# *α1-Antitrypsin* Mutations in NAFLD: High Prevalence and Association With Altered Iron Metabolism But Not With Liver Damage

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**Hyperferritinemia, a common feature of nonalcoholic fatty liver disease (NAFLD), has been associated with steatohepatitis and fibrosis. Heterozygosity for *α1-antitrypsin* (*AAT*) mutations is a cofactor of liver damage, and AAT influences inflammation and iron metabolism. This study evaluated the prevalence of the common *AAT* PiS/PiZ mutants in 353 patients with NAFLD, 195 of whom had hyperferritinemia, versus 114 matched controls and their influence on iron metabolism and the severity of liver damage in the 212 patients submitted to biopsy. PiS and PiZ alleles were searched for by restriction analysis. Thirty-eight patients (10.8%) carried non-MM genotypes versus 4/114 (3.5%) controls ($P = .02$). Patients carrying *AAT* mutations had higher ferritin (573 [454-966] vs. 348 [201-648]; $P = .001$) with similar transferrin saturation. The difference was more evident in males ($P < .0001$) and significant in patients not carrying *HFE* genotypes associated with iron overload ($P = .015$). The prevalence of non-MM genotypes was higher in patients with hyperferritinemia than in those without (28/195, 14% vs. 10/158, 6%, $P = .016$), and *AAT* mutations were associated with higher prevalence of sinusoidal siderosis (17/27, 63% vs. 70/180, 39%; $P = .02$), and sinusoidal/total iron score (46.3 ± 38% vs. 25.1 ± 35%, $P = .01$). Although ferritin was independently associated with fibrosis ($P = .047$), *AAT* mutations favoring sinusoidal iron deposition did not affect liver damage. In conclusion, *AAT* mutations are associated with hyperferritinemia and sinusoidal iron accumulation, but not with more severe liver damage in NAFLD.** (HEPATOLOGY 2006;44:857-864.)

Nonalcoholic fatty liver disease (NAFLD), the leading cause of liver disease in Western countries, includes a spectrum of clinical entities ranging from pure fatty liver to nonalcoholic steatohepatitis (NASH) with possible evolution to cirrhosis and hepatocarcinoma.[1-3] Diabetes, obesity, and dyslipidemia are the main risk factors for NAFLD, with insulin resistance as the key pathogenic event.[4,5]

Hyperferritinemia associated with nonparenchymal iron overload in the presence of nearly normal transferrin saturation[6-8] represents a common clinical presentation of NAFLD, involving up to one third of unselected cases,[9] shares clinical features with the insulin resistance–hepatic iron overload syndrome (IR-HIO),[6] and is related to mutations in the *HFE* gene responsible for hereditary hemochromatosis (HHC) only in a minority of cases.[9-11] Although increased oxidative stress is possibly implicated,[12] the reasons why only a subset of subjects with metabolic liver disease shows alterations in iron parameters is at present unclear, but hyperferritinemia has bee reported to represent a risk factor for steatohepatitis and fibrosis.[11,13,14]

It can be speculated that genetic factors influencing hepatocellular damage, inflammation and iron handling result in hyperferritinemia and affect the progression of

*Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; IR-HIO, insulin resistance–hepatic iron overload; HHC, hereditary hemochromatosis; AAT, α1-antitrypsin; TfR, transferrin receptor.*

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NAFLD. Alpha1-antitrypsin (AAT), the principal serum protease inhibitor synthesized by the liver, potentially represents one of such factors. Several variants of this gene have been described, the most common being the PiZ (Glu342Lys) and PiS (Glu264Val) alleles, whose prevalence is about 1% and 4%, respectively, in Northern Italy, and show a decreasing gradient from north to south in Europe.[15,16] These amino acid substitutions lead to abnormal folding and spontaneous protein polymerization, determining endoplasmic reticulum stress and hepatocellular damage. Heterozygosity for the PiZ, and to a lesser extent for the PiS[17] allele has been associated with cirrhosis and hepatocarcinoma in the presence of other hepatotoxic factors in adulthood,[18,19] and increased prevalence of the PiZ allele has been reported in patients with cryptogenic cirrhosis, now recognized to be frequently related to NASH,[17-19] and in HHC,[20,21] although evidence is controversial.[15,22]

AAT is involved in the regulation of the innate immunity and its deficiency may promote a pro-inflammatory status.[23] This protein interacts with transferrin receptor (TfR) inducing ferritin synthesis in myelo-monocytic cells, which compared to parenchymal cells are not strictly dependent on TfR for their iron uptake,[24-26] and could play a role in the diversion of iron trafficking from parenchymal to monocytic cells during inflammation.[27] The aim of this study was to determine the prevalence of PiZ and PiS *AAT* alleles in a large series of patients with NAFLD and to define their influence on iron metabolism and on the severity of liver damage.

