The human liver lipidome is significantly related to the lipid composition and aggregation susceptibility of low-density lipoprotein (LDL) particles

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ABSTRACT

Background and aims: The susceptibility of low-density lipoprotein (LDL) to aggregation predicts atherosclerotic cardiovascular disease. However, causes of interindividual variation in LDL lipid composition and aggregation susceptibility remain unclear. We examined whether the lipid composition and aggregation susceptibility of LDL reflect the lipid composition of the human liver.
Methods: Liver biopsies and blood samples for isolation of LDL particles were obtained from 40 obese subjects (BMI 45.9 ± 6.1 kg/m^2, age 43 ± 8 years). LDL was isolated using sequential ultracentrifugation and lipidomic analyses of liver and LDL samples were determined using ultra-high performance liquid chromatography–mass spectrometry. LDL aggregation susceptibility *ex vivo* was analyzed by inducing aggregation by human recombinant secretory sphingomyelinase and following aggregate formation.
Results: The composition (acyl carbon number and double bond count) of hepatic triglycerides, phosphatidylcholines, and sphingomyelins (SMs) was closely associated with that of LDL particles. Hepatic dihydroceramides and ceramides were positively correlated with concentrations of the corresponding SM species in LDL as well with LDL aggregation. These relationships remained statistically significant after adjustment for age, sex, and body mass index.
Conclusions: Lipid composition of LDL reflects that of the human liver in obese patients. Changes in hepatic sphingolipid metabolism may contribute to interindividual variation of LDL lipid composition and susceptibility to aggregation.

1. Introduction

The main cause of atherosclerotic cardiovascular disease (ASCVD) is accumulation of cholesterol carried in low density lipoprotein (LDL) particles to the arterial wall [1]. A high concentration of LDL-cholesterol is the major risk factor for ASCVD, but the qualities of LDL particles, including their aggregation susceptibility, also contribute [2]. Differences in the susceptibility of LDL particles to aggregation can be determined using an *ex vivo* assay mimicking LDL modification in the arterial wall [3–6]. LDL aggregability predicts independently ASCVD

events [4] and mortality [3], but does not seem to correlate with known ASCVD risk factors such as age, sex, body mass index (BMI), or the concentration of serum LDL cholesterol [3,4]. The reason(s) for the interindividual variation in LDL aggregability remain poorly understood.

LDL originates from triglyceride (TG)-rich very-low-density lipoprotein (VLDL), which is synthesized and secreted by the liver. In peripheral tissues, VLDL undergoes lipolysis and becomes depleted of TGs and enriched with cholesteryl esters (CEs), thereby gradually increasing in density as it is transformed into LDL [7]. In addition to these

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hydrophobic, nonpolar core lipids, the surface of LDL is composed of surface-bound apolipoproteins, phospholipids, mainly phosphatidyl-cholines (PCs) and sphingomyelin (SMs), and free unesterified cholesterol that stabilize the lipid assembly into the surface monolayer [8]. These data raise the possibility that lipid composition of LDL may reflect that of the liver.

The human liver lipidome exhibits marked interindividual variation [9]. We have previously shown that a change in diet composition changes that in the liver as judged from the composition of VLDL [10]. A saturated as compared to a polyunsaturated diet increases saturated fatty acids and circulating ceramides, which are synthetized in the liver from saturated fatty acids via the *de novo* ceramide synthetic pathway [10]. There are no previous data examining whether the lipid composition of the human liver and that of LDL particles are interrelated, or whether they associate with the susceptibility of LDL to aggregation.

Given that the aggregation susceptibility of LDL is related to its lipid composition, especially the sphingolipidome [3,5,11], we hypothesized that a higher LDL aggregation susceptibility may be associated with a distinct hepatic lipidomic profile. To this end, in obese volunteers undergoing a liver biopsy, w e profiled the hepatic lipidome while simultaneously analyzing the lipid composition and aggregation susceptibility of circulating LDL particles.

2. Materials and methods

2.1. Subjects

The study subjects were recruited amongst those referred for obesity surgery in the Helsinki University Hospital. Subjects were eligible if they met the following criteria: (a) age 18–75 years; (b) no known acute or chronic disease except for obesity (BMI >35 kg/m^2) or hypertension on the basis of medical history, physical examination, and standard laboratory tests (complete blood count, serum creatinine, electrolyte concentrations); (b) no use of antihyperglycemic or lipid-lowering medications; (c) no clinical or biochemical evidence of liver disease; (e) no history of use of toxins or drugs associated with liver steatosis. Elevated liver enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) were not exclusion criteria. The study protocol has been priorly approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa on research on humans. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each patient included in the study.

