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Research Article

Predictive Value of Serum Ferritin in Combination with Alanine Aminotransferase and Glucose Levels for Non-invasive Assessment of NAFLD: Fatty Liver in Obesity (FLiO) Study

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ABSTRACT

Background and objective: Non alcoholic fatty liver disease (NAFLD) is a major cause of liver morbidity. The identification of affordable and reproducible non-invasive biomarkers for the diagnosis and characterization of NAFLD as feasible alternative to liver biopsy is a major challenge for the research community. This study aimed to explore the usefulness of ferritin as a proxy biomarker of NAFLD condition, alone or in combination with other routine biochemical parameters.

Methods: Subjects with overweight/obesity and ultrasound-confirmed liver steatosis (n=112) from the Fatty Liver in Obesity (FLiO) study were assessed. Hepatic evaluation considered magnetic resonance imaging (MRI), ultrasonography and credited routine blood liver biomarkers. Anthropometry and body composition, dietary intake (by means of a validated 137-item food frequency questionnaire) and specific biochemical markers of nutritional status were also determined. Serum ferritin levels were analyzed using a Chemiluminescent Microparticle Immunoassay (CMIA) kit.

Results: Lower serum ferritin concentrations were associated with a general better liver health and nutritional status. The evaluation of ferritin as a surrogate of liver damage by means of quantile regression analyses showed a positive association with alanine aminotransferase (ALT) (β =19.21; p=<0.001), liver fat content (β =8.70; p=0.008) and hepatic iron by MRI (β =3.76; p=<0.001), after adjusting for potential confounders. In ROC analyses, the panel combination of blood ferritin, glucose and ALT showed the best prediction for liver fat mass (AUC 0.82).On the other hand, a combination of ferritin and ALT showed the higher predictive ability for estimating liver iron content (AUC 0.73).

Conclusion: This investigation demonstrated the association of serum ferritin with liver health as well as with glucose and lipid metabolism markers in subjects with NAFLD. Current findings led to the identification of ferritin as a potential non-invasive predictive biomarker of NAFLD, whose surrogate value increased when combined with other routine biochemical measurements such as glucose/ALT.

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Keywords: Fatty liver; Insulin resistance; Iron; Obesity; Ferritin; Biomarker; Glucose; Metabolism

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a condition defined by an excessive triglyceride accumulation in liver cells that is not caused by heavy alcohol consumption [1]. NAFLD is a worldwide major cause of liver disease [2] which potentially contributes to a burden of extra-hepatic disturbances. Indeed, NAFLD is considered a multi-organ failure linked to obesity, cardiovascular disease (CVD), insulin resistance (IR)or metabolic syndrome (MetS)features [2-5].This morbid condition can lead to non-alcoholic steatohepatitis (NASH), advanced fibrosis, cirrhosis, and finally, hepatocellular carcinoma [6]. Multiple environmental and genetic factors are involved in the onset and progression of NAFLD [7]. Concerning NAFLD treatments, weight loss induced by energy-restricted diets, physical activity promotion and other lifestyle modifications have exhibited promising results leading to a better hepato-metabolic status [8,9]. In this context, liver biopsy has been recognized as the reference method for hepatic steatosis quantification. However, liver biopsy is an invasive and expensive procedure with some inherent surgical risks and only represents around 1/50000 of the total hepatic volume [2,10]. In this context, non-invasive liver biomarkers and reproducible surrogate routine laboratory tests are seeking as feasible alternatives to liver biopsy. Therefore, research is focusing on more efficient diagnostic and predictive biomarkers for identifying NAFLD features at early stages [2,10-12].

Novel investigations evidenced that iron metabolism related parameters may be suitable predictors of liver disease outcomes [13].The liver is the major iron storage organ and plays a key role in the metabolism of this nutrient[14]. Thus, iron has been involved in cellular oxidative stress and IR, key features of NAFLD pathogenesis, and hepatic iron accumulation has been linked to advanced fibrosis [15,16]. Ferritin is the chief iron storage protein, regulated post-transcriptionally by cellular iron status via iron-responsive elements in its messenger RNA. Thus, higher intracellular iron concentrations result in increased ferritin expression, whereas iron deficiency inhibits its expression [17]. During liver injury, ferritin releases from hepatocytes and plasma concentrations rise. Serum ferritin appears to be chiefly derived from macrophages [18] and does not contain storage iron but reflects body iron stores and ferritin levels in the liver and other tissues [19]. Ferritin is also an acute-phase protein and serum concentrations are increased in inflammatory conditions [20]. In this context, it remains unclear if serum ferritin reflects liver damage and accompanying inflammation features, increased body iron stores or a combination of these factors [21]. Interestingly, mild to moderate serum ferritin levels are identified in NAFLD, involving up to 30% of affected individuals [22,23]. Iron status as reflected by increased serum ferritin has been related to a higher risk of type 2 diabetes, IR and CVD, conditions inherently related to NAFLD [24]. Hyperferritinemia is also associated with more advanced NAFLD and increased mortality [25].

