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Original article

Insulin resistance and NAFLD: Relationship with intrahepatic iron and serum TNF-α using 1H MR spectroscopy and MRI

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ABSTRACT

Aim. – The association of non-alcoholic fatty liver disease (NAFLD) with insulin resistance (IR) is well established, yet little is known of their possible relationship with intrahepatic iron and serum tumour necrosis factor (TNF)-α concentrations in adults without diabetes. Thus, this study looked at the relationship of intrahepatic iron and serum TNF-α with intrahepatic triglycerides and IR in non-diabetic adults.
Methods. – In this cross-sectional study of 104 healthy non-diabetic Caucasians, a quantitative magnetic resonance (MR) imaging T2 gradient-echo technique was used to measure hepatic iron, with 1H-MR spectroscopy used to measure hepatic triglycerides. HOMA-IR was calculated to determine IR.
Results. – The prevalence of hepatic iron overload (HIOL) was 26.6% in individuals with NAFLD vs. 0% in those without. IR was present in 87.5% of subjects with both NAFLD and HIOL, in 45.4% of those with NAFLD but not HIOL, and in 4.5% of those with neither. HOMA-IR was positively correlated with hepatic triglycerides ($r = 0.56$, $P < 0.001$) and hepatic iron ($r = 0.52$, $P < 0.001$), whereas serum TNF-α concentrations correlated with intrahepatic triglyceride levels ($r = 0.28$, $P < 0.04$), but not with intrahepatic iron. Hepatic triglycerides, serum TNF-α and age were the only significant determinants of IR in regression analyses.
Conclusion. – IR is closely associated with intrahepatic triglycerides and, to a lesser extent, intrahepatic iron, with some interplay between them. High serum TNF-α concentrations may contribute to the association between NAFLD and IR, while increased hepatic triglycerides appear to be a determinant of the development of HIOL in non-diabetic subjects without haemochromatosis.

Introduction

Over the past two decades, non-alcoholic fatty liver disease (NAFLD) has become a major public-health problem in the developed countries and been linked to obesity, type 2 diabetes mellitus (T2DM) and the metabolic syndrome (MetS), although it is also found in non-obese individuals. Patients with NAFLD generally have an increased risk of death from cardiovascular disease rather than from chronic liver disease [1,2]. It is well established that insulin resistance is closely associated to NAFLD, and hepatic iron overload has emerged as a possible new factor involved not only in NAFLD, but also in insulin resistance [3,4]. Studies of liver biopsies have observed hepatic stainable iron in around one-third of adults [5] and one-quarter of children with biopsy-proven NAFLD [6]. Epidemiological studies have reported that excessive dietary iron intakes and high body iron stores may also increase the risk of T2DM [7]. Likewise, laboratory and animal studies have found that

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iron overload induces insulin resistance [8,9,10], while one human study concluded that insulin sensitivity could be partly related to iron accumulation in the liver [11]. It has also been observed that a reduction in total iron body content through phlebotomy can improve insulin resistance in patients with NAFLD and hyperferritinaemia [4,12], as well as in those with normal serum ferritin concentrations [4,13]. However, two large-scale prospective controlled trials of the impact of phlebotomy in patients with NAFLD reported no improvement in either insulin resistance or hepatic steatosis [14,15], and an autopsy study found no differences in hepatic iron content between those with and without T2DM [16].

Thus, based on the above data, the relationship between iron accumulation in liver and insulin resistance remains controversial. There have also been no definitive conclusions as to the pathogenic relevance of the association between NAFLD and hepatic iron overload with insulin resistance. For this reason, our present study hypothesized that subclinical chronic inflammation may be involved in the association of intrahepatic triglycerides and intrahepatic iron with insulin resistance. Given this background, the objectives of our study were to look at the relationships between insulin resistance and intrahepatic triglycerides, as quantified by 3 Tesla (3T) ^{1}H magnetic resonance spectroscopy (MRS), and intrahepatic iron, as measured by magnetic resonance imaging (MRI), and to analyze their possible relationship with serum tumour necrosis factor (TNF)-α and adiponectin concentrations and lipid profile. The study was conducted in non-diabetic Caucasians to avoid the presence of T2DM as a confounding factor.

Design and methods

Study population

The study included 104 healthy non-diabetic Caucasian adults aged 25 to 70 years. Participants were consecutively recruited from people undergoing examinations at the Occupational Risk Prevention Unit in Granada (Southern Spain) for their routine annual general check-ups. Study exclusion criteria were: history of daily alcohol intakes > 20 g (men) or > 10 g (women), based on their responses to a validated questionnaire on alcohol consumption and confirmation of the results by a family member; presence of hepatitis B virus (HBV)/hepatitis C virus (HCV) serological markers, autoimmune hepatitis, primary biliary cirrhosis, Wilson's disease, cancer, diabetes, or endocrine, cardiac, renal or pulmonary disease; use of drugs that might cause steatosis (such as corticosteroids, amiodarone, methotrexate, tamoxifen); body mass index (BMI) < 18.5 or ≥ 40 kg/m^2; and use of a pacemaker or other device not compatible with ^{1}H-MRS.

