

*patient-oriented and epidemiological research*Lipoprotein subclass metabolism in nonalcoholic steatohepatitis[§]

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Abstract Nonalcoholic steatohepatitis (NASH) is associated with increased synthesis of triglycerides and cholesterol coupled with increased VLDL synthesis in the liver. In addition, increased cholesterol content in the liver associates with NASH. Here we study the association of lipoprotein subclass metabolism with NASH. To this aim, liver biopsies from 116 morbidly obese individuals [age 47.3 ± 8.7 (mean ± SD) years, BMI 45.1 ± 6.1 kg/m², 39 men and 77 women] were used for histological assessment. Proton NMR spectroscopy was used to measure lipid concentrations of 14 lipoprotein subclasses in native serum samples at baseline and after obesity surgery. We observed that total lipid concentration of VLDL and LDL subclasses, but not HDL subclasses, associated with NASH [false discovery rate (FDR) < 0.1]. More specifically, total lipid and cholesterol concentration of VLDL and LDL subclasses associated with inflammation, fibrosis, and cell injury (FDR < 0.1), independent of steatosis. Cholesterol concentration of all VLDL subclasses also correlated with total and free cholesterol content in the liver. All NASH-related changes in lipoprotein subclasses were reversed by obesity surgery. **High total lipid and cholesterol concentration of serum VLDL and LDL subclasses are linked to cholesterol accumulation in the liver and to liver cell injury in NASH.**—Männistö, V. T., M. Simonen, P. Soinen, M. Tiainen, A. J. Kangas, D. Kaminska, S. Venesmaa, P. Käkälä, V. Kärjä, H. Gylling, M. Ala-Korpela, and J. Pihlajamäki. **Lipoprotein subclass metabolism in nonalcoholic steatohepatitis.** *J. Lipid Res.* 2014. 55: 2676–2684.

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Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the Western world (1). It is estimated to affect 10–50% of the population (2). The disease presents primarily as steatosis contributing to metabolic alterations such as insulin resistance, hyperglycemia, and hyperlipidemia (3–5). However, NAFLD can also lead to nonalcoholic steatohepatitis (NASH) with a potential of leading to liver cirrhosis and liver failure (6).

Increased triglyceride synthesis and accumulation are characteristic in a steatotic liver (7). In addition, cholesterol synthesis is also increased in NAFLD (8), and cholesterol accumulation has been suggested to contribute to liver cell injury in NASH (9). The related overproduction of VLDL particles in the metabolic syndrome (10) and NAFLD (4) has been thought to be a consequence of this

Abbreviations: *CYP51A1*, cytochrome P450 family 51 subfamily A polypeptide 1; *DHCR7*, 7-dehydrocholesterol reductase; *DHCR24*, 24-dehydrocholesterol reductase; FDR, false discovery rate; GLC, gas-liquid chromatography; *HMGCR*, HMG-CoA reductase; *LDLR*, LDL receptor; *LSS*, lanosterol synthase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; *PNPLA3*, patatin-like phospholipase domain containing 3; RYGB, Roux-en-Y gastric bypass; *SCAMOL*, methylsterol monooxygenase; *SC5D*, sterol-C5-desaturase; *SREBP1a*, sterol regulatory element binding transcription factor 1a; *SREBP1c*, sterol regulatory element binding transcription factor 1c; *SREBP2*, sterol regulatory element binding transcription factor 2; *TM7SF2*, transmembrane 7 superfamily member 2; *XBPI*, X-box binding protein 1.

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increased lipid production in the liver. It has also been suggested that high LDL cholesterol relates to NASH and liver fibrosis (11, 12), suggesting that the association of NASH with serum lipids is not only related to increased VLDL synthesis.

We investigated the association between serum lipoprotein subclasses and NASH. Our hypotheses were that dyslipidemia in NASH includes altered lipoprotein subclass lipid profile, and specifically that changes in cholesterol metabolism associate with liver inflammation and injury. To answer these questions, we investigated serum lipoprotein subclasses using NMR spectroscopy (13) in 116 obese individuals with detailed liver histology. The main finding was that the levels of total lipids and cholesterol in VLDL and LDL subclasses were linked to inflammation and cell injury in NASH, but not to simple steatosis. These changes were corrected with obesity surgery.

MATERIALS AND METHODS

Subjects

All patients undergoing obesity surgery in Kuopio University Hospital are recruited into our ongoing study investigating metabolic consequences of obesity surgery (Kuopio Obesity Surgery Study) (14, 15). The study group included 116 consecutive subjects [age 47.3 ± 8.7 (mean \pm SD) years, BMI 45.1 ± 6.1 kg/m², 39 men and 77 women], who were accepted for the Roux-en-Y gastric bypass (RYGB) operation over the years 2005–2010. Fifty-nine patients had serum NMR data available also 1 year after the surgery. The study protocol was approved by the Ethics Committee of the Northern Savo Hospital District (54/2005,104/2008, and 27/2010), and it was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the subjects.

Laboratory determinations

Diabetes was defined by WHO's criteria of diabetes (16). Plasma glucose, insulin, serum total cholesterol, HDL cholesterol, and triglyceride levels were analyzed as described before (14, 15).

