methods

Lipid profiling of FPLC-separated lipoprotein fractions by electrospray ionization tandem mass spectrometry[S]

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Abstract **Glycerophospholipid and sphingolipid species and their bioactive metabolites are important regulators of lipoprotein and cell function. The aim of the study was to develop a method for lipid species profiling of separated lipoprotein classes. Human serum lipoproteins VLDL, LDL, and HDL of 21 healthy fasting blood donors were separated by fast performance liquid chromatography (FPLC) from 50 μl serum. Subsequently, phosphatidylcholine (PC), lysophosphatidylcholine, sphingomyelin (SM), ceramide (CER), phosphatidylethanolamine (PE), PE-based plasmalogen (PE-pl), cholesterol, and cholesteryl ester (CE) content of the separated lipoproteins was quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS). Analysis of FPLC fractions with PAGE demonstrated that albumin partially coelutes with HDL fractions. However, analysis of an HDL deficient serum (Tangier disease) showed that only lysophosphatidylcholine, but none of the other lipids analyzed, exhibited a significant coelution with the albumin containing fractions. Approximately 60% of lipoprotein CER were found in LDL fractions and 60% of PC, PE, and plasmalogens in HDL fractions. VLDL, LDL, and HDL displayed characteristic lipid class and species pattern. The developed method provides a detailed lipid class and species composition of lipoprotein fractions and may serve as a valuable tool to identify alterations of lipoprotein lipid species profiles in disease with a reasonable experimental effort.—** Wiesner, P., K. Leidl, A. Boettcher, G. Schmitz, and G. Liebisch. **Lipid profiling of FPLC-separated lipoprotein fractions by electrospray ionization tandem mass spectrometry.** ***J. Lipid Res.*** **2009.** 50: **574–585.**

Supplementary key words lipid species • lipoprotein fractionation • glycerophospholipids • sphingolipids • cholesterol • VLDL • LDL • HDL • fast performance liquid chromatography

Major lipoprotein components beside free cholesterol (FC), cholesteryl esters (CE), and triglycerides are phospholipids, particularly phosphatidylcholine (PC) and sphingomyelin (SM). These lipid classes are not only important structural components, but also modulate the function of lipoproteins including their metabolism or activity of related enzymes. Moreover, PC and SM serve as precursors for a variety of regulatory molecules including lysophosphatidylcholine (LPC) (1, 2) and ceramide (CER) (3). The LCAT reaction in reconstituted HDL is inhibited by SM addition (4, 5), and CER has the ability to alter the substrate specificity of LCAT and favors the synthesis of unsaturated CE at the expense of saturated esters (5). An increased SM to PC ratio enhances the susceptibility of LDL to secretory sphingomyelinase, which leads to CER generation and the formation of aggregated LDL exhibiting a high atherogenic potential (6). In contrast, the antiatherogenic potential of HDL (i.e., the cholesterol uptake capacity) may be enhanced by an enrichment of PC and SM (7). Additionally, there is a relationship between HDL mediated cellular cholesterol efflux, phospholipid acyl chain length, and degree of unsaturation (8). Recently, LPC plasma levels were discussed as a biomarker in ovarian (9) and colorectal (10) cancer as well as sepsis (11). A disease-specific species pattern was also observed for CER (11) in sepsis patients. Taken together, lipid class composition as well as the species pattern of lipoprotein fractions may be important for lipoprotein function and may be altered in various disorders.

To gain insight into lipid class and species composition of lipoproteins, fractionation is required prior to analysis. Classical lipoprotein isolation techniques by ultracentrifugation (12) or preparative free-solution isotachophoresis (13) are too tedious for large studies and may alter the composition of lipoproteins (14, 15). In contrast, fast performance liquid chromatography (FPLC) offers rapid and

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Abbreviations: apoA-I, apolipoprotein A-I; CE, cholesteryl ester; CER, ceramide; ESI-MS/MS, electrospray ionization tandem mass spectrometry; FC, free cholesterol; FPLC, fast performance liquid chromatography; GC, gas chromatography; LPC, lysophosphatidylcholine; NBD, 7-nitrobenz-2-oxa-1,3-diazole; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PE-pl, PE-based plasmalogen; PL, total phospholipids; SM, sphingomyelin; TC, total cholesterol.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of ten figures and four tables.

reproducible separation of lipoproteins by size (16). This technique has been proven to be reproducible and reliable for cholesterol determination in lipoproteins and does not affect lipoprotein composition (17, 18).

In order to generate a comprehensive lipid pattern including species information from a small amount of serum, we analyzed FPLC-fractions using well-established methods for quantitative lipid analysis based on electrospray ionization tandem mass spectrometry (ESI-MS/MS) (19–22). Application of this sensitive and fast technique provides detailed cholesterol, glycerophospholipid, and sphingolipid composition of the separated lipoprotein fractions and may help to identify novel biomarkers in disorders of lipid and lipoprotein metabolism.

MATERIALS AND METHODS

Reagents

Methanol (HPLC grade) and chloroform (freshly purchased, analytical grade, stabilized with 0.6–1.0 % ethanol) were purchased from Merck (Darmstadt, Germany). Lipid standards for quantitative lipid mass spectrometry were obtained from Avanti Polar Lipids (Alabaster, AL) and Sigma (Deisenhofen, Germany) with purities higher than 99% as described previously (19–22). Ammonium acetate and acetyl chloride of the highest analytical grade available were purchased from Fluka (Buchs, Switzerland).

Characteristics of blood donors

Lipoprotein fractions were prepared from 21 healthy blood donors (Caucasians, 10 female, 11 male with mean age of 28 ± 6). All donors underwent a complete physical examination and laboratory medicine screening to exclude diseases especially related to infectious causes. Donors did not take any medication within 2 weeks before blood drawing. We received informed consent of all donors in written form. Serum lipoprotein levels analyzed by routine diagnostic assays were as follows: VLDL: 16 ± 14 [mg/dl]; LDL: 106 ± 21 [mg/dl]; HDL: 62 ± 12 [mg/dl] (mean ± SD; n = 21).

Lipoprotein separation by FPLC

VLDL, LDL, and HDL were isolated from serum of 21 healthy and overnight fasting human blood donors as previously described (18). In brief, a Pharmacia Smart System® FPLC equipped with a Superose 6 PC 3.2/30 column (GE Healthcare Europe GmbH, Munich, Germany) was used with Dulcobecco's PBS containing 1 mM EDTA as a running buffer. After loading 50 μl serum the system was run with a constant flow of 40 μl/min, and fractionation was started after 18 min with 80 μl per fraction. Fractions 1–20 containing the human serum lipoproteins were used for further analysis on a Cobas Integra 400 (Roche Diagnostic, Penzberg, Germany) to determine cholesterol and triglyceride levels of each fraction and for mass spectrometric analysis as described below. The cholesterol and triglyceride determination assays are standard enzymatic, colorimetric methods, which are also used in routine diagnostics.

Nondenaturing PAGE

Ten microliters of each FPLC fraction were mixed separately with 15 μl Optiprep™ (Axis-Shield, Oslo, Norway) and 7-nitrobenz-2-oxa-1,3-diazole (NBD)-CER, dissolved in 0.1 mg/ml ethylene glycol and 10% methanol. (NBD)-CER was shown to stain serum lipoproteins (23). Afterwards, 10 μl of the mixture of fraction 6–15, containing LDL, were separated on a 3–8% Tris-acetate polyacrylamide gradient gel (Ready Gels; Invitrogen, Karlsruhe, Germany) at 20 mA per gel for 18 h at 4°C. Ten microliters of the mixture of fraction 12–19, containing HDL particles were separated on 4–20% Tris-glycine polyacrylamide gradient gels (Ready Gels; Bio-Rad, Munich, Germany) at 20 mA per gel for 4 h at 4°C. Electrophoresis was carried out in 20 mM Tris/150 mM glycine buffer. PAGE-gels were scanned on a Typhoon fluorescence scanner (GE healthcare, Freiburg, Germany) with an excitation of 488 nm and emission of 520 nm to detect the fluorescence dye bound to the serum lipoproteins.

Denaturing SDS-PAGE

Five microliters of each FPLC fraction were mixed with 15 μl NuPAGE® LDS sample buffer (Invitrogen, Karlsruhe, Germany) and incubated for 10 min at 70°C in the presence of 50 mM DTT. Samples were run on 4–12% Bis-Tris gels (Ready Gels; Invitrogen, Karlsruhe, Germany) with NuPAGE MOPS SDS running buffer (Invitrogen, Karlsruhe, Germany) at 200 V per gel. Proteins separated within the gels were subsequently transferred to a PVDF membrane. Incubation with antibodies against apolipoprotein A-I (apoA-I) and albumin was performed in 1% nonfat dry milk in PBS and 0.1% Tween-20. The immune complexes were detected with an ECL plus (GE Healthcare, Freiburg, Germany). Primary rabbit anti-human apoA-I and anti-human albumin antibodies were purchased from Calbiochem (Darmstadt, Germany) and secondary peroxidase conjugated anti-rabbit antibody from Jackson Immuno Research (Hamburg, Germany).

