Research Article

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# *Beta-globin* mutations are associated with parenchymal siderosis and fibrosis in patients with non-alcoholic fatty liver disease☆

Luca Valenti[1,*], Elena Canavesi[1], Enrico Galmozzi[1], Paola Dongiovanni[1], Raffaela Rametta[1], Paolo Maggioni[1], Marco Maggioni[2], Anna Ludovica Fracanzani[1], Silvia Fargion[1,**]

[1]*Department of Internal Medicine, Università degli Studi Milano, UO Medicina Interna 1B, Fondazione Ospedale Policlinico MaRE IRCCS, Milan, Italy;* [2]*UOC Anatomia Patologica, Ospedale San Paolo, Fondazione Ospedale Policlinico MaRE IRCCS, Milan, Italy*

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**Background & Aims**: Parenchymal liver siderosis is associated with increased fibrosis in patients with non-alcoholic fatty liver disease (NAFLD). The aim of this study was to assess whether a panel of genetic variants previously reported to influence iron metabolism, including the C282Y/H63D *HFE*, the PiZ/PiS *alpha1-antitrypsin*, the IVS1–24 *ferroportin* polymorphisms, and the beta-thalassemia trait, may be able to predict the presence of parenchymal siderosis and of progressive fibrosis in NAFLD.

**Methods**: We considered 274 Italian patients with biopsy-proven NAFLD. Genetic polymorphisms were searched for by sequence allele specific-polymerase chain reaction and restriction analysis, whereas beta-trait was determined according to blood count and $HbA_2$ determination.

**Results**: Parenchymal iron deposition was predominantly observed in 32 (11.7%) patients. Heterozygosity for the C282Y (OR 1.87, 95% CI 1.04–3.25), homozygosity for the H63D *HFE* (OR 2.31, 95% CI 1.04–4) mutations, and the beta-thalassemia trait (OR 2.57 95% CI 1.49–4.47) were all predominantly associated with parenchymal siderosis, independently of age, sex, body mass index, alcohol intake, ferritin, and transferrin saturation. Sixty-three percent of patients with hepatocellular siderosis were positive for at least one of the aforementioned genetic variants. The beta-thalassemia trait had the highest positive and the lowest negative likelihood ratios for predominantly parenchymal iron accumulation (5.05 and 0.74, respectively), and was independently associated with moderate/severe fibrosis (OR 2.50, 95% CI 1.26–5.19).

**Conclusions**: In patients with NAFLD, predominant hepatocellular iron deposition is often related to genetic factors, among which *beta-globin* mutations play a major role, predisposing to parenchymal iron accumulation and to progressive liver fibrosis.


Keywords: Genetics; Iron overload; Non-alcoholic steatohepatisis; Thalassemia.
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* Corresponding author. Tel.: +39 02 55033301; fax: +39 02 50320296.
** Corresponding author. Tel.: +39 02 55033301; fax: +39 02 50320296.
*E-mail addresses:* luca.valenti@unimi.it (L. Valenti), silvia.fargion@unimi.it (S. Fargion).

*Abbreviations:* AAT, alpha1-antitrypsin; ALT, alanine transaminase; BMI, body mass index; CI, confidence interval; FPN, ferroportin-1; GGT, gamma-glutamyl-transferase; Hb, hemoglobin; HDL, high density lipoprotein; HFE, hemochromatosis gene; HOMA-IR, homeostasis metabolic assessment-insulin resistance index; IVS, intervening sequence; LR, likelihood ratio; MCV, mean curpuscular volume; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; SAS-PCR, sequence allele specific-polymerase chain reaction; SNP, single nucleotide polymorphism.

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease that affects 20–34% of the population [1]. Its presentation is strictly related to the metabolic syndrome [2] and hepatic insulin resistance [3]. Although in most cases fatty liver does not progress to severe liver disease, the presence of non-alcoholic steatohepatitis (NASH) increases the risk of developing cirrhosis, liver failure [4], and hepatocellular carcinoma [5].

The susceptibility to develop NASH is influenced by inherited factors [6], as single nucleotide polymorphisms in genes involved in inflammation, oxidative stress, and fibrogenesis have been associated with the severity of liver damage [7,8].