Thus, the objective of this research was to explore the usefulness of ferritin as a predictive surrogate biomarker of NAFLD condition, alone or in combination with other routine biochemical parameters.

MATERIALS AND METHODS

Participants

The current research is an ancillary cross-sectional analysis includingbaseline data of the FLiO study (Fatty Liver in Obesity), a randomized controlled trial (www.clinicaltrials.gov; NCT03183193). The study included 112 (65 Male and 47 Female) adults (aged 40-80 years) with overweight/obesity [Body Mass Index (BMI) ≥ 27.5 kg/m2 to <40 kg/m2], and ultrasound-confirmed hepatic steatosis.

Exclusion criteria included the presence of known liver disease (other than NAFLD), ≥ 3 kg body weight loss in the last 3 months, elevated alcohol consumption (>21 and >14 units of alcohol per week for men and women, respectively) [26], endocrine disorders (hyperthyroidism or uncontrolled hypothyroidism), pharmacological treatment with immune-suppressants, cytotoxic agents, systemic corticosteroids (or other drugs that could potentially cause hepatic steatosis or alteration of liver tests), active autoimmune diseases or requiring pharmacological treatment, acute infections, the use of weight modifiers, the presence of severe psychiatric disorders and inability to follow the diet (food allergies, intolerances) as well as difficulties to follow scheduled visits.

All the procedures performed were in accordance with the ethical guidelines of the Declaration of Helsinki. The study protocol and informed consent document were approved by the Research Ethics Committee of the University of Navarra on 24 April 2015 (ref. 54/2015).All participants gave written informed consent prior to inclusion.

Anthropometrics, body composition and biochemical assessment

Anthropometric measurements (body weight, height and waist circumference), body composition (DXA, Lunar iDXA, encore 14.5, Madison, WI, USA), and blood pressure (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, The Netherlands) were determined in fasting state following standardized procedures [27]. BMI was calculated as weight (kg) divided by the square of height (m).

Blood samples were properly collected at baseline after 12 hovernight fast, processed (15 min; 3500 rpm; 5°C), and stored at-80°C until the biochemical analyses were performed. Blood glucose, glycated hemoglobin (HbA1c), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) were analyzedon asuitable autoanalyzer (Pentra C-200; HORIBA ABX, Madrid,

Spain) with routine validated procedures. Insulin, adiponectin, fibroblast growth factor 21 (FGF-21), retinol binding protein 4 (RBP-4)and dipeptidyl-peptidase 4 (DPP4) concentrations were quantified using specific ELISA kits (Demeditec; Kiel-Wellsee, Germany) in a Triturus autoanalyzer (Grifols, Barcelona, Spain). Serum ferritin levels were analyzed by an external certified laboratory (Eurofins Megalab S.A, Madrid, Spain) using a Chemiluminescent Microparticle Immunoassay (CMIA) technology (Abbott Architect Ferritin Assay).The low-density lipoprotein (LDL-c) levels were calculated using the Friedewald formula: LDL-c=TC-HDLc-TG/5 [28]. The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) [29], the TyG index (Ln(TG[mg/dL]x glucose [mg/dL]/2) [30] and the TG/HDL-c index (TG (mg/dL)/HDL-c (mg/dL) were also calculated as described elsewhere [31].

Physical activity was classified in 4 different categories (sedentary, mild, moderated or elevated).

Hepatic imaging techniques

The entire hepatic assessment was determined under fasting conditions by qualified staff at the University of Navarra Clinic. The presence of hepatic steatosis was determined by Ultrasonography (Siemens ACUSON S2000 and S3000) in accordance with the previously described methodology [32]. Magnetic Resonance Imaging (MRI) (Siemens Aera 1.5 T) was performed to quantify the fat and iron content of the liver and the hepatic volume (HISTO technique), as described elsewhere [33].

Dietary intake estimate

Dietary intake was assessed with a validated semi quantitative 137-item food frequency questionnaire (FFQ) as described elsewhere [34]. Each item in the questionnaire included a typical portion size. For each food item, daily food consumption was estimated by multiplying the portion size by the consumption frequency and dividing as described elsewhere [35].The nutrient composition of the food items was derived from accepted Spanish food composition tables.

