Journal of Chromatography B, 990 (2015) 52–63

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Lipidomic analysis of plasma, erythrocytes and lipoprotein fractions of cardiovascular disease patients using UHPLC/MS, MALDI-MS and multivariate data analysis

Michal Holčapek[a,*], Blanka Červená[a], Eva Cífková[a], Miroslav Lísa[a], Vitaliy Chagovets[a], Jitka Vostálová[b], Martina Bancířová[b], Jan Galuszka[c], Martin Hill[d]

[a] Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, 53210 Pardubice, Czech Republic
[b] Palacký University, Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, 77515 Olomouc, Czech Republic
[c] University Hospital Olomouc, I. P. Pavlova 185/6, 77520 Olomouc, Czech Republic
[d] Institute of Endocrinology, Národní 8, 11694 Prague 1, Czech Republic

ARTICLE INFO

Article history:
Received 25 December 2014
Accepted 17 March 2015
Available online 23 March 2015

Keywords:
Lipids
Lipidomics
Cardiovascular diseases
Lipoprotein fractions
UHPLC/MS
Multivariate data analysis

ABSTRACT

Differences among lipidomic profiles of healthy volunteers, obese people and three groups of cardiovascular disease (CVD) patients are investigated with the goal to differentiate individual groups based on the multivariate data analysis (MDA) of lipidomic data from plasma, erythrocytes and lipoprotein fractions of more than 50 subjects. Hydrophilic interaction liquid chromatography on ultrahigh-performance liquid chromatography (HILIC-UHPLC) column coupled with electrospray ionization mass spectrometry (ESI-MS) is used for the quantitation of four classes of polar lipids (phosphatidylethanolamines, phosphatidylcholines, sphingomyelins and lysophosphatidylcholines), normal-phase UHPLC—atmospheric pressure chemical ionization MS (NP-UHPLC/APCI-MS) is applied for the quantitation of five classes of nonpolar lipids (cholesteryl esters, triacylglycerols, sterols, 1,3-diacylglycerols and 1,2-diacylglycerols) and the potential of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is tested for the fast screening of all lipids without a chromatographic separation. Obtained results are processed by unsupervised (principal component analysis) and supervised (orthogonal partial least squares) MDA approaches to highlight the largest differences among individual groups and to identify lipid molecules with the highest impact on the group differentiation.

1. Introduction

Lipids are hydrophobic or amphipathic small molecules that may originate entirely or in part by the carbanion based condensation of ketoacylthioesters and/or the carbocation based condensation of isoprene units according to the Lipid MAPS classification system. Lipids are divided into eight categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids and prenol lipids [1–3]. For the chromatographic analysis of lipids, it is useful to divide them into groups of polar and nonpolar lipids. Lipids have several important functions in a human organism, such as a source of energy (triacyglycerols, TG), building blocks of cell membrane (glycerophospholipids), signaling molecules between organelles or cells (glycerophospholipids, diacylglycerols and fatty acyl derivatives), hormones (derivatives of cholesterol) and the role in the immune system (glycerolipids) [4–7]. Lipids are transported by lipoprotein particles in the human body due to their hydrophobicity. Dietary

Abbreviations: APCI, atmospheric pressure chemical ionization; BMI, body mass index; CE, cholesteryl esters; CVD, cardiovascular disease; DG, diacylglycerols; DHB, 2,5-dihydroxybenzoic acid; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; g1, group 1; g2, group 2; g3, group 3; g4, group 4; g5, group 5; FWHM, full width at half maximum; HDL, high-density lipoproteins; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; IS_1, internal standard 1 (sphingosyl PE d17:1/12:0); IS_2, internal standard 2 (dioleoyl ethylene glycol); LC, liquid chromatography; LDL, low-density lipoproteins; LPC, lysophosphatidylcholines; LVEF, left ventricular ejection fraction; MALDI, matrix-assisted laser desorption/ionization; MDA, multivariate data analysis; MS, mass spectrometry; NP, normal phase; OPLS, orthogonal partial least squares; PC, phosphatidylcholines; PCA, principal component analysis; PE, phosphatidylethanolamines; PLS, partial least squares; SM, sphingomyelins; TG, triacylglycerols; UHPLC, ultrahigh-performance liquid chromatography; VLDL, very low-density lipoproteins.

* Corresponding author. Tel.: +420 466 037 087; fax: +420 466 037 068.
E-mail address: Michal.Holcapek@upce.cz (M. Holčapek).

http://dx.doi.org/10.1016/j.jchromb.2015.03.010
1570-0232/

cholesterol, glycerophospholipids and TG are absorbed in the small intestine, transported by chylomicrons to the blood circulation, where the TG part of chylomicrons is degraded to fatty acids, which could be used as the source of energy. Liver generates very low-density lipoproteins (VLDL) containing TG, glycerophospholipids, cholesterol, cholesteryl esters (CE) and protein parts. The main function of VLDL is the transport of TG to extrahepatic tissues. Low-density lipoproteins (LDL) are responsible for the transfer of cholesterol and CE to cells. High-density lipoproteins (HDL) are the smallest lipoprotein particles and they transfer overflowing cholesterol from cells back to the liver and the interchange of CE, TG and glycerophospholipids with VLDL. HDLs have also numerous additional properties, such as the apoprotein source for other lipoproteins, anti-inflammatory, antioxidant and antithrombotic properties [8–10]. The dysregulation of lipid metabolism could lead to the development of CVD, cancer, Alzheimer disease, etc. [11].

The prevalence of major risk factors for CVD is increasing in the major population of countries of the developing world with subsequently increased rates of coronary and cerebrovascular events [12]. The major risk factors of CVD are obesity, high level of circulating lipids, age, gender, smoking, diabetes mellitus, hyperhomocysteinemia, etc. [5,11,13–16]. The obesity has a strong connection with the development of CVD [17]. Dietary fatty acids are accumulated in the adipose tissue until the storage capacity becomes saturated in case of the obesity, which induces a combined state of inflammation and insulin resistance [13,17,18]. The term CVD does not represent a single disease, but it includes, for example coronary heart disease, strokes, valvular heart disease and cardiomyopathy [19]. Lipids have a crucial role in the development of CVD and related diseases [20]. The downregulation of PC and plasmalogen-PE and the increase of PC/lysophosphatidylcholines (LPC) ratio have been reported for hyperlipidemic patients [21].

Two main analytical techniques are used for the lipidomic quantitation, shotgun without any chromatographic separation [22,23] and high-performance liquid chromatography–mass spectrometry (HPLC/MS) or UHPLC/MS approaches. Advantages of shotgun are an easier automation and higher throughput, while HPLC/MS is more prone to ion suppression effects and can provide more detailed information of various types of lipid isomerism. HILIC can be used for the separation of individual classes of polar lipids [24–28], while NP-HPLC is more convenient for nonpolar lipid classes [29–32]. The lipid species separation according to the acyl chain length and the number of the double bonds can be achieved by reversed phase (RP)-HPLC [25,33–36], while silver-ion HPLC is more convenient for nonpolar lipid regioisomers or double bond positional isomers [37,38]. MALDI provides the fast analysis without the requirement of chromatographic separation [39], which could be useful for high-throughput clinical screening.

MDA methods are typically used for the group differentiation in the lipidomic analysis using either unsupervised (principal component analysis, PCA) or supervised (partial least squares, PLS, and OPLS) methods [40]. PCA is used to reduce primary variables to the latent variables, which are called principal components due to the decreasing model dimensionality. Principal components are linear combinations of x_i, which are mutually uncorrelated. PLS relates two data matrices, *X* and *Y*, to each other by a linear multivariate model, which works with a maximum covariance between matrices *X* and *Y* [41,42]. OPLS divides a systematic variation in matrices *X* into two model parts, one part of the model expresses correlations between *X* and *Y* matrices and another part of the model expresses the variation that is not related (orthogonal) to *Y* [42,43]. Results of MDA are typically presented in in two forms, score plots and loading plots. The score plot displays two score vectors plotted against each other for the visualization of objects, *e.g.*, samples or patients. The loading plot is constructed using two loading vectors plotted against each other to visualize characteristic variables, such as lipid species [44]. The S-plot is a variant of the loading plot, where *X* axis is the variable of magnitude and *Y* axis is the reliability [44].

The main goal of this work is a lipidomic study of differences among CVD, healthy normal and healthy obese subjects using three MS-based methods: HILIC-UHPLC/ESI-MS for the analysis of polar lipid classes, NP-UHPLC/APCI-MS for the analysis of nonpolar lipid classes and MALDI-MS for the fast lipidomic screening. MDA methods (PCA and OPLS) are used for a better visualization of group differences.

2. Materials and methods

2.1. Materials

Acetonitrile, 2-propanol, methanol (all HPLC/MS grade), hexane (HPLC grade), chloroform (HPLC grade, stabilized with 0.5–1% ethanol), ammonium acetate, 2,5-dihydroxybenzoic acid (DHB), NaCl, KBr and EDTA $Na_2 \cdot 2H_2O$ were purchased from Sigma–Aldrich (St. Louis, MO, USA). Deionized water was prepared with Demiwa 5-roi purification system (Watek, Ledeč nad Sázavou, Czech Republic) and by ultra CLEAR UV apparatus (SG, Hamburg, Germany). Standards of polar lipids containing oleoyl acyls (PE, PC, SM and LPC), internal standards for polar lipids (sphingosyl PE d17:1/12:0, IS_1) and for nonpolar lipids (dioleoyl ethylene glycol, IS_2) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Standards of CE, cholesterol, TG, 1,3-DG and 1,2-DG containing oleoyl acyls were purchased from Sigma–Aldrich. Biological samples (plasma, erythrocytes, VLDL, LDL and HDL) were obtained from healthy volunteers and CVD patients in cooperation with the Faculty Hospital Olomouc based on the approval of the ethical committee at the Faculty Hospital Olomouc.

