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Lipoprotein subclasses are associated with Hepatic steatosis: insights from the prospective multicenter imaging study for the evaluation of chest pain (PROMISE) clinical trial

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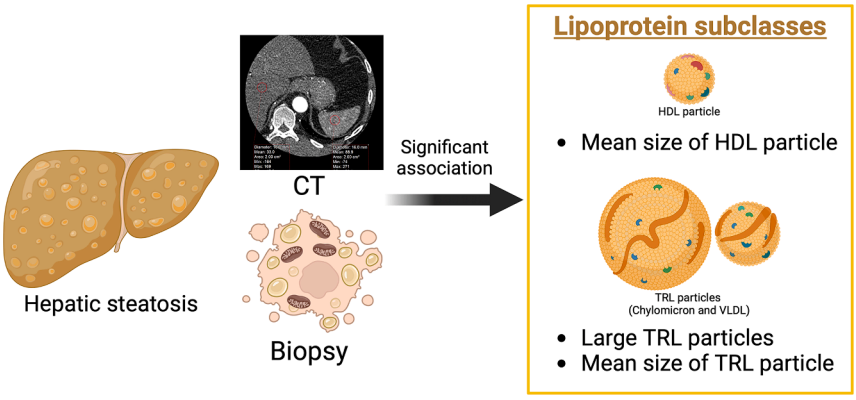
HIGHLIGHTS

- The nr1 cause of morbidity and mortality in hepatic steatosis (HS) is CV disease.
- Clinically, HS is associated with dyslipidemia and coronary artery disease (CAD).
- Lipoprotein particle number/size are associated with CAD and CV events.
- We analyzed the association lipoprotein particle size/number and HS on CT/biopsy.
- Large TRL, mean sizes of TRL-, and HDL were associated with HS on CT/biopsy.
- The use of lipoprotein subclasses may improve CV risk assessment in patients with HS.

GRAPHICAL ABSTRACT



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ABSTRACT

Objectives: To determine the relationship between lipoprotein particle size/number with hepatic steatosis (HS), given its association with traditional lipoproteins and coronary atherosclerosis.
Methods: Individuals with available CT data and blood samples enrolled in the PROMISE trial were studied. HS was defined based on CT attenuation. Lipoprotein particle size/number were measured by nuclear magnetic resonance spectroscopy. Principal components analysis (PCA) was used for dimensionality reduction. The association of PCA factors and individual lipoprotein particle size/number with HS were assessed in multivariable regression models. Associations were validated in an independent cohort of 59 individuals with histopathology defined HS.
Results: Individuals with HS (n=410/1,509) vs those without (n=1,099/1,509), were younger (59±8 vs 61±8 years) and less often females (47.6 % vs 55.9 %). All PCA factors were associated with HS: factor 1 (OR:1.36, 95 %CI:1.21–1.53), factor 3 (OR:1.75, 95 %CI:1.53–2.02) and factor 4 (OR:1.49; 95 %CI:1.32–1.68) were weighted heavily with small low density lipoprotein (LDL) and triglyceride-rich (TRL) particles, while factor 2 (OR:0.86, 95 %CI:0.77–0.97) and factor 5 (OR:0.74, 95 %CI:0.65–0.84) were heavily loaded with high density lipoprotein (HDL) and larger LDL particles. These observations were confirmed with the analysis of individual lipoprotein particles in PROMISE. In the validation cohort, association between HS and large TRL (OR: 8.16, 95 % CI:1.82–61.98), and mean sizes of TRL- (OR: 2.82, 95 %CI:1.14–9.29) and HDL (OR:0.35, 95 %CI:0.13–0.72) were confirmed.
Conclusions: Large TRL, mean sizes of TRL-, and HDL were associated with radiographic and histopathologic HS. The use of lipoprotein particle size/number could improve cardiovascular risk assessment in HS.

1. Introduction

Hepatic steatosis (HS) impacts approximately 25-30 % of adults in the United States and is the leading cause of liver-related morbidity and mortality around the world [1-3]. The presence of HS is also associated with an increased risk for coronary artery disease (CAD) and incident cardiovascular events independent of traditional cardiovascular risk factors [4-6]. Concomitantly, cardiovascular disease is the leading cause of morbidity and mortality among individuals with HS [7].

The gold standard diagnosis of HS remains the histopathologic assessment of liver tissue obtained by liver biopsy; however, due to its invasive nature, non-invasive diagnostic testing alternatives are increasingly utilized [8]. In recent years, computed tomography (CT) imaging has emerged as a non-invasive method for detecting elevated lipid content of the liver. In fact, cardiac CT imaging can be used to phenotype HS, as upper aspects of the liver and spleen used for the diagnosis of HS are most often visible [4,9,10].

