Neciljana lipidomika kod muškaraca s prekomjernom tjelesnom masom i kardiovaskularnim bolestima - utjecaj statusa pušenja

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FACULTY OF CHEMISTRY AND TECHNOLOGY
GRADUATE STUDY OF CHEMISTRY
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UNTARGETED LIPIDOMICS IN OVERWEIGHT MALES WITH CARDIOVASCULAR DISEASES -INFLUENCE OF SMOKING STATUS

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UNTARGETED LIPIDOMICS IN OVERWEIGHT MALES WITH CARDIOVASCULAR DISEASES -INFLUENCE OF SMOKING STATUS

Viktorija Jurić,285

Abstract:

Cigarette smoking is one of the main causes of the death and it is responsible for development of cardiovascular disease. One of the reason for the disease lies in the distruption of the serum lipid profile and lipoprotein levels. Lipids are biomolecules that are involved in the different biological processes. They are playing a key role in the membranes integrity and signaling pathways. It is important to identify lipid species in order to understand biological systems. Lipidomics is growing analytical technique and is currently in the centar of the attention of the scientific research due to the importance of lipids. The aim of this study was to evaluate the effect of cigarette smoking on lipid profile in overweight patients with already developed cardiovascular disease by liquid chromatography (LC) coupled online to mass spectrometry (MS). The overall observation was that, there was an increase in concentration of fatty acids, and some specific triglycerides (TG) and diglycerides (DG) and decrease in the concentration of colesteryl esters (both saturated and unsaturated) phosphatidylcholines (especially unsaturated) and lysophosphatidycolines (especially saturated), and triglycerides saturated in smokers compared to nonsmokers.Thus, it can be said based on the present study that smoking affects and deranges the lipid profile, but in patients with already existing cardiovascular diseases and many confounding factors smoking may not have such significant influence as before disease development

Keywords: cardiovascular disease, tobacco smoking, lipidomics, lipids

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3. Maša Buljac-PhD, Assistant Professor member - mentor

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Viktorija Jurić , 285

Sažetak:

Pušenje duhana odgovorno je za prerani razvoj kardiovaskularnih bolesti različitim mehanizmima, a abnormalne razine lipidnih seruma i razina lipoproteina jedna su od posljedica. Lipidi su biomolekule koje igraju vitalnu ulogu u raznim fiziopatologijama. Kako bi se otkrila funkcija lipida, od iznimne je važnosti identificirati i kvantificirati pojedinačne molekularne vrste lipida u složenim biološkim sustavima. Lipidomika je brzo razvijajuća analitička tehnika sposobna mjeriti stotine lipida i trenutno je na čelu znanstvenih istraživanja zbog važnosti lipida u zdravlju i bolesti. Cilj ove studije bio je procijeniti učinak pušenja cigareta na profil lipida u pacijenata s prekomjernom tjelesnom težinom s već razvijenom kardiovaskularnom bolešću pomoću tekuće kromatografije (LC) spojene online s masenom spektrometrijom (MS). Sveukupno zapažanje ove studije bilo je da je došlo do povećanja koncentracije masnih kiselina i nekih specifičnih triglicerida (TG) i diglicerida (DG) te do smanjenja koncentracije kolesterol estera (i zasićene i nezasićene) , fosfatidilkolina (osobito nezasićene) i lizofosfatidikolina (osobito zasićene), i TG zasićen u pušača u usporedbi s nepušačima. Stoga se na temelju ove studije može reći da pušenje utječe i narušava profil lipida, ali kod pacijenata s već postojećim kardiovaskularnim bolestima i mnogim dodatnim čimbenicima pušenje možda nema tako značajan utjecaj kao prije razvoja bolesti

Ključne riječi: kardiovaskularne bolesti, pušenje duhana, lipidomika, lipidi

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Viktorija

OBJECTIVES OF THE THESIS

Lipids are biomolecules that are involved in the cellular membrane structure and fluidity.

Lipids are extracted from serum samples by using a methanol, methyl tertbutyl ether and water the method for extration was described in Matyash et al.(2008).

The aim of this study was to evaluate the effect of cigarette smoking on lipid profile in overweight patients with already developed cardiovascular disease by liquid chromatography (LC) coupled online to mass spectrometry (MS).

SUMMARY

Cigarette smoking is one of the main causes of the death and it is responsible for development of cardiovascular disease. One of the reason for the disease lies in the distruption of the serum lipid profile and lipoprotein levels.

Lipids are biomolecules that are involved in the different biological processes. They are playing a key role in the cellular membranes integrity, energy sources, and signaling pathways.

It is important to identify lipid species in order to understand biological systems. Lipidomics is growing analytical technique and is currently in the centar of the attention of the scientific research due to the importance of lipids.

The aim of this study was to evaluate the effect of cigarette smoking on lipid profile in overweight patients with already developed cardiovascular disease by liquid chromatography (LC) coupled online to mass spectrometry (MS).

Lipids were extracted from serum samples by using methanol, methyl tertbutyl ether and water with the method that was explained in the in Matyash et al.(2008).

The overall observation of the present study was that, there was an increase in concentration of fatty acids, and some specific triglycerides (TG) and diglycerides (DG) and decrease in the concentration of colesteryl esters (both saturated and unsaturated) phosphatidylcholines (especially unsaturated) and lysophosphatidycolines (especially saturated), and triglycerides saturated in smokers compared to nonsmokers.

Thus, it can be said based on the present study that smoking affects and deranges the lipid profile, but in patients with already existing cardiovascular diseases and many confounding factors smoking may not have such significant influence as before disease development.

SAŽETAK

Pušenje je jedan od vodećih uzroka mortaliteta koji obično počinje u adolescenciji i nastavlja se u odrasloj dobi. Pušenje duhana odgovorno je za razvoj kardiovaskularnih bolesti, a abnormalni profil lipida i lipoproteina u serumu su glavni uzorak.

Lipidi su male biomolekule koje igraju važnu ulogu u raznim fizio-patološkim događajima, služeći kao sastavni dijelovi staničnih membrana, staničnih barijera, transdukcije signala, izvora energije i posrednika u signalnim putovima.

Kako bi se otkrila funkcija lipida, od iznimne je važnosti identificirati i kvantificirati pojedinačne molekularne vrste lipida u složenim biološkim sustavima.

Lipidomika je brzo razvijajuća analitička tehnika sposobna mjeriti stotine lipida i trenutno je glavna tema znanstvenih istraživanja zbog važnosti lipida u zdravlju i bolesti kod ljudi.

Cilj ove studije bio je procijeniti učinak pušenja cigareta na profil lipida u pacijenata s prekomjernom tjelesnom težinom s već razvijenom kardiovaskularnom bolešću pomoću tekuće kromatografije (LC) spojene online s masenom spektrometrijom (MS).

Lipidi su ekstrahirani iz uzoraka seruma korištenjem dvofaznog sustava otapala hladnog metanola, MTBE i vode.

Sveukupno zapažanje ove studije bilo je da je došlo do povećanja koncentracije masnih kiselina i nekih specifičnih triglicerida (TG) i diglicerida (DG) te do smanjenja koncentracije kolesterol estera (i zasićene i nezasićene) , fosfatidilkolina (osobito nezasićene) i lizofosfatidikolina (osobito zasićene), i TG zasićen u pušača u usporedbi s nepušačima. Stoga se na temelju ove studije može reći da pušenje utječe i narušava profil lipida, ali kod pacijenata s već postojećim kardiovaskularnim bolestima i mnogim dodatnim čimbenicima pušenje možda nema tako značajan utjecaj kao prije razvoja bolesti.

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LIST OF ABBREVIATIONS

CE	Cholesteryl ester
Cer_NS	Ceramide non-hydroxyfatty acid-sphingosine
CL	Cardiolipin
DG	Diacylglycerol
Ether PE	Ether-phosphatidylethanolamine
EtherOxPE	Ether oxidized phosphatidylethanolamine
EtherPC	Ether-phosphatidylcholine
FA	Fatty acyls
FAA	Free Fatty acid
GL	Glycerolipids
GLP	Glycerophospholipids
LPC	Lysophophatidylcholine
MG	Monoacylglycerol
NAE	N-acyl ethanolamines
OxTG	Oxidized triglyceride
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PK	Polyketides
PR	Prenol lipids
SL	Saccharolipids
SM	Sphingomyelin
SP	Sphingolipids
ST	Sterol lipids
TG	Triglycerides

INTRODUCTION

Smoking cigaretts significantly elevating the risk of a diferent range of health problems and it has been listed as one of the most common addictions that contributing to approximately 6 million deaths annually.[1]

Tobacco smoke is consistent of the compounds such as nicotine, carbon monoxide, benzene, tar and other harmful substances. These substances are a major risk factor for development of cardiovascular diseases like aortic atherosclerosis, cerebrovascular disease, coronary heart disease and peripheral artery disease.[1]

Nicotine and carbon monoxide may lead to alterations in serum lipids such as increases the free fatty acid, total cholesterol, triglycerides, LDL-C and lowers the level of HDL-C.[2] It has been shown that nicotine activates release of hormones catecholamines and triggers sympathetic adrenal system. From the liver to the bloodstream free fatty acids , TG and cholesterol are released.[3] This cascade can potentially lead to the development of cardiovascular disease. However, the influence of tobacco smoke on the human body and there influence on the lipids and lipid environment still remains not fully elucidated.

Lipidome is contained of the different lipid species that are found in a humans, animals and plants. Interaction of the polar headgroups and fatty acyl chains is defining lipids properties.[4] Lipids are small molecules that play a crutial role as a energy sources and signaling pathways. They are also important molecules that contribute to the integrity and consistency of cellular membranes. Lipid are also involved in the different physiological and pathological processes in the system.[5] To fully understand lipid function both health and disease, it is importan to identify, but also quantify lipid species. In this study we used analytical technique called lipidomics that can quantify numerous lipid species.[6]

The objective of this study was to ivestigate the effect of cigarette smoking on lipid profile in overweight patients with already developed cardiovascular disease by liquid chromatography (LC) coupled online to mass spectrometry (MS).

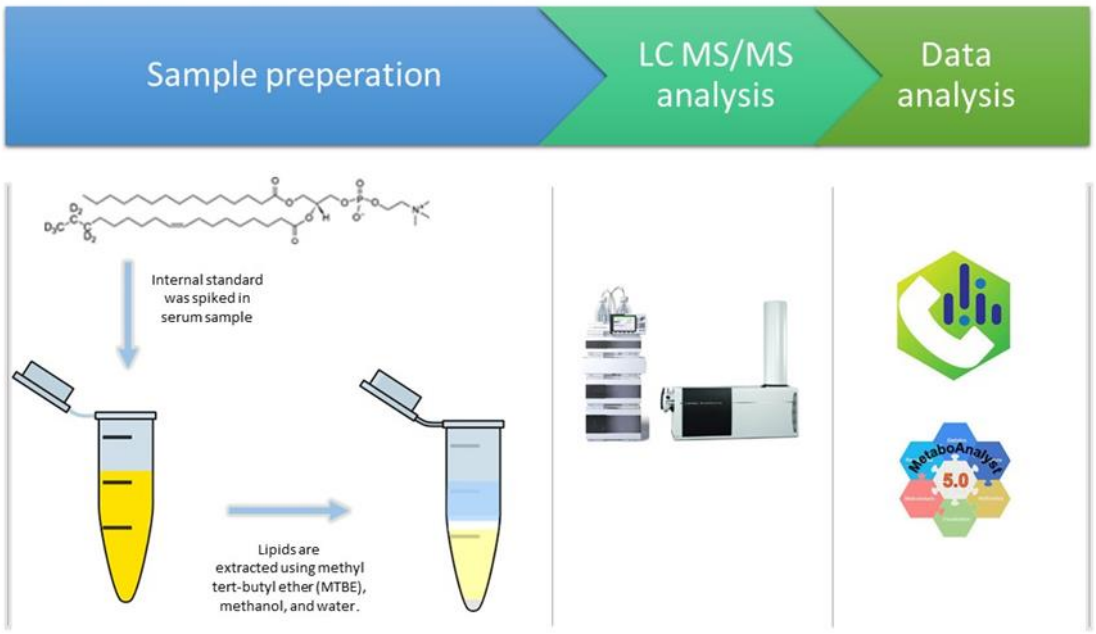


Figure 1. Schematic overview of the LC–MS-lipidomics workflow and identification of lipid species by using a open tool software MSDial combined with the integrated LipidBlast in-silico MS/MS library.[7]

1. LIPIDS IN BIOLOGICAL SYSTEMS

Lipids are vital small biomolecules that can have hydrophobic or amphipathic nature.[8] Combination of the polar head group and hydrophobic tails serves in formation of lipid bilayers. Different lipids are playing key roles in energy storage**,** hormone production, cellular membranes and cell signaling and cell metabolism.[9]