2.2. Study design

The subjects were invited to Clinical Research Unit after an overnight fast, approximately one week prior to obesity surgery. A history and physical examination were performed as described in detail previously [12]. Blood samples were drawn for the measurement of complete blood count, serum concentrations of glucose and insulin and HbA$_{1c}$, creatinine, sodium and potassium, LDL- and HDL cholesterol and TG, and activities of AST, ALT, glutamyltransferase and alkaline phosphatase, and for the isolation of LDL particles and measurement of the LDL lipidome and aggregation susceptibility of LDL. A liver biopsy was obtained during the surgery as described in Ref. [9]. Part of the biopsy was sent to the pathologist, while the remaining material was snap-frozen in liquid nitrogen and used for lipidomic analysis. Liver histology was analyzed by an experienced hepatopathologist (J.A.) in a blinded fashion as proposed by Brunt et al. [13]. Liver fat percentage was defined based on the percentage of hepatocytes containing macrovesicular lipid droplets [14].

2.3. LDL isolation and measurement of the susceptibility of LDL to aggregation

LDL (d = 1.019–1.063 g/mL) was isolated from plasma samples by D$_2$O-based sequential ultracentrifugation [15]. An aliquot of 300 μL of plasma was used for isolation, and 300 μL of LDL was collected after ultracentrifugation. The concentration of protein in LDL was measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The susceptibility of isolated LDL particles to aggregation was measured as previously described in detail [16,17]. In brief, LDL samples were diluted to a concentration of 200 μg of LDL protein/mL, and aggregation was induced by addition of an in-house produced [18] human recombinant acid sphingomyelinase (SMase) to the LDL samples. LDL aggregation was determined by dynamic light scattering using Wyatt DynaPro Plate Reader II (Wyatt Technology, Goleta, CA) with paraffin coating. LDL aggregate size was followed as a time-varying parameter, and the inflection point of the aggregate size vs. time curve (EC50) was used as the measure of LDL aggregation. Thus, the faster the aggregation, the lower EC50.

2.4. Lipidomic analysis of LDL particles

LDL samples were extracted using a modified version of the previously published Folch procedure [19]. Briefly, 10 μL of 0.9% NaCl, 40 μL of CHCl$_3$:MeOH (2:1, v/v), and 80 μL of the 2.25 μg/mL internal standard solution of chosen lipid standards were added to each 10 μL LDL sample. The internal standard solution contained the following compounds: PE(17:0/17:0), SM(d18:1/17:0), Cer(d18:1/17:0), PC (17:0/17:0), LPC(17:0), and PC(16:0/d31/18:1) from Avanti Polar Lipids, Inc. (Alabaster, AL), and TG(17:0/17:0/17:0) from Larodan AB (Solna, Sweden). Samples were vortexed and incubated on ice for 30 min after which they were, along with 60 μL from the lower layer of each sample, collected, and 60 μL of CHCl$_3$:MeOH (2:1, v/v) added to each sample. The UHPLC-QTOFMS analyses were performed as described earlier [20] with some modifications. The UHPLC-QTOFMS (Agilent Technologies, Santa Clara, CA) combined the 1290 Infinity system and 6545 QTOFMS, interfaced with a dual jet stream electrospray (dual ESI) ion source. MassHunter B.06.01 software (Agilent Technologies, Santa Clara, CA) was used for all data acquisition and MZmine 2 was used for data processing [21]. Lipid identification was based on an in-house spectral library with retention times. The lipid species were analyzed as percentages of surface lipids (glycerophospholipids (GPLs) and sphingolipids (SLs)) and percentages of neutral core lipids (CEs and TGs).

2.5. Lipidomic analysis of liver tissue

The liver lipidome was analyzed using UHPLC-MS at the VTT Technical Research Centre of Finland (Espoo, Finland) as described in detail earlier in Ref. [9]. Briefly, liver tissue was first homogenized when still frozen (Covaris, CryoPrep CP02, MA), and an aliquot (20 μL) of an internal standard mixture was added. The lipids were extracted using a mixture of HPLC-grade chloroform and methanol (2:1; 400 μL). Then, 50 μL of 0.9% NaCl was added and the lower phase (200 μL) was collected, and 20 μL of an internal standard mixture containing labeled PC(16:1/0:0-D3), PC(16:1/16:1-D6) and TG(16:0/16:0/16:0–13C3) was added. The extracts were run on a Waters Q-TOF Premier mass spectrometer combined with an Acquity UHPLC. The lipid profiling was carried out using electrospray ionization mode. The data processing included alignment of peaks, peak integration, normalization, and identification using an internal spectral library. The data were normalized using one or more internal standards representative of each class.

2.6. Statistical analyses

Normal distribution of the data was analyzed by the Kolmogorov-

Smirnov test and the equality of variances was tested with the Levene's test. Data are shown as mean ± standard deviation. Spearman correlation coefficients were calculated to analyze the relationships between the continuous variables. *Post-hoc*, multiple linear regression analysis adjusted by age, sex and BMI was used to confirm the identified significant associations between the hepatic lipid species and LDL aggregation. The analyses were performed using GraphPad Prism version 9.0.1 [22] and R version 4.0.2 (R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Characteristics of the subjects

Characteristics of the study subjects are shown in Table 1. The mean age of the 40 subjects (30 women, 10 men) was 43 ± 8 years, and mean BMI 45.9 ± 6.1 kg/m^2 (range 35.4–59.1 kg/m^2) (Table 1). Thus, all subjects were obese (BMI >30 kg/m^2) and 31 (78%) were severely obese (BMI>40 kg/m^2). Plasma TGs averaged 1.3 ± 0.7 mmol/l, HDL cholesterol 1.2 ± 0.3 mmol/l, and LDL cholesterol 2.9 ± 0.7 mmol/l. Fasting plasma glucose averaged 5.4 ± 0.7 mmol/l, HbA$_{1c}$ 5.6 ± 0.3 mmol/mol, and serum insulin 13 ± 7 mU/L. None of the subjects had type 2 diabetes or used antihyperglycemic or lipid-lowering medications. The median liver fat percentage was 5% (interquartile range 0–23%).