Haemochromatosis was excluded with the following protocol. Patients with serum ferritin levels > 300 ng/mL (men) or > 150 ng/mL (women) and transferrin saturation > 45% underwent screening for *HFE* (haemochromatosis) gene mutations; those with *HFE* genotypes potentially predisposing to iron overload (C282Y/H63D, H63D/H63D, C282Y/wt) were excluded. No C282Y homozygotes were found.

The present study was approved by the local ethics committee of the Hospital San Cecilio in Granada. All participants were given complete information about the study and gave their written informed consent to participate.

Study design and anthropometric evaluations

All subjects recruited into the study underwent a full medical history, physical examination, complete blood analyses and ultrasound examination as part of the screening process. Their weight and height were also recorded, and their BMI (kg/m^2) calculated; waist circumference was also measured using a soft tape placed midway between the lowermost rib and iliac crest in standing position.

Laboratory analyses

Blood was drawn in the morning after overnight fasting. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were determined by a kinetic method using a cobas analyzer (Roche Diagnostics GmbH, Rotkreuz, Switzerland), serum glucose by the glucose oxidase (enzymatic) method using a Roche/Hitachi Modular Analytics System (Roche Diagnostics), adiponectin concentrations by radioimmunoassay (LINCO Research, St. Charles, MO, USA), serum insulin by electrochemiluminescence immunoassay (Elecsys 2010, Roche Diagnostics), serum TNF-α by human TNF-alpha enzyme-linked immunosorbent assay (ELISA; BioSource Europe, Nivelles, Belgium), serum cholesterol by an enzymatic method (Roche Diagnostics) and serum ferritin concentrations by immunochemiluminescence using an Architect i2000 analyzer (Abbott Laboratories, Abbott Park, IL, USA). Insulin resistance was calculated according to the homoeostasis model assessment method for insulin resistance (HOMA-IR) formula: fasting insulin (mU/L) × fasting glucose (mmol/L)/22.5 [17]. The presence of insulin resistance was defined as HOMA-IR values > 95th percentile in lean subjects (HOMA-IR > 2.8). Coefficients of variation for the biochemical tests ranged from 3.1–9.9%.

3T MRI and 1H-MRS examinations

Non-contrast multiparametric 3T MRI was performed as described elsewhere [18–20], using an Achieva system (Royal Philips, Amsterdam, Netherlands). Liver MRI comprised a multi-echo chemical-shift-based encoded gradient-echo (MECSE-MR) sequence using 12 echoes [echo time (TE) = 0.99–8.69; short echo spacing = 0.7 ms; repetition time (TR) = 10 ms] with a 10º flip angle to minimize T1 bias. The whole liver was covered under end-expiratory phase single-breath-hold acquisition (34 slices; voxel dimensions: 3 × 3 mm; slice thickness: 7 mm; 0.3-mm gap; reconstruction voxel size: 2 × 2 mm; field of view: 375 × 302 mm; parallel imaging effective acceleration factor: 1.8; and bandwidth: 2433 Hz/pixel) [19].

A three-plane localizer was used to plan the ^{1}H-MRS, using spectra obtained with the scanner's body coil and monitoring breath-hold with a respiratory belt. A single voxel of 27 cm^3 (30 × 30 × 30 mm) was selected in segment-VI normal liver tissue, avoiding the edge of the liver, diaphragm and major blood vessels. All spectra were obtained using the following stimulated echo acquisition mode (STEAM) sequence: TR = 8000 ms; TE = 20, 40 and 60 ms; number of signal averages = 4; no water suppression; and bandwidth = 2000 Hz. Data were acquired during one breath-hold. T2 correction was applied and field homogeneity was adjusted automatically for each voxel.

3T MRI and 1H-MRS analyses

Images were exported as raw data to quantify iron-related R2* measurements using QLiver software [Quantitative Imaging Biomarkers in Medicine (QUIBIM), Valencia, Spain], based on least-squares analysis using the Levenberg-Marquardt algorithm, which performs fitting of water and fat sinusoidal signals modulated by exponential decays corresponding to the R2* of water and R2* of fat. Only the former component was considered for the R2* iron measurements, given the negligible R2* fat component, and used to estimate iron content by averaging the mean value of three regions of interest (ROIs) within the right liver lobe, excluding vessels [20].

Table 1
Characteristics of all study participants and as classified by hepatic triglyceride content.