Risk factors for HS include age, diabetes mellitus, metabolic syndrome, and traditional lipid measures including high levels of triglycerides and low-density lipoprotein (LDL) cholesterol [11]. These traditional lipid measurements are also associated with an increased risk of cardiovascular events [12-15]. Thus, prevention guidelines recommend the measurement of standard blood lipids for cardiovascular risk assessment [16]. However, data suggest that cardiovascular disease risk prediction is improved with the utilization of more detailed blood lipid measures. For example, lipoprotein particle number and size are associated with CAD and cardiovascular events incremental to traditional lipid measures [17], with smaller LDL particle size and higher LDL particle number associated with increased risk [18-20]. Further, as previously demonstrated in the PROspective Multicenter Imaging Study for Evaluation of chest pain (PROMISE) study, high density lipoprotein (HDL) cholesterol subclasses are associated with decreased risk [21]. However, the relationship between HS and granular measures of blood lipids has not been previously evaluated.

As such, given the association between HS with traditional lipids and CAD, and the association between granular lipid measurements and CAD, we sought to evaluate whether lipoprotein particle size and number are associated with radiographic HS as detected on cardiac CT imaging.

2. Methods

2.1. Study Populations and HS Phenotyping

2.1.1. PROMISE cohort

The study design of the PROMISE trial has been described previously (ClinicalTrials.gov NCT01174550) [22,23]. Briefly, the PROMISE trial was a pragmatic comparative effectiveness trial of non-invasive cardiovascular testing. PROMISE enrolled 10,003 outpatients without prior known CAD who presented with stable chest pain and required noninvasive cardiovascular testing across North America, and randomized individuals to anatomic assessment with coronary CT angiography (CTA) vs. standard of care (usually functional stress testing). Local or central institutional review boards approved the study protocol at each coordinating center and enrolling sites. All participants provided written informed consent.

The current substudy of the PROMISE trial included individuals who were randomized to coronary CTA, received both non-contrast and contrast-enhanced CT evaluation with diagnostic image quality, and consented to participate in the PROMISE biomarker sub-study (Fig. 1).

The CT definition of HS in PROMISE has been reported previously [4]. Briefly, five core laboratory readers analyzed non-contrast CT images in a randomly assigned, blinded fashion. Hepatic and splenic CT attenuations were measured on three cross-sections obtained at different levels by drawing circular regions of interest with an area of at least 2 cm^2 avoiding areas of vascular and biliary structures [4,9,10,24]. Hepatic and splenic attenuation were calculated as the mean of the three measurements. HS was defined using the following criteria: 1) hepatic CT attenuation minus splenic CT attenuation of <1 HU; 2) the mean CT number ratio of liver-to-spleen parenchyma of ≤ 1; or 3) absolute hepatic CT attenuation <40 HU.

2.1.2. Laval validation cohort

The validation cohort included individuals undergoing liver biopsy at Laval University with available clinical-, histopathology-, and lipoprotein data [25]. Patients in the validation cohort consisted of individuals of European ancestry with severe obesity (BMI>35 kg/m^2) from the eastern provinces of Canada who underwent bariatric surgery at Institut Universitaire de Cardiologie et de Pneumologie de Québec (QHLI). In this analysis, we evaluated 59 individuals with lipoprotein data available from the QHLI Obesity Biobank with severe obesity (BMI >35 kg/m^2) who had liver biopsy specimens obtained and concomitantly had advanced lipoprotein analysis. Liver biospecimens of the validation cohort were analyzed and graded for the severity of HS as well as categorized to HS vs no HS by a pathologist according to the methods of Brunt *et al* [26].

2.2. Lipoprotein Profiling

Non-fasting blood samples were collected via peripheral venous phlebotomy in EDTA tubes and immediately processed and frozen at -80°C. Lipoproteins were measured in EDTA plasma samples at Labcorp (Morrisville, NC) by nuclear magnetic resonance (NMR) on a Vantera® Clinical Analyzer as previously described [27]. The NMR MetaboProfile analysis, which reports lipoprotein particle concentrations and sizes was performed using the LipoProfile-4 lipoprotein profile deconvolution algorithm [28]. Linear regression of the lipoprotein subclass signal areas against serum lipid levels measured chemically in a large reference range study population provided the conversion factors to generate NMR-derived concentrations of TRL, LDL and HDL fractions (Table 1). Mean triglyceride-rich lipoproteins (TRL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) particle sizes are weighted averages derived from the sum of the diameter of each subclass multiplied by its relative mass percentage. NMR-derived concentrations of these parameters are highly correlated ($r \geq 0.95$) with those measured by standard methods [29].

