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Article type : Original Articles

Editor : Isabelle Leclercq

Evaluating the association of serum ferritin and hepatic iron with disease severity in non-alcoholic fatty liver disease

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List of abbreviations: NAFLD, non-alcoholic fatty liver disease; MetS, metabolic syndrome; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; SF, serum ferritin; HC, hepatocellular; RES, reticuloendothelial; DM, type 2 diabetes mellitus; IFG, impaired fasting glucose; TG, triglycerides; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; BMI, body mass index; PLT, platelet count; ALT, alanine aminotransferases; AST, aspartate aminotransferases; GGT, gamma-glutamiltranspeptidase; HOMA, homeostatic model assessment index; HbA1c, glycosylated hemoglobin; TIBC, total iron binding capacity; TSAT, transferrin saturation; CRN, clinical research network; SD, standard deviation; IQR, interquartile range; CRP, C reactive protein; NAS, NASH activity score; OR, odds ratio; GLC, glucose.

Conflicts of interest: none

Acknowledgments: E.B. was supported by a fellowship-grant from AIGO-M.I.M.I. (Associazione Italiana Gastroenterologi Ospedalieri-Montecatini Interactive Medicine International); A.P. was recipient of a Telethon grant (#GGP14285)

ABSTRACT

Background and aims. Hyperferritinemia, with or without increased hepatic iron, represents a common finding in non-alcoholic fatty liver disease (NAFLD). However, it is unclear whether it reflects hepatic inflammation or true iron-overload and, in case the latter is confirmed, whether this influences disease progression. We therefore explored the association between serum ferritin, degree and pattern of hepatic iron deposition and liver disease severity in patients with NAFLD.

Methods. We selected 468 patients with biopsy-proven NAFLD from two European centres. Iron, hepatic and metabolic parameters were collected at the time of liver biopsy. Iron deposits in hepatocytes and reticuloendothelial cells were assessed and graded. Diagnosis of non-alcoholic steatohepatitis (NASH) and fibrosis staging were performed.

Results. 122 (26%) patients had hyperferritinemia, whereas stainable hepatic iron was found in 116 (25%) patients (38% predominantly in hepatocytes, 20% in reticuloendothelial cells and 42% in both). Subjects with stainable hepatic iron, particularly those with a mixed pattern, had higher serum ferritin and transaminases but only a mixed pattern of iron deposition was among the variables significantly associated with presence of NASH. Serum ferritin was not associated with presence of NASH, however it increased with worsening fibrosis stage (F3 compared to F0-F1), and significantly decreased in stage F4.

Conclusions. A mixed pattern of hepatic iron deposition is associated with the presence of steatohepatitis, while serum ferritin increases with worsening fibrosis up to pre-cirrhotic stage. In individual NAFLD patients, serum ferritin could be evaluated as part of non-invasive diagnostic panels but not on its own.

Key words: NAFLD, iron, ferritin, histology

Lay summary: There are contrasting results on the association of serum ferritin and presence or pattern of hepatic iron deposition with disease severity in people with fatty liver. Therefore, we reviewed liver biopsies of NAFLD patients in order to provide a conclusive answer to the above question. We showed that iron deposition both in hepatocytes and macrophages (mixed pattern) was associated with non-alcoholic steatohepatitis, which is the progressive form of fatty liver. Serum ferritin alone could not be used as a marker of liver scarring (fibrosis).

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is increasingly prevalent in Western countries, affecting approximately 30% of unselected population[1]. It is usually diagnosed in patients with metabolic syndrome (MetS), but also in a small percentage of subjects with normal weight (7%)[2]. NAFLD encompasses a wide spectrum of histological conditions, from simple steatosis to steatohepatitis or NASH (characterized by ballooning and lobular inflammation) with subsequent development of fibrosis that can lead to cirrhosis. Despite its high prevalence, only a small

proportion of subjects with NAFLD develop NASH and face a higher risk of liver disease progression[3].

A high serum ferritin (SF) is a common finding in NAFLD, involving up to 30% of affected subjects, and it is often the biochemical abnormality that leads to medical attention. However, it is still unclear whether it simply reflects hepatic inflammation or represents true hepatic iron-overload[4,5]. In this context, SF has been proposed as a marker of both NASH and liver fibrosis with contrasting results[6-8] and has been incorporated in panels for liver fibrosis assessment[9]. More recently, SF has been proposed as an independent predictor of long-term mortality in NAFLD[10].