The dietary total antioxidant capacity (TAC) score was calculated by computing the individual TAC values from the ferric reducing antioxidant power assay of each food. The mean TAC value of the foods contained in each item was used to calculate the dietary TAC score from the FFQ [36].

The adherence to the Mediterranean diet was assessed with a 17-point screening questionnaire, with a final score ranging from 0 to 17 and a higher score indicating a better adherence to the Mediterranean diet [37].

Glycemic Index (GI) values for single food items on the food frequency questionnaire were derived from the "International Tables of Glycemic Index and Glycemic Load Values" as previously reported [36]. Total dietary GI was estimated by multiplying the amount of available carbohydrate (g) of each food item by its GI. The sum of these products was divided by the total carbohydrate intake. Glycemic Load (GL) was also calculated, which represents the amount of carbohydrates multiplied by the average GI [36].

Statistical analyses

The normal distribution of the continuous variables was assessed using the Shapiro-Wilk test. The data were expressed as a mean ± standard deviation for continuous traits and percentage for categorical variables. Participants were classified according to sex-specific serum ferritin tertiles(women: T1: <31.8, T2: ≥ 33.5 to <76.6, and T3: ≥ 80.4; men: T1: <109.8, T2: ≥ 116.1 to<263.7, and T3: ≥ 272.1).Differences in anthropometric data, body composition, biochemical variables, hepatic status and dietary characteristics among the three ferritin sex-specific tertiles were tested by analysis of variance and the $\chi 2$ test for categorical variables. Spearman correlations were performed to further explore the association between serum ferritin levels and both liver and glucose state. Multivariable adjusted quantile regression models were performed to evaluate the association between serum ferritin (in tertiles) and liver status variables (ALT, liver fat mass and hepatic iron content). We run first an unadjusted Model 1. Model 2 was minimally adjusted by age and sex. Model 3 was adjusted by age, sex, Mediterranean diet adherence score, physical activity and BMI. Receiver Operating Characteristic (ROC) curves were applied to calculate the power of prediction of serum ferritin for liver fat and hepatic iron content (NAFLD). Also, combination panels were created to calculate the power of prediction including glucose, ALT and TG.

Statistical analyses were performed using Stata version 12.1 (StataCorp 2011, College Station, TX, USA). All p values presented are two-tailed, and differences were considered statistically significant at $p<0.05$.

RESULTS

The average age of study subjects was 51 ± 9 years old and 42% were women. The mean BMI of the participants was 34 ± 4 kg/m^2 with a waist circumference of 110 ± 8 cm. Subjects were categorized according to serum ferritin sex-specific tertiles. An overview on anthropometric data, body composition, glucose and lipid metabolism, liver markers, and dietary characteristics, considering serum ferritin tertiles, are given in Tables 1 and 2, respectively. Anthropometric and body composition variables showed no mentionable statistical differences among serum ferritin groups. No significant differences were either observed in any glucose or lipid marker among ferritin tertiles. Regarding liver health status, participants in the third ferritin tertile had increased ALT, AST and GGT concentrations, higher liver fat mass and hepatic iron contentthan subjects from the other groups ($p<0.05$), shown in Table 1.

Table 1: Baseline characteristics of participants according to sex specific serum ferritin tertiles.

Characteristics	Overall (n=112)	Serum ferritin tertiles			p value
		Tertile 1	Tertile 2	Tertile 3	
		(n=38)	(n=38)	(n=36)	