2.2. Characterization of studied subjects

Fifty eight men between 40 and 55 years were selected for the study with the following characterization of individual groups (details in Table S1): group 1 (g1): healthy subjects with body mass index (BMI) with the mean value of 25.1 kg/m^2 without medication; group 2 (g2): healthy subjects with BMI between 30 and 35 kg/m^2 without medication; group 3 (g3): subjects with the non-ischemic dilated cardiomyopathy and negative coronographic findings, chronic heart failure, left ventricular ejection fraction (LVEF) below 35% with stable therapy (diuretics, angiotensin-converting enzyme inhibitors, beta-blockers and acetylsalicylic acid); group 4 (g4): subjects with the chronic form of atrial fibrillation/flutter without valvular heart disease, myocardial infarction or chronic heart failure, LVEF more than 35% with antiarrhythmic agents, anticoagulants, statins and radiofrequency ablation therapy; group 5 (g5): subjects with ischemic heart diseases, post myocardial infarction, without the chronic heart failure, LVEF more than 35% with stable therapy of beta-blockers, statins and acetylsalicylic acid.

2.3. Sample preparation

Blood was collected to heparin-lithium tubes and centrifuged to obtain erythrocytes and plasma. Erythrocytes were washed three times by phosphate buffered saline. Plasma was further separated into lipoprotein fractions by the ultracentrifugation. The following ultracentrifugation steps were applied to obtain lipoprotein fractions (VLDL, LDL and HDL) according to literature [45]. The volume of plasma was divided into two polycarbonate tubes for the ultracentrifugation and refilled with the density solvent 1 (1.1 g NaCl + 37.6 mg EDTA $Na_2 \cdot 2H_2O$ dissolved in 100 mL of water). Samples were centrifuged for 11 h at 10 °C and 45,000 × *g* and then the VLDL fraction was collected. The remaining solution was mixed

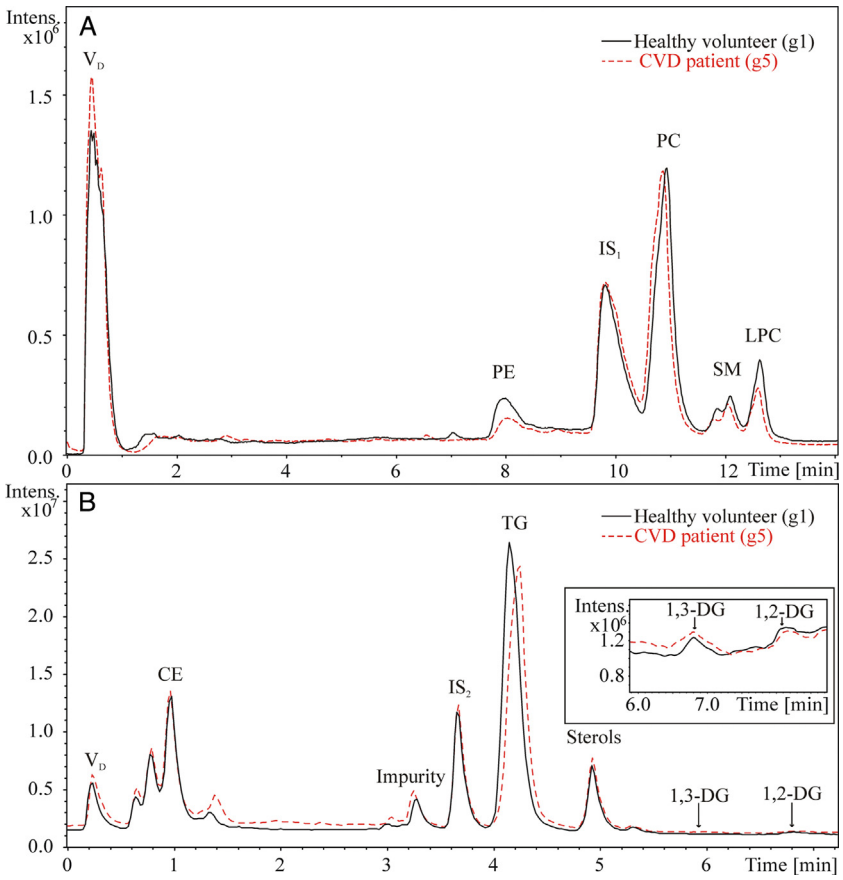

Fig. 1. Chromatograms of plasma samples of healthy volunteer (g1) and CVD patient (g5). (A) HILIC-UHPLC/ESI-MS separation of polar lipid classes (PE, PC, SM and LPC) and the internal standard IS_1 (sphingosyl PE d17:1/12:0). *Conditions*: Acquity UPLC HILIC column (50 mm × 2.1 mm, 1.7 μm), flow rate 0.5 mL/min, column temperature 40 °C, gradient 0 min; 0.5% A + 99.5% B, 20 min 20.5% A + 79.5% B, where A is a mixture of 5 mmol/L aqueous ammonium acetate and methanol (9:1, v/v) and B is acetonitrile. (B) NP-UHPLC/APCI-MS separation of nonpolar lipid classes (CE, TG, sterols, 1,3-DG and 1,2-DG) and the internal standard IS_2 (dioleoyl ethylene glycol). *Conditions*: Acquity UPLC HILIC column (50 mm × 2.1 mm, 1.7 μm), flow rate 1 mL/min, column temperature 30 °C, gradient 0 min; 99% A + 1% B, 20 min 32% A + 68% B, where A is hexane and B is the mixture of hexane–2-propanol–acetonitrile (96:2:2, v/v/v).

with 0.5 mL of the density solvent 2 (9.2 g KBr + 37.6 mg EDTA $Na_2 \cdot 2H_2O$ dissolved in 100 mL water). Samples were centrifuged again for 12 h at 10 °C and 45,000 × *g* and then the LDL fraction was collected. The remaining solution was mixed with 0.5 mL of the density solvent 3 (34.8 g KBr + 188.0 mg EDTA $Na_2 \cdot 2H_2O$ dissolved in 100 mL of water). Samples were centrifuged for 48 h at 10 °C and 20,000 × *g* and finally the HDL fraction was collected.

The total lipid extract was prepared according to the modified Folch method [46]. Five hundred microliter of sample with 50 μL (3.3 mg/mL) of IS_1 was homogenized with 10 mL of chloroform–methanol mixture (2:1, v/v). This mixture was filtered using a rough filter paper. Then 2 mL of 1 mol/L NaCl was added and centrifuged for 3 min at 2500 rpm. The chloroform layer containing lipids was evaporated by a gentle stream of nitrogen and dissolved in chloroform–2-propanol (1:1, v/v) for the HILIC analysis.

Nonpolar lipid extracts were prepared according to the previously developed method [32]. Fifty microliter of the sample with 5 μL (3.3 mg/mL) of IS_2 was mixed with 150 μL of hexane–methanol (98:2, v/v). The mixture was incubated for 10 min, then 300 μL of methanol–water mixture (95:5, v/v) and 300 μL of hexane–methanol mixture (98:2, v/v) were added. The sample was centrifuged for 1 min at 2500 rpm and then the upper hexane layer was collected. The lower layer was washed twice with 300 μL of hexane–methanol and the upper hexane layer was collected again and combined. Hexane solution containing nonpolar lipids was evaporated by the gentle stream of nitrogen, then dissolved in hexane and used for NP-UHPLC/APCI-MS analysis.

Ten microliter of the total lipid extract was mixed with 10 μL of 0.5 mol/L DHB in methanol and 0.7 μL of this mixture was deposited on the stainless steel sample plate and dried using the gentle stream of nitrogen. Each sample was deposited in six wells and used for the MALDI-MS analysis.

2.4. HILIC-UHPLC/ESI-MS conditions [32]

Experiments were performed with a liquid chromatograph Agilent 1290 Infinity Series (Agilent Technologies, Santa Clara, CA, USA). Acquity UPLC HILIC column (50 mm × 2.1 mm, 1.7 μm, Waters, Milford, MA, USA) was used for the separation of polar classes of lipids. The flow rate was 0.5 mL/min, the column temperature was 40 °C, the mobile phase gradient was 0 min: 0.5% A + 99.5% B; 20 min: 20.5% A + 79.5% B, where A was the mixture of 5 mmol/L aqueous ammonium acetate and methanol (9:1, v/v), B was acetonitrile. Hybrid quadrupole–time-of-flight mass spectrometer (MicroTOF-Q, Bruker Daltonics, Bremen, Germany) operating in the positive-ion ESI mode was used for the determination of polar lipid classes (PE, PC, SM and LPC) and IS_1 under the following conditions: capillary voltage 4.5 kV, pressure of nebulizer gas 1.6 bar, flow rate of drying gas 9 mL/min, drying temperature 220 °C and mass range *m/z* 50–1500.