	Total participants	Non-alcoholic fatty liver disease (NAFLD)		P[a]
	(n = 104)	Without (42.31%) (n = 44)	With (57.69%) (n = 60)	
Gender (male/female)	59/45	19/25	40/20	0.017
Age (years)	45.11 ± 10.03	41.64 ± 8.89	47.65 ± 10.11	0.002
Body mass index (kg/m^2)	27.81 ± 4.31	25.26 ± 3.42	29.71 ± 3.92	0.001
Waist circumference (cm)	97.25 ± 14.38	88.02 ± 12.06	104.26 ± 11.88	0.001
Cholesterol (mg/dL)	195.27 ± 41.69	192.36 ± 42.87	197.40 ± 41.03	0.545
HDL-C (mg/dL)	55.35 ± 15.64	61.45 ± 15.17	50.87 ± 14.53	0.001
LDL-C (mg/dL)	117.59 ± 37.33	112.89 ± 34.91	121.03 ± 38.93	0.274
Triglycerides (mg/dL)	110.86 ± 71.40	85.20 ± 65.12	130.00 ± 70.36	0.001
Serum AST (IU/L)	26.04 ± 9.89	22.00 ± 7.86	29.00 ± 10.23	0.001
Serum GGT (IU/L)	41.50 ± 37.81	28.32 ± 23.94	51.17 ± 43.06	0.001
Serum ALT (IU/L)	34.75 ± 22.64	19.98 ± 6.91	45.58 ± 24.05	0.001
Fasting glucose (mg/dL)	92.54 ± 9.36	91.66 ± 6.52	93.19 ± 11.00	0.377
Fasting insulin (μU/mL)	9.37 ± 6.18	6.26 ± 2.55	13.72 ± 7.15	0.001
HOMA-IR	2.17 ± 1.52	1.41 ± 0.61	3.24 ± 1.78	0.001
Serum iron (μg/dL)	93.67 ± 35.68	86.16 ± 34.65	99.35 ± 35.75	0.090
Ferritin (ng/mL)	142.36 ± 128.84	70.42 ± 68.25	197.09 ± 137.49	0.001
Transferrin saturation (%)	24.86 ± 12.37	18.00 ± 10.64	27.50 ± 12.32	0.150
Hepatic iron R2[a] (s^{-1})	57.95 ± 18.38	47.68 ± 10.82	66.21 ± 19.11	0.001
Hepatic triglycerides (%)	10.87 ± 10.20	2.54 ± 1.45	16.99 ± 9.51	0.001
TNF-α (pg/mL)	164.29 ± 42.98	150.5 ± 31.35	174.39 ± 47.58	0.003
Adiponectin (μg/mL)	11.07 ± 9.09	15.47 ± 9.53	7.84 ± 7.28	0.001

HDL-C/LDL-C: high-density/low-density lipoprotein cholesterol; AST/ALT: aspartate/alanine aminotransferase; GGT: gamma-glutamyl transferase; HOMA-IR: homoeostasis model assessment of insulin resistance; TNF-α: tumour necrosis factor-alpha.
[a] With vs. without NAFLD.

MRS images were reconstructed using Extended MR WorkSpace software (Royal Philips). Raw data were zero-filled once with no filter, and then phase-corrected, Fourier-transformed, baseline-corrected and averaged. A Marquardt curve was fitted using a combined Lorentzian-Gaussian model to calculate the area under the curve (AUC) of the fat and water peaks. Spectra were referenced to the residual water and dominant methylene lipid (-CH2) peaks at δ = 4.47 ppm and δ = 1.43 ppm, respectively. Fat fraction (FF) percentage was defined as FA/(FA + WA) × 100, where FA is the area under the fat peak and WA is the area under the water peak.

All MRI and ^{1}H-MRS data were interpreted by an experienced radiologist who was blinded to the biochemical results. In addition, NAFLD was defined as an hepatic triglyceride content > 5.56% (5.56 g triglyceride/100 g wet liver tissue) as previously proposed, using the 95th percentile in lean subjects as reference [21], while iron overload was defined by the 95th percentile of R2* values in lean subjects (R2* > 75.7 seg^{-1}).

Statistical analyses

Results are expressed as means ± standard deviation (SD). The Kolmogorov-Smirnoff test was used to check the normality of the data distribution. Mean values were compared across groups by either unpaired two-tailed Student's *t*-test or non-parametric Mann–Whitney *U*-test and one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test, as appropriate. The chi-square test was used for non-continuous variables and to compare percentages. Correlations were examined by either Pearson's standard linear regression analysis (normal distribution) or Spearman's test (non-normal distribution). Backward stepwise regression analyses were conducted for evaluation of the main predictors of insulin resistance (hepatic triglyceride and iron contents) in our population of non-diabetic individuals by entering the following variables: age; gender; HOMA-IR (except for HOMA-IR analysis); hepatic triglyceride content (except for hepatic triglyceride analysis); hepatic iron R2* (except for intrahepatic iron analysis); waist circumference; BMI; and serum ALT, triglyceride, ferritin, adiponectin and high-density lipoprotein cholesterol (HDL-C) values. Only variables with P-values < 0.05 were retained in the final regression model. SPSS software for Windows version 22 (IBM Corp., Armonk, NY, USA) was used for all data analyses.

