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ORIGINAL ARTICLE

Associations between higher plasma ferritin and hepcidin levels with liver stiffness in patients with type 2 diabetes: An exploratory study

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Abstract

Background: Currently, there is no information about the association between circulating levels of ferritin and hepcidin and liver fibrosis in patients with type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD).

Methods: We enrolled 153 patients with T2DM with no known liver diseases, who consecutively attended our diabetes outpatient service and who underwent liver ultrasonography and liver stiffness measurement (LSM) by vibration-controlled transient elastography (Fibroscan® for the non-invasive assessment of liver fibrosis). Plasma ferritin and hepcidin concentrations were measured with an electrochemiluminescence immunoassay and mass spectrometry-based assay, respectively.

Results: After stratification of patients by LSM tertiles [1st tertile median LSM: 3.6 (interquartile range: 3.3–4.0) kPa, 2nd tertile: 5.3 (4.9–5.9) kPa and 3rd tertile: 7.9 (6.7–9.4) kPa], we found that plasma ferritin and hepcidin concentrations increased across LSM tertiles [median ferritin: 68.7 (interquartile range: 25.1–147) vs. 85.8 (48.3–139)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; GGT, gamma-glutamyl transferase; HOMA-IR, homeostasis model assessment—insulin resistance; hs-CRP, high-sensitivity C-reactive protein; LSM, liver stiffness measurement; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain-containing-3; T2DM, type 2 diabetes mellitus.

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vs. 111 (59.3–203) μg/L, $p=0.021$; median hepcidin: 2.5 (1.1–5.2) vs. 4.4 (2.5–7.3) vs. 4.1 (1.9–6.8) nmol/L, $p=0.032$]. After adjustment for age, sex, diabetes duration, waist circumference, haemoglobin A1c, HOMA-insulin resistance score, triglycerides, haemoglobin, presence of hepatic steatosis on ultrasonography and patatin-like phospholipase domain-containing-3 (*PNPLA3*) rs738409 genetic variant, higher plasma ferritin levels were associated with greater LSM values (adjusted-odds ratio 2.10, 95% confidence interval 1.23–3.57, $p=0.005$). Higher plasma hepcidin levels were also associated with greater LSM values (adjusted-odds ratio 1.90, 95% confidence interval 1.15–3.13, $p=0.013$).

Conclusions: Higher levels of plasma ferritin and hepcidin were associated with greater NAFLD-related liver fibrosis (assessed by LSM) in patients with T2DM, even after adjustment for established cardiometabolic risk factors, diabetes-related variables and other potential confounders.

KEYWORDS

fatty liver, ferritin, hepcidin, liver stiffness measurement, non-alcoholic fatty liver disease, type 2 diabetes

1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a metabolic liver disease that is tightly linked with obesity, insulin resistance and type 2 diabetes mellitus (T2DM).[1,2] To date, NAFLD has become the most common liver disease worldwide,[1–3] affecting up to ~30% of adults in the general population[4] and up to ~70% of patients with T2DM.[5,6] Compared with individuals without diabetes, patients with T2DM are more likely to develop the more severe histological forms of NAFLD, such as non-alcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis.[7,8] A 2019 meta-analysis of observational studies reported that the global prevalence rates of biopsy-confirmed NASH and advanced fibrosis in people with T2DM were 37% and 17%, respectively.[5]

NAFLD is commonly asymptomatic and frequently detected incidentally by serum liver enzyme levels or imaging methods (such as liver ultrasonography) performed for other reasons. Mild-to-moderate hyperferritinemia may be present in nearly 20%–25% of patients with NAFLD.[9] Ferritin is an iron storage protein reflecting body iron stores.[9] Ferritin is also an acute-phase protein, and its circulating levels may be increased in cardiometabolic disorders, such as obesity, metabolic syndrome and cardiovascular disease,[9] which are conditions often observed in people with NAFLD. In the last years, accumulating evidence suggested that higher plasma ferritin levels are also associated with more severe liver fibrosis,[10–17] vascular damage[18] and increased all-cause mortality[19] in cohorts of patients with biopsy-proven NAFLD. Similarly, obesity-related metabolic disorders are associated with an increased production of hepcidin,[20,21] which is a peptide hormone that is released mainly from hepatocytes and acts as a key regulator of the entry of iron into the circulation.[21] In individuals not affected by haemochromatosis, higher plasma hepcidin levels are associated with higher plasma ferritin levels and tissue iron overload.[22] It is thought that tissue (hepatic) iron deposition may also trigger low-grade inflammation and oxidative stress, thus promoting the development of NASH and liver fibrosis.[9,23] Although there are still conflicting results, higher circulating hepcidin levels seem to be also associated with advanced NAFLD in some studies.[20,24–28]

To our knowledge, however, it is currently unknown whether an association also exists between circulating levels of ferritin and hepcidin with liver fibrosis among patients with T2DM and NAFLD. We believe that this topic is of clinical importance because the non-invasive identification of T2DM patients with NAFLD and elevated iron storage biomarkers might identify a subgroup of individuals at substantially higher risk of developing fibrotic NAFLD and cardiovascular disease (i.e. the leading cause of mortality in this patient population[6,29]).