Mild-to-moderate hepatic iron accumulation is encountered in a number of liver diseases including chronic hepatitis C, alcoholic and non-alcoholic fatty liver disease and cirrhosis[11], but the underlying mechanism is unclear. Also, although it is known that iron excess can damage the liver by inducing oxidative stress and lipid peroxidation[12], in these settings the effect of iron accumulation is not completely understood.

Moreover, the relationship between SF levels, the pattern of iron deposition (hepatocellular (HC), reticuloendothelial (RES) or mixed)[13] and liver disease stage in NAFLD has not been elucidated.

The aim of the present study was therefore to assess the presence and pattern of hepatic iron accumulation in patients with biopsy-proven NAFLD, and to examine whether such a finding is associated with more severe/progressive liver disease. Furthermore, we analysed the role of serum ferritin as predictor of liver disease severity.

PATIENTS AND METHODS

2.1.Patients population

We retrospectively evaluated all consecutive outpatients (aged ≥18 years) with a liver biopsy showing NAFLD, irrespective of fibrosis severity, seen at the Hepatology clinic of the Royal Free Hospital (London, United Kingdom) or Policlinico Giaccone (Palermo, Italy) in a time frame of 20 and 12 years respectively, and finally included 468 out of 477 patients. Histological and clinical criteria for NAFLD definition and inclusion in the present study were: presence of steatosis in more than 5% of hepatocytes in the absence of features characteristic of other etiologies of liver disease[14]; alcohol intake lower than 20/30 g/day in females/males; absence of chronic liver disease of other etiologies such as viral hepatitis, autoimmune hepatitis, primary biliary cholangitis, hereditary hemochromatosis (evidence of iron overload and relevant genetic testing showing C282Y homozygosity, C282Y/H63D compound heterozygosity, H63D homozygosity), Wilson's disease, alpha-1-antitrypsin deficiency based on appropriate testing. We excluded causes of secondary hepatic steatosis, such as use of fatty liver-inducing drugs or previous gastro-intestinal by-pass surgery. Clinical information including presence of hypertension (blood pressure ≥ 140/90 mmHg measured in two different occasions and/or

antihypertensive drug treatment), type 2 diabetes mellitus (DM) or impaired fasting glucose (IFG), dyslipidemia (elevated triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), decreased high-density lipoprotein cholesterol (HDL-C)[15] or use of a lipid-lowering agent) and body mass index (BMI) were collected from clinical documentation recorded within 6 months from liver biopsy.

MetS was diagnosed according to the National Cholesterol Education Program Adult Treatment Panel III criteria[16], when at least three of the followings were present: enlarged waist circumference (≥102-88 cm, males-females), TG ≥1.7 mmol/L and/or medication use for elevated TG, reduced HDL cholesterol (<1.0-1.3 mmol/L, males-females) and/or medication use for reduced HDL cholesterol, blood pressure ≥130/85 mmHg and/or medication use for hypertension, fasting glucose ≥5.6 mmol/L and/or medication use for elevated glucose. Biochemical parameters included platelet count (PLT), aminotransferases (ALT, AST), bilirubin, serum albumin, gamma-glutamiltranspeptidase (GGT), fasting glucose, HOMA (homeostatic model assessment) index and glycosylated hemoglobin (HbA1c) when available.

2.2.Iron status

Serum iron, SF and total iron binding capacity (TIBC) and/or transferrin saturation (TSAT) were determined using automated biochemical methods. *HFE* genetic test had been performed in patients with hyperferritinemia (above 200 µg/L in females and 300 µg/L in males or menopause females) and an abnormal transferrin saturation (TSAT>45%) and in selected patients with hyperferritinemia and normal TSAT according to the physicians' discretion, by PCR-based techniques.