Results

Table 1 presents the anthropometric and biochemical data from all participants, and the characteristics of those with and without NAFLD. In comparison to individuals without NAFLD, patients with NAFLD had significantly greater age, BMI, waist circumference, HOMA-IR, and serum ALT, glucose, fasting insulin and TNF-α concentrations, but significantly lower serum adiponectin and

Table 2
Characteristics of participants according to hepatic iron content (n = 104).

	Hepatic iron overload		P[a]
	Without (84.79%) (n = 88)	With (15.21%) (n = 16)	
Gender (male/female)	52/36	9/7	0.899
Age (years)	43.81 ± 10.14	54.00 ± 7.05	0.001
Body mass index (kg/m^2)	27.22 ± 4.47	29.06 ± 2.10	0.038
Waist circumference (cm)	95.27 ± 15.06	103.14 ± 4.26	0.050
Cholesterol (mg/dL)	194.51 ± 44.24	198.64 ± 32.30	0.431
HDL-C (mg/dL)	54.92 ± 15.57	51.50 ± 15.49	0.453
LDL-C (mg/dL)	117.45 ± 39.23	118.86 ± 29.74	0.625
Triglycerides (mg/dL)	106.85 ± 70.18	145.43 ± 80.82	0.040
Serum AST (IU/L)	25.88 ± 10.51	28.79 ± 8.93	0.101
Serum GGT (IU/L)	40.08 ± 39.92	45.00 ± 30.12	0.150
Serum ALT (IU/L)	32.09 ± 20.22	49.43 ± 33.35	0.007
Fasting glucose (mg/dL)	92.31 ± 8.66	94.57 ± 11.63	0.493
Fasting insulin (μU/mL)	7.84 ± 4.83	21.45 ± 4.54	0.002
HOMA-IR	1.81 ± 1.23	5.06 ± 1.20	0.003
Serum iron (μg/dL)	92.73 ± 35.01	115.22 ± 40.32	0.101
Ferritin (ng/mL)	133.18 ± 115.34	285.52 ± 178.05	0.011
Transferrin saturation (%)	23.75 ± 11.32	37.90 ± 5.23	0.104
Hepatic iron R2[a] (s^{-1})	52.01 ± 11.65	91.05 ± 13.07	0.001
Hepatic triglycerides (%)	7.63 ± 6.70	19.97 ± 10.84	0.001
TNF-α (pg/mL)	162.98 ± 37.26	159.98 ± 36.05	0.728
Adiponectin (μg/mL)	11.87 ± 9.56	5.96 ± 4.08	0.023

HDL-C/LDL-C: high-density/low-density lipoprotein cholesterol; AST/ALT: aspartate/alanine aminotransferase; GGT: gamma-glutamyl transferase; HOMA-IR: homoeostasis model assessment of insulin resistance; TNF-α: tumour necrosis factor-alpha.

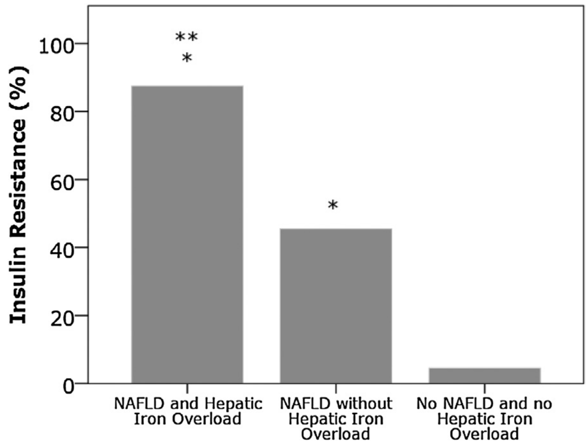

Fig. 1. Percentages of participants with insulin resistance in three different study groups. No (0/44) participant had hepatic iron overload without non-alcoholic fatty liver disease (NAFLD). **$P < 0.001$ vs. no NAFLD and no hepatic iron overload; *$P < 0.01$ vs. NAFLD without hepatic iron overload and vs. no NAFLD and no hepatic iron overload.

HDL-C concentrations. The same trend was observed in the comparison between those with and without hepatic iron overload (Table 2), except for no differences in serum TNF-α and HDL-C concentrations. Hepatic iron overload was recorded in 26.6% (16/60) of those with NAFLD vs. 0% (0/44) of those without. Table 3 compares individuals with and without insulin resistance, and shows that mean age, BMI, waist circumference, intrahepatic iron, intrahepatic triglycerides and serum ALT, triglycerides, fasting insulin, ferritin and TNF-α concentrations were significantly higher, whereas serum adiponectin and HDL-C concentrations were significantly lower, in those with *vs* without insulin resistance. Fig. 1 depicts the proportions of participants with insulin resistance in the different study groups, revealing insulin resistance in 4.54% (2/44) of those with neither NAFLD nor hepatic iron overload, in 45.45% (20/44) of those with NAFLD but no hepatic iron overload, and in 87.5% (14/16) of those with both NAFLD and hepatic iron overload.

