ORIGINAL ARTICLE

Metabolic subtypes of patients with NAFLD exhibit distinctive cardiovascular risk profiles

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Abstract

Background and Aims: We previously identified subsets of patients with NAFLD with different metabolic phenotypes. Here we align metabolomic signatures with cardiovascular disease (CVD) and genetic risk factors.

Approach and Results: We analyzed serum metabolome from 1154 individuals with biopsy-proven NAFLD, and from four mouse models of NAFLD with impaired VLDL-triglyceride (TG) secretion, and one with normal VLDL-TG secretion. We identified three metabolic subtypes: A (47%), B (27%), and C (26%). Subtype A phenocopied the metabolome of mice with impaired VLDL-TG secretion; subtype C phenocopied the metabolome of mice with normal VLDL-TG; and subtype B showed an intermediate signature. The percent of patients with NASH and fibrosis was comparable among subtypes, although subtypes B and C exhibited higher liver enzymes. Serum VLDL-TG levels and secretion rate were lower among subtype A compared with subtypes B and C. Subtype A VLDL-TG and VLDL–apolipoprotein B concentrations were independent of steatosis, whereas subtypes B and C showed an association with these parameters. Serum TG, cholesterol, VLDL, small dense $LDL_{5,6}$, and remnant lipoprotein cholesterol were lower among subtype A compared with subtypes B and C. The 10-year high risk of CVD, measured with the Framingham risk score, and the frequency of patatin-like phospholipase domain-containing protein 3 NAFLD risk allele were lower in subtype A.

Conclusions: Metabolomic signatures identify three NAFLD subgroups, independent of histological disease severity. These signatures align with known CVD and genetic risk factors, with subtype A exhibiting a lower CVD risk profile. This may account for the variation in hepatic versus cardiovascular outcomes, offering clinically relevant risk stratification.

INTRODUCTION

NAFLD represents a spectrum of progressive liver disease characterized by increased intrahepatic triglyceride (IHTG) content in the absence of excessive alcohol consumption.[1–3] NAFLD ranges from simple steatosis to NASH, fibrosis, and ultimately cirrhosis and HCC. NAFLD is strongly associated with obesity, dyslipidemia, type 2 diabetes mellitus, and the metabolic syndrome. Importantly, cardiovascular disease (CVD) is the leading cause of mortality in patients with NAFLD.[4] Although NAFLD has been shown to be an independent risk factor for CVD, the mechanisms by which NAFLD contributes to CVD are unknown.

NAFLD represents a complex heterogeneous disease in which the histological features result from an imbalance in liver lipid metabolism caused by multiple nutritional and environmental exposures on a susceptible genetic background.[3,5–7] A better understanding of NAFLD heterogeneity and its biology will facilitate the development of personalized treatments. Overproduction of VLDL into circulation has been observed in patients with NAFLD and is thought to be, in part, a consequence of increased *de novo* lipogenesis (DNL) promoting hepatic triglyceride (TG) synthesis.[8–10] NAFLD is characterized by increased serum levels of TG and cholesterol (CL) in VLDL and intermediate-density lipoproteins (IDL), increased concentration of smaller LDL subclasses, and decreased content of larger LDL subclasses, all of which are features associated with increased CVD risk.[11,12] Moreover, it has been suggested that increased levels of total lipids and CL in VLDL and LDL subclasses may be associated with NAFLD progression.[13] On the other hand, VLDL-TG secretion is relatively impaired in a subset of patients with NAFLD and cannot compensate for increases in both fatty acid (FA) influx and DNL.[14] Genetic defects (e.g., microsomal triglyceride transfer protein [MTTP], transmembrane 6 superfamily member 2 [TM6SF2] and apolipoprotein B, [APO-B]), which impair hepatic VLDL secretion, are also associated with reduced LDL-CL levels and a favorable CVD risk profile, yet they still cause steatosis that may progress to NASH with fibrosis, even without obesity or insulin resistance.[15–20] In addition, the therapeutic benefits of LDL reduction in patients treated with antisense APO-B or MTTP inhibitors are associated with hepatic steatosis, inflammation and fibrosis, which limits their utility.[21–23]

Our published data, using high-throughput metabolomics, suggested that patients with NAFLD could be subclassified into two main metabolic subtypes.[24] One of these subtypes (which we here refer to as subtype A) corresponds to specific serum metabolic alterations that correlated with those observed in methionine adenosyltransferase 1A knockout (*Mat1a*-KO) mice,[24] a model of NASH characterized by a reduction in hepatic S-adenosylmethionine (SAMe),[25] impaired phosphatidylcholine (PC) synthesis, and VLDL secretion.[26] Importantly, patients with NAFLD often exhibit downregulation of MAT1A expression[27,28] and low SAMe.[29] The comparison of the serum lipidomic profile of LDL receptor (*Ldlr*)–KO mice fed a high-fat diet[30] with the lipidomic profile of the cohort of patients with NAFLD mentioned previously revealed the existence of a second NAFLD subtype, referred to here as subtype C. The deletion of *Ldlr* in mice results in increased serum level of VLDL-TG and LDL-CL.[31–33] These findings raised the corollary question of whether patients with NAFLD with subtype A show impaired secretion rate (SR) of VLDL compared to patients with NAFLD with subtype C, which would show VLDL hypersecretion, and, consequently, both NAFLD subtypes could present different CVD risks.

Based on these concepts, here we investigated the association between NAFLD subtypes with the main classes (VLDL, IDL, LDL, and HDL) and subclasses of serum lipoproteins, as well as with several common single-nucleotide polymorphisms associated with hepatocyte TG accumulation and VLDL secretion.

MATERIALS AND METHODS

Study participants

NAFLD cases

Patients with biopsied NAFLD (n = 1154) were recruited at several clinical centers from Europe (Spain, UK, Italy, the Czech Republic), the USA (California and Florida), and Chile, as well as patients recruited by Galmed Pharmaceuticals (Israel) (Table 1). Patients fulfilled the following inclusion criteria: (1) age 18−75 years; (2) no known acute or chronic disease except for obesity, dyslipidemia, hypertension, or type 2 diabetes based on medical history, physical examination, and standard laboratory tests; and (3) alcohol consumption lower than 20 g/day for women and 30 g/day for men. Exclusion criteria included patients with NAFLD with cirrhosis and other causes of liver disease. The institutional review board of the participating hospitals approved the study, and written informed consent was obtained from all patients.[34]

Diagnoses were established histologically in liver biopsy specimens. The histological diagnosis of NAFLD was established by a single liver pathologist in each hospital using the scoring system defined by Kleiner/Brunt.[35,36] Following assessment, patients were classified into two histological groups: (1) biopsies with only steatosis, steatosis with inflammation but without ballooning, or steatosis with ballooning but without inflammation were classified as nonalcoholic fatty liver, and (2) the histopathologic definition of NASH was determined by the joint presence of steatosis, lobular inflammation, and hepatocellular ballooning, independently of the total NAFLD activity score.