Lipid mass spectrometry

FPLC fractions were extracted according to the method by Bligh and Dyer (24) in the presence of nonnaturally occurring lipid species used as internal standards (PC 14:0/14:0, PC 22:0/22:0, PE 14:0/14:0, PE 20:0/20:0 (di-phytanoyl), LPC 13:0, LPC 19:0, Cer 14:0, Cer 17:0, D_7-FC, CE 17:0 and CE 22:0).

Lipid species were quantified by ESI-MS/MS using methods validated and described previously (19–22). In brief, samples were analyzed by direct flow injection on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Manchester, UK) by direct-flow injection analysis using a HTS PAL autosampler (Zwingen, Switzerland) and an Agilent 1100 binary pump (Waldbronn, Germany) with a solvent mixture of methanol containing 10 mM ammonium acetate and chloroform (3:1, v/v). A flow gradient was performed starting with a flow of 55 μl/min for 6 s followed by 30 μl/min for 1.0 min and an increase to 250 μl/min for another 12 s.

A precursor ion scan of *m/z* 184 specific for phosphocholine containing lipids was used for PC, SM (21), and lysophosphatidylcholine (LPC) (20). A neutral loss scan of *m/z* 141 was used for phosphatidylethanolamine (PE) (25), and PE-based plasmalogens (PE-pl) were analyzed according to the principles described by Zemski-Berry (26). In brief, fragment ions of *m/z* 364, 380, and 382 were used for PE p16:0, p18:1, and p18:0 species, respectively. CER was analyzed similar to a previously described methodology (19) using N-heptadecanoyl-sphingosine as internal standard. Free cholesterol (FC) and CE species were determined after selective acetylation of FC (22).

Quantification was achieved by calibration lines generated by addition of naturally occurring lipid species (19-22) to plasma (extraction of 20 μl 5-fold diluted plasma for single FPLC fractions or 20 μl undiluted plasma for pooled lipoprotein fractions). All lipid classes were quantified with internal standards belonging to the same lipid class, except SM (PC internal standards) and PE-pl (PE internal standards). Calibration lines were generated for the following naturally occurring species: PC 34:1, 36:2,

38:4, 40:0, and PC O 16:0/20:4; SM 34:2, 36:2, 36:1; LPC 16:0, 18:1, 18:0; PE 34:1, 36:2, 38:4, 40:6; and PE p16:0/20:4; Cer 16:0, 18:0, 20:0, 24:1, 24:0; FC, CE 16:0, 18:2, 18:1, 18:0. These calibration lines were also applied for not calibrated species, as follows: concentrations of saturated, monounsaturated, and polyunsaturated species were calculated using the closest related saturated, monounsaturated, and polyunsaturated calibration line slope, respectively. For example PE 36:2 calibration was used for PE 36:1, PE 36:3, and PE 36:4; PE 38:4 calibration was used for PE 38:3, PE 38:5, and so on. Ether-PC species were calibrated using PC O 16:0/20:4 and PE-pl were quantified independent from the length of the ether linked alkyl chain using PE p16:0/20:4.

Correction of isotopic overlap of lipid species as well as data analysis was preformed by self programmed Excel macros for all lipid classes according to the principles described previously (21).

Preparation of lipoproteins by ultracentrifugation

Lipoprotein fractions were isolated from sera of individual normo-lipidemic volunteers by sequential ultracentrifugation as described previously (27).

Statistical analysis for mass spectrometry results

Statistical analysis was performed with SPSS© (SPSS Inc., Chicago, IL). We used a Wilcoxon signed-rank test as a nonparametric alternative to a paired Student's *t*-test (n = 21 donors).

RESULTS

Validation of the lipoprotein separation

We prepared 20 fractions from 50 μl serum of a healthy blood donor by FPLC-size exclusion chromatography similar to the method of Innis-Whitehouse et al. (18). In order to check whether the major lipoprotein classes were properly separated, total cholesterol (TC) and triglyceride concentrations were analyzed by routine clinical chemistry assays (**Fig. 1A**). As shown previously (18), three major peaks were found, representing the lipoprotein classes VLDL, LDL, and HDL, respectively. From the same fractions we quantified PC, SM, lysophosphatidylcholine (LPC), CER, PE, PE-pl, FC, and CE including their fatty acid species by previously published tandem mass spectrometric assays (19–22). As expected, most of these lipids revealed a distribution representing the three main lipoprotein classes (Fig. 1B).

However, 60% of total LPC were found in fractions 14–17 (Fig. 1B). Because it is known that LPC can bind to albumin (28), this fraction shift may represent LPC bound albumin. To identify the albumin containing fractions, FPLC fractions were analyzed by nondenaturing PAGE and SDS-PAGE. Corresponding to the main LPC fractions 14 to 17, we found a protein band at a MW of 66 kDa identified as albumin (**Fig. 2C**). Another question was whether other lipids than LPC coelute with albumin containing fractions. Therefore, we subjected a HDL-deficient serum of a Tangier patient (29, 30) to our method. Consistent with the HDL deficiency, fractions 13 to 17 contained only traces of PC, SM, CER, PE, PE-pl, and cholesterol, but more than 50% of LPC (Fig. 1C). In summary, we could show a clear overlap of albumin and LPC containing fractions, which is in accordance with previous studies (28, 31). Additionally, we could conclude from the analysis of HDL deficient serum that only LPC but none of the other lipid classes analyzed coelutes significantly with albumin containing fractions.

We also tested whether the applied FPLC separation had the ability to separate lipoprotein subclasses. For that purpose, we subjected LDL and HDL containing fractions of a serum sample to nondenaturing PAGE to identify the actual size of the lipoproteins, as shown before (32). Prior to PAGE we stained the lipoproteins with NBD-CER (23). In accordance with the principle of FPLC separation, a difference in particle size between fractions was observed for LDL (**Fig. 3**) and HDL (Fig. 2). Although, no clear separation of these LDL subfractions was observed, fraction 8 contained an increased content of large LDL particles (Fig. 3, band a) and fraction 10 an increased content of small dense LDL (Fig. 3, band b). In contrast to LDL, HDL showed a continuous size gradient (Fig. 2A).

These size gradients could be useful especially for the analysis of abnormal lipoprotein compositions. Because we could not separate defined lipoprotein subclasses, we decided to determine in a first step a detailed lipid species pattern of the major lipoprotein classes VLDL (fractions 3 to 6), LDL (fractions 7 to 11), and HDL (fractions 12 to 17) by fraction pooling. A prerequisite for fraction pooling is a reproducible lipoprotein fractionation. For that reason, we separated serum of a healthy donor in three independent runs. Analysis of the total cholesterol concentration (TC) showed very low variation between the runs (see supplementary Fig. I). Although these data indicate a reproducible separation and fraction pooling, TC profiles were measured for another 10 runs and afterwards in every third run to control the FPLC separation of lipoprotein classes. No shift in the lipoprotein separation has been observed (data not shown).

Validation of mass spectrometric analysis

To show, that the previously validated assays (19–22) were applicable for the mass spectrometric analysis of lipoprotein fractions, a number of experiments were performed. Pooled fractions of three independent FPLC runs were analyzed by ESI-MS/MS and coefficients of variation below 6% (except PE-pl in VLDL, which was close to the limit of quantification) were found for the lipid class composition of VLDL, LDL, and HDL (see supplementary Table I). Additionally, we compared the results of the analysis of single fractions and pooled fractions. Summation of single fractions was in good accordance with the analysis of pooled fractions (see supplementary Table II).