Correlation studies

The results of these studies are shown in full in Table 4, as only significant correlations are reported in the following text.

HOMA-IR correlations

HOMA-IR was significantly and positively correlated with hepatic triglyceride content ($r = 0.56$, $P < 0.001$), hepatic iron content ($r = 0.52$, $P < 0.001$), age ($r = 0.63$, $P < 0.001$), waist circumference ($r = 0.64$, $P < 0.001$), BMI ($r = 0.60$, $P < 0.001$), serum ferritin ($r = 0.28$, $P < 0.05$), ALT ($r = 0.27$, $P < 0.05$), serum TNF-α ($r = 0.28$, $P < 0.04$) and serum triglyceride ($r = 0.38$, $P < 0.05$), but significantly and inversely correlated with serum adiponectin concentrations ($r = -0.35$, $P < 0.01$).

Correlations of hepatic triglyceride content

Hepatic triglyceride content was significantly and positively correlated with HOMA-IR ($r = 0.56$, $P < 0.001$), hepatic iron content ($r = 0.57$, $P < 0.001$), age ($r = 0.33$, $P < 0.01$), waist circumference ($r = 0.59$ $P < 0.001$), BMI ($r = 0.56$, $P < 0.001$), and serum concentrations of ferritin ($r = 0.41$, $P < 0.01$), ALT ($r = 0.78$, $P < 0.001$), TNF-α ($r = 0.28$, $P < 0.01$) and triglycerides ($r = 0.34$, $P < 0.01$), but was inversely correlated with serum adiponectin ($r = -0.41$, $P < 0.001$) and HDL-C ($r = -0.3$, $P < 0.02$) concentrations.

Table 3
Characteristics of participants according to insulin resistance ($n = 104$).

	Insulin resistance		P[a]
	Without (75%) ($n = 78$)	With (25%) ($n = 26$)	
Gender (male/female)	39/39	15/11	0.620
Age (years)	38.50 ± 7.52	51.75 ± 9.46	0.001
Body mass index (kg/m^2)	25.59 ± 3.64	31.45 ± 4.51	0.001
Waist circumference (cm)	87.49 ± 11.98	107.50 ± 10.65	0.000
Cholesterol (mg/dL)	183.33 ± 39.46	197.50 ± 35.61	0.284
HDL-C (mg/dL)	58.19 ± 14.17	48.17 ± 13.86	0.035
LDL-C (mg/dL)	109.53 ± 39.12	122.75 ± 32.46	0.194
Triglycerides (mg/dL)	76.77 ± 35.07	129.67 ± 57.36	0.003
Serum AST (IU/L)	26.86 ± 12.65	25.42 ± 3.92	0.431
Serum GGT (IU/L)	27.83 ± 22.94	52.08 ± 44.59	0.008
Serum ALT (IU/L)	28.03 ± 16.99	38.17 ± 11.07	0.008
Fasting glucose (mg/dL)	89.43 ± 8.26	96.42 ± 7.05	0.018
Fasting insulin (μU/mL)	6.30 ± 2.62	18.58 ± 4.23	0.001
HOMA-IR	1.42 ± .65	4.42 ± 1.07	0.001
Serum iron (μg/dL)	88.18 ± 32.62	106.67 ± 41.21	0.204
Ferritin (ng/mL)	95.17 ± 88.33	215.06 ± 148.84	0.009
Transferrin saturation (%)	16.97 ± 4.64	40.20 ± 13.72	0.083
Hepatic iron R2[a] (s^{-1})	47.53 ± 9.44	67.20 ± 17.49	0.001
Hepatic triglycerides (%)	5.71 ± 6.87	16.85 ± 10.91	0.001
TNF-α (pg/mL)	162.24 ± 49.37	191.32 ± 52.39	0.051
Adiponectin (μg/mL)	17.21 ± 10.03	11.40 ± 10.75	0.043

HDL-C/LDL-C: high-density/low-density lipoprotein cholesterol; AST/ALT: aspartate/alanine aminotransferase; GGT: gamma-glutamyl transferase; HOMA-IR: homoeostasis model assessment of insulin resistance; TNF-α: tumour necrosis factor-alpha.
[a] With vs. without insulin resistance.

Correlations of hepatic iron content

Hepatic iron content was significantly and positively correlated with HOMA-IR ($r = 0.52$, $P < 0.001$), hepatic triglyceride content ($r = 0.57$, $P < 0.001$), age ($r = 0.50$, $P < 0.001$), waist circumference ($r = 0.49$ $P < 0.001$), BMI ($r = 0.39$, $P < 0.01$), and serum ferritin ($r = 0.62$, $P < 0.001$), ALT ($r = 0.47$, $P < 0.001$) and triglyceride ($r = 0.26$, $P < 0.05$) concentrations, but was inversely correlated with serum adiponectin ($r = -0.45$, $P < 0.001$) and HDL-C ($r = -0.22$, $P < 0.05$) concentrations.