Blood was drawn under fasting conditions within 30 days of the diagnostic liver biopsy was performed. Serum was stored at −80°C until analysis as described.[34] Clinical data were collected retrospectively using patient records and laboratory values obtained at the time of biopsy. Serum samples from all 1154 patients with NAFLD were used to identify the main metabolic NAFLD subtypes by analyzing their serum metabolomic profiles. A subset of 197 patients with NAFLD from this cohort was used to study the association of the NAFLD metabolic subtype with the serum lipoprotein profile (Table S1). This cohort was determined by the availability of sufficient serum to perform the lipoprotein profiling (0.5 ml). For details see the Supporting Methods.

TABLE 1 Clinical and biochemical characteristics of the patients with NAFLD included in the lipidomic study

		Total	Subtype A	Subtype B	Subtype C	*p* value
Demographics						
n		1154	541	305	308	
Age (years)		50.0 ± 11.9 (1154)	47.9 ± 12.2 (541)	52.0 ± 11.3 (305)	51.7 ± 11.3 (308)	0.182
Female (%)		576 (50%)	266 (49%)	151 (49%)	159 (52%)	0.778
BMI (kg/m^2)		34.6 ± 7.7 (1154)	36.4 ± 9.0 (541)	32.7 ± 6.0 (305)	33.4 ± 5.9 (308)	0.028
Biochemistry						
TG (mg/dl)		165.4 ± 106.1 (1109)	140.6 ± 94.5 (525)	177.5 ± 86.7 (292)	197.6 ± 130.2 (292)	2.84E-17
Cholesterol (mg/dl)		189.3 ± 44.4 (862)	175.8 ± 41.1 (392)	188.0 ± 37.3 (226)	212.3 ± 46.4 (244)	2.86E-16
ALT (U/l)		56.6 ± 44.9 (1112)	53.1 ± 45.5 (509)	56.3 ± 41.4 (297)	62.6 ± 46.5 (306)	6.20E-04
AST (U/l)		38.7 ± 28.2 (1113)	35.2 ± 25.1 (509)	40.3 ± 29.3 (297)	43.1 ± 31.3 (307)	8.00E-06
GGT (U/l)		66.5 ± 95.6 (687)	60.3 ± 104.5 (328)	70.5 ± 88.0 (190)	73.9 ± 85.0 (169)	2.09E-04
HbA1c (%)		6.5 ± 1.2 (722)	6.5 ± 1.3 (259)	6.4 ± 1.0 (220)	6.5 ± 1.1 (243)	0.854
HOMA-IR		7.0 ± 7.7 (597)	6.7 ± 7.9 (204)	7.1 ± 6.5 (186)	7.3 ± 8.5 (207)	0.412
Albumin (g/dl)		4.4 ± 0.4 (897)	4.4 ± 0.4 (379)	4.5 ± 0.3 (261)	4.4 ± 0.4 (257)	3.49E-04
INR		1.0 ± 0.2 (419)	1.1 ± 0.1 (138)	1.1 ± 0.3 (146)	1.0 ± 0.1 (135)	5.00E-06
Medication						
Lipid-lowering medication	N	685 (79.4%)	331 (76.3%)	193 (82.5%)	161 (82.6%)	0.076
	Y	178 (20.6%)	103 (23.7%)	41 (17.5%)	34 (17.4%)	
CVD risk (FRS)						
Intermediate–high (>10%)		46 (12.8%)	28 (12.1%)	8 (13.1%)	10 (14.7%)	0.851
High (>15%)		21 (5.8%)	10 (4.3%)	6 (9.8%)	5 (7.4%)	0.221
Histology						
NASH (%)		735 (64%)	359 (66%)	187 (61%)	189 (61%)	0.208
Steatosis grade (%)	1	48.31%	44.92%	52.13%	46.75%	0.118
	2	31.81%	34.01%	30.82%	29.22%	
	3	19.88%	21.07%	17.05%	24.03%	
Inflammation grade (%)	0	12.39%	13.31%	12.13%	11.36%	0.059
	1	46.94%	50.65%	46.56%	41.88%	
	2	33.97%	29.57%	34.75%	41.23%	
	3	6.68%	6.47%	6.56%	5.52%	
Ballooning grade (%)	0	29.54%	25.88%	35.08%	35.71%	0.013
	1	45.73%	49.17%	42.30%	43.83%	
	2	24.71%	24.95%	22.62%	20.45%	
Fibrosis stage (%)	0	25.92%	26.80%	29.51%	28.25%	0.447
	1	35.66%	38.26%	37.70%	37.34%	
	2	19.32%	22.74%	18.36%	18.18%	
	3	12.88%	12.20%	14.43%	16.23%	

Note: Values for age, BMI, HbA1c, TG, ALT, AST, GGT, HbA1c, HOMA-IR, albumin, and INR are given as means ± SD. Statistical significance was determined by Chi-square test for categorical variables and Kruskal-Wallis H-test for continuous variables.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular disease; FRS, Framingham risk score; GGT, gamma-glutamyl transferase; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis assessment model for insulin resistance; INR, international normalized ratio.

Controls

As a control for comparison of the lipoprotein profiling data, we included 350 serum samples from the general population[37] with similar gender and age to the cohort of patients with NAFLD used to determine the serum lipoprotein profile, and normal glucose, TG, CL, and alanine aminotransferase (ALT) (Table S2). For details, see the Supporting Methods.

Subjects with plasma VLDL-TG secretion rate

Serum samples from women (*n* = 30) for which the hepatic VLDL-TG metabolism and kinetics were previously studied[38] were used to determine the association of the NAFLD metabolic subtypes with the VLDL-TG secretion rate (SR). Three groups were considered of the following characteristics: lean subjects with low VLDL-TG SR (*n* = 10), obese subjects with NAFLD with

low VLDL-TG SR (*n* = 10), and obese subjects with NAFLD with high VLDL-TG SR (*n* = 10).

Animals

Serum samples from five mouse models of NASH, all of them previously described, were used to compare their metabolomic profile with that of the patients with NAFLD mentioned previously: *Mat1a*-KO mice,[24] methionine (0.1%) and choline-deficient diet (0.1MCD)–fed mice,[39] *Mttp*-LKO mice,[40] *Tm6sf2*-LKO mice,[41] and LDLR-KO mice fed a high-fat diet (*Ldlr−/−*.Leiden/HFD).[30]

Metabolomics

Serum metabolomic profiles were semi-quantified by liquid chromatography coupled with mass spectrometry.[24] For details see the Supporting Methods.