Because ESI-MS/MS analysis may be affected by the individual lipid content of different samples materials, the species response in different lipoprotein classes was analyzed. To provide sufficient material for calibration lines, LDL, HDL_2, and HDL_3 were prepared by ultracentrifugation. The response variation was below 10% (CV) for a number of PC, SM, and PE species in LDL, HDL_2, and HDL_3 of two different donors (see supplementary Table III). In contrast to PC, PE species showed a strong dependence

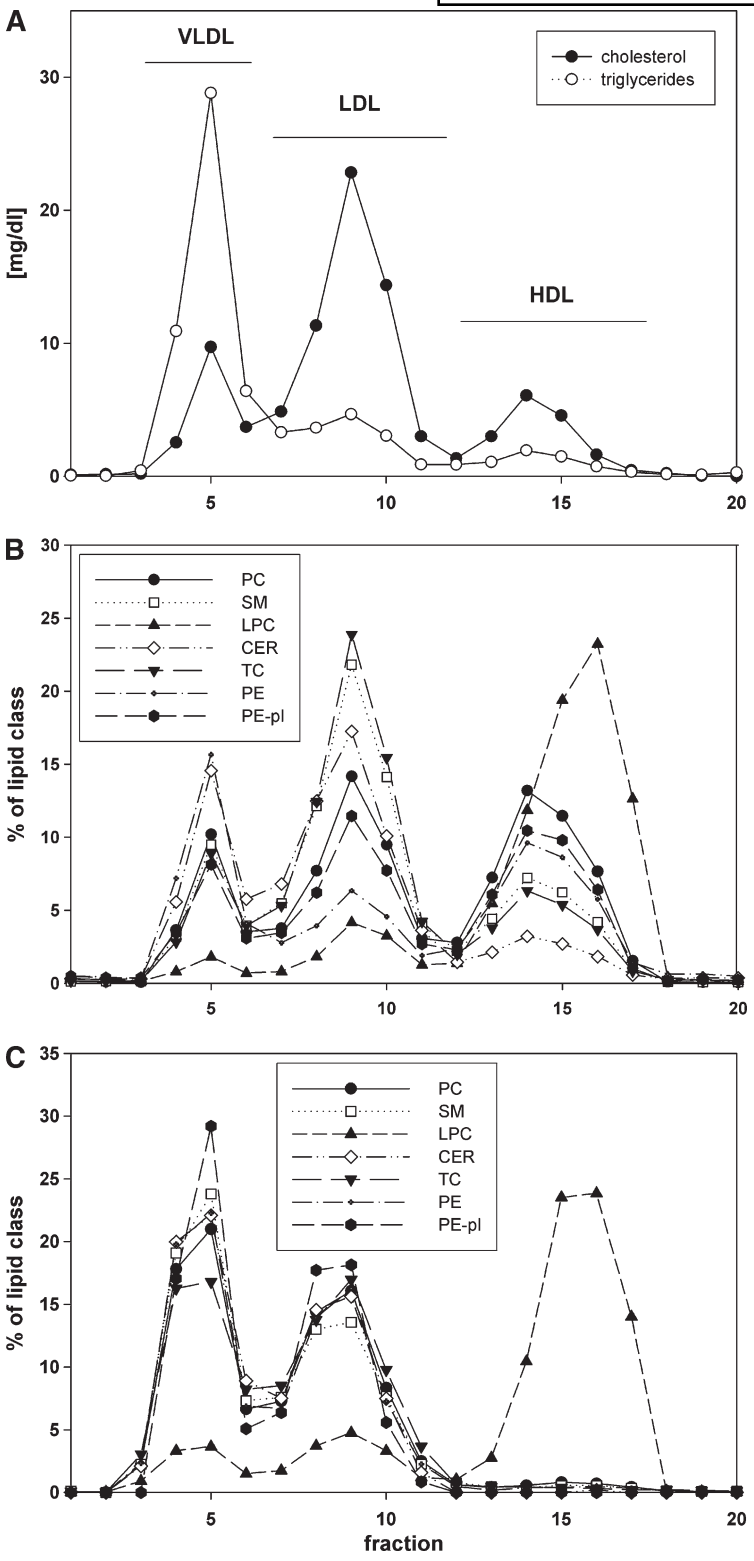

Fig. 1. A: Total cholesterol (TC) and triglyceride level of fast performance liquid chromatography (FPLC) fractions. The graph shows cholesterol and triglyceride concentrations in mg/dl of FPLC fractions from human serum determined by routine enzymatic assays. Fractions 3 to 6 contain VLDL, fractions 7 to 11 contain LDL, and fractions 12 to 17 contain HDL. B: Glycerophospholipid and sphingolipid profile of FPLC fractions. Glycerophospholipids and sphingolipids were quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) from the fractions shown in A. Displayed are percentage of each fraction related to total lipid class concentration for phosphatidylcholine (PC), sphingomyelin (SM), lysophosphatidylcholine (LPC), ceramide (CER), phosphatidylethanolamine (PE), PE-based plasmalogen (PE-pl), and TC, respectively. C: Glycerophospholipid and sphingolipid profile of a HDL-deficient serum as described in B. The glycerophospholipid and sphingolipid concentrations were determined from serum of a Tangier disease patient, an ABCA1 deficiency syndrome resulting in HDL deficiency (30).

on chain length and number of double bonds. Consequently, it is necessary to use different species per lipid class for calibration to ensure an accurate quantification (see Materials and Methods). A low variation between different lipoprotein fractions permits the use of one calibration for different lipoprotein fractions. Finally, species profiles of undiluted and 5-fold diluted samples strongly resembled each other (for an example, see supplementary Fig. VIII–X). In summary, these data clearly indicate that the previously validated methods are also applicable for lipoprotein fractions.

Furthermore, the use of two internal standards for each lipid class permits a quality control for each sample. As internal standards were added in a constant ratio, an

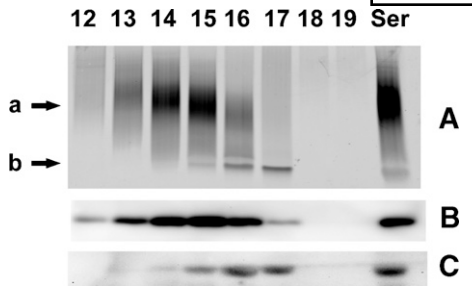

Fig. 2. Native and SDS-PAGE of HDL containing fractions. A: After prelabeling with NBD-CER FPLC fractions 12–19 (fractions 12–17 contain HDL) as well as an unseparated serum were analyzed by native PAGE and subsequently visualized by fluorescence scanning. The broad band (a) represents HDL particles and band (b) indicates the NBD-Cer bound to albumin (51). SDS-PAGE and Western blot against apolipoprotein A-I (apoA-I) of fractions 12–19 and original serum with subsequent Western blotting against apoA-I (B) and albumin (C).

abnormal internal standard ratio may indicate a disturbed species response (see also supplementary Table IV).

Glycerophospholipid and sphingolipid class distribution across lipoprotein classes

We analyzed the pooled lipoprotein fractions of 21 fasting healthy blood donors. First, we calculated the distribution of the lipid classes across the main lipoprotein classes (**Table 1**). As expected, approximately 60% of PC and 40% of SM were found in HDL, whereas LDL carried 50% of lipoprotein SM and 60% of CER, respectively. Moreover, the HDL fraction contained 60% of PE and PE-pl. As shown above, this FPLC separation does not provide a complete separation of albumin and HDL; therefore albumin-bound LPC was included into the HDL fraction. Whereas LDL and HDL revealed low variations in their lipid class percentages between the different donors, VLDL displayed high variations most probably due to its low concentration and dependency of the fasting status of the blood donor.

Lipid composition of lipoprotein classes

To evaluate the glycerophospho- and sphingolipid composition in more detail, we determined the lipid composition of each lipoprotein class either including FC and CE (**Fig. 4A**) or without cholesterol (Fig. 4B). LDL revealed the highest CE content with approximately 55%, whereas VLDL and HDL had a 20% lower CE fraction. Overall,

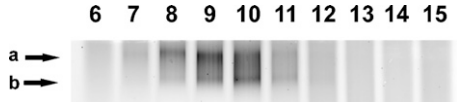

Fig. 3. Native PAGE of LDL containing fractions. After prelabeling with NBD-CER the LDL containing fractions 7–11 (fractions 6 and 12–15 are included as control) were analyzed by native poly-acrylamide gel electrophoresis (PAGE) and subsequently visualized by fluorescence scanning. The two main bands (a) and (b) represent the sizes extremes. (a) indicates large LDL particles, whereas (b) indicates small LDL particles.

TABLE 1. Distribution of lipid classes on major lipoprotein classes

	Conc [μM]	VLDL [%]	LDL [%]	HDL [%]
PC	1986 ± 727	8.1 ± 6.4	29.9 ± 6.6	62 ± 10.3
SM	415 ± 141	7.2 ± 6.8	50.4 ± 7.5	42.6 ± 10.7
LPC	330 ± 168	1.7 ± 1.5	11 ± 4.5	87.3 ± 5.4[a]
PE	35.6 ± 20.8	18.6 ± 9.4	21.3 ± 5.6	60.1 ± 11.4
PE-pl	31.9 ± 13.9	11.6 ± 5.3	28.5 ± 6.5	59.9 ± 8.8
CER	8.1 ± 3.4	15.6 ± 9.9	60.3 ± 6.7	24.1 ± 7.4

PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PE-pl, PE-based plasmalogen; CER, ceramide. Major lipoprotein classes were pooled from fast performance liquid chromatography (FPLC) fractions as indicated in Fig. 1A. Displayed are total serum concentrations (Conc) of the lipid classes in μM and their distribution on the respective lipoprotein class in percent (mean ± SD, n = 21 different donors).