Data are shown as number (%), mean (standard deviation), or median (25th–75th percentile), as appropriate. [a]Chi-square test, Student's *t*-test and Mann–Whitney *U* test were used for statistical testing. ALT, alanine aminotransferase; AST, aspartate aminotransferase; B, blood; BMI, body mass index; DBP, diastolic blood pressure; f, fasting; GGT, gamma-glutamyltransferase; HDL-C, HDL cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; LDL-C, LDL cholesterol; n, number; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; P, plasma; SBP, systolic blood pressure; TG, triglyceride.

3.2. Lipid composition of the liver and LDL particles

A total of 324 individual lipid species were identified in the liver biopsies (Supplementary Figure IA) and 171 in LDL particles (Supplementary Figure IB). The relative composition of main lipid classes in liver and LDL are shown in Fig. 1. Liver lipids consisted mainly of GPLs (51.0 ± 8.3%) and LDL lipids of CEs (85.0 ± 4.9%). With the exception of CEs, relative amounts of all the lipid subclasses shown in Fig. 1 were significantly higher in the liver as compared to LDL (Supplementary Table I).

3.3. The acyl chain composition of the liver and LDL lipids are strongly interrelated

The total acyl carbon number and double bond count of liver and LDL TGs, SMs and PCs were closely correlated (Fig. 2). Hepatic concentrations of TGs containing more than 5 double bonds (Fig. 2A), and TGs with the shortest or longest total carbon number (<51 or ≥54–56) closely correlated with respective species in LDL. In the case of SMs, the species with two double bonds (Fig. 2C) and a total (acyl and sphingosine) chain length of more than 39 carbons (Fig. 2D) were significantly interrelated. Similarly, PCs with more than 4 double bonds (Fig. 2E) and a total chain length of more than 38 carbons (Fig. 2F) were significantly interrelated.

The relationships between the total acyl carbon number and double bond count of liver and LDL TGs, SMs and PCs are shown separately for women and men in Supplementary Figures II and III for double bonds and acyl carbon numbers, respectively. Overall, the associations were similar in women and men with respect to double bond count (Supplementary Figure II) and acyl carbon number (Supplementary Figure III) in TGs, SMs and PCs.

3.4. LDL aggregation susceptibility associates with distinct changes in hepatic and LDL sphingomyelins

3.4.1. LDL aggregation and the liver lipidome

Relative hepatic concentrations of very-long chain TGs and polyunsatured PC species were associated with decreased LDL aggregation (Fig. 3A), while several dihydroceramide and ceramide species, two ether-linked PC species, two PE plasmalogens, and DG(36:4), were associated with increased LDL aggregation (Fig. 3A). Almost all hepatic dihydroceramide and ceramide species showed a trend towards a positive association with faster LDL aggregation (Fig. 4A). Several dihydroceramides and ceramides including Cer(d18:0/16:0), Cer(d18:0/

Table 1
Clinical characteristics of the study subjects.