Serum lipoprotein subclass analysis by NMR spectroscopy

Fasting concentrations of lipoprotein subclass particles and their main lipid components were analyzed by proton NMR spectroscopy in native serum samples (13, 17, 18). The details of this methodology have been described previously (13, 19), and this platform has recently been applied in various large-scale epidemiological and genetic studies (20–22). The lipoprotein subclass data available are as follows: chylomicrons and largest VLDL particles (average particle diameter at least 75 nm); five different VLDL subclasses: very large VLDL (average particle diameter of 64.0 nm), large VLDL (53.6 nm), medium VLDL (44.5 nm), small VLDL (36.8 nm), and very small VLDL (31.3 nm); IDL (28.6 nm); three LDL subclasses: large LDL (25.5 nm), medium LDL (23.0 nm), and small LDL (18.7 nm); and four HDL subclasses: very large HDL (14.3 nm), large HDL (12.1 nm), medium HDL (10.9 nm), and small HDL (8.7 nm). The following components of the lipoprotein particles were quantified: phospholipids, triglycerides, and cholesterol. Due to resolution and concentration issues, all of these components are not available for every subclass (17, 18, 20). The VLDL cholesterol concentration

was calculated by subtracting the LDL, IDL, and HDL cholesterol from the total cholesterol. The total cholesterol content of chylomicrons and extremely large VLDL, very large VLDL and very small VLDL was calculated by subtracting the triglyceride and phospholipid concentrations from the total lipids of each subclass. Lipid composition as a percentage of each available lipid of the total lipid content in each lipoprotein subclass was also calculated.

Liver histology

Liver biopsies were obtained using a Tru-Cut needle (Rapid AB, Uppsala, Sweden) during elective gastric bypass operations. Overall histological assessment of liver biopsy samples was performed by one pathologist according to the standard criteria (23, 24), and histological diagnosis was divided into three categories: 1) normal liver without any steatosis, inflammation, ballooning, or fibrosis; 2) simple steatosis (steatosis >5%) without evidence of hepatocellular ballooning, inflammation, or fibrosis; and 3) NASH (supplementary Tables I, II). When analyzing the effects of the gastric bypass operation, a different histological characterization was used to separate normal from probable and definite NASH (supplementary Table III): steatosis was graded into four categories (<5, 5–33, 33–66, and >66%); lobular inflammation was graded into four categories (no foci, <2, 2–4, and >4 per 200 \times field); fibrosis was staged from 1 to 4 and ballooning from 0 to 2. When all four variables were present, the diagnosis was “definite steatohepatitis,” and when variables one to three were positive, the diagnosis was “probable steatohepatitis.” In the absence of lobular inflammation and steatosis, the diagnosis was “not steatohepatitis.” All patients with alcohol consumption >2 doses per day were excluded from the study. Chronic hepatitis B and C (HBV and HCV) were excluded using serology if alanine aminotransferase values were elevated prior to surgery (HCV and HBV infections are rare in Finland). Hemochromatosis was excluded by histological analysis of liver biopsies, and by normal serum ferritin levels in subjects that had an elevated serum alanine aminotransferase level.

Liver cholesterol content with gas-liquid chromatography

Liver total cholesterol content (per 100 mg liver tissue) was quantified with gas-liquid chromatography (GLC) on a 50 m long capillary column (Ultra 2, Agilent Technologies, Wilmington, DE) using 5 α -cholestane as the internal standard (25).

Liver free cholesterol content with NMR spectroscopy

Liver samples (approximately 50 mg) were homogenized in 1.5 ml Eppendorf tubes in NaCl solution (150 μ l of 150 mM NaCl in D₂O) by pestle. After homogenization, 300 μ l of CD₃OD and 600 μ l of CDCl₃ were added and samples were mixed vigorously using a vortex mixer and sonicated for 15 min (indirect sonication) in an ice bath. After mixing, the samples were centrifuged (5,000 g, 10 min, 4°C) to separate the organic and water phase. The lower organic phase was recovered and the aqueous layer was extracted again, first with 600 μ l and then with 300 μ l CDCl₃ to standardize the yield. The separated organic layers were combined and evaporated to dryness under a gentle flow of dried air. Prior to NMR analysis, the extracted lipids were redissolved into 600 μ l of CDCl₃ containing 0.03% of tetramethylsilane as a reference substance.

¹H NMR spectra of extracted lipids were recorded on a Bruker Avance III HD 600 NMR spectrometer operating at 600.28 MHz and equipped with a Prodigy TCI 5 mm cryogenically cooled probe head. Standard 1D ¹H NMR spectra were recorded with 96,000 data points using 32 transients and applying a standard Bruker zg pulse sequence. The acquisition time was 5 s and the relaxation delay was 15 s. The spectra were measured at 295,000.

Data processing. The free induction decays (FIDs) with 96,000 data points were zero-filled to 256,000 data points and multiplied by an exponential window function with a 0.3 Hz line-broadening. The areas of the known lipid resonances (26) in the spectra were determined using a constrained total-lineshape fitting approach to enable quantitative analysis of severely overlapping peaks and to increase the quantification accuracy (27). This methodology allowed us to get information on the amounts of several lipid components in the extracted samples, e.g., free cholesterol and total triglycerides. The PERCH NMR software was used for all the lineshape fitting analyses.

Liver gene expression and genotyping

Quantitative real-time PCR was carried out in the Applied Biosystems 7500 real-time PCR system using KAPA SYBR FAST qPCR Universal Master Mix (Kapa Biosystems, Woburn, MA). Primers are available in supplementary Table III. We genotyped rs738409 SNP of patatin-like phospholipase domain containing 3 (*PNPLA3*) (28) (using the TaqMan SNP genotyping assay (Applied Biosystems) according to the manufacturer's protocol.