[a] The HDL fraction also contains LPC bound to albumin.

HDL showed a phospholipid to cholesterol ratio of 1.09, which was significantly higher than in VLDL (0.64) and LDL (0.35) ($P < 0.001$). In relation to the sum of all analyzed glycerophospholipids and sphingolipids, PC was by far the most abundant lipid class ranging from 65 to 74% (Fig. 4B). Compared with VLDL (14%) and HDL (10%), LDL displayed a high content of SM (25%) ($P < 0.001$). Moreover, LDL and VLDL (both approximately 0.6%) had a more than 5-fold higher content of CER than HDL (0.1%) ($P < 0.001$). PE and PE-pl had almost equal amounts within one lipoprotein class, but about a three-times higher content was observed in VLDL (3%) compared with LDL (1%) and HDL (1%) ($P < 0.001$). Due to a significant contribution of albumin-bound LPC to the HDL fractions, lipid class composition was additionally calculated without LPC (see supplementary Fig. II).

Lipid species pattern of lipoprotein classes

Biological function of lipoproteins may not only be related to lipid class composition but also to their lipid molecular species pattern. Therefore, we calculated the proportion of each species related to the total concentration of the lipid class in each lipoprotein fraction (**Fig. 5**). The performed mass spectrometric analysis only allows the determination of the total number of carbon atoms and double bonds in the fatty acid moiety for lipid classes containing two fatty acids esterified to the glycerol backbone (PC, PE). For example a PC 34:1 may represent different combination of fatty acids such as 18:0/16:1, 16:0/18:1, etc. Moreover, the assignment to a bond type (acyl or ether) is based on the assumption that fatty acids with odd-numbered carbon-atoms constitute a negligible fraction. For PE-pl, mass spectrometric analysis allows to differentiate ether and fatty acid linked to the glycerol backbone. SM species assignment includes sphingoid base and N-linked fatty acid, because plasma SM contains beside sphingosine d18:1 (62%), other sphingoid bases, mainly sphingadienine d18:2 (14%) and hexadecasphingosine d16:1 (10%), in considerable amounts (33). Lysophospholipids (LPC), CER, and CE contain one fatty acid denominated by the species nomenclature.

The proportion of the major PC species did not show a huge variation between the lipoprotein classes (Fig. 5A; see

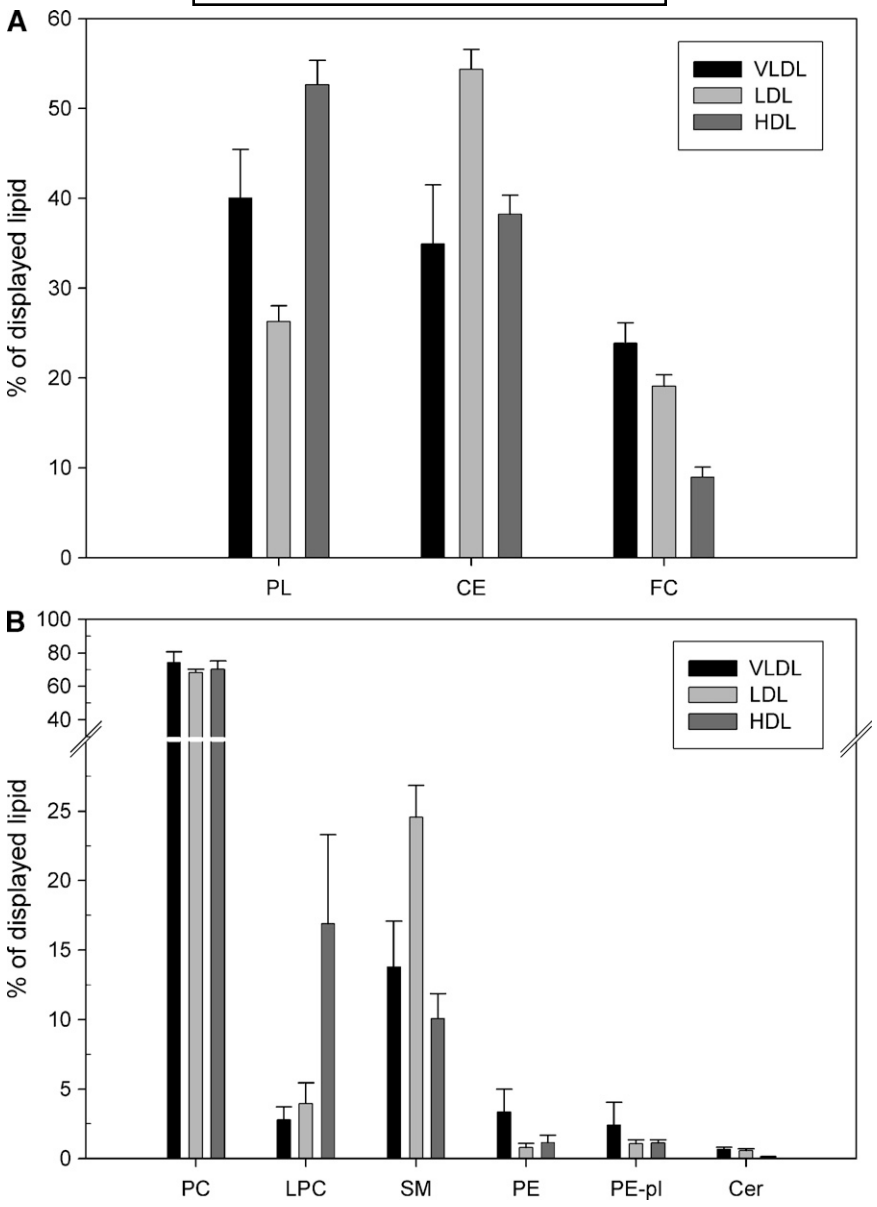

Fig. 4. Lipid composition of pooled lipoprotein classes. Displayed are % mol of the lipid classes related to the lipid content of the respective lipoprotein class (mean ± SD, n = 21 different donors). In A, free cholesterol (FC) and cholesteryl ester (CE) were included into the calculation; phospholipids were displayed as the sum of PC, SM, LPC, CER, PE, and PE-pl [total phospholipids (PL)]. B shows % mol related to the sum of the displayed lipid classes. The lipoprotein classes were pooled as indicated in Fig. 1.

supplementary Fig. IV). Comparison of LDL and HDL revealed a systematic difference between highly unsaturated (three and more double bonds) and mono-/diunsaturated PC species. Thus, HDL exhibited a higher proportion of polyunsaturated species (35 ± 3% vs. 28 ± 4%, $P < 0.001$) and lower fraction of mono-/diunsaturated PC species (54 ± 4% vs. 58 ± 4%, $P < 0.001$) compared with LDL. This difference was most remarkable for PC 36:4 with 11.1 ± 1.2% and 8.5 ± 1.5% for HDL and LDL, respectively ($P < 0.001$).

In contrast to PC, LPC showed a pronounced lipoprotein specific lipid species pattern (Fig. 5A; see supplementary Fig. V). Remarkable differences were found in saturated LPC 16:0 (VLDL 45 ± 6%; LDL 51 ± 6%; HDL/albumin 59 ± 5%; $P < 0.001$), LPC 18:0 (VLDL 32 ± 6%, LDL 32 ± 7%, HDL/albumin 17 ± 2%; $P < 0.001$, difference VLDL and LDL not significant) and unsaturated LPC 18:2 (VLDL 7.4 ± 3%, LDL 5.0 ± 2%; HDL/albumin 11.4 ± 3%; $P < 0.001$).

The major SM species (Fig. 5B; see supplementary Fig. IV) in all three lipoprotein classes was SM 34:1 with a slightly decreased proportion in HDL compared with VLDL and LDL (VLDL 30 ± 3%; LDL 29 ± 3%; HDL 25 ± 1%, $P < 0.001$). Although not very pronounced, most SM species exhibited a lipoprotein specific distribution. Differences in minor species could be of particular interest like SM 38:1, which showed an approximately 5-fold increased proportion in LDL compared with HDL (LDL 2.4 ± 0.4%; HDL 0.5 ± 0.5%; $P < 0.001$).