	All	Women	Men	*p*-value[a]
Number	40	30	10	
Age, years	43 (8)	43 (9)	45 (6)	0.536
BMI, kg/m^2	45.9 (6.1)	46.0 (6.2)	45.5 (5.8)	0.847
SBP, mmHg	132 (18)	132 (18)	132 (20)	0.928
DBP, mmHg	89 (11)	89 (12)	87 (9)	0.631
fP-Cholesterol, mmol/L	4.5 (0.8)	4.6 (0.9)	4.2 (0.6)	0.208
fP-HDL-C, mmol/L	1.24 (0.30)	1.27 (0.33)	1.14 (0.16)	0.263
fP-LDL-C, mmol/L	2.9 (0.8)	2.9 (0.8)	2.7 (0.7)	0.539
fP-TGs, mmol/L	1.13 (0.89–1.38)	1.18 (0.89–1.37)	0.93 (0.90–1.38)	0.731
fP-Glucose, mmol/L	5.4 (0.7)	5.4 (0.6)	5.3 (0.9)	0.684
B-HbA$_{1c}$, %	5.6 (0.4)	5.6 (0.4)	5.5 (0.2)	0.238
fS-Insulin, mU/L	13.4 (7.3)	12.7 (7.6)	15.7 (5.7)	0.267
HOMA-IR	3.1 (1.7–4.5)	2.9 (1.5–4.1)	3.3 (2.7–4.9)	0.288
P-ALT, U/L	32 (20–47)	27 (18–48)	41 (33–45)	0.138
P-AST, U/L	28 (25–36)	27 (24–35)	30 (27–36)	0.453
P-GGT, U/L	25 (20–36)	22 (17–31)	33 (24–45)	**0.038**
Liver fat, %	5 (0–23)	5 (0–28)	5 (0.0–9)	0.518
Lobular inflammation, n (0/1/2/3)	35/5/0/0	27/3/0/0	8/2/0/0	0.783
Grade of activity, n (0/1/2/3)	33/7/0/0	25/5/0/0	8/2/0/0	1.000
Fibrosis stage, n (0/1/2/3/4)	26/14/0/0/0	19/11/0/0/0	7/3/0/0/0	1.000
NAFLD, n (%)	3 (30)	16 (40)	13 (43)	0.709
NASH, n (%)	2 (20)	7 (18)	5 (17)	1.000
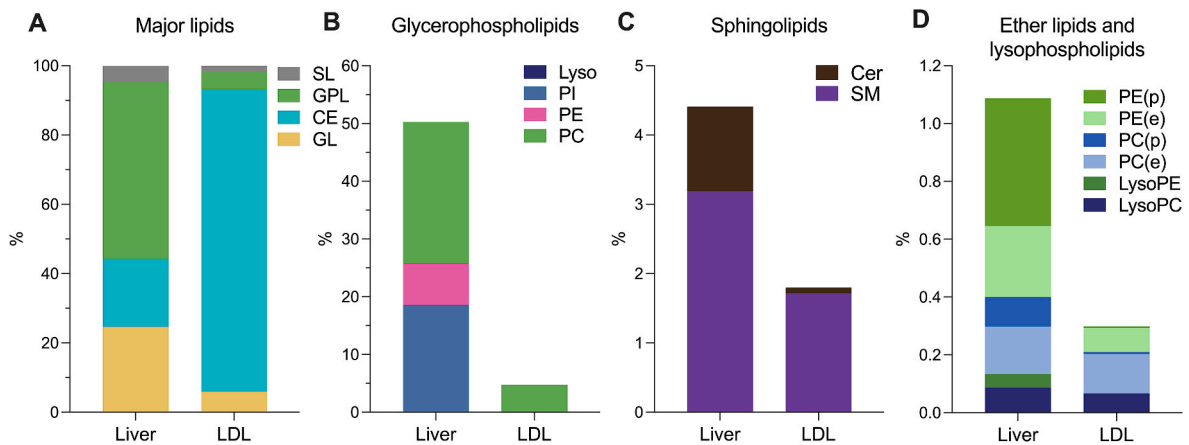
Fig. 1. Hepatic and low-density lipoprotein (LDL) particle lipid compositions.
Graphical presentation of (A) all major lipids, (B) glycerophospholipids (GPL), (C) sphingolipids (SL), and (D) ether lipids and lysophospholipids in the liver and in LDL particles. The y-axis denotes the % of given lipid of total liver or LDL lipids. Lipidomic analyses were performed using ultra-high performance liquid chromatography–mass spectrometry. The data are shown numerically in Supplementary Table I. CE, cholesteryl ester; Cer, ceramide; GPL, glycerophospholipid; LDL, low-density lipoprotein; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PC(e), ether-linked phosphatidylcholine; PC (p), phosphatidylcholine plasmalogen; PE, phosphatidylethanolamine; PE(e), ether-linked phosphatidylethanolamine; PE(p), phosphatidylethanolamine plasmalogen; SL, sphingolipid; SM, sphingomyelin; TG, triglyceride.
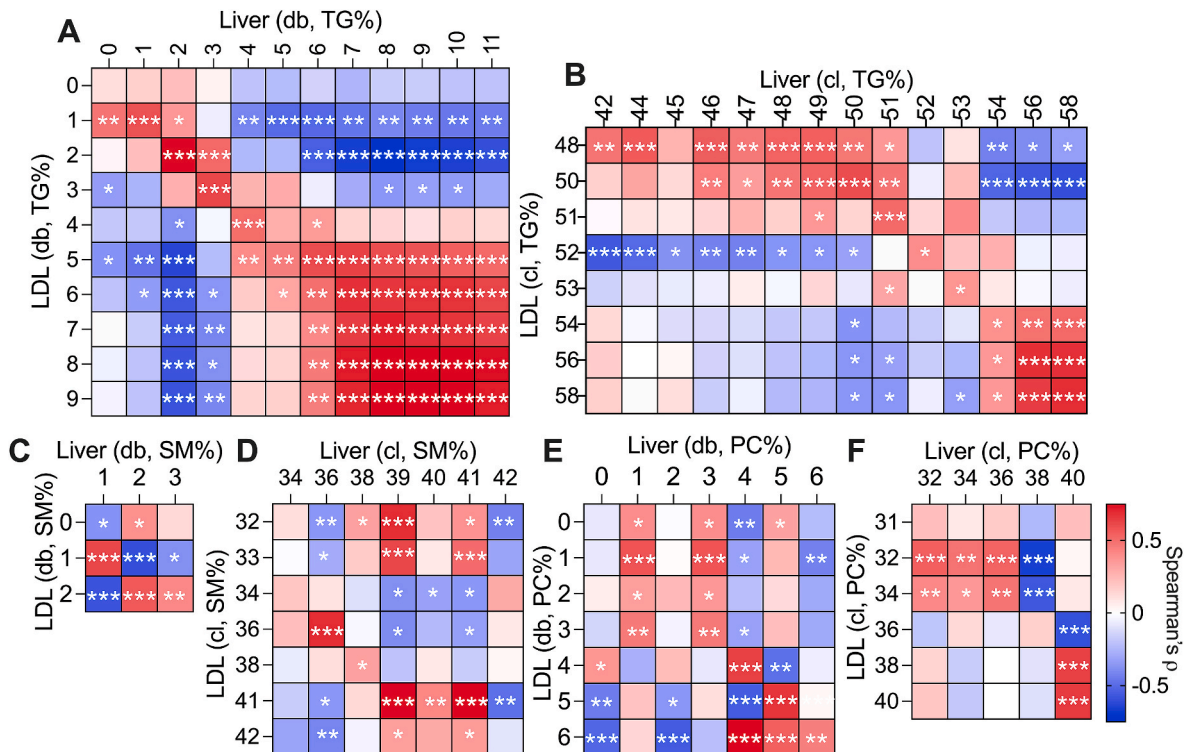