Lipoprotein analyses

Nuclear magnetic resonance lipoprotein analyses were performed in a subcohort of patients with biopsied NAFLD (*n* = 197; Supporting Table S1) and controls (*n* = 350; Supporting Table S2) as described.[37] For details, see the Supporting Methods.

Statistical analysis

Continuous variables were described by means ± SDs. Comparisons between pairs of groups were performed by two-tailed independent Student *t* test or Kruskal-Wallis H-test. Chi-square test was applied for categorical variables. Unadjusted *p* values were considered as statistically significant below 0.05.

Hierarchical clustering algorithms based on metabolites were used to visualize the differences in signatures between samples, considering the Ward's minimum variance as the agglomeration method. The maximum of the average of the individual silhouette widths was calculated for the clusters.

A multinomial logistic regression classifier analysis was applied to generate a predictive metabolic signature capable of discriminating among NAFLD subtypes. A forward stepwise method was used as a variable selection criterion, in which the process started with a null model and optimum variables were added in each step until the best model was achieved. Logarithmic transformation of the data was applied. Validation process was performed through 5-fold cross validation. References for libraries are included in the Supporting Methods.

RESULTS

The serum lipidome of patients with NAFLD with subtype A is similar to that of mouse models of NAFLD exhibiting defective VLDL secretion

Comparison of the serum metabolomic profile of the patients with NAFLD (*n* = 1154; Table 1) with that of different mouse models of NASH showing impaired (*Mat1a*-KO, 0.1MCD, *Mttp*-KO, and *Tm6sf2*-KO) or increased (*Ldlr−/−*.Leiden/HFD) VLDL-TG secretion using the experimental procedure previously described[24] resulted in the identification of three major metabolic NAFLD subtypes (A, B, and C) (Figure 1A, Figure S1). Patients with NAFLD (541, 47%) reflecting metabolomic features common to the four mouse models of NASH showing impaired VLDL secretion were classified as subtype A. Of the remaining subjects, 308 (27%) patients with NAFLD resembled the metabolic profile of the *Ldlr−/−*.Leiden/HFD NASH mouse model and were classified as subtype C, and the remaining 305 (26%) patients were classified as subtype B (Figure 1B, Table 1).

We then searched for lipid species that best differentiated among A, B, and C NAFLD subtypes (Figure 1C), and generated a statistical model in which these given lipids were assigned a relative weight depending on their importance for the classification of patients with NAFLD into subtypes (Table S3). This set of 12 NAFLD subtype biomarkers was gender-independent. We also identified signatures that could differentiate steatosis from NASH for each subtype. These signatures included glycerolipids, glycerophospholipids, and sphingolipids (Figure S2).