In biological system, lipids have diferent physiochemical and biological properties that come from variations in the fatty acyl chain length, double bond location, *cis-trans* geometric isomerism, the type of the covalent bond and type of the head groups.[3, 10]

Acording to the LIPID MAPS datebase lipids are classified in eight categories and they are listed and explained in the Table 1. [3]

Table 1. Lipid clases

Lipid class (abbr)	Characteristic
Fatty acyls (FA)	FA they are involved in many physiological processes and are composed of the hydrocarbon chain and carboxyl group. They can be bound to albumin or in a form of complex lipids. Additonaly they are involved in cell signaling also main source of the energy and structural support in the cells.[11]
Glycerolipids (GL)	GL they are components of the membrane structure, varying by organelle type and they serve as the energy storage.[12]
Glycerophospholipids (GPL)	GPLs located in biological membranes and contributing to the cellular integrity, function, fluidity and permeability. They have glycerol backbone and there polar head group linked with fatty acyl chains.[13]
Sphingolipids (SP)	SP are engaged in tissue growth, cell communication and they are involved in the cellular protection and immunity.[14]

Sterols (ST)	ST are the cell membrane components and they have an important role in membrane function by interacting with other proteins.[15]
Prenol lipids (PR)	PR are class of lipids contributing synthesis of carotenoids in some bacterias they are synthesized through mevalonic acid pathway. Some examples of the sterols are retionids and carotenoids.[16]
Saccharolipids (SL)	SL consist sugar backbone that is connected to a fatty acids this allows saccharolipids easy integrate in bilayers and with this it contributes to the stabilization of the membranes[17]
Polyketides (PK)	PK are found as the most abundant class of secondary metabolites produced by fungi and with the many biological activites, they are used in the medicine. [18,19]

2. MATERIALS AND METHODS

2.1. Reagent and Chemicals

SPLASH® LIPIDOMIX® Mass Spec Standard was acquired from Avanti Polar Lipids (Alabaster, AL, USA) while acetonitrile, prop-2-anol, methanol, chloroform, formic acid, ammonium acetate, and ammonium format were bought from Sigma-Aldrich (St. Louis, MO, USA).[20]

2.2. Sample collection

The research was carried out on 40 patients of the Clinic of Cardiology of the Wroclaw Medical University who were admitted to the hospital in 2013-2015 due to suspected myocardial infarction, which was finally excluded. They were overweight males (body mass index, BMI 27.47 ± 1.23), aged above 50 (56.80 ± 6.71) with at least one of the following cardiovascular disease: hypertension (50%), atherosclerosis (90%), stable coronary artery disease (50%), chronic heart failure (85%). Patients were routinely treated with hypotensive (angiotensin converting enzyme inhibitors), hypolipemic (statins) and antiplatelet drugs (acetylsalicylic acid). The presence of diabetes was an exclusion criteria. Based on the medical interview collected during admission to the hospital, patients were divided into smokers (S) and nonsmokers (NS). The detailed lipid profile of examined subjects is described in table 1 (data is presented as mean ± SD, the statistical significance of differences between S and NS is calculated by Mann-Whitney U test).

Table 2. Detailed lipid profile of examined subjects

Parameter	All (n=40)	Smokers (n=20)	Nonsmokers (n=20)	*P* value
Age [years]	56.80 ± 6.71	59.60 ± 8.41	54.00 ± 2.29	0.03
BMI	27.47 ±1.24	27.33 ± 1.31	27.60 ± 1.18	ns
Total cholesterol [mg/dL]	185.92 ± 53.01	157.25 ± 36.23	214.60 ± 52.19	<0.001
LDL [mg/dL]	111.05 ± 41.60	89.45 ± 29.68	132.65 ± 41.10	0,002
HDL [mg/dL]	42.62 ± 11.47	39.95 ± 11.74	45.30 ± 10.81	ns
Triglycerides [mg/dL]	159.72 ± 76.67	139.15 ± 55.13	180.30 ± 90.21	ns

2.3. Sample preparation

Solvent that were used for the extraction of the lipids from the human serum samples were cold methanol, methyl tertbutyl ether (MTBE), and water.

Firstly, serum samples were thawed at 4˚C and then vortexed. 20 µL of serum was aliquoted per sample and transferred into tubes. The extraction solvent contained MeOH: MTBE in the ratio 3:10.

Then, 975 µL of extraction solvent mixture was added to the each serum sample. Tubes with the samples containig the extration solvent were vortex for 30 seconds, and then shaken for 6 minutes at 4°C on the mixer. 5 µL of *SPLASH™ LIPIDOMIX®* internal standard, was added to each Eppendorf tube (Table 3).

Table 3. SPLASH™ LIPIDOMIX® Quantitative Mass Spec Internal Standard contains 14 lipid internal standards.[21]

Mixture Component	Chemical Formula	Target Conc. µg/mL	Target Conc. µM	Exact Mass	M-H	M+H	$M+NH_4$	M+AcO
15:0-18:1(d7) PC	$C_{41}H_{73}D_7NO_8P$	160.7	213	752.6061	×	753.6134	×	811.6199
18:1(d7) Lyso PC	$C_{26}H_{45}D_7NO_7P$	25.5	48	528.3921	×	529.3994	×	587.4059
15:0-18:1(d7) PE	$C_{38}H_{67}D_7NO_8P$	5.7	8	710.5591	709.5519	711.5664	×	×
18:1(d7) Lyso PE	$C_{23}H_{39}D_7NO_7P$	5.3	11	486.3451	485.3379	487.3524	×	×
15:0-18:1(d7) PG	$C_{39}H_{68}D_7O_{10}P$	29.1	38	741.5537	740.5464	×	759.5875	×
15:0-18:1(d7) PI	$C_{42}H_{72}D_7O_{13}P$	9.1	11	829.5698	828.5625	×	847.6036	×
15:0-18:1(d7) PS	$C_{39}H_{67}D_7NO_{10}P$	4.2	5	754.5490	753.5417	755.5562	×	×
15:0-18:1(d7)-15:0 TAG	$C_{51}H_{89}D_7O_6$	57.3	71	811.7646	×	×	829.7985	×
15:0-18:1(d7) DAG	$C_{36}H_{61}D_7O_5$	9.4	16	587.5506	×	×	605.5844	×
18:1(d7) MAG	$C_{21}H_{33}D_7O_4$	2	6	363.3366	×	364.3429	381.3704	422.3504
18:1(d7) Chol Ester	$C_{45}H_{71}D_7O_2$	356.1	541	657.6441	×	×	675.6779	×
d18:1-18:1(d9) SM	$C_{41}H_{72}D_9N_2O_6P$	30.9	42	737.6397	×	738.6470	×	796.6536
15:0-18:1(d7) PA	$C_{36}H_{61}D_7NaO_8P$	7.4	11	667.5181	666.5097	×	×	×
Cholesterol-d7	$C_{27}H_{39}D_7O$	98.4	248	393.3988	×	394.4061	411.4326	

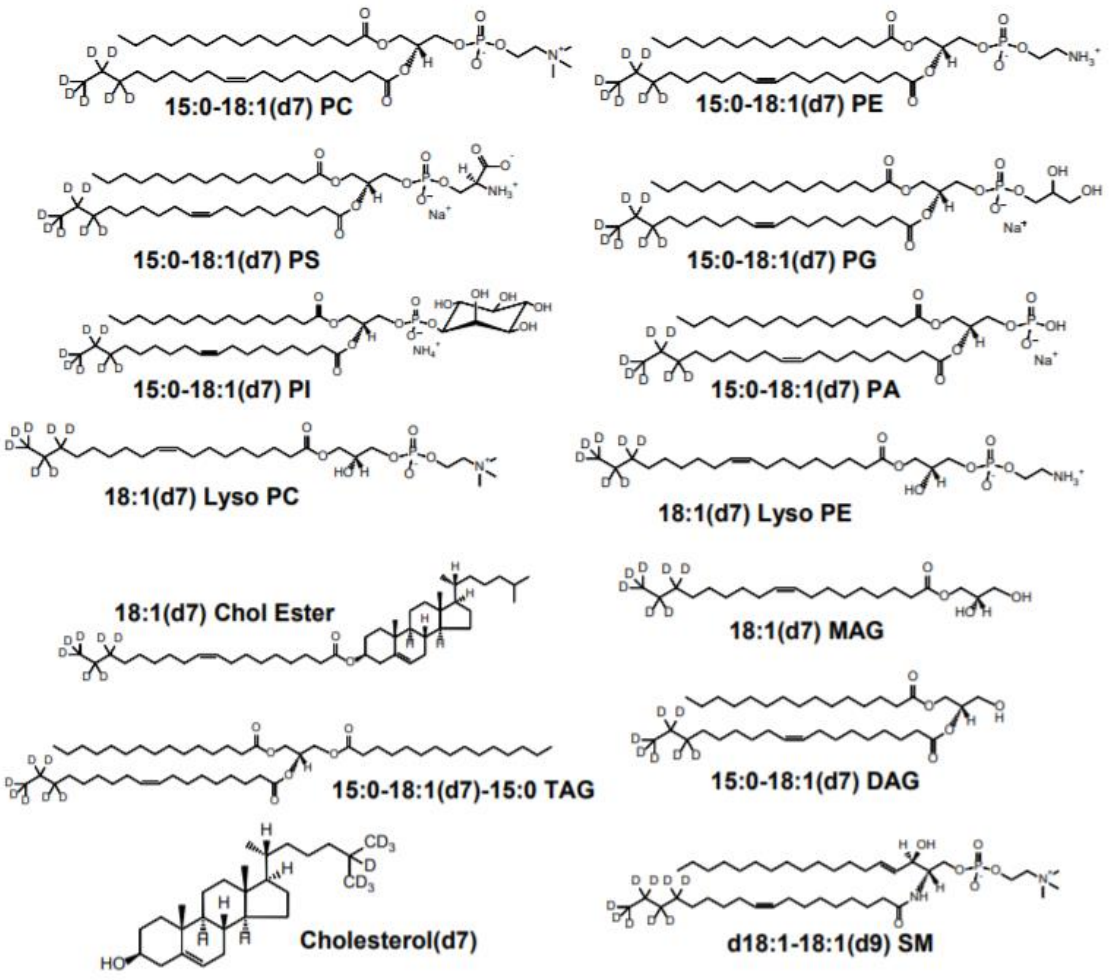


Figure 2. Chemical structure of 14 deuterated lipid internal standards contained in SPLASH LipidoMIX™ Internal Standards[22]

To separte organic and aqueous phase 188 µL LC-MS grade water was added to the each tube. Samples were vortex for 20 seconds and then centrifuge @ 14,000 rcf (12300 rpm) for 2 minutes. The separated upper organic phase contains non-polar lipids, while the bottom aqueous phase contains more polar metabolites. For untargeted LC-MS/MS lipidomics analysis 350 µL of the upper organic phase was transferred to two separate vials and the samples were dried down under the nitrogen steam. Dried samples were immediately stored at -80˚C until the analysis.[23,24]

Samples that were previously dried down under the nitrogen steam were resuspended in resuspension solution MeOH: Toluen (9:1). Resuspended serum samples were then

vortex for 20 seconds. After the vortexing they were sonicated for 5 minutes, and then centrifuge for 2 minutes @ 16,100 g on the room temeperature. Resuspended aliquots of the lipids were transferred to the inserts and subjected to analysis.[23,24]

2.4. LC MS Analaysis

Mass spectrometry detection (ESI-Q-TOF, model Compact, Bruker Daltonics, Germany) was made in positive and negative ion mode with auto MSMS measurements.

Calibration of the instrument was done with solution of sodium formate in a HPLC calibration method in the scan range from 100 to 1700 m/z. Nitrogen drying gas was set to 2.0 bar and flow rate was set to 8 L/min. The capillary temperature was set at 210°C and spray voltage was 4.5 kV.

All serum samples were messured in positive and negative mode with ESI electrospray ionization.[24]

Column that was used for untargeted lipidomics analysis was Acquity UPLC CSH C18 (100 × 2.1 mm; 1.7 μm) with the Acquity UPLC CSH C18 VanGuard precolumn (5 × 2.1 mm; 1.7 μm) (Waters, Milford, MA). Experimental conditions were set as folow temperature of the column was 65 °C and flow-rate was 0.4 mL/min. Mobile phases and the gradient that was used for untargeted lipidomics analysis were described in Cajka et.al.[24]

2.5. Quality control

Quality control (QC) for the LC-MS mesurments was assured by the injection of the pool (QC) sample after every 10 injection. Pool (QC) sample was done by pooling 5 μL aliqoute from each vial. Then by randomization of the smaple sequence and injection of 3 pooled serum samples to equilibrate the LC−MS system before the sample sequence To assure that there is no caryover blank samples that have resuspension solution MeOH: Tol (9:1) were injected before and after every pool (QC) injection.[24]

2.6. Data Processing

Raw .d files from Bruker were first converted to ABF format by using Reifycs Abf (Analysis Base File) Converter (http://www.reifycs.com/AbfConverter /).[24]

Then raw .d Bruker files were porcesed in MS-DIAL (v. 4.48) software. Identification of the lipid species was done with the integrated LipidBlast library.[24]

Normalization with internal standards was done in MSDial and the it was reported as a concentrations (µmol/ml).

$$c(lipid) = c(IS) \cdot \frac{h(lipid)}{h(IS)}$$

c(lipids)- lipids concentration

c(IS)- Internal Standard concentration

h(lipids)-peak height of lipids

h(IS)- peak height of Splash internal standard

In the case of isomeric lipid species, the sum of their area were calculated beacuse determination of the exact stereochemistry could not be resolve with used LC-MS instrument.[25]

Data was then filtered for blank samples caryover if the signal in the blank was higer then the fold change >10 this feature was removed from the further analysis. Lipid species that had a coefficient of variation (CV%) ≥30% in the pool (QC) smaples were also excluded from the further statistical analysis.[26]

Data was exported from the MSDial in the Excel file and filtered according to following manner: rev dot product ≥700 and the dot product ≥350 all the species that were below this values were excluded from the list.