2.3.Histological assessment

All liver biopsy specimens were obtained by percutaneous or trans-jugular route, with a median length of 19 mm (6-58 mm). Specific criteria for liver biopsy were: length of at least 10 mm, comprising at least 6 portal tracts or less if cirrhotic or considered of sufficient quality for a diagnosis and staging by the pathologist. We included six biopsies <10 mm (6, 7, 8, 9, 9 and 9 mm respectively) presenting simple steatosis since the histopathologist was satisfied with the representativity of the sample and the results of the analysis were not changed by their removal (data not shown). Liver sections were routinely stained with hematoxylin/eosin, silver reticulin, blue aniline or Sirius red for collagen, Perls' Prussian blue for iron. Liver biopsies were centrally reviewed by a single pathologist in each centre. NAFLD lesions were scored according to the NASH Clinical Research Network (CRN) NAS scoring system[17]. NASH was diagnosed in the presence of the combination of any degree of hepatic steatosis, hepatocellular ballooning and lobular inflammation[18,19]. Hepatic fibrosis was staged on a 5-point scale (0=absence of fibrosis, 1=zone 3 perisinusoidal/perivenular fibrosis, 2=zone 3 and periportal fibrosis, 3=septal/bridging fibrosis, 4=cirrhosis[17]). Significant and advanced fibrosis were defined as stages ≥F2 and ≥F3, respectively. Advanced fibrosis was chosen as one of the main variables of interest based on previous studies demonstrating that it is associated with long-term clinical outcomes and increased mortality in NAFLD patients[20,21]. Inter-observer agreement regarding histological evaluation by the two pathologists was tested on a set of 30 slides by weighted Cohen's kappa, with a resulting k value for fibrosis of 0.76, meaning excellent agreement.The presence of iron was assessed both in hepatocytes and reticuloendothelial cells and the degree

of liver siderosis was classified according to a modified Scheuer's system (Table 1-S, Supporting Information[13,22]).

2.4 Ethical approval

Blood tests and liver biopsy were performed as part of the standard or routine care. Both centres had the approval from the local ethical committee to use registered parameters and liver biopsies for studies. The study was carried out in accordance with the principles of the Helsinki Declaration.

3. Statistical analysis

Continuous data were presented as mean ± standard deviation if parametric or median and interquartile range if nonparametric. Categorical data were presented as number and percentage. Comparisons between frequencies or percentages were performed by using the chi-square test or the Fisher's Exact Test. Between-group comparisons of continuous variables were performed using the Student's t-test or Analysis of Variance for normally distributed variables, and the Mann-Whitney or Kruskal-Wallis tests for non-normally distributed variables.

Multiple logistic regression analysis, stepwise approach, was used to examine the relationship between serum ferritin, presence and pattern of hepatic iron deposition and presence of NASH or fibrosis. All the variables that were associated with NASH at the univariate analysis with a statistical significance corresponding to a *p* value up to 0.1 were included in the multivariate analysis. Similarly, logistic regression

analysis was performed to find variables associated to advanced fibrosis. A two-sided *p* value <0.05 was considered significant. All analyses were performed using IBM SPSS (22.0, IBM, New York, USA).

RESULTS

4.1.Characteristics of the NAFLD population

Of the 477 patients initially considered, two were subsequently excluded because of suboptimal biopsy sample, three because of missing clinical and/or biochemical data and four because of compound C282Y/H63D heterozygosity at the *HFE* gene test. The demographic, clinical, biochemical, and histological details of the 468 patients included in the study are shown in Table 1: the mean age was 47 years, 76% of patients were of Caucasian ethnicity and 38% were females. The mean BMI was 30.4 kg/m^2; a history of IFG was observed in 19% patients and DM in 29% of patients, of which 65% were on hypoglycemic agents, mainly metformin. There was a high percentage of patients affected by dyslipidemia (68%), of which only 25% were on statin treatment. Hypertension had already been diagnosed in 32% of subjects, and 62% were treated mainly with renin-angiotensin system inhibitors. Female subjects were more likely to have diabetes and hypertension (45% versus 29%, p<0.001, and 39% versus 27%, p=0.009, respectively). None of the included patients had radiological or histological evidence of HCC at the time of liver biopsy.

Histological criteria for NASH were fulfilled in 247 (53%) patients, while advanced fibrosis was prevalent in 89 (19%) patients (81 of which having NASH and 8 with likely 'burnt-out NASH'). The British cohort had a higher prevalence of obesity (mean BMI 31 vs 29, p=0.003), hypertension (37 vs 24%, p=0.003) and dyslipidemia than the Italian cohort (77 vs 55%, p<0.0001) and this was reflected by a higher prevalence of patients who fulfilled the criteria for MetS (37 vs 20%, p<0.0001). On the other hand, a higher proportion of patients with NASH (83 vs 33%, p<0.0001) and hepatic iron deposition (36 vs 17%, p<0.0001) was found in the Italian cohort. No difference in the proportion of patients with advanced fibrosis was found when comparing the two cohorts (18 vs 20%, p=0.53).