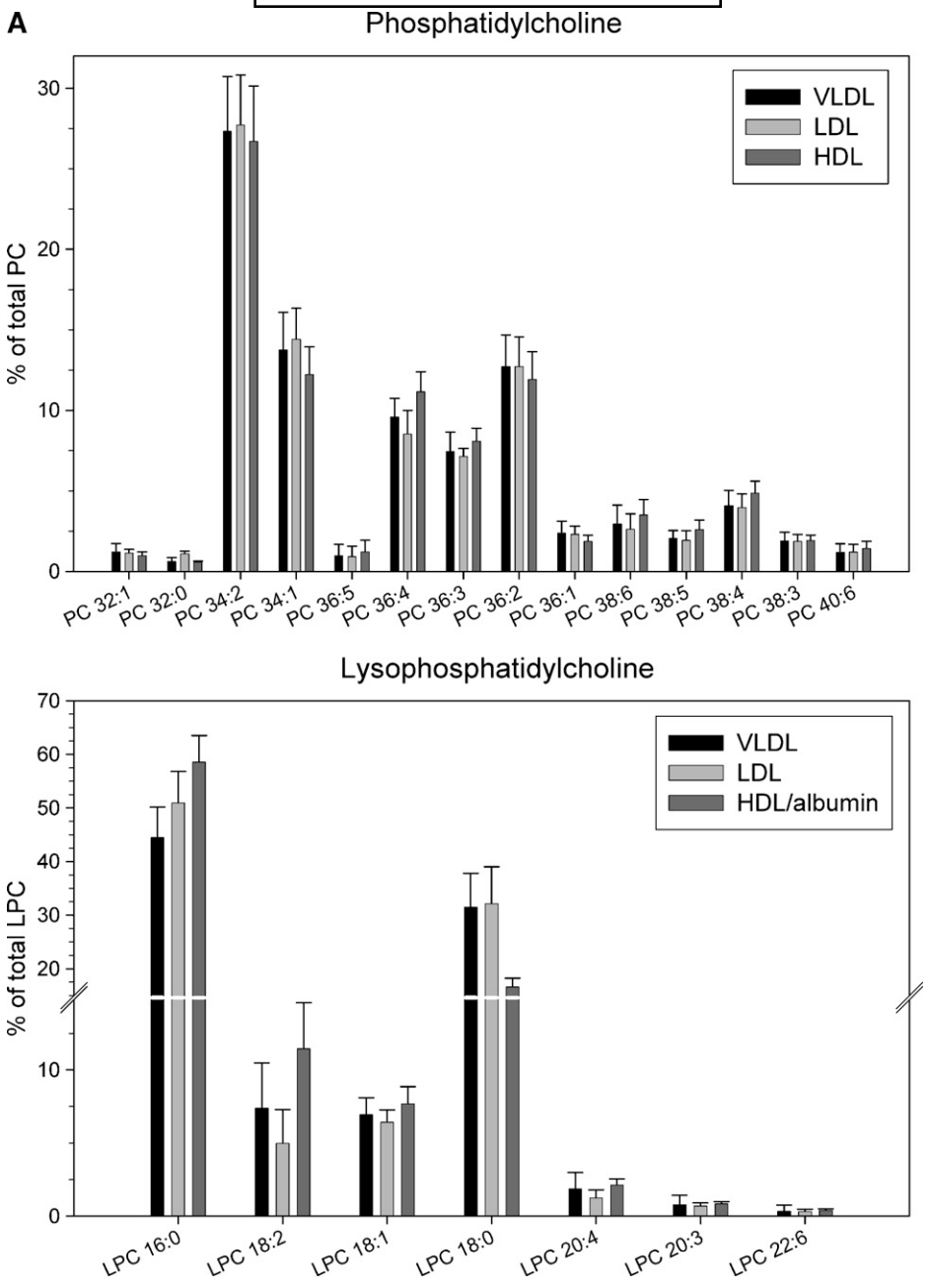

Fig. 5. Lipid species pattern of lipoprotein classes. Lipid species pattern were calculated as % mol for each species related to the total lipid class concentration in a lipoprotein class (mean ± SD, n = 21 different donors). The lipoprotein classes are pooled as indicated in Fig. 1. Only peaks above 0.5% were displayed. The species assignment for glycerophospholipids includes both glycerol-linked fatty acids (e.g., PC 36:3 = 36 carbon atoms and 3 double bonds, representing combinations like 18:0/18:3, 18:1/18:2, etc.). SM species assignment comprises sphingoid base and amide-linked fatty acid. Species pattern is displayed for phosphatidylcholine, lysophosphatidylcholine (A), sphingomyelin, ceramide (B) and phosphatidylethanolamine, PE-based plasmalogens (C).

The CER species pattern (Fig. 5B) was similar for VLDL and LDL, whereas HDL differed significantly. Thus, the percentage of CER 16:0 was almost doubled (VLDL 7.4 ± 2.0%; LDL 8.4 ± 2.0%; HDL 15 ± 2%, $P < 0.001$) and the proportion of CER 24:0 was found more than 10% reduced (VLDL 43 ± 4%; LDL 46 ± 3%; HDL 31 ± 2%; $P < 0.001$) in HDL compared with VLDL and LDL, respectively.

PE and PE-pl revealed a relative high variation between the blood donors (Fig. 5C; see supplementary Fig. VI). Interestingly, HDL contained a higher fraction of PE-pl containing 20:4 with 40% compared with 33% and 27% for VLDL and LDL, respectively ($P < 0.001$). This was accompanied by a reduced proportion of PE-pl containing 18:1 and 18:2 (VLDL 24%, LDL 27%, HDL 27%; difference

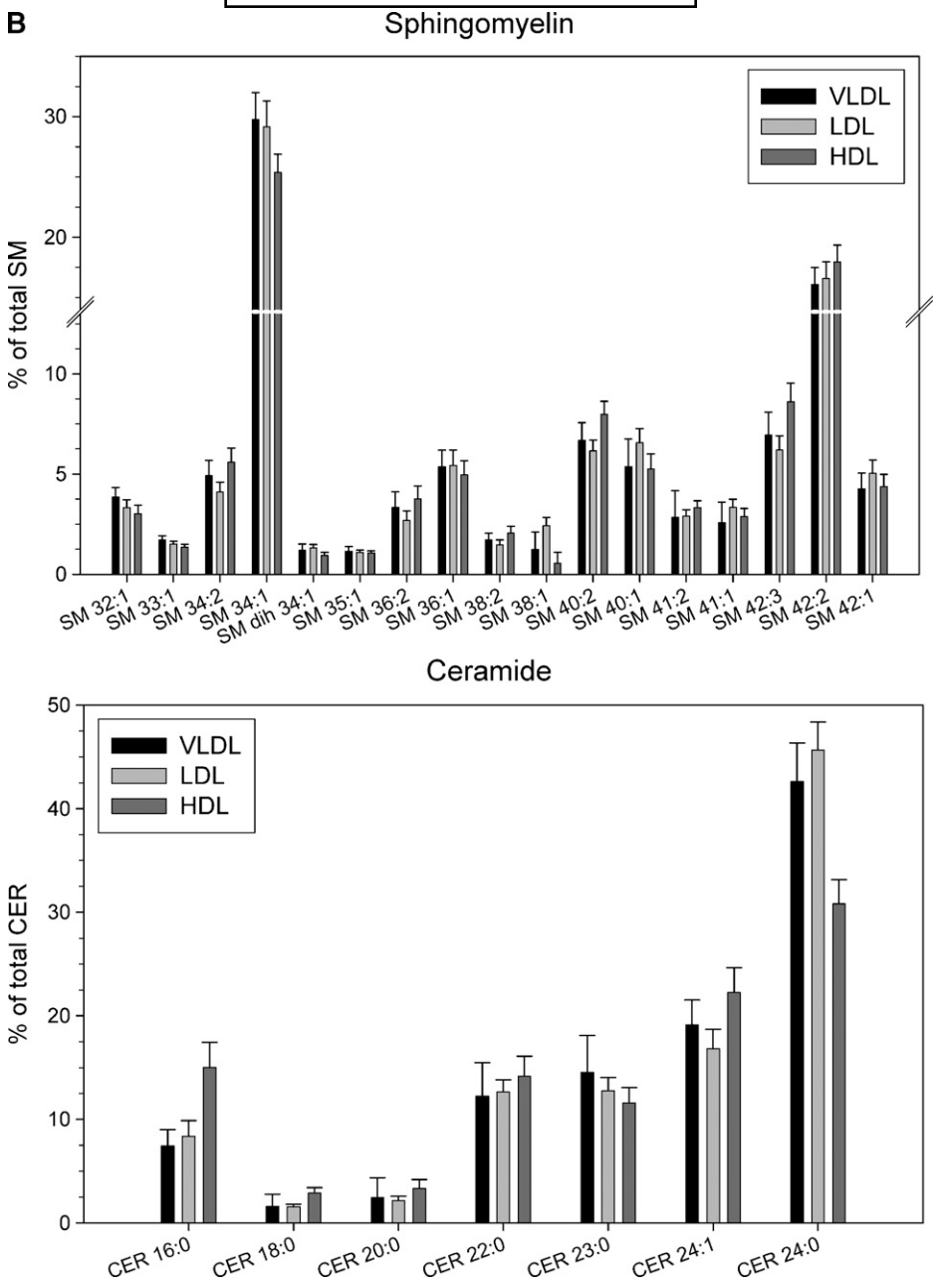

Fig. 5.—*Continued.*

VLDL to HDL and LDL significant, $P < 0.001$). Analysis of CE species pattern showed no major differences between lipoprotein fractions (see supplementary Figs. III and VII).

