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

Jurić, Viktorija

Master's thesis / Diplomski rad

2021

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Split, Faculty of Chemistry and Technology / Sveučilište u Splitu, Kemijsko-tehnološki fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:167:647454

UNIVERSITY OF SPLIT
FACULTY OF CHEMISTRY AND TECHNOLOGY
GRADUATE STUDY OF CHEMISTRY
ORIENTATION: ORGANIC CHEMISTRY AND BIOCHEMISTRY

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

DIPLOMA THESIS

VIKTORIJA JURIĆ
Index number:
Split, October 2021

UNIVERSITY OF SPLIT
FACULTY OF CHEMISTRY AND TECHNOLOGY
GRADUATE STUDY OF CHEMISTRY
ORGANIC CHEMISTRY AND BIOCHEMISTRY

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

DIPLOMA THESIS

VIKTORIJA JURIĆ
Index number: 285
Split, October 2021

SVEUČILIŠTE U SPLITU
KEMIJSKO-TEHNOLOŠKI FAKULTET
DIPLOMSKI STUDIJ KEMIJE
ORGANSKA KEMIJA I BIOKEMIJA

NECILJANA LIPIDOMIKA KOD MUŠKARACA S PREKOMJERNOM TJELESNOM MASOM I KARDIOVASKULARNIM BOLESTIMA-UTJECAJ STATUSA PUŠENJA

DIPLOMSKI RAD

VIKTORIJA JURIĆ
Matični broj: 285
Split, Listopad 2021

BASIC DOCUMENTATION CARD

DIPLOMA THESIS

University of Split

Faculty of Chemistry and Technology Split

Study Chemistry

Scientific area: Natural Sciences

Scientific field: Chemistry

Thesis subject was approved by Faculty Council of Faculty of Chemistry and Technology, session no.6.

Mentor: Maša Buljac-PhD, Assistant Professor

Advisore: dr n farm Ewa Żurawska-Płaksej

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

Viktorija Jurić,285

Abstract:

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. Lipids are crucial small biomolecules and play vital roles in a variety of physio-pathological events. In order to unravel lipid function, it is of utmost importance to identify and quantify single lipid molecular species in complex biological systems. Lipidomics is a rapidly evolving analytical technique capable of measuring hundreds of lipids and is currently at the forefront of scientific research due to the importance of lipids in health and disease. 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 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.

Keywords: cardiovascular disease, tobacco smoking, lipidomics, lipids

Thesis contains: 52 pages, 12 figures, 8 tables, 0 supplements, 45 references

Original in: english

Defence committee:

1. Ivica Blažević- PhD, Associate Professor chair person

2. Franko Burčul- PhD, Assistant Professor member

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

Defence date: 28.10.2021.

Printed and electronic (pdf format) version of thesis is deposed in Library of Faculty of Chemistry and Technology Split, Ruđera Boškovića 35.

TEMELJNA DOKUMENTACIJSKA KARTICA

DIPLOMSKI RAD

Sveučilište u Splitu

Kemijsko-tehnološki fakultet u Splitu

Diplomski studij Kemija

Znanstveno područje: prirodne znanosti

Znanstveno polje: kemija

Tema rada : je prihvaćena na 6. sjednici Fakultetskog vijeća Kemijsko-tehnološkog fakulteta

Mentor: doc.dr.sc. Maša Buljac

Pomoć pri izradi: dr n farm Ewa Żurawska-Płaksej

NECILJANA LIPIDOMIKA KOD MUŠKARACA S PREKOMJERNOM TJELESNOM MASOM I KARDIOVASKULARNIM BOLESTIMA-UTJECAJ STATUSA PUŠENJA

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

Rad sadrži: 52 stranica, 12 slika, 8 tablica, 0 priloga, 45 literaturnih referenci

Jezik izvornika: engleski

Sastav povjerenstva:

1. izv. prof. dr. sc. Ivica Blažević - predsjednik
2. doc. dr. sc Franko Burčul - član
3. doc. dr. sc. Maša Buljac - član-mentor

Datum obrane: 28.10.2021.

Rad je u tiskanom i elektroničkom(pdf formatu) obliku pohranjen u Knjižnici Kemijsko-tehnološkog fakulteta Split, Ruđera Boškovića 35

Reaserch was done at the Wroclaw Medical University under the supervisor of dr Ewa Żurawska-Płaksej and supervisor doc.dr.sc. Maša Buljac from Faculty of Chemistry and Technology in Split-Croatia, in the time period from March to September 2021.

I would like to express my special appreciation and thanks to my advisor dr Ewa Żurawska-Płaksej for beeing a tremendous mentor and for encouraging my research.
Also, special thanks to dr. Hanna Czapor-Irzabek for all the help with the LC MS/MS.

I would especially like to thank to my friends Karla, Veronika, Lea, Lucija R, Lucija G, Luka, Ana K, Ana J, Valentina, Luana, Barbara, Tassos and Eugenia, Marijan for being there to support me during my faculty years and for always cheering me up.
I would also like to take this opportunity to thank all the coworkers at Institutes in Vienna and Wroclaw for support and help during my internship.

A special thanks goes to my family the words can't express how grateful I am for have them always by my side. Mum, dad and sis you are the most amazing family I could have asked for. Without you this would be impossible.