The characteristics of the 247 patients with NASH are shown in Table 2. They were more likely to be older, have a higher BMI and be affected by diabetes when compared to patients without NASH. Interestingly, the prevalence of MetS was not different between the two groups. Diabetic patients with NASH had a higher probability of suboptimal glycemic control if compared to diabetic patients without NASH as shown by a significantly different distribution of HbA1c values (54(42-96) vs 44(38-55) mmol/mol, p=0.02), despite a similar proportion of patients on hypoglycemic treatment (data not shown); also, the HOMA index was higher in NASH patients (4.1 vs 1.7, p=0.001), reflecting higher insulin resistance. Serum transaminases were significantly higher in patients with NASH, with a median value of 1.5-2xULN. When considering general markers of inflammation (CRP), no difference was found between patients with and without NASH.

4.2.Hepatic iron deposition

Stainable hepatic iron was found in 116 (25%) patients: the pattern of iron deposition was mainly HC in 42 patients (36%), mainly reticuloendothelial in 24 patients (21%) and mixed in 50 (43%) patients. An iron grade 2 was found in 11 (9%) patients and grade 3 in 3 (3%) patients; none had an iron grade 4.

Clinical and laboratory data of subjects according to the presence of stainable hepatic iron and to the pattern of iron deposition are shown in Table 3: subjects with stainable hepatic iron were more likely to be male and have a lower BMI, had increasingly higher levels of SF (particularly Mixed>RES>HC, $p<0.0001$), serum iron (23.4 vs. 17 µmol/L, $p<0.0001$) and TSAT (37 vs 27 %, $p=0.04$), with a higher proportion of patients with hyperferritinemia (60% vs 15%, $p<0.0001$) and TSAT >45% (27% vs 10%, $p=0.003$, data not shown). In addition, they had significantly higher levels of ALT ($p=0.007$).

4.3.SF, iron deposition and liver disease severity

Hyperferritinemia was found in 122 (26%) patients, with no significant difference between the two cohorts (34 vs 28%, $p=0.17$).

Patients with hyperferritinemia had no difference in sex (p=0.23), ethnicity (p=0.64), cohort (0.17), age (0.39), BMI (p=0.83), prevalence of diabetes and or/IFG compared to non hyperferritinemics. When looking at mild alcohol consumption, an increasing trend in SF values was found for increasing alcohol intake; however, the prevalence

of hyperferritinemia was not different between abstinent vs mild drinkers (p=0.1), even when considering subclasses of alcohol intake (p=0.097, Table 2-S, Supporting Information).

Looking at the single components of histological NASH, a positive association was found between SF and increasing degree of steatosis (p=0.008) but not with hepatocellular ballooning or lobular inflammation and overall SF did not associate with diagnosis of NASH (Table 2). Interestingly, SF showed a peculiar pattern throughout fibrosis stages, increasing from F0-F1 to F3, and subsequently decreasing in cirrhosis (Figure 1). When comparing the inter-group SF distribution, it was significantly higher for F3 compared to F0-F1 (p=0.024), with an average increase of 14% from F0-F1 to F2 and of 61% from F2 to F3 and a decrease of 43% from F3 to F4.

On the other hand, patients with a mixed pattern of iron deposition were more likely to have NASH if compared to patients with other patterns of iron deposition or to patients without hepatic iron. No difference was seen between patients with and without hepatic stainable iron when considering the individual components of histological NASH, NAS >3 or >5 (data not shown).

No association was found between presence of stainable hepatic iron and fibrosis (mild, significant, advanced).

As shown in Table 4, at the multivariate analysis, BMI (OR 1.06, 95% CI 1.02-1.11, p=0.005), presence of diabetes (OR 2.13, 95% CI 1.31-3.45, p=0.01), ALT levels (OR 1.012, 95% CI 1.006-1.017, p<0.0001) and a mixed pattern of hepatic iron deposition, (OR 2.23, 95% CI 1.08-4.6, p=0.03) were independently associated with the presence of NASH. Such variables remained the only significantly associated even after removing patients with TSAT higher than 45% and correcting for cohort and sex or when including alcohol as a categorical or continuous variable but not when considering each cohort separately (data not shown).

Advanced fibrosis was associated with advanced age (OR 1.04, 95% CI 1.01-1.06, p=0.01), presence of diabetes (OR 3.09, 95% CI 1.65-5.79, p<0.0001) and low platelet count (OR 0.987; 95% CI 0.982-0.992, p<0.0001) but not with serum ferritin.

5. Discussion

In this study, we showed that a mixed pattern of hepatic iron deposition is associated with the presence of NASH, while ferritin levels increase with worsening of fibrosis up to a pre-cirrhotic stage but are not independent predictors of advanced fibrosis.