Fig. 2. Triglyceride (TG), sphingomyelin (SM), and phosphatidylcholine (PC) total double bond and carbon number compositions in the liver and low-density lipoprotein (LDL) particles.
Heatmaps depicting relationships (Spearman) between liver and low-density lipoprotein (LDL) particle total double bond count and carbon number of TGs (panels A and B, respectively), SMs (panels C and D, respectively) and PCs (panels E and F, respectively). Positive correlations are shown in red and negative correlations in blue. Asterisks denote statistically significant relationships. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

18:0), Cer(d18:0/23:0), Cer(d18:1/23:0), Cer(d18:1/24:0), and Cer (d18:1/25:0), were positively correlated with corresponding SM species in LDL (SM(d18:0/16:0), SM(d18:1/24:0), SM(d33:1), SM(d36:0), SM (d36:1), SM(d36:2), SM(d41:1)), which in turn positively associated with LDL aggregation (Fig. 4B). For example, hepatic Cer(d18:0/24:0) correlated significantly with hepatic SM(d18:1/24:0) (R = 0.430, *p* = 0.006), which in turn correlated with SM(d18:1/24:0) in LDL (R =

0.536, *p* < 0.0001) (Supplementary Figure IV). Multiple linear regression adjusted by age, sex, and BMI was used *post-hoc* to confirm the identified associations between the hepatic dihydroceramide and ceramide species and LDL aggregation. The relationships remained significant after adjustment for age, sex, and BMI (Supplementary Figure V).

Relationships between concentrations of hepatic dihydroceramide and ceramide species, and SM species of LDL are shown separately for
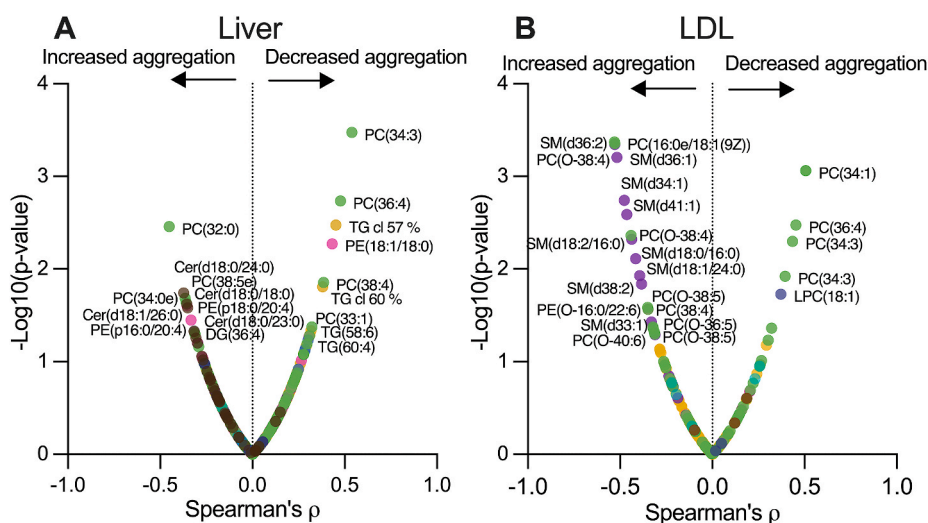
Fig. 3. LDL (low-density lipoprotein) aggregation susceptibility and lipid compositions of the liver and LDL particles.