2.3. Statistical methods

Continuous variables are presented as mean $\pm$ standard deviation, while categorical values are presented as absolute and relative frequencies in percentages. Baseline variables were compared between subjects with and without HS using Pearson's chi-squared test for binary variables and Student's t-test for continuous variables.

Principal components analysis (PCA) was used for dimensionality reduction on the lipoproteins given collinearity between lipoprotein subclasses to reduce the burden of multiple comparisons. All particle size subclasses of TRL particle, LDL particle, and calibrated HDL particle concentrations, as well as mean TRL, LDL and HDL lipoprotein size variables were included in PCA. Analytes with >25 % of values below

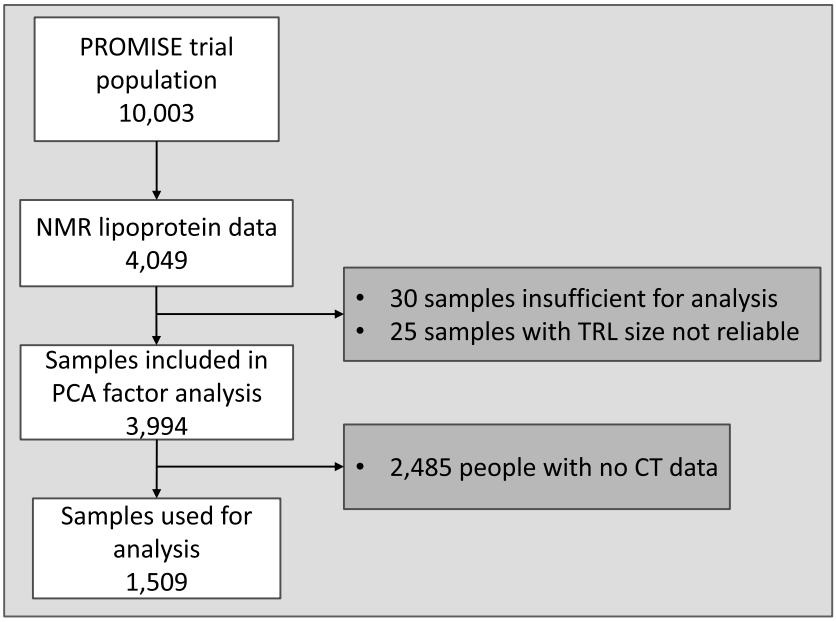

Fig. 1. Consort diagram. CT: Computed tomography; NMR: Nuclear magnetic resonance; TRL: Triglyceride-rich lipoproteins.

Table 1
NMR spectroscopy derived lipoprotein subclasses.

Analyte Category	Analyte Description	Analyte	Size Range (nm)	Unit of measure
Triglyceride-Rich Lipoprotein Particle (TRLP) Concentrations	*TRLP Subclasses*	Very Large TRLP	90-240	nmol/L
		Large TRLP	50-89	nmol/L
		Medium TRLP	37-49	nmol/L
		Small TRLP	30-36	nmol/L
		Very Small TRLP	24-29	nmol/L
LDL Particle (LDLP) Concentrations	*LDLP Subclasses*	Large LDLP	21.5-23	nmol/L
		Medium LDLP	20.5-21.4	nmol/L
		Small LDLP	19-20.4	nmol/L
Calibrated HDL Particle (cHDLP) Concentrations	*cHDLP Subclasses*	H7P	12	umol/L
		H6P	10.8	umol/L
		H5P	10.3	umol/L
		H4P	9.5	umol/L
		H3P	8.7	umol/L
		H2P	7.8	umol/L
		H1P	7.4	umol/L
Mean Lipoprotein Sizes	*Particle Sizes*	TRL Size	30-100	nm
		LDL Size	19-22.5	nm
		HDL Size	7.4-13	nm

lower limits of quantification of the assay were not included in PCA and were instead analyzed as binary variables (present/absent: very large TRL particle (TRLP) and calibrated HDL particle ["H7P"]). Lipoproteins input for PCA were centered and scaled, and principal components were created using the prcomp function in R. Five resulting eigenvectors with an eigenvalue >1 were carried forward to create varimax-rotated factors. The association between lipoprotein PCA factors with HS was analyzed using a univariable logistic regression model, adjusting for multiple comparisons by using false discovery rates (FDR) p-value <0.1. Individual lipoproteins heavily loaded (having an absolute factor loading >0.4) on significant factors were extracted. We then tested the association of these lipoproteins with HS using univariable and multivariable (adjusted for age, sex, hypertension, diabetes, body mass index [BMI], smoking status, statin use, metabolic syndrome, and traditional LDL-, TG- and HDL cholesterol measures) logistic regression models. In a sensitivity analysis we further adjusted for inflammatory biomarkers (i.e. IL-6 and adiponektine) as well as ApoB and total cholesterol to explore whether observed associations were independent of inflammation and TRL related impacts. Nominal significance was considered at $p<0.05$. All individual lipoproteins were scaled before analysis.