## Patients and Methods

***Patients.*** 353 consecutive unrelated patients with NAFLD. Other causes of liver disease were excluded, including increased alcohol intake (>30 g/day for males, >20 g/day for females), aceruloplasminemia, dyserythropoietic and hemolytic anemias, oral iron intake, repeated blood transfusions, HBV and HCV chronic viral hepatitis, autoimmune hepatitis, Wilson's disease, and drug-induced liver disease. We also excluded patients with a previous diagnosis of HHC and with severe AAT deficiency, as determined by serum protein electrophoresis. Part of this group has previously been described,[28] with 311 (88%) from Northern Italy and 42 (12%) with at least one ancestor from Central and Southern Italy. Demographic and clinical features at diagnosis available for each patient are shown in Table 1. Patients were defined to have increased ferritin in the presence of values >320 ng/mL in males and >240 ng/mL in females.

AAT serum levels (as determined by nephelometric analysis) were available in 188 (53%) patients. Patients with moderate sinusoidal hepatic iron stores[29] negative for the C282Y *HFE* mutation (n = 7) were screened for and all resulted to be negative for mutations in all exons and the promoter deletion of *Ferroportin1*. Mutation in *TfR2* (all exons), *Hepcidin* (all exons) and *Hemojuvelin* (exons 3 and 4) genes were also excluded in patients with moderate parenchymal siderosis negative for the C282Y *HFE* mutation (n = 7).[30] TNF$\alpha$ serum levels were measured in duplicate by ELISA (Quantikine, R&D Systems, Minneapolis, MN), in the 77 patients for whom a serum sample was available. Clinical features of subjects with measured TNF$\alpha$ were not significantly different from those of the others.

***Histological Data.*** Liver biopsy, performed in 212 (60%) cases because of persistent elevation of liver enzymes confirmed the presence of steatosis in all cases. Liver biopsy was not performed in 9 cases with altered tests because of the lack of patient consent. Tissue sections were stained with hematoxylin and eosin, impregnated with silver for reticulin framework, and stained with PAS for glycogen, periodic Schiff diastase for nonglycogen proteins, Perls for iron, and trichrome for collagen. A single expert pathologist unaware of clinical and genetic data reviewed all the available biopsies (207/212, 98%). Iron deposits were assessed semiquantitatively according to Deugnier et al.[29] The sinusoidal iron score/total iron score ratio was calculated for each case. Steatosis was graded as I (10-33%), II (33-66%), and III (>66%). The presence of NASH was assessed according to Brunt et al.[31] PAS-positive diastase-resistant globules were searched for in patients positive for mutant *AAT* alleles.

***Controls.*** The control group included 114 subjects (blood donors) without clinical and biochemical evidence of liver disease and without iron overload at the first evaluation of the same geographical origin. Eighty-nine percent were from Northern Italy, and 11% had at least one ancestor from Central and Southern Italy. Metabolic features were available in 89 of these subjects (Table 1).

Informed written consent was obtained from each patient and control subject, and the study conforms to the ethical guidelines of the 1975 declaration of Helsinki.

***Genetic Data.*** *AAT* and *HFE* genotype were determined in each patient by restriction analysis. Genomic DNA was extracted from EDTA-preserved whole blood using the phenol-chloroform method. The PiS and PiZ *AAT* alleles were detected after DNA amplification by polymerase chain reaction by restriction with TaqI.[32,33] The finding of mutated alleles was confirmed by sequencing, which was consistent with restriction analysis in each case.