Volcano plots showing relationships (Spearman correlation coefficients) between the susceptibility of LDL to aggregation and (A) lipid composition of the liver, and (B) lipid composition of LDL. LDL aggregation was measured by inducing aggregation with human recombinant sphingomyelinase and following aggregation size at various time points. The inflection point (EC50) of the curve relating aggregate size to time was used as the measure of aggregation susceptibility. Thus, the faster the aggregation, the lower the EC50. Lipid compositions of the liver and LDL particles were determined as described under Fig. 1 and Materials and methods section. Lipid species that correlated significantly with LDL aggregation have been written out. Cer, ceramide; DG, diacylglycerol; LPC, lysophosphatidylcholine; LDL, low-density lipoprotein; PC, phosphatidylcholine; SM, sphingomyelin; TG, triglyceride.
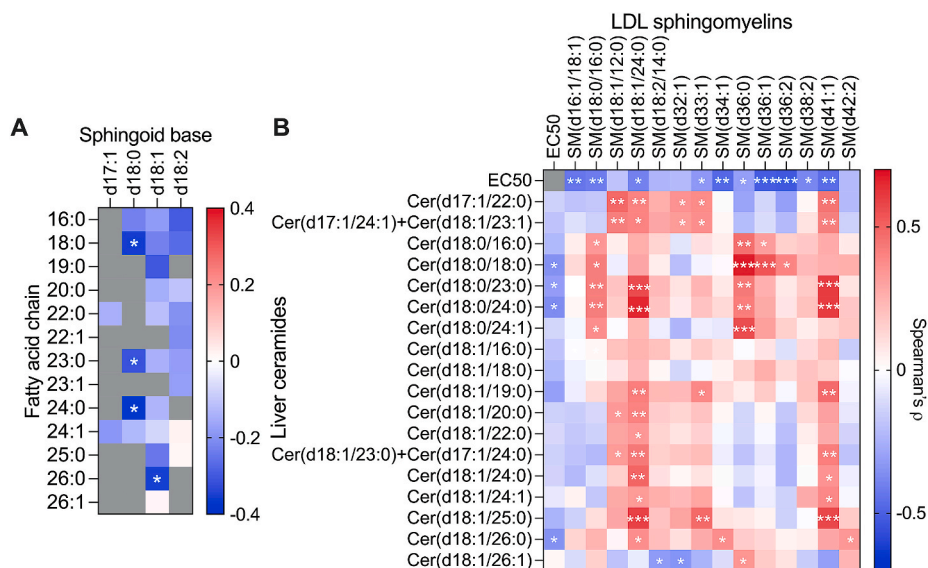
Fig. 4. Hepatic dihydroceramides and ceramides, and low-density lipoprotein (LDL) aggregation susceptibility and sphingomyelins (SMs).

(A) Heatmap of Spearman's correlation coefficients between concentrations of hepatic dihydroceramide and ceramide species and susceptibility of LDL to aggregation (EC50). The red color denotes a positive correlation to EC50 (negative correlation to LDL aggregation) and the blue color denotes a negative correlation to EC50 (positive correlation to LDL aggregation). Individual values for the four significant relationships after adjustment for age, sex and body mass index are shown in Supplementary Figure V. (B) Heatmap of Spearman's correlation coefficients between concentrations hepatic dihydroceramide and ceramide species, and SM species of LDL. The species significantly associated to LDL aggregation are highlighted. The correlation coefficient between hepatic Cer(d18:0/18:0) and SM(d36:0) in LDL was 0.663 ($p < 0.0001$), and between hepatic Cer(d18:0/24:0) and SM(d18:1/24:0) in LDL 0.645, $p < 0.0001$. Concentrations of hepatic and LDL lipid species and LDL aggregation were measured as described under Fig. 1 and Methods. The absolute values of these lipid species are shown in Supplementary Figure I. Relationships between concentrations of hepatic dihydroceramide and ceramide species, and SM species of LDL are shown separately for women and men in Supplementary Figure VI. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Cer, ceramide; LDL, low-density lipoprotein; SM, sphingomyelin.

women and men in Supplementary Figure VI. The associations were similar in both sexes (Supplementary Figure VI). Hepatic lipidome in subjects with a BMI above the median of 45.3 kg/m^2 (BMI 46.6 ± 2.9 kg/m^2) was enriched with ceramide and ether-linked GPL species and depleted in polyunsaturated PC and SM species as compared to those with a BMI of 45.3 kg/m^2 or less (BMI 50.9 ± 3.5 kg/m^2) (Supplementary Figure VII).