Correlations of serum TNF-α

Serum TNF-α concentrations were significantly and positively correlated with HOMA-IR ($r = 0.28$, $P < 0.04$), hepatic triglyceride content ($r = 0.28$, $P < 0.01$), waist circumference ($r = 0.38$, $P < 0.02$), BMI ($r = 0.39$, $P < 0.02$), and serum ALT ($r = 0.2$, $P < 0.05$) and triglyceride ($r = 0.22$, $P < 0.03$) concentrations, but inversely correlated with serum adiponectin ($r = -0.23$, $P < 0.04$) and HDL-C ($r = -0.51$, $P < 0.001$).

Correlations of serum ferritin

Serum ferritin concentrations were significantly and positively correlated with HOMA-IR ($r = 0.28$, $P < 0.05$), hepatic triglyceride content ($r = 0.41$, $P < 0.01$), hepatic iron content ($r = 0.62$, $P < 0.001$), age ($r = 0.26$, $P < 0.01$), waist circumference ($r = 0.54$, $P < 0.001$), BMI ($r = 0.38$, $P < 0.001$), and ALT ($r = 0.62$, $P < 0.001$) and triglyceride ($r = 0.22$, $P < 0.02$) concentrations, but inversely correlated with serum adiponectin ($r = -0.50$, $P < 0.001$) and HDL-C ($r = -0.38$, $P < 0.001$) concentrations.

Regression analyses

In the backward stepwise multiple regression analysis of the main determinants of insulin resistance (HOMA-IR), hepatic triglyceride content was the most important predictor [B coefficient = 0.144, standard error (SE) = 0.044, $P < 0.003$], although

Table 4
Results of correlation studies.

	HOMA-IR	Hepatic TR content	Hepatic iron content	Serum TNF-α	Serum ferritin
HOMA-IR	–	r = 0.56 $P < 0.001$	r = 0.52 $P < 0.001$	r = 0.28 $P < 0.04$	r = 0.28 $P < 0.05$
Hepatic TR content (%)	r = 0.56 $P < 0.001$	–	r = 0.57 $P < 0.001$	r = 0.28 $P < 0.01$	r = 0.41 $P < 0.01$
Hepatic iron content (s^{-1})	r = 0.52 $P < 0.001$	r = 0.57 $P < 0.001$	–	r = 0.026 $P < 0.793$	r = 0.62 $P < 0.001$
Age (years)	r = 0.63 $P < 0.001$	r = 0.33 $P < 0.01$	r = 0.50 $P < 0.001$	r = −0.140 $P < 0.126$	r = 0.26 $P < 0.01$
WC (cm)	r = 0.64 $P < 0.001$	r = 0.59 $P < 0.001$	r = 0.49 $P < 0.001$	r = 0.38 $P < 0.02$	r = 0.54 $P < 0.001$
BMI (kg/m^2)	r = 0.60 $P < 0.001$	r = 0.56 $P < 0.001$	r = 0.39 $P < 0.01$	r = 0.39 $P < 0.02$	r = 0.38 $P < 0.001$
Serum ferritin (ng/mL)	r = 0.28 $P < 0.05$	r = 0.41 $P < 0.01$	r = 0.62 $P < 0.001$	r = 0.005 $P < 0.966$	–
ALT (IU/L)	r = 0.27 $P < 0.05$	r = 0.78 $P < 0.001$	r = 0.47 $P < 0.001$	r = 0.2 $P < 0.05$	r = 0.62 $P < 0.001$
Serum TNF-α (ρg/mL)	r = 0.28 $P < 0.04$	r = 0.28 $P < 0.01$	r = 0.026 $P < 0.793$	–	r = 0.005 $P < 0.966$
Serum TR (mg/dL)	r = 0.38 $P < 0.05$	r = 0.34 $P < 0.01$	r = 0.26 $P < 0.05$	r = 0.22 $P < 0.03$	r = 0.22 $P < 0.02$
Serum adipo (μg/mL)	r = −0.35 $P < 0.01$	r = −0.41 $P < 0.001$	r = −0.45 $P < 0.001$	r = −0.23 $P < 0.04$	r = −0.50 $P < 0.001$
HDL-C (mg/dL)	r = −0.204 $P < 0.139$	r = −0.3 $P < 0.02$	r = −0.22 $P < 0.05$	r = −0.51 $P < 0.001$	r = −0.38 $P < 0.001$

HOMA-IR: homoeostasis model assessment of insulin resistance; TR: triglyceride; TNF-α: tumour necrosis factor alpha; WC: waist circumference; BMI: body mass index; ALT: alanine aminotransferase; adipo: adiponectin; HDL-C: high-density lipoprotein cholesterol.

Table 5
Backward stepwise multiple regression analysis of the main determinants of insulin resistance (by HOMA-IR).