To validate the significant association of lipoprotein subclasses with HS observed in PROMISE, we used the Laval cohort consisting of subjects with biopsy-confirmed HS. Using similar rules to PROMISE, we treated medium calibrated LDL particle (analyzed as continuous in PROMISE) and very large TRL particle as binary variables. The largest calibrated HDL particle (H7P), analyzed as a binary variable in PROMISE, was treated as a continuous variable in this cohort. We defined HS as having hepatic steatosis grade from 1-3, and HS = 0 otherwise. A univariate logistic regression model was used to assess the association with HS for the lipoproteins that were significant in PROMISE. All lipoproteins were scaled before analysis. We did not perform a multivariate model due to the small sample size, especially in the group without HS. Nominal significance was considered at $p<0.05$.

3. Results

3.1. Baseline characteristics

Of 10,003 individuals included in the PROMISE trial, 1,509 individuals with complete lipoprotein data and CT images of diagnostic quality were evaluated for HS (Fig. 1). Baseline characteristics of the studied population are presented in Table 2, stratified by the presence/absence of HS. Individuals with HS (n=410/1,509) were younger (59.4 ±7.9 vs 60.9±8.1; p=0.002), less often female (47.5 % vs 56.0 %; p=0.004) and had higher BMI (32.3±5.7 vs 29.7±5.7 kg/m^2; p<0.001) compared with those without HS (n=1,099/1,509). Patients with HS were at higher median [IQR] 10-year atherosclerotic cardiovascular disease (ASCVD [30]) risk (12.6 % [7.4, 20.0] vs 10.3 % [5.9, 18.3]; p=0.001) and the mean number of cardiovascular risk factors among those with HS was significantly higher compared to those without (2.6 ±1.1 vs 2.3±1.1; p<0.001). Further, HS subjects were more likely to be on an ACE inhibitor or ARB compared to non-HS subjects (49.0 % vs 39.7 %; p=0.002) but other preventive medication use, including lipid lowering therapy (i.e. statins) was similar between the groups.

3.2. Lipoprotein subclasses are associated with HS

A total of 16 lipoprotein subclasses were included in creating PCA factors (4 TRL particles, 3 LDL particles, 6 HDL particles and mean lipoprotein sizes of TRL, LDL and HDL), Table 1). PCA reduced these correlated subclasses into five orthogonal factors (Table 3): Factor 1 was heavily loaded with small to medium size LDL particles and mean LDL size; factor 2 consisted of small to medium size HDL particles (H1P and H3P); factor 3 was heavily loaded with small to large size HDL particles

Table 2
Demographic characteristics of patients in PROMISE.

n	No HS (n=1099)	HS (n=410)	p
Age, mean (SD)	60.86 (8.06)	59.41 (7.93)	**0.002**
Female Sex, n (%)	614 (55.9)	195 (47.6)	**0.005**
Body-mass index, mean (SD)	29.68 (5.52)	32.30 (5.68)	**<0.001**
Race, n (%)			**0.019**
Asian	16 (1.5)	10 (2.4)	
Black	94 (8.6)	26 (6.4)	
Other	27 (2.5)	2 (0.5)	
White	955 (87.5)	371 (90.7)	
Risk factors			
Comorbidities, n (%)			
Hypertension	691 (62.9)	290 (70.7)	**0.005**
Diabetes	164 (14.9)	129 (31.5)	**<0.001**
Dyslipidemia	712 (64.8)	299 (72.9)	**0.003**
Family history of premature CAD	371 (33.8)	144 (35.1)	0.68
Peripheral arterial/ cerebrovascular disease	62 (5.6)	16 (3.9)	0.22
CAD risk equivalent	213 (19.4)	141 (34.4)	**<0.001**
Metabolic syndrome	332 (30.2)	219 (53.4)	**<0.001**
Current or past tobacco use	588 (53.5)	219 (53.4)	1.00
Sedentary lifestyle	488 (44.5)	213 (52.0)	**0.012**
History of depression	252 (22.9)	106 (25.9)	0.26
No risk factors	32 (2.9)	4 (1.0)	**0.045**
Number of risk factors per patient, mean (SD)	2.30 (1.05)	2.64 (1.05)	**<0.001**
ASCVD risk ≥7.5 %, n (%)	711 (65.2)	301 (73.6)	**0.002**
ASCVD risk score, median [IQR]	10.30 [5.85, 18.29]	12.55 [7.36, 20.03]	**0.001**
Medication use, n (%)			
Beta-blocker	269 (25.2)	112 (28.4)	0.24
ACE inhibitor or ARB	424 (39.7)	194 (49.1)	**0.001**
Statin	459 (42.9)	185 (46.8)	0.20
Aspirin	503 (47.1)	190 (48.1)	0.77

ACE: Angiotensin converting enzyme; ARB: Aldosterone receptor blocker; ASCVD: Atherosclerotic cardiovascular disease; CAD: Coronary artery disease; HS: Hepatic steatosis.

