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BRIEF ARTICLES

Iron homeostasis and H63D mutations in alcoholics with and without liver disease

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Abstract

AIM: To evaluate the prevalence of *HFE* gene mutation and indices of disturbed iron homeostasis in alcoholics with and without liver disease.

METHODS: One hundred and fifty-three heavy drinkers (defined as alcohol consumption > 80 g/d for at least 5 years) were included in the study. These comprised 78 patients with liver disease [liver disease alcoholics (LDA)] in whom the presence of liver disease was confirmed by liver biopsy or clinical evidence of hepatic decompensation, and 75 subjects with no evidence of liver disease, determined by normal liver tests on two occasions [non-liver disease alcoholics (NLDA)], were consecutively enrolled. Serum markers of iron status and HFE C282Y and H63D mutations were determined. *HFE* genotyping was compared with data obtained in healthy blood donors from the same geographical area.

RESULTS: Gender ratio was similar in both study groups. LDA patients were older than NLDA patients (52 ± 10 years *vs* 48 ± 11 years, $P = 0.03$). One third and one fifth of the study population had serum transferrin saturation (TS) greater than 45% and 60% respectively. Serum iron levels were similar in both groups. However, LDA patients had higher TS (51 ± 27 *vs* 36 ± 13, $P < 0.001$) and ferritin levels (559 ± 607 ng/mL *vs* 159 ± 122 ng/mL, $P < 0.001$), and lower total iron binding capacity (TIBC) (241 ± 88 μg/dL *vs* 279 ± 40 μg/dL, $P = 0.001$). The odds ratio for having liver disease with TS greater than 45% was 2.20 (95% confidence interval (CI): 1.37-3.54). There was no difference in C282Y allelic frequency between the two groups. However, H63D was more frequent in LDA patients (0.25 *vs* 0.16, $P = 0.03$). LDA patients had a greater probability of carrying at least one *HFE* mutation than NLDA patients (49.5% *vs* 31.6%, $P = 0.02$). The odds ratio for LDA in patients with H63D mutation was 1.57 (95% CI: 1.02-2.40).

CONCLUSION: The present study confirms the presence of iron overload in alcoholics, which was more severe in the subset of subjects with liver disease, in parallel with an increased frequency of H63D *HFE* mutation.

Key words: Alcoholic liver disease; Iron; *HFE* gene; H63D; Hemochromatosis

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INTRODUCTION

Alcohol consumption and iron overload have long been found to be associated with each other. In 1896, the condition we now recognize as hereditary hemochromatosis was considered a variant of alcoholic cirrhosis[1], and even in the 1960s was believed to be a nutritional disorder related to alcohol intake, in which excess iron originated from the diet and iron content in

red wine[2].

Alcohol may increase iron absorption and cellular iron uptake by several possible mechanisms: (1) increased absorption *via* a non-carrier-mediated paracellular route[3]; (2) iron absorption is stimulated by anemia secondary to ineffective erythropoiesis due to alcohol-induced folic acid deficiency[4,5]; and (3) alcohol consumption is associated with decrease in enterocyte turnover through mitosis inhibition[6], which may reduce the already limited intestinal iron excretion. Recently, it has been shown that alcohol down-regulates hepcidin transcription, which leads to increased duodenal iron absorption *via* a divalent metal transporter-1 (with enhanced luminal import) and ferroportin protein expression (with enhanced basolateral translocation to the circulation)[7,8]. Furthermore, it has been shown that alcohol abolishes the iron-induced up-regulation of both liver hepcidin transcription and the DNA-binding activity of C/EBP alpha[9], thus negating the protective effect of hepcidin.

Suzuki *et al*[10] also demonstrated, an up-regulation of transferrin receptor expression in the hepatocytes of liver disease alcoholics (LDAs), which may promote hepatocyte iron accumulation.