2.7. Statistical and Data Analysis

Statistical differences in lipid metabolisms between overweight smokers and non-smokers, were compared by t-test with GraphPad Prism 7.0 (GraphPad Software, Inc, La Jolla, CA, USA).[25]

First, outliers were removed by using the GraphPad Prism. Data tables with the lipids identified under the smokers and non-smokers detected in positive and negative set were uploaded to the MetaboAnalyst server (version 4.0).[27,28]

In MetaboAnalyst, data was processed in the following mannare: row wise normalization to constant sum, log transformation (base 10) and Pareto scaling was applied. Univariate analysis methods were used to compare overweight smokers and overweight non-smokers. Fold Change (FC) analysis, t-tests method, PCA and PLS-DA analysis and cluster analysis (heatmaps) were also preformed by using MetaboAnalyst.

3. RESULTS

In examined cohort of overweight participants 232 lipid were annotated and detailed list of detected compounds is provided in table 4.

Table 4. List of annotated and detected compounds

Average Rt(min)	Average Mz	Metabolite name	Adduct type	Fill %	Formula	Ontology
13.9	640.6	CE 16:1	[M+NH4]+	0.824	C43H74O2	CE
14.5	670.7	CE 18:0	[M+NH4]+	0.809	C45H80O2	CE
14.2	668.6	CE 18:1	[M+NH4]+	0.824	C45H78O2	CE
13.9	666.6	CE 18:2	[M+NH4]+	0.824	C45H76O2	CE
13.6	664.6	CE 18:3	[M+NH4]+	0.824	C45H74O2	CE
13.7	690.6	CE 20:4	[M+NH4]+	0.824	C47H76O2	CE
13.4	688.6	CE 20:5	[M+NH4]+	0.824	C47H74O2	CE
13.5	714.6	CE 22:6	[M+NH4]+	0.824	C49H76O2	CE
12.8	714.6	CE 22:6	[M+NH4]+	0.765	C49H76O2	CE
12.2	632.6	Cer 42:1;2O|Cer 18:1;2O/24:0	[M+H-H2O]+	0.809	C42H83NO3	Cer_NS
12.2	650.6	Cer 42:1;2O|Cer 18:1;2O/24:0	[M+H]+	0.809	C42H83NO3	Cer_NS
12.2	694.6	Cer 42:1;2O|Cer 18:1;2O/24:0	[M+HCOO]-	0.902	C42H83NO3	Cer_NS
12.2	694.6	Cer 42:1;2O|Cer 18:1;2O/24:0	[M+HCOO]-	0.902	C42H83NO3	Cer_NS
11.6	692.6	Cer 42:2;2O|Cer 18:1;2O/24:1	[M+HCOO]-	0.118	C42H81NO3	Cer_NS
11.6	692.6	Cer 42:2;2O|Cer 18:1;2O/24:1	[M+HCOO]-	0.745	C42H81NO3	Cer_NS
11.6	692.6	Cer 42:2;2O|Cer 18:1;2O/24:1	[M+HCOO]-	0.118	C42H81NO3	Cer_NS
11.6	692.6	Cer 42:2;2O|Cer 18:1;2O/24:1	[M+HCOO]-	0.745	C42H81NO3	Cer_NS
8.1	531.4	DG 28:2	[M+Na]+	1	C31H56O5	DG
9.3	563.5	DG 30:0	[M+Na]+	1	C33H64O5	DG
8.1	553.4	DG 30:5	[M+Na]+	1	C33H54O5	DG
3.2	551.4	DG 30:6	[M+Na]+	0.809	C33H52O5	DG
10.7	591.5	DG 32:0	[M+Na]+	1	C35H68O5	DG
10.1	589.5	DG 32:1	[M+Na]+	0.794	C35H66O5	DG
11.4	619.5	DG 34:0	[M+Na]+	1	C37H72O5	DG
10.8	617.5	DG 34:1	[M+Na]+	0.824	C37H70O5	DG
10.1	615.5	DG 34:2	[M+Na]+	0.574	C37H68O5	DG
12.0	647.6	DG 36:0	[M+Na]+	0.971	C39H76O5	DG
11.5	645.5	DG 36:1	[M+Na]+	0.779	C39H74O5	DG

10.8	643.5	DG 36:2	[M+Na]+	0.824	C39H72O5	DG
10.8	638.6	DG 36:2|DG 18:1_18:1	[M+NH4]+	0.824	C39H72O5	DG
10.3	641.5	DG 36:3	[M+Na]+	0.824	C39H70O5	DG
11.3	647.5	DG 37:7	[M+Na]+	1	C40H64O5	DG
14.5	675.6	DG 38:0	[M+Na]+	0.809	C41H80O5	DG
10.1	665.5	DG 38:5	[M+Na]+	0.794	C41H70O5	DG
11.3	669.4	DG 39:10	[M+Na]+	1	C42H62O5	DG
12.2	699.6	DG 40:2	[M+Na]+	1	C43H80O5	DG
12.1	705.5	DG 41:6	[M+Na]+	0.044	C44H74O5	DG
13.1	721.6	DG 42:5	[M+Na]+	0.5	C45H78O5	DG
12.2	721.6	DG 42:5	[M+Na]+	1	C45H78O5	DG
12.8	719.6	DG 42:6	[M+Na]+	0.809	C45H76O5	DG
12.7	743.6	DG 44:8	[M+Na]+	0.647	C47H76O5	DG
12.2	757.6	DG 45:8	[M+Na]+	0.029	C48H78O5	DG
12.4	835.6	DG 51:11	[M+Na]+	0.015	C54H84O5	DG
6.8	283.3	FA 18:0	[M-H]-	0.902	C18H36O2	FA
6.1	281.2	FA 18:1	[M-H]-	0.804	C18H34O2	FA
5.3	279.2	FA 18:2	[M-H]-	0.549	C18H32O2	FA
9.0	409.3	FA 28:7	[M-H]-	0.02	C28H42O2	FA
2.6	518.3	LPC 16:0	[M+Na]+	0.809	C24H50NO7P	LPC
2.9	518.3	LPC 16:0_1	[M+Na]+	0.824	C24H50NO7P	LPC
2.6	540.3	LPC 16:0_2	[M+HCOO]-	0.824	C24H50NO7P	LPC
4.8	546.4	LPC 18:0	[M+Na]+	0.809	C26H54NO7P	LPC
4.7	568.4	LPC 18:0	[M+HCOO]-	0.804	C26H54NO7P	LPC
4.8	568.4	LPC 18:0	[M+HCOO]-	0.098	C26H54NO7P	LPC
3.3	544.3	LPC 18:1	[M+Na]+	0.809	C26H52NO7P	LPC
3.3	566.3	LPC 18:1	[M+HCOO]-	0.078	C26H52NO7P	LPC
3.3	566.3	LPC 18:1	[M+HCOO]-	0.824	C26H52NO7P	LPC
2.3	542.3	LPC 18:2	[M+Na]+	0.809	C26H50NO7P	LPC
2.3	564.3	LPC 18:2	[M+HCOO]-	0.118	C26H50NO7P	LPC
2.2	564.3	LPC 18:2	[M+HCOO]-	0.784	C26H50NO7P	LPC
2.2	566.3	LPC 20:4	[M+Na]+	0.75	C28H50NO7P	LPC
5.7	408.3	MG 21:5	[M+NH4]+	0.853	C24H38O4	MG
5.1	376.3	NAE 22:4	[M+H]+	0.618	C24H41NO2	NAE
3.8	376.3	NAE 22:4	[M+H]+	0.588	C24H41NO2	NAE
4.3	374.3	NAE 22:5	[M+H]+	0.059	C24H39NO2	NAE

4.0	374.3	NAE 22:5	[M+H]+	0.044	C24H39NO2	NAE
4.1	374.3	NAE 22:5	[M+H]+	0.471	C24H39NO2	NAE
7.4	428.4	NAE 26:6	[M+H]+	1	C28H45NO2	NAE
9.4	778.6	PC 32:0|PC 16:0_16:0	[M+HCOO]-	0.902	C40H80NO8P	PC
9.5	782.6	PC 34:1	[M+Na]+	0.824	C42H82NO8P	PC
9.1	780.6	PC 34:2	[M+Na]+	0.029	C42H80NO8P	PC
9.1	780.6	PC 34:2	[M+Na]+	0.029	C42H80NO8P	PC
8.9	802.6	PC 34:2	[M+HCOO]-	0.118	C42H80NO8P	PC
8.9	802.6	PC 34:2	[M+HCOO]-	0.784	C42H80NO8P	PC
8.9	780.6	PC 34:2|PC 0:0_34:2	[M+Na]+	0.824	C42H80NO8P	PC
8.2	800.5	PC 34:3	[M+HCOO]-	0.098	C42H78NO8P	PC
9.3	816.6	PC 35:2|PC 17:0_18:2	[M+HCOO]-	0.235	C43H82NO8P	PC
10.2	832.6	PC 36:1|PC 18:0_18:1	[M+HCOO]-	0.078	C44H86NO8P	PC
10.2	832.6	PC 36:1|PC 18:0_18:1	[M+HCOO]-	0.824	C44H86NO8P	PC
9.0	808.6	PC 36:2	[M+Na]+	0.794	C44H84NO8P	PC
9.6	808.6	PC 36:2	[M+Na]+	0.824	C44H84NO8P	PC
9.6	830.6	PC 36:2|PC 18:0_18:2	[M+HCOO]-	0.902	C44H84NO8P	PC
9.1	806.6	PC 36:3	[M+Na]+	0.824	C44H82NO8P	PC
9.3	828.6	PC 36:3|PC 18:0_18:3	[M+HCOO]-	0.118	C44H82NO8P	PC
9.1	828.6	PC 36:3|PC 18:1_18:2	[M+HCOO]-	0.902	C44H82NO8P	PC
8.8	828.6	PC 36:3|PC 18:1_18:2	[M+HCOO]-	0.804	C44H82NO8P	PC
9.0	828.6	PC 36:3|PC 18:1_18:2	[M+HCOO]-	0.745	C44H82NO8P	PC
9.0	828.6	PC 36:3|PC 18:1_18:2	[M+HCOO]-	0.098	C44H82NO8P	PC
8.8	804.6	PC 36:4	[M+Na]+	0.824	C44H80NO8P	PC
8.8	826.6	PC 36:4	[M+HCOO]-	0.902	C44H80NO8P	PC
8.4	826.6	PC 36:4|PC 18:2_18:2	[M+HCOO]-	0.078	C44H80NO8P	PC
8.4	826.6	PC 36:4|PC 18:2_18:2	[M+HCOO]-	0.569	C44H80NO8P	PC
8.3	802.5	PC 36:5	[M+Na]+	0.824	C44H78NO8P	PC
8.3	824.5	PC 36:5|PC 16:0_20:5	[M+HCOO]-	0.902	C44H78NO8P	PC
9.8	836.6	PC 38:2	[M+Na]+	0.765	C46H88NO8P	PC
9.8	834.6	PC 38:3	[M+Na]+	0.824	C46H86NO8P	PC
9.8	856.6	PC 38:3|PC 18:0_20:3	[M+HCOO]-	0.902	C46H86NO8P	PC
9.3	854.6	PC 38:4|PC 18:0_20:4	[M+HCOO]-	0.412	C46H84NO8P	PC
9.5	854.6	PC 38:4|PC 18:0_20:4	[M+HCOO]-	0.902	C46H84NO8P	PC
9.0	852.6	PC 38:5|PC 18:0_20:5	[M+HCOO]-	0.902	C46H82NO8P	PC
8.6	852.6	PC 38:5|PC 18:1_20:4	[M+HCOO]-	0.902	C46H82NO8P	PC