Stainable hepatic iron was found in 25% of patients, in line with previous studies reporting a prevalence ranging from 14% to 50%[23-25]. The degree of iron accumulation was rarely above a mild to moderate degree and never above grade 3: this can be considered a typical characteristic of NAFLD patients with stainable hepatic iron, and would be one of the reasons explaining why many studies of

phlebotomy in NAFLD have failed to show a benefit or improvement in liver histology[26,27].

A mixed pattern of iron deposition was the most common, accounting for 43% of patients with stainable hepatic iron and this is consistent with data from the literature[23,24,5]. Noteworthy, a mixed pattern of iron deposition was independently associated to the presence of histological NASH. However, hepatic iron was not associated with fibrosis, which was recently identified as the main determinant of prognosis in NAFLD patients. This would suggest that NAFLD patients who tend to accumulate iron in both hepatocytes and macrophages for multiple factors (genetics, local effect of inflammation on iron metabolism, leaking from dying hepatocytes and subsequent phagocytosis by liver macrophages[28]) are at higher risk to have a more active form of liver disease, but might still require a further hit to trigger and maintain fibrogenesis, as per the 'multiple hit' hypothesis[3].

Our results are different from those previously presented by other groups. In 849 patients enrolled in the NASH CRN[24], despite similar rates of hepatic iron and mixed pattern deposition, it was only RES iron which was associated with advanced fibrosis. At variance to these data, in another study by Valenti et al.[23] including 587 NAFLD patients, hepatocellular iron was associated with a 1.7-fold higher risk of fibrosis ≥F1.

These discrepancies are difficult to explain. Preliminary data from next generation sequencing studies have shown how variants/polymorphisms of genes involved in iron metabolism, other than *HFE*, could have an impact on SF levels and hepatic iron in NAFLD patients[29,30], therefore differences linked to ethnicity and geographical provenience are to be expected.

As regards to SF, the prevalence of hyperferritinemia was 26% in our cohort. SF was higher in patients with detectable hepatic iron, particularly those with a mixed pattern of iron deposition. Similarly, in a study by Ryan et al.[31], SF correlated with liver iron determined by MRI in 51 NAFLD patients, and with iron deposition in RES in a retrospective cohort of 404 NAFLD patients.

Interestingly, in our study SF was associated with increasing degree of steatosis but not with NASH and, more importantly, SF was not independently associated with fibrosis. Previous studies have indeed tried to understand the meaning of a raised SF in NAFLD patients, particularly examining the role of SF as a noninvasive marker of NASH and/or fibrosis, some underlying its positive correlation with increasing fibrosis [7] [6] , others obtaining opposite results [8]. In a study including 1201 patients with biopsy-proven NAFLD, SF was associated with presence of steatosis, lobular inflammation and ballooning, but had suboptimal diagnostic performance for detecting significant or advanced fibrosis[32].

When addressing the potential effect of mild-moderate alcohol intake on SF, a previous observational study on more than 12000 patients showed that consumption of two or more alcoholic drinks per day was associated with significant risk of iron overload, whereas consumption of a lesser amount was associated with reduced risk of iron deficiency[33]. In our cohort, SF increased with increasing degree of alcohol intake, but only patients who drank more than 10g/day of alcohol showed significantly higher SF when compared to total abstinent and lower alcohol intake subgroups. Moreover, there was no association between alcohol intake and hepatic iron deposition, NASH or fibrosis, suggesting that in NAFLD patients, the effect of mild alcohol consumption on iron metabolism and NASH is negligible, and different pathways are involved.

Few studies have explored the role of diet and its influence on SF and iron levels: although there are not many solid data, the consumption of red meat or insufficient amount of vegetables, common in MetS patients, seems to be associated with higher SF levels[34]. Unfortunately, we did not have such information from our cohort.

Considering our results, and the fact that ferritin levels in NAFLD patients tend to be higher than in hemochromatosis patients with the same amount of hepatic iron[35], hyperferritinemia in NAFLD might be due both to increased iron stores (without excluding the direct release by macrophage/hepatocytes in response to necrosis and inflammation-mediated mechanisms) and to the systemic inflammatory and metabolically altered status typical for these patients rather than reflect hepatic disease severity. This would be true in our NAFLD population even if raised SF was

not associated with high CRP (a suboptimal marker of inflammatory activity) or single MetS components. Moreover, this would explain the reduction of SF seen in cirrhotic patients, where the extinguishing inflammation, the decrease in liver synthetic activity and the development of portal hypertension, could concur in reducing iron adsorption and ferritin synthesis.