Statistical analysis

Data are presented as mean \pm SD. Differences between the study groups were examined with the χ^2 test (in categorical variables) and by nonparametric Kruskal-Wallis test (continuous variables). Spearman rank correlation was used for correlation analysis. General linear model analysis was used for studying the independent contributions of variables on liver histology. Analyses were conducted with the SPSS version 19 (IBM SPSS). Multiple testing of several related lipid phenotypes was corrected using false discovery rate (FDR) calculated by the Benjamini-Hochberg method as implicated in the "p adjust method" in R

(version 2.0.1) (29). FDR values <0.1 were considered to indicate a statistically significant difference.

RESULTS

Subject characteristics

Seventy-six out of the 116 patients had a clearly defined liver phenotype: 32 had normal liver, 19 had simple steatosis, and 25 had NASH (Table 1). Levels of fasting insulin and total and LDL cholesterol ($P = 0.006$, $P = 0.004$, and $P = 0.010$, Kruskal-Wallis test) differed between study groups. Total and LDL cholesterol were higher in individuals with NASH compared to those with simple steatosis ($P = 0.002$ and $P = 0.007$). The results were essentially the same if individuals using cholesterol-lowering medication ($n = 21$) were excluded (supplementary Table IV).

Serum lipids in relation to steatosis, inflammation, and fibrosis

Next, we investigated to determine whether the association between NASH and serum lipids is related to steatosis, inflammation, or fibrosis in the liver. To this aim, obese patients were divided into four groups based on severity of steatosis (steatosis grades: $<5\%$, $5\text{--}33\%$, $33\text{--}66\%$, and $>66\%$; supplementary Table II, upper part); into three groups based on lobular inflammation (no inflammatory cells, <2 cells per $200\times$ field, and $2\text{--}4$ cells per $200\times$ field; supplementary Table II, middle part); and into three groups based on fibrosis stage (by combining stages $2\text{--}4$;

TABLE 1. Clinical characteristics based on liver phenotype

	Normal Liver (n = 32)	Simple Steatosis (n = 19)	NASH (n = 25)	<i>P</i> ^a
Sex (male/female)	11/21	4/15	10/15	0.404
Age (y)	47.9 \pm 9.7	45.8 \pm 9.8	46.7 \pm 8.0	0.725
Weight (kg)	127.2 \pm 19.4	126.2 \pm 14.8	132.1 \pm 24.5	0.676
BMI (kg/m ²)	44.1 \pm 6.8	44.8 \pm 4.3	44.3 \pm 6.9	0.716
Fasting glucose (mmol/l)	6.1 \pm 0.9	6.3 \pm 1.2	6.5 \pm 1.6	0.929
Fasting insulin (mU/l)	14.5 \pm 9.0	19.7 \pm 10.1	25.4 \pm 17.1 ^b	0.006
Total cholesterol (mmol/l)	4.23 \pm 0.8	3.80 \pm 0.9	4.74 \pm 1.0 ^{b,c}	0.004
LDL cholesterol (mmol/l)	2.48 \pm 0.7	2.11 \pm 0.8	2.89 \pm 1.0 ^c	0.010
HDL cholesterol (mmol/l)	1.07 \pm 0.3	1.02 \pm 0.2	1.06 \pm 0.4	0.539
Total triglycerides (mmol/l)	1.49 \pm 0.7	1.46 \pm 0.6	1.74 \pm 0.6	0.103
Non-HDL cholesterol (mmol/l)	2.23 \pm 0.6	2.02 \pm 0.6	2.67 \pm 0.8 ^{b,c}	0.012
Steatosis grade (n)				8.0×10^{-17}
<5%	32	0	0	
5–33%	0	15	10	
33–66%	0	2	10	
>66%	0	2	5	
Lobular inflammation (n)				1.2×10^{-15}
None	32	19	0	
<2 Foci per 200 \times field	0	0	17	
2–4 Foci per 200 \times field	0	0	8	
>4 Foci per 200 \times field	0	0	0	
Fibrosis (n)				6.6×10^{-11}
0	32	19	4	
1	0	0	19	
2	0	0	1	
3	0	0	0	
4	0	0	1	

^a*P* value is over all groups. Kruskal-Wallis test for continuous variables and chi-square test for categorical variables.

^bNormal liver versus NASH, $P < 0.05$.

^cSteatosis versus NASH, $P < 0.05$.

supplementary Table II, lower part). Steatosis associated with higher fasting insulin levels ($P = 0.002$), but not with serum lipids (supplementary Fig. I). In contrast, lobular inflammation and stage 1 fibrosis associated with total and LDL cholesterol ($P = 0.0001-0.022$; supplementary Fig. IB, C). In addition, individuals with stage 1 fibrosis had higher total triglycerides ($P = 0.008$) than individuals without any sign of fibrosis (supplementary Fig. IC). There was no difference when comparing individuals without fibrosis to those with grades 2-4 fibrosis, suggesting a decline in serum lipids when moving from stage 1 to a more advanced stage of fibrosis.

VLDL and LDL lipid concentration associates with inflammation, fibrosis, and liver cell injury

The serum lipid and lipoprotein analysis was extended to a more detailed lipoprotein subclass analysis using NMR spectroscopy (13, 19) (Fig. 1A). Total lipid concentration of VLDL (excluding very small VLDL) and medium and small LDL associated with NASH (FDR < 0.1, Table 2). More specifically, total lipid concentration of VLDL and LDL subclasses was increased in individuals

with NASH, but not significantly in those with simple steatosis (Fig. 1A).