8.8	852.6	PC 38:5|PC 18:1_20:4	[M+HCOO]-	0.902	C46H82NO8P	PC
8.6	828.6	PC 38:6	[M+Na]+	0.824	C46H80NO8P	PC
8.6	850.6	PC 38:6	[M+HCOO]-	0.902	C46H80NO8P	PC
9.3	856.6	PC 40:6	[M+Na]+	0.824	C48H84NO8P	PC
9.3	878.6	PC 40:6|PC 18:0_22:6	[M+HCOO]-	0.804	C48H84NO8P	PC
9.3	878.6	PC 40:6|PC 18:0_22:6	[M+HCOO]-	0.098	C48H84NO8P	PC
9.5	804.6	PC O-34:2;1O|PC O-17:0_17:2;1O	[M+HCOO]-	0.902	C42H82NO8P	EtherOxPC
9.2	812.6	PC O-36:4|PC O-16:0_20:4	[M+HCOO]-	0.902	C44H82NO7P	EtherPC
9.2	838.6	PC O-38:5|PC O-18:1_20:4	[M+HCOO]-	0.882	C46H84NO7P	EtherPC
10.4	746.6	PE 36:1	[M+H]+	0.471	C41H80NO8P	PE
9.8	742.5	PE 36:2|PE 18:0_18:2	[M-H]-	0.745	C41H78NO8P	PE
9.7	744.6	PE 36:2|PE 18:1_18:1	[M+H]+	0.132	C41H78NO8P	PE
9.7	766.5	PE 38:4|PE 18:0_20:4	[M-H]-	0.902	C43H78NO8P	PE
9.7	768.6	PE 38:4|PE 19:2_19:2	[M+H]+	0.809	C43H78NO8P	PE
9.3	722.5	PE O-36:5|PE O-16:1_20:4	[M-H]-	0.686	C41H74NO7P	EtherPE
10.0	750.5	PE O-38:5|PE O-18:1_20:4	[M-H]-	0.824	C43H78NO7P	EtherPE
9.3	748.5	PE O-38:6|PE O-18:2_20:4	[M-H]-	0.627	C43H76NO7P	EtherPE
10.0	752.6	PE P-38:4|PE P-18:0_20:4	[M+H]+	0.809	C43H78NO7P	EtherPE
9.0	885.5	PI 38:4|PI 18:0_20:4	[M-H]-	0.902	C47H83O13P	PI
7.9	697.5	SM 32:1;2O	[M+Na]+	0.809	C37H75N2O6P	SM
8.7	725.6	SM 34:1;2O	[M+Na]+	0.824	C39H79N2O6P	SM
8.7	747.6	SM 34:1;2O|SM 18:1;2O/16:0	[M+HCOO]-	0.804	C39H79N2O6P	SM
8.0	723.5	SM 34:2;2O	[M+Na]+	0.809	C39H77N2O6P	SM
8.0	745.5	SM 34:2;2O	[M+HCOO]-	0.765	C39H77N2O6P	SM
10.9	809.7	SM 40:1;2O	[M+Na]+	0.824	C45H91N2O6P	SM
11.2	823.7	SM 41:1;2O	[M+Na]+	0.809	C46H93N2O6P	SM
11.5	837.7	SM 42:1;2O	[M+Na]+	0.809	C47H95N2O6P	SM
10.8	835.7	SM 42:2;2O	[M+Na]+	0.824	C47H93N2O6P	SM
10.8	857.7	SM 42:2;2O|SM 18:1;2O/24:1	[M+HCOO]-	0.902	C47H93N2O6P	SM
11.5	656.6	TG 36:0|TG 10:0_12:0_14:0	[M+NH4]+	0.118	C39H74O6	TG

12.1	684.6	TG 38:0|TG 10:0_12:0_16:0	[M+NH4]+	0.382	C41H78O6	TG
8.0	701.6	TG 39:1|TG 10:0_16:0_13:1	[M+Na]+	0.824	C42H78O6	TG
12.7	712.6	TG 40:0|TG 10:0_14:0_16:0	[M+NH4]+	0.662	C43H82O6	TG
12.2	710.6	TG 40:1|TG 10:0_12:0_18:1	[M+NH4]+	0.103	C43H80O6	TG
13.2	740.7	TG 42:0|TG 12:0_14:0_16:0	[M+NH4]+	0.838	C45H86O6	TG
12.7	738.7	TG 42:1|TG 8:0_16:0_18:1	[M+NH4]+	0.588	C45H84O6	TG
12.2	736.6	TG 42:2|TG 12:0_12:0_18:2	[M+NH4]+	0.044	C45H82O6	TG
13.6	768.7	TG 44:0|TG 14:0_14:0_16:0	[M+NH4]+	1	C47H90O6	TG
13.2	766.7	TG 44:1|TG 12:0_14:0_18:1	[M+NH4]+	0.809	C47H88O6	TG
12.7	764.7	TG 44:2|TG 12:0_14:0_18:2	[M+NH4]+	0.647	C47H86O6	TG
12.3	762.7	TG 44:3|TG 12:0_14:0_18:3	[M+NH4]+	0.044	C47H84O6	TG
10.9	787.7	TG 45:0|TG 11:0_11:0_23:0	[M+Na]+	0.824	C48H92O6	TG
13.8	782.7	TG 45:0|TG 14:0_15:0_16:0	[M+NH4]+	1	C48H92O6	TG
14.0	796.7	TG 46:0|TG 14:0_16:0_16:0	[M+NH4]+	0.971	C49H94O6	TG
10.5	799.7	TG 46:1|TG 12:0_12:0_22:1	[M+Na]+	0.397	C49H92O6	TG
13.6	794.7	TG 46:1|TG 12:0_16:0_18:1	[M+NH4]+	0.838	C49H92O6	TG
13.2	792.7	TG 46:2|TG 12:0_16:0_18:2	[M+NH4]+	0.824	C49H90O6	TG
12.8	790.7	TG 46:3|TG 10:0_18:1_18:2	[M+NH4]+	0.485	C49H88O6	TG
12.8	790.7	TG 46:3|TG 12:0_16:0_18:3	[M+NH4]+	0.162	C49H88O6	TG
11.1	815.7	TG 47:0|TG 13:0_13:0_21:0	[M+Na]+	0.765	C50H96O6	TG
11.5	815.7	TG 47:0|TG 14:0_14:0_19:0	[M+Na]+	0.824	C50H96O6	TG
14.1	810.8	TG 47:0|TG 15:0_16:0_16:0	[M+NH4]+	1	C50H96O6	TG
10.8	813.7	TG 47:1|TG 9:0_11:0_27:1	[M+Na]+	0.824	C50H94O6	TG
14.0	822.8	TG 48:1|TG 14:0_16:0_18:1	[M+NH4]+	1	C51H96O6	TG
13.6	820.7	TG 48:2|TG 14:0_16:0_18:2	[M+NH4]+	0.824	C51H94O6	TG
13.2	820.7	TG 48:2|TG 14:0_16:1_18:1	[M+NH4]+	0.765	C51H94O6	TG
13.2	818.7	TG 48:3|TG 12:0_18:1_18:2	[M+NH4]+	0.824	C51H92O6	TG
12.8	816.7	TG 48:4|TG 12:0_18:2_18:2	[M+NH4]+	0.662	C51H90O6	TG
12.4	814.7	TG 48:5|TG 12:0_18:2_18:3	[M+NH4]+	0.029	C51H88O6	TG
13.9	838.8	TG 49:0|TG 16:0_16:0_17:0	[M+NH4]+	0.059	C52H100O6	TG
14.3	838.8	TG 49:0|TG 16:0_16:0_17:0	[M+NH4]+	1	C52H100O6	TG
14.1	836.8	TG 49:1|TG 15:0_16:0_18:1	[M+NH4]+	1	C52H98O6	TG
13.8	834.8	TG 49:2|TG 15:0_16:0_18:2	[M+NH4]+	0.824	C52H96O6	TG
14.4	852.8	TG 50:0|TG 16:0_16:0_18:0	[M+NH4]+	1	C53H102O6	TG
14.1	852.8	TG 50:0|TG 9:0_19:0_22:0	[M+NH4]+	0.059	C53H102O6	TG
14.0	850.8	TG 50:1|TG 16:0_16:0_18:1	[M+NH4]+	0.824	C53H100O6	TG

14.2	850.8	TG 50:1|TG 16:0_16:0_18:1	[M+NH4]+	1	C53H100O6	TG
14.0	848.8	TG 50:2|TG 16:0_16:1_18:1	[M+NH4]+	1	C53H98O6	TG
13.7	846.8	TG 50:3|TG 14:0_18:1_18:2	[M+NH4]+	0.824	C53H96O6	TG
13.3	844.7	TG 50:4|TG 16:1_16:1_18:2	[M+NH4]+	0.824	C53H94O6	TG
12.9	842.7	TG 50:5|TG 14:0_18:2_18:3	[M+NH4]+	0.515	C53H92O6	TG
13.1	842.7	TG 50:5|TG 16:0_16:1_18:4	[M+NH4]+	0.088	C53H92O6	TG
14.3	864.8	TG 51:1|TG 16:0_17:0_18:1	[M+NH4]+	0.824	C54H102O6	TG
14.1	862.8	TG 51:2|TG 16:0_17:1_18:1	[M+NH4]+	0.838	C54H100O6	TG
13.8	860.8	TG 51:3|TG 15:0_18:1_18:2	[M+NH4]+	0.824	C54H98O6	TG
14.4	878.8	TG 52:1|TG 16:0_18:0_18:1	[M+NH4]+	1	C55H104O6	TG
14.2	878.8	TG 52:1|TG 16:0_18:0_18:1	[M+NH4]+	0.824	C55H104O6	TG
14.0	876.8	TG 52:2|TG 16:0_18:1_18:1	[M+NH4]+	0.824	C55H102O6	TG
14.2	876.8	TG 52:2|TG 16:0_18:1_18:1	[M+NH4]+	1	C55H102O6	TG
12.7	890.8	TG 52:3;1O |TG 16:0_18:1 _18:2;1O	[M+NH4]+	0.059	C55H100O7	OxTG
13.0	922.8	TG 52:3;3O|TG 17:1_17:1_ 18:1;3O	[M+NH4]+	0.309	C55H100O9	OxTG
14.0	874.8	TG 52:3|TG 16:0_18:1_18:2	[M+NH4]+	1	C55H100O6	TG
13.4	872.8	TG 52:4|TG 16:0_18:2_18:2	[M+NH4]+	0.779	C55H98O6	TG
13.7	872.8	TG 52:4|TG 16:1_18:1_18:2	[M+NH4]+	0.824	C55H98O6	TG
13.5	870.8	TG 52:5|TG 16:0_16:1_20:4	[M+NH4]+	0.103	C55H96O6	TG
13.4	870.8	TG 52:5|TG 16:0_18:2_18:3	[M+NH4]+	0.824	C55H96O6	TG
13.2	868.7	TG 52:6|TG 16:0_18:2_18:4	[M+NH4]+	0.176	C55H94O6	TG
13.0	868.7	TG 52:6|TG 16:1_18:2_18:3	[M+NH4]+	0.074	C55H94O6	TG
12.9	868.7	TG 52:6|TG 16:1_18:2_18:3	[M+NH4]+	0.044	C55H94O6	TG
13.1	868.7	TG 52:6|TG 16:1_18:2_18:3	[M+NH4]+	0.471	C55H94O6	TG
12.9	866.7	TG 52:7|TG 16:0_18:3_18:4	[M+NH4]+	0.176	C55H92O6	TG
14.5	892.8	TG 53:1|TG 17:0_18:0_18:1	[M+NH4]+	0.676	C56H106O6	TG
14.3	890.8	TG 53:2|TG 17:0_18:1_18:1	[M+NH4]+	0.824	C56H104O6	TG
14.1	888.8	TG 53:3|TG 17:0_18:1_18:2	[M+NH4]+	0.824	C56H102O6	TG
10.4	906.8	TG 54:1|TG 18:0_18:0_18:1	[M+NH4]+	0.044	C57H108O6	TG
10.1	906.8	TG 54:1|TG 18:0_18:0_18:1	[M+NH4]+	0.015	C57H108O6	TG
10.5	906.8	TG 54:1|TG 18:0_18:0_18:1	[M+NH4]+	0.015	C57H108O6	TG
14.2	904.8	TG 54:2|TG 18:0_18:1_18:1	[M+NH4]+	0.882	C57H106O6	TG
14.4	904.8	TG 54:2|TG 18:0_18:1_18:1	[M+NH4]+	0.985	C57H106O6	TG
14.0	902.8	TG 54:3|TG 18:1_18:1_18:1	[M+NH4]+	0.824	C57H104O6	TG
14.2	902.8	TG 54:3|TG 18:1_18:1_18:1	[M+NH4]+	1	C57H104O6	TG