Table 1 lists the main clinical data of patients with NAFLD classified by their metabolic subtype. No differences were found between the percentage of patients with NASH or stage of fibrosis and the NAFLD subtype. Moreover, no gender differences were indicated between subtypes. Patients with metabolic subtypes B and C were slightly older, with lower body mass index (BMI), and had more frequently higher TG, CL, ALT, aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT), and less ballooning than subjects with subtype A. The homeostasis model assessment of insulin resistance (HOMA-IR) index, glycated hemoglobin (HbA1c), albumin, and international normalized ratio (INR) were similar among the three subtypes. The Framingham CVD risk score (FRS) was calculated for 360 patients with NAFLD, who were chosen based on availability of clinical data. The percent of patients at intermediate CVD risk (FRS ≥ 10% at 10 years) was similar in subtypes A (12%), B (13%), and C (15%). When the percentage of patients at high CVD risk (FRS ≥ 15% at 10 years) was compared, patients with subtype A showed a lower score (4%) than patients with subtypes B (10%) or C (7%). We also found that the percent of patients with NAFLD
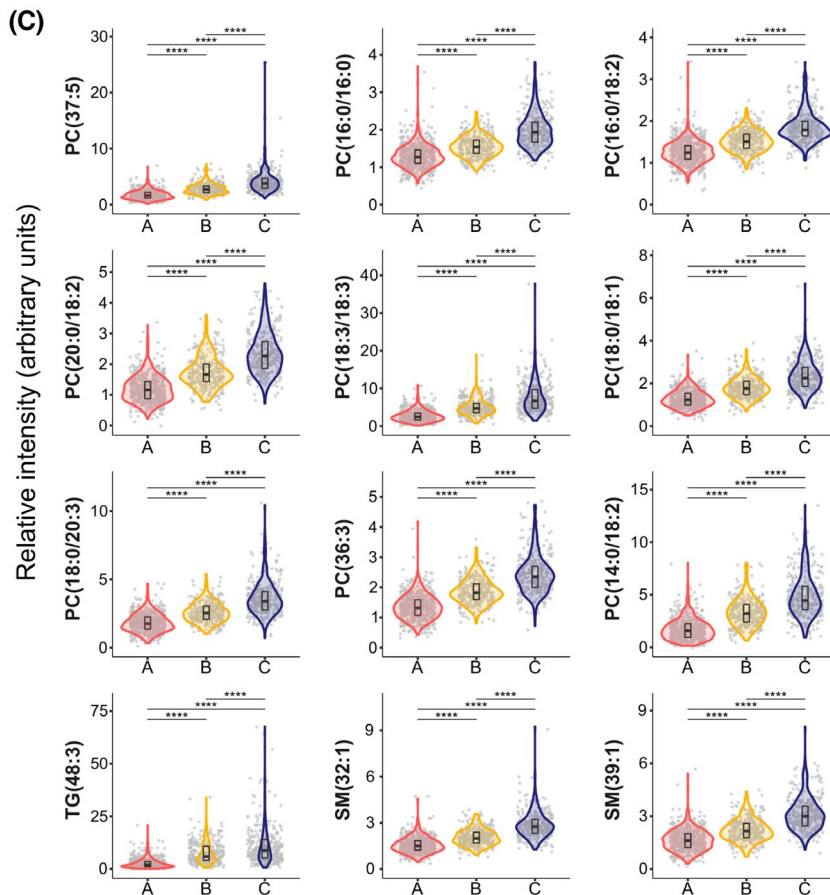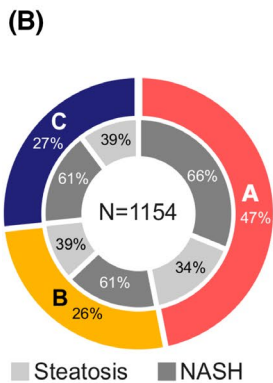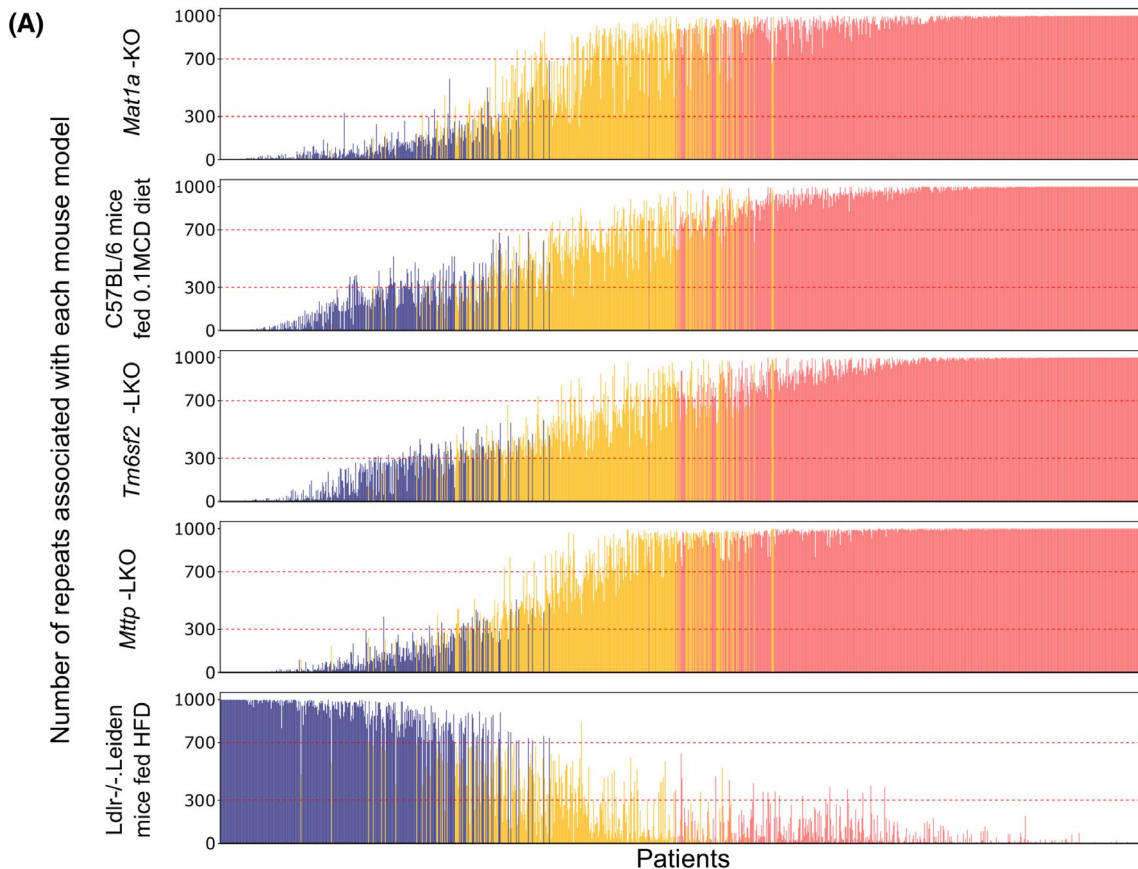
F I G U R E 1 Classification of patients with NAFLD (*n* = 1242) into subtypes. (A) The frequency distribution of the patients with NAFLD (*n* = 1154) according to five mouse models of NAFLD. Frequencies were obtained after (1) random patient partition (50/50) into two cohorts with equal proportional representation of nonalcoholic fatty liver (NAFL)/NASH and males/females; (2) clustering analysis based on the 50 more significantly serum metabolites that differentiated more significantly between the mouse model of NAFLD and the respective control mice; (3) 1000-fold repetition of the random partition with equal representation of NAFL/NASH and males/females. Representation of the number of repeats associated with methionine adenosyltransferase 1A knockout (*Mat1a*-KO) mice, C57BL/6 mice fed 0.1% methionine and choline deficient diet (0.1MCD), liver-specific microsomal triglyceride transfer protein KO (*Mttp*-LKO), liver-specific transmembrane 6 superfamily member 2 KO (*Tm6sf2*-LKO), and germline Ldlr-deficient (*Ldlr−/−*.Leiden) KO mice fed high-fat diet (HFD). Patients with a frequency over 70% for *Mat1a*-KO, 0.1MCD, *Mttp*-LKO and *Tm6sf2*-LKO, and lower than 70% for *Ldlr−/−*.Leiden/HFD, were classified as subtype A (colored in red). Patients with a frequency lower than 70% for *Mat1a*-KO, 0.1MCD, *Mttp*-LKO and *Tms6f2*-LKO, and higher than 70% for the *Ldlr−/−*.Leiden/HFD, were classified as subtype C (colored in blue). Patients not classified as either subtype A or C were classified as subtype B (colored in yellow). (B) Percentage of patients classified as subtypes A, B, and C. Percentage of patients with NAFL and NASH per subtype is also indicated. (C) Serum levels of lipids that discriminate among NAFLD subtypes A, B, and C. Gray points in the background indicate the real values with a minimal random displacement to avoid overplotting. Densities are represented as violin plots, while main distributions (medians and first and third quartiles) are represented as internal box plots. Horizontal black lines are statistical comparisons (*t* test) between two groups, with the unadjusted *p* values as symbols above the lines (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, ≥ 0.05). ns, not significant; PC, phosphatidylcholine; SM, sphingomyelin; TG, triglycerides

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treated with lipid-lowering medication (statins and/or fibrates) was higher in subtype A (24%) than in subtypes B (17%) and C (17%), although it did not reach statistical significance (Table 1). These results indicate that lipid-lowering medication has limited relevance to a particular NAFLD subtype.

Serum VLDL-TG concentration is lower among patients with NAFLD with subtype A compared with the subtype C

Because the lipidomic profile of patients with NAFLD with subtype A resembled that of mouse models of NAFLD deficient in VLDL-TG secretion, we hypothesized that the serum concentration of VLDL-TG and its subclasses would also be lower in patients with NAFLD with subtype A as compared to patients with NAFLD with subtype C, and that patients with NAFLD with subtype B would show an intermediate content of total VLDL-TG and subclasses.