In summary, we could demonstrate that the main lipoprotein fractions VLDL, LDL, and HDL are characterized by specific lipid class and lipid species pattern.

DISCUSSION

A number of studies have shown that lipoprotein function may be modulated by glycerophospholipids and sphingolipids (4–8). Recently, a variety of lipid species were discovered as novel biomarkers in different diseases (9–11). So far, most studies used unseparated plasma or serum samples. Due to an overlap of lipoprotein classes, this may not allow the identification of lipid species involved in the regulation of specific lipoprotein functions. The chance to discover lipid species suitable as biomarkers may be improved considerably by lipoprotein fractionation. Consequently, the aim of this study was to present a simple method for lipoprotein fractionation combined with lipid species analysis.

We could show that lipoprotein separation by FPLC was reproducible to allow pooling of VLDL, LDL, and HDL as a necessary step to reduce mass spectrometric analysis time. Analysis of HDL deficient serum revealed that beside

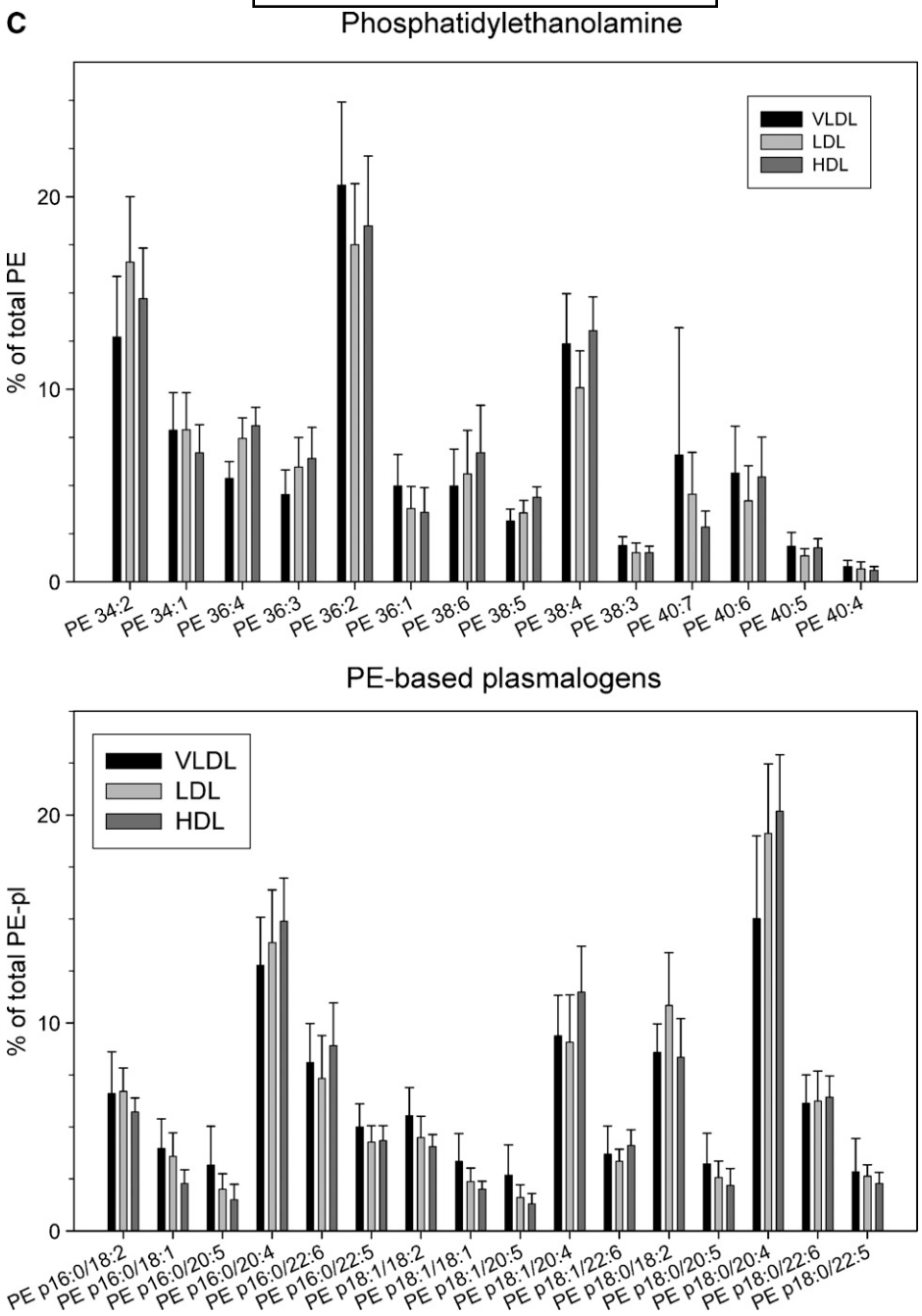

Fig. 5.—*Continued.*

LPC (28, 31), none of the other analyzed lipid classes bind to albumin substantially (Fig. 1C). However, data interpretation should keep in mind that albumin-bound LPC, but none of the other analyzed lipids, overlaps with HDL fractions prepared by FPLC (Fig. 2). Similarly, other lipoprotein separation techniques like density gradient centrifugation may suffer from albumin contamination in HDL, as shown in a recent study by Yee et al. (34) using an iodoxanol gradient.

Additionally, particle-size gradients were observed for HDL and LDL, which provide a basis for the analysis of lipid species composition of enriched lipoprotein subclasses from single FPLC fractions. Lipoprotein subclasses may exhibit a high pathophysiological relevance, as shown for small HDL and small dense LDL particles and their association with myocardial infarction (35) and the metabolic syndrome (36), respectively. Taken together, this approach could help to unravel lipid profiles of lipoprotein subclasses with clinical relevance.

In our study, we presented for the first time a comprehensive, quantitative glycerophospholipid and sphingolipid species profile of VLDL, LDL, and HDL from 21 fasting healthy blood donors. Comparison to previous studies using TLC and gas chromatography (GC) to analyze lipoproteins prepared by ultracentrifugation showed a good correlation of lipid class proportions with our data.

Kuksis et al. (37) (% weight were converted to % mol using an average molecular weight) determined for VLDL a proportion of 41% total phospholipids (PL), 35% CE and 24% FC (present study: 40% PL, 35% CE, and 24% FC); for LDL a proportion of 28% PL, 53% CE, and 19% FC (present study: 26% PL, 54% CE, and 19% FC); for HDL a proportion of 47% PL, 42% CE, and 11% FC (present study: 48% PL without LPC, 42% CE, 10% FC). Skipski et al. (38) analyzed lipoprotein classes by TLC and reported the following PL proportions (% mol related to analyzed PL): LDL contained 67% PC, 27% SM, 2.8% LPC, and 2.3% PE (present study: 68% PC, 25% SM, 4.0% LPC and 1.9% PE including plasmalogens); HDL (calculation without LPC) contained 82% PC, 14% SM and 3.3% PE (present study: 85% PC, 12% SM, and 2.7% PE including plasmalogens). Myher et al. determined species profiles of the lipoprotein classes by TLC separation, scraping and GC-MS (39). Profiles of this study were comparable to our data, especially the study group on saturated fat diet showed a good agreement to our data. Major species of both studies revealed similar tendencies between lipoprotein classes: PC 34:2 (VLDL 32%, LDL 31%, HDL_3 28%; our study VLDL 27%, LDL 28%, HDL 27%), PC 36:2 (VLDL 15%, LDL 19%, $HDL_3$17%; our study VLDL 13%, LDL 13%, HDL 12%), PC 36:4 (VLDL 11%, LDL 10%, HDL_3 13%; our study VLDL 10%, LDL 9%, HDL 11%), and PC 34:1 (VLDL 11%, LDL 10%, HDL_3 9%; our study VLDL 14%, LDL 14%, HDL 12%). Comparable to our study, Myher et al. (39) found a increased proportion of polyunsaturated PC in the HDL fraction, for instance PC 36:4 (see above) and PC 38:4 (VLDL 5.8%, LDL 6.5%, HDL 8.7%; our study VLDL 4.1%, LDL 4.0%, HDL 4.8%).