Thus, the main aim of our exploratory cross-sectional study was to assess whether circulating levels of ferritin and hepcidin are associated with greater NAFLD-related liver fibrosis, as non-invasively assessed by vibration-controlled transient elastography (using the Fibroscan® device), in adult individuals with T2DM.

Lay Summary

Higher levels of plasma ferritin and hepcidin were associated with greater NAFLD-related liver fibrosis in patients with type 2 diabetes mellitus. These associations remained statistically significant after adjusting for established cardiometabolic risk factors, diabetes-related variables and other potential confounding factors.

2 | MATERIALS AND METHODS

2.1 | Participants

We enrolled 153 individuals with established T2DM, who consecutively attended our diabetes outpatient service during a 6-month period, and who accepted to undergo liver ultrasonography and vibration-controlled transient elastography for diagnosing and staging NAFLD. We excluded patients with: (a) significant alcohol consumption (defined as >20 grams of alcohol per day) and other known causes of chronic liver diseases (e.g. virus, drugs, or autoimmunity); (b) prior history of cirrhosis of any aetiology, active cancer, end-stage kidney disease (defined as estimated glomerular filtration rate [e-GFR] <15 mL/min/1.73 m^2 or chronic dialysis) and impaired thyroid function; (c) chronic use of potentially hepatotoxic drugs, such as nonsteroidal anti-inflammatory drugs, steroids, tamoxifen, amiodarone, methotrexate or use of hormone replacement treatment (for women only); and (d) treatment with insulin. According to the technical limitations of the vibration-controlled transient elastography, patients with congestive heart failure or free abdominal fluid were also excluded from the study. No participants had chronic blood losses, chronic intestinal diseases, or were chronically treated with blood transfusions or assumed iron supplementation. Most participants enrolled in the present study were also included in our previous study exploring the prevalence of NAFLD and its associations with chronic vascular complications of diabetes.[30]

2.2 | Clinical and laboratory data

Body mass index (BMI) was measured as kilograms divided by the square of height in meters. Waist circumference was measured at the midpoint between the lowest rib and the iliac crest. Blood pressure was measured with a standard sphygmomanometer after the subject had been seated quietly for at least 5 min. Subjects were considered to have hypertension if their blood pressure was ≥140/90 mmHg or if they were taking any anti-hypertensive agents.

Venous blood samples were collected in the morning after an overnight fast. Complete blood count, liver enzymes (aspartate aminotransferase [AST], alanine aminotransferase [ALT], gamma-glutamyl-transferase [GGT]), lipids, creatinine, high-sensitivity C-reactive protein (hs-CRP) and other biochemical blood parameters were measured using standard laboratory procedures at the central Laboratory of our hospital, using relative reference techniques and a Cobas® 8000 modular analyser (Roche Diagnostics GmbH, Mannheim, Germany). Haemoglobin A1c (HbA1c) was measured using the high-performance liquid chromatography analyzer Tosoh-G7 (Tosoh Bioscience Inc., Tokyo, Japan). Serum insulin concentration was measured using a chemiluminescent immunoassay (LIAISON, Diasorin, Saluggia, Italy). Homeostasis model assessment (HOMA-IR) score was used for estimating insulin resistance.

Glomerular filtration rate was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.[31] Chronic kidney disease (CKD) was defined as the presence of e-GFR <60 mL/min/1.73 m^2. The presence of ischemic heart disease was defined as a documented history of myocardial infarction, angina pectoris or coronary revascularization procedures. The presence of diabetic retinopathy, diagnosed with fundoscopy after pupillary dilation, was also recorded in all participants.

2.3 | Measurements of plasma iron storage biomarkers

Blood samples for measurement of plasma iron, transferrin, ferritin and hepcidin were collected into lithium heparin tubes and centrifuged immediately after their arrival in the laboratory. Plasma samples were then stored at −80°C until analysis. An expert laboratory technician, who was blinded to participants' clinical details, performed the measurements of plasma iron, transferrin, ferritin and hepcidin concentrations and the calculation of transferrin saturation. These assays, except for hepcidin, were performed by the fully automated analyser Cobas® 8000 using standard laboratory methods: ferritin by electrochemiluminescence (ECLIA), transferrin by immunoturbidimetry and iron by photometry (ferrozine reactif). The transferrin saturation was calculated using the following formula: transferrin saturation [%] = plasma iron [μmol/L]/plasma transferrin [g/L] × 3.984. All solvents, reagents and kits needed for these tests were purchased from Roche Diagnostics (Monza, Italy).

Plasma hepcidin concentration was assessed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) after minor modifications of a method included in an international harmonization study.[32] Hepcidin-25 standards, both native and isotopic-labelled internal standards (Asp-Thr-His-[13C9,15N] Phe-Pro-Ile-Cys-Ile-[13C9,15N]Phe-Cys-Cys-[15 N]Gly-Cys-Cys-His-Arg-Ser-Lys-Cys-Gly-Met-Cys-Cys-Lys-Thr (Mr2810.2)), were purchased from Peptide International (Louisville, Kentucky, USA). Samples were treated by solid-phase extraction using Oasis hydrophilic–lipophilic balanced reversed-phase cartridges (Waters, Milan, Italy). High-performance liquid chromatography (HPLC) was performed using an X-Terra MS C182.5 μm (Waters), and detection was obtained using a Triple Quad LC–MS/MS (Agilent Technologies, Santa Clara, CA, USA).