The strengths of this study are as follows: first, the number of included patients and the multicentric structure (allowing inclusion of patients from Northern and Southern Europe); secondly, the precise histological review, performed by one dedicated histopathologist in each centre using pre-determined, widely used criteria.

The limitations are linked to the retrospective type of the study, therefore some missing clinical data, with a possible underestimation of MetS, the lack of *HFE* genetic analysis in patients with SF within the range of normal, and the lack of quantitative measurement of hepatic iron concentration (HIC) and cytokines, which would have permitted a more accurate description of the hepatic iron content and inflammatory status of our patients.

In conclusion, the findings presented here allow us to identify two possible "iron signatures": 1) in NAFLD patients SF is frequently high, but in most cases iron does not accumulate in the liver: therefore SF likely reflects a systemic dysmetabolic/inflammatory state; 2) a minority of NAFLD patients with high SF display hepatic iron accumulation in RES cells and hepatocytes (mixed pattern) and

have NASH: here hepatic iron accumulation likely results from more pronounced intrahepatic necro-inflammatory events leading to an “iron retention phenomenon” possibly driven by hepcidin induction.

Do increased iron and ferritin have a direct causative role in NASH? Unfortunately, answering such an important question was beyond the aim of the study; still, an hypothesis can be made: the damaging and pro-inflammatory activity of hepatocellular iron has been already established by a number of studies (with a less clear role of RES iron)[36]; interestingly, also ferritin has been reported to act as a proinflammatory cytokine able to activate hepatic stellate cells involved in liver fibrosis[37].

These hypotheses need corroboration; however, we think that our results provide a further confirmation of the complex relationship between NAFLD and iron metabolism, likely regulated by different and antagonistic pathways: this is intriguing and deserves to be explored by future studies.

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Table 1. Demographic, clinical data and laboratory findings of the 468 patients with nonalcoholic fatty liver disease.

	British cohort (n=282)	Italian cohort (n=186)	*p*	Total (n=468)
Age, years	49±13	46±13	0.03	47±13
Caucasian ethnicity, n (%)	168 (60)	186 (100)	<0.0001	354 (76)
Females, n (%)	112 (40)	65 (35)	0.29	177 (38)
BMI, Kg/m²	31±6	29±6	0.003	30.4±5.8
Alcohol>10g/day, n (%)	20 (7)	7 (4)	0.13	27 (5.8)
Hypertension, n (%)	104 (37)	45 (24)	0.003	149 (32)
Dyslipidemia, n (%)	217 (77)	103 (55)	<0.0001	320 (68)
Diabetes, n (%)	90 (32)	43 (23)	0.03	133 (29)
IFG, n (%)	58 (21)	32 (17)	0.36	90 (19)
MetS, n (%)	105 (37)	38 (20)	<0.0001	143 (31)
Platelets , x 10^9/L	238 (195-280)	228 (190-266)	0.18	233 (192-277)
ALT, U/L	62 (34-90)	69 (39-98)	0.03	65 (37-93)
AST, U/L	41 (26-55)	38 (25-51)	0.26	40 (26-54)
GGT, U/L	77 (24-130)	66 (19-113)	0.009	73 (24-121)
Bilirubin, µmol/L	11 (7-14)	10 (7-13)	0.97	11 (7-14)
Albumin, g/dL	4.6 (4.4-4.8)	4.6 (4.4-4.9)	0.98	4.6 (4.4-4.8)
INR >1.2, n (%)	14 (5)	1 (0.5)	0.0061	15 (3.3)
Cholesterol, mmol/L	5.4 (4.5-6.3)	5.1 (4.4-5.8)	0.3	5.2 (4.4-6)
HDL, mmol/L	1.2 (0.9-1.5)	1.3 (1.1-1.5)	0.9	1.2 (1-1.4)
TG, mmol/L	1.8 (1-2.4)	1.4 (0.9-2)	<0.0001	1.6 (1-2.2)
GLC, mmol/L	5.4 (4.4-6.4)	5.2 (4.6-5.8)	0.035	5.3 (4.6-6)
Ferritin, µg/L	187 (54-320)	189 (82-295)	0.98	188 (61-314)
Ferritin>ULN, n (%)	78 (36)	44 (26)	0.05	122 (32)
TSAT, %	32 (23-41)	20 (13-27)	<0.0001	29 (20-38)
NASH, n (%)	92 (33)	155 (83)	<0.0001	247 (53)
F0, n (%)	158 (56)	49 (26)	<0.0001	207 (44)
F1, n (%)	52 (18)	52 (28)	0.02	104 (22)
F2, n (%)	21 (7)	47 (25)	<0.0001	68 (15)
F3, n (%)	16 (6)	25 (13)	0.004	41 (9)
F4, n (%)	35 (12)	13 (7)	0.06	48 (10)
≥ F3, n (%)	51 (18)	38 (20)	0.53	89 (19)
Hepatic iron, n (%)	49 (17)	67 (36)	<0.0001	116 (25)
HC, n (%)	22 (8)	20 (11)	0.27	42 (9)
RES, n (%)	14 (5)	10 (5)	0.84	24 (5)
Mixed, n (%)	13 (5)	37 (20)	<0.0001	50 (11)