***Statistical Analysis.*** The sample size was calculated (http://calculators.stat.ucla.edu/powercalc/) on the basis

**Table 1. Demographic, Anthropometric, and Clinical Features of 353 Patients With NAFLD**

| Parameter | NAFLD (n = 353) Mean/Distribution | Range | Controls (n = 89) | Reference Values (Female/Male) |
|---|---|---|---|---|
| Sex (M/F) | 280/73 (79%/21%) | – | 72/17 (81%/19%) | – |
| Age (years) | 49.2 ± 11 | 25–77 | 48.7 ± 11 | – |
| BMI (kg/m$^2$) | 27.3 ± 3.5 | 17.6–40 | 25.8 ± 2.3 | ≤25 |
| Total cholesterol (mg/dL) | 212 ± 45 | 102–416 | 209 ± 47 | <200 |
| HDL cholesterol (mg/dL) | 44 ± 12 | 16–98 | – | >60 |
| Triglycerides (mg/dL) | 159 ± 93 | 22–564 | 100 ± 56 | <160 |
| LDL (mg/dL) | 136 ± 38 | 33–257 | – | <110 |
| Uric acid (mg/dL) | 5.5 ± 1.4 | 2.4–9.8 | – | <5.4F/<6M |
| Glucose (mg/dL) | 98.4 ± 21 | 72–175 | – | <110 |
| Homa-R | 3.9 ± 3.1 | 0.7–25.2 | 2.6 ± 1 | ≤2.7 |
| Glucose tolerance status (Normal/IGT/diabetes) | 278/44/31 (79%/12%/9%) | – | 89/0/0 | Normal |
| Hypertension | 125 (35%) | – | – | 0 |
| ALT (IU/mL) | 57 ± 44 | 11–316 | – | <42 |
| GGT (IU/mL) | 75.8 ± 161 | 22–1687 | – | <40 |
| Ferritin* (ng/mL) | 368 (220–693) | 11–3151 | – | <200F/<320M |
| Transferrin saturation % | 37 ± 14.6 | 2–92 | – | <40F/<45M |
| *HFE* C282Y mutation | 307/39/7† | – | 87/2/0 | – |
| *HFE* H63D mutation | 216/112/25† | – | 67/20/2 | – |
| Serum AAT (mg/dL) | 126 ± 30 | 43–272 | 155 ± 52 | >90 |
| NASH‡ | 103/212 (48%) | – | – | – |
| Steatosis grade (I/II/III) | 115/73/24 (54%/34%/11%) | – | – | 0 |
| Grade‡ (0/1/2/3) | 110/75/24/3 (52%/35%/11%/1%) | – | – | 0 |
| Fibrosis stage‡ (0/1/2/3/4) | 131/48/21/6/6 (62%/22%/10%/3%/3%) | – | – | 0 |
| Iron overload score§ | | | | |
| Parenchymal (0-36) | 3.2 ± 4.8 | | | 0 |
| Sinusoidal (0-12) | 1.9 ± 2.3 | | | 0 |
| Portal (0-12) | 0.1 ± 0.6 | | | 0 |
| Total (0-60) | 5.3 ± 6.2 | | | 0 |
| SIS/TIS ratio % | 48 ± 36 | | | – |
| TIS > 0 | 122/205 (59%) | | | |

Abbreviations: SIS, sinusoidal iron score; TIS, total iron score.
*The geometric mean and interquartile range are shown.
†7 patients were C282Y+/+ and 13 were C282Y/H63D compound heterozygotes.
‡According to Brunt et al.[31]
§According to Deugnier et al.[29]

of the expected relative risk of the mutant allele versus the wild-type allele, the desired power, and significance. The sample size had 85% power of detecting an odds ratio (OR) of 2.5 for NAFLD and 65% power of detecting an OR of 2.5 for fibrosis in patients with NAFLD with a significance of 5%. Except for serum ferritin, results are expressed as means ± standard deviation and considered significant when $P < .05$ (two-tailed). For serum ferritin levels, analysis was done after log transformation, and the geometric mean and interquartile range are reported. Iron scores were approximated to continuous variables for quantitative analysis. Mean values were compared by Student *t* test. Frequencies were compared by Fisher's exact test. Logistic regression analysis was performed to determine variables associated with fibrosis in the 212 patients submitted to biopsy. Female sex, age (years), body mass index (BMI, kg/m$^2$), LDL cholesterol (mg/dL), ALT (IU/L), ferritin (log ng/mL), and Homa-R index were considered as independent variables. Analyses were carried out with JMP 5.1 statistical analysis software (SAS Institute Inc., Cary, NC).