		5.3 to	33.5 to	80.4 to	
Serum ferritin level (ng/mL)	150.1 (130)	<109.9	<263.6	<588.1	
Age (years)	51.1 (9)	54.5 (10)	47.5 (8)*	51.3 (8)	0.004
Sex (male/female)	65/47	22/16	22/16	21/15	0.999
BMI (kg/m2)	33.7 (4)	34.6 (4)	33.6 (4)	33.0 (4)	0.208
Physical activity (%)					
Never	41.1	47.4	34.2	41.7	0.439
Mild	23.2	26.3	21	22.2	
Moderated	23.2	13.2	31.6	25	
Elevated	12.5	13.2	13.2	11.1	
Cardiometabolic Risk Factors					
Waist Circumference (cm)	109.8 (10)	112.3 (10)	108.8 (10)	108.4 (9)	0.166
Total Fat Mass (kg)	39.5 (9)	41.2 (9)	39.7 (8)	37.5 (8)	0.196
Visceral Fat Mass (g)	2371 (1071)	2504 (954)	2340 (1036)	2268 (1222)	0.636
Systolic blood pressure (mmHg)	131.3 (14)	134.1 (15)	130.1 (14)	129.6 (13)	0.329
Diastolic blood pressure (mmHg)	86.9 (9)	87.7 (9)	87.5 (10)	85.4 (7)	0.476
Glucose Metabolism Variables					
Glucose (mg/d)	109.0 (32)	108.8 (20)	111.3 (45)	106.7 (24)	0.823
Insulin (mU/L)	18.3 (11)	18.5 (9)	15.8 (10)	20.6 (13)	0.161
HbA1c (%)	5.9 (1)	6.0 (1)	5.9 (1)	5.8 (1)	0.643
HOMA-IR	5.2 (5)	5.0 (3)	4.9 (6)	5.8 (5)	0.713
TyG index	8.8 (0.6)	8.8 (0.5)	8.8 (0.7)	8.8 (0.6)	0.987
TG/HDL-c index	3.1 (3)	2.7 (2)	3.4 (3)	3.2 (3)	0.541
Lipid Metabolism Variables					
Total cholesterol (mg/dL)	197.0 (39)	195.0 (43)	198.1 (44)	197.9 (29)	0.929
LDL cholesterol (mg/dL)	117.7 (35)	115.9 (37)	117.3 (39)	119.9 (27)	0.889
HDL cholesterol (mg/dL)	52.1 (14)	54.0 (13)	52.5 (17)	49.6 (11)	0.381
Triglyceride (mg/dL)	137.8 (79)	130.4 (62)	141.2 (90)	142.1 (85)	0.781
Liver Status Variables					
ALT (IU/L)	33.2 (18)	26.0 (12)	33.8 (21)	40.2 (17) #	0.002
AST (IU/L)	24.5 (10)	21.3 (6)	23.9 (10)	28.5 (12) #	0.004
GGT (IU/L)	37.6 (25)	30.7 (18)	35.9 (22)	46.5 (32) #	0.023
Liver fat mass (%)	11.7 (8)	8.4 (10)	7.0 (7)	12.5 (11) †	0.048
Liver Iron (%)	26.9 (4)	31.9 (8)	33.1 (7)	44.2 (18) #†	<0.001
Hepatic Volume (mL)	1813 (530)	1904 (479)	1768 (571)	1768 (536)	0.466
Eco Steatosis Degree (1-3 range)	1.6 (0.7)	1.6 (0.7)	1.4 (0.6)	1.7 (0.7)	0.196

* p values are represented as Mean (SD). Abbreviations: BMI: Body Mass Index; HbA1c: Glycosylated Hemoglobin; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; TyG index: Triglyceride-Glucose Index; TG/HDL-c index: Triglyceride/High-Density Lipoprotein cholesterol index;ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; GGT: Gamma-Glutamyl Transferase. p was significant between T1 y T2; # p was significant between T1 y T3; † p was significant between T2 y T3.

Concerning dietary characteristics, no statistically significant differences were observed in total energy intake and macronutrient distribution among serum ferritin tertiles (Table 2). When food groups were evaluated, main differences were observed in meat, whose consumption was increased in participants with higher serum ferritin ($p<0.01$). On the other hand, subjects from the third tertile consumed less fish than subjects from the other two tertiles ($p<0.05$). No differences were showed in dietary quality indicators (GI, GL and TAC) although a tendency in the Mediterranean dietary score was

observed among serum ferritin tertiles since the adherence to the MedDiet pattern is reduced as serum ferritin increased, shown in Table 2.

Table 2: Description of the nutrient and food consumption according to sex specific serum ferritin tertiles.