2.5. NP-UHPLC/APCI-MS conditions [32]

Experiments were performed with a liquid chromatograph Agilent 1200 Infinity Series (Agilent Technologies). Acquity UPLC HILIC

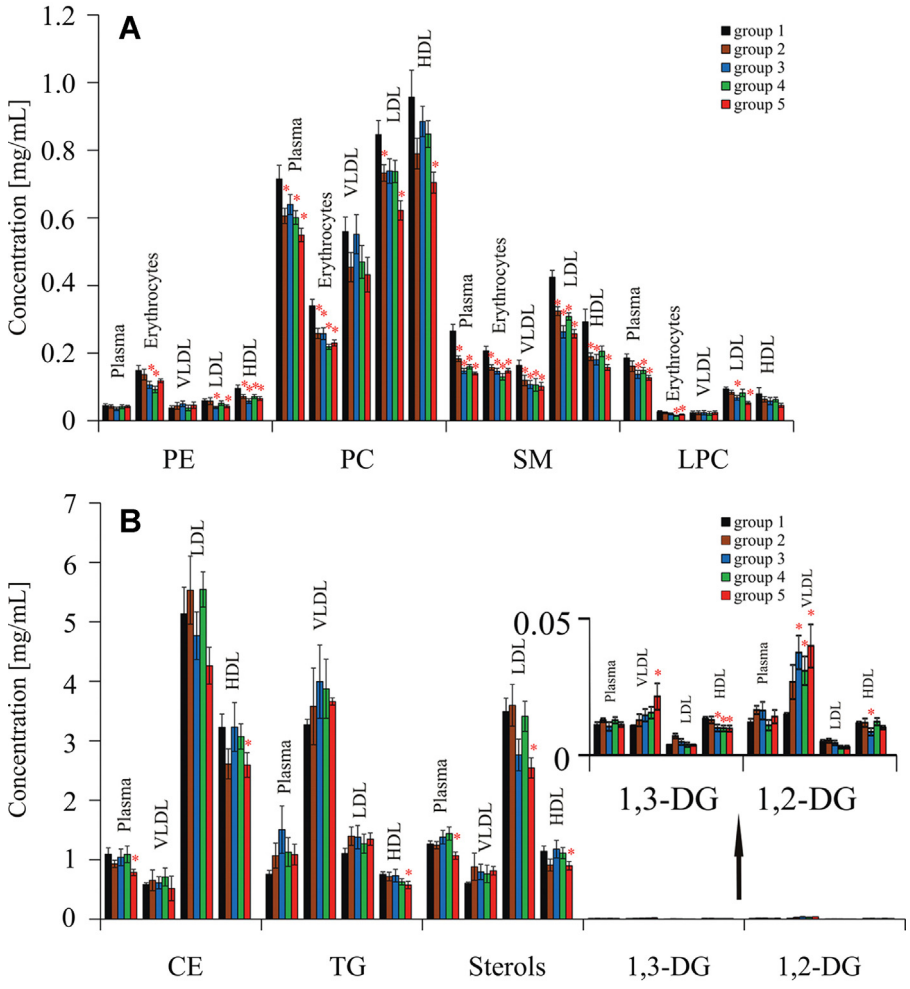

Fig. 2. Comparison of mean group concentrations with standard error bars for: (A) PE, PC, SM and LPC as polar lipid classes, and (B) CE, TG, sterols, 1,3-DG and 1,2-DG as nonpolar lipid classes determined in plasma, erythrocytes, VLDL, LDL and HDL. Lipid class concentrations significantly different from the group 1 ($p \leq 0.05$) according to the Student's *t*-test are labelled by an asterisk.

(50 mm × 2.1 mm, 1.7 μm, Waters) column was used for the separation of nonpolar classes of lipids. The flow rate was 1 mL/min, the column temperature was 30 °C, the mobile phase gradient was 0 min: 99% A + 1% B; 20 min: 32% A + 68% B, where A was hexane and B was the mixture of hexane–2-propanol–acetonitrile (96:2:2, v/v/v). Esquire 3000 ion trap mass spectrometer (Bruker Daltonics) operating in the positive-ion APCI mode was used for the determination of nonpolar lipid classes under the following conditions: corona current 4000 nA, pressure of nebulizer gas 65 psi, flow rate of drying gas 3 L/min, drying temperature 350 °C, vaporizer temperature 375 °C, target mass *m/z* 500 and mass range *m/z* 50–1000.

2.6. MALDI-MS conditions

Experiments were performed with hybrid linear ion trap–orbitrap mass analyzer LTQ Orbitrap XL (Thermo Scientific, Waltham, MA, USA) operating in the positive-ion MALDI in the mass range *m/z* 300–2000 with the resolving power of 100,000 and the laser energy of 15 μJ per laser shot. The mass spectrum from one point was obtained as a summation of 3 laser shots. Spectra for each well were measured from 50 randomly distributed positions. The final spectrum for each patient was obtained by averaging of 300 spectra from six wells to obtain the most representative spectrum. Mass spectra were converted with msConvert tool [47] and preliminarily processed with home-made program based on the MALDIquant package [48].

2.7. MDA of lipidomic data

MS data were evaluated by the Data Analysis software (Bruker Daltonics), the Progenesis QI software (Waters) and the Simca 13.0 software (Umetrics, Umeå, Sweden). MS data were processed by the Progenesis QI software using the high resolution positive-ion MS for polar lipids and the low resolution positive-ion MS for nonpolar lipids. The alignment, peak picking and identification of lipids were performed. Data sheets from Progenesis QI software were obtained and absolute intensities of all identified compounds were recalculated to relative abundances of lipid molecules. The data were transformed using the logarithmic transformation to obtain a Gaussian normal distribution and the Pareto scaling was used for final statistical models. The data were processed by unsupervised PCA and supervised OPLS methods to obtain group clusters. Lipid molecules with the highest impact on the group clustering were identified in S-plots.

3. Results and discussion

3.1. UHPLC/MS methods

The goal of this study is finding differences among the lipidomic composition of blood fractions (plasma, erythrocytes, VLDL, LDL and HDL) of three types of CVD patients (g3, g4 and g5) and healthy controls (g1). The obesity plays a rather important role in the

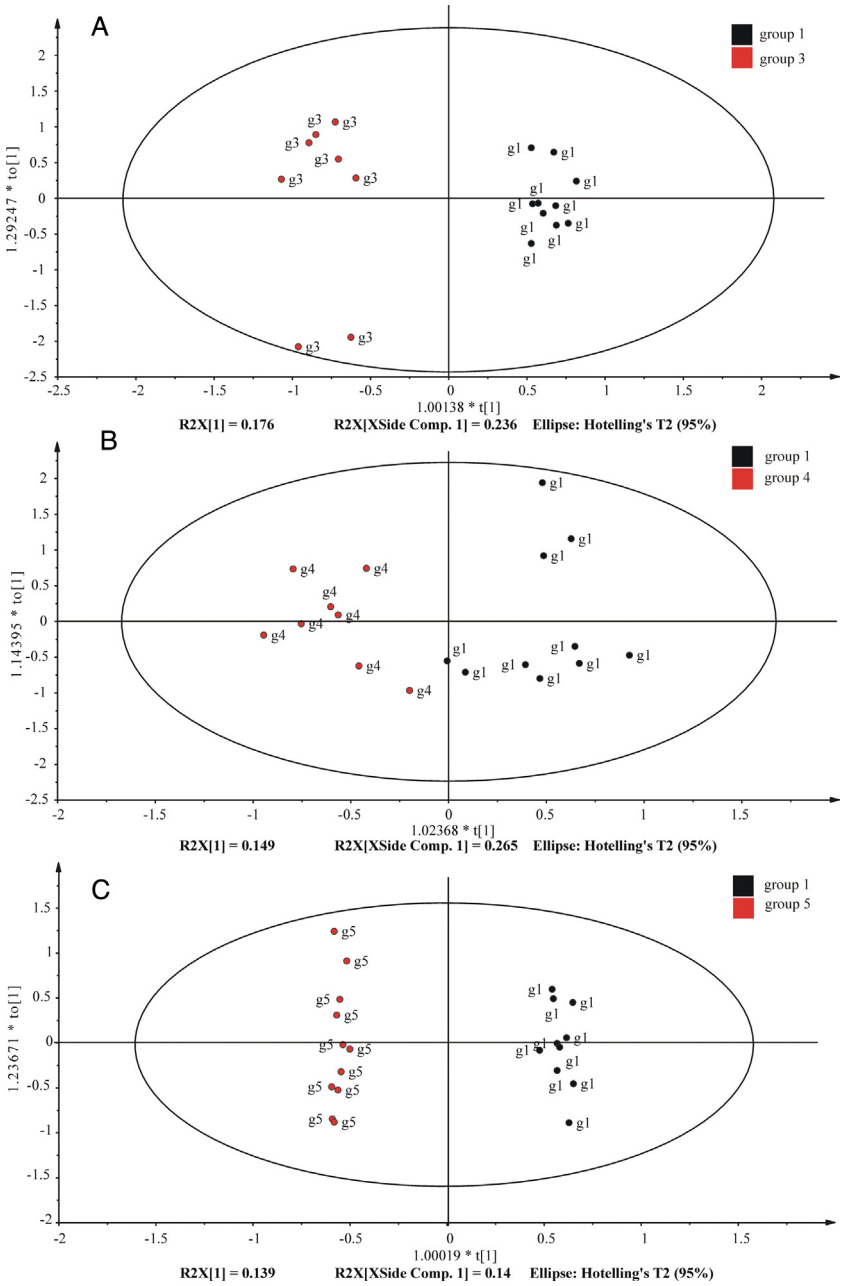

Fig. 3. OPLS score plots for polar and nonpolar lipid classes in human plasma samples: (A) g1 *vs.* g3, (B) g1 *vs.* g4, and (C) g1 *vs.* g5.

development of CVD [17] so the group of obese healthy control (g2) is included as well to differentiate effects of CVD and obesity on the lipidome. Details on the selection and size of individual groups, characteristic biochemical and anthropometric parameters are summarized the Experimental part and Table S1.