14.0	900.8	TG 54:4|TG 18:1_18:1_18:2	[M+NH4]+	0.956	C57H102O6	TG
13.9	898.8	TG 54:5|TG 16:0_18:1_20:4	[M+NH4]+	0.809	C57H100O6	TG
13.7	898.8	TG 54:5|TG 18:1_18:2_18:2	[M+NH4]+	0.824	C57H100O6	TG
13.6	896.8	TG 54:6|TG 16:0_18:2_20:4	[M+NH4]+	0.824	C57H98O6	TG
13.4	896.8	TG 54:6|TG 18:1_18:2_18:3	[M+NH4]+	0.824	C57H98O6	TG
13.2	894.8	TG 54:7|TG 16:0_18:2_20:5	[M+NH4]+	0.176	C57H96O6	TG
13.3	894.8	TG 54:7|TG 16:0_18:2_20:5	[M+NH4]+	0.647	C57H96O6	TG
13.0	894.8	TG 54:7|TG 18:2_18:2_18:3	[M+NH4]+	0.559	C57H96O6	TG
12.9	892.7	TG 54:8|TG 16:1_18:2_20:5	[M+NH4]+	0.632	C57H94O6	TG
12.6	892.7	TG 54:8|TG 18:2_18:3_18:3	[M+NH4]+	0.059	C57H94O6	TG
14.2	920.9	TG 55:1|TG 18:0_19:0_18:1	[M+NH4]+	0.824	C58H110O6	TG
14.4	920.9	TG 55:1|TG 18:0_20:0_17:1	[M+NH4]+	0.824	C58H110O6	TG
14.2	918.8	TG 55:2|TG 18:0_18:1_19:1	[M+NH4]+	0.897	C58H108O6	TG
12.8	934.9	TG 56:1|TG 18:0_20:0_18:1	[M+NH4]+	0.015	C59H112O6	TG
12.8	916.7	TG 56:10|TG 16:0_18:4_22:6	[M+NH4]+	0.015	C59H94O6	TG
12.6	916.7	TG 56:10|TG 16:1_18:3_22:6	[M+NH4]+	0.015	C59H94O6	TG
14.4	930.8	TG 56:3|TG 18:1_18:1_20:1	[M+NH4]+	0.824	C59H108O6	TG
14.1	926.8	TG 56:5|TG 18:0_18:1_20:4	[M+NH4]+	0.824	C59H104O6	TG
13.9	924.8	TG 56:6|TG 16:0_18:1_22:5	[M+NH4]+	0.824	C59H102O6	TG
13.6	924.8	TG 56:6|TG 16:0_20:3_20:3	[M+NH4]+	0.809	C59H102O6	TG
13.7	922.8	TG 56:7|TG 16:0_18:1_22:6	[M+NH4]+	0.824	C59H100O6	TG
13.2	922.8	TG 56:7|TG 16:0_18:1_22:6	[M+NH4]+	0.176	C59H100O6	TG
13.6	922.8	TG 56:7|TG 16:0_18:2_22:5	[M+NH4]+	0.824	C59H100O6	TG
13.4	920.8	TG 56:8|TG 16:0_18:2_22:6	[M+NH4]+	0.824	C59H98O6	TG
13.2	920.8	TG 56:8|TG 18:1_18:2_20:5	[M+NH4]+	0.338	C59H98O6	TG
13.0	918.8	TG 56:9|TG 16:0_18:3_22:6	[M+NH4]+	0.5	C59H96O6	TG
13.0	918.8	TG 56:9|TG 16:1_18:2_22:6	[M+NH4]+	0.147	C59H96O6	TG
12.9	918.8	TG 56:9|TG 18:2_18:2_20:5	[M+NH4]+	0.029	C59H96O6	TG
14.4	946.9	TG 57:2|TG 18:0_21:0_18:2	[M+NH4]+	0.824	C60H112O6	TG
13.0	944.8	TG 58:10|TG 18:2_18:2_22:6	[M+NH4]+	0.735	C61H98O6	TG
12.9	942.8	TG 58:11|TG 16:0_20:5_22:6	[M+NH4]+	0.015	C61H96O6	TG
12.7	942.8	TG 58:11|TG 18:1_18:4_22:6	[M+NH4]+	0.015	C61H96O6	TG
12.6	942.8	TG 58:11|TG 18:2_18:3_22:6	[M+NH4]+	0.044	C61H96O6	TG
12.3	940.7	TG 58:12|TG 18:2_18:4_22:6	[M+NH4]+	0.015	C61H94O6	TG
13.1	968.8	TG 60:12|TG 16:0_22:6_22:6	[M+NH4]+	0.324	C63H98O6	TG
12.5	966.8	TG 60:13|TG 18:2_20:5_22:6	[M+NH4]+	0.015	C63H96O6	TG

Cholesteryl ester (CE), Ceramide non-hydroxyfatty acid-sphingosine (Cer_NS), Diacylglycerol (DG), Fatty Acyls (FA), Lysophophatidylcholine (LPC), Monoacylglycerol (MG), N-acyl ethanolamines (NAE), phosphatidylcholine (PC), Ether-linked oxidized phosphatidylethanolamine (EtherOxPE), Ether-phosphatidylcholine (EtherPC), Ether-phosphatidylethanolamine (EtherPE), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Sphingomyelin, (SM), OxTG Oxidized triglyceride (OxTG) , Triacylglycerol(TG).

3.1. Lipid Classes

LS-MS analysis allow to identify lipids from several different classes. The most abundant lipid group was triglycerides (TG) and the least abundant was monoacylglycerol (MG).

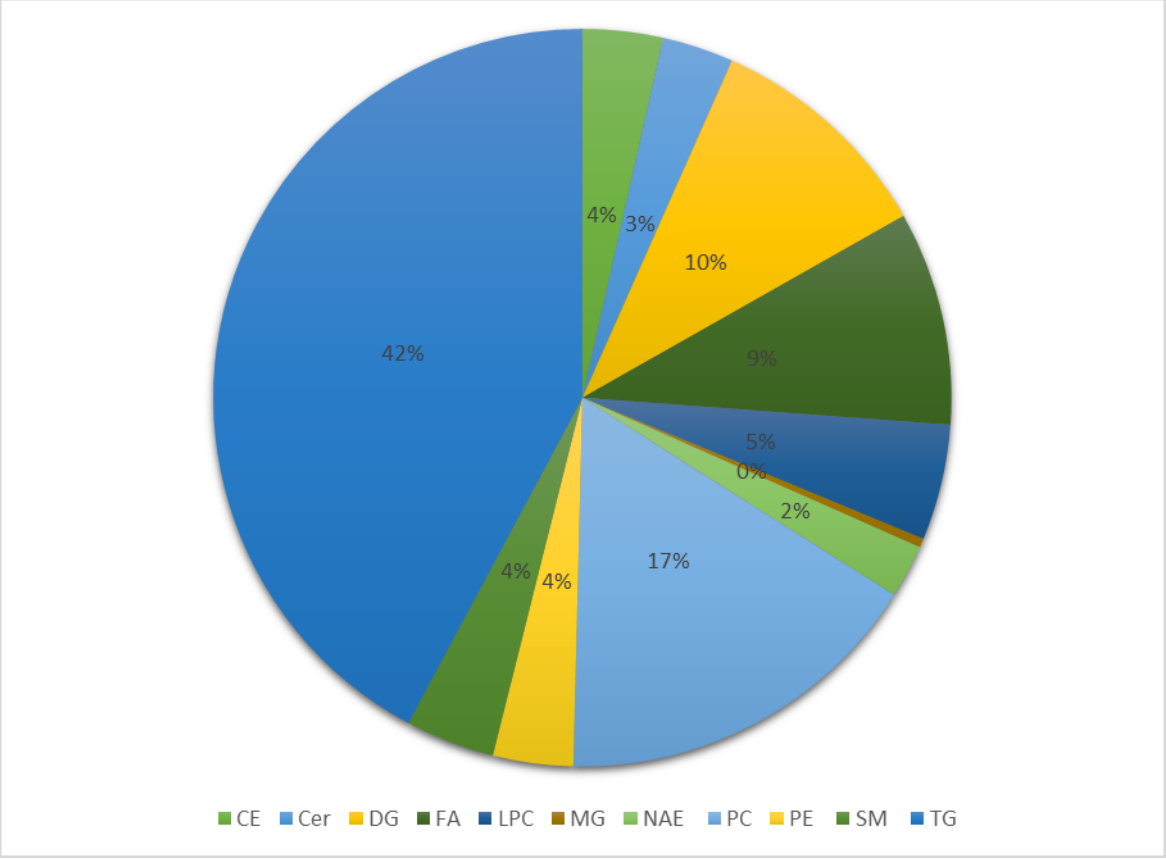


Figure 3. Pie chart showing different lipid classes identified by Untargeted LC MS analysis. The graph represents the number of lipids that are detected per group.

Cholesteryl ester (CE), Diacylglycerol (DG), Fatty Acyls (FA), Lysophophatidylcholine (LPC), Monoacylglycerol (MG), N-acyl ethanolamines (NAE), phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Sphingomyelin, (SM), Triacylglycerol (TG).

Comparing smokers and non-smokers some lipid classes were considerably changed as revealed by fold change analysis. Those classes are listed in the table.

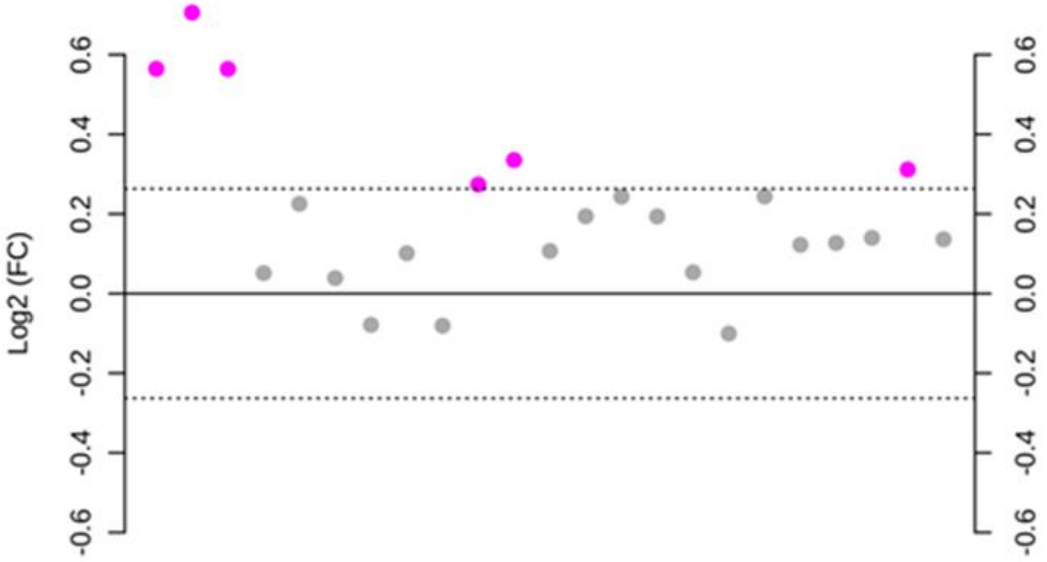


Figure 4: Lipid species selected by fold-change analysis with threshold 1.2. Direction of comparisons non-smokers (NS) vs smokers (S). [27]

Tabel 5: Important lipid species selected by fold-change analysis with threshold 1.2.

Lipid groups	**Fold Change**	**log2(FC)**
CE (total)	1.4788	-0.56446
CE saturated	1.631	-0.70572
CE unsaturated	1.4784	-0.56407
LPC (total)	1.209	-0.27379
LPC saturated	1.2617	-0.33535
TG saturated	1.2411	-0.31157

Cholesteryl ester (CE), Lysophophatidylcholine (LPC), Triacylglycerol (TG)

T-test showed many significant differences in lipid classes among smokers and non-smokers. A general trend of decrease was observed in lipid classes in smokers. Exceptionally, fatty acids were upregulated in S vs NS.