Next, we investigated the association of total lipoprotein lipid concentration with steatosis, inflammation, or fibrosis (detailed results in supplementary Tables V-VII). No significant associations were observed between subclass lipid concentration and steatosis (FDR > 0.1, Fig. 1B), while total lipid concentration in all VLDL, IDL, and LDL subclasses (excluding very small VLDL) was increased by 20-80% in relation to inflammation (Fig. 1C) and grade 1 fibrosis (Fig. 1D). Stage 2-4 fibrosis was characterized with lower lipoprotein lipid concentrations than grade 1 (Fig. 1D). Furthermore, the total lipid concentration of all VLDL and LDL particles associated with the NAFLD activity score [that measures histological injury in NASH combining information about steatosis, inflammation, and liver cell injury (24)] and with ballooning [a histological marker of liver cell injury in NASH (FDR < 0.1, data not shown)]. The total lipid concentration of HDL subclasses was not altered in relation to steatosis or inflammation, but stage 1 fibrosis related to higher HDL lipid concentration (Fig. 1D).

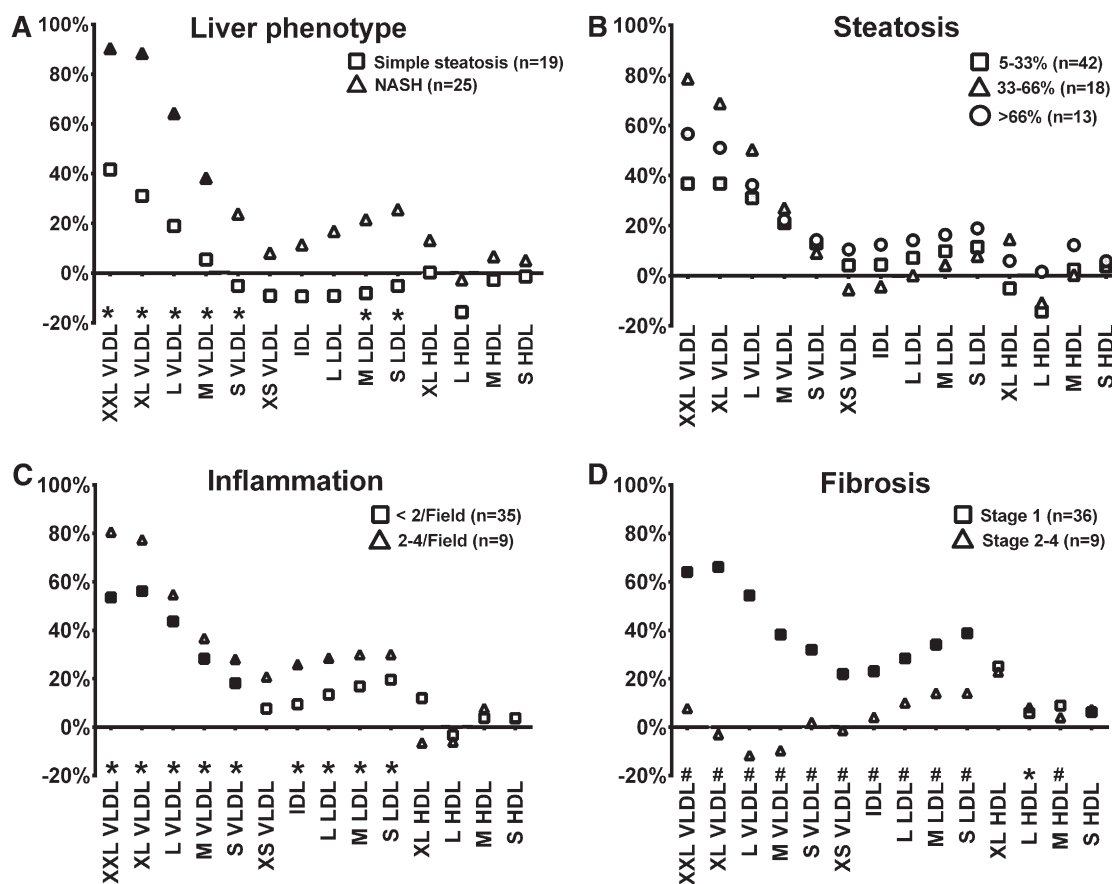


Fig. 1. Lipoprotein subclass lipid concentration in individuals divided into groups by liver phenotype divided to those with normal liver histology ($n = 32$), simple steatosis without inflammation and cell injury ($n = 19$) and to those with NASH ($n = 25$) (A), steatosis grade (B), lobular inflammation (C), and fibrosis stage (D). Percentage changes comparing to the group without the pathology (set to 0%) have been calculated. Statistical significance over all groups (normal and all degrees of pathology in each panel) are visualized with FDR < 0.05 (*) and FDR < 0.01 (#) compared with individuals without the pathology below the horizontal axis in each panel. The color of the symbol indicates subgroup analysis comparing a given group to the group without pathology (gray indicates $P < 0.05$, black indicates $P < 0.01$).

