)	2.06 ± 0.29	2.46 ± 1.17
Iron (µg/dl)	134.33 ± 43.19	95.83 ± 28.41
Transferrin saturation (%)	44.33 ± 11.01	33.1 ± 12.7
Ferritin (male) (µg/dl)	764.33 ± 487.64	348.9 ± 166.36*
Ferritin (female) (µg/dl)	-	219.8 ± 163.8
Histopathological profile		
Necroinflammatory activity		
mild (grades 1 and 2)	3	17
severe (grades 3 and 4)	0	12
Architectural damage		
mild (grades 1 and 2)	3	19
severe (grades 3 and 4)	0	10
<i>HFE</i> mutations (m/T)		
C282Y +/-	0/3	1/28
H63D +/-	1/3	6/28
H63D +/+	0/3	1/28

m: carriers of the mutation; T: total number of subjects studied; xNL: number of times the upper limit of the normal range. Biochemical data are reported as means ± SD.

\* $P = 0.01$  compared to patients with hepatic siderosis (Fisher exact test).

## Discussion

In our cohort of 32 patients there was no evidence of the association between NASH and hepatic iron overload suggested in recent publications (7,14,15,18). Siderin pigment in liver tissue was not significant in frequency or intensity by Perls' staining. The *HFE* gene mutation frequencies were compatible with the characteristics of the local general population (19).

Hyperferritinemia was found in less than a quarter of our patients, in contrast to the majority of the patients reported by the French group from Rennes (18,20,21). According to the cited authors, the phenomena of hyperferritinemia, normal transferrin saturation and hepatic siderosis were predominant in patients with NASH and insulin resistance syndrome. Insulin resistance syndrome was defined by the presence of one or more of the following features: obesity, hyperlipidemia

and abnormal glucose metabolism. In the present study, however, only 9 (36%) of the 25 patients with insulin resistance syndrome had hyperferritinemia but no hepatic iron overload. In contrast, three patients with hepatic iron overload had significantly higher ferritin levels and two of them had insulin resistance syndrome. This divergence could be partially attributed to the difference between the databases: the patients reported by the cited investigators were selected among subjects with hepatic iron overload, while ours were from the population routinely attended at a Gastroenterology Outpatient Clinic. Since hepatic steatosis, insulin resistance syndrome and iron overload can occur independently or in combination, the hypothesis that postulates insulin resistance as the pivotal abnormality leading to iron overload and subsequently to steatohepatitis (20) is acceptable for the subgroup affected by all of these conditions. This narrows down our population to only two subjects.

The predominant profile of our NASH patients agrees with that reported by Younossi et al. (22) for patients from Cleveland, OH, USA. These authors did not observe significant iron accumulation in cases with nonalcoholic fatty liver, including those with NASH. They carefully excluded alcoholism, by defining 20 g as the upper limit of acceptable daily ethanol consumption by reviewing the charts and following up patients when necessary. Our criteria were similarly strict about this aspect, a fact that could explain the scarcity of iron deposition also in our cohort, since alcohol induces secondary iron overload.

The absence of Africans and the same proportion of Mulattoes and Asians (12%) with NASH was conspicuously different from the ethnic distribution of the local population, described as 6.7, 28.4 and 0.5%, respectively, according to Brazilian government statistics in 1999 and referring to the southeast region of the country (<http://www1.ibge.gov.br/english/estatistica/>

[populacao/condicao/indicadores/minimos/tabela1.shtm#a112](#)). The interpretation of the ethnic profile of Brazilians is more difficult because of the intense pluriracial mixing which has been taking place in the last five centuries, involving natives, Africans, Caucasians and others (23). To our knowledge, patients of African origin having NASH have not been reported in the literature, and the lower proportion of Mulattoes suggests that an ethnically related protective effect might need to be investigated. The higher proportion of Asians in our NASH population could be attributed to the alcohol intolerance genetically determined among them (24), facilitating the determination of alcohol consumption. All Asians were of Japanese origin and all of them had glucose intolerance. Interestingly, it is known that western acculturation increases the frequency of diabetes among the Japanese (25). Since NASH patients in Japan are not excessively diabetic (26), an eventual major susceptibility to developing liver alterations secondary to a glucose intolerance condition might be further studied in this group.

Three patients, two of whom were hypercholesterolemic, had been continuously exposed to fumes while working in a steel factory. They were neither obese nor diabetic, nor did they have any hepatic iron overload. Even though the occupational etiology was not established in this study, Cotrim et al. (27) have already demonstrated in Brazil that chronic exposure to volatile petrochemical products can lead to NASH. Others have also recognized the association between environmental toxicity and NASH (8,28).

Another patient who did not have any of the clinical conditions associated with NASH had been submitted to an extensive enterectomy ten years before because of an abdominal trauma, and had developed short bowel syndrome, that had been well compensated for in the last eight years. Experimental studies using animal models have demonstrated

that this kind of surgery leads to an increase in permeability of the remaining bowel (29) and bacterial translocation (30). These phenomena could be related to the absorption of endotoxins and bacterial lipopolysaccharides, increasing TNF- $\alpha$  levels and finally leading to steatohepatitis (31).

The allelic frequencies of C282Y (1.6%) and H63D (14.5%) observed among Brazilian NASH patients were compatible with those described for the local population: C282Y allelic frequency ranges from 1.1 to 1.4% and H63D allelic frequency from 6.4 to 20.3% according to the population groups analyzed (19). Only one of the three subjects with hepatic iron deposition carried an *HFE* mutation (H63D +/-). Moreover, it is known that hereditary hemochromatosis and *HFE* mutations are almost absent among Japanese people (26), although they were well represented in our NASH series. These findings provide evidence that *HFE* mutations are not necessarily involved in NASH pathogenesis, although they seem to play an important role

in Australian and North American Caucasians (8,14,15).

For those populations with high allelic frequencies of *HFE* mutations, secondary hepatic iron overload might determine oxidative stress and inflammation. In Brazil, however, NASH patients comprise a heterogeneous group with many associated conditions such as hyperinsulinism, environmental hepatotoxin exposure and drugs - but not hepatic iron overload, and their disease susceptibility could be related to genetic and environmental features other than *HFE* mutations.

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