UHPLC/MS methods for the analysis of large series of clinical samples have been developed in our previous works [26,32,49]. Briefly, HILIC-UHPLC/ESI-MS method (Fig. 1A) is used for the determination of polar lipid classes (PE, PC, SM and LPC) using the IS_1 (sphingosyl PE d17:1/12:0) and response factors approach [26,32] to normalize different ionization efficiencies of individual lipid classes. IS_1 is not present in biological samples and well separated from chromatographic peaks of other lipid classes. Nonpolar lipid classes (CE, TG, ST, 1,3-DG and 1,2-DG) are determined in a similar fashion, but their separation is performed in NP-UHPLC/APCI-MS (Fig. 1B) mode, because they elute in the void volume using the previously mentioned HILIC method [25]. Dioleoyl ethylene glycol is used as IS_2. The quantitation of polar lipid classes in the HILIC mode and nonpolar lipid classes in NP mode is done by the multiplication of lipid class peak areas by response factors of this class and normalized to the IS_1 for polar lipids and IS_2 for nonpolar lipids to obtain absolute lipid class concentrations shown in Table S2. Then the lipid species composition is determined from the overall mass spectra of chromatographic peaks of lipid classes. Relative concentrations of lipid species are determined based on the assumption that differences in relative responses of individual lipid species can be neglected. Most lipidomic clinical studies are based on the comparison of healthy and disease states, so basically relative changes are measured and the extent of such changes is statistically evaluated by MDA methods. Mean group concentrations for individual lipid classes with their standard error bars are summarized in Fig. 2. Another way of data presentation is the use of relative concentrations in pie chart graphs (Fig. S1), which can better visualize some trends in concentration changes among

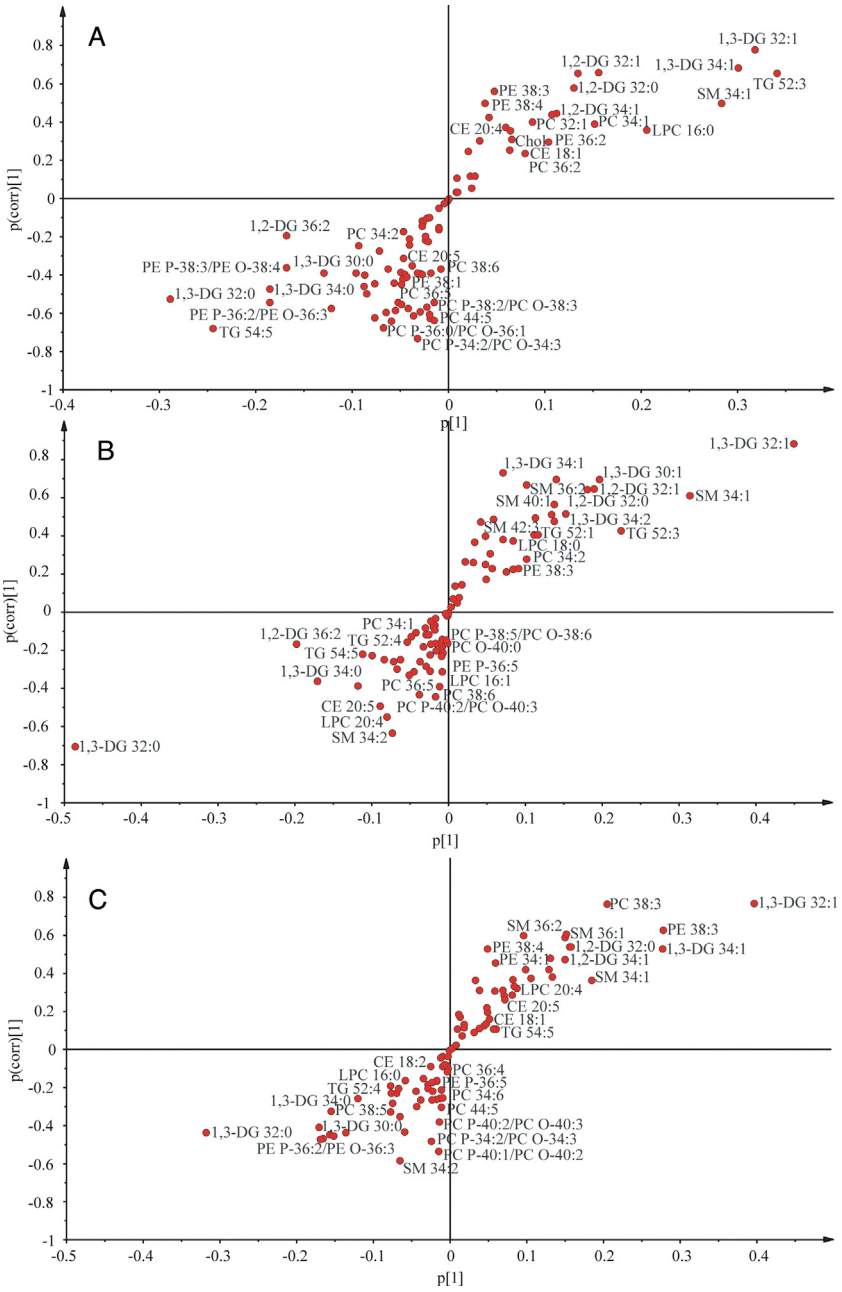

Fig. 4. S-plots from OPLS analysis (see Fig. 3) for lipid classes in human plasma samples with the most important upregulated lipid molecules in the upper right corner and downregulated lipid molecules in the lower left corner: (A) g1 *vs.* g3, (B) g1 *vs.* g4, and (C) g1 *vs.* g5.

individual lipid classes. Fig. S2 illustrates two selected examples of lipid molecules with large changes in concentrations among individual groups. The concentration of PC 32:0 (Fig. S2A) is downregulated in CVD patients, where the highest decrease but also high variation is observed for g3. The concentration of TG 52:3 (Fig. S2B) is upregulated in CVD patients, where the largest change is observed for g3, but again with the largest variability for this group.

3.2. MALDI-MS

MALDI-MS has the potential for the high-throughput clinical analysis of numerous samples without a chromatographic separation. The high-resolution (100,000 FWHM) full scan positive-ion mode is used for our measurements to reduce the risk of peak overlaps caused by missing chromatography. At the beginning, all important lipid molecules are identified based on the mass accuracy better than 1 ppm, verified by MS/MS spectra and also correlated with identified lipids in UHPLC/MS experiments. For routine measurements, only absolute intensities of monitored *m/z* values are recorded and their mass accuracies are checked. Then, absolute intensities are normalized to IS_1 in a similar way as described for UHPLC/MS experiments and processed by MDA methods. Conditions of MALDI measurements are carefully optimized in terms of selected matrix, laser energy and extensive signal averaging to obtain the highest possible robustness for quantitative MALDI measurements. MALDI-MS analysis is tested simultaneously with UHPLC/MS analysis to compare the potential of UHPLC/MS with MALDI-MS for the quantitative comparison of large data sets of samples. Some advantages but mainly limitations of MALDI-MS are observed in our study, such as lower robustness of the quantitation and more demanding data interpretation due to the lack