## Results

***Mutated AAT Alleles Are More Represented in NAFLD.*** The prevalence of PiS and PiZ mutated *AAT* alleles in patients and in controls is shown in Table 2. Thirty-eight patients (10.8%) carried non-MM genotypes (2 PiS/PiS, 30 PiS/wt, 5 PiZ/wt, 1 PiS/PiZ) compared to 4/114 (3.5%) healthy controls ($P = .02$ for the prevalence of non-MM genotypes, $P = .03$ for the PiS/wt genotype). The latter prevalence is in agreement with previous reports in the Italian population.[16] The frequency of the PiZ allele was not significantly different (0.007 vs. 0.009), whereas the frequency of the PiS allele was signif-

**Table 2. Prevalence of *AAT* Mutant Alleles in Patients With NAFLD Versus Healthy Controls**

| | n | wt/wt | PiZ/wt | PiS/wt | PiS/PiS | PiS/PiZ |
|---|---|---|---|---|---|---|
| Patients | 353 | 315 (89%) | 5 (1.4%) | 30 (8.8%) | 2 (0.5%) | 1 (0.3%) |
| Controls | 114 | 110 (96%) | 1 (1%) | 3 (3%) | 0 | 0 |
| *P* | | 0.02 | 1 | 0.03 | 1 | 1 |

icantly higher in patients than controls (0.050 vs. 0.013; $P = .01$, OR 3.9, 95% CI 1.2-12.8].

Ten patients had decreased (<90 mg/dL) serum AAT (87-43 mg/dL); their clinical and demographic characteristics were not significantly different compared to those of subjects with normal AAT levels. Of these 10 subjects, 1 carried the PiS allele, 1 the PiZ allele, and 1 was a PiS/PiZ compound heterozygote. There was a trend for lower serum AAT levels in patients carrying the PiS and PiZ AAT alleles, (117 ± 34 vs.127 ± 29 mg/dL; $P = .09$).

***AAT Mutated Alleles Associated With Increased Ferritin and Sinusoidal Hepatic Siderosis in the Presence of Normal Transferrin Saturation.*** Clinical features of patients positive for the PiS/PiZ allele compared to those who were negative are shown in Table 3. Demographic, anthropometric, as well as metabolic parameters did not differ between the 2 groups. Patients positive for the *AAT* mutations had higher ferritin than negative ones ($P = .001$). Ferritin levels were 348 (201-468) in negative subjects (n = 315), 554 (298-1003) in PiS/wt (n = 30), 764 (600-1024) in PiZ/wt (n = 5), and 898 (745-1120) in PiS/PiS and PiS/PiZ subjects (n = 3). The difference was even more marked in male patients, with serum ferritin levels of 791 (454-995) in mutation carriers versus 451 (221-665) in wild-type subjects ($P < .0001$), whereas no significant difference was observed in females (73 patients, 68 wild-type and 5 carrying *AAT* mutations). The prevalence of *HFE* mutations ($P = .13$ for the C282Y and $P = .72$ for the H63D mutation) and transferrin saturation were not significantly different between patients positive or negative for *AAT* mutations. Inflammatory indices (C-reactive protein and $\alpha$1-acid glycoprotein) performed at diagnosis in a subset of patients (n = 52, 10 with *AAT* mutations) were in the normal range and did not show significant differences between patients positive or negative for *AAT* mutations. Serum TNF$\alpha$ levels were lower in patients positive than in those negative for *AAT* mutations (1.38 ± 1.39, n = 20 vs. 2.94 ± 2.61, n = 57 pg/mL respectively; $P = .01$).

Histological features of the 212 patients submitted to liver biopsy subdivided according to the presence of *AAT* PiS and PiZ alleles are shown in Table 4. The proportion