Data are reported as number and percentage (%), mean ± standard deviation, median and interquartile range (IQR). Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; INR, international normalised ratio; HDL, high density lipoprotein;

TG, triglyceride; GLC, serum glucose; TSAT, transferrin saturation; NASH, nonalcoholic steatohepatitis; HC, hepatocellular iron deposition; RES, reticuloendothelial system cells iron deposition.

Table 2. Comparison of patients according to the presence of NASH.

	NASH (n=247)	No NASH (n=221)	*p*
British/Italian, n(%)	92(37)/155(63)	190(86)/31(14)	<0.0001
Age, years	50 ±13	45±12	0.014
Females, n (%)	99 (40)	78 (35)	0.29
BMI, Kg/m²	31±6	29±6	0.014
Hypertension, n (%)	87 (35)	62 (29)	0.12
Dyslipidemia, n (%)	162 (66)	158 (72)	0.14
Diabetes, n (%)	114 (46)	49 (23)	<0.0001
IFG, n (%)	88 (39)	79 (40)	0.74
MetS, n (%)	81 (33)	62 (28)	0.26
Alcohol, g/day	1.3 ± 4	2.1 ± 5	0.055
Alcohol>10g/d, n(%)	11 (4.5)	16 (7)	0.19
HOMA	4.1 ± 3.7	1.7 ± 1.2	0.001
WCC, x10^9/L	6.56 (5.5-7.6)	6.8 (5.5-8.1)	0.63
ALT, U/L	74 (43-105)	58 (34-72)	<0.0001
GGT, U/L	68 (25-111)	81 (29-133)	0.13
Bilirubin, µmol/L	10 (7-13)	11 (8-14)	0.64
Cholesterol, mmol/l	5.2 (4.5-6)	5.2 (4.3-6.1)	0.75
HDL (mmol/L)	1.2 (1-1.4)	1.3 (1-1.6)	0.15
TG, mmol/L	1.5 (0.9-2.1)	1.7 1.1-2.3)	0.41
CRP, mg/L	4.5 (0.5-8.5)	3 (0.5-5.5)	0.24
Serum Iron, µmol/L	17.3 (12-22)	18.6 (13-23)	0.25
Ferritin, µg/L	198 (53-343)	181 (64-298)	0.42
Ferritin>ULN, n (%)	72 (26)	50 (23)	0.11
TSAT, %	24 (16-32)	33 (24-42)	0.001
Hepatic Iron, n (%)	66 (27)	50 (23)	0.31
HC	20 (8)	22 (10)	0.48
RES	11 (4)	13 (6)	0.49
Mixed	35 (14)	15 (7)	0.01

Data are reported as number and percentage (%), mean ± standard deviation, median and interquartile range (IQR). Abbreviations: BMI, body mass index; HOMA IR, homeostatic model for assessment of insulin resistance; WCC, white cells count; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase, HDL, high density lipoprotein; TG, triglyceride; CRP, C reactive protein; TSAT, transferrin saturation; HC, hepatocellular iron deposition; RES, reticuloendothelial system cells iron deposition.

Table 3. Demographic, clinical data and laboratory findings of the 468 patients with nonalcoholic fatty liver disease according to the presence of stainable iron in the liver and the pattern of iron deposition.