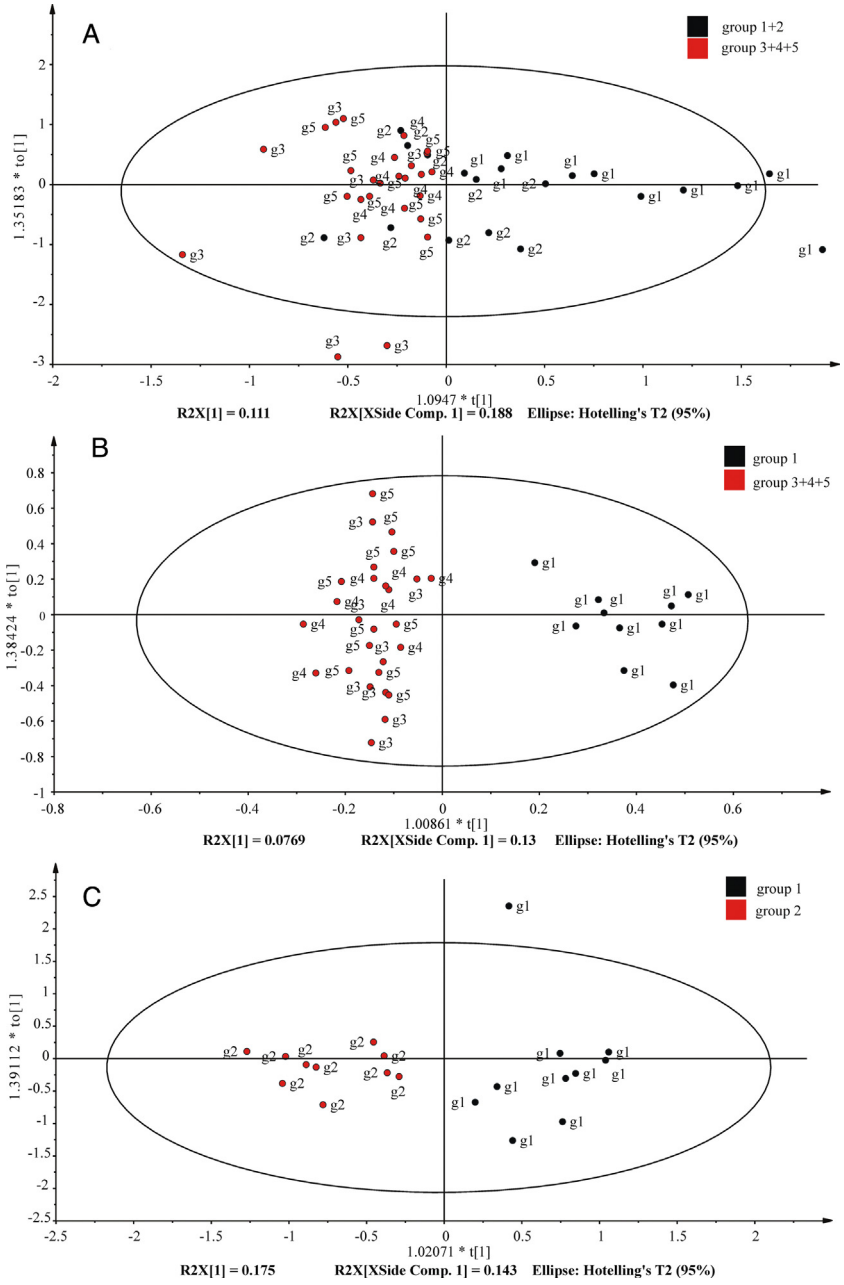

Fig. 5. OPLS score plots for polar and nonpolar lipid classes in human plasma samples: (A) g1 + g2 *vs.* g3 + g4 + g5, (B) g1 *vs.* g3 + g4 + g5, and (C) g1 *vs.* g2.

of retention time in UHPLC/MS, where the lipid class is easily determined by the characteristic retention of particular group. The main advantage of MALDI in our study is a reduced fragmentation in comparison to APCI, which has to be used in NP-UHPLC/MS setup, because mobile phases used in NP systems do not allow the use of ESI. This advantage of MALDI over APCI is dramatic in case of TG, where the significant fragmentation is observed with APCI (relative abundances of $[M+H]^+$ lower than 2% for saturated TG) compared to base peaks of molecular adducts in case of MALDI. Unfortunately, the MDA of MALDI-MS data is slightly worse compared to UHPLC/MS (Fig. S3), as concluded from the worse group clustering. Our conclusion on MALDI-MS *vs.* UHPLC/MS comparison is that UHPLC/MS is superior technique for the quantitative lipidomic studies due to the lower signal variation, easier species identification (possible combination of accurate *m/z* values and class characteristic retention times) and improved grouping in MDA.

3.3. Development of statistical models

Tables of absolute intensities of individual lipid species obtained by the Progenesis QI software are processed using the Simca 13.0 statistical software. First, the absolute intensity of each lipid molecule is normalized to the absolute intensity of the IS_1 for polar lipid classes and IS_2 for nonpolar lipid classes. The development of the best statistical model is explained on the example of plasma samples. First, an unsupervised PCA method (Fig. S4) is always applied to see the natural grouping of samples, as shown on examples of g1 + g2 *vs.* g3 + g4 + g5 using different ways of data scaling and normalization. Distinct group clusters can be expected only for samples with significantly different values of certain parameters, which is often not a case of lipidomic or metabolomic studies. Then, the supervised OPLS method is applied to improve the group clustering (Fig. S5). The important parameter is the way of data scaling. The conventional unit variance (UV) scaling does not provide the

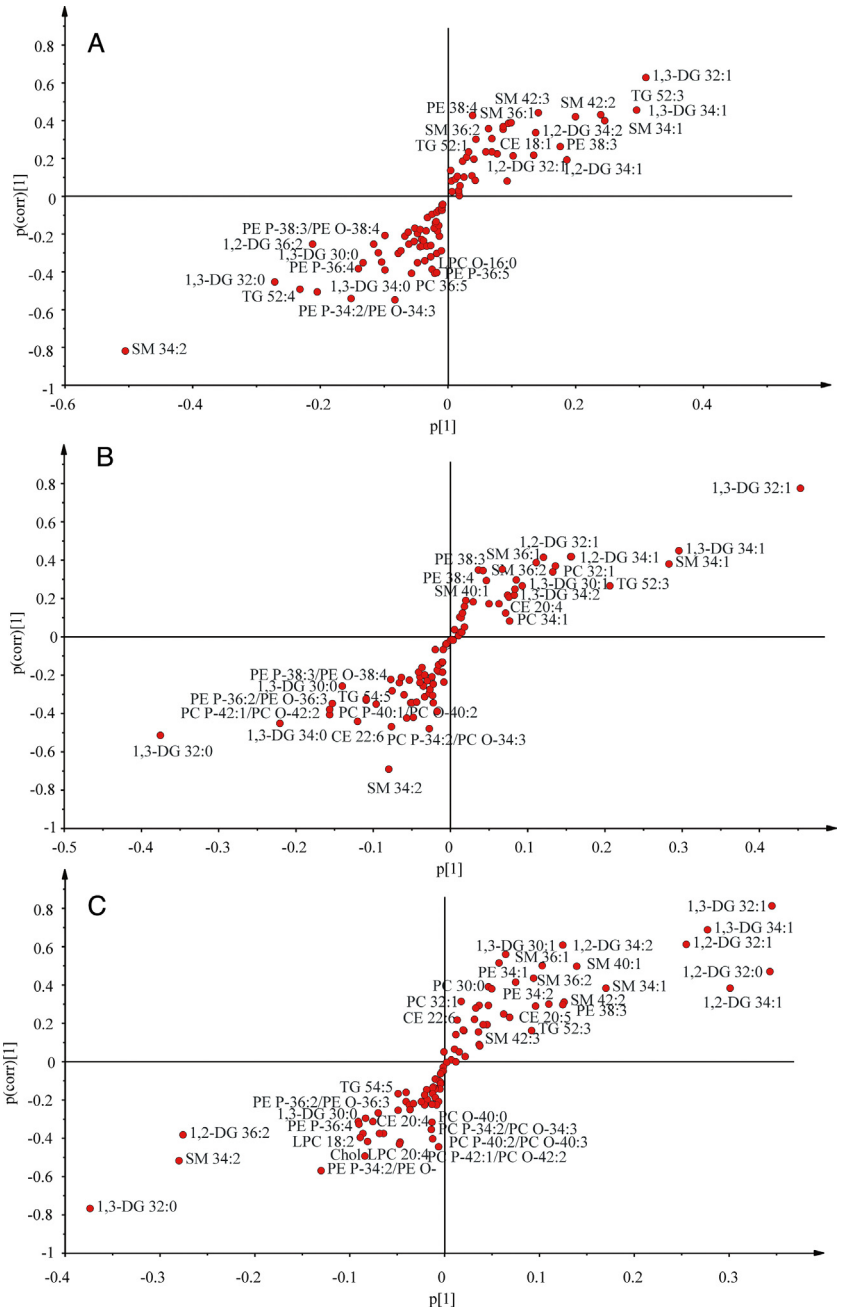

Fig. 6. S-plots from OPLS analysis (see Fig. 5) for lipid classes in human plasma samples with the most important upregulated lipid molecules in the upper right corner and downregulated lipid molecules in the lower left corner: g1 + g2 *vs.* g3 + g4 + g5, (B) g1 *vs.* g3 + g4 + g5, and (C) g1 *vs.* g2.

best results for the metabolomic data (Figs. S4A, S4B, S5A and S5B), where the Pareto scaling is recommended [42] and also in our case the group clustering is better with the Pareto scaling used in the rest of this work. Tables S3 and S4 show basic parameters of statistical models used in figures presented in this paper, where nonpolar and polar lipids can be processed either separately or jointly depending on quality of the statistical group differentiation.