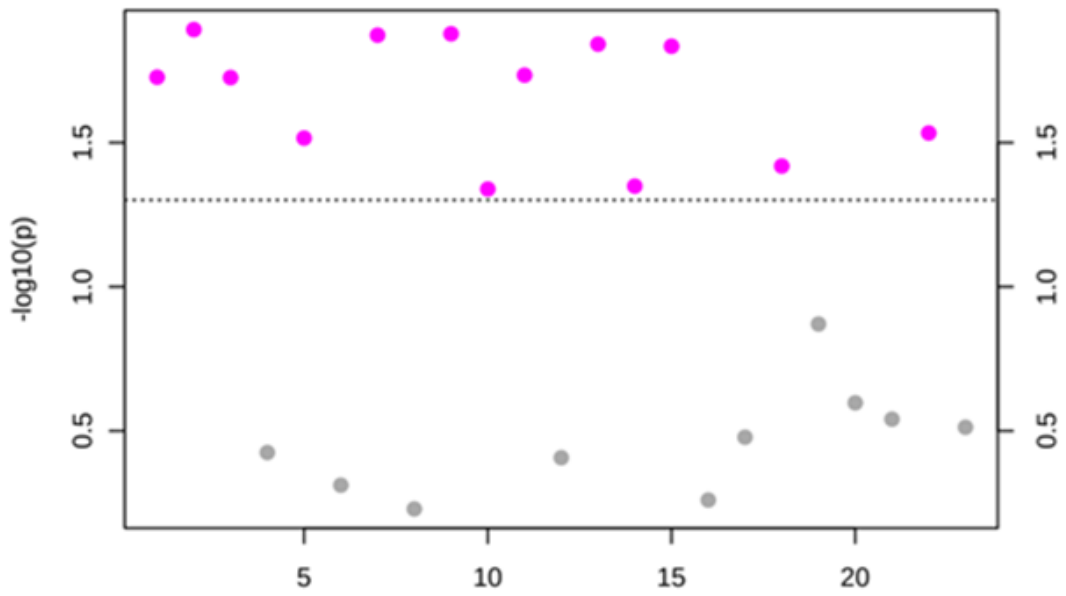


Figure 5: Lipid groups selected by t-tests with threshold 0.05.[27]

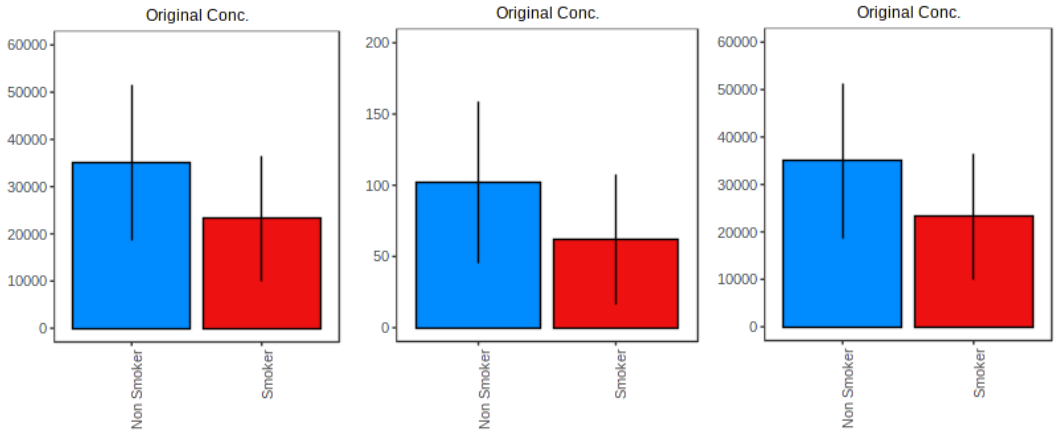
Table 6: Lipid groups and there respective p. values.

Lipids	t.stat	p.value	-log10(p)
CE saturated	2.6163	0.012812	1.8924
FA unsatureted	-26022	0.013257	1.8775
FA	-25979	0.0134	1.8729
PC	2.5695	0.014397	1.8417
PC unsaturated	2.5628	0.014638	1.8345
LPC saturated	2.4698	0.01845	1.734
CE	2.4588	0.01875	1.727
CE unsaturated	2.4575	0.018807	1.7257

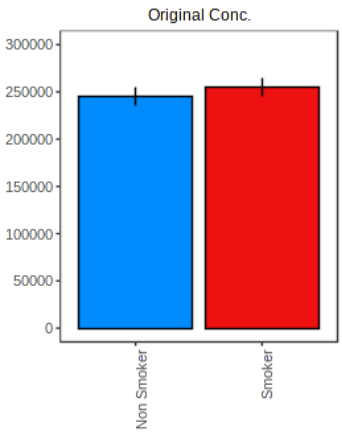
TG saturated	2.2712	0.029288	1.5333
DG saturated	2.2505	0.030492	1.5158
Ether PE	2.1577	0.038129	1.4187
PC saturated	2.0778	0.044755	1.3492
LPC	2.0676	0.045819	1.339

Cholesteryl ester (CE), Diacylglycerol (DG), Fatty Acyls (FA), Lysophophatidylcholine (LPC), Phosphatidylcholine (PC), Ether-phosphatidylethanolamine (EtherPE), Phosphatidylethanolamine (PE), Triacylglycero l(TG).

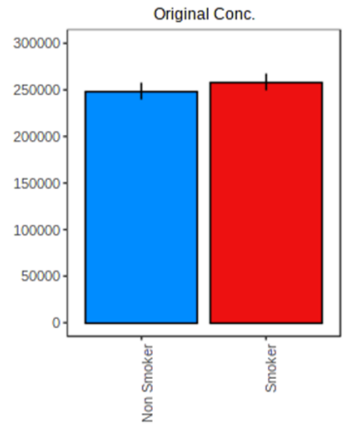
Exact concentrations of significantly different lipid classes are shown in graphs (figure 6). Most of them are decreased in smokers in comparison to non-smokers.



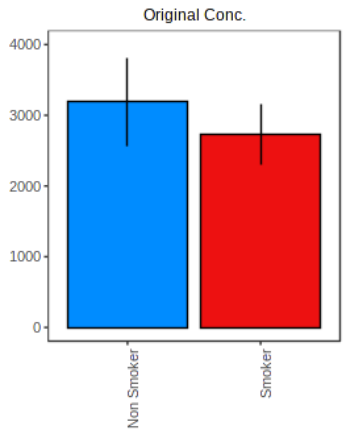
CE **Saturated CE** **Unsaturated CE**



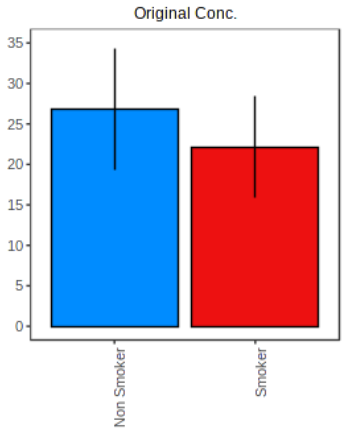
Unsaturated FA



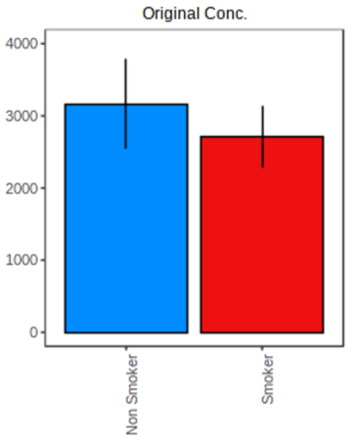
FA



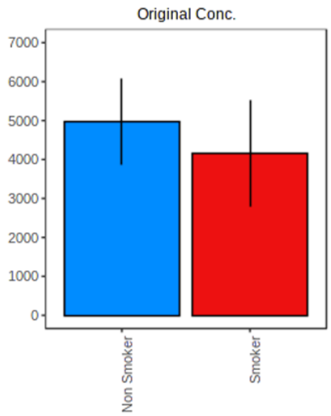
PC



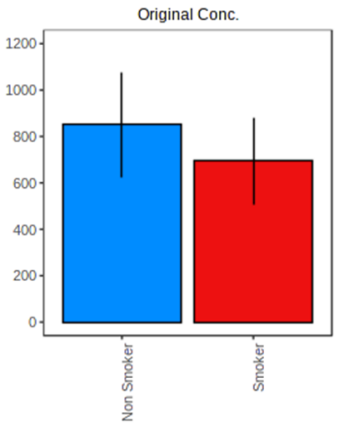
Saturated PC



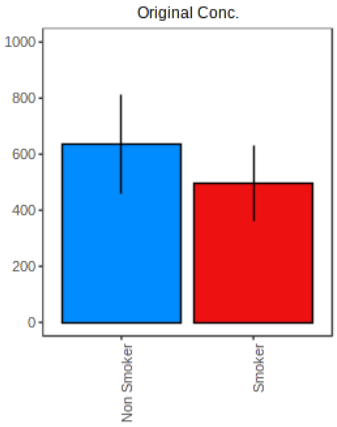
Unsaturated PC



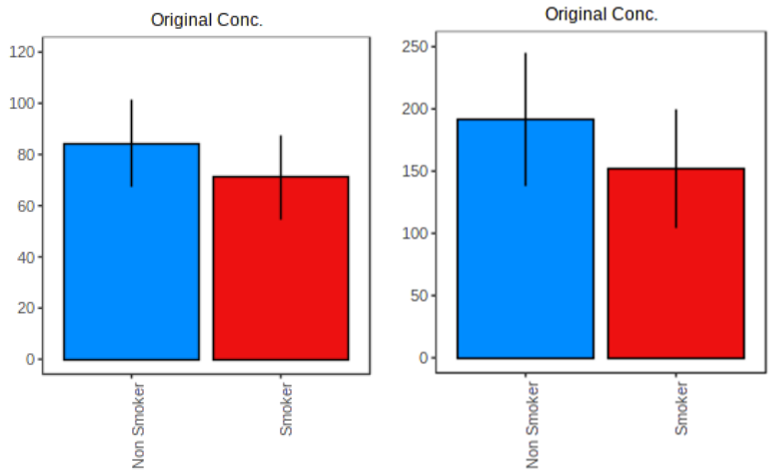
Ether PE



LPC



Saturated LPC



Saturated DG **Saturated TG**

Figure 6. Lipid comparison between non-smoker samples vs smokers. Graphs represent the lipid amount of lipids in µmol/ml, mean ± SD which indicates the sum of the metabolites within a class after normalization by internal standard. [27]

Cluster analysis do not show any obvious diversity between smoking and non-smoking group (heatmap is presented in figure 7).

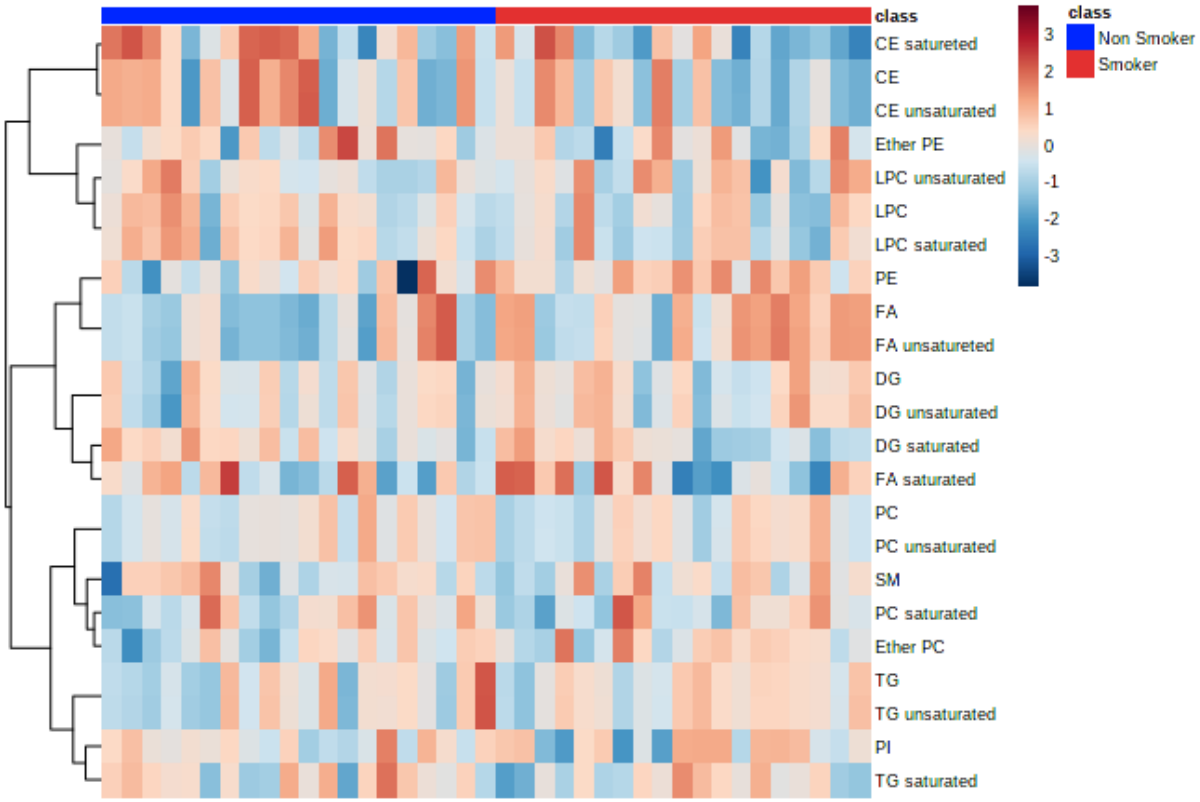


Figure 7: Compersion of the Non- Smoker and Smoker where shown as heatmap where distance measure was done by using correlation and clustering algorithm using ward.D. [27]

3.2. Individual lipid species

Comparing smokers and non-smokers for individual lipids. Fold change analysis shown that the lipid were changed in the two groups. Graphical representation of fold change analysis is shown in figure 8 and list of important features is in Table 7.