2.4 | Liver ultrasonography and vibration-controlled transient elastography

A single expert physician blinded to participants' clinical and biochemical details performed both liver ultrasonography and vibration-controlled transient elastography, on the same day, in all participants.

Hepatic steatosis was diagnosed by ultrasonography using a 4 MHz probe (MyLab 70, Esaote Group, Genova, Italy) according to specific ultrasonographic features, such as diffuse hyperechogenicity of the liver relative to kidneys, ultrasonographic beam

attenuation and poor visualization of intra-hepatic vessel borders and diaphragm.[30]

Liver stiffness measurement (LSM) was performed in all patients after at least 8 hours of fasting, using Fibroscan® (Echosens, Paris, France) with an M probe. We did not have the Fibroscan® XL probe for subjects with severe obesity. The accuracy of the M probe to detect significant fibrosis is excellent in individuals with mild or moderate obesity (BMI ≤35 kg/m^2). In our study, only a few patients had a BMI >35 kg/m^2. Our Fibroscan® device was not equipped with a controlled attenuation parameter (CAP) to non-invasively assess hepatic steatosis.[30] Each patient's LSM was considered adequate if it included at least 10 valid measurements, with a success rate >60% and measurement variability <30% of the median.[30,33,34] The presence of clinically significant fibrosis or advanced fibrosis was defined by the presence of either LSM ≥7.0 kPa (corresponding to Kleiner stage $F \geq 2$ fibrosis on histology) or LSM ≥8.7 kPa (corresponding to $F \geq 3$ fibrosis on histology).[30]

2.5 | Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Germany).[30] Genotyping of rs738409 C>G in patatin-like phospholipase domain-containing protein-3 (*PNPLA3*) gene, encoding for the p.I148M aminoacidic substitution, was carried out by a predesigned TaqMan probe (Applied Biosystem, California, USA), according to the manufacturer's protocol.[30] Polymorphism genotyping was performed using 7900 HT Real-Time PCR (Applied Biosystem, California, USA).[30]

2.6 | Statistical analysis

Continuous variables were expressed as means ± SD or medians (inter-quartile ranges [IQR]), and categorical variables as percentages. The one-way analysis of variance (ANOVA) for normally distributed continuous variables, the Kruskal–Wallis test for non-normally distributed variables (i.e. diabetes duration, plasma levels of iron, transferrin, ferritin, hepcidin, ferritin-to-hepcidin ratio, triglycerides, insulin, GGT and HOMA-IR score) and the chi-squared test for categorical variables were used to examine differences in clinical and biochemical characteristics among patients stratified by increasing LSM tertiles.

The association between plasma ferritin levels (logarithmically transformed before analysis) and Fibroscan®-assessed LSM values was assessed using logistic regression analyses where LSM was modelled as 1st tertile versus 3rd and 2nd LSM tertiles combined. In addition to an unadjusted logistic regression model, we performed three progressive forced-entry adjusted models. Regression model 1 was adjusted for age and sex; model 2 was adjusted for the same covariates included in model 1 *plus* waist circumference, HOMA-IR score, plasma triglycerides, haemoglobin, presence of hepatic steatosis on ultrasonography and *PNPLA3* rs738409 variant; and, finally, model 3 was additionally adjusted for duration of diabetes and HbA1c. The same logistic regression models were repeated for testing the association between plasma hepcidin levels and Fibroscan®-assessed LSM values. We also examined the independent associations of plasma ferritin or hepcidin concentrations with LSM tertiles using multinomial logistic regression analyses. In these multinomial regression analyses the dependent variable was the LSM tertiles that were included as follows: 1st tertile (i.e. the reference category) versus 2nd tertile versus 3rd tertile. Covariates included in all these logistic regression models were chosen as potential confounding variables based on their biological plausibility or statistical associations with Fibroscan®-assessed LSM in univariate analyses. We did not perform separate logistic regression analyses for men and women because the interaction term by sex was not significant ($p > 0.10$) in these logistic regression models. In addition, the total number of men ($n = 79$) and women ($n = 74$) was too small to perform separate fully adjusted logistic regression models (that included 10 covariates in adjusted model 3).

All statistical tests were two-sided and a p-value <0.05 was considered statistically significant. Statistical analyses were performed using STATA software, version 17.0 (STATA, College Station, Texas, USA).

3 | RESULTS

Among the 153 (79 men and 74 women) patients with T2DM without known liver diseases included in the study, the proportion of patients with NAFLD (hepatic steatosis) on ultrasonography was 70.6%, while the proportion of those who had significant or advanced fibrosis was 19.6% ($n = 30$ with an LSM cut-off value ≥7 kPa) or 12.4% ($n = 19$ with an LSM cut-off value ≥8.7 kPa), respectively. In the whole sample of participants, the medians and IQRs of plasma iron, ferritin, hepcidin and transferrin saturation were 14.2 μmol/L (IQR: 11.1–17.6 μmol/L), 84.6 μg/L (44.2–161.0 μg/L), 3.8 nmol/L (1.9–6.1 nmol/L) and 21.2% (15.9–17.1%), respectively. Only 13.1% ($n = 20$) of participants had metabolic hyperferritinemia (defined as a plasma ferritin level >200 μg/L in women and >300 μg/L in men, respectively).