TABLE 2. Serum lipoprotein subclass data according to liver phenotype

	Normal Liver (n = 32)	Simple Steatosis (n = 19)	NASH (n = 25)	<i>P</i> ^a	FDR
Total lipids in (mmol/l)					
Chylomicrons and extremely large VLDL	0.025 ± 0.021	0.036 ± 0.031	0.048 ± 0.026	0.0007	0.0065
Very large VLDL	0.069 ± 0.050	0.090 ± 0.069	0.129 ± 0.061	0.0004	0.0065
Large VLDL	0.277 ± 0.153	0.330 ± 0.211	0.456 ± 0.185	0.0017	0.0074
Medium VLDL	0.566 ± 0.198	0.597 ± 0.266	0.783 ± 0.246	0.0022	0.0077
Small VLDL	0.629 ± 0.145	0.596 ± 0.159	0.778 ± 0.205	0.0013	0.0065
Very small VLDL	0.508 ± 0.124	0.462 ± 0.096	0.549 ± 0.172	0.1255	0.1515
Total IDL	1.033 ± 0.255	0.936 ± 0.237	1.151 ± 0.344	0.0651	0.0872
Large LDL	1.206 ± 0.301	1.095 ± 0.310	1.409 ± 0.416	0.0154	0.0250
Medium LDL	0.694 ± 0.181	0.638 ± 0.203	0.844 ± 0.258	0.0095	0.0179
Small LDL	0.457 ± 0.120	0.433 ± 0.156	0.574 ± 0.184	0.0097	0.0179
Very large HDL	0.266 ± 0.135	0.267 ± 0.160	0.301 ± 0.193	0.6399	0.6399
Large HDL	0.463 ± 0.264	0.390 ± 0.249	0.451 ± 0.300	0.3278	0.3585
Medium HDL	0.844 ± 0.175	0.821 ± 0.107	0.901 ± 0.188	0.2808	0.3170
Small HDL	1.110 ± 0.090	1.095 ± 0.168	1.167 ± 0.128	0.0781	0.0976
Total cholesterol in (mmol/l)					
Large VLDL	0.062 ± 0.035	0.071 ± 0.046	0.100 ± 0.040	0.0011	0.0065
Medium VLDL	0.160 ± 0.053	0.159 ± 0.063	0.214 ± 0.066	0.0028	0.0082
Small VLDL	0.232 ± 0.060	0.207 ± 0.054	0.277 ± 0.082	0.0028	0.0082
Total IDL	0.633 ± 0.156	0.567 ± 0.148	0.703 ± 0.205	0.0440	0.0616
Large LDL	0.797 ± 0.213	0.712 ± 0.222	0.937 ± 0.293	0.0157	0.0250
Medium LDL	0.453 ± 0.131	0.406 ± 0.107	0.558 ± 0.188	0.0096	0.0179
Small LDL	0.283 ± 0.083	0.260 ± 0.103	0.357 ± 0.127	0.0141	0.0247
Very large HDL	0.151 ± 0.080	0.161 ± 0.095	0.175 ± 0.093	0.5106	0.5256
Large HDL	0.217 ± 0.118	0.183 ± 0.130	0.208 ± 0.162	0.2098	0.2448
Medium HDL	0.415 ± 0.094	0.401 ± 0.063	0.439 ± 0.098	0.3646	0.3867
Triglycerides in (mmol/l)					
Chylomicrons and extremely large VLDL	0.019 ± 0.015	0.027 ± 0.022	0.035 ± 0.019	0.0009	0.0065
Very large VLDL	0.045 ± 0.032	0.060 ± 0.044	0.084 ± 0.040	0.0004	0.0065
Large VLDL	0.163 ± 0.089	0.198 ± 0.125	0.269 ± 0.111	0.0019	0.0074
Medium VLDL	0.292 ± 0.111	0.321 ± 0.156	0.415 ± 0.139	0.0035	0.0094
Small VLDL	0.253 ± 0.067	0.255 ± 0.087	0.329 ± 0.088	0.0012	0.0065
Very small VLDL	0.119 ± 0.028	0.112 ± 0.023	0.142 ± 0.040	0.0095	0.0179
Total IDL	0.120 ± 0.032	0.111 ± 0.020	0.138 ± 0.041	0.0313	0.0457
Very large HDL	0.010 ± 0.004	0.009 ± 0.004	0.013 ± 0.005	0.0079	0.0179
Small HDL	0.042 ± 0.010	0.042 ± 0.010	0.048 ± 0.011	0.0673	0.0872
Apolipoproteins (g/l)					
Apolipoprotein A-I	1.367 ± 0.200	1.311 ± 0.186	1.476 ± 0.231	0.0179	0.0272
Apolipoprotein B	0.860 ± 0.179	0.821 ± 0.189	1.023 ± 0.246	0.0060	0.0150

^aKruskal-Wallis test. FDR with Benjamini-Hochberg method.

We also evaluated whether the lipid composition (as a percentage of individual lipids from total lipids) would alter in NASH. There were no differences in the lipid composition of any lipoprotein subclass in relation to steatosis and inflammation, and only small changes in relation to fibrosis (supplementary Table VIII).

VLDL and LDL cholesterol associates with liver inflammation independent of steatosis and serum triglycerides

The liver cholesterol accumulation has been associated with NASH (9, 12, 30), and our traditional lipid analysis supported an association between cholesterol metabolism and NASH (supplementary Fig. I). The cholesterol concentration of VLDL (except in small VLDL), IDL, and LDL associated with inflammation and fibrosis (FDR < 0.05; supplementary Fig. II; supplementary Tables V–VII), but not with steatosis. Importantly, in multivariate analysis the association of VLDL and LDL cholesterol concentration with liver inflammation was independent of steatosis and serum total triglycerides (*P* = 0.024–0.049 for small, medium, and large VLDL and for large and medium

LDL subclasses). We also demonstrated that the association of lipoprotein cholesterol concentrations with liver inflammation remained significant when adjusted for fasting insulin (*P* = 0.012–0.045).