Viktorija Juric

OBJECTIVES OF THE THESIS

Lipids are crucial small biomolecules and play vital roles in a variety of physio-pathological events by serving as constituents of cellular membranes, cellular barriers, signal transduction, energy sources, and intermediates in signaling pathways.

Lipids are extracted from serum samples by using a biphasic solvent system of cold methanol, methyl tertbutyl ether (MTBE), and water with some modifications.

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 leading causes of preventable morbidity and mortality that usually starts in adolescence and continues into adult life. 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.

Lipids are crucial small biomolecules and play vital roles in a variety of physio-pathological events by serving as constituents of cellular membranes, cellular barriers, signal transduction, energy sources, and intermediates in signaling pathways.

In order to unravel lipid function, it is of utmost importance to identify and quantify single lipid molecular species in complex biological systems.

Lipidomics is a rapidly evolving analytical technique capable of measuring hundreds of lipids and is currently at the forefront of scientific research due to the importance of lipids in health and disease.

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 a biphasic solvent system of cold methanol, methyl tertbutyl ether (MTBE), and water.

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 morbiditeta i 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.

TABLE OF CONTENTS

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

Cigarette smoking is one of the most potent and prevalent addictive habits. It is associated with increased risk of a variety of health problems and causes about 6 million deaths worldwide every year.[1]

Nicotine, carbon monoxide, and other toxic substances from tobacco smoke are absorbed through the lungs into the bloodstream and are distributed throughout the body. In particular, smoking is considered a major risk factor for development of cardiovascular diseases (especially atherosclerosis and coronary heart disease).[1]

In particular, it may lead to changes in normal plasma lipid profile. It increases the concentration of serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG), and decreases the level of high-density lipoprotein cholesterol (HDL-C) circulating in the blood vessels, making serum lipid profile more atherogenic.[2] Nicotine causes secretion of catecholamines and stimulation of sympathetic adrenal system results in increased lipolysis - hepatic free fatty acids and TG along with very low-density lipoprotein cholesterol are released into the bloodstream.[3] However, the influence of tobacco smoke on other lipid species in human body is not well understood.

Lipidome in biological systems consists of hundreds of thousands of individual lipid molecules that possess complex structures, multiple categories, and diverse physicochemical properties assembled by different combinations of polar headgroups and hydrophobic fatty acyl chains.[4] Lipids are crucial small biomolecules and play vital roles in a variety of physio-pathological events by serving as constituents of cellular membranes, cellular barriers, signal transduction, energy sources, and intermediates in signaling pathways.[5] In order to unravel lipid function, it is of utmost importance to identify and quantify single lipid molecular species in complex biological systems. Lipidomics is a rapidly evolving analytical technique capable of measuring hundreds of lipids and is currently at the forefront of scientific research due to the importance of lipids in health and disease.[6]

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).

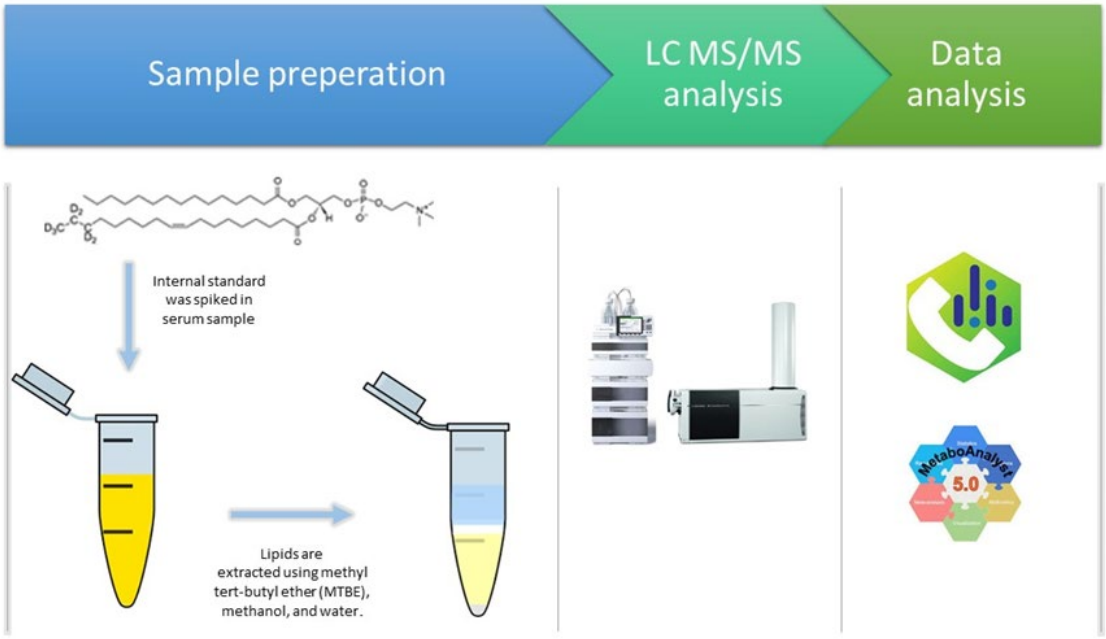


Figure 1. Workflow of LC–MS-based lipidomics and automated identification of lipids using the LipidBlast in-silico MS/MS library.[7]