**Table 3. Demographic and Clinical Characteristics of 353 Italian Patients With NAFLD Subdivided According to the Presence or Absence of *AAT* PiS/PiZ Mutations**

| | *AAT* PiS/PiZ | |
|---|---|---|
| Parameter | Present (n = 38) | Absent (n = 315) |
| Age (years) | 50.2 ± 11 | 49 ± 11 |
| Sex (F) | 5 (13%) | 68 (21%) |
| BMI (kg/m$^2$) | 27.6 ± 3.8 | 27.3 ± 3.6 |
| LDL cholesterol (mg/dL) | 139 ± 37 | 136 ± 38 |
| HDL cholesterol (mg/dL) | 41.3 ± 10 | 44.4 ± 12 |
| Triglycerides (mg/dL) | 156 ± 81 | 159 ± 94 |
| Uric acid (mg/dL) | 5.6 ± 1.4 | 5.5 ± 1.4 |
| Glucose (mg/dL) | 97.1 ± 21 | 98.6 ± 21 |
| Homa-R | 3.6 ± 2.4 | 3.9 ± 2.6 |
| Glucose tolerance status | | |
| Normal/IFG/diabetes | 30/5/3 (78%/13%/8%) | 248/39/28 (79%/12%/8%) |
| Hypertension | 12 (32%) | 113 (36%) |
| ALT (IU/mL) | 60 ± 51 | 56 ± 44 |
| GGT (IU/mL) | 62 ± 66 | 77 ± 139 |
| Ferritin (ng/mL) | 573 (454-966)* | 348 (201-648)* |
| Transferrin saturation % | 37 ± 15 | 37 ± 15 |
| C282Y *HFE* mutation | | |
| (no/hetero/homozygous) | 30/7/1 (79%/18%/3%) | 277/32/6 (88%/10%/2%) |
| H63D *HFE* mutation | | |
| (no/hetero/homozygous) | 22/11/5 (59%/29%/12%) | 194/101/20 (62%/32%/6%) |
| Serum AAT (mg/dL) | 117 ± 34 (n = 32) | 127 ± 29 (n = 156) |

*$P = .001$.

**Table 4. Histological Features of the 212 Patients Submitted to Liver Biopsy Subdivided According to the Presence of the PiS/PiZ *AAT* Alleles**

| Parameter | AAT PiS/PiZ Present (n = 27) | AAT PiS/PiZ Absent (n = 185) |
|---|---|---|
| Steatosis | | |
| Grade I | 16 (59%) | 99 (54%) |
| Grade II | 8 (30%) | 65 (35%) |
| Grade III | 3 (11%) | 21 (11%) |
| NASH* | 12 (44%) | 92 (50%) |
| Grade 1 | 8 (30%) | 69 (38%) |
| Grade 2 | 3 (11%) | 21 (11%) |
| Grade 3 | 1 (4%) | 2 (1%) |
| Fibrosis | 7 (26%) | 74 (40%) |
| Perivenular | 2 (7%) | 46 (25%) |
| Bridging | 5 (19%) | 22 (12%) |
| Cirrhosis | 0 | 6 (3%) |
| Siderosis† | 21/27‡ (78%) | 101/180‡ (56%) |
| Parenchymal | 16/27 (59%) | 79/180 (44%) |
| Sinusoidal | 17/27§ (63%) | 70/180§ (39%) |
| Portal | 2/27 (7%) | 8/180 (4%) |

NOTE. The prevalence of non-MM genotypes was 27/212, 12.7% versus 4/114, 3.5% in controls ($P = .005$).

*According to Brunt et al.[31]

†according to Deugnier et al.[29] available in 207/212 (98%) patients.

‡$P = .038$.

§$P = .02$.

of those submitted to liver biopsy was not significantly different between patients positive and negative for *AAT* mutations (27/38, 71% vs. 185/315, 59% respectively; $P = .16$). No significant difference in the severity of steatosis, inflammation, and liver damage was observed between patients with and without *AAT* mutations. Liver biopsies of the patients positive for the PiS or PiZ alleles were negative for PAS-positive diastase-resistant globules.

Patients carrying *AAT* mutations had higher prevalence of total ($P = .03$), and sinusoidal ($P = .02$) hepatic siderosis, but not of parenchymal and portal siderosis. *AAT* mutation carriers had higher total tissue iron score (8.6 ± 7 vs. 4.7 ± 5.8; $P = .01$), parenchymal (PIS) (3.2 ± 2.1 vs. 1.7 ± 2.3; $P = .001$), and sinusoidal iron score (3.2 ± 2 vs. 1.7 ± 2.3; $P = .001$), but not significantly higher portal iron score (0.15 ± 0.6 vs. 0.1 ± 0.6; $P =$ ns). Ratio of sinusoidal/total iron score was significantly higher in patients positive compared to those negative for *AAT* mutations (46.3 ± 38% vs. 25.1 ± 35%, respectively, $P = .01$). Liver iron concentration was available in only 34 cases and did not show significant differences between patients with (n = 4) and without *AAT* mutations (177 ± 206 vs. 233 ± 190 μg/100 mg dry weight).