VLDL cholesterol correlates with liver total and free cholesterol content

To explain the link between lipoprotein cholesterol metabolism and liver disease, we analyzed the association between lipoprotein cholesterol concentrations and liver cholesterol content (n = 52). Liver cholesterol content measured by GLC correlated with lobular inflammation (*r*_s = 0.393, *P* = 0.004), as expected, but not significantly with steatosis (*r*_s = 0.258, *P* = 0.065) and fibrosis (*r*_s = -0.186, *P* = 0.221). Interestingly, liver cholesterol content also correlated with VLDL cholesterol, but not with LDL and HDL cholesterol (Fig. 2B). To further study the interaction between liver cholesterol metabolism and NASH, we measured liver free cholesterol content with NMR in the available liver samples (n = 45). First, our observation that liver triglyceride content measured with NMR correlated

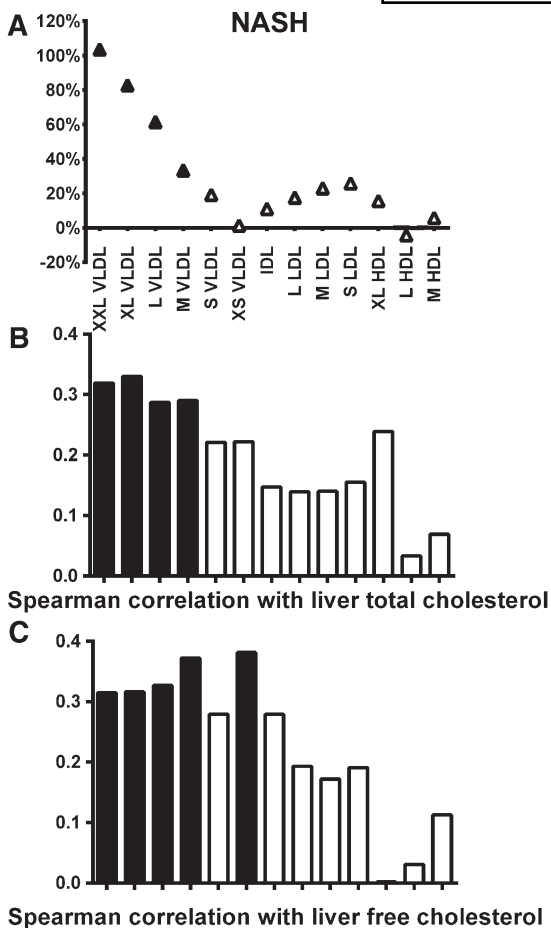


Fig. 2. A: Lipoprotein subclass cholesterol concentration in individuals with NASH compared with those with normal liver histology. Percentage changes comparing to the group without the pathology (set to 0%) have been calculated and statistical significance is shown as in Fig. 1. Spearman correlation between lipoprotein subclass cholesterol concentration and liver cholesterol content (B) and liver free cholesterol content (C) (black bars, $P < 0.05$).

with steatosis determined by histology validates our NMR methodology ($r_s = 0.772$, $P = 5.4 \times 10^{-10}$). Second, free cholesterol content measured with NMR correlated with liver total cholesterol content measured with GLC ($r_s = 0.419$, $P = 0.024$), but not with steatosis, lobular inflammation, or fibrosis (supplementary Table IX). Similarly to total liver cholesterol, liver free cholesterol correlated with all VLDL subclasses ($r_s = 0.315$ – 0.381 , $P < 0.005$), except with small VLDL ($r_s = 0.279$, $P = 0.063$), and not with LDL and HDL cholesterol (Fig. 2C).

Because high VLDL cholesterol is linked with increased cholesterol synthesis in the liver, we also explored mRNA expression of genes regulating cholesterol synthesis (*CYP51A1*, *DHCR7*, *DHCR24*, *HMGCR*, *LSS*, *SC4MOL*, *SC5D*, *SREBP1a*, *SREBP1c*, *SREBP2*, and *TM7SF2*) and uptake (*LDLR*) in the liver. Although mRNA expression of some genes regulating synthesis tended to correlate with VLDL and LDL cholesterol subclasses (supplementary Table X), we could not show any association between the expression of cholesterol synthesis genes and steatosis,

inflammation, or fibrosis (all $P > 0.3$, data not shown). Thus, the transcriptional upregulation of the cholesterol synthesis genes does not explain the association between VLDL cholesterol and NASH. Interestingly, we found that X-box binding protein 1 (*XBPI*) splicing in the liver correlated with lobular inflammation ($r_s = 0.272$, $P = 0.014$) and ballooning ($r_s = 0.243$, $P = 0.029$), but not with steatosis ($r_s = 0.080$, $P = 0.479$). Because *XBPI* splicing also associated with cholesterol concentration of small VLDL, IDL, LDL subclasses and medium HDL ($r_s = 0.220$ – 0.313 , $P < 0.05$), endoplasmic reticulum stress could potentially be a link between cholesterol metabolism and NASH.

Although the *PNPLA3* genotype has been associated with liver fat (15, 31), and also tended to associate with steatosis in our study ($P = 0.080$, χ^2 test), there was no association of the *PNPLA3* genotype with serum lipids (all $P > 0.2$, data not shown).

Obesity surgery normalizes NASH-related lipoprotein abnormalities

To determine whether obesity surgery normalizes NASH-related lipoprotein abnormalities, we analyzed serum NMR results before and after RYGB. The effect of RYGB on total lipid concentration of different lipoprotein subclasses is shown in Fig. 3A (more detailed in supplementary Table XI). The VLDL and LDL lipid accumulations that were associated with NASH (Fig. 3B) decreased significantly after surgery. In contrast, HDL lipid concentration that was not associated with simple steatosis or NASH clearly increased with weight loss. To investigate whether obesity surgery corrects NASH-related dyslipidemia, we divided patients not using cholesterol lowering medication to those without NASH ($n = 27$) at baseline and to those with possible/definite NASH ($n = 20$, for definition see Materials and Methods) at baseline. The increases in VLDL, IDL, and LDL cholesterol and serum triglycerides in individuals with NASH normalized after surgery to levels comparable with the levels in individuals without NASH (all changes $P < 0.05$) (Table 3).