1. LIPIDS IN BIOLOGICAL SYSTEMS

Lipids are defined as hydrophobic or amphipathic small molecules that originate entirely or, in part, from carbanion-based condensation of thioesters (ketoacyl groups) and carbocation-based condensation of isoprene units (isoprene groups).[8] The polar head group of lipids in combination with the hydrophobic tails provides the basis for the energy-driven formation of lipid bilayers within the aqueous, polar cellular environment. Lipid species play key roles in cellular membranes, cell signaling, and cell metabolism.[9] Lipids in biological systems consist of tens to hundreds of thousands distinct chemical entities with wide diversities in structures and physiochemical properties.[10]

The structural diversity of the lipidome arises via variations in the type of the head groups, the fatty acyl chain length, the level of unsaturation, double bond location, *cis-trans* geometric isomerism, branched functional groups in the fatty acyl chains, the type of the covalent bond, i.e., ester (acyl-), ether (alkyl-) and vinyl-ether (alkenyl-), linked to the head groups.[3]

Currently, LIPID MAPS Structure Database has enrolled 46 285 unique lipid structures dispersed in eight categories, including fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR derived from condensation of isoprene), saccharolipids (SL), and polyketides (PK derived from condensation of ketoacyl subunits).[3]

Brief characteristic of each group is provided in table 1.

Table 1. Lipid clases

Lipid class (abbr)	Characteristic
Fatty acyls (FA)	circulates as free FAs bound to albumin or form components of complex lipids.[11]
Glycerolipids (GL)	abundant cellular lipids with physiological roles in energy storage (primarily in the form of triacylglycerol) and membrane structure (phospholipids and other lipids, varying by organelle type).[12]

Glycerophospholipids (GPL)	fatty acid diglycerides with a phosphatidyl ester attached to the terminal carbon; the terminal ester groups are mainly ethanolamine, choline, serine, or inositol; GPLs are highly amphiphilic and normally are components of cellular or vesicle membranes.[13]
Sphingolipids (SP)	composed of an sphingoid base backbone to which a fatty acid may be attached through an amide bond and a head group at the primary hydroxyl; the head groups range from a simple hydrogen to more complex species, such as the phosphocholine moiety of sphingomyelin (SM) and the simple to complex glycans of glycosphingolipids.[14]
Sterols (ST)	ubiquitous and essential membrane components in all eukaryotes, affecting membrane rigidity, fluidity, and permeability by interacting with other lipids and proteins within the membranes.[15]
Prenol lipids (PR)	synthesized from the 5-carbon precursors isopentenyl diphosphate and dimethylallyl diphosphate; the simple isopreno- ids (linear alcohols, diphosphates, and so on) are formed by the successive addition of C5 units, and are classified according to the number of these terpene units. This class includes the carotenoids, which are precursors of vitamin A and also possess antioxidant effects. PR containing more than 40 carbon atoms are termed as polyterpenes.16
Saccharolipids (SL)	compounds in which fatty acids are linked directly to a sugar backbone, forming structures that are compatible with membrane bilayers.[17]

Polyketides (PK)	bioactive natural products isolated from diverse microorganisms.[18] Polyketides are the amplest class of fungal secondary metabolism and are biosynthesized by type I polyketide synthases (PKSs).[19]

2. MATERIALS AND METHODS

2.1. Reagent and Chemicals

SPLASH® LIPIDOMIX® Mass Spec Standard was purchased from Avanti Polar Lipids (Alabaster, AL, USA). The chemicals acetonitrile, prop-2-anol, methanol, chloroform, formic acid, ammonium acetate, and ammonium formate were purchased by 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

Extraction of serum lipids was carried out using a biphasic solvent system of cold methanol, methyl tertbutyl ether (MTBE), and water with some modifications.

Plasma samples were thawed at 4˚C and vortex briefly to homogenize. Aliquot 20 µL of plasma per sample into pre-labeled 1.5 mL eppendorf tubes.

Then, 975 µL 3:10 extraction solvent mixture was added to each aliquot, keeping the extraction solvent on the ice during the procedure. Samples were vortex samples for 10 seconds, and then shake for 6 minutes at 4°C on the orbital mixer. 5 µL of internal standard, methanol solution was added to each eppendorf tube (Table 3).

Table 3. Avanti's SPLASH™ LIPIDOMIX® Quantitative Mass Spec Internal Standard. Each sealed ampule of SPLASH™ contains 1mL of methanol solution with 14 deuterated lipid internal standards at concentrations relative to human plasma lipid ratios. The concentrations are verified and based on the isotopic purity of each individual compound.[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	