	B coefficient	SE	Significance	95% CI
Hepatic TR content (%)	0.144	0.044	0.003	0.054–0.234
Age (years)	0.095	0.034	0.009	0.025–0.165
Serum TNF-α (ρg/mL)	0.048	0.026	0.047	0.997–1.103

HOMA-IR: homoeostasis model assessment of insulin resistance; SE: standard error; 95% CI: 95% confidence interval; TR: triglyceride; TNF-α: tumour necrosis factor-alpha.

Table 6
Backward stepwise multiple regression analysis of the main determinants of hepatic triglyceride content.

	B coefficient	SE	Significance	95% CI
Hepatic iron content (pg/mL)	0.293	0.076	0.0001	0.139–0.446
Serum TNF-α (pg/mL)	0.047	0.021	0.032	0.004–0.090

SE: standard error; 95% CI: 95% confidence interval; TNF-α: tumour necrosis factor-alpha.

Table 7
Backward stepwise multiple regression analysis of the main determinants of hepatic iron content.

	B coefficient	SE	Significance	95% CI
Hepatic TR content (%)	0.985	0.251	0.0001	0.475–1.494
Serum ferritin (ng/mL)	0.029	0.013	0.030	0.003–0.055

SE: standard error; 95% CI: 95% confidence interval; TR: triglyceride.

age (B coefficient = 0.095, SE = 0.034, $P < 0.009$) and serum TNF-α were also significant (B coefficient = 0.048, SE = 0.026, $P < 0.047$). No other variable reached $P < 0.05$ significance (Table 5).

In the backward stepwise multiple regression analysis of the main determinants of hepatic triglyceride content, hepatic iron content was the most important determinant (B coefficient = 0.293, SE = 0.076, $P < 0.0001$), while serum TNF-α was also significant (B coefficient = 0.047, SE = 0.021, $P < 0.032$). All other significant ($P < 0.05$) variables are presented in Table 6.

In the backward stepwise multiple regression analysis of the main determinants of hepatic iron content, hepatic triglyceride content was the most important predictor (B coefficient = 0.985, SE = 0.251, $P < 0.0001$), but serum ferritin (B coefficient = 0.029, SE = 0.013, $P < 0.030$) was also significant. No other variable reached $P < 0.05$ significance (Table 7).

Discussion

In the present study of individuals without T2DM, insulin resistance was closely associated with hepatic triglyceride content and, to a lesser extent, with hepatic iron content, and revealed some degree of interplay between them. In addition, high serum TNF-α concentrations may also contribute to the association between NAFLD and insulin resistance. However, as hepatic iron overload was found in only one-third of our subjects with NAFLD and in none of those without NAFLD, this suggests that increased intrahepatic triglycerides may be especially important for the development of hepatic iron overload in patients with neither T2DM nor haemochromatosis.

It has been widely debated whether insulin resistance is a cause or a consequence of NAFLD: however, it is now generally accepted that insulin resistance both causes and appears to be a consequence of NAFLD [22–24]. The association of liver fat accumulation with insulin resistance is well established, although there is substantial between-patient variation, mainly attributed to genetic and environmental factors [22]. These include iron overload, which has been implicated in the pathogenesis of insulin resistance [9,25,26]. This is consistent with our present findings of greater hepatic iron content in subjects with *vs* without insulin resistance, and the significant positive correlation between hepatic iron and HOMA-IR values. However, the amount of iron in the liver was not a significant determinant of insulin resistance in our study, as insulin resistance was observed in 45.45% of those with NAFLD but without hepatic iron overload vs. 87.5% of those with both NAFLD and liver iron overload.

In fact, these data suggest that insulin resistance is more strongly associated with NAFLD than with hepatic iron overload and that there is some interplay between them, as previously suggested as regards insulin sensitivity [11]. In addition, unlike intrahepatic iron, intrahepatic triglyceride content proved to be a significant determinant of insulin resistance on regression analyses, thereby supporting the greater impact of NAFLD, although the pathogenic link between NAFLD, intrahepatic iron and insulin resistance remains unclear.

Our study hypothesis was that increased intrahepatic triglycerides and/or iron might be contributing to insulin resistance through changes in the secretion of cytokines commonly found in various chronic liver diseases [27,28] and in NAFLD [24,29] as an expression of subclinical inflammation. Interestingly, in our study, serum TNF-α concentrations were higher in individuals with vs. without NAFLD and in those with *vs* without insulin resistance, whereas there was no significant difference between those with vs. without hepatic iron overload. Furthermore, serum TNF-α values were positively correlated with intrahepatic triglyceride content, but not with intrahepatic iron content, whereas regression analyses showed that serum TNF-α concentrations were significant determinants of both intrahepatic triglyceride content and insulin resistance. Taken together, these findings suggest that serum TNF-α might play a role in the association of insulin resistance with NAFLD rather than intrahepatic iron. Likewise, serum TNF-α concentrations had a negative influence on serum adiponectin and lipid profiles.