The SM profiles observed by Myher et al. (39) showed a higher discrepancy to the present study especially for HDL. The major species SM 34 (VLDL 33%, LDL 38%, HDL_3 20%; our study VLDL 36%, LDL 35%, HDL 32%), SM 42 (VLDL 25%, LDL 26%, HDL_3 37%; our study VLDL 27%, LDL 28%, HDL 31%) and SM 40 (VLDL 11%, LDL 11%, HDL_3 15%; our study VLDL 12%, LDL 13%, HDL 13%) were in good agreement for VLDL and LDL, but not for HDL. One reason for this discrepancy may be that Myher et al. (39) investigated HDL_3, and our data comprise the whole HDL fraction including other HDL subclasses like HDL_2. Another explanation for differences to other studies may be related to diet, which has a pronounced effect on PC species profiles (39). In general, we observed a good agreement of previous studies with data generated by FPLC fractionation and ESI-MS/MS, which strongly supports the validity of the presented method for lipid class and species analysis of lipoproteins.

One novel finding is that LDL is the main carrier of CER in fasting condition (Table 1). Another study by Lightle et al. (40) using ultracentrifugation followed by a TLC/HPLC reported a high contribution of VLDL to total plasma CER level. However, it is not mentioned, whether fasting or nonfasting blood donors were investigated. Therefore, we analyzed two blood donors under nonfasting conditions with higher VLDL concentrations (40 mg/dl cholesterol) and calculated the CER distribution across the lipoprotein classes like described in Table 1. Under these conditions, VLDL contains 38 ± 5%, LDL 48 ± 2%, and HDL 14 ± 3% of total plasma CER, which closely resembles the results found by Lightle et al. (40) (VLDL 42%, LDL 38%, HDL 15%, LPDS 5%). LDL as the main carrier of CER fits well to data describing plasma CER level as a risk factor for atherosclerosis (41). Furthermore, half of serum SM is located in the LDL fraction (Table 1), which may be reflected in a positive correlation of plasma SM level to coronary heart disease (42, 43).

Pathophysiological relevance of CER may arise from a 10–50-fold higher CER content of lesional LDL compared with plasma LDL (44) and a CER binding to the LPS receptor CD14, which induces clustering of coreceptors in membrane microdomains (45). Whole plasma concentrations of coronary artery disease and stroke patients showed an increased concentration of CER 24:0 compared with controls without considerable changes of other CER species (45). Interestingly, the major CER species of LDL from healthy blood donors was CER 24:0 (Fig. 5), which may indicate high LDL and low HDL level in CAD and stroke patients. In a further study, we compared control with sepsis patients and found CER 23:0 and 24:0 decreased, whereas CER 16:0 and 24:1 increased in sepsis patients (11). Comparison of the CER species profiles of main lipoprotein fractions (Fig. 5B) with alterations observed in sepsis patients suggests that sepsis patients exhibit not only an imbalance of major lipoprotein fractions, but also major changes in CER species profiles of lipoprotein classes. This hypothesis is supported also by the finding that CER elevation during sepsis is mainly related to an increase in VLDL and LDL associated CER (40). Further evidence for this assumption comes from a study showing an increased PC/SM ratio and more saturated and less polyunsaturated PC species in acute phase compared with control HDL (46).

These studies clearly indicate the potential of the presented method to contribute with a lipoprotein-specific lipid classes and species pattern to the understanding of lipid related pathophysiology in diseases like sepsis. Combining lipoprotein separation by FPLC (18) with sensitive high throughput assays for quantitative lipid species analysis (19–22), allows for the first time a detailed lipid species pattern of lipoprotein fractions from a small sample volume of 50 μl serum with a reasonable analytical effort. Altogether, this approach is applicable for routine analysis and large patient studies. Furthermore, FPLC fractions could be analyzed by other lipid mass spectrometric approaches (47, 48), which may provide even more detailed lipid species pattern (49, 50). In conclusion, the presented method for lipoprotein fractionation followed by high throughput lipid profiling may be a useful tool for research on lipoprotein metabolism, diagnostics of lipid related disorders, and biomarker search.