Table 1 shows the clinical and biochemical characteristics of participants stratified by increasing LSM tertiles. Compared with those in 1st tertile who had a median LSM of 3.6 kPa (IQR 3.3–4.0 kPa), those with increased liver fibrosis (so belonging to 3rd LSM tertile), who had a median LSM of 7.9 kPa (IQR 6.7–9.4 kPa), were more likely to be younger, centrally obese, more insulin-resistant (as reflected by greater HOMA-IR score) and had higher levels of serum liver enzymes and triglycerides, as well as a higher prevalence of hepatic steatosis (NAFLD) on ultrasonography. Notably, patients with increased liver fibrosis also had significantly higher plasma ferritin and hepcidin levels but comparable values of plasma iron, transferrin, transferrin saturation and ferritin-to-hepcidin ratio compared with

TABLE 1 Baseline clinical and biochemical characteristics of patients with T2DM stratified by LSM tertiles.

	1st LSM tertile ($n=52$) median LSM: 3.6 (IQR 3.3–4.0) kPa	2nd LSM tertile ($n=49$) median LSM: 5.3 (4.9–5.9) kPa	3rd LSM tertile ($n=52$) median LSM: 7.9 (6.7–9.4) kPa	*p*-value
Age (years)	71 ± 8	70 ± 10	67 ± 9	**0.025**
Men (%)	40.0	55.1	61.5	0.084
BMI (kg/m^2)	27.9 ± 5.6	27.2 ± 3.7	30.1 ± 3.8	**<0.005**
Waist circumference (cm)	96.6 ± 12.5	97.7 ± 12.9	105.9 ± 9.6	**<0.005**
Diabetes duration (years)	11 (7–19)	11 (5–19)	10 (7–16)	0.457
Current smokers (%)	8.0	12.2	17.3	0.731
Systolic blood pressure (mmHg)	136 ± 15	133 ± 17	135 ± 17	0.593
Diastolic blood pressure (mmHg)	75 ± 9	75 ± 9	78 ± 10	0.182
Hypertension (%)	78.0	79.6	82.7	0.833
Haemoglobin (g/L)	134 ± 15	140 ± 11	140 ± 13	0.060
Platelet count (×100000/mm^3)	241 ± 59	236 ± 59	233 ± 62	0.810
Iron (μmol/L)	12.6 (9.9–17.8)	15.4 (12.0–18.6)	14.4 (11.8–17.3)	0.157
Ferritin (μg/L)	68.7 (25.1–147)	85.8 (48.3–139)	111 (59.3–203)	**0.021**
Transferrin (g/L)	2.8 (2.4–3.0)	2.6 (2.4–3.0)	2.7 (2.4–3.0)	0.794
Hepcidin (nmol/L)	2.5 (1.1–5.2)	4.4 (2.5–7.3)	4.1 (1.9–6.8)	**0.032**
Transferrin saturation (%)	20.8	23.5	23.2	0.261
Ferritin-to-hepcidin ratio	8.0 (6.2–13.9)	8.9 (4.6–14.5)	10.5 (7.6–19.8)	0.063
hs-C-reactive protein (mg/L)	1.2 (0.6–2.7)	0.9 (0.4–2.6)	1.5 (0.7–3.9)	0.179
Fasting glucose (mg/dL)	129 ± 30	125 ± 24	134 ± 29	0.284
Haemoglobin A1c (%)	6.9 ± 0.8	6.8 ± 0.6	7.0 ± 0.8	0.524
Total cholesterol (mg/dL)	158 ± 37	159 ± 37	151 ± 31	0.448
LDL-cholesterol (mg/dL)	83 ± 34	81 ± 32	72 ± 24	0.157
HDL-cholesterol (mg/dL)	55 ± 12	54 ± 15	52 ± 17	0.576
Triglycerides (mg/dL)	94 (71–132)	112 (86–169)	124 (96–173)	**<0.005**
Fasting insulin (mUI/L)	5.6 (3.5–9.7)	4.5 (3.1–8.7)	9.3(4.3–15.9)	**<0.005**
HOMA-IR score	1.8 (1.0–3.0)	1.6 (1.0–2.4)	3.0 (1.4–5.9)	**0.001**
AST (IU/L)	23 ± 7	23 ± 6	27 ± 10	**0.021**
ALT (IU/L)	14 ± 7	13 ± 6	18 ± 9	**0.050**
GGT (IU/L)	15 (11–24)	19 (13–30)	27 (17–39)	**<0.001**
Creatinine (μmol/L)	74 ± 19	78 ± 25	81 ± 23	0.335
e-GFR$_{CKD-EPI}$ (mL/min/1.73 m^2)	79 ± 17	80 ± 17	79 ± 18	0.956
Ischemic heart disease (%)	16.0	8.2	17.3	0.361
Diabetic retinopathy (%)	10.4	16.7	12.0	0.639
Chronic kidney disease (%)	10.0	14.3	17.3	0.564
Metformin users (%)	84.0	83.7	92.3	0.345
Sulfonylureas users (%)	24.0	30.6	28.9	0.748
Pioglitazone users (%)	2.0	12.2	13.5	0.093
DPP-4 inhibitor users (%)	28.0	20.4	26.9	0.642
GLP-1 receptor agonist users (%)	18.0	22.5	19.2	0.849
SGLT-2 inhibitor users (%)	8.0	8.2	17.3	0.234