Comparison of the serum lipoprotein profile of a subset of 197 samples from the NAFLD cohort (Table S4) confirmed this hypothesis. Table S1 lists the main clinical data of this subset of patients with NAFLD classified by their metabolic subtype. As expected, our results indicate that, independently of their subtype, patients with NAFLD have higher serum TG levels (*p* < 0.0001) than controls (Figure 2, Table S4). Our data also show that the concentration of serum TG was higher (*p* < 0.0001) among patients with NAFLD with subtype C than among subjects with NAFLD with subtype A, with intermediate levels of TG in patients with NAFLD with subtype B (Figure 2, Table S4). In accordance with former studies in patients with NAFLD,[10,13] we observed that, independently of the subtype, the concentration of TG in VLDL was higher in the patients with NAFLD (*p* < 0.0001) compared with controls (Figure 2, Table S4). Furthermore, our results show that the concentration of TG in VLDL, IDL, and LDL was lower (*p* < 0.0001) in

patients with subtype A than among patients with subtype C, with intermediate levels of VLDL-TG, IDL-TG, and LDL-TG in patients with NAFLD with subtype B (Figure 2). The concentration of serum VLDL-Apo-B (a surrogate for VLDL particle number) was higher among patients with NAFLD with subtype C than among subjects with subtype A (*p* < 0.0001), with intermediate levels of VLDL-Apo-B in patients with subtype B (Figure 2). The difference in serum IDL-Apo-B concentration between the A and C subtypes, although statistically significant (*p* < 0.0001), was less pronounced than for VLDL-Apo-B (Figure 2). For LDL-Apo-B, patients with subtype C showed higher levels than patients with subtype A (*p* < 0.0001), with intermediate levels in patients with subtype B (Figure 2). The concentration of LDL-Apo-B was higher among the control group than among patients with subtype A (*p* < 0.0001), and lower than in the cases with subtype C (Figure 2). This observation is reflected when the total concentration of Apo-B was determined, which follows the order: subtype A < control group < subtype C (Figure 2). Given that a reduction in VLDL-TG in patients with NAFLD with subtype A might reflect partitioning of acetyl–coenzyme A (CoA) toward ketone body synthesis, we determined the serum concentration of acetoacetate, β-hydroxybutyrate, and acetone in the control group and the NAFLD subtypes. Our results showed that there are no differences in the concentration of ketone bodies among subtypes (Figure S3).

In patients with NAFLD with subtype A, the serum concentration of VLDL-TG and VLDL-Apo-B was independent of the degree of steatosis

We next examined the relationship between serum VLDL-TG and VLDL-Apo-B concentrations and the grade of steatosis for each NAFLD subtype. Liver steatosis was graded histologically based on the percentage
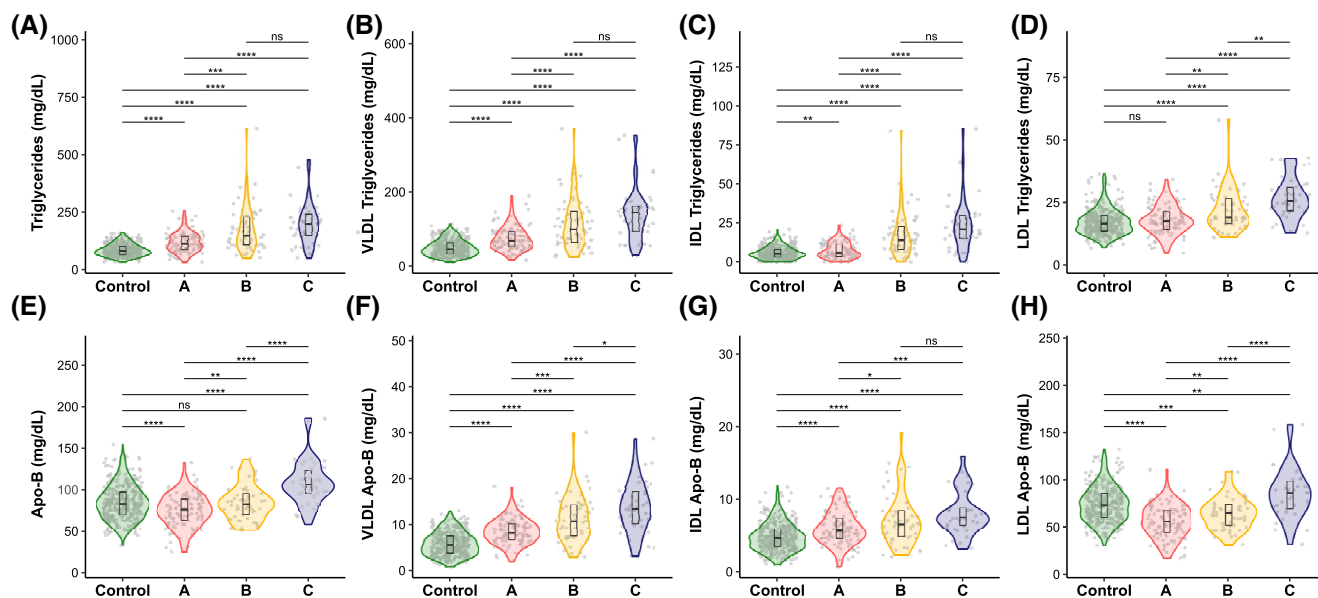
FIGURE 2 Blood lipoprotein TG (A–D) and apolipoprotein B (Apo-B) (E–H) concentrations for NAFLD subtypes and control subjects. Gray points in the background indicate the real values with a minimal random displacement to avoid overplotting. Densities are represented as violin plots, while main distributions (medians and first and third quartiles) are represented as internal box plots. Horizontal black lines are statistical comparisons (*t* test) between two groups, with the unadjusted *p* values as symbols above the lines (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, ≥ 0.05). IDL, intermediate-density lipoprotein

of hepatocytes showing fat accumulation[36]: S0 (normal, <5%), S1 (mild, 5%–33%), S2 (moderate, 34%–66%), and S3 (severe, >66%). When the serum concentrations of VLDL-TG and VLDL-Apo-B from patients with NAFLD (*n* = 197) and the control group (*n* = 350) were plotted against the grade of steatosis for each NAFLD subtype, we found that patients with subtype C showed a curvilinear relationship between these parameters with a peak at S2, whereas in patients with subtype B increased linearly from S1 to S3 (Figure 3). In contrast, patients with subtype A exhibited lower concentrations of serum VLDL-TG and VLDL-Apo-B, regardless of the steatosis grade (Figure 3). Serum albumin and INR were comparable among subtypes (Table 1), indicating that altered hepatic VLDL production alone does not account for the differences in hepatic steatosis in subtype A.