Alcoholic liver disease (ALD) is often associated with elevated serum iron indices and hepatic iron overload[11-14]. Iron is also believed to be central in the pathogenesis of ALD, and some reports show iron overload as a predictive indicator of higher mortality[15], and development of hepatocellular carcinoma[16]. In fact, iron overload and alcohol have a synergistic effect on the production of oxidative stress[17-20].

The fact that only a minority of alcohol abusers, develop advanced liver disease such as steatohepatitis, fibrosis, and cirrhosis, prompted the search for genetic predisposing factors[21], such as C282Y and H63D mutations in the hemochromatosis protein HFE, which increases iron overload. However, no association has been found between C282Y *HFE* gene mutation and ALD, and there are conflicting reports on the association between H63D and ALD[22-27]. On the other hand, it is clear that the phenotypic expression of HFE C282Y homozygosity (the prototype for the genetic hemochromatosis syndrome) is low, and it increases markedly in patients with excessive alcohol consumption[28-30], which suggests that alcohol may act as a potential modifier of the (genetically determined) hemochromatosis phenotype.

The aim of the present study was to evaluate the prevalence of *HFE* mutations, and indices of disturbed iron homeostasis in alcoholics with and without liver disease.

MATERIALS AND METHODS

The study was approved by the Institutional Ethics Committees and written informed consent was obtained from the study subjects. A total of 284 heavy drinkers, defined as alcohol consumption > 80 g/d for at least five years were included in the study. The subjects consisted of consecutive patients seen in the Liver Unit (ambulatory or hospitalized) of a University Hospital, with suspected ALD; and consecutive referrals to two Alcohol Addiction Units, with psychiatric alcohol dependency, and no previous suspicion of liver disease.

Lifetime alcohol intake was assessed in all subjects, using a semi-structured questionnaire. Subjects were excluded from the study if they had any of the following: serological evidence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections or autoimmune liver disease, histological evidence of other liver diseases, or "mild" abnormalities of liver tests (bilirubin, aminotransferases, alkaline phosphatase, less than twice the upper limit of normal) in the absence of clinical signs of liver disease. Drinkers without clinical manifestations, and with normal liver tests on one occasion were excluded if it was not possible to obtain a second blood sample for reconfirmation.

Based on the above mentioned criteria, the subjects were divided into two categories: LDA and non-liver disease alcoholics (NLDA). The criteria for inclusion in the LDA group were the presence of either laboratory/clinical evidence of hepatic decompensation (e.g. ascites, varices, and encephalopathy) or liver histology compatible with LDA of severity greater than steatosis. Percutaneous liver biopsy specimens were evaluated blindly according to standard procedures[31]; only 11 subjects underwent this procedure since in patients with evidence of hepatic decompensation, such as ascitis, encephalopathy, or signs of portal hypertension, liver biopsy was considered unnecessary. Inclusion criteria for NLDA consisted of lack of clinical signs of liver disease and normal liver tests on two occasions (aminotransferases, prothrombin time, albumin and bilirubin) with the exception of an isolated rise in γ-glutamyl transferase[32]; no liver biopsy was performed in this group as it was considered unethical.

Laboratory tests

After a 12-h overnight fast, blood samples were collected and biochemical tests were done on the same day by routine methods, in the central pathology laboratory. The tests included: aminotransferases, bilirubin, γ-glutamyl transpeptidase, protein electrophoresis, prothrombin time, renal functions, cholesterol, triglycerides, ceruloplasmin, α-1 antitrypsin, anti-nuclear, anti-mitochondrial, anti-smooth muscle antibodies, and serological markers of HBV and HCV infections. Serum iron indices: iron, ferritin, total iron binding capacity (TIBC) and % transferrin saturation were also determined.

Genotyping

To detect the C282Y and H63D mutations, genomic DNA, extracted from the buffy coat fraction of whole blood was amplified by polymerase chain reaction as previously described[29,33]. The C282Y mutation creates a new *Rsa*I restriction site and the H63D mutation abolishes a *Mbo*I site allowing identification by restriction enzyme digestion.