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REFERENCES

1. Meyer zu, H. D., and K. H. Jakobs. 2007. Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism. *Biochim. Biophys. Acta.* **1768:** 923–940.
2. Mueller, R. B., A. Sheriff, U. S. Gaipl, S. Wesselborg, and K. Lauber. 2007. Attraction of phagocytes by apoptotic cells is mediated by lysophosphatidylcholine. *Autoimmunity.* **40:** 342–344.
3. Tani, M., M. Ito, and Y. Igarashi. 2007. Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. *Cell. Signal.* **19:** 229–237.
4. Bolin, D. J., and A. Jonas. 1996. Sphingomyelin inhibits the lecithin-cholesterol acyltransferase reaction with reconstituted high density lipoproteins by decreasing enzyme binding. *J. Biol. Chem.* **271:** 19152–19158.
5. Subbaiah, P. V., P. Horvath, and S. B. Achar. 2006. Regulation of the activity and fatty acid specificity of lecithin-cholesterol acyltransferase by sphingomyelin and its metabolites, ceramide and ceramide phosphate. *Biochemistry.* **45:** 5029–5038.
6. Schissel, S. L., X. Jiang, J. Tweedie-Hardman, T. Jeong, E. H. Camejo, J. Najib, J. H. Rapp, K. J. Williams, and I. Tabas. 1998. Secretory sphingomyelinase, a product of the acid sphingomyelinase gene, can hydrolyze atherogenic lipoproteins at neutral pH. Implications for atherosclerotic lesion development. *J. Biol. Chem.* **273:** 2738–2746.
7. Yancey, P. G., L.-M. de, S. Swarnakar, P. Monzo, S. M. Klein, M. A. Connelly, W. J. Johnson, D. L. Williams, and G. H. Rothblat. 2000. High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. *J. Biol. Chem.* **275:** 36596–36604.
8. Davidson, W. S., K. L. Gillotte, S. Lund-Katz, W. J. Johnson, G. H. Rothblat, and M. C. Phillips. 1995. The effect of high density lipoprotein phospholipid acyl chain composition on the efflux of cellular free cholesterol. *J. Biol. Chem.* **270:** 5882–5890.
9. Okita, M., D. C. Gaudette, G. B. Mills, and B. J. Holub. 1997. Elevated levels and altered fatty acid composition of plasma lysophosphatidylcholine(lysoPC) in ovarian cancer patients. *Int. J. Cancer.* **71:** 31–34.
10. Zhao, Z., Y. Xiao, P. Elson, H. Tan, S. J. Plummer, M. Berk, P. P. Aung, I. C. Lavery, J. P. Achkar, L. Li, et al. 2007. Plasma lysophosphatidylcholine levels: potential biomarkers for colorectal cancer. *J. Clin. Oncol.* **25:** 2696–2701.
11. Drobnik, W., G. Liebisch, F. X. Audebert, D. Frohlich, T. Gluck, P. Vogel, G. Rothe, and G. Schmitz. 2003. Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients. *J. Lipid Res.* **44:** 754–761.
12. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
13. Bottcher, A., J. Schlosser, F. Kronenberg, H. Dieplinger, G. Knipping, K. J. Lackner, and G. Schmitz. 2000. Preparative free-solution isotachophoresis for separation of human plasma lipoproteins: apolipoprotein and lipid composition of HDL subfractions. *J. Lipid Res.* **41:** 905–915.
14. Herbert, P. N., T. M. Forte, R. S. Shulman, M. J. La Piana, E. L. Gong, R. I. Levy, D. S. Fredrickson, and A. V. Nichols. 1975. Structural and compositional changes attending the ultracentrifugation of very low density lipoproteins. *Prep. Biochem.* **5:** 93–129.
15. Kunitake, S. T., and J. P. Kane. 1982. Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. *J. Lipid Res.* **23:** 936–940.
16. Ha, Y. C., and P. J. Barter. 1985. Rapid separation of plasma lipoproteins by gel permeation chromatography on agarose gel Superose 6B. *J. Chromatogr.* **341:** 154–159.
17. Marz, W., R. Siekmeier, H. Scharnagl, U. B. Seiffert, and W. Gross. 1993. Fast lipoprotein chromatography: new method of analysis for plasma lipoproteins. *Clin. Chem.* **39:** 2276–2281.
18. Innis-Whitehouse, W., X. Li, W. V. Brown, and N. A. Le. 1998. An efficient chromatographic system for lipoprotein fractionation using whole plasma. *J. Lipid Res.* **39:** 679–690.
19. Liebisch, G., W. Drobnik, M. Reil, B. Trumbach, R. Arnecke, B. Olgemoller, A. Roscher, and G. Schmitz. 1999. Quantitative measurement of different ceramide species from crude cellular extracts by electrospray ionization tandem mass spectrometry (ESI-MS/MS). *J. Lipid Res.* **40:** 1539–1546.
20. Liebisch, G., W. Drobnik, B. Lieser, and G. Schmitz. 2002. High-throughput quantification of lysophosphatidylcholine by electrospray ionization tandem mass spectrometry. *Clin. Chem.* **48:** 2217–2224.
21. Liebisch, G., B. Lieser, J. Rathenberg, W. Drobnik, and G. Schmitz. 2004. High-throughput quantification of phosphatidylcholine and sphingomyelin by electrospray ionization tandem mass spectrometry coupled with isotope correction algorithm. *Biochim. Biophys. Acta.* **1686:** 108–117.
22. Liebisch, G., M. Binder, R. Schifferer, T. Langmann, B. Schulz, and G. Schmitz. 2006. High throughput quantification of cholesterol and cholesteryl ester by electrospray ionization tandem mass spectrometry (ESI-MS/MS). *Biochim. Biophys. Acta.* **1761:** 121–128.
23. Schmitz, G., C. Mollers, and V. Richter. 1997. Analytical capillary isotachophoresis of human serum lipoproteins. *Electrophoresis.* **18:** 1807–1813.
24. Bligh, E. G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
25. Brugger, B., G. Erben, R. Sandhoff, F. T. Wieland, and W. D. Lehmann. 1997. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *Proc. Natl. Acad. Sci. USA.* **94:** 2339–2344.
26. Zemski Berry, K. A., and R. C. Murphy. 2004. Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. *J. Am. Soc. Mass Spectrom.* **15:** 1499–1508.
27. Drobnik, W., H. Borsukova, A. Bottcher, A. Pfeiffer, G. Liebisch, G. J. Schutz, H. Schindler, and G. Schmitz. 2002. Apo AI/ABCA1-dependent and HDL3-mediated lipid efflux from compositionally distinct cholesterol-based microdomains. *Traffic.* **3:** 268–278.
28. Thumser, A. E., J. E. Voysey, and D. C. Wilton. 1994. The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. *Biochem. J.* **301:** 801–806.
29. Orso, E., C. Broccardo, W. E. Kaminski, A. Bottcher, G. Liebisch, W. Drobnik, A. Gotz, O. Chambenoit, W. Diederich, T. Langmann, et al. 2000. Transport of lipids from golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. *Nat. Genet.* **24:** 192–196.
30. Bodzioch, M., E. Orso, J. Klucken, T. Langmann, A. Bottcher, W. Diederich, W. Drobnik, S. Barlage, C. Buchler, M. Porsch-Ozcurumez, et al. 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat. Genet.* **22:** 347–351.
31. Brousseau, T., V. Clavey, J. M. Bard, and J. C. Fruchart. 1993. Sequential ultracentrifugation micromethod for separation of serum lipoproteins and assays of lipids, apolipoproteins, and lipoprotein particles. *Clin. Chem.* **39:** 960–964.
32. Blom, D. J., P. Byrnes, S. Jones, and A. D. Marais. 2003. Non-denaturing polyacrylamide gradient gel electrophoresis for the diagnosis of dysbetalipoproteinemia. *J. Lipid Res.* **44:** 212–217.
33. Karlsson, K. A. 1970. Sphingolipid long chain bases. *Lipids.* **5:** 878–891.
34. Yee, M. S., D. V. Pavitt, T. Tan, S. Venkatesan, I. F. Godsland, W. Richmond, and D. G. Johnston. 2008. Lipoprotein separation in a novel iodixanol density gradient, for composition, density and phenotype analysis. *J. Lipid Res.* **49:** 1364–1371
35. Zeller, M., D. Masson, M. Farnier, L. Lorgis, V. Deckert, J-P. Pais de Barros, C. Desrumaux, P. Sicard, J. Grober, D. Blache, et al. 2007. High serum cholesteryl ester transfer rates and small high-density lipoproteins are associated with young age in patients with acute myocardial infarction. *J. Am. Coll. Cardiol.* **50:** 1948–1955.
36. Rizzo, M., and K. Berneis. 2007. Small, dense low-density-lipoproteins and the metabolic syndrome. *Diabetes Metab. Res. Rev.* **23:** 14–20.
37. Kuksis, A., J. J. Myher, K. Geher, W. C. Breckenridge, G. J. Jones, and J. A. Little. 1981. Lipid class and molecular species interrelationships among plasma lipoproteins of normolipemic subjects. *J. Chromatogr.* **224:** 1–23.
38. Skipski, V. P., M. Barclay, R. K. Barclay, V. A. Fetzer, J. J. Good, and F. M. Archibald. 1967. Lipid composition of human serum lipoproteins. *Biochem. J.* **104:** 340–352.
39. Myher, J. J., A. Kuksis, J. Shepherd, C. J. Packard, J. D. Morrisett, O. D. Taunton, and A. M. Gotto. 1981. Effect of saturated and unsaturated fat diets on molecular species of phosphatidylcholine and sphingomyelin of human plasma lipoproteins. *Biochim. Biophys. Acta.* **666:** 110–119.
40. Lightle, S., R. Tosheva, A. Lee, J. Queen-Baker, B. Boyanovsky, S. Shedlofsky, and M. Nikolova-Karakashian. 2003. Elevation of ceramide in serum lipoproteins during acute phase response in humans and mice: role of serine-palmitoyl transferase. *Arch. Biochem. Biophys.* **419:** 120–128.
41. Ichi, I., K. Nakahara, Y. Miyashita, A. Hidaka, S. Kutsukake, K. Inoue, T. Maruyama, Y. Miwa, M. Harada-Shiba, M. Tsushima,

et al. 2006. Association of ceramides in human plasma with risk factors of atherosclerosis. *Lipids.* **41:** 859–863.
42. Jiang, X. C., F. Paultre, T. A. Pearson, R. G. Reed, C. K. Francis, M. Lin, L. Berglund, and A. R. Tall. 2000. Plasma sphingomyelin level as a risk factor for coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.* **20:** 2614–2618.
43. Schlitt, A., S. Blankenberg, D. Yan, G. H. von, M. Buerke, K. Werdan, C. Bickel, K. J. Lackner, J. Meyer, H. J. Rupprecht, et al. 2006. Further evaluation of plasma sphingomyelin levels as a risk factor for coronary artery disease. *Nutr. Metab. (Lond).* **3:** 5.
44. Schissel, S. L., J. Tweedie-Hardman, J. H. Rapp, G. Graham, K. J. Williams, and I. Tabas. 1996. Rabbit aorta and human atherosclerotic lesions hydrolyze the sphingomyelin of retained low-density lipoprotein. Proposed role for arterial-wall sphingomyelinase in subendothelial retention and aggregation of atherogenic lipoproteins. *J. Clin. Invest.* **98:** 1455–1464.
45. Pfeiffer, A., A. Bottcher, E. Orso, M. Kapinsky, P. Nagy, A. Bodnar, I. Spreitzer, G. Liebisch, W. Drobnik, K. Gempel, et al. 2001. Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. *Eur. J. Immunol.* **31:** 3153–3164.
46. Pruzanski, W., E. Stefanski, F. C. de Beer, M. C. de Beer, A. Ravandi, and A. Kuksis. 2000. Comparative analysis of lipid composition of normal and acute-phase high density lipoproteins. *J. Lipid Res.* **41:** 1035–1047.
47. Han, X., and R. W. Gross. 2003. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. *J. Lipid Res.* **44:** 1071–1079.
48. Pulfer, M., and R. C. Murphy. 2003. Electrospray mass spectrometry of phospholipids. *Mass Spectrom. Rev.* **22:** 332–364.
49. Ejsing, C. S., E. Duchoslav, J. Sampaio, K. Simons, R. Bonner, C. Thiele, K. Ekroos, and A. Shevchenko. 2006. Automated identification and quantification of glycerophospholipid molecular species by multiple precursor ion scanning. *Anal. Chem.* **78:** 6202–6214.
50. Hermansson, M., A. Uphoff, R. Kakela, and P. Somerharju. 2005. Automated quantitative analysis of complex lipidomes by liquid chromatography/mass spectrometry. *Anal. Chem.* **77:** 2166–2175.
51. Frijters, C. M. G., C. J. Tuijn, R. Ottenhoff, B. N. Zegers, A. K. Groen, and R. P. J. O. Elferink. 1999. The role of different P-glycoproteins in hepatobiliary secretion of fluorescently labeled short-chain phospholipids. *J. Lipid Res.* **40:** 1950–1958.