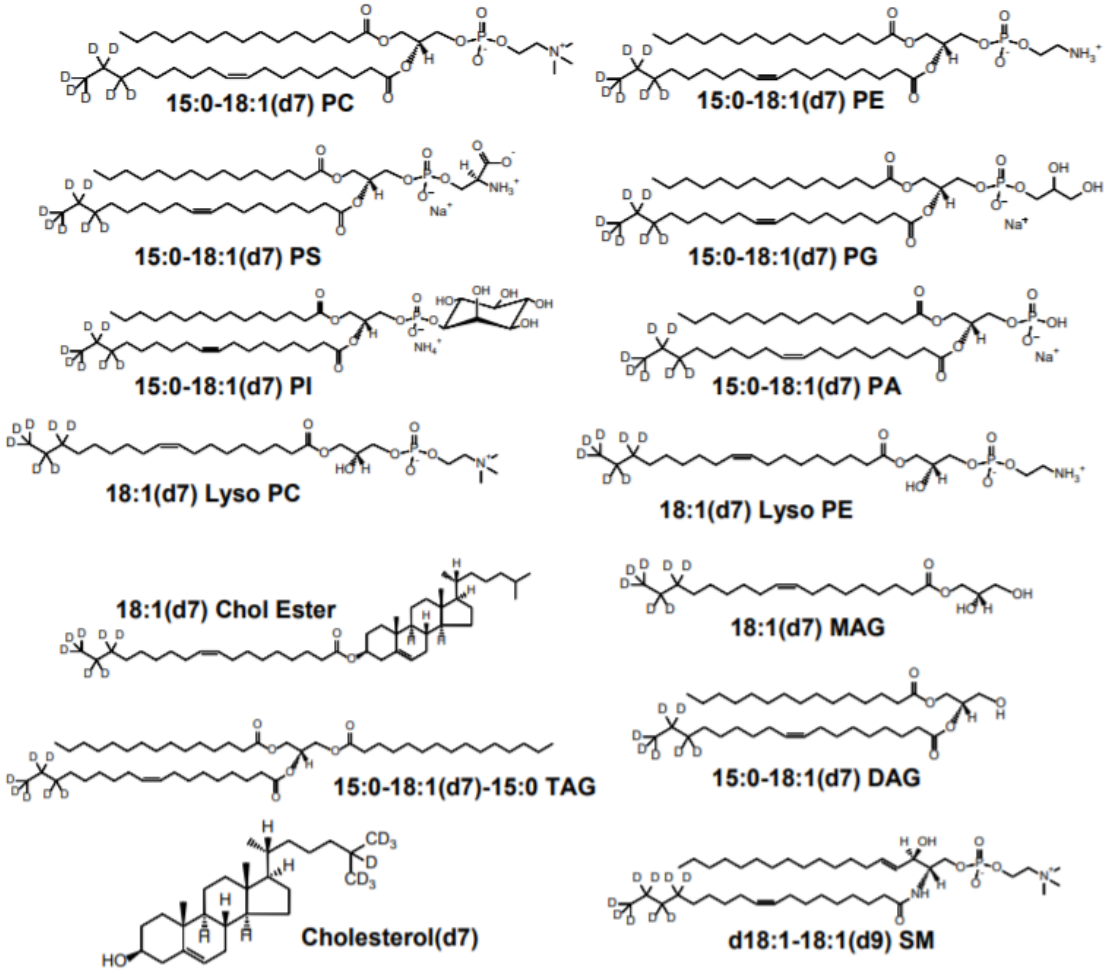


Figure 2. Chemical structure of SPLASH LipidoMIX™ Internal Standards[22]

Phase separation was induced by adding 188 µL room temperature LC-MS grade water to each tube. Tubes were vortex for 20 seconds and then centrifuge @ 14,000 rcf (12300 rpm) for 2 minutes. The upper organic phase was transferred to two separate tubes (350 µL/each tube) for lipidomics analysis. Samples were dried down under the nitrogen steam. Tubes were stored at -80˚C until ready for analysis.[23,24]

Resuspend dry samples in 110 µL resuspension solution MeOH: Tol (9:1). Resuspended samples were vortex for 10 seconds. Sonicated at room temperature for 5 minutes, and then centrifuge for 2 minutes @ 16,100 g. The volume of 50 µL was transferred from each tube to two separate amber glass vials with micro-inserts.[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. The instrument was calibrated with solution of sodium formate in a HPLC calibration method and the scan range was 100-1700 m/z. Nitrogen was used as a drying gas at 2.0 bar and flow rate of 8 L/min. The capillary temperature was set at 210°C and spray voltage was 4.5 kV.

All samples were analyzed in duplicate in both positive and negative mode with electrospray ionization.[24]

Each LC system consisted of a pump, a column oven and an autosampler. Lipids were separated on an Acquity UPLC CSH C18 column (100 × 2.1 mm; 1.7 μm) coupled to an Acquity UPLC CSH C18 VanGuard precolumn (5 × 2.1 mm; 1.7 μm) (Waters, Milford, MA). The column was maintained at 65 °C at a flow-rate of 0.4 mL/min. The mobile phases consisted of (A) 60:40 (v/v) acetonitrile:water with ammonium formate (10 mM) and formic acid (0.1%) and (B) 90:10 (v/v) isopropanol:acetonitrile with ammonium formate (10 mM) and formic acid (0.1%). The separation was conducted under the following gradient: 0 min 15% (B); 0−2 min 30% (B); 2−2.5 min 48% (B); 2.5−11 min 82% (B); 11−11.5 min 99% (B); 11.5−12 min 99% (B); 12−12.1 min 15% (B); and 12.1−15 min 15% (B).[24]

2.5. Quality control

Quality control was assured by randomization of the sequence, injection of 3 pooled samples to equilibrate the LC−MS system before the actual sequence of samples; injection of pool samples at the beginning each 10 actual samples.[24]

2.6. Data Processing

In case of the instrument, the .d files were centroided and by converting files to ABF format. Raw data files were converted to ABF format using Reifycs Abf (Analysis Base File) Converter (accessible at: http://www.reifycs.com/AbfConverter/).[24]

For data processing, MS-DIAL (v. 4.48) software program was used.