A sub-group of 11 patients, all belonging to

Table 1 Clinical and laboratory characteristics of LDA and NLDA

	LDA (n = 78)	NLDA (n = 75)	P value
Age (yr)	52.3 ± 10.1	48.5 ± 10.7	0.03
Number of men (%)	66 (85)	62 (83)	NS
Alcohol consumption (g/d)	217 ± 195	327 ± 311	0.004
Presence of ascitis (%)	38 (52.1)	-	-
Presence of encephalopathy (%)	17 (34.7)	-	-
Alanine aminotransferase (r.v. 0-37 IU/L)	53 ± 60	17 ± 6	< 0.05
Aspartate aminotransferase (r.v. 0-41 IU/L)	81 ± 128	15 ± 5	< 0.05
Alkaline phosphatase (r.v. 40-129 IU/L)	143 ± 75	91 ± 21	< 0.05
γ-Glutamil transpeptidase (r.v. 8-61 IU/L)	225 ± 239	47 ± 48	< 0.05
Albumin (g/L)	36 ± 8	43 ± 3	< 0.05
Bilirubin (mg/dL)	4.0 ± 7.2	0.7 ± 0.9	< 0.05
Prothrombin time (seconds prolonged from control)	3.2 ± 2.7	0.5 ± 1.2	< 0.05
Cholesterol (mg/dL)	160.0 ± 84	209.1 ± 41.7	< 0.05
Triglycerides (mg/dL)	125.6 ± 111.3	162.8 ± 121.0	NS
Glucose (mg/dL)	119.9 ± 40.5	97 ± 13.6	NS
Iron (r.v. 65-175 μg/dL)	115 ± 64	99.4 ± 39	NS
TIBC (r.v. 250-425 μg/dL)	241 ± 88	279 ± 40	0.001
Transferrin saturation (%)	51 ± 27	36 ± 13	< 0.001
Ferritin (r.v. 23-236 ng/mL)	559 ± 607	159 ± 122	< 0.001

r.v.: Reference value; TIBC: Total iron binding capacity.

the LDA, had a liver biopsy; the degree of hepatic parenchymal siderosis was identified by Perl's iron stain, and graded from 0 to 4.

Statistical analysis

Basic descriptive statistics, means, standard deviation (SD), ranges and percentages, were used to characterize the populations. Categorical variables were analyzed by chi squared test and paired parametric numerical variables were compared, using the Student's t test. Correlations between several variables were evaluated through Spearman correlation coefficient.

Odds ratio analysis was used to explore interactions between iron overload and genetic mutations in the pathogenesis of ALD *vs* non-liver disease: the odds and 95% confidence intervals of having LDA outcome *vs* NLDA outcome were determined. LDA and NLDA were always the dependent variables and transferrin saturation > 45% or the presence of genetic mutations were evaluated as risk factors. All analyses were adjusted for patient's age.

The computer software used was Statistical Program for Social Sciences (SPSS) for Windows 12.0 (SPSS Inc., Chicago, USA, 2004). All P values were two-sided; for all statistics, significance was accepted at the 5% probability level.

RESULTS

Based on the predefined inclusion and exclusion criteria, 153 heavy drinkers were included, 78 in the LDA group and 75 in the NLDA group. Clinical and biochemical characteristics of the study groups are summarized in Table 1. The gender ratio was similar in both groups; LDA patients were older (52.3 ± 10.1 years *vs* 48.5 ± 10.7 years, P = 0.03); and alcohol consumption was lower in LDA compared to NLDA (217 ± 195 g/d *vs* 327 ± 311 g/d, P = 0.004).