Characteristics	Overall (n=112)	Serum ferritin tertiles			p value
		Tertile 1 (n=38)	Tertile 2 (n=37)	Tertile 3 (n=37)	
Serum ferritin level (ng/mL)	150.1 (130)	5.31 to <68.70	68.70 to <177.13	177.13 to <588.12	
Nutrients					
Energy intake (kcal/day)	2689 (1014)	2625 (765)	2655 (1089)	2792 (1171)	0.759
Carbohydrates (%E)	43.2 (7)	43.9 (7)	41.3 (7)	44.5 (7)	0.099
Proteins (%E)	17.3 (4)	17.1 (4)	18.1 (3)	16.7 (3)	0.201
Lipids (%E)	37.1 (7)	36.4 (7)	38.3 (7)	36.4 (6)	0.397
Fibre (g/day)	24.7 (9)	25.4 (8)	25.0 (9)	23.6 (8)	0.652
Alcohol intake (g/day)	8.8 (11)	9.4 (12)	8.5 (12)	8.6 (10)	0.933
Iron (mg/day)	18.4 (6)	18.9 (7)	17.8 (6)	18.4 (6)	0.733
Vitamin C (mg/day)	199.7 (115)	208.5 (146)	203.8 (75)	186.1 (114)	0.683
Food Groups					
Vegetables (g/day)	303.5 (143)	308.4 (160)	314.5 (133)	286.7 (138)	0.687
Fruits (g/day)	289.4 (195)	302.2 (199)	315.1 (194)	248.8 (191)	0.306
Legumes (g/day)	19.7 (9)	19.4 (8)	20.2 (12)	19.3 (7)	0.898
Cereals (g/day)	187 (123)	194.5 (142)	166.3 (100)	200.6 (123)	0.438
Dairy products (g/day)	397.2 (415)	368.4 (358)	466.4 (589)	354.5 (189)	0.448
Meat (g/day)	189.8 (75)	161.9 (60)	216.7 (93)*	191.0 (57)	0.005
Fish (g/day)	91.1 (44)	102.6 (44)	93.2 (49)	76.8 (33) #	0.036
Nuts (g/day)	12.8 (19)	15.0 (20)	11.8 (17)	11.4 (20)	0.674
Dietary Quality Indices					
Glycemic Index	53.7 (7)	53.2 (7)	53.4 (5)	54.7 (8)	0.586
Glycemic Load	161.7 (81)	0.356 (73)	149.2 (72)	176.4 (97)	0.357
Total Antioxidant Capacity (mmol/day)	10.7 (4)	10.4 (4)	11.3 (5)	10.5 (4)	0.646
Mediterranean Diet score (points)	6.3 (3)	6.8 (3)	6.5 (3)	5.4 (2)	0.112

Note: Values are represented as Mean (SD). * p was significant between T1 y T2; # p was significant between T1 y T3; † p was significant between T2 y T3.

Further analyses were performed regarding dietary intake and food group consumption. In addition to the previous results, fruit consumption and adherence to the Mediterranean diet were inversely proportional to the levels of serum ferritin.

The link between serum ferritin levels and liver, lipid and glucose metabolism was further explored. Positive associations of serum ferritin with HOMA-IRand TyG index were found concerning glucose metabolism (Table 3). When lipid parameters were evaluated, positive correlations of serum ferritin concentrations with TG and TG/HDL index were observed whereas HDL-c was negatively associated with ferritin. Regarding hepatic status, serum ferritin was positively correlated with ALT, AST, GGT, hepatic fat, liver iron, hepatic volume and steatos is degree (Table 3). When analyzing cytokines, significant positive associations of ferritin with DPP4 and RBP4 were observed, shown in Table 3.

Table 3: Correlation analyses between serum ferritin levels with liver status and glucose metabolism related markers.

	Ferritin (ng/mL)	
	r	p
Glucose Metabolism Variables		
Glucose (mg/d)	0.088	0.358
Insulin (mU/L)	0.185	0.05
HbA1c (%)	0.05	0.605
HOMA-IR	0.21	0.026

TyG index	0.279	0.003
Lipid Metabolism Variables		
Total cholesterol (mg/dL)	0.018	0.848
LDL cholesterol (mg/dL)	0.011	0.905
HDL cholesterol (mg/dL)	-0.367	<0.001
Triglyceride (mg/dL)	0.275	0.003
TG/HDL-c index	0.342	<0.001
Liver Status Variables		
ALT (IU/L)	0.575	<0.001
AST (IU/L)	0.477	<0.001
GGT (IU/L)	0.498	<0.001
Liver fat mass (%)	0.426	<0.001
Liver Iron (%)	0.435	<0.001
Hepatic Volume (mL)	0.222	0.022
Eco Steatosis Degree (1-3)	0.195	0.039
Blood Cytokines Concentrations		
FGF21	0.07	0.465
DPP4	0.334	<0.001
Adiponectin	-0.139	0.145
RBP4	0.272	0.004

Abbreviations: HbA1c: Glycosylated Hemoglobin; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; TyG index: Triglyceride-Glucose Index; TG/HDL-c index: Triglyceride/High-Density Lipoprotein cholesterol index;ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; GGT: Gamma-Glutamyl Transferase; FGF-21: Fibroblast Growth Factor 21; DPP4: Dipeptidyl-Peptidase 4;RBP-4: Retinol Binding Protein 4.

Multivariable quantile regression models were performed with NAFLD markers (ALT, liver fat mass and liver iron) as dependent factors and serum ferritin (in tertiles) as the independent variable, shown in Table 4.

Table 4: Quantile regression models with NAFLD markers as dependent factors and serum ferritin tertiles as the independent variable among study participants.