The following parameters for data collection, peak detection, identification and alignment were used: retention time begin, 0 min; retention time end, 15 min; mass range begin, 0 Da; mass range end, 2000 Da; MS1 (centroiding) tolerance 0.01 Da MS2 (centroiding) tolerance, 0.025 Da; smoothing method: Linear Weighted Moving Average, smoothing level, 3 scans; minimum peak height 1000; mass slice width, 0.1 Da; retention time tolerance for retention time−m/z (tR−m/z) library, 100 min; accurate mass tolerance (MS1) 0.01, accurate mass tolerance (MS2) 0.05, retention time tolerance 0.1, accurate mass tolerance 0.01, identification score cut off 85.[24]

For lipid identification, accurate mass and MS/MS matching was used with the public LipidBlast library of over 200000 MS/MS spectra.[24]

Normalization was preformed in MSDial and by using class-specific internal standards and reported "estimated" 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 internal standard

If multiple isomeric lipid species were detected, the sum of their abundances would be further considered. This operation is driven by the fact that the exact position and stereochemistry of the unsaturations could not be deduced from this kind of experiment.[25]

Data were then filtered for blank samples signals with a fold change >10. Lipids that presented a coefficient of variation (CV%) ≥30% in the QC were excluded for further investigation.[26]

Data was exported in Excel file and filtered according to following manner:MS/MS true, rev dot product ≥700 te dot product ≥350.

2.7. Statistical and Data Analysis

As a first approach to evidence differences in lipid metabolisms between overweight smokers and non-smokers, the different classes (sum of the concentrations of the species) were compared by t-test with GraphPad Prism 7.0 (GraphPad Software, Inc, La Jolla, CA, USA).[25]

GraphPad Prism was also used to remove outliers from the data set and then, for biomarker discovery, data tables with the lipids identified under both smokers and non-smokers detected in positive and negative set were formatted as .csv files and uploaded to the MetaboAnalyst server (version 4.0).[27,28]

In Metabo Analyst server data was processed as follows: row wise normalization to constant sum, log transformation (base 10) and Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable) was applied. Univariate analysis methods were used for exploratory data analysis, specifically for two-group data, Fold Change (FC) analysis and t-tests method was performed. Multivariate statistics (PCA and PLS-DA analysis) and cluster analysis (heatmaps) were also done.

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 was triglycerides (TG) and the least abundant was monoacylglycerol (MG).

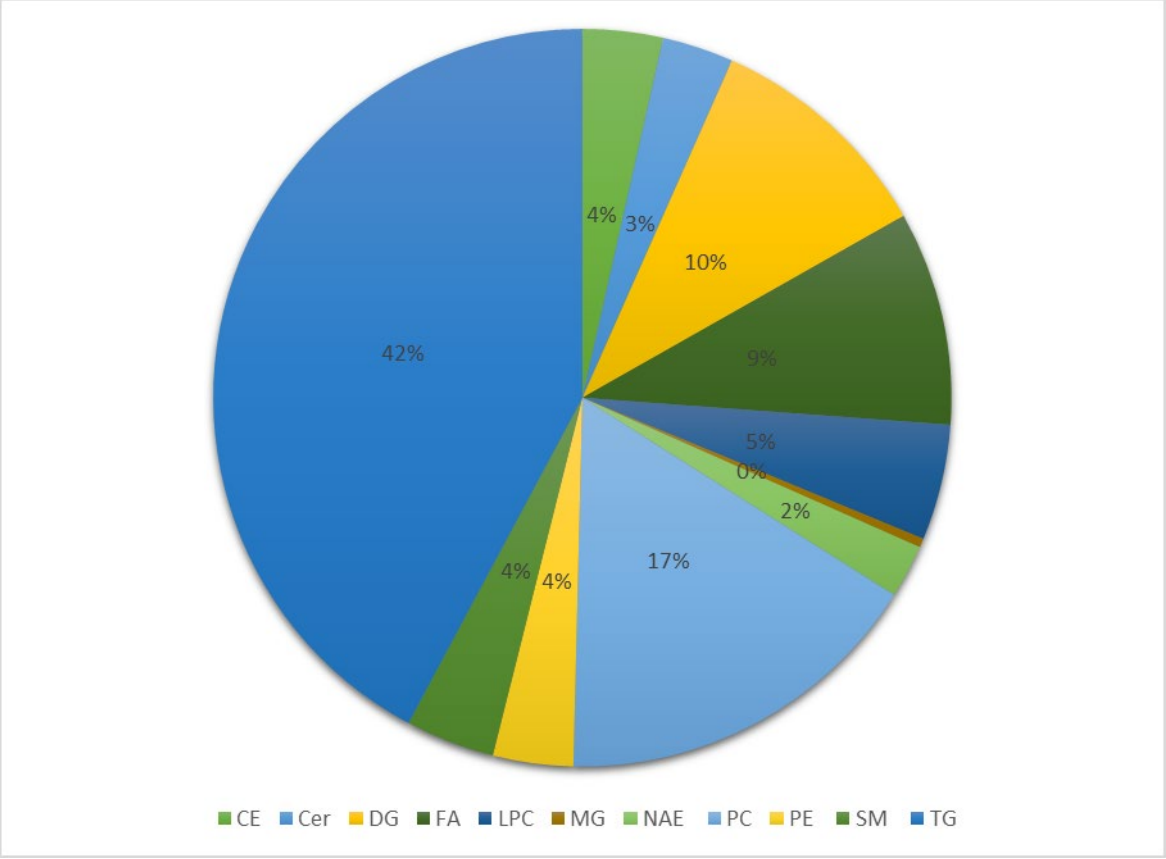


Figure 3. Different lipid classes identified by LC MS analysis. The graph represents the number of lipids that are detected per group.