Cardiovascular risk factors are lower among patients with NAFLD with subtype A compared with subtype C

Compared to the control group, patients with NAFLD with subtype C exhibited an increase in the serum concentration of VLDL and small dense LDL (LDL₅ and LDL₆) particles, lower total HDL-CL, higher LDL-CL/HDL-CL and Apo-B/Apo-A1 ratios, and increased concentration of remnant lipoprotein cholesterol (RLP-CL, the sum of the CL associated with VLDL and IDL) (Figure 4, Table S4), which are all markers of CVD risk and have previously been associated with NAFLD.[4] Our data also show that the concentration of VLDL and small dense LDL (LDL₅ and LDL₆) particles, the
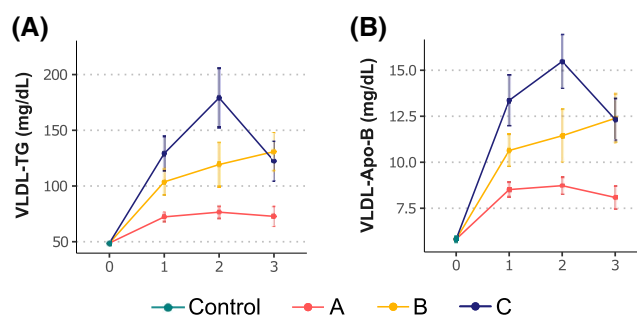
FIGURE 3 VLDL-TG and VLDL-Apo-B concentration by grade of steatosis for each NAFLD subtype and control cohort. (A,B) Total VLDL-TG (A) and VLDL-Apo-B (B). Values are represented as means (points) and standard errors (vertical lines)

HDL-CL content, and the RLP-CL concentration were lower among patients with subtype A than among subjects with subtype C, whereas the LDL-CL/HDL-CL ratio showed similar values in both subtypes (Figure 4). Patients with B subtype exhibited intermediate values for these parameters. These results indicate that, compared to the C subtype, patients with NAFLD with subtype A exhibited a favorable CVD risk profile.

VLDL-TG SR is lower in subjects with NAFLD with subtype A compared to the B and C subtypes

To address the question of whether patients with NAFLD with subtype A have lower VLDL-TG SR than patients with non-A subtype, we examined the

relationship between the NAFLD subtype, the plasma VLDL-TG concentration, and the VLDL-TG SR in a group of obese women (*n* = 20) with IHTG content assessed by MR spectroscopy. A group of lean women (*n* = 10) with low VLDL-TG SR and normal IHTG content was included for comparison (Table 2). These subjects are a subgroup of a larger cohort previously used to study VLDL-TG SR kinetics.[38] Subtyping of the obese subjects with NAFLD was performed using the classification algorithm described in Table S2. Of the 20 obese subjects with NAFLD studied, 12 fell into subtype A, 5 into subtype B, and 3 into subtype C (Table 2). Subjects with subtypes B and C were grouped together for comparative reasons. Obese subjects with NAFLD with subtype A had similar age, BMI, and IHTG content than patients with subtypes B and C. As hypothesized, VLDL-TG SR was higher (2.7-fold) among obese subjects with NAFLD with subtypes B and C than in obese subjects with NAFLD with subtype A (Table 2). The fractional catabolic rate (FCR, the fraction of the intravascular pool of VLDL catabolized per hour) was similar among obese subjects with NAFLD with subtype A that in the patients with subtypes B and C (Table 2). Obese patients with NAFLD with subtype A had similar

plasma TG, VLDL-TG, and VLDL-TG SR than lean subjects (Table 2).

Relevance of the genotype to a particular NAFLD subtype

To study the relevance of the genotype to a particular NAFLD metabolic phenotype, we tested the association with the three NAFLD subtypes for four of the major known genetic risk factors, namely, rs9992651 (17β-hydroxysteroid dehydrogenase type 13), rs641738 (membrane-bound O-acyltransferase domain-containing protein 7), rs738409 (patatin-like phospholipase domain-containing protein 3 [*PNPLA3*]), and rs58542926 (*TM6SF2*).[5] Only one variant (*PNPLA3*) associated with metabolic subtype distributions (*p* = 0.0003) (Table 3), being that this allele is overrepresented in individuals with NAFLD subtypes B and C. The I148M protein variant of *PNPLA3* accounts for the largest proportion of genetic predisposition to NAFLD in all populations.[42–44] This variant has been associated with increased hepatic fat and elevated liver enzymes, which goes in accordance with the biological
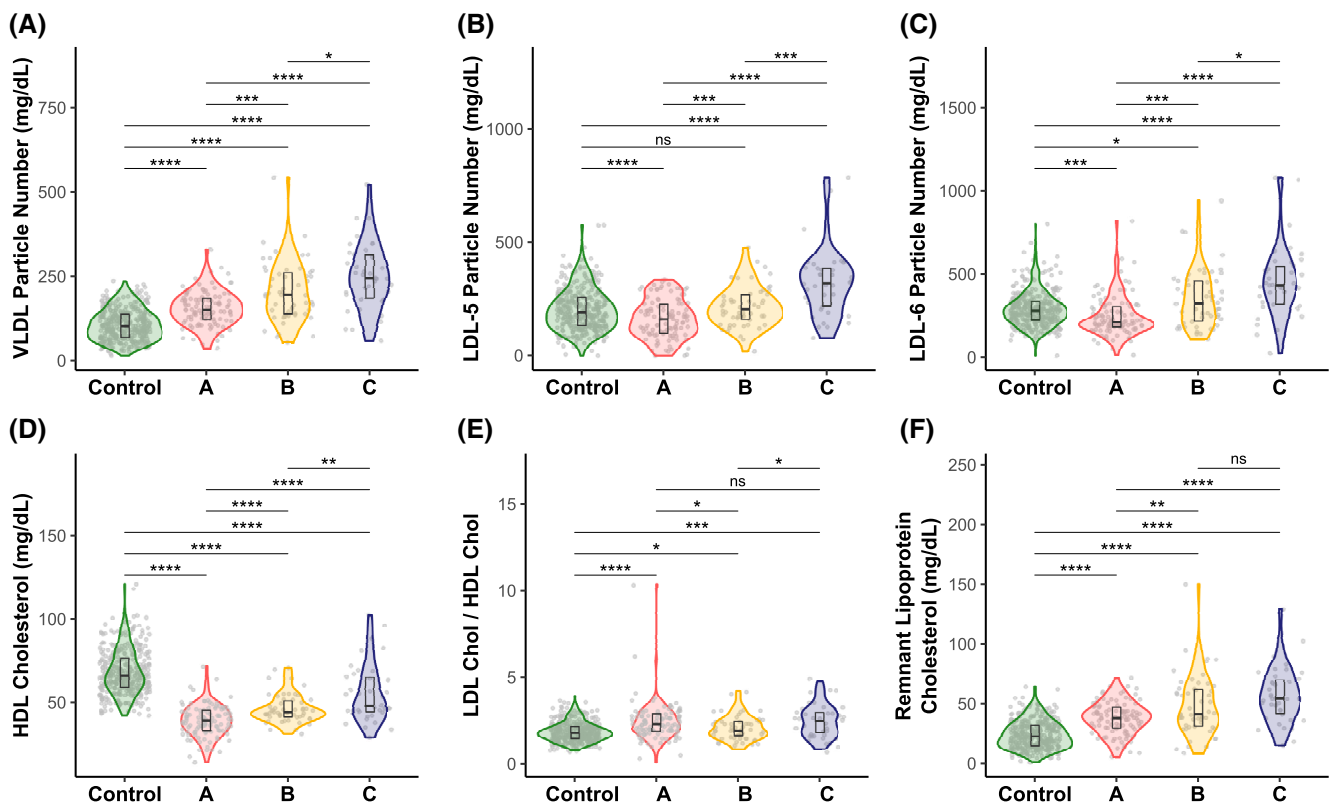
FIGURE 4 Blood lipoprotein particle numbers and cholesterol concentrations for NAFLD subtypes (A, B, and C) and control subjects. (A) VLDL particle number. (B) LDL-5 particle number. (C) LDL-6 particle number. (D) HDL cholesterol. (E) LDL cholesterol/HDL cholesterol ratio. (F) Remnant lipoprotein cholesterol. Gray points in the background indicate the real values with a minimal random displacement to avoid overplotting. Densities are represented as violin plots, while main distributions (medians and first and third quartiles) are represented as internal box plots. Horizontal black lines are statistical comparisons (*t* test) between two groups, with the unadjusted *p* values as symbols above the lines (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, ≥ 0.05)