3.4. *Effects of CVD and obesity on lipidomic profiles*

Various statistical correlations among individual groups are studied in our lipidomic data set and the most interesting results are presented here with the emphasis on the differentiation of healthy and CVD groups by MDA methods. The following main trends are observed (Fig. 2) for comparison of mean lipid class concentrations of individual groups in comparison to normal healthy group (g1). In general, quantitative data obtained by biochemical measurements (Table S1) and UHPLC/MS data (Fig. 2) are in a good agreement. PC and SM decrease in all fractions in comparison to g1. This decrease is statistically significant according to *t*-test for SM in most fractions and for PC in plasma and erythrocytes. PE decreases in many cases, the most pronounced and statistically relevant decrease is observed for HDL (g3, g4 and g5) and LDL (g3 and g5) in agreement with the previous work [50] and also for erythrocytes (g3 and g4). The decrease of LPC concentration is observed for all fractions except for VLDL. The statistically significant changes are reported for g3, g4 and g5 in plasma, g4 and g5 in erythrocytes, g3 and g5 in LDL. LPC with anti-inflammatory properties is decreased in obese and CVD patients [51]. LPC (18:2) decrease observed in obese patients is in agreement with the

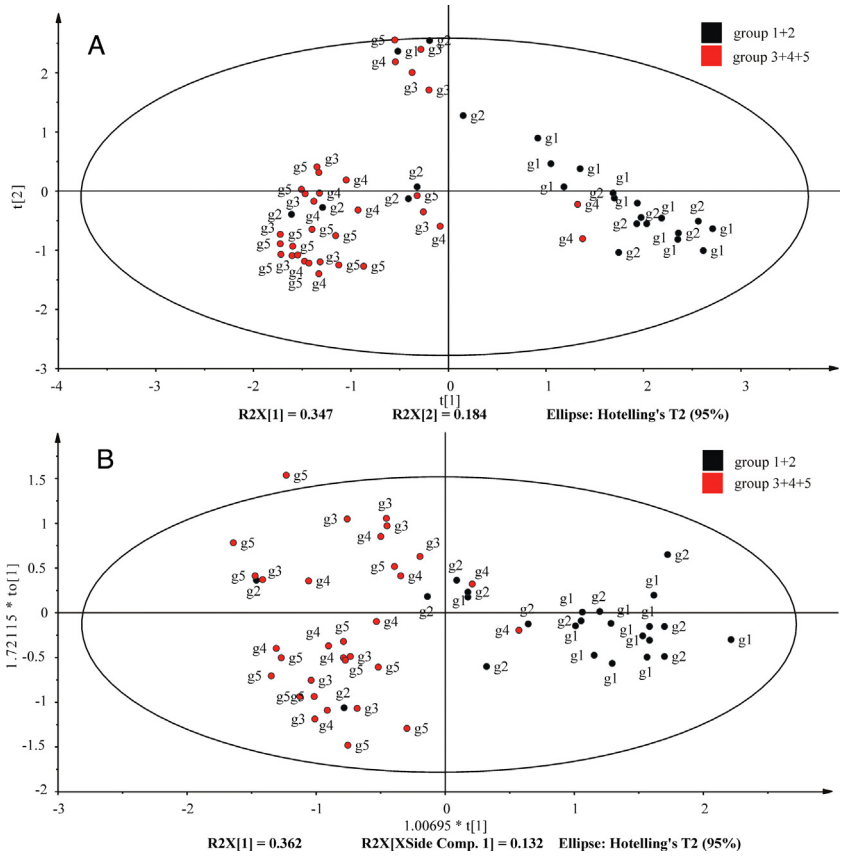

Fig. 7. Statistical plots for nonpolar lipid classes in HDL samples: (A) PCA score plot, and (B) OPLS score plot.

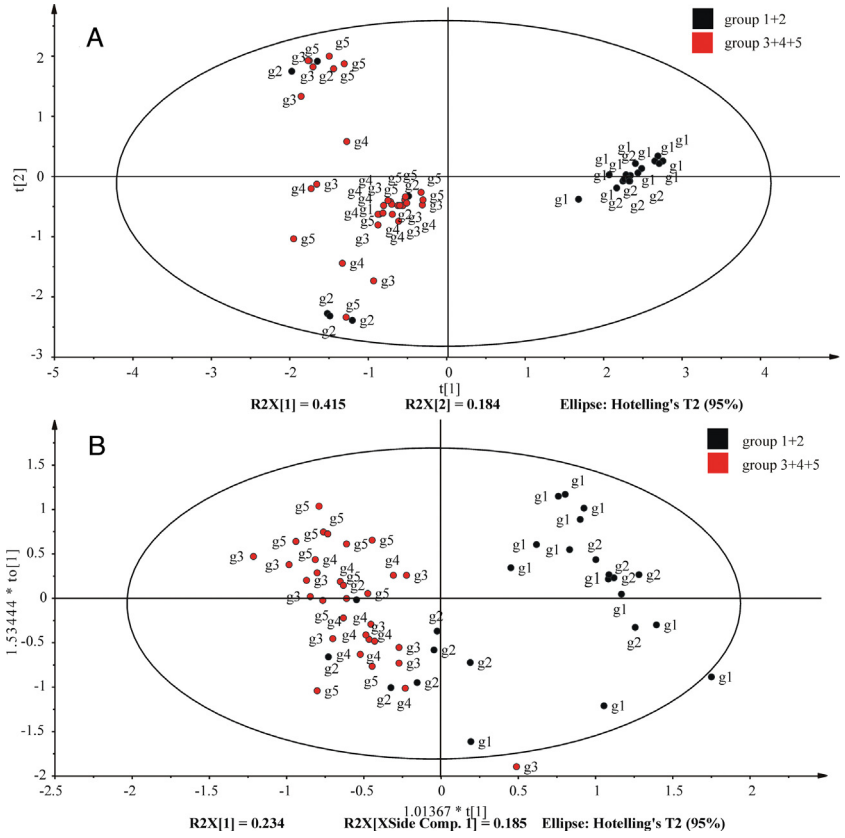

Fig. 8. Statistical plots for nonpolar lipid classes in LDL samples: (A) PCA score plot, and (B) OPLS score plot.

previous work [52]. Other works have reported the opposite effects, such as increased LPC concentration in the sperm of obese man [53] or in human plasma of rheumatoid arthritis patients [54], but this observation does not correspond to our data for plasma of CVD patients. Table S5 shows that ratios of PC/LPC and PC/SM are increased for CVD groups (g3, g4 and g5) compared to the healthy control in case of plasma, LDL and mainly HDL, which is in agreement with the previously published work [55], but another work [56] reports the increased cardiovascular risk for the elevated concentration of lipoprotein-associated phospholipase A2.

For nonpolar lipid classes, the concentration increase is observed for both 1,2- and 1,3-DG in VLDL, but not for other blood fractions. 1,3-DG concentrations even decrease in HDL (g3, g4 and g5). Concentrations of TG in most fractions (except for HDL) are increased, which is in agreement the previous study as well [57]. Large variations in TG concentrations are probably associated with the different diet and living style of individual objects. Changes are mostly not statistically significant due to large variations, but anyway the following trends are apparent: increase of TG concentration (the largest for g3) in plasma, VLDL and LDL, but TG concentration decreases in HDL (statistically significant for g5). Concentration trends for sterols and CE measured by UHPLC/MS (Fig. 2B) are in a full agreement with biochemical measurements (Table S1). The reduced concentration of most lipid classes is observed for g5 (Fig. 2), in many cases these decreases are statistically significant according to *t*-test, such as decreases of CE in plasma and HDL, TG in HDL, sterols in plasma, LDL and HDL, PC, SM and LPC in most fractions. This trend is probably related to the drug lowering therapy with statins, because g5 has a stable therapy of beta-blockers and statins. High blood TG level is associated with the increased risk of CVD [58]. The large increase of 1,2-DG and 1,3-DG in VLDL (Fig. 2B) could be associated with TG degradation by the lipoprotein lipase. Concentration profiles of DG and TG in VLDL show similar profiles.

The first step in MDA is the comparison of CVD groups separately for individual types of CVD (g3, g4 or g5) with healthy controls (g1) excluding healthy obese at this stage (Figs. S6 and 3). Unsupervised PCA score plots (Fig. S6) do not provide a clear group separation, but OPLS score plots (Fig. 3) show the distinct group separation of healthy and disease groups in all cases and there are also similarities in the most influential lipid molecules on the group separation visualized in S-plots (Fig. 4). The common features are the downregulation of some saturated DG (1,3-DG 32:0, 1,3-DG 34:0 and 1,3-DG 30:0) and several plasmalogen/ether PE and PC, the upregulation of some monounsaturated DG (*e.g.*, 1,3-DG 32:1, 1,3-DG 34:1, 1,2-DG 32:1). Another features are observed only for some CVD groups, such the upregulation of TG 52:3 for g3 (Fig. 4A), SM 34:1 for g3 (Fig. 4A) and g4 (Fig. 4B), PC 38:3 and PE 38:3 for g5 (Fig. 4C). When all three CVD groups are combined (g3 + g4 + g5) and correlated with combined healthy normal and healthy obese groups (g1 + g2), then the group clustering is not so clear (Fig. 5A) as in previous examples, but anyway all healthy normal and CVD subjects are clearly distinguished the score plot, but obese subjects (g2) are almost randomly distributed in this graph. The lipidomic composition of healthy obese is somewhere in between healthy state and CVD state in agreement with previous works and known facts that the obesity leads to proinflammatory conditions with the possible development of CVD [17]. If the obese group is excluded from the OPLS model, then the correlation g1 *vs.* g3 + g4 + g5 shows the excellent group separation again (Fig. 5B). Fig. 5C shows that healthy normal and healthy obese can also be easily differentiated by their lipidomic plasma composition. Lipid species with the highest impact on the group clustering are highlighted in S-plots (Fig. 6) showing similarities with the previous correlation of individual disease types (Fig. 4) as expected, but some features are better visualized here. The upregulation of SM 34:1 in g3 and g4, and of SM 36:1 and SM 36:2 in patients of all CVD is observed (Fig. 4). Obese participants (g2) have elevated SM 34:2, SM 36:1 and SM 36:2 (Fig. 6C).