Hyperferritinemia is observed in up to one third of NAFLD cases [9,10], and has been associated with more advanced liver damage [11], oxidative stress, and a typical pattern of mild iron accumulation, involving both hepatocytes and sinusoidal cells [12,13]. Increased liver iron may directly promote fibrogenesis by inducing oxidative stress and stimulating hepatic stellate cells activation through ferritin release [14], but increased iron stores have also been shown to promote hepatic insulin resistance by reducing glucose clearance [15]. Furthermore, iron depletion improved insulin resistance more than only lifestyle changes in patients with NAFLD [16].

A recent study in 480 Italian patients with NAFLD demonstrated a strong association between hepatocellular iron deposition and advanced liver fibrosis. However, the C282Y and H63D mutations of the *HFE* gene for hereditary hemochromatosis, the main cause of inherited parenchymal iron overload in individuals of European ancestry [17], were not associated with fibrosis due to the weak association with iron overload [18].

Thus, we hypothesized that other genes are implicated in determining the individual susceptibility to developing hepatocellular siderosis in patients with NAFLD, and sought to evaluate a wider panel of inherited factors.

Among the possible candidates, heterozygosity for the PiZ and PiS *alpha1-antitrypsin* (*AAT*) mutations has previously been associated with hyperferritinemia and sinusoidal iron in patients with NAFLD [19].

Also, *beta-globin* mutations were associated with both hepatic iron and fibrosis in patients with chronic hepatitis C [20]. Furthermore, patients with C282Y HFE hemochromatosis and beta-thalassemia trait have higher rates of iron accumulation and develop more severe iron-related complications [21].

Another potential candidate is the single nucleotide polymorphism IVS1–24 C>G of the iron exporter ferroportin (FPN), which has recently been associated with the severity of iron overload and the presence of liver disease in Southern European patients with hemochromatosis [22].

The aim of this study was thus to determine in 274 Italian patients with biopsy-proven NAFLD, whether a panel of selected gene mutations including *HFE*, *AAT*, *FPN*, and *beta-globin* variants are associated with severe liver damage by predisposing to hepatocellular iron accumulation.

## Methods

### *Patients*

We considered 274 out of 295 (92.9%) Italian patients with biopsy-proven NAFLD, whose DNA samples and complete clinical data were available. Other causes of liver disease were excluded, including increased alcohol intake (>30/20 g/day for M/F), as confirmed by at least one family member and carboxydesialylated transferrin determination, hereditary hemochromatosis (*HFE* C282Y+/+ and C282Y/H63D), hereditary iron overload due to *FPN* mutations, *AAT* deficiency (PiZ/Z, PiZ/S), chronic viral and autoimmune hepatitis, Wilson disease, and celiac disease. Part of this group had previously been described [7]. BMI and metabolic parameters, including glucose and lipid levels, ferritin, and liver enzymes (AST, ALT, and GGT), and evaluation of acquired causes of iron overload, were available for all patients. Serum hepcidin levels, available in a subset of patients, were determined as previously described [23]. Demographic and clinical features are shown in Table 1.

### *Controls*

The control group, matched for age, sex, and geographical origin, included 179 Italian subjects out of a larger series of 482 blood donors from Northern Italy without clinical and biochemical evidence of liver and metabolic disease and no alcohol abuse, and without alterations in iron parameters and anemia. We excluded subjects with ALT >35/30 IU/ml in males/females, GGT >35 IU/ml, BMI >28, abdominal circumference >100 cm, glucose levels $\geqslant$100 mg/dl, triglycerides $\geqslant$150 mg/dl, HDL $\leqslant$45/55 in M/F, or a fatty liver index >40, a value with high specificity to rule out NAFLD in the general population [24].

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 and was approved by our Institution Review Board.

### *Histological assessment*

Tissue sections were stained with hematoxylin and eosin, impregnated with silver for reticulin framework, and stained with trichrome for collagen. One expert pathologist unaware of clinical and genetic data reviewed all biopsies for fibrosis stage and the presence and pattern of liver siderosis. The presence of NASH was assessed according to Kleiner et al. [25]. The minimum biopsy size was 1.7 cm and the number of portal areas was 10. Histological re-evaluation of liver siderosis was performed by one expert pathologist according to Scheuer et al. [26].