	Model 1		Model 2		Model 3		Model 4		Model 5		Model 6	
Variables	**β (95% CI)**	**p value**	**β (95% CI)**	**p value**	**β (95% CI)**	**p value**	**β (95% CI)**	**p value**	**β (95% CI)**	**p value**	**β (95% CI)**	**p value**
ALT level (n=112)												
Serum ferritin level (ng/mL)												
Tertile 1 (5.31-68.69)	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Tertile 2 (68.70-177.12)	6.00 (-1.19; 13.19)	0.101	5.28 (-1.66; 12.22)	0.134	4.79 (-3.11; 12.69)	0.232	5.66 (-2.62; 13.96)	0.178	5.38 (-2.28; 13.04)	0.167	5.40 (-2.58; 13.38)	0.183
Tertile 3 (177.13-588.12)	18.76 (10.70; 26.82)	<0.001	22.10 (13.89; 30.31)	<0.001	20.76 (11.79; 29.74)	<0.001	20.57 (11.07; 30.07)	<0.001	19.21 (10.39; 28.03)	<0.001	21.19 (12.14; 30.24)	<0.001
Liver fat (n=112)												

Serum ferritin level (ng/mL)												
Tertile 1 (5.31-68.69)	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Tertile 2 (68.70-177.12)	4.06 (-1.27; 9.39)	0.134	2.82 (-2.0;7.66)	0.249	2.84 (-2.31; 8.00)	0.277	2.41 (-1.71; 6.54)	0.249	3.69 (-1.94; 9.32)	0.196	2.22 (-2.59; 7.04)	0.361
Tertile 3 (177.13-588.12)	9.09 (3.19; 14.99)	0.003	9.81 (4.16; 15.47)	0.001	10.04 (4.29; 15.78)	0.001	6.42 (1.77; 11.08)	0.007	8.70 (2.37; 15.03)	0.008	8.71 (3.35; 14.06)	0.002
Liver iron (n=112)												
Serum ferritin level (ng/mL)												
Tertile 1 (5.31-68.69)	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Tertile 2 (68.70-177.12)	1.61 (-0.44; 3.66)	0.122	1.08 (-0.91; 3.06)	0.285	1.16 (-0.52; 2.85)	0.174	1.11 (-0.65; 2.88)	0.212	1.26 (-0.45; 2.97)	0.147	0.84 (-1.18; 2.86)	0.41
Tertile 3 (177.13-588.12)	3.44 (1.18; 5.71)	0.003	2.61 (0.29; 4.93)	0.028	3.69 (1.81; 5.57)	<0.001	3.72 (1.73; 5.71)	<0.001	3.76 (1.84; 5.69)	<0.001	4.20 (1.96; 6.45)	<0.001

Quantile regression model. Model 1 was adjusted for sex and age. Model 2 was adjusted for age, sex, Mediterranean Diet adherence score, physical activity and body mass index. Model 3 was adjusted for age, sex, meat consumption, physical activity and body mass index. Model 4 was adjusted for age, sex, meat consumption, physical activity and HOMA-IR. Multivariable adjusted Model 5 was adjusted for age, sex, meat consumption, physical activity and DPP4. Model 6 was adjusted for age, sex, meat consumption, physical activity and RBP4.

Minimally adjusted (Model 1: age and sex) and multiple adjusted (Model 2: age, sex, Mediterranean diet adherence score, physical activity and BMI; Model 3: age, sex, meat consumption, physical activity and body mass index; Model 4: age, sex, meat consumption, physical activity and HOMA-IR; Model 5: age, sex, meat consumption, physical activity and DPP4; Model 6: age, sex, meat consumption, physical activity and RBP4) models exhibited positive associations between the lowest to highest tertile of serum ferritin concentrations and ALT, liver fat mass and hepatic iron content.

In order to further analyse the potential usefulness of serum ferritin as a predictor of NAFLD, the Receiver Operating Characteristic (ROC) curves for ferritin were calculated, using the MRI technique as the reference method to quantify the liver fat and hepatic iron. The areas under the curve (AUC) of serum ferritin were 0.73 and 0.68 for liver fat and hepatic iron content, respectively. We also investigated whether its combination with other biochemical parameters might improve the AUC of serum ferritin alone. Forward-selection procedures identified the combination of ferritin, glucose and ALT (AUC0.82) as the best predictive score for liver fat mass, followed by a combination panel formed of ferritin and glucose (AUC0.80). On the other hand, a panel combination of ferritin and ALT showed the major predictive ability for liver iron content (AUC0.73) followed by a panel designed with ferritin and TG (AUC0.72), shown in Figures 1A and 1B.