***Interaction Between AAT and HFE Genotypes in the Regulation of Iron Metabolism.*** To determine whether the effect of *AAT* mutations on iron metabolism was influenced by mutations in the *HFE* gene responsible for HHC, we analyzed serum iron parameters (ferritin and transferrin saturation) and the presence and distribution of hepatic siderosis in patients subdivided according to *HFE* genotypes (Table 5). We considered three classes of *HFE* genotypes based on the effect of *HFE* on iron status as determined in epidemiological studies:[34] class I (wt/wt and wt/H63D), class II (wt/C282Y and H63D/H63D), and class III (H63D/C282Y and C282Y/C282Y).

In the absence of *HFE* genotypes associated with iron overload (n = 282, class I), patients positive for *AAT* mutations had significantly higher ferritin than did negative patients ($P = .015$) in the presence of normal trans-

**Table 5 Effect of *AAT* Gene Mutations on Iron Metabolism in Patients Subdivided According to *HFE* Genotypes**

| | *HFE* genotype | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | wt/wt, H63D/wt | | | C282Y/wt, H63D/H63D | | | C282Y/H63D, C282Y/C282Y | | |
| *AAT* muts | Present | Absent | *P* | Present | Absent | *P* | Present | Absent | *P* |
| No. of cases | 25 | 257 | | 7 | 40 | | 6 | 18 | |
| Ferritin* | 735 (259–995) | 327 (168–602) | 0.015 | 829 (548–885) | 494 (189–646) | 0.0004 | 675 (375–957) | 488 (432–664) | ns |
| TS % | 37 ± 15 | 34 ± 13 | ns | 33 ± 6 | 44 ± 13 | 0.03 | 41 ± 20 | 55 ± 22 | ns |
| No. of cases | 19 | 145 | | 5 | 21 | | 3 | 14 | |
| TIS > 0 | 13 (68%) | 71 (49%) | 0.1 | 5 (100%) | 17 (81%) | ns | 2 (66%) | 13 (93%) | ns |
| TIS | 8.3 ± 7 | 4 ± 5 | 0.03 | 13.4 ± 5 | 6.3 ± 5 | 0.02 | 4.2 ± 2 | 9.6 ± 4 | 0.02 |
| PIS > 0 | 9 (47%) | 49 (34%) | ns | 5 (100%) | 16 (76%) | ns | 2 (66%) | 14 (93%) | ns |
| PIS | 4.9 ± 6 | 2.4 ± 4 | 0.049 | 10.2 ± 4 | 3.7 ± 4 | 0.014 | 0.8 ± 1 | 7.1 ± 7 | ns |
| SIS > 0 | 13 (68%) | 51 (35%) | 0.009 | 2 (40%) | 11 (42%) | ns | 2 (66%) | 7 (50%) | ns |
| SIS | 3.2 ± 2 | 1.6 ± 2 | 0.004 | 3 ± 2 | 2.2 ± 2 | ns | 3.5 ± 1 | 2.3 ± 2 | ns |
| SIS/TIS% | 44 ± 39 | 23 ± 35 | 0.019 | 23 ± 19 | 37 ± 37 | ns | 88 ± 25 | 33 ± 35 | 0.005 |

Abbreviations: Muts, mutations; No., number; TS, transferrin saturation; TIS, tissue iron score; PIS, parenchymal iron score; SIS, sinusoidal iron score; ns, not significant.

*Geometric mean and interquartile range (in parentheses) are shown.