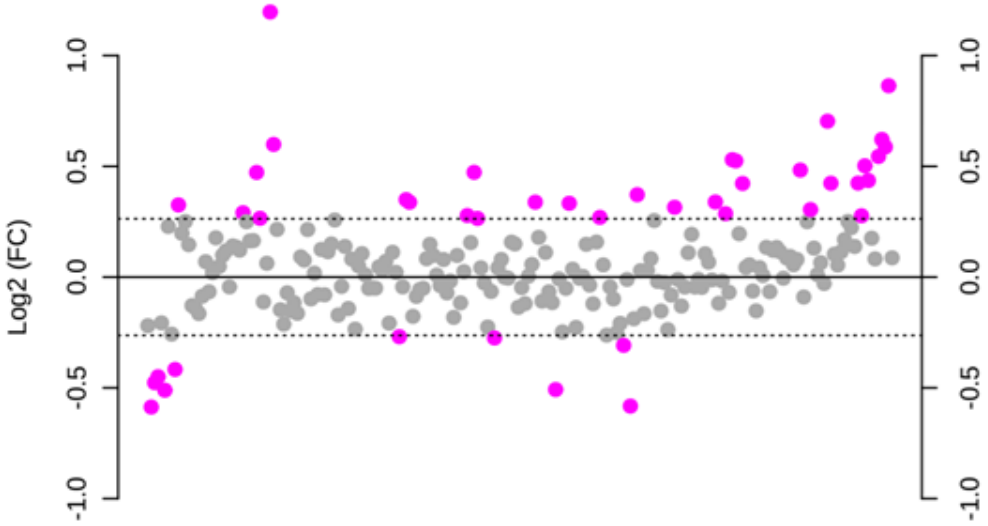


Figure 8: Individual lipid species that were identified by fold-change analysis with threshold 1.2. [27]

Table 7: Important lipid features identified by fold change analysis

Metabolite name	Fold Change	log2(FC)
CE 18:0	0.66585	-0.58673
CE 18:1	0.71866	-0.47663
CE 18:2	0.73214	-0.44982

CE 20:4	0.70188	-0.5107
CE 22:6.1	0.74902	-0.41692
DG 28:2	1.2533	0.32569
DG 41:6	1.223	0.2904
DG 44:8	1.3875	0.47251
DG 45:8	1.2019	0.26536
FA 18:1	2.292	1.1966
FA 18:2	1.5145	0.5988
PC 36:4|PC 18:2_18:2	0.82986	-0.26906
PC 36:5	1.2741	0.34953
PC 36:5|PC 16:0_20:5	1.2638	0.33776
PE 36:1	1.2123	0.27773
PE 36:2|PE 18:1_18:1	1.3879	0.47288
PE 38:4|PE 18:0_20:4	1.2015	0.2648
PE P-38:4|PE P-18:0_20:4	0.8265	-0.27492
TG 36:0|TG 10:0_12:0_14:0	1.2643	0.3383
TG 42:1|TG 8:0_16:0_18:1	0.70337	-0.50764
TG 44:2|TG 12:0_14:0_18:2	1.2597	0.33311
TG 46:3|TG 12:0_16:0_18:3	1.2054	0.26951
TG 48:2|TG 14:0_16:1_18:1	0.8077	-0.3081
TG 48:4|TG 12:0_18:2_18:2	0.6679	-0.58231
TG 49:0|TG 16:0_16:0_17:0	1.2941	0.37192
TG 50:5|TG 14:0_18:2_18:3	1.2441	0.31505
TG 52:4|TG 16:0_18:2_18:2	1.2649	0.33904
TG 52:5|TG 16:0_18:2_18:3	1.2193	0.28611
TG 52:6|TG 16:1_18:2_18:3	1.444	0.53006
TG 52:6|TG 16:1_18:2_18:3.1	1.4386	0.52466
TG 52:7|TG 16:0_18:3_18:4	1.3403	0.42253
TG 54:7|TG 16:0_18:2_20:5.1	1.3975	0.48289
TG 54:8|TG 18:2_18:3_18:3	1.2351	0.30467
TG 56:10|TG 16:0_18:4_22:6	1.6286	0.70366
TG 56:10|TG 16:1_18:3_22:6	1.3412	0.42355
TG 56:8|TG 16:0_18:2_22:6	1.3419	0.42425
TG 56:8|TG 18:1_18:2_20:5	1.2114	0.27667
TG 56:9|TG 16:0_18:3_22:6	1.4167	0.50257
TG 56:9|TG 16:1_18:2_22:6	1.3527	0.43587
TG 58:10|TG 18:2_18:2_22:6	1.4586	0.54459
TG 58:11|TG 16:0_20:5_22:6	1.5383	0.6213
TG 58:11|TG 18:1_18:4_22:6	1.5027	0.58759
TG 58:11|TG 18:2_18:3_22:6	1.8199	0.86384

Cholesteryl ester (CE), Diacylglycerol (DG), Fatty Acids (FA), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Triacylglycerol (TG).

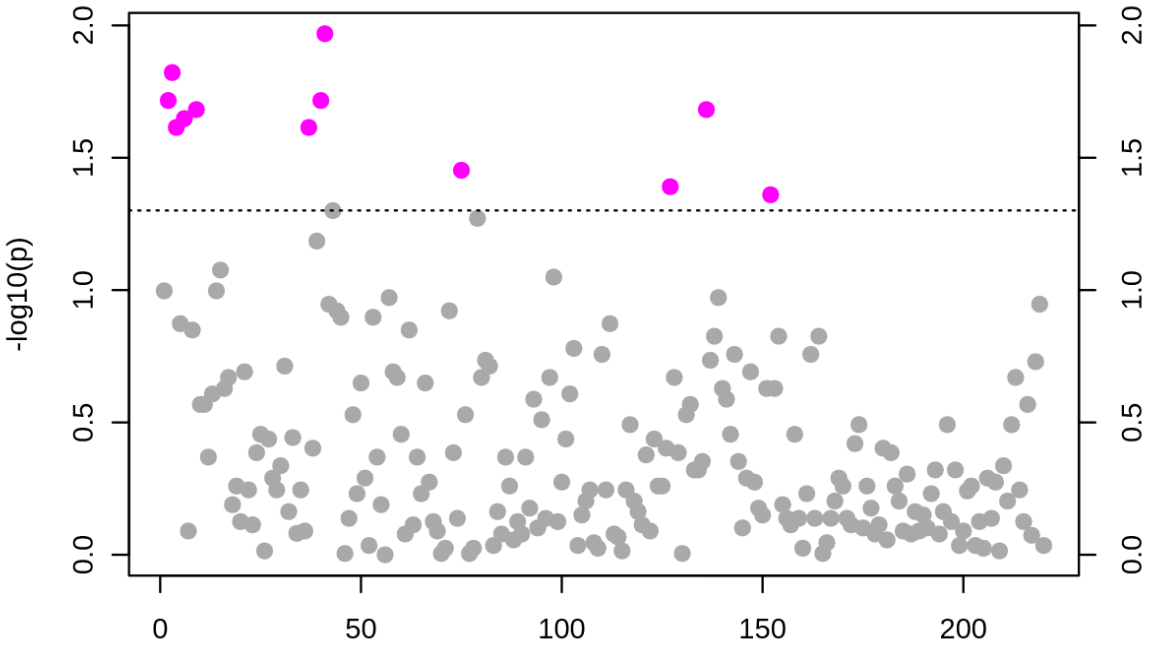


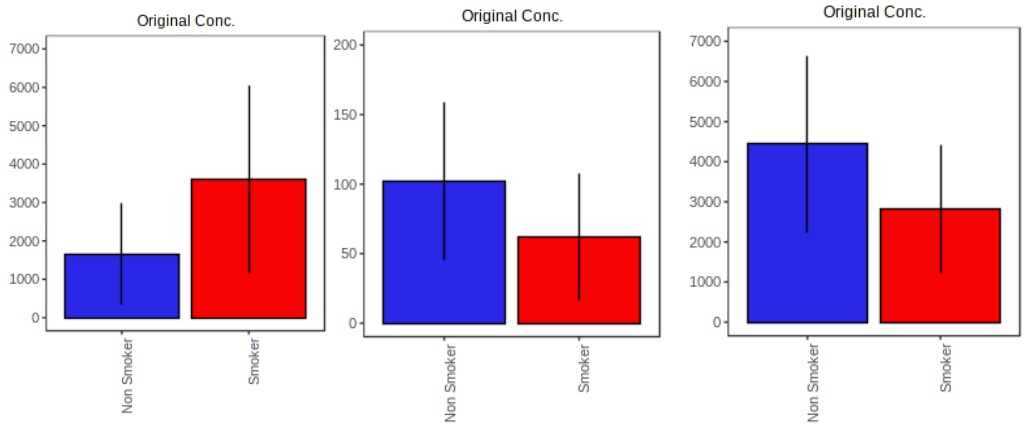
Figure 9. Lipid species selected by t-tests with threshold 0.05. [27]

Tabel 8: Important features selected by t-tests with threshold 0.05.

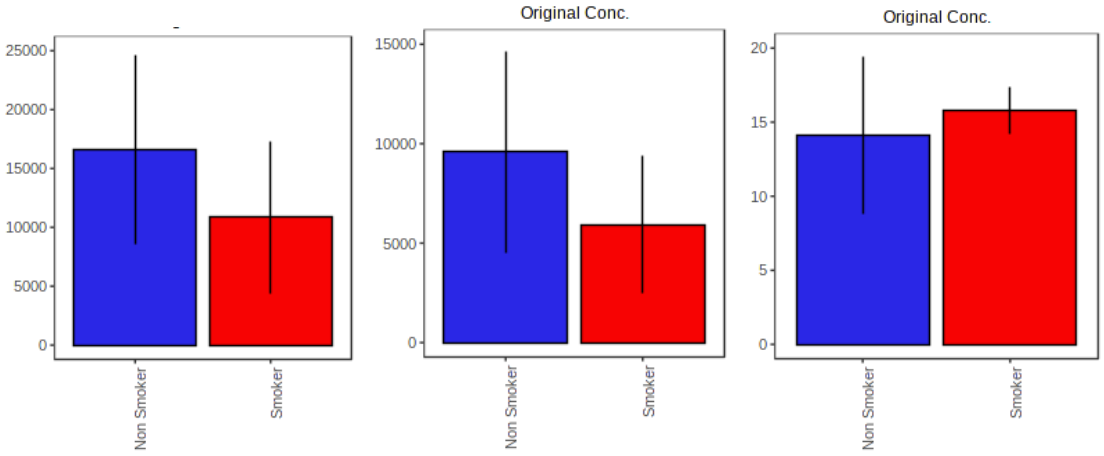
Metabolite name	t.stat	p.value	-log10(p)
CE 18:0	2.5453	0.015218	1.8176
CE 18:1	2.7428	0.009335	2.0299
CE 18:2	2.6418	0.012012	1.9204
CE 20:4	2.7628	0.008874	2.0519
DG 28:2	-2.1246	0.040364	1.394
FA 18:1	-2.9115	0.006061	2.2174
TG 52:7|TG 16:0_18:3_18:4	-2.2582	0.029917	1.5241
TG 54:7|TG 16:0_18:2_20:5.1	-2.3723	0.022987	1.6385
TG 56:10|TG 16:0_18:4_22:6	-2.4101	0.021036	1.677
TG 58:11|TG 18:2_18:3_22:6	-2.0272	0.049896	1.3019

Cholesteryl ester (CE), Diacylglycerol (DG), Fatty Acyls (FA), Triacylglycerol (TG).

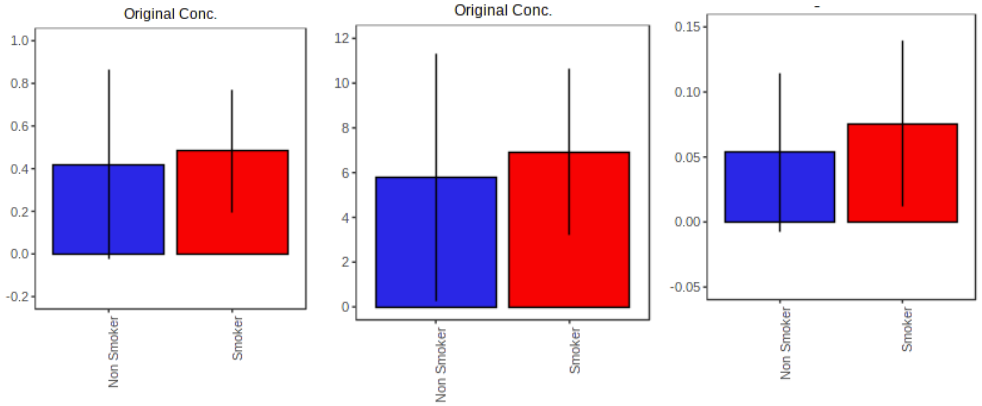
Exact concentrations of significantly different individual lipid are shown in graphs (figure 10). Most of them are decreased in smokers in comparison to non-smokers.