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.

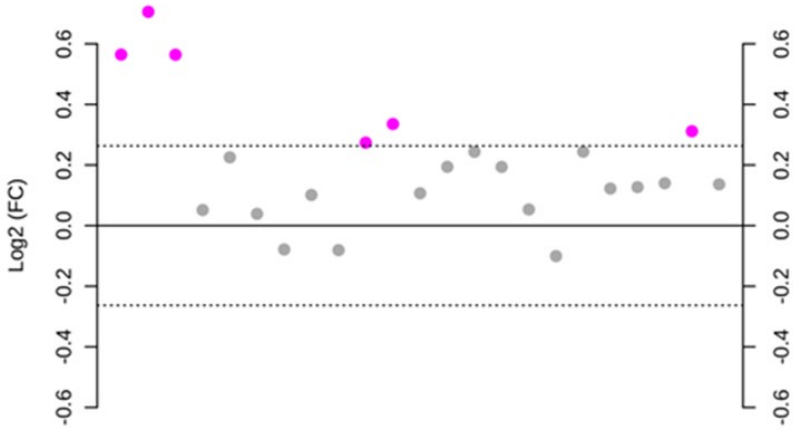


Figure 4: Important features selected by fold-change analysis with threshold 1.2. The purpled circles represent features above the threshold. The values are on log scale, so that both up-regulated and downregulated features are identified (direction of comparisons: NS vs S). [27]

Tabel 5: Important features 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 to decrease was observed in lipid classes in smokers. Exceptionally, fatty acids were upregulated in S vs NS.

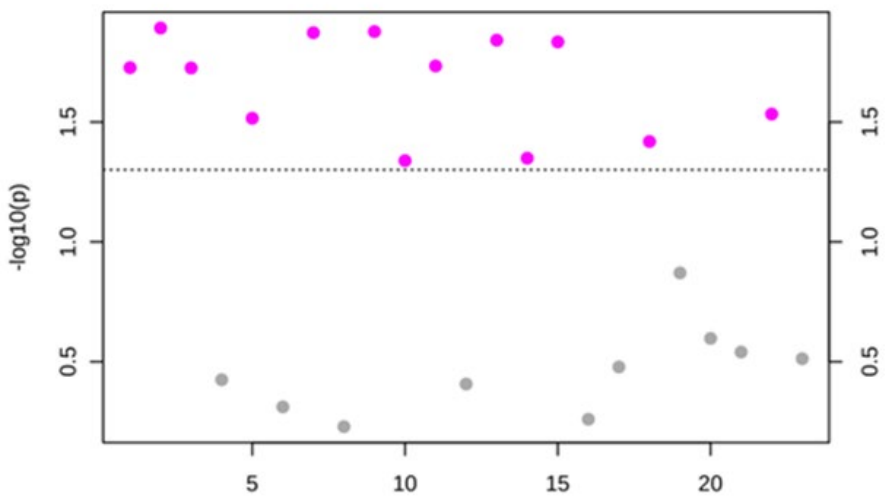


Figure 5: Important features selected by t-tests with threshold 0.05. The red circles represent features above the threshold. [27]

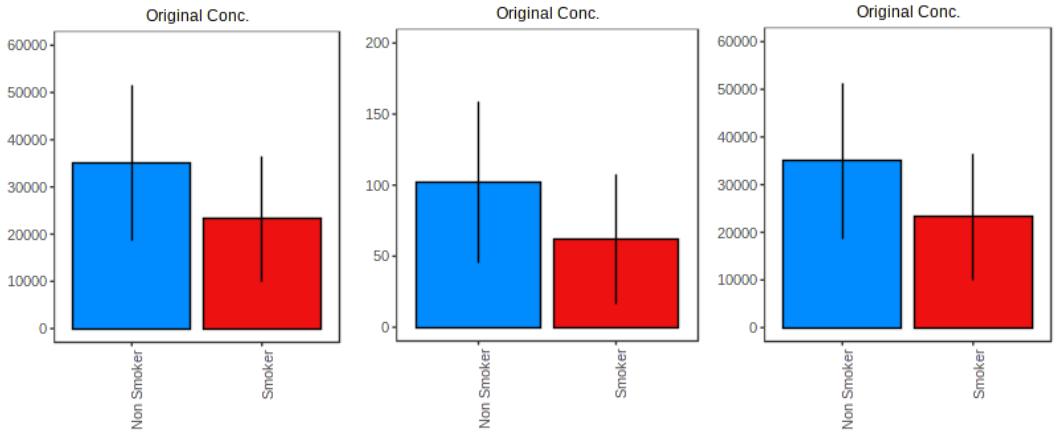
Table 6: Important features selected by t-tests with threshold 0.05.