TABLE 2   Relationship between NAFLD subtypes and plasma VLDL-TG secretion rate in obese women with NAFLD (*n* = 20) and lean women (*n* = 10)

	Lean	Obese A	Obese B/C	*p* value		
	n = 10	*n* = 12	*n* = 8	Lean vs. obese A	Lean vs. obese B/C	Obese A vs. obese B/C
Age (years)	42.50 ± 20.10	39.50 ± 10.28	38.75 ± 11.46	0.6759	0.626161	0.88344
BMI (kg/m²)	21.71 ± 1.89	36.14 ± 4.33	36.48 ± 4.74	2.02e-08	1.9e-05	0.87137
IHTG (%)	1.02 ± 0.37	12.09 ± 13.90	13.76 ± 8.90	0.0329	0.004851	0.76145
Plasma TG (mg/dl)	80.90 ± 21.43	92.08 ± 49.21	161.25 ± 79.62	0.4879	0.024660	0.05158
VLDL-TG (mmol/l)	0.21 ± 0.12	0.37 ± 0.43	0.83 ± 0.47	0.2592	0.007241	0.04268
VLDL-TG SR (μmol/l/h)	113.18 ± 43.91	169.70 ± 134.47	467.72 ± 189.10	0.1924	0.000974	0.00241
FCR (pools/h)	0.63 ± 0.30	0.76 ± 0.44	0.74 ± 0.37	0.4017	0.506677	0.89100

Note: Obese women classified as NAFLD subtypes B and C were group together for comparative reasons. For each group, the variables are summarized as means and SDs. *p* values between pairs of groups were calculated from two-tailed independent Student's *t* test.

Abbreviations: FCR, fractional catabolic rate; IHTG, intrahepatic triglycerides; SR, secretion rate.

TABLE 3   Percent of risk alleles by single-nucleotide polymorphism (gene) and subtype

SNP (gene) risk alleles	Subtype A	Subtype B	Subtype C	*p* value	*n*
rs9992651-G or rs72613567-T (*HSD17B13*)				0.6358	391
0	16 (6.18%)	3 (4.35%)	2 (3.17%)		
1	74 (28.57%)	22 (31.88%)	14 (22.22%)		
2	169 (65.25%)	44 (63.77%)	47 (74.60%)		
rs641738-T (*MBOAT7*)				0.7730	335
0	62 (28.05%)	16 (30.77%)	21 (33.87%)		
1	111 (50.23%)	28 (53.85%)	30 (48.39%)		
2	48 (21.72%)	8 (15.38%)	11 (17.74%)		
rs738409-G (*PNPLA3*)				0.0003	433
0	131 (46.13%)	30 (39.47%)	17 (23.29%)		
1	96 (33.80%)	20 (26.32%)	26 (35.62%)		
2	57 (20.07%)	26 (34.21%)	30 (41.10%)		
rs58542926-T (*TM6SF2*)				0.2939	410
0	234 (85.71%)	66 (94.29%)	61 (91.04%)		
1	35 (12.82%)	4 (5.71%)	5 (7.46%)		
2	4 (1.47%)	0 (0.00%)	1 (1.49%)		

Note: Risk alleles are indicated next to SNP code. *p* values were calculated with Fisher's exact test.

Abbreviations: *HSD17B13*, 17β-hydroxysteroid dehydrogenase type 13; *MBOAT7*, membrane-bound O-acyltransferase domain-containing protein 7; PNPLA3, patatin-like phospholipase domain-containing protein 3; SNP, single-nucleotide polymorphism.

inferences observed in patients with NAFLD with subtypes B and C.

DISCUSSION

FAs in the liver largely originate from the blood derived from lipolysis of TG in adipose tissue, and from glucose derived from DNL. FAs can be re-esterified into lipids or be transported to the mitochondria to be oxidized into acetyl-CoA, which in turn can be condensed with oxaloacetate to form citrate entering the tricarboxylic acid (TCA) cycle for complete oxidation, or enter the ketogenic pathway, where it is converted to acetoacetate and β-hydroxybutyrate. Alternatively, citrate may be redirected to DNL when the amount of acetyl-CoA exceeds the capacity of the TCA cycle to oxidize it. Some of the TG synthesized by the liver combine with Apo-B to form VLDL, and are exported into the blood stream. IDL and LDL are formed after lipases hydrolyze VLDL-TG for delivery of FAs to peripheral tissues. TGs that are not secreted into the blood form lipid droplets in hepatocytes, whose accumulation is a defining feature of NAFLD.[45] Understanding to which extent alterations in these metabolic pathways contribute to NAFLD development and its progression to NASH is crucial not only in providing granularity to understand its pathogenesis, but also to understand to which extent different

mechanisms leading to disease may contribute to the clinical manifestations of other pathologies associated with NAFLD, such as CVD.[2,3,5,6]