TABLE 1 (Continued)

	1st LSM tertile ($n=52$) median LSM: 3.6 (IQR 3.3–4.0) kPa	2nd LSM tertile ($n=49$) median LSM: 5.3 (4.9–5.9) kPa	3rd LSM tertile ($n=52$) median LSM: 7.9 (6.7–9.4) kPa	*p*-value
Anti-platelet users (%)	57.1	35.4	46.2	0.100
Beta-blocker users (%)	32.7	25.0	32.7	0.635
ARB/ACE inhibitors (%)	61.2	66.7	65.4	0.841
Calcium-channel blockers users (%)	16.3	16.7	32.7	0.076
Diuretic users (%)	36.7	22.9	34.6	0.287
Statin users (%)	80.0	81.3	73.1	0.564
NAFLD (steatosis) on ultrasound (%)	56.0	59.2	94.2	**<0.001**
PNPLA3 rs738409				0.385
CC genotype (%)	48.0	46.9	55.8	
CG genotype (%)	48.0	44.9	34.6	
GG genotype (%)	4.0	8.2	9.6	

Note: Sample size: $n=153$. Data are expressed as means ± SD, medians and interquartile ranges (IQRs) (in parenthesis) or percentages. Differences among the three patient groups were tested by the chi-squared test for categorical variables, the one-way ANOVA for normally distributed continuous variables and the Kruskal–Wallis test for non-normally distributed variables (i.e. diabetes duration, ferritin, iron, transferrin, hepcidin, ferritin-to-hepcidin ratio, triglycerides, fasting insulin, HOMA-IR score and GGT levels). For the sake of clarity, significant *p*-values are highlighted in bold.

Abbreviations: ACE, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DPP-4, dipeptidyl peptidase-4; e-$GFR_{CKD\text{-}EPI}$, estimated glomerular filtration rate calculated by the CKD-Epidemiology Collaboration equation; GGT, gamma-glutamyl transferase; GLP-1, glucagon-like peptide-1; HOMA-IR, homeostasis model assessment—insulin resistance; NAFLD, non-alcoholic fatty liver disease; PNPLA3, patatin-like phospholipase domain-containing protein 3; SGLT-2, sodium/glucose cotransporter-2.

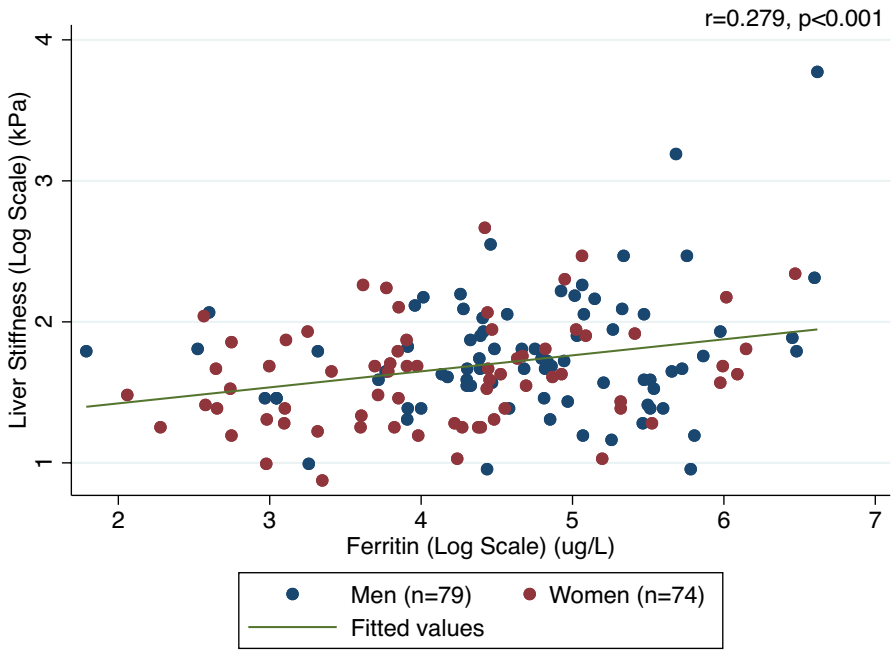

FIGURE 1 Univariable linear correlation between plasma ferritin levels and Fibroscan®-assessed LSM values in patients with T2DM, stratified by sex. The correlation was tested using the Pearson's correlation analysis. Note: Both plasma ferritin levels and LSM values were logarithmically transformed before statistical analysis.

those belonging to the 1st LSM tertile. No significant differences were found in sex distribution, diabetes duration, HbA1c, smoking history, blood pressure, haemoglobin, platelet count, hs-CRP and e-GFR, as well as in the prevalence of ischemic heart disease, diabetic retinopathy and use of glucose-lowering, anti-hypertensive or lipid-lowering medications among the three patient groups. Moreover, there were no significant differences in the distribution of the *PNPLA3* rs738409 genotypes among the groups.