When detected, hepatic iron accumulation was defined as hepatocellular or non-parenchymal according to the prevalent distribution pattern of siderosis [18].

**Table 1. Demographic and clinical features of 179 Italian healthy control subjects with normal liver enzymes, metabolic parameters, without alterations in iron metabolism, and erythropoiesis and 274 patients with NAFLD with available re-evaluation of histological siderosis.**

| Number | Controls 179 | NAFLD 274 |
|---|---|---|
| Sex F | 38 (21) | 49 (18) |
| Age years | 48.4 ± 13 | 48.7 ± 11.5 |
| BMI Kg/m$^2$ | 25.1 ± 2.7 | 27.1 ± 3.5*** |
| Hb g/dl | > 13 ^ | 14.4 ± 1.5 |
| MCV fl | > 85 ^ | 87 ± 9.2 |
| LDL cholesterol mg/dl | 118.7 ± 29 | 124.3 ± 42.3 |
| HDL cholesterol mg/dl | 55.2 ± 13 | 47.3 ± 14.2*** |
| Triglycerides mg/dl | 90.1 ±44 | 151.3 ± 89.3*** |
| Glucose mg/dl | 89.0 ± 10 | 97.7 ± 23.8*** |
| HOMA-IR | 2.7 ± 1.6 | 4.1 ± 3.2*** |
| ALT UI/ml | 21.8 ± 7 | 54.4 ± 40.5*** |
| GGT UI/ml | 23.7 ± 16 | 71.5 ± 79.7*** |
| Fibrosis stage | | |
| F0 | - | 140 (51) |
| F1 | - | 92 (33) |
| F2 | - | 20 (8) |
| F3 | - | 10 (4) |
| F4 | - | 12 (4) |
| *HFE* | | |
| C282Y +/- | 10 (5.6) | 28 (10.2) |
| H63D +/+ | 7 (3.%) | 13 (4.7) |
| Beta-thalassemic trait | 0 (0) ^ | 25 (9.1)*** |
| *AAT* PiZ or PiS | 7 (4) | 30 (10.9)* |
| *FPN* IVS1-24 C>G (n = 207) | 66 (36.7) | 69 (33.3) |

*** $p$ <0.0001 between patients and controls, * $p$ <0.05 between patients and controls, ^as enrolment criterium. ( ):% values; BMI: body mass index; Hb: hemoglobin concentration; MCV mean corpuscular volume.

### *Genetic analysis*

DNA was extracted from peripheral blood by the phenol–chloroform method. *HFE* genotype was genotyped by analysis of single nucleotide polymorphism with sequence allele specific-polymerase chain reaction (SAS-PCR) by personnel unaware of patients' clinical status. *FPN* genotype was assessed at first by analysis of SNP with SAS-PCR and then with a 5′nuclease TaqMan SNP genotyping assay. The primers and protocols used for the PCR reactions are shown in Supplementary Table S1.

The *AAT* genotype was genotyped by restriction analysis [19]. Random samples were confirmed by direct sequencing that provided concordant results in all cases. Quality controls were performed to verify the reproducibility of the results. Valid genotypic data were obtained for over 99% of subjects analyzed.

Patients with mean corpuscular volume value less than 78 fl, in the absence of iron deficiency (ferritin >12 ng/ml), and with increased $HbA_2$ levels (>3.5%), were considered heterozygous for *beta-globin* gene mutations (henceforth beta-trait) [27].

### *Statistical analysis*

Results are expressed as means ± standard deviation and considered significant when $p$ <0.05 (two-tailed). Mean values were compared by ANOVA and post hoc tests and frequencies by chi-square test.

The association between inherited variants and the presence of siderosis and fibrosis was evaluated by logistic regression analysis adjusted for confounders (as reported in the Results). Analyses were carried out with JMP 6.0 statistical analysis software (SAS Institute Inc, Cary, NC).