Table 3
Associations between PCA lipoprotein factors with HS in the PROMISE cohort.

Factors	Lipoprotein subclasses*	Factor load	OR	95 % CI	FDR-adjusted p
Factor 1	Small LDL particle (LDLP)	0.88	1.36	1.21 – 1.53	<0.001
	Mean LDL size (LDLP)	-0.79			
	Medium LDL particle (LDLP)	-0.75			
	Medium TRL particle (TRLP)	0.65			
	Large TRL particle (TRLP)	0.61			
Factor 2	Small HDL particle (H1P)	0.80	0.86	0.77 – 0.97	0.011
	Medium HDL particle (H3P)	-0.75			
Factor 3	Mean HDL size	-0.86	1.75	1.53 – 2.02	<0.001
	Large HDL particle (H6P)	-0.75			
	Small HDL particle (H2P)	0.61			
	Large LDL particle (LDLP)	-0.60			
	Medium HDL particle (H4P)	-0.55			
Factor 4	Large HDL particle (H5P)	-0.73	1.49	1.32 – 1.68	<0.001
	Very small TRL particle (TRLP)	0.51			
	Medium HDL particle (H4P)	0.51			
	Mean TRL size	0.44			
Factor 5	Small TRL particle (TRLP)	0.90	0.74	0.65 – 0.84	<0.001
	Medium TRL particle (TRLP)	0.45			
	Large LDL particle (LDLP)	0.40			

* Lipoprotein subclasses with high loads on the associated factors (i.e. absolute value of factor load >0.4).

FDR: False discovery rate; TRLP: Triglyceride-Rich Lipoprotein Particle; LDLP: LDL Particle; OR: Odds ratio; HXP: HDL X Particle.

(H2P, H4P and H6P) and mean HDL size; factor 4 was heavily loaded with medium to large size HDL particles (H4P and H5P); and factor 5 was heavily loaded with small size TRL particle.

In univariate analyses, all factors were significantly associated with radiographic HS (factor 1: odds ratio (OR) 1.36, 95 % CI: 1.21 – 1.53, FDR-adjusted p<0.001; factor 2: OR=0.86, 95 % CI: 0.77 – 0.97, FDR-adjusted p=0.01; factor 3: OR=1.75, 95 % CI: 1.53 – 2.02, FDR-adjusted p<0.001; factor 4: OR=1.49, 95 % CI: 1.32 – 1.68, FDR-adjusted p<0.001; and factor 5: OR=0.74, 95 % CI: 0.65 – 0.84, p<0.001) (Table 3).

Individual lipoprotein subclass analysis demonstrated that large TRL (OR: 1.64, 95 % CI: 1.32 – 2.03; p<0.001), medium TRL (OR: 0.66, 95 % CI: 0.54 – 0.80; p<0.001), small TRL (OR: 0.76, 95 % CI: 0.66 – 0.87; p<0.001), very small TRL (OR: 1.23, 95 % CI: 1.08 – 1.41; p=0.002), large LDL (OR: 0.81, 95 % CI: 0.68 – 0.97; p=0.023), large (H5P) HDL particle (OR: 0.79, 95 % CI: 0.68 – 0.92; p=0.002), small (H2P) HDL particle (OR: 1.38, 95 % CI: 1.21 – 1.59; p<0.001), and mean sizes of TRL (OR: 1.87, 95 % CI: 1.60 – 2.18; p<0.001), and HDL (OR: 0.55, 95 % CI: 0.43 – 0.71; p<0.001) were significantly associated with radiographic HS, independent of age, sex, hypertension, diabetes, BMI, smoking status, statin, metabolic syndrome and traditional LDL-, TG- and HDL cholesterol measures. These represent most of the individual components of factors 1, 3, and 4 and all individual lipoprotein subclasses of factor 5. Small (H1P) and medium (H3P) size HDL particles, which were the individual components of factor 2, were not independently associated with HS (Table 4). Further, we assessed analytes not included in the PCA studied as binary variables (i.e. H7P and very large TRLP). Of these, none showed a significant association with HS in multivariate models. We observed similar associations in the sensitivity analysis.