As shown in Figure 1, there was a significant positive correlation between plasma ferritin levels and Fibroscan®-assessed LSM values in the whole group of participants (Pearson's r coefficient = 0.279, $p<0.001$). Moreover, plasma ferritin levels were significantly

associated with LSM values in both women (Pearson's $r=0.271$, $p=0.023$) and men (Pearson's $r=0.224$, $p=0.045$).

Table 2 shows the association between plasma ferritin levels and Fibroscan®-assessed LSM values. In these logistic regression analyses where LSM values were modelled as tertiles (i.e. 1st tertile vs. 2nd and 3rd LSM tertiles combined), we found that higher plasma ferritin levels were significantly associated with greater LSM values both in the unadjusted regression model (odds ratio 1.68, 95% CI 1.18–2.39, $p=0.005$) and in the regression model 1 after adjustment for age and sex. The strength of this association was not weakened by additional adjustment for waist circumference, HOMA-IR score, plasma triglycerides, haemoglobin, presence of hepatic steatosis on ultrasound and the *PNPLA3* rs738409 genetic variant (model 2). Again, the association between plasma ferritin levels and LSM values remained significant even after further adjustment for diabetes duration and HbA1c (model 3: adjusted-odds ratio 2.10, 95% CI 1.23–3.57, $p=0.005$). Other variables that were independently associated with greater LSM values were higher plasma triglycerides ($p=0.005$) and the presence of hepatic steatosis on ultrasonography ($p=0.044$).

Table 3 shows the association between plasma hepcidin levels and LSM values. In a similar way to what we observed above with plasma ferritin levels, we found that higher plasma hepcidin levels were significantly associated with higher LSM values both in the unadjusted regression model (odds ratio 1.58, 95% CI 1.10–2.25, $p=0.005$) and in the three progressive forced-entry adjusted models (model 3: adjusted-odds ratio 1.90, 95% CI 1.15–3.13, $p=0.013$).

Supporting Information Table S1 shows the association between plasma ferritin concentrations and Fibroscan®-assessed LSM tertiles (i.e. 1st tertile [the reference category] vs. 2nd tertile vs. 3rd tertile) using a multinomial logistic regression analysis. In the unadjusted model, higher plasma ferritin levels were significantly associated with a ~1.5-fold and 1.9-fold odds of 2nd LSM tertile and 3rd LSM tertile, respectively. These results remained statistically significant in the three progressive forced-entry adjusted models.

Supporting Information Table S2 shows the association between plasma hepcidin concentrations and Fibroscan®-assessed LSM tertiles using a multinomial logistic regression analysis. These results were essentially comparable to those observed above for plasma ferritin levels, although the magnitude of associations between plasma hepcidin concentrations and LSM tertiles were less strong, both in the unadjusted regression analysis and in the three progressive forced-entry adjusted models.

Finally, we also conducted stratified analyses to better examine whether the observed associations between plasma ferritin, plasma hepcidin and Fibroscan®-assessed LSM values were mainly attributed to hepatic fibrosis or steatosis. When participants were divided into three subgroups, that is subjects without hepatic steatosis, those with hepatic steatosis alone, and those with hepatic steatosis and LSM ≥7.0 kPa, we found that the highest levels of plasma ferritin and hepcidin were those of subjects with hepatic steatosis and LSM ≥7.0 kPa (median ferritin 146 μg/L [IQR 75–231]; hepcidin 4.2 nmol/L [2.1–6.5]). Conversely, subjects without steatosis (ferritin 83 μg/L [40–140]; hepcidin 3.7 nmol/L [2.0–6.1]) and subjects with hepatic steatosis alone (ferritin 81 μg/L [41–153]; hepcidin 3.9 nmol/L [1.8–6.5]) had comparable levels of plasma ferritin and hepcidin.

TABLE 2 Association between plasma ferritin concentrations and Fibroscan®-assessed LSM values.

	Logistic regression analysis ($Y=$ 1st tertile vs. 2nd and 3rd LSM tertiles combined)		
	Odds ratio(s)	**95% CI**	***p*-value**
Unadjusted model			
Ferritin (Log scale), μg/L	1.68	1.18–2.39	0.005
Adjusted model 1			
Ferritin (Log scale), μg/L	1.56	1.08–2.24	0.018
Adjusted model 2			
Ferritin (Log scale), μg/L	1.89	1.19–3.01	0.005
Adjusted model 3			
Ferritin (Log scale), μg/L	2.10	1.23–3.57	0.005

Note: Sample size, $n=153$. Data are expressed as odds ratios and 95% confidence intervals (CI) by using logistic regression analysis. The dependent variable (Y) for all logistic regression models was Fibroscan®-assessed LSM value. Other covariates included in adjusted models, along with plasma ferritin concentrations (log-transformed before analysis), were as follows: *model 1*: adjusted for age and sex; *model 2*: adjusted for the same variables included in model 1 *plus* waist circumference, HOMA-IR score, plasma triglycerides, haemoglobin, presence of NAFLD on ultrasound and *PNPLA3* rs738409 variant; and *model 3*: additionally adjusted for duration of diabetes and HbA1c.