The current study confirms and extends prior observations on NAFLD subtypes[24] with extensive metabolomic profiling of a large international cohort of 1154 biopsy-confirmed patients with NAFLD. Here we demonstrate the existence of three distinctive, clinically potentially relevant NAFLD subtypes characterized by serum lipidomic signatures. Among these, subtype A, which accounts for 47% of patients with NAFLD, shows a lipidomic signature like that observed in four different mouse models with defective VLDL-TG secretion. Alterations in these four models ranged from the deletion of *Mttp* (the gene encoding MTTP, a protein playing a central role in VLDL assembly)[40] and *Tm6sf2* (the gene encoding TM6SF2, a protein involved in the lipidation of nascent VLDL particles)[41] to the deletion of *Mat1a* (a protein-encoding gene that catalyzes the synthesis of SAMe, which plays a necessary role in the liver synthesis of PC,[46] the major phospholipid component of VLDL) and the induction of NAFLD by feeding mice a MCD diet, which also results in a lower PC/phosphatidylethanolamines (PE) ratio.[47] The geographical distribution of NAFLD cases among subtypes A, B, and C varied significantly between Europe (higher proportion of patients with subtype A), the United States (higher proportion of patients with subtype C), and Israel and Chile (with similar proportions among subtypes) (Table S5). Based on these results, we propose that subtype A stems from multiple nutritional and environmental factors that, acting on a susceptible genetic background, converge in reduced synthesis and export of VLDL-TG, resulting in lower circulating levels of both VLDL-Apo-B and VLDL-TG.

Consistent with this hypothesis, we found that the concentration of TG, VLDL-TG, IDL-TG, and LDL-TG was lower among patients with subtype A compared to patients with subtypes B or C, and similar to that observed in controls. The same pattern was also observed when the content of phospholipids, total CL, and CL esters in VLDL, IDL, and LDL was analyzed (Table S4). Moreover, the content of VLDL-Apo-B, IDL-Apo-B, and LDL-Apo-B was also lower among patients with subtype A compared to patients with subtypes B and C, and similar to controls. The ratio of VLDL-TG (TG content in the particle) to VLDL Apo-B (number of particles) was similar among the three subtypes, suggesting that secreted VLDL particles are similar in size and lipid composition. It is tempting to speculate that PC availability for VLDL assembly is lower in patients with subtype A, as is the case in *Mat1a*-KO and 0.1MCD mice,[47,48] and VLDL synthesis is therefore saturated at a lower IHTG content than in the subtypes B and C. That speculation, however, will require formal evaluation.

The finding that the concentration of VLDL-TG and VLDL-Apo-B in the serum of patients with subtype A was independent of the grade of steatosis, whereas in subjects with subtypes B or C the concentration of serum VLDL-TG and VLDL-Apo-B increased with the grade of steatosis, also supports the hypothesis that VLDL-TG SR is saturated in patients with subtype A at a lower IHTG concentration.

Evidence that patients with NAFLD with subtype A exhibit reduced VLDL-TG SR was obtained by subtyping a cohort of 20 obese women with elevated IHTG and different VLDL-TG SR. VLDL-TG SR was 2.7-fold lower in obese individuals with NAFLD classified as subtype A compared to the obese subjects with NAFLD with subtypes B and C. The FCR was similar among subjects with subtype A compared to subtypes B and C, which indicates that reduced catabolism of VLDL-TG does not contribute to the higher concentration of VLDL-TG in subjects with subtypes B and C. Finally, subjects with NAFLD with subtype A showed similar VLDL-TG SR, VLDL-TG, and TG compared with lean subjects, despite having a 12-fold increase in IHTG, which further supports the hypothesis that the VLDL-TG SR in these subjects likely becomes saturated at low levels of IHTG.

NAFLD is associated with increased CVD risk in association with alterations in circulating lipid levels and lipoprotein metabolism.[11,13] We observed that patients with NAFLD with subtype C display hypertriglyceridemia, elevated VLDL-TG, and VLDL-Apo-B, increased small dense LDL particles (LDL_5 and LDL_6), and RLP-CL, compared with control subjects. All of these features were significantly lower among patients with NAFLD with subtype A as compared to subtype C, indicating that patients with subtype A exhibited a favorable CVD risk profile, likely the result of reduced VLDL secretion. The percentage of patients with NAFLD at high CVD risk (FRS ≥ 15% at 10 years) was lower in subtype A than in subtypes B and C. This reduction in FRS did not reach statistical significance, probably due to the small number of high-risk CVD cases. The percent of patients receiving lipid-lowering medication was not statistically different between subtypes. Liver enzymes (ALT, AST, and GGT), which are also associated with increased risk of CVD,[49] were higher in patients with subtype C than in subjects with subtype A. The HOMA-IR index and HbA1c, two independent CVD risk factors,[50,51] were similar among the three subtypes. NASH lipidomic signatures were characterized by changes in TG, PC, and sphingomyelins (SM). Up to 56% of TG (49 of 84), 24% of PC (23 of 92), and 19% of SM (6 of 31) analyzed changed significantly in patients with NASH with subtype C. Diglycerides (DG; 9 of 13), PE (4 of 24), phosphatidylinositols (PI; 2 of 3), and ceramides (Cer; 1 of 12) also changed significantly in patients with NASH with subtype C. Compared with subtype C, NASH lipidomic signatures in patients with subtype A displayed fewer changes. These included TG (23 of 84), DG (6 of 13), PC (6 of 92), PE (4 of 24), and

SM (1 of 31). NASH lipidomic signature in patients with subtype B showed changes in TG (23 of 84), DG (5 of 13), PC (15 of 92), PE (4 of 24), PI (1 of 3), Cer (1 of 12), and SM (18 of 31). These data provide evidence of the existence of distinct metabolic mechanisms associated with NAFLD progression varying between subtypes.

We also acknowledge some limitations in the interpretation of our findings. The small number of genotyped individuals limits our ability to characterize in depth the role of genetics in NAFLD metabolic subtypes. Despite this limitation, the association of *PNPLA3* risk allele with NAFLD subtypes B and C is robust. The high frequency of these subtypes in the Chilean cohort, nonetheless, introduces a note of caution regarding the actual strength of this association. This cohort likely harbors genetic differences with the other European counterparts; hence, larger studies that are focused on homogeneous genetic ancestry settings will be useful to validate our observation. The main limitation of our study is the lack of CVD outcomes and the relatively small number of cases (360 individuals) for which the FRS was available. The lower content of serum TG, VLDL, LDL, and Apo-B, and the lower FRS ≥ 15% at 10 years, suggest an improved CVD risk profile in patients with NAFLD with subtype A compared to subjects with subtypes B and C, which can provide clinically useful information in the management of patients with NAFLD.[52] Further work should be carried out to confirm this hypothesis. We further emphasize a strength of this study, which is the inclusion of large international cohorts of patients with biopsy-proven NAFLD/NASH. This study shows NAFLD/NASH lipidomic phenotyping according to CVD risks profile, the latter representing the leading cause of death in patients with NAFLD/NASH.

CONFLICT OF INTEREST

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