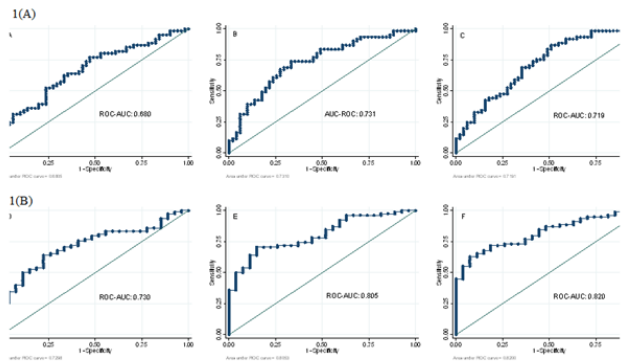

Figure 1: (A) Receivers Operating Curves between Liver Iron percentage by Magnetic Resonance Imaging (MRI) and A: Ferritin; B: Ferritin and ALT; C: Ferritin and Triglycerides. (B) Receivers Operating Curves between Liver Fat percentage by Magnetic Resonance Imaging (MRI) and D: Ferritin; E: Ferritin and glucose; F: Ferritin, glucose and ALT.

DISCUSSION

The current research involving the Fatty Liver in Obesity (FLiO) project demonstrated the association of serum ferritin concentrations with liver health as well as glucose and lipid metabolism in the presence of NAFLD. The analysis of ferritin by means of quantile regression showed a positive association with ALT, liver fat content and hepatic iron. Our data have also driven to assess ferritin as a predictive biomarker of NAFLD. Remarkably, serum ferritin allowed to predict the liver fat deposition and hepatic iron content by MRI, alone or in combination with other routine biochemical parameters as TG, ALT and glucose.

NAFLD is a clinical syndrome increasing globally, and it is a leading cause of chronic liver disease [2]. The liver is the major site of systemic iron regulation and is central to its metabolism [38]. Hepatocytes constitute the major parenchymal iron storage pool and contain large amounts of ferritin, the primary iron storage protein [39]. Iron is an essential, but potentially toxic element that may promote the onset and progression of NAFLD by increasing oxidative stress and altering insulin signalling and lipid metabolism [14-16,40,41]. Iron overload is observed in approximately one-third of adults with NAFLD, but the mechanisms underlying iron deposition are not entirely understood [42]. In NAFLD, damage to hepatic cells induces an increase in circulating ferritin concentrations, released into the bloodstream [18,19]. As ferritin is also an acute phase reactant, it may be elevated in inflammatory processes [20,21]. Mild to moderate serum ferritin concentrations are found in NAFLD, involving up to 30% of affected subjects [22,23]. Researchers found that hyperferritinemia has been associated with markers of liver injury [25,43,44]. In accordance with previous reports, our results showed that serum ferritin was strongly associated with ALT and liver fat content suggesting a close connection between high serum ferritin levels and impaired liver metabolism. In addition, a positive association was also found between serum ferritin and hepatic iron content. In line with our results, Ryan et al. reported a strong association between ferritin and hepatic iron content by MRI in 129 participants with NAFLD [45]. Most of the study subjects did not have significant hyperferritinemia or iron overload. Instead, slight changes in iron status were evident, which correlated with disease stage. Regarding possible explanations of these clinical manifestations, the major discussed mechanism is that iron induced oxidative stress resulting in cellular damage [14,15,16].

Increased iron stores are intimately connected to β-cell dysfunction, impaired glucose metabolism, DNA damage and lipid peroxidation [40,41]. Furthermore, iron overload, estimated as ferritin levels, has been associated with hyperinsulinemia, type 2 diabetes, IR and MetS features, conditions inherently related to NAFLD [24,41]. Interestingly, we found that serum ferritin was significantly associated with high HOMA-IR, a marker of IR. As a novelty, our results have shown that serum ferritin levels were positively correlated with DPP4 and RBP4, giving new molecular pathways that could explain the link between iron homeostasis and IR. The mechanisms by which iron metabolism may affect glucose and insulin traits are currently unknown, although some hypotheses have been suggested to explain this possible association. Recent data suggest that iron induced reactive oxygen species (ROS), initiating an oxidative stress cascade causing lipid peroxidation and disturbances in insulin signalling [46]. Increased free radicals may contribute to insulin resistance via increased free fatty acids oxidation and reduction of glucose uptake by the muscle [47]. High hepatic iron stores also decrease insulin extraction and affecting insulin signalling [48,49]. Furthermore, ferritin has been associated with reduced adiponectin, a key mediator of insulin sensitivity [45]. However, we did not find significant associations between serum ferritin and adiponectin. Interestingly, we found that the association between ferritin and liver markers remained significant after adjustment for IR (HOMA-IR, RBP4 and DPP4). The above points imply that association between ferritin and liver status is not entirely explained by the associations with hyperinsulinemia and IR, and more studies concerning this issue are needed.