4 | DISCUSSION

The main and novel findings of our exploratory cross-sectional study that was performed on an outpatient cohort of T2DM individuals with no known liver diseases (except for NAFLD), are as follows: (a) higher circulating levels of ferritin and hepcidin were significantly associated with greater NAFLD-related liver fibrosis, as non-invasively assessed by Fibroscan®; and (b) these associations remained statistically significant even after adjustment for multiple established cardiometabolic risk factors and potential confounders, such as age, sex, diabetes duration, HbA1c, waist circumference, HOMA-estimated insulin resistance, plasma triglycerides, haemoglobin, presence of hepatic steatosis on ultrasonography and *PNPLA3* rs738409 C>G polymorphism (i.e. the genetic variant most robustly associated with greater susceptibility to NAFLD and NASH).

Presently, most published studies examining the associations between plasma ferritin levels, hepatic iron deposition and liver fibrosis have enrolled cohorts of patients with histologically confirmed NAFLD at tertiary care gastroenterology centres.[10–17] For instance, in a multicentre study involving 587 Italian patients with NAFLD and 184 control subjects, Valenti et al. reported that iron deposition, predominantly in hepatocytes, was associated with a higher risk of liver fibrosis stage, compared with the absence of siderosis (even after adjustment for age, BMI, glucose tolerance status

TABLE 3 Association between plasma hepcidin concentrations and Fibroscan®-assessed LSM values.

	Logistic regression analysis (Y = 1st tertile vs. 2nd and 3rd LSM tertiles combined)		
	Odds ratio(s)	**95% CI**	***p*-value**
Unadjusted model			
Hepcidin (Log scale), nmol/L	1.58	1.10–2.25	0.005
Adjusted model 1			
Hepcidin (Log scale), nmol/L	1.50	1.03–2.17	0.032
Adjusted model 2			
Hepcidin (Log scale), nmol/L	1.75	1.12–2.74	0.015
Adjusted model 3			
Hepcidin (Log scale), nmol/L	1.90	1.15–3.13	0.013

Note: Sample size, $n = 153$. Data are expressed as odds ratios and 95% confidence intervals (CI) by using logistic regression analysis. The dependent variable (*Y*) for all logistic regression models was Fibroscan®-assessed LSM value. Other covariates included in adjusted models, along with plasma hepcidin concentrations (log-transformed before analysis), were as follows: *model 1*: adjusted for age and sex; *model 2*: adjusted for the same variables included in model 1 *plus* waist circumference, HOMA-IR score, plasma triglycerides, haemoglobin, presence of NAFLD on ultrasound and *PNPLA3* rs738409 variant; and *model 3*: additionally adjusted for duration of diabetes and HbA1c.

and serum ALT levels).[10] In another multicentre study involving 468 obese individuals with biopsy-proven NAFLD, Buzzetti et al. reported that plasma ferritin levels increased with the worsening of liver fibrosis up to pre-cirrhotic stages.[17] In a small single-centre study involving 51 patients with NAFLD, 30 patients with chronic viral hepatitis and 20 control subjects, Ryan et al. found that higher plasma ferritin levels were associated with advanced histological features of NAFLD (especially higher liver fibrosis stages), whereas circulating hepcidin levels and hepatic iron deposition were the strongest predictors of plasma ferritin levels.[15] Studies examining the association between circulating hepcidin levels and advanced NAFLD have provided conflicting results.[24–28] For instance, in a case–control study of 88 patients with biopsy-proven NAFLD and 88 control subjects, Senates et al. reported that patients with NAFLD had significantly higher circulating hepcidin levels than control subjects.[25] However, in that study the association between hepcidin levels and histological features of NAFLD became non-significant after adjusting for potential confounding factors.[25] In another small study of 105 patients undergoing bariatric surgery (most of whom had liver histology assessment) and 60 healthy blood donors, the histological features of NAFLD were not associated with higher serum hepcidin levels.[26]

Collectively, therefore, the results of our cross-sectional study corroborate and expand previous findings, showing that higher circulating ferritin and hepcidin levels are significantly associated with greater NAFLD-related liver fibrosis in T2DM outpatients with no known liver diseases, even after adjustment for multiple cardiometabolic risk factors, diabetes-related variables, presence of hepatic steatosis, *PNPLA3* rs738409 variant and other potential confounders.