	Hepatic Iron (n=116)	No hepatic Iron (n=352)	*p*	HC iron (n=42)	RES iron (n=24)	Mixed (n=50)	*p*
Age, years	47±13	48±13	0.79	47 ±14	48±15	47±11	0.97
Females,n(%)	22 (19)	155 (44)	<0.001	8 (19)	5 (21)	9 (18)	0.95
BMI, Kg/m²	29 ± 5	31 ± 6	0.03	29±5	30.6±4	29±5	0.14
Alcohol, g/day	2.1±	1.5±4.5	0.23	2.2±5	1.6±4	2.3±5	0.61
Alcohol>10g/d,n(%)	9 (7)	18 (5)	0.28	4 (9)	1 (4)	4 (8)	0.58
HTN, n (%)	31 (27)	118 (33.8)	0.17	17 (40)	7 (30)	7 (14)	0.016
DM, n (%)	37 (32)	126 (36)	0.44	14 (33)	6 (26)	17 (34)	0.6
GLC, mmol/L	5.2(4.5-6)	5.3(2.5-6.1)	0.11	5.3 (4.6-6)	4.9(4.2-6)	5 (4.3-6)	0.6
HOMA-IR	3.2±2.9	4.4±3.8	0.14	3.5±3.1	3.8±3.2	4±2.7	0.35
PLT, x10^9/L	212 (78)	240 (76)	0.001	230 (116)	221 (53)	206 (58)	0.48
ALT, U/L	77(43-111)	64 (37-91)	0.007	77 (41-112)	70 (43-97)	76(40-111)	0.99
Albumin, g/dL	4.7 (4.4-5)	4.6(4.3-4.8)	0.05	4.7 (4.4-5)	4.7(4.5-5)	4.7(4.5-5)	0.67
Iron, µmol/L	22 (19-25)	17 (12-21)	<0.001	25 (21-28)	19 (15-23)	22 (17-27)	<0.001
SF, µg/L (IQR)	427(490)	146 (160)	<0.001	388 (372)	427 (517)	501 (453)	<0.001
SF>ULN,n (%)	70 (60)	52 (15)	<0.001	19 (45)	12 (50)	39 (78)	0.003
TSAT, %	37 (29-45)	27 (15-39)	0.01	47 (32-62)	33 (25-41)	31 (20-42)	0.04
≥ F1, n (%)	67 (58)	193 (55)	0.58	23 (55)	12 (50)	32 (64)	0.61
≥ F2, n (%)	41 (35)	115 (33)	0.6	16 (38)	7 (29)	18 (36)	0.84
≥ F3, n (%)	24 (21)	65 (19)	0.59	11 (26)	4 (17)	9 (18)	0.66
F4, n (%)	11 (9)	37 (10)	0.67	5 (11)	1 (5)	5 (10)	0.79
NASH, n (%)	66 (57)	181 (51)	0.31	20 (48)	11 (46)	35 (70)	0.05
NAS	3.5±1.8	3.4±1.7	0.49	3.4±1.9	3.1±1.9	3.9±1.8	0.24

Data are reported as number and percentage (%), mean ± standard deviation, median and interquartile range (IQR). Abbreviations: BMI, body mass index; HTN, hypertension; DM, type 2 diabetes mellitus; GLC, serum glucose; HOMA IR, homeostatic model for assessment of insulin resistance; PLT, platelets; ALT, alanine aminotrans-

ferase; TSAT, transferrin saturation; HC, hepatocellular iron deposition; RES, reticuloendothelial system cells iron deposition; NASH, nonalcoholic steatohepatitis; NAS, NASH activity score.

Table 4. Predictors of NASH at univariate and multivariable analysis

	OR (95% CI)	***p***	**OR (95% CI)**	***p***
Age	1.01 (1.003-1.03)	0.01		ns
Sex	1.22 (0.84-1.78)	0.28		ns
BMI	1.06 (1.02-1.1)	0.002	1.06 (1.02-1.11)	0.005
Hypertension	1.4 (0.92-2.01)	0.1		ns
Diabetes	2.11 (1.39-3.21)	<0.0001	2.13 (1.31-3.45)	0.01
ALT	1.007 (1.003-1.01)	<0.0001	1.01 (1.006-1.017)	<0.0001
Ferritin	1.001 (1-1.001)	0.07		ns
Iron pattern				
HC iron	0.86 (0.45-1.63)	0.64	0.72 (0.32-1.63)	0.43
RES iron	0.79 (0.35-1.83)	0.59	0.64 (0.24-1.72)	0.12
Mixed iron	2.21 (1.16-4.18)	0.01	2.23 (1.08-4.6)	0.03

Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; HC, hepato-cellular iron deposition; RES, reticuloendothelial system cells iron deposition; OR, odds ratio; CI, confidence interval.