3.3. *Validation of association of lipoprotein subclasses with histopathologic HS*

To validate the associations between lipoprotein subclasses and HS, using the gold standard biopsy-confirmed diagnosis for HS, we performed NMR lipoprotein profiling in N=59 patients with histopathologic assessment for HS. In univariate analysis, association observed in PROMISE of the concentration of large TRLP (OR: 8.16, 95 % CI:1.82 – 61.98; p=0.018), mean TRL size (OR: 2.82, 95 % CI: 1.14 – 9.29; p=0.047), and mean HDL size (OR: 0.35, 95 % CI: 0.13 – 0.72; p=0.012) with HS were confirmed as associations between lipoprotein subclasses and histopathologic HS (Table 4). Associations between medium TRL, small TRL, very small TRL, large (H5P) and small (H2P) HDL particles, seen in the PROMISE trial, were not validated among individuals with biopsy-proven HS. Further, associations between larger HDL particle (H6P) concentration (OR: 0.29, 95 %CI: 0.09 – 0.63, p=0.009) and mean LDL particle size (OR: 0.21, 95 %CI: 0.05 – 0.64; p=0.017) and histopathological HS were not significant in the large cohort of patients with radiographic HS.

4. Discussion

Using a detailed analysis of serum lipoproteins, we identified lipid particles that were associated with CT-defined radiographic HS in PROMISE, a large clinical trial of cardiovascular imaging with CT and biospecimens. We subsequently validated the associations between lipoprotein characteristics and HS in a cohort of subjects with histopathologically confirmed HS. We found that lipoprotein particles previously shown to be associated with CAD and cardiovascular disease events, were also associated with HS independent of traditional risk factors and traditional measures of blood lipids. These lipoproteins included large size TRL particles and mean TRL size, which were associated with HS, and mean HDL size, which was inversely associated with HS in the discovery PROMISE and validation Laval cohorts. These results highlight a possible mechanistic link in the association between HS and cardiovascular disease.

Lipoprotein profiling allows for the differentiation and quantification of various subtypes of lipoproteins and thus offers a more comprehensive risk assessment for CAD compared to traditional lipid parameters. NMR spectroscopy is a powerful technique used for lipoprotein analysis, providing detailed information about lipoprotein composition, size, distribution, and concentration [31]. This detailed analysis involves identifying and quantifying lipoprotein subtypes and delivering information on TRL-, LDL- and HDL sub-particles. Previously, this technique has been shown to improve cardiovascular risk assessment compared to traditional lipid assessment. In the PROMISE trial, large (H6P) and medium (H4P) HDL particles and HDL size were associated with a lower risk for high-risk coronary atherosclerosis, and greater concentrations of medium-size HDL particles (H3P) were associated with a lower risk of incident major adverse cardiovascular events [21].

Prior studies of lipoprotein subclasses showed similar associations with HS as observed in our study. TRL were associated with an increased risk for cardiovascular disease and the treatment of individuals with increased TRL reduced the risk for cardiovascular events [32-34]. Beyond its association with cardiovascular risk, TRL was shown to be correlated with HS. In a cohort of 280 patients (median age 61 years [IQR:52,66], 49 % female) large TRL as detected with NMR spectroscopy was associated with non-invasive fatty liver disease indices (i.e. fatty liver index and fibrosis 4 score) [35]. Our study extends our understanding of the association of radiographic HS (which is an easily obtainable measure on CT datasets) and large TRL and mean TRL size.

Table 4
Associations between individual lipoprotein subclass concentration and size and radiographic and histopathologic HS.