Speculatively, the most obvious explanation for our findings is that the association between plasma ferritin and hepcidin levels with higher liver fibrosis is simply an epiphenomenon of shared cardiometabolic risk factors, genetic factors, poor glycaemic control and coexisting severe hepatic steatosis. However, it should be noted that in our study the significant associations of plasma ferritin and hepcidin levels with Fibroscan®-assessed LSM values were not attenuated after adjustment for age, sex, waist circumference, diabetes duration, HbA1c, HOMA-IR score, plasma triglycerides, haemoglobin, presence of hepatic steatosis on ultrasonography and *PNPLA3* rs738409 genetic variant. Thus, although further mechanistic studies are needed, it is possible to hypothesize that higher circulating levels of ferritin and hepcidin might play a direct role in the progression of NAFLD to NASH and advanced fibrosis. Ferritin is the main intracellular iron storage protein and is a reliable biomarker of low-grade inflammation.[9] Circulating ferritin levels are also increased in obesity-related metabolic disorders.[9] The iron-regulatory hormone hepcidin regulates iron influx into the bloodstream from duodenal enterocytes and macrophages, but low-grade inflammation may promote hepcidin synthesis.[9,21] Excess fatty acid levels, which happens during diets rich in fat, sugar or processed foods, may reduce the hepcidin's ability to limit intestinal iron absorption.[9] As a result, there is an increased hepcidin production, and iron accumulates in cells expressing ferroportin, especially macrophages and hepatocytes.[9] In the presence of systemic insulin resistance and low-grade inflammation, iron accumulates not only in hepatocytes, but also in Kupffer and hepatic stellate cells, thus leading to hepatocellular damage, ferroptosis, inflammation and hepatic fibrosis.[9,35] Higher plasma ferritin levels have also been associated with iron overload in adipose tissue, which may worsen insulin sensitivity and adiponectin secretion, and promote the release of multiple proinflammatory cytokines, thereby leading to liver damage.[9]

Our findings may have important clinical implications as the identification of T2DM patients with NAFLD and mildly elevated plasma ferritin levels might identify a subset of individuals at higher

risk of having fibrotic NAFLD. In addition, it should be noted that higher plasma ferritin levels are associated with an increased risk of adverse cardiovascular[18,36,37] and renal outcomes.[38] Therefore, the results of our study highlight the need to early assess and adequately treat all coexisting cardiometabolic risk factors in T2DM patients with NAFLD and elevated ferritin levels, as also supported by a recent international consensus on the definition and classification of metabolic hyperferritinemia.[9] Currently, lifestyle intervention (achieving a 5% to 10% weight loss through increased physical activity and a hypocaloric diet with a Mediterranean-style dietary pattern) is the cornerstone of the treatment of NAFLD and may also improve cardiometabolic risk factors and reduce metabolic hyperferritinemia.[1,2,9]

Our study has some important limitations that should be mentioned. Firstly, the cross-sectional design of the study limits our ability to establish temporal or causal associations between circulating ferritin and hepcidin levels and Fibroscan®-assessed LSM values. Secondly, our exploratory study included a relatively small cohort of Caucasian individuals with metabolically well-controlled T2DM without known liver diseases, who regularly attended a diabetes outpatient service. Hence, these results could not be generalizable to other patient groups with T2DM, and additional larger studies are certainly needed to corroborate these results in other cohorts of patients with T2DM and also to examine possible sex differences in the relationship between plasma ferritin or hepcidin levels with NAFLD-related liver fibrosis in people with T2DM. Thirdly, we did not perform the *HFE* (human homeostatic iron regulator protein) genetic analysis and the quantitative measurement of intrahepatic iron content that would have permitted an accurate quantification of hepatic iron deposition in our patients. Fourthly, we did not perform a liver biopsy or magnetic resonance elastography for staging liver fibrosis. Liver biopsy assessment is difficult to justify in subjects with fairly normal serum aminotransferase concentrations (such as those observed in most of our participants). Consequently, we cannot compare the results of liver stiffness obtained by Fibroscan® with liver histology data. However, vibration-controlled transient elastography (Fibroscan®) is a reliable method for non-invasively staging liver fibrosis in NAFLD.[1,2,34] The 2023 American Diabetes Association guidelines recommended that people with T2DM who have either elevated serum liver enzymes or hepatic steatosis on imaging should be evaluated for presence of liver fibrosis, using both blood-based fibrosis biomarkers and transient elastography.[39] Finally, we cannot exclude definitely the possibility that other unmeasured factors might at least partly explain the observed associations.

Notwithstanding these limitations, our study has important strengths, such as the completeness of the database, the consecutive enrolment of the study population and the exclusion of patients with important comorbidities (such as cirrhosis, active cancer, advanced renal disease or impaired thyroid function), as we believe that the inclusion of patients with such comorbidities might have confounded the interpretation of data. Additionally, both liver transient elastography and ultrasonography were performed in all patients by a single trained physician, blinded to participants' clinical and biochemical details, thereby eliminating possible assessment bias and inter-observer variability.

In conclusion, the results of this exploratory cross-sectional study showed for the first time that higher circulating ferritin and hepcidin levels are associated with greater NAFLD-related liver fibrosis in ambulatory patients with T2DM. Notably, these associations remained statistically significant even after adjustment for established cardiometabolic risk factors, HOMA–IR score, diabetes-related variables, presence of hepatic steatosis on ultrasonography and *PNPLA3* rs738409 genetic variant. Larger studies are required to further validate these findings in other independent cohorts of patients with T2DM and to better elucidate the complex biological mechanisms underpinning the association between iron storage biomarkers and NAFLD-related liver fibrosis in diabetes.

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CONFLICT OF INTEREST STATEMENT

The authors have no potential conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

Individual participant sensitive data cannot be rendered public based on the terms of the informed consent of the study and regulations by the Italian Guarantor for Privacy. All data, codes and materials used in the analyses are available upon reasonable request for collaborative studies regulated by materials/data transfer agreements (MTA/DTAs) to the corresponding author.

ETHICS APPROVAL STATEMENT

The local Ethics Committee approved the study protocol. All participants gave their written informed consent to participate in the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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