**Table 6. Variables Independently Associated With the Risk of Fibrosis (Stage > 0 According to Brunt) in 212 Patients With NAFLD Submitted to Liver Biopsy**

| Parameter | Values | | *P |
|---|---|---|---|
| | No Fibrosis | Fibrosis | |
| ALT (IU/l) | 57 ± 44 | 73 ± 49 | .002 |
| Homa-R index | 3.3 ± 2.2 | 4.9 ± 3.7 | .035 |
| Ferritin (ng/mL)† | 399 (162–647) | 505 (234–735) | .047 |

*At logistic regression analysis considering sex (F), age (years), BMI (kg/m$^2$), LDL cholesterol (mg/dL), ALT (IU/L), ferritin (log ng/mL), and Homa-R index as independent variables. †Geometric mean and interquartile range (in parentheses) are shown.

ferrin saturation, and increased prevalence of sinusoidal siderosis ($P = .006$). We also observed higher total ($P = .03$), parenchymal ($P = .05$), and sinusoidal ($P = .004$) iron scores, and ratio of sinusoidal/total iron score ($P = .019$). In patients with class II *HFE* genotype (n = 47) subjects carrying *AAT* mutations had higher ferritin ($P = .0004$) and lower transferrin saturation ($P = .03$). Subjects carrying *AAT* mutations had higher total ($P = .02$) and parenchymal ($P = .01$) iron scores. In patients with class III *HFE* genotype (n = 24), no significant difference was observed in ferritin and transferrin saturation levels, although there was a tendency for higher ferritin and lower transferrin saturation, associated with lower tissue iron score ($P = .02$), and higher ratio of sinusoidal/total tissue iron score in *AAT* mutation-positive than in mutation-negative patients ($P = .005$).

When we subdivided patients according to the presence of hyperferritinemia, the prevalence of non-MM genotypes was higher in patients with increased than in those with normal ferritin levels (28/195, 14% vs. 10/158, 6%, $P = .016$).

***Interaction Between HFE and AAT Genotype and Liver Damage.*** In patients submitted to biopsy, ferritin was higher in AAT mutation carriers than in noncarriers (791 [289-912] vs. 409 [165-648]; $P = .01$), and was independently associated with the presence of fibrosis ($P = .047$) (Table 6).

Patients with class II and III *HFE* genotypes were not more likely to undergo liver biopsy compared to those with class I genotypes (43/71, 60.6% vs. 169/282, 59.9%). Patients with fibrosis carrying class II and III *HFE* genotypes were leaner (BMI 26.2 ± 2.8 vs. 28.7 ± 2.4 $P = .006$) and less insulin resistant (Homa-R 2.8 ± 1.3 vs. 5.3 ± 3.9, $P = .0003$) than those with class I genotypes, but the degree of liver steatosis, age, sex distribution, and serum lipids were not significantly different.

Because being overweight has been reported to decrease the penetrance of *HFE* mutations,[35] and in this series *AAT* mutations appeared to modify the phenotypic expression of *HFE* mutations, we analyzed the prevalence of liver fibrosis in patients subdivided according to *AAT* and *HFE* genotypes and the presence of severe overweight condition (arbitrarily defined as BMI > 27.5). In patients with BMI < 27.5 and negative for *AAT* mutations, those carrying class II and III *HFE* genotypes had significantly higher parenchymal iron score than patients with class I HFE genotypes (6.2 ± 5 vs. 2.4 ± 4; $P < .0001$). This relationship was not significant in patients with AAT mutations, and in patients with BMI > 27.5. In patients without severe overweight (BMI < 27.5; 146/212, 68.9%), patients with class II and III *HFE* genotypes and no *AAT* mutations had significantly higher prevalence of fibrosis than those with class I *HFE* genotypes and no *AAT* mutations (14/27, 51.8% vs. 28/97, 28.7%; $P = .037$). In contrast, *HFE* and *AAT* genotypes were not significantly associated with the presence of fibrosis in patients with BMI > 27.5. No significant difference in the prevalence of fibrosis was observed in *AAT* positive subjects with or without *HFE* mutations.

## Discussion

In this study, we analyzed the prevalence of the PiZ and PiS mutations in the *Serpina1* gene, previously known as *AAT*,[23] in Italian patients with NAFLD, and their influence on iron metabolism and the severity of liver disease. Our results indicate that the mutated *AAT* alleles are prevalent in patients with NAFLD and evidence of altered liver enzymes, but are not associated with more severe liver disease. In addition, our data indicate that an interaction occurs between *AAT* and iron metabolism.