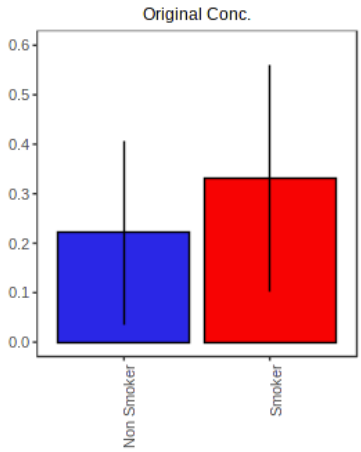
FA 18:1 **CE 18:0** **CE 18:1**



CE 18:2 **CE 20:4** **DG 28:2**



TG 52:7 **TG 54:7** **TG 56:10**



TG 58:11

Figure 10. Individual lipid species comparison between non- smokers vs smokers. Graphs represent the lipid amount of lipids in µmol/ml, mean ± SD which indicates the sum of the metabolites within a class after normalization by internal standard. [27]

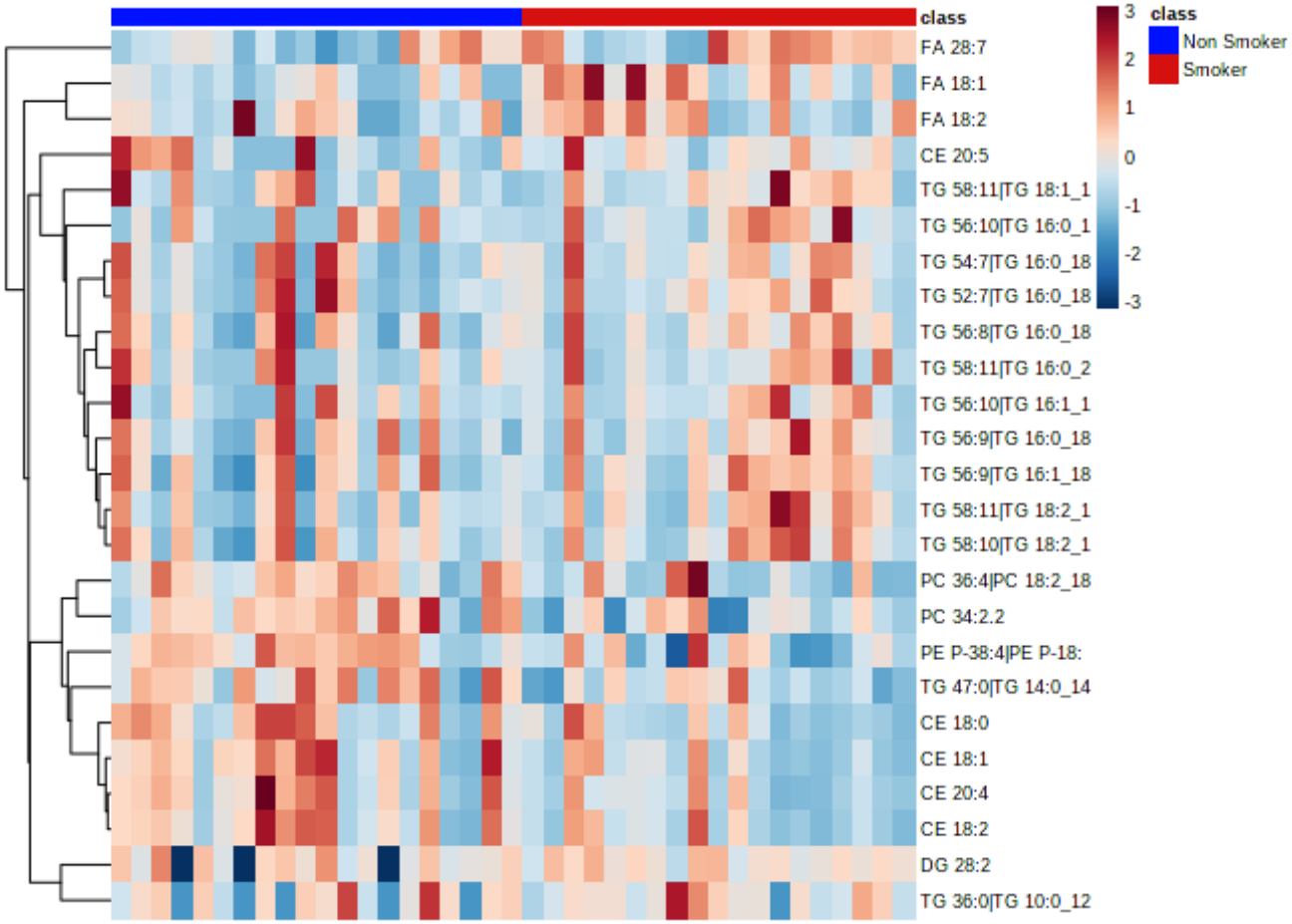


Figure 11: Graph representing the Heatmap where the distance was measure by using Euclidean and clustering algorithm was done by using ward.D. [27]

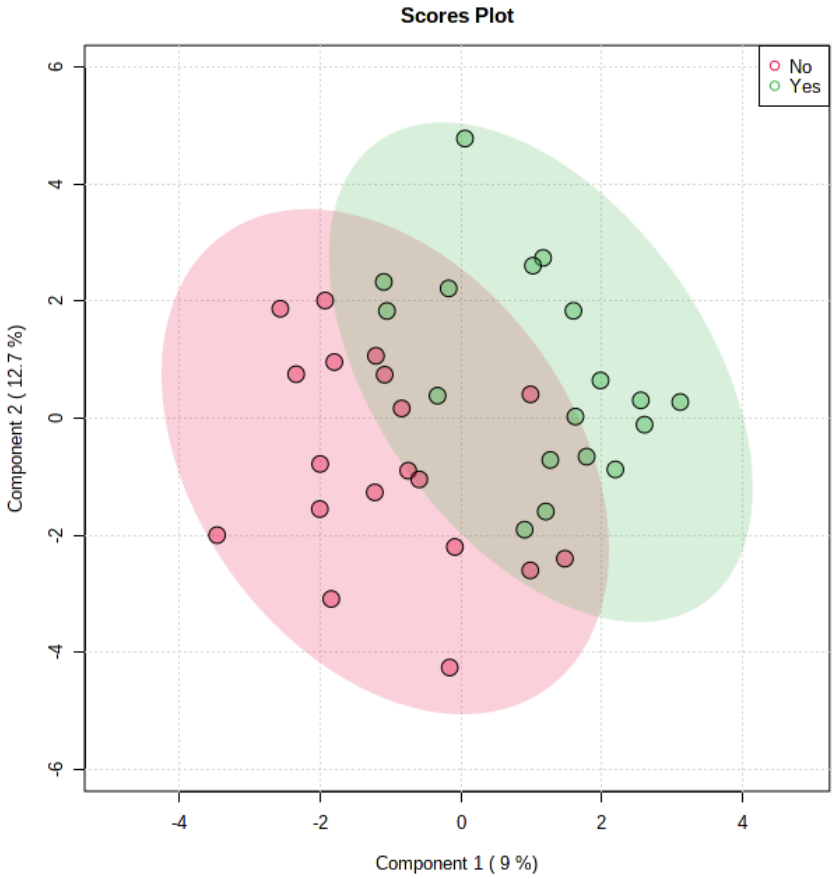


Figure 12- Partial Least Squares Discriminant Analysis (PLS-DA) for the groups where red color represents non- smokers and the green color represents smokers.[27]

4. DISCUSSION

Cigarette smoking is one of the leading causes of preventable morbidity and mortality that usually starts in adolescence and continues into adult life.[29,30] Tobacco smoking is responsible for the premature development of cardiovascular disease by various mechanisms, and abnormal serum lipid profile and lipoprotein levels are one of the consequences.[31,32]

Participants showed an increase in fatty acids in smoker groups compared with non-smokers. It is consistent with the available data on the effect of intravenous nicotine on the increase of free fatty acids in the plasma through enhanced lipolysis resulting from sympathoadrenal stimulation.[33] In these studies, one of the detected species of faty acids, oleic acid (FA18:1) in particular changed significantly. It is known that smoking can reduce the conversion of short-chain unsaturated fatty acids to long-chain derivatives, resulting in the accumulation of the former. *In vitro* studies have shown that oleic acid may promote oxidative stress and lipid accumulation in hepatocytes,[34] so it can be concluded that this compound causes unfavorable phenomena in human metabolism. Recently it has been shown that higher circulating oleic acid levels are related to greater risks of cardiovascular events and all cause mortality.[35] Some authors reported that after smoking cessation concentration of this compound tends to decrease.[36]

In turn, cholesterol esters were significantly downregulated in smokers (both saturated and unsaturated), which is difficult to explain as their contribution to atherosclerosis is well-documented.[37] In the examined cohort total cholesterol concentration was also significantly decreased in smokers and it was not an effect of treatment or co-morbid conditions as checked by regression analysis. However, HDL-C was also decreased. CE, along with phosphatidylcholines, is one of the most abundant lipid pool not only in low-density, but also in high-density lipoprotein.[38] Thus, decreased concentration of CE in smokers may somehow reflect decreased HDL-C in these patients, but this hypothesis needs verification by lipidomic analysis in separated lipoprotein fractions. Many authors considered HDL-C fraction as the most susceptible to changes during smoking.[39] It should be underlined that multiple mechanism of adverse effects of smoking on HDL-C

particle has been documented, such as inhibition of lecithin: cholesterol acyltransferase (LCAT) and/or altering cholesterol ester transfer protein (CETP) and hepatic lipase activity, which attributes to its impact on HDL metabolism and HDL subfractions distribution.[39] Analyzing changes in individual compounds some CE species were shown as important: CE 18:0, 18:1, 18:2 (all were decreased in smokers). This may again result from deficient conversion of free cholesterol to CE (catalyzed by LCAT), but it is somehow inconsistent. Particles enriched with monounsaturated CE (CE 18:1) are considered as more active in binding to arterial proteoglycans, leading to the subsequent formation of atherosclerotic lesions, while CE with linoleic acid (CE 18:2) are thought to be less atherogenic.

Thus smoking appears to have at least two lipid effects that may promote atherosclerosis and coronary artery disease : increased plasma FFA and decreased plasma high-density lipoprotein cholesterol fraction.[40]

Although I did not reach statistical significance in lipid groups diacylglycerol (DAG) and triacylglycerol (TAG) showed increase in male smokers as individual metabolites: TG 58:11, TG 56:10, TG 52:7, TG 54:7 and DG 28:2.

Komiya et al. reported smokers with Brinkman index ≥ 554 (defined as the number of cigarettes smoked per day multiplied by duration of smoking in years) to have 1.657 times the odds of having abnormal triglyceride (TG) levels among Japanese males aged 24–68 years.[41]

A possible mechanism of how cigarette smoking may alter lipid levels in serum has been suggested.[42] As mentioned above, absorption of nicotine induces lipolysis of stored TG and release of free fatty acids. This, in turn, results in increased hepatic synthesis of TG and VLDL.[43] My results stay in accordance with available scientific reports. Titz et al. reported that TAG 52:2 levels were positively associated with smoking and CE 22:6 and LPC 18:0 levels were positively associated with non-smoking.[44]

Also, I observed general downregulation of PC class and such profile is associated with coronary artery disease.[45]

Surprisingly, lipid profile was better for smokers than nonsmokers (lover total cholesterol, LDL and trigliceride concentration), only HDL concentration was higher in

nonsmokers (but without statistical significance). Received hypolipemic treatment did not differ between subgroups.

The overall observation of the present study was that, there was an increase in the concentration of fatty acids, and some specific triglycerides and diglycerides and a decrease in the concentration of CE (both saturated and unsaturated) PC (especially diunsaturated, data not shown), and LPC (especially saturated), and TG saturated in smokers compared to nonsmokers. Thus, it can be said based on the present study that smoking affects and deranges the lipid profile, but in patients with already existing cardiovascular diseases and many confounding factors smoking may not have such significant influence as before disease development.

5. CONCLUSIONS

- The tobacco smoking is responsible for premature development of cardiovascular disease by various mechanism, and abnormal serum lipid profile and lipoprotein levels are one of the consequences.[31,32]
- Participants showed an increase in fatty acids in smoker groups compared with non smokers.
- Oleic acid (FA18: 1) in particular changed significantly. It is known that smoking can reduce the conversion of short chain unsaturated fatty acids to long chain derivatives, resulting in the accumulation of the former.
- Cholesterol esters were significantly downregulated in smokers (both saturated and unsaturated).
- There was an increase in concentration of fatty acids, and some specific triglycerides and diglycerides and decrease in the concentration of CE (both saturated and unsaturated) PC (especially diunsaturated, data not shown), and LPC (especially saturated), and TG saturated in smokers compared to nonsmokers.

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