About lipid metabolism, serum ferritin was significantly correlated with high triglycerides and low HDL-c levels. In line with our results, there is a growing body of evidence that iron may affect lipid metabolism, possibly via hepcidin [50]. Some researchers reported a positive association between hepatic hepcidin expression and TC, TG and LDL-c concentrations in NAFLD [51]. In a meta-analysis evaluated the association between ferritin and MetS. Remarkably, they reported that high triglycerides and glucose were the components more strongly linked to ferritin [52]. Additionally, numerous proteomic and hepatic gene expression studies have found a link between iron homeostasis and lipid status although more research is needed to further elucidate this relationship in the context of NAFLD and progression to NASH [50,51,53].

When dietary intake and food groups were explored, we evidenced that meat consumption was increased in participants with higher serum ferritin. These results were in accordance with the literature, since numerous evidences have suggested that some meat components as heme-iron, sodium and

preservatives could be potentially harmful for health and, specifically, liver function [40,54,55]. In this context, some studies found an association of meat or heme-iron intake with higher serum ferritin, leading to necro inflammation and fibrosis, both hallmarks of NAFLD [56]. On the other hand, fish was associated with lower concentrations of ferritin. In this context, the omega-3 PUFAs contained in fatty fish might exert beneficial effects over ferritin levels. Several studies have shown that omega-3 PUFAs are inversely associated with NAFLD, by decreasing pro inflammatory molecules, TG and improving liver histology [50,57]. In addition, fish contains lower heme-iron when compared with red meat, thus explaining the results obtained in this study [58]. Fish could be proposed as a healthier dietary alternative whereas meat consumption should be controlled in the management of NAFLD.

Liver biopsy is the reference method for diagnose of NAFLD [42,59]. However, liver biopsy presents various limitations since it is an invasive technique and surgical expensive, and only represents approximately 1/50000 of the hepatic volume [2,10]. In this context, robust non-invasive tests are needed to quantify fat and iron content [7,11,46]. Therefore, research is focusing on more efficient diagnostic and predictive biomarkers for identifying NAFLD features at early stages [2,10,11,12,60]. Recent studies evidenced that iron metabolism related parameters may be suitable predictors of liver disease outcomes [13]. In this research, we hypothesized that serum ferritin could constitute a marker of fatty liver in subjects with NAFLD. In fact, serum ferritin, intimately connected to liver health condition, IR and MetS [52], allows to predict the liver fat deposition and hepatic iron content by MRI, alone or in combination with other routine biochemical parameters link to lipid, glucose or hepatic metabolism such as TG, ALT and glucose. Indeed, the combination of ferritin, glucose and ALT showed the best prediction for liver fat mass with an accuracy of 82%. On the other hand, a panel combination of ferritin and ALT showed the major predictive ability for liver iron content(AUC0.73).

The design of different predictive models for NAFLD through blood biomarkers have many advantages, although further investigation and consensus are needed concerning the utility of non-invasive liver markers.

This assay adds further insights and knowledge about the link between iron metabolism and NAFLD. Serum ferritin levels showed a relevant impact on both liver health and general metabolism, being a key factor to be considered in the management of NAFLD. Our results also suggested the possible clinical use of ferritin as an indicator of NAFLD, alone or in combination with other routine biochemical measures.

The current study presents some limitations. Firstly, the cross-sectional nature of the study which does not allow the establishment of causality. Thus, longitudinal studies are needed to determine whether ferritin might be a good predictor of the progression of the disease or it is just a consequence of the liver function alteration. Secondly, dietary data was evaluated using self-reported information of the participants, and thus, the results are susceptible to some degree of bias. Thirdly, we did not analyse other iron metabolism parameters such as transferrin, serum iron or hepcidin that could provide complementary information. On the other hand, some strengths can be mentioned. In this way, participants have been carefully selected following exclusion and inclusion criteria to avoid a heterogeneous sample. Participants have been well characterized and the methodology used to evaluate liver health was ultrasonography and MRI.

CONCLUSION

The present study demonstrated the association of serum ferritin with liver health(ALT, liver fat content and hepatic iron) as well as glucose and lipid metabolism in individuals with NAFLD. Also, this research identified ferritin as a potential biomarker of NAFLD, enabling to predict the liver fat deposition and hepatic iron content by MRI, alone or in combination with other routine biochemical parameters as TG, ALT and glucose.

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