3.4.2. LDL aggregation and the LDL lipidome
LDL aggregated faster in subjects with a high content of SMs and a low content of PCs in LDL (Fig. 3B)
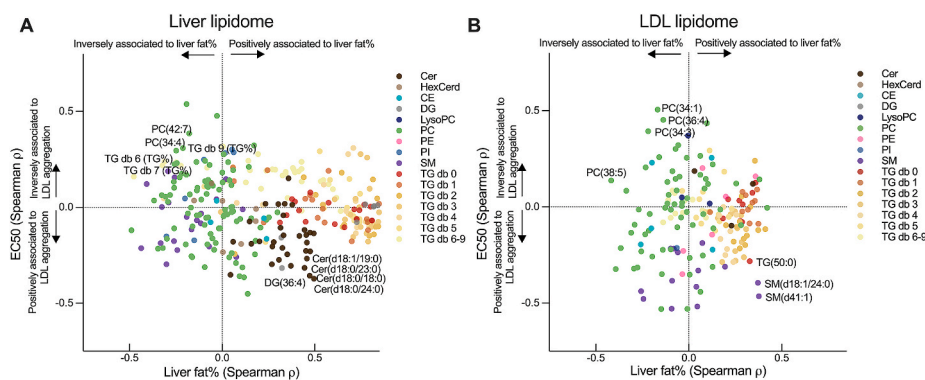
3.4.3. DL aggregation, liver fat, and lipid compositions of the liver and LDL particles

The Spearman correlation coefficients between liver fat content, LDL aggregation susceptibility, and hepatic and LDL lipid compositions are shown in Fig. 5. In the scatter plot, each dot corresponds to an individual

lipid species colored according to its class, and its relationship with liver fat % is shown on the x-axis and with LDL aggregation susceptibility (EC50) on the y-axis. Thus, lipids located in the higher left quadrant are associated with lower liver fat % and decreased LDL aggregation susceptibility, while lipids located in the lower right quadrant are associated with higher liver fat % and increased LDL aggregation.

Several hepatic ceramides, DGs, and saturated TGs were associated with both higher liver fat % and increased LDL aggregation, while the relative proportion of polyunsaturated TG and PC species were associated with lower liver fat % and decreased LDL aggregation (Fig. 5A). As expected, liver fat % showed a strong positive association with almost all hepatic TGs (Fig. 5A).

Regarding LDL lipid composition, SMs were positively and some PC species negatively associated with increased liver fat % and faster LDL aggregation (Fig. 5B).

Fig. 5. Relationships between liver fat, LDL aggregation, and lipid compositions of the liver and low-density lipoprotein (LDL) particles.
Spearman's correlation coefficient plot of (A) hepatic lipid species and (B) LDL lipid species showing the relationships between individual lipids and liver fat % on the x-axis and LDL aggregation (EC50) on the y-axis. Each dot corresponds an individual lipid species, which is colored according to its class. The lipids located in the higher left quadrant are inversely associated to both liver fat % and LDL aggregation, and the lipids located in the lower right quadrant positively associated to both liver fat % and LDL aggregation. The lipid species that most significantly either associated or dissociated between liver fat % and LDL aggregation have been written out. CE, cholesteryl ester; db, double bond; DG, diacylglycerol; Cer, ceramide; HexCer, hexosylcer-

amide; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; TG, triglyceride.

4. Discussion

To the best of our knowledge, this is the first study to examine how the lipid composition of the human liver is related to that of isolated LDL particles and the susceptibility of LDL to aggregation. We show that the total double bond count and acyl carbon number in TGs, SMs, and PCs in the liver and in LDL isolated from plasma are closely interrelated in obese patients. Hepatic dihydroceramides and ceramides were positively correlated with concentrations of the corresponding SM species in LDL as well with LDL aggregation susceptibility. These relationships remained significant after adjustment for age, sex, and BMI.

Analysis of the lipidome by UHPLC-MS enables comprehensive characterization of lipids and their structure in biological samples [23]. The human liver contained more TGs, SLs, GPLs, and lysoPCs than LDL, while LDL, as expected, was enriched with CEs. These observations are in line with multiple studies showing that the TG-rich VLDL becomes depleted of TGs and enriched with CEs upon transformation to LDL via the action of lipases and the cholesteryl ester transfer protein [24]. We also showed that the fatty acid composition, i.e. double bond count and total acyl carbon number of major lipid species including TGs, SMs, and PCs of human LDL particles closely resembled those of the liver. This implies that changes in liver lipid composition will change that of VLDL and ultimately LDL. In direct support of this, we recently showed that overconsumption of saturated as compared to polyunsaturated fat by human volunteers increased saturated fatty acids in VLDL-TGs [10] and SMs in LDL, and also the susceptibility of LDL to aggregation [5].

ASCVD is often accompanied by insulin resistance. The latter is characterized by an excess of bioactive lipids such as ceramides and diacylglycerols in the human liver [25,26]. The increase in hepatic ceramides seems to reflect specifically the activation of *de novo* ceramide synthesis from saturated fatty acids [9,10]. This pathway is causally related to the development of hepatic steatosis and insulin resistance. For example, animals lacking the enzyme converting dihydroceramides to ceramides are resistant to high-fat diet-induced hepatic steatosis and insulin resistance [27]. Circulating ceramides have also been linked with ASCVD and may even outperform traditional predictors of ASCVD [25, 28]. In addition, a recent study by Poss et al. using targeted metabolomics showed that in addition to ceramides, circulating SMs and dihydroceramides, predict ASCVD independent of cholesterol [29]. Serum SM(d18:1/24:0) was one of the final components in the proposed novel risk-predicting scores developed by machine learning approaches [29]. This is in line with the present data, which identified liver Cer (d18:0/24:0), the precursor of Cer(d18:1/24:0) and SM(d18:1/24:0), to be significantly associated with the aggregation susceptibility of LDL (Fig. 4, Supplementary Figure V). The corresponding sphingomyelin SM (18:1/24:0) both in the liver and in LDL was similarly associated with

LDL aggregation susceptibility (Supplementary Figure IV). Accordingly, multiple hepatic ceramides were highly significantly associated with SM (18:1/24:0) in LDL (Fig. 4B).