Both groups had similar mean iron concentrations (Table 1), however, LDA patients had lower TIBC (241 ± 88 μg/dL *vs* 279 ± 40 μg/dL, P = 0.001), and higher levels of ferritin (559 ± 607 ng/mL *vs* 159 ± 122 ng/mL, P < 0.001) and serum transferrin saturation (51% *vs* 36%, P < 0.001). Overall, among the 153 heavy drinkers, 33% had serum transferrin saturation greater than 45%, while 20% had greater than 60%; transferrin saturation higher than 45% and higher than 60% were more frequent in LDA patients (47.4% *vs* 18.1%, P < 0.001, and 34.6% *vs* 5.3%, P < 0.001, respectively). Furthermore, in subjects with transferrin saturation higher than 45%, the odds ratio for having LDA was 3.90 (95% confidence interval (CI): 1.59-4.54, P < 0.0001).

In the 11 patients who had a liver biopsy, there was a significant association between serum ferritin levels and the degree of hepatic parenchymal siderosis, as identified by Perl's iron stain (r = 0.692, P = 0.02). Five of seven patients (71%) with Perl's staining > 1, had H63D mutation, compared with two of four (50%) in those with a score of 1 or less (r = 0.217, P = 0.547). The distribution of C282Y and H63D genotypes is shown in Table 2. Allelic frequency of H63D mutation was higher in LDA than in NLDA patients (0.25 *vs* 0.16, P = 0.032). Furthermore, allelic frequencies of H63D mutation in NLDA subjects were similar to that seen in the general population from the same geographical area, based on the data on healthy blood donors[34]. There were no differences in the allelic frequency of C282Y between the two groups.

The odds ratio of having LDA and H63D mutation was 1.75 (95% CI: 1.02-2.40, P < 0.03), while the odds ratio of carrying at least one *HFE* mutation was 1.56 (95% CI: 1.05-2.32, P < 0.03).

The serum transferrin saturation and ferritin levels were higher in subjects carrying at least one *HFE* mutation

Table 2 Comparison of *HFE* genotypes, with C282Y or H63D allelic frequencies in LDA *vs* NLDA subjects and a control population *n* (%)

	LDA (*n* = 78)	NLDA (*n* = 75)	Blood donors[34] (*n* = 133)
wt/wt	39 (50)	52 (68)	92 (69)
wt/H63D	31 (39.7)	19 (25.3)	27 (20)
wt/C282Y	3 (3.8)	2 (3.7)	7 (5)
H63D/H63D	3 (3.8)	2 (3.7)	6 (4)
C82Y/H63D	2 (2.5)	1 (1.3)	1 (1)
C282Y allelic frequency	0.032	0.02	0.03
H63D allelic frequency[1]	0.25	0.16	0.15
Any mutation[1]	39 (50)	24 (32)	41 (31)

wt: Wild type; [1]$P = 0.02$; There were no other statistically significant differences between the various groups.

compared with subjects without *HFE* mutation (49% ± 24% *vs* 39% ± 23%, $P = 0.02$ and 499 ± 600 ng/mL *vs* 258 ± 339 ng/mL, $P = 0.005$, respectively) (Table 3). Moreover, the presence of one H63D mutation in patients with transferrin saturation > 45% increased the odds ratio for having LDA to 2.17 (95% CI: 1.42-3.32, $P < 0.01$).

DISCUSSION

In the present study, heavy drinking was frequently associated with iron overload, as suggested by elevated serum ferritin levels and transferrin saturation, in the absence of hemochromatosis[35]. Moreover, iron overload was more intense in the presence of liver disease, as shown by higher serum concentrations of ferritin and transferrin saturation.

Although ferritin and transferrin saturation may be questioned as markers of iron overload in the presence of liver disease, since ferritin elevation may result from necroinflammatory activity, and decreased hepatic protein production may occur secondary to liver disease[36], resulting in lower TIBC and higher transferrin saturation, previous studies in patients with liver disease have shown significantly higher ferritin levels in patients with alcohol-related liver disease[12]. Furthermore, in the present study, we observed a positive correlation between serum ferritin and the degree of hepatic iron deposition in patients who had a liver biopsy.