To the best of our knowledge, this is the first published study of the relationship of intrahepatic triglycerides and iron content with insulin resistance and serum TNF-α concentrations in Caucasian adults without T2DM. In addition, despite having no ready explanation for the potential negative effect of hepatic iron accumulation on insulin resistance, our finding of no association between hepatic iron and serum TNF-α concentrations suggest that intrahepatic iron-associated subclinical inflammation may not be playing a role in the development of insulin resistance, although the possible role of oxidative stress cannot be ruled out [30].

Serum ferritin is an established biomarker of not only body iron stores, but also of inflammation, and elevated concentrations have been associated with insulin resistance [31]. In the present study, serum ferritin concentrations were positively correlated with insulin resistance, intrahepatic triglycerides and intrahepatic iron, but not with serum TNF-α concentrations and, more importantly, proved to be a significant determinant of intrahepatic iron content in regression analyses. Together with the positive relationship of serum ferritin concentrations with serum triglyceride and ALT concentrations, and their negative relationship with serum adiponectin and HDL-C concentrations, these findings support the idea that serum ferritin concentrations in NAFLD patients reflect increases not only in intrahepatic iron, and also possibly in insulin resistance and in the metabolic consequences of NAFLD rather than subclinical inflammation [32].

As already noted, clinical studies have reported discordant results regarding the relationship of increased body iron stores and serum ferritin concentrations with T2DM [16,33,34], and the effect of phlebotomy on insulin resistance [4,12,14,15]. These discrepancies may be explained, at least in part, by our finding that less than one-third of patients with NAFLD also have hepatic iron overload and that increased iron in the liver appears to play only a minor role in insulin resistance, although it may have an influence on the association between NAFLD and insulin resistance. It can therefore be speculated that only a specific subgroup of patients with NAFLD are likely to benefit from phlebotomy.

Our present study also looked at the association of HOMA-IR with BMI and waist circumference, and found positive univariate correlations of HOMA-IR with hepatic triglyceride content as well as BMI and waist circumference; nevertheless, in the multivariate regression analysis after adjusting for hepatic triglyceride content, neither BMI nor waist circumference were independent predictors of HOMA-IR, which suggests that their relationship with HOMA-IR might be mediated mostly by an increase in intrahepatic triglycerides. Also, the percentage of participants with NAFLD was higher than previously described in the general population with a similar use of MRS for liver triglyceride quantification [21]. These discrepancies could be due, at least in part, to ethnic differences in the populations studied, as our study involved only Caucasians whereas the other study included black subjects (about 48%), who are known to have a significantly lower prevalence of NAFLD than their white counterparts [35]. In addition, in our study, there were greater proportions of overweight (51.46%) and obese (24.27%) vs. lean (24.27%) participants and thus, given the clear association between NAFLD and BMI, our proportion of patients with NAFLD was also higher. However, this was unlikely to have influenced our results regarding the link between insulin resistance, iron overload and liver fat content, as all of the participants in our sample were healthy and free of diabetes.

Nevertheless, in addition to its cross-sectional design, our study limitations must also include, first, the lack of liver biopsy and histological data, although none of our participants had overt liver disease on ultrasound examination and hepatic MRI, and assessment of liver injury was not our primary endpoint. Second, while abdominal visceral adipose tissue would have been more accurately measured by MRI, waist circumference is a simple indirect method for measuring visceral adiposity and, while it may represent visceral and subcutaneous fat, it has nonetheless been described as the most reliable surrogate marker of visceral adiposity [36], with a strong correlation reported between waist circumference and visceral adiposity assessed by MRI [37]. Third, HOMA-IR was used to measure insulin resistance instead of the more accurate euglycaemic clamp method. However, in non-diabetic subjects, the HOMA-IR can serve as a surrogate of insulin resistance, and is therefore an acceptable alternative to more expensive and time-consuming dynamic testing [17,38]. Finally, although our sample size provided adequate statistical power, further studies of larger samples are nevertheless warranted.

Our study strengths include the use of MRI to quantify hepatic iron content and ^{1}H-MRS to measure triglyceride content, and the adoption of strict exclusion criteria (presence of T2DM) to avoid possible confounding factors. Furthermore, all anthropometric, biochemical, MRI and ^{1}H-MRS data were gathered within a 48-h period.

In conclusion, our present results indicate that insulin resistance is closely associated with hepatic triglyceride content and, to a lesser extent, with hepatic iron content. Some interplay was also observed between NAFLD and hepatic iron overload, with the former having a greater impact, while high serum TNF-α concentrations may be contributing to the association between NAFLD and insulin resistance. Finally, increased intrahepatic triglyceride accumulation appears to be a determinant of the development of hepatic iron overload in non-diabetic subjects without haemochromatosis.

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Authors' contributions

JLMR researched data. JGC wrote the manuscript and researched data. AGC reviewed/edited the manuscript. LMB and AAG contributed to the discussion and reviewed/edited the manuscript. TGC researched data and contributed to the discussion. JLGC wrote the manuscript.

Disclosure of interest

The authors declare that they have no competing interest.

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