Although ceramides synthesized via the *de novo* ceramide synthetic pathway are important regulators and markers of liver health [27,28], the mechanisms by which hepatic ceramides may be linked to ASCVD and accumulation of LDL-derived cholesterol in the arterial wall are complex. First, in the Golgi apparatus of hepatocytes, ceramides are converted to SMs, some of which are incorporated into VLDL and are thus ultimately found in LDL [30]. In line with this, we observed positive associations between several hepatic ceramides and corresponding LDL-SMs (Fig. 4B). Second, the SM content of LDL particles was closely related with the susceptibility of these particles to aggregate, which is in line with several previous studies [3,5,6,31]. Enhanced LDL aggregation susceptibility has the potential to increase accumulation of LDL-derived lipids in the atherosclerotic arterial wall. LDL particles aggregate in the arterial intima after the hydrolysis of LDL-SM by the local action of secretory SMase, leading to accumulation of ceramides in LDL particles [32]. In the present study, the ceramide content of LDL was low, in keeping with a previous study directly comparing concentrations of ceramides between plasma LDL and human atherectomy specimens [33]. These findings support the view that ceramides, which are abundant in aggregated LDL in the human atherosclerotic plaques [33], need to be generated locally in the arterial wall via the hydrolysis of liver-derived LDL-SM [34,35]. In accordance with this hypothesis, SMase knock-out mice are protected against atherosclerosis and are virtually unable to trap LDL in the vessel wall [34]. This was also shown by Deevska et al. who induced an accumulation of circulating SMs and LDL aggregation in LDL receptor-null mice fed an atherogenic diet, and then deleted SMase which dramatically decreased ceramides and LDL aggregation [35].

The acyl carbon number and double bond count of PCs in the liver and LDL were significantly interrelated (Fig. 2). PCs abundant in LDL (Supplementary Figure I) were also the PCs most significantly associated with decreased aggregation (Fig. 3). It is noteworthy that we defined the concentration of the individual PCs in LDL by calculating their relative amounts of total surface lipids. Our findings are in agreement with previous data showing that a high SM/PC ratio is the most important factor in determining the susceptibility of atherogenic lipoproteins to aggregation *in vitro* [11]. Consistent with this, we previously showed that *in vivo* administration of PC-containing vesicles into $APOB^{100}$ transgenic/$Ldlr^{-/-}$ mice decreased the SM/PC ratio and rendered LDL nearly completely resistant to aggregation *ex vivo.* [3] Furthermore, treatment of LDL particles from healthy donors with PC vesicles *in vitro* makes them less susceptible to aggregation [3]. As both of these manipulations change the SM/PC ratio, we can infer that the ratio of these

two lipids, rather than the amount of either SM or PC, controls the susceptibility of LDL particles to aggregate.

The present study has some limitations. Because it is unethical to perform liver biopsies in healthy individuals, the results of subjects with obesity at relatively young age may not be directly applicable to normal-weight individuals. Second, although the sample size is large considering that liver biopsies were obtained and state-of the art measurements of the liver and LDL lipidomes were performed, it is small for epidemiological purposes. The study is cross-sectional and as such does not prove cause and effect. In addition, we did not collect information of the diets of the subjects, and thus cannot rule out its effects on the results.

We conclude that the human liver lipidome is significantly related to the lipid composition and aggregation susceptibility of LDL particles in obese patients. The distribution of double bonds and acyl carbon number in TGs, SMs and PCs of LDL particles closely resemble those of the liver. Qualitative changes in sphingolipids are seen on the surface of LDL particles and in the liver in individuals with increased LDL aggregation susceptibility. Increased hepatic dihydroceramides and ceramides are related to aggregation-prone SM-rich LDL particles. These changes in hepatic sphingolipid metabolism may contribute to an increased risk of ASCVD.

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CRediT authorship contribution statement

Mari Lahelma: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Sami Qadri:** Conceptualization, Methodology, Writing – review & editing. **Noora Ahlholm:** Data gathering, Writing – review & editing. **Kimmo Porthan:** Data gathering, Writing – review & editing. **Maija Ruuth:** Data gathering, Methodology, Writing – review & editing. **Anne Juuti:** Data gathering, Methodology, Writing – review & editing. **Matej Orešič:** Methodology, Formal analysis, Writing – review & editing. **Tuulia Hyötyläinen:** Methodology, Formal analysis, Writing – review & editing. **Katariina Öörni:** Conceptualization, Methodology, Writing – review & editing, Project administration. **Hannele Yki-Järvinen:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: K.Ö. and M.R. have a patent pending on the LDL aggregation method.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2022.11.018.

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