In two cases, lipidomic differences between g1 + g2 *vs.* g3 + g4 + g5 are large enough that even unsupervised PCA yields the visible separation of group clusters, as shown for nonpolar lipid classes in Fig. 7A for HDL and in Fig. 8A for LDL. Only few obese subjects from g2 and two g4 subjects in case of HDL are incorrectly classified in these score plots. OPLS improves the group clustering only in part, but anyway the analysis of nonpolar lipids in HDL and LDL may be considered as the possible target in the future searches for CVD biomarkers. Another example of successful g1 + g2 *vs.* g3 + g4 + g5 MDA separation is shown for polar lipid classes in erythrocytes and VLDL, but the supervised OPLS has to be used for such group clustering (Fig. S7).

4. Conclusions

Three different MS-based methods are used for the lipidomic characterization of five groups containing healthy control, obese and three groups of different types of CVD patients. HILIC-UHPLC/ESI-MS provides the quantitative data on polar lipid classes and NP-HPLC/APCI-MS on nonpolar lipid classes. In both cases, relative lipid species concentrations are determined as well from mass spectra of chromatographic peaks of individual classes and further used for MDA. MALDI-MS is tested for the determination of all lipids without the chromatographic separation, but this approach is not superior to UHPLC/MS methods in terms of analytical information and also the quality of statistical differentiation of group clusters by MDA methods. The biological variability among individual people causes that unsupervised PCA method provides in most cases only a partial group clustering in PCA score plots, so the supervised OPLS has to be used for the better differentiation. CVD is a typical multifactorial type of diseases, where individual types of CVD exhibit also differences in their lipidomic composition. Another risk factor is the obesity, therefore the group of healthy obese is often in between healthy and CVD groups. If the obese group is excluded from our data, then the differentiation between healthy and disease samples is unambiguous in all cases, but such picture would be artificial. Finally, lipid molecules with the highest impact on the group clustering in OPLS score plots are identified using S-plots and increased/decreased concentrations of these lipids can be correlated with the development of CVD and the possible future research of their biomarker potential. Two most upregulated lipids in CVD groups are 1,3-DG 32:1 and 1,3-DG 34:1 and most downregulated species are SM 34:2 and 1,3-DG 32:0.

Acknowledgments

This work was supported by the project No. 206/11/0022 sponsored by the Czech Science Foundation. E. C. and V. C. acknowledge the support of the grant project No. CZ.1.07/2.3.00/30.0021 sponsored by the Ministry of Education, Youth and Sports of the Czech Republic.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.03.010.

References

[1] E.A. Dennis et al., http://www.lipidmaps.org/, downloaded December 2014.
[2] E. Fahy, S. Subramaniam, R.C. Murphy, M. Nishijima, C.R.H. Raetz, T. Shimizu, F. Spener, G. van Meer, M.J.O. Wakelam, E.A. Dennis, Update of the lipid maps comprehensive classification system for lipids, J. Lipid Res. 50 (2009) S9–S14.