	PROMISE								Laval			
	Unadjusted				Adjusted**				Unadjusted			
Lipoprotein	OR	95 % CI		p	aOR	95 %CI		p	OR	95 % CI		p
TRLP, nmol/L												
Very Large TRLP*	1.27	1.00	1.62	0.05	1.28	0.99	1.67	0.06	0.50	0.07	2.43	0.42
Large TRLP	1.78	1.57	2.03	**<0.001**	1.64	1.32	2.03	**<0.001**	8.16	1.82	61.98	**0.018**
Medium TRLP	1.16	1.04	1.29	**0.007**	0.66	0.54	0.80	**<0.001**	1.19	0.57	2.92	0.66
Small TRLP	0.74	0.65	0.85	**<0.001**	0.76	0.66	0.87	**<0.001**	0.79	0.38	1.67	0.52
Very Small TRLP	1.37	1.23	1.54	**<0.001**	1.23	1.08	1.41	**0.002**	2.06	0.78	9.20	0.25
LDLP, nnmol/L												
Large LDLP	0.65	0.57	0.75	**<0.001**	0.81	0.68	0.97	**0.023**	0.46	0.18	0.98	0.06
Medium LDLP	0.94	0.84	1.06	0.32	0.97	0.82	1.14	0.71	1.38	0.30	6.46	0.67
Small LDLP	1.41	1.27	1.58	**<0.001**	1.11	0.97	1.28	0.14	4.63	1.73	17.25	0.66
HDLP, umol/L												
H7P*	0.50	0.40	0.63	**<0.001**	0.77	0.58	1.03	0.08	0.55	0.21	1.02	0.10
H6P	0.66	0.57	0.77	**<0.001**	0.85	0.68	1.06	0.15	0.29	0.09	0.63	**0.009**
H5P	0.73	0.64	0.83	**<0.001**	0.79	0.68	0.92	**0.002**	0.80	0.39	1.70	0.54
H4P	0.80	0.71	0.91	**<0.001**	1.04	0.89	1.21	0.63	1.39	0.63	3.80	0.47
H3P	0.85	0.76	0.96	**0.007**	0.98	0.84	1.15	0.82	0.69	0.33	1.44	0.31
H2P	1.48	1.32	1.67	**<0.001**	1.38	1.21	1.59	**<0.001**	1.38	0.64	3.53	0.46
H1P	0.95	0.85	1.06	0.38	0.97	0.85	1.11	0.65	0.96	0.46	2.14	0.92
Mean particle size, nm												
TRL Size	2.09	1.85	2.37	**<0.001**	1.87	1.60	2.18	**<0.001**	2.82	1.14	9.29	**0.047**
LDL Size	0.67	0.60	0.75	**<0.001**	0.87	0.73	1.02	0.09	0.21	0.05	0.64	**0.017**
HDL Size	0.57	0.49	0.65	**<0.001**	0.55	0.43	0.71	**<0.001**	0.35	0.13	0.72	**0.012**

* Analytes with >25 % of values below lower limits of quantification of the assay were analyzed as binary variables.
** Adjusted for age, sex, hypertension, diabetes, BMI, smoking status, statin use, metabolic syndrome, traditional LDL-, TG- and HDL cholesterol measures.
TRLP: Triglyceride-Rich Lipoprotein Particle; LDLP: LDL Particle HXP: HDL Particle.

We further were able to confirm this association with our analysis of large TRL sub particles and the mean size of TRL with histopathologic HS. In HS, dysregulated lipid metabolism and atherogenic dyslipidemia are present in many cases, given the liver's central role in the metabolism of triglyceride and cholesterol and lipoprotein particle production. Traditionally, the hallmark of HS is high TRL concentration [36]. Therefore, these results may underscore the significance of TRL as a common mechanistic pathway in HS and increased cardiovascular risk observed in patients with HS.

We further identified mean HDL size to be inversely associated with both radiographic and histopathologic HS. An association between mean HDL size and cardiovascular risk is typically inverse and similarly, larger HDL size often displays an inverse relationship with cardiovascular risk. As assessed in PROMISE and other cohorts, mean HDL size, as well as greater concentrations of large and medium sized HDL subclasses, were inversely associated with high risk coronary plaque phenotype and major adverse cardiovascular events [37]. The GENES (Génétique et Environnement en Europe du Sud) study assessing 214 male participants (45-74 years) found that the strongest predictor of all-cause and cardiovascular mortality was mean HDL particle size, which had an inverse association with these endpoints [38]. In our analysis, PCA-determined factor 2, heavily loaded with small size HDL subclasses, was inversely associated with HS, while other factors (i.e. factors 3 and 4), heavily negatively loaded with medium to large HDL subclasses among other lipoprotein particles, were negatively associated with an increased risk for HS. Further, larger size HDL subclasses were associated with a decreased risk of HS in the individual lipoprotein subclass analysis among PROMISE patients and in the validation dataset. These findings corroborate prior evidence describing that higher levels of large HDL subfractions are inversely associated with HS. For example, Corey *et al.* described that among individuals with histopathologically-confirmed non-alcoholic steatohepatitis, larger HDL particle concentration was significantly lower when compared to those without non-alcoholic steatohepatitis [39].