Since iron plays an important pathological role in ALD[37], and alcoholics are more prone to develop iron overload, it is conceivable that alcoholics who tend to absorb and store more iron are at an increased risk of liver disease. The presence of mutations in the hemochromatosis *HFE* gene may serve as a predisposing factor for the development of liver disease. However, five previous studies failed to show a relationship between ALD and the presence of such mutations[22-26]. On the other hand, Ropero Gradilla *et al*[27], in Spain, observed an association between H63D mutation (but not with C282Y mutation) and the risk of advanced liver disease. In the present study, individuals carrying at least one *HFE* mutation had a significantly higher probability of having liver disease, which suggested an association between *HFE* mutation and increased susceptibility to ALD. However, it is possible that our observation of an increased prevalence of *HFE* mutations may be a casual finding (type I error).

Table 3 Serum iron indices according to *HFE* status

	At least one *HFE* mutation (*n* = 63)	No *HFE* mutation (*n* = 90)	*P* value
Iron (μg/dL) (r.v. 65-175)	118 ± 52	100 ± 55	NS
TIBC (μg/dL) (r.v. 250-425)	262 ± 83	259 ± 63	NS
Transferrin saturation (%)	49 ± 24	39 ± 22	0.02
Ferritin (ng/mL) (r.v. 23-236)	499 ± 600	258 ± 339	< 0.001

r.v.: Reference value; TIBC: Total iron binding capacity.

The extent to which H63D mutation predisposes to iron overload has been the subject of much debate. Such an association has been observed in homozygosity studies[38,39], and also with the findings that serum transferrin saturation is significantly increased in H63D homozygotes and heterozygotes as compared with wild-type individuals[40]. To reinforce the importance of the *HFE* mutations as risk factors for liver disease, the presence of these mutations should be associated with significantly higher iron parameters. Indeed, the present study showed that transferrin saturation and ferritin concentration were higher in patients with at least one *HFE* mutation, with no difference in the TIBC values. However, even in the sub-group of individuals with increased iron saturation, the presence of H63D mutation was associated with a higher probability of liver disease, suggesting that H63D mutation may be a risk factor independent of the associated iron overload.

In conclusion, the present study has confirmed previous reports of the presence of iron overload in alcoholics, which is more severe in the subset of subjects with liver disease, and is associated with an increased frequency of H63D *HFE* mutation. Our findings indicate that H63D *HFE* mutation, by further increasing iron overload, is a risk factor for liver disease, through the synergistic damaging effects of alcohol and iron. Further research is needed to evaluate if the progression of the liver disease in alcoholic patients with iron overload is associated with a worse prognosis.

COMMENTS

Background

Alcohol abuse enhances iron absorption and may play a crucial role in the pathogenesis of alcoholic liver disease (ALD). Thus, conditions that enhance iron uptake may have a synergistic role in the development of ALD. Currently, the relevance of hemochromatosis-associated gene mutations and/or iron status in ALD is unclear.

Innovations and breakthroughs

The fact that only a minority of alcohol abusers, develop advanced liver disease such as steatohepatitis, fibrosis, and cirrhosis, prompted the search for genetic predisposing factors, such as C282Y and H63D mutations in the hemochromatosis protein HFE, which increases iron overload. However, no association has been found between C282Y *HFE* gene mutation and ALD, and there are conflicting reports on the association between H63D and ALD. The aim of the present study was to evaluate the prevalence of *HFE* mutations,

and indices of disturbed iron homeostasis in alcoholics with and without liver disease.

Applications

The research reported that H63D HFE mutation, by further increasing iron overload, is a risk factor for liver disease, through the synergistic damaging effects of alcohol and iron.

Peer review

The paper is interesting and is focused on original topic. Title reflects the content of the article. Results and discussion provide accurate informations and lead to conclusions

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