In this series, the prevalence of the PiS and PiZ alleles was higher in NAFLD than in controls, in particular in those presenting with hyperferritinemia, being 14% in this last group. Patients with the *AAT* mutations, more evidently males, had higher ferritin levels than did patients without mutations in the presence of normal transferrin saturation. We hypothesized that the association between *AAT* mutants and increased serum ferritin could be due to (a) increased liver damage, (b) increased inflammatory activity, and/or (c) altered iron metabolism in carriers of *AAT* mutations compared to the other patients. Patients with and without non-MM genotypes did not differ in biochemical and histological indices of liver damage: AAT globules were not detected in the hepatocytes of subjects carrying *AAT* mutants, thus excluding increased liver damage as a likely explanation for increased ferritin. Inflammatory indices, including CRP and TNF$\alpha$ levels, performed in a subset of patients with and without mutations did not differ, but we cannot exclude that patients positive for *AAT* mutations had subtle alterations in in-

flammatory activity in the hepatic microenvironment undetectable at the systemic level.

On the other hand, we found a higher prevalence and degree of sinusoidal hepatic siderosis in patients carrying *AAT* mutations. To better characterize these findings, we determined the effect of *AAT* mutations on iron metabolism according to *HFE* genotype. The presence of *AAT* mutations was associated with higher ferritin in all *HFE* classes, but the difference was not significant in patients carrying *HFE* genotypes consistent with HHC. In addition, the percentage of transferrin saturation did not increase significantly with increasing *HFE* class in patients carrying *AAT* mutations, and indeed it was lower in patients carrying *HFE* genotypes usually associated with iron overload, reaching significance in subjects carrying the C282Y/wt or H63D/H63D genotypes. The histological picture mirrored what we observed at biochemical level: in patients negative for *HFE* genotypes usually predisposing to iron overload, those positive for *AAT* mutations had a higher prevalence and degree of sinusoidal siderosis.

Thus, *AAT* mutations are prevalent and associated with the typical abnormalities in iron metabolism observed in NAFLD and in the so-called IR-HIO syndrome, i.e., increased serum ferritin in the presence of normal transferrin saturation. It is possible that the coexistence of multiple genetic abnormalities contribute to this atypical pattern of iron overload,[6-9] and it could be speculated that *AAT* affects the susceptibility to develop biochemical abnormalities related to NAFLD by altering iron metabolism, redox status and possibly cytokine profile.[36]

*AAT* is not in linkage with any gene known to regulate iron metabolism, but AAT has been demonstrated to interact with TfR inducing ferritin synthesis.[24-26] Since this interaction has been reported to differentially influence iron metabolism in myelo-monocytic compared to other cells, it has been suggested to play a role in the phenotypic expression of iron overload disorders and in the diversion of iron trafficking between different cellular compartments upon activation of inflammatory pathways.[27] It is tempting to speculate that subtle alterations in AAT activity interfere with iron trafficking between hepatocytes and Kupffer cells in NAFLD,[36] but this hypothesis requires further confirmation.

Heterozygosity for *AAT* mutations was not a risk factor for more severe liver damage, but consistently with iron redistribution from hepatocytes to sinusoidal cells, was possibly protective in subjects carrying *HFE* genotypes associated with parenchymal iron accumulation. In fact, parenchymal iron overload characteristic of hereditary hemochromatosis is a known cause of liver damage and has been shown to promote fibrogenesis synergizing with steatosis,[37] whereas the pure sinusoidal siderosis, such as that observed in subjects with some *Ferroportin1* mutations, has not been associated with progressive liver disease.[38]

These data suggest that, although *HFE* mutations do not represent a major determinant of liver damage in obese subjects with NAFLD,[11] evaluation of the cellular distribution of iron stores is more important than that of total hepatic iron. Thus, *HFE* genotypes consistent with hereditary hemochromatosis are not sufficient *per se* to predict the phenotype (*i.e.*, parenchymal iron overload) possibly at risk for liver damage, but requires the co-determination of permissive genetic, such as *AAT*, as well as acquired factors.

The limitations of this study include the fact that we could not ascertain the effect of *AAT* mutations on iron parameters in controls, given the relatively low prevalence of mutated alleles. Moreover, we did not measure liver iron concentration in the majority of patients at diagnosis, but, since *AAT* genotype influenced more iron compartmentalization than total stores, we think that the availability of these data in a higher number of subjects would not have changed the main results. Also, we cannot exclude that patients with decreased serum AAT carried rare mutations in this gene.

In conclusion, the present study indicates that *AAT* mutant alleles are prevalent in patients with NAFLD and associated with hyperferritinemia related to sinusoidal iron accumulation, but do not affect liver damage.

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