Given that cardiovascular disease is the leading cause of morbidity and mortality among subjects with HS, there is an unmet need for an improved cardiovascular risk assessment. We believe that in important clinical implication of these results is that advanced lipoprotein profiling with NMR-spectroscopy of individuals with HS could be considered to be implemented in the everyday clinical practice which should be followed by a referral of patients to follow up in preventive cardiology clinic to enhance advanced decision making on further risk modification [7]. Thus, an important consideration of these results in the light of prior evidence suggesting a potential critical role of lipoprotein subclasses in the estimation of risk for coronary artery disease and adverse cardiovascular events [21], is that the findings of our study may suggest that lipoprotein subclasses could improve cardiovascular risk assessment among patients with HS. Associations between lipoprotein particle concentrations and sizes observed only in the PROMISE cohort including patients with radiographic HS, but not further validated in patients with histopathologic HS, as well as significant associations only observed among patients with biopsy confirmed HS but not in patients with HS on CT, warrant further investigation to better understand their significance. Further, for clinical use future research should aim to determine thresholds that should inform providers on the need to take action and initiate advanced preventive cardiology workup and risk mitigation.

4.1. *Strengths and limitations*

The strengths of this analysis include a large study sample as we utilized data from a large clinical trial of outpatients who had well-phenotyped cardiovascular disease characterized on CT imaging at baseline. We replicated our findings in a cohort of individuals who underwent histopathologic assessment of HS, making these results robust to provide evidence for future trials. With this approach, we were able to demonstrate that sub particles of blood lipids were independently associated with HS, not only independently of traditional cardiovascular risk factors, but also from traditional lipid measures.

There are limitations of this study. First, in the PROMISE trial the diagnosis of HS was based on CT findings and thus the relationship between lipoprotein particle number/size and steatosis, non-alcoholic steatohepatitis, fibrosis and cirrhosis could not be assessed. However, the inclusion of a validation cohort with histologically confirmed HS status supports the findings in the PROMISE, thus suggesting that this

limitation potentially influenced our results minimally. Second, blood testing was not required to be performed in a fasting state, which may have impacted the measured lipid parameters in this study. However, we note that the associations with lipoprotein characteristics and HS seen here are consistent with other studies. To fully understand the impact of pre- and postprandial lipoprotein states, with special regards to the atherogenic post-prandial TRL metabolism, further research is needed with more strict requirements on fasting vs non-fasting. Third, lipoprotein subclasses were determined via NMR spectroscopy and there is a possibility that other methods to quantify lipoprotein components may render different results. Fourth, in the PROMISE trial, a history of alcohol consumption was not collected. Based on prior data on the relatively low prevalence of alcoholic fatty liver disease compared to NAFLD in the general population (alcoholic fatty liver disease prevalence 4 % [40] vs NAFLD 30-37 % [1,3]), we presume that the majority of the individuals included here had NAFLD, but emphasize that our analysis is for hepatic steatosis of any cause. Fifth, patients in the Laval cohort, used for the validation of our findings, were at higher risk for more severe HS, given that they had clinical indications for liver biopsy as compared to the asymptomatic individuals who were identified with radiographic HS in the PROMISE trial.

5. Conclusion

We found an association of between large TRL, as well as mean sizes of TRL-, and HDL with radiographic HS phenotyped by CT in the PROMISE trial and validated these associations with histologically confirmed HS. Given their known association with CAD and cardiovascular events, we conclude that the use of these lipoprotein subclasses could potentially improve cardiovascular risk assessment in patients with HS.

CRediT authorship contribution statement

Julia Karady: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Robert W McGarrah:** Writing – review & editing, Methodology, Conceptualization. **Maggie Nguyen:** Writing – review & editing, Formal analysis, Data curation. **Stephanie N Giamberardino:** Writing – review & editing, Formal analysis, Data curation. **Nandini Meyersohn:** Writing – review & editing, Investigation. **Michael T Lu:** Writing – review & editing, Investigation, Conceptualization. **Pedro V Staziaki:** Writing – review & editing, Investigation, Conceptualization. **Stefan B Puchner:** Writing – review & editing, Investigation. **Daniel O Bittner:** Writing – review & editing, Investigation. **Borek Foldyna:** Writing – review & editing, Project administration, Investigation, Data curation. **Thomas Mayrhofer:** Writing – review & editing, Formal analysis, Data curation. **Margery A Connelly:** Writing – review & editing, Software. **Andre Tchernof:** Writing – review & editing, Validation. **Phillip J White:** Writing – review & editing, Investigation. **Khurram Nasir:** Writing – review & editing, Investigation. **Kathleen Corey:** Writing – review & editing, Investigation. **Deepak Voora:** Writing – review & editing, Investigation, Conceptualization. **Neha Pagidipati:** Writing – review & editing, Investigation, Conceptualization. **Geoffrey S Ginsburg:** Writing – review & editing, Investigation, Conceptualization. **William E Kraus:** Writing – review & editing, Conceptualization. **Udo Hoffmann:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Pamela S Douglas:** Writing – review & editing, Resources, Methodology, Investigation, Data curation, Conceptualization. **Svati H Shah:** Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Maros Ferencik:** Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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