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

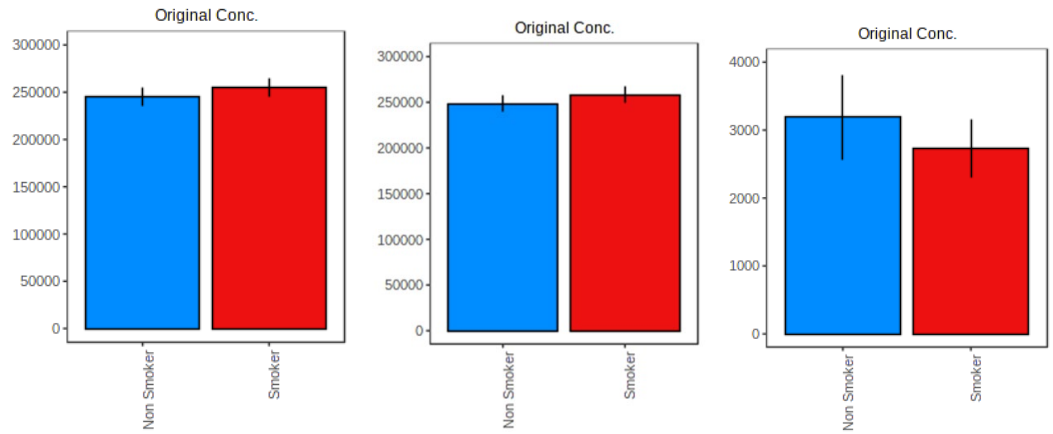
Ether PE	2.1577	0.038129	1.4187
PC saturated	2.0778	0.044755	1.3492
LPC	2.0676	0.045819	1.339

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

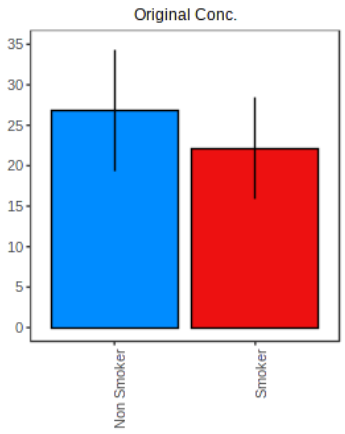
Exact concentrations of significantly different lipid classes are shown in graphs (figure 6). Most of them are decreased in smokers in comparison to non-smokers.