DISCUSSION

Our study was set to assess whether lipoprotein subclass metabolism is linked with NAFLD or NASH. High serum VLDL and LDL lipid concentration related to liver inflammation and fibrosis more than to steatosis (Fig. 1). More specifically, we explored the association of cholesterol metabolism with liver injury in NASH (9, 12, 32). Both VLDL and LDL cholesterol concentrations associated with liver inflammation and fibrosis (Fig. 2), and VLDL subclass cholesterol concentrations associated also with liver cholesterol content. This association was independent of steatosis and serum triglycerides (Fig. 2 and multivariate analysis) suggesting an independent link between VLDL and LDL cholesterol and NASH.

A key finding in this study was that high serum VLDL and LDL lipid concentration related to lobular inflammation in the liver independent of steatosis (Table 2, Fig. 1C,

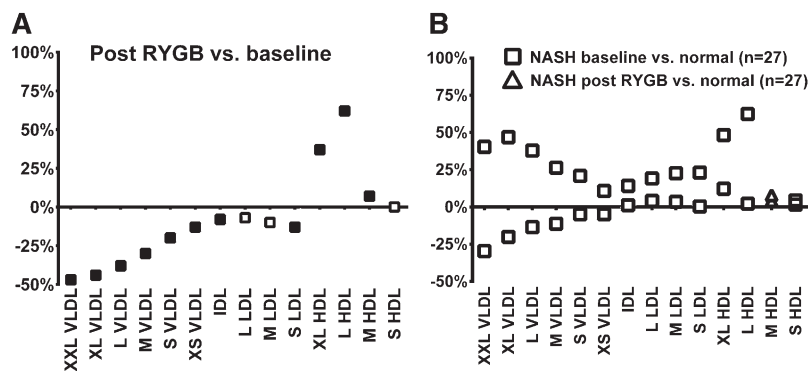


Fig. 3. Changes in lipoprotein subclass lipid concentrations (A) in response to RYGB in the whole study group compared with baseline. B: Shows the effect of RYGB specifically in individuals with NASH by showing the difference between the NASH group and the normal group at baseline (squares) and the NASH group after RYGB compared with the normal group at baseline (triangles). The color of the symbol indicates subgroup analysis comparing a given group to the group without pathology (A) or to baseline (B) (gray indicates $P < 0.05$, black indicates $P < 0.01$).

and multivariate analysis). We also found a link between VLDL and LDL lipid concentration and cell injury in NASH, quantified by the NAFLD score and ballooning (24, 33). These data give a more detailed view on lipoprotein subclass metabolism in NASH than earlier findings demonstrating increased VLDL synthesis in steatosis (4, 8), leading to the suggestion that high serum VLDL and LDL lipid concentrations are also related to the progression from simple steatosis to NASH. This is in line with

previous findings suggesting that nonHDL cholesterol (VLDL + LDL) is a biomarker for NASH (34).

One potential link between the VLDL and LDL lipid contents and NASH is altered cholesterol metabolism in the liver. Accumulation of liver cholesterol has been suggested to be important in the pathophysiology of NASH (9). Cellular free cholesterol, but not free fatty acids or triglycerides, sensitizes to TNF- and Fas-induced steatohepatitis (35). The possible explanation is that free cholesterol

TABLE 3. Effect of obesity surgery on NASH-related alterations in cholesterol concentration of different lipoproteins and serum total triglycerides in patients not using cholesterol lowering medication

	Not NASH (n = 27)	Possible/Definite NASH (n = 20)	P^b
Total cholesterol			
Before	4.27 ± 0.9	4.98 ± 0.9	0.009
After	4.48 ± 1.0	4.43 ± 0.8	0.914
Change	4.9%	-11.0%	0.008
P value for change ^a	0.189	0.016	
VLDL cholesterol			
Before	0.76 ± 0.2	0.82 ± 0.2	0.237
After	0.69 ± 0.3	0.63 ± 0.2	0.813
Change	-9.2%	-23.2%	0.169
P value for change ^a	0.073	3.0×10^{-5}	
IDL cholesterol			
Before	0.66 ± 0.2	0.78 ± 0.2	0.023
After	0.67 ± 0.2	0.64 ± 0.2	0.621
Change	1.5%	-17.9%	0.013
P value for change ^a	0.709	0.003	
LDL cholesterol			
Before	1.67 ± 0.5	2.04 ± 0.6	0.019
After	1.65 ± 0.6	1.57 ± 0.5	0.949
Change	-1.2%	-23.0%	0.022
P value for change ^a	0.752	0.001	
HDL cholesterol			
Before	1.18 ± 0.2	1.34 ± 0.3	0.037
After	1.48 ± 0.3	1.60 ± 0.3	0.085
Change	25.4%	19.4%	0.111
P value for change ^a	3.0×10^{-6}	0.0002	
Triglycerides			
Before	1.60 ± 0.5	1.75 ± 0.5	0.143
After	1.25 ± 0.6	1.16 ± 0.4	0.966
Change	-21.9%	-33.7%	0.288
P value for change ^a	0.002	2.1×10^{-5}	

^aPaired samples *t*-test for within subjects comparison.


^bKruskal-Wallis independent samples test for between groups comparison.

is cytotoxic and can activate both Fas-independent and Fas-triggering mitochondrial dysfunction in macrophages (36). Thus, it has been proposed that hepatic accumulation of free cholesterol results in cytotoxicity which mediates transition from steatosis to NASH (37). It has recently been suggested that the cause for cholesterol accumulation in NASH is increased cholesterol synthesis (30). Our gene expression findings in the liver support the suggestion that endoplasmic reticulum stress may be a link between cholesterol metabolism and NASH. We observed that altered splicing of XBP1 related to both NASH and to changes in cholesterol metabolism linked with NASH. Earlier, the IRE α /XBP1 pathway has been suggested to regulate hepatic lipid homeostasis and its dysregulation has been associated with NASH (38).