[3] E. Fahy, D. Cotter, M. Sud, S. Subramaniam, Lipid classification, structures and tools, Biochim. Biophys. Acta Mol. Cell Biolog. Lipids 1811 (2011) 637–647.
[4] Y. Liu, V.A. Bankaitis, Phosphoinositide phosphatases in cell biology and disease, Prog. Lipid Res. 49 (2010) 201–217.
[5] F. Gunstone, J. Harwood, A.J. Dijkstra (Eds.), The Lipid Handbook with CD-ROM, third ed., CRC, Press, Boca Raton, FL, USA, 2007.
[6] O. Quehenberger, A.M. Armando, A.H. Brown, S.B. Milne, D.S. Myers, A.H. Merrill, S. Bandyopadhyay, K.N. Jones, S. Kelly, R.L. Shaner, C.M. Sullards, E. Wang, R.C. Murphy, R.M. Barkley, T.J. Leiker, C.R.H. Raetz, Z.Q. Guan, G.M. Laird, D.A. Six, D.W. Russell, J.G. McDonald, S. Subramaniam, E. Fahy, E.A. Dennis, Lipidomics reveals a remarkable diversity of lipids in human plasma, J. Lipid Res. 51 (2010) 3299–3305.
[7] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat. Rev. Mol. Cell Biol. 9 (2008) 112–124.
[8] M. Navab, S.T. Reddy, B.J. Van Lenten, A.M. Fogelman, HDL and cardiovascular disease: atherogenic and atheroprotective mechanisms, Nat. Rev. Cardiol. 8 (2011) 222–232.
[9] R. Martinez-Beamonte, J.M. Lou-Bonafonte, M.V. Martinez-Gracia, J. Osada, Sphingomyelin in high-density lipoproteins: structural role and biological function, Int. J. Mol. Sci. 14 (2013) 7716–7741.
[10] K.A. Rye, P.J. Barter, Cardioprotective functions of HDL, J. Lipid Res. 55 (2014) 168–179.
[11] E.Y. Cho, J.E. Manson, M.J. Stampfer, C.G. Solomon, G.A. Colditz, F.E. Speizer, W.C. Willett, F.B. Hu, A prospective study of obesity and risk of coronary heart disease among diabetic women, Diabetes Care 25 (2002) 1142–1148.
[12] D.S. Celermajer, C.K. Chow, E. Marijon, N.M. Anstey, K.S. Woo, Cardiovascular disease in the developing world, J. Am. Coll. Cardiol. 60 (2012) 1207–1216.
[13] M. Bastien, P. Poirier, I. Lemieux, J.-P. Després, Overview of epidemiology and contribution of obesity to cardiovascular disease, Prog. Cardiovasc. Dis. 56 (2014) 369–381.
[14] M. Boban, V. Persic, Z. Jovanovic, A. Brozina, B. Miletic, A. Rotim, N. Drinkovic, S. Manola, G. Laskarin, L. Boban, Obesity dilemma in the global burden of cardiovascular diseases, Int. J. Clin. Pract. 68 (2014) 173–179.
[15] A.M. Kanaya, D. Grady, E. Barrett-Connor, Explaining the sex difference in coronary heart disease mortality among patients with type 2 diabetes mellitus—a meta-analysis, Arch. Intern. Med. 162 (2002) 1737–1745.
[16] X.H. Qin, Y. Huo, D. Xie, F.F. Hou, X.P. Xu, X.B. Wang, Homocysteine-lowering therapy with folic acid is effective in cardiovascular disease prevention in patients with kidney disease: a meta-analysis of randomized controlled trials, Clin. Nutr. 32 (2013) 722–727.
[17] P. Mathieu, P. Pibarot, E. Larose, P. Poirier, A. Marette, J.P. Despres, Visceral obesity and the heart, Int. J. Biochem. Cell Biol. 40 (2008) 821–836.
[18] P. Codoner-Franch, S. Tavarez-Alonso, M. Porcar-Almela, M. Navarro-Solera, A. Arilla-Codoner, E. Alonso-Iglesias, Plasma resistin levels are associated with homocysteine, endothelial activation, and nitrosative stress in obese youths, Clin. Biochem. 47 (2014) 44–48.
[19] C. Mathers, J. Salomon, M. Ezzati, S. Begg, S. Vander-Hoorn, A. Lopez, Global Burden of Disease and Risk Factors, Oxford University Press, New York, NY, USA, 2006.
[20] A. Reis, A. Rudnitskaya, P. Chariyavilaskul, N. Dhaum, V. Melville, J. Goddard, D.J. Webb, A.R. Pitt, C.M. Spickett, Top-down lipidomics of low density lipoprotein reveal altered lipid profiles in advanced chronic kidney disease, J. Lipid Res. (2015), in press.
[21] G. Stubiger, E. Aldover-Macasaet, W. Bicker, G. Sobal, A. Willfort-Ehringer, K. Pock, V. Bochkov, K. Widhalm, O. Belgacem, Targeted profiling of atherogenic phospholipids in human plasma and lipoproteins of hyperlipidemic patients using MALDI-QIT-TOF-MS/MS, Atherosclerosis 224 (2012) 177–186.
[22] X.L. Han, R.W. Gross, Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples, Mass Spec. Rev. 24 (2005) 367–412.
[23] C. Papan, S. Penkov, R. Herzog, C. Thiele, T. Kurzchalia, A. Shevchenko, Systematic screening for novel lipids by shotgun lipidomics, Anal. Chem. 86 (2014) 2703–2710.
[24] B. Barroso, R. Bischoff, LC-MS analysis of phospholipids and lysophospholipids in human bronchoalveolar lavage fluid, J. Chromatogr. B 814 (2005) 21–28.
[25] M. Lísa, E. Cífková, M. Holčapek, Lipidomic profiling of biological tissues using off-line two-dimensional high-performance liquid chromatography–mass spectrometry, J. Chromatogr. A 1218 (2011) 5146–5156.
[26] E. Cífková, M. Holčapek, M. Lísa, M. Ovčačíková, A. Lyčka, F. Lynen, P. Sandra, Nontargeted quantitation of lipid classes using hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry with single internal standard and response factor approach, Anal. Chem. 84 (2012) 10064–10070.
[27] U. Sommer, H. Herscovitz, F.K. Welty, C.E. Costello, LC-MS-based method for the qualitative and quantitative analysis of complex lipid mixtures, J. Lipid Res. 47 (2006) 804–814.
[28] M. Pulfer, R.C. Murphy, Electrospray mass spectrometry of phospholipids, Mass Spec. Rev. 22 (2003) 332–364.
[29] P. Olsson, J. Holmback, B. Herslof, Separation of lipid classes by HPLC on a cyanopropyl column, Lipids 47 (2012) 93–99.
[30] P.M. Hutchins, R.M. Barkley, R.C. Murphy, Separation of cellular nonpolar neutral lipids by normal-phase chromatography and analysis by electrospray ionization mass spectrometry, J. Lipid Res. 49 (2008) 804–813.
[31] D.G. McLaren, P.L. Miller, M.E. Lassman, J.M. Castro-Perez, B.K. Hubbard, T.P. Roddy, An ultraperformance liquid chromatography method for the normal-phase separation of lipids, Anal. Biochem. 414 (2011) 266–272.
[32] M. Holčapek, E. Cífková, B. Červená, M. Lísa, J. Vostálová, J. Galuszka, Determination of nonpolar and polar lipid classes in human plasma, erythrocytes and plasma lipoprotein fractions using ultrahigh-performance liquid chromatography–mass spectrometry, J. Chromatogr. A 1377 (2015) 85–91.
[33] E.J. Ahn, H. Kim, B.C. Chung, G. Kong, M.H. Moon, Quantitative profiling of phosphatidylcholine and phosphatidylethanolamine in a steatosis/fibrosis model of rat liver by nanoflow liquid chromatography/tandem mass spectrometry, J. Chromatogr. A 1194 (2008) 96–102.
[34] O. Berdeaux, P. Juaneda, L. Martine, S. Cabaret, L. Bretillon, N. Acar, Identification and quantification of phosphatidylcholines containing very-long-chain polyunsaturated fatty acid in bovine and human retina using liquid chromatography/tandem mass spectrometry, J. Chromatogr. A 1217 (2010) 7738–7748.
[35] K. Sandra, A.D.S. Pereira, G. Vanhoenacker, F. David, P. Sandra, Comprehensive blood plasma lipidomics by liquid chromatography/quadrupole time-of-flight mass spectrometry, J. Chromatogr. A 1217 (2010) 4087–4099.
[36] Y. Sato, I. Suzuki, T. Nakamura, F. Bernier, K. Aoshima, Y. Oda, Identification of a new plasma biomarker of Alzheimer's disease using metabolomics technology, J. Lipid Res. 53 (2012) 567–576.
[37] M. Holčapek, H. Dvořáková, M. Lísa, A.J. Girón, P. Sandra, J. Cvačka, Regioisomeric analysis of triacylglycerols using silver-ion liquid chromatography atmospheric pressure chemical ionization mass spectrometry: comparison of five different mass analyzers, J. Chromatogr. A 1217 (2010) 8186–8194.
[38] M. Lísa, K. Netušilová, L. Franěk, H. Dvořáková, V. Vrkoslav, M. Holčapek, Characterization of fatty acid and triacylglycerol composition in animal fats using silver-ion and non-aqueous reversed-phase high-performance liquid chromatography/mass spectrometry and gas chromatography/flame ionization detection, J. Chromatogr. A 1218 (2011) 7499–7510.
[39] B. Fuchs, Analysis of phospolipids and glycolipids by thin-layer chromatography-matrix-assisted laser desorption and ionization mass spectrometry, J. Chromatogr. A 1259 (2012) 62–73.
[40] S. Wagner, K. Scholz, M. Sieber, M. Kellert, W. Voelkel, Tools in metabonomics: an integrated validation approach for LC-MS metabolic profiling of mercapturic acids in human urine, Anal. Chem. 79 (2007) 2918–2926.
[41] S. Wold, J. Trygg, A. Berglund, H. Antti, Some recent developments in PLS modeling, Chemom. Intell. Lab. Syst. 58 (2001) 131–150.
[42] L. Errikson, T. Byrne, E. Johansson, J. Trygg, C. Vikstrom, Multi- and Megavariate Data Analysis (Basic Principles and Application), Sweden, UMETRICS ACADEMY, Malmo, 2013.
[43] J. Trygg, S. Wold, Orthogonal projections to latent structures (O-PLS), J. Chemom. 16 (2002) 119–128.
[44] E.L. Donovan, S.M. Pettine, M.S. Hickey, K.L. Hamilton, B.F. Miller, Lipidomic analysis of human plasma reveals ether-linked lipids that are elevated in morbidly obese humans compared to lean, Diabetol. Metab. Syndr. 5 (2013) 24–38.
[45] R.J. Havel, H.A. Eder, J.H. Bragdon, Distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum, J. Clin. Invest. 34 (1955) 1345–1353.
[46] J. Folch, M. Lees, G.H.S. Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[47] M.C. Chambers, B. Maclean, R. Burke, D. Amodei, D.L. Ruderman, S. Neumann, L. Gatto, B. Fischer, B. Pratt, J. Egertson, K. Hoff, D. Kessner, N. Tasman, N. Shulman, B. Frewen, T.A. Baker, M.-Y. Brusniak, C. Paulse, D. Creasy, L. Flashner, K. Kani, C. Moulding, S.L. Seymour, L.M. Nuwaysir, B. Lefebvre, F. Kuhlmann, J. Roark, P. Rainer, S. Detlev, T. Hemenway, A. Huhmer, J. Langridge, B. Connolly, T. Chadick, K. Holly, J. Eckels, E.W. Deutsch, R.L. Moritz, J.E. Katz, D.B. Agus, M. MacCoss, D.L. Tabb, P. Mallick, A cross-platform toolkit for mass spectrometry and proteomics, Nat. Biotechnol. 30 (2012) 918–920.
[48] S. Gibb, K. Strimmer, MALDIquant A versatile R package for the analysis of mass spectrometry data, Bioinformatics 28 (2012) 2270–2271.
[49] E. Cífková, M. Holčapek, M. Lísa, Nontargeted lipidomic characterization of porcine organs using hydrophilic interaction liquid chromatography and off-line two-dimensional liquid chromatography-electrospray ionization mass spectrometry, Lipids 48 (2013) 915–928.
[50] I. Yang, K.H. Kim, J.Y. Lee, M.H. Moon, On-line miniaturized asymmetrical flow field-flow fractionation-electrospray ionization-tandem mass spectrometry with selected reaction monitoring for quantitative analysis of phospholipids in plasma lipoproteins, J. Chromatogr. A 1324 (2014) 224–230.
[51] S. Heimerl, M. Fischer, A. Baessler, G. Liebisch, A. Sigruener, S. Wallner, G. Schmitz, Alterations of plasma lysophosphatidylcholine species in obesity and weight loss, PLoS ONE 9 (2014) e111348–e111354.
[52] M.N. Barber, S. Risis, C. Yang, P.J. Meikle, M. Staples, M.A. Febbraio, C.R. Bruce, Plasma lysophosphatidylcholine levels are reduced in obesity and type 2 diabetes, PLoS ONE 7 (2012) e41456–e41467.
[53] A. Nimptsch, S. Pyttel, U. Paasch, C. Mohr, J.-M. Heinrich, J. Schiller, A MALDI MS investigation of the lysophosphatidyl-choline/phosphatidylcholine ratio in human spermatozoa and erythrocytes as a useful fertility marker, Lipids 49 (2014) 287–293.
[54] B. Fuchs, J. Schiller, U. Wagner, H. Häntzschel, K. Arnold, The phosphatidylcholine/lysophosphatidylcholine ratio in human plasma is an indicator of the severity of rheumatoid arthritis: Investigations by 31P NMR and MALDI-TOF MS, Clin. Biochem. 38 (2005) 925–933.

[55] S.K. Byeon, J.Y. Lee, S. Lim, D. Choi, M.H. Moon, Discovery of candidate phospholipid biomarkers in human lipoproteins with coronary artery disease by flow field-flow fractionation and nanoflow liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 1270 (2012) 246–253.
[56] I. Gonçalves, A. Edsfeldt, N.Y. Ko, H. Grufman, K. Berg, H. Björkbacka, M. Nitulescu, A. Persson, M. Nilsson, C. Prehn, J. Adamski, J. Nilsson, Evidence supporting a key role of Lp-PLA2-generated lysophosphatidylcholine in human atherosclerotic plaque inflammation, Arterioscler. Thromb. Vasc. Biol. 32 (2012) 1505–1512.
[57] M.J. Stampfer, R.M. Krauss, J. Ma, P.J. Blanche, L.G. Holl, F.M. Sacks, C.H. Hennekens, A prospective study of triglyceride level, low-density lipoprotein particle diameter, and risk of myocardial infarction, JAMA - J. Am. Med. Assoc. 276 (1996) 882–888.
[58] P.N. Hopkins, M. Nazeem Nanjee, L.L. Wu, M.G. McGinty, E.A. Brinton, S.C. Hunt, J.L. Anderson, Altered composition of triglyceride-rich lipoproteins and coronary artery disease in a large case-control study, Atherosclerosis 207 (2009) 559–566.