Although both VLDL and LDL cholesterol associated with inflammation and fibrosis, only VLDL cholesterol correlated significantly with the liver cholesterol content in our study (Fig. 2B, C). This suggests that increased synthesis of VLDL particles, rather than decreased release of VLDL (39), is the key finding in NASH. Although we could not link liver cholesterol accumulation, or increased VLDL cholesterol concentrations, to increased expression of genes regulating cholesterol metabolism, as recently suggested (30, 40), our results support the view that changes in lipoprotein subclass metabolism in NASH relate to inflammation and cell injury in NASH (8, 9, 30, 40–42).

Importantly, all NASH-related lipoprotein abnormalities were abolished by obesity surgery (Fig. 3). The decrease in serum LDL cholesterol and total triglycerides, and the increase HDL cholesterol, in response to RYGB has been well-characterized (43). In this study we demonstrate that the lipoprotein abnormalities related to NASH can be corrected with RYGB, also at a subclass level. We assume that this is related to the amelioration of NASH. Unfortunately, we had only 11 liver biopsies taken at 1 year follow-up. As expected (44–46), they consistently showed improved histology (data not shown).

Although the study population was limited to 116 patients, the detailed histological characterization is more extensive than in most previous studies (9, 11, 12, 30). In addition, the analysis of serum concentrations of lipoprotein subclasses, as opposed to using only standard enzymatic methods, was a strength in our study. For example, we could not identify the expected association between serum total triglycerides and NASH (Table 1), but could clearly demonstrate that triglyceride levels of all VLDL subclasses had association with NASH diagnosis (Table 2). Because of ethical difficulties to obtain liver biopsies in healthy lean individuals, we only had biopsies from obese individuals. On the other hand, by studying obese individuals, we increased the possibility to find subjects with NASH.

We conclude that VLDL and LDL subclass lipid concentrations are associated with lobular inflammation and liver cell injury in NASH. More specifically, our results support an independent link between VLDL cholesterol, liver cholesterol, and NASH. Finally, we demonstrated that lipoprotein subclass abnormalities in NASH can be reversed with obesity surgery. 

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The authors of “Lipoprotein subclass metabolism in nonalcoholic steatohepatitis” (*J. Lipid Res.* 2014, 55: 2676–2684) have informed the *Journal* that the following errors appeared in the following figures in their article: Fig. 1 (p. 2679)

The symbols on the graphs in Fig. 1A–D were too small and the symbol colors were unclear. The symbol size is now enlarged and the color of the symbols is now visible.

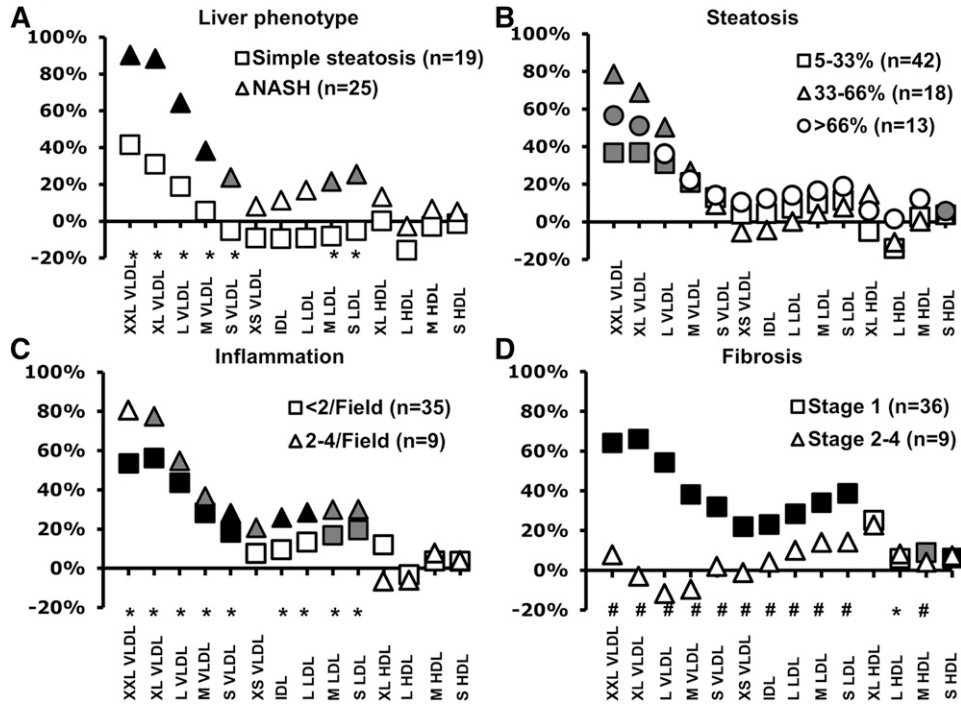


Fig. 3 (p. 2682)

Two data points on Fig. 3A, L LDL and M LDL, appeared as white squares and should be gray squares. In Fig. 3B, symbols for both NASH baseline and NASH post RYGB were presented as squares. However, NASH post RYGB should be presented as triangles, as correctly stated in the figure legend. These errors have been corrected. Symbols on both graphs in Fig. 3A and B have been enlarged for clarity. These corrections do not affect the interpretations of the results or the conclusions of the article.