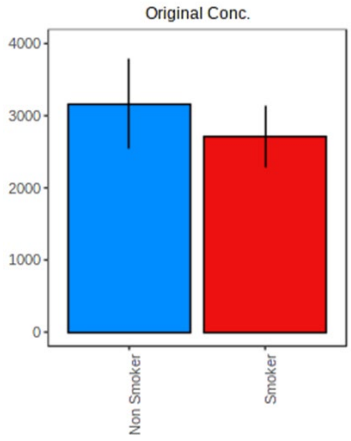
CE **Saturated CE** **Unsaturated CE**



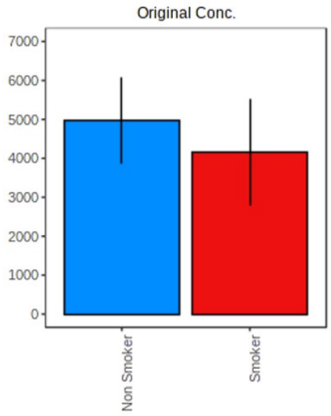
Unsaturated FA **FA** **PC**



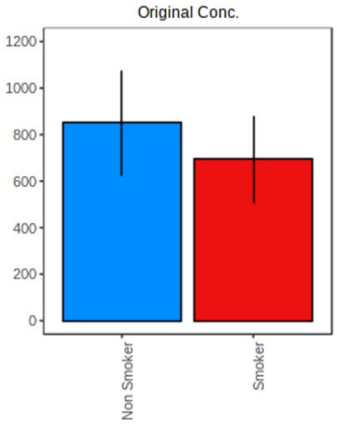
Saturated PC



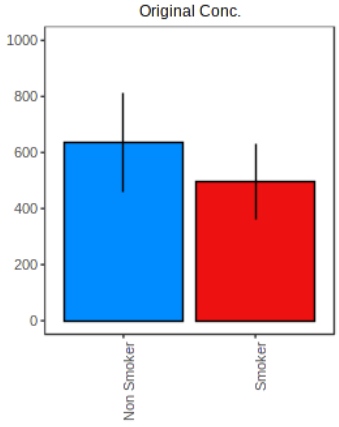
Unsaturated PC



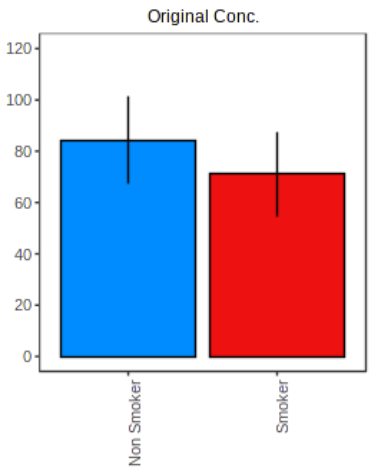
Ether PE



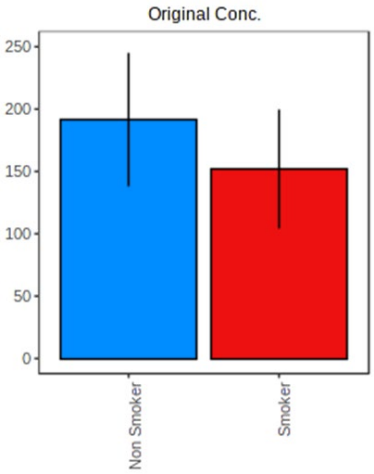
LPC



Saturated LPC



Saturated DG



Saturated TG

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

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

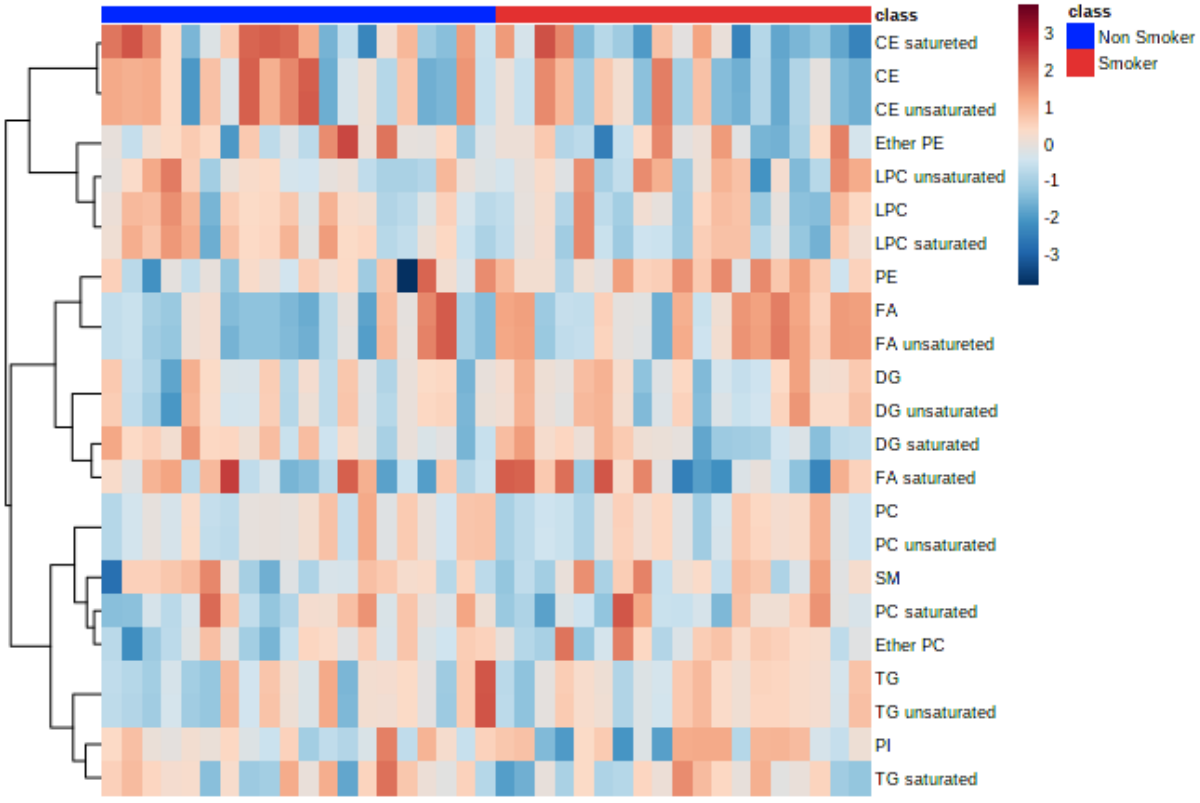


Figure 7: Clustering result shown as heatmap (distance measure using correlation, and clustering algorithm using ward.D). [27]

3.2. Individual lipid species

Comparing smokers and non-smokers some lipids were considerably changed as revealed by fold change analysis. Graphical representation of fold change analysis is shown in figure 8 and list of important features is in Table 7.

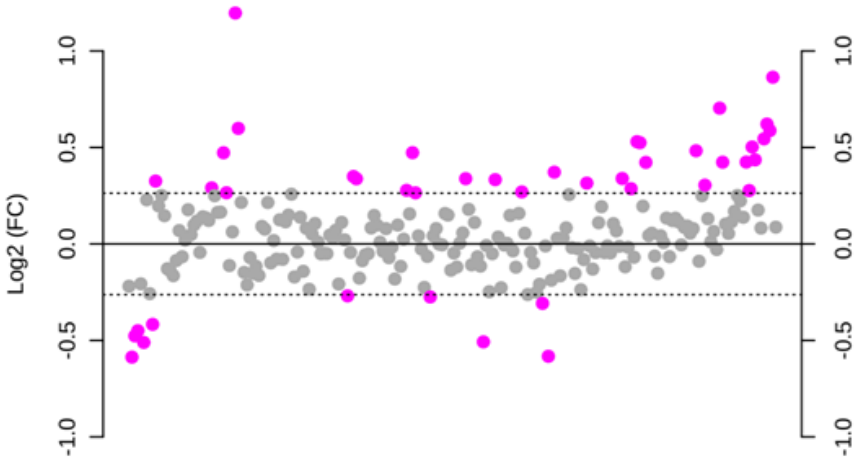


Figure 8: Important features selected by fold-change analysis with threshold 1.2. The purpled circles represent features above the threshold. The values are on log scale, so that both up-regulated and downregulated features are identified. [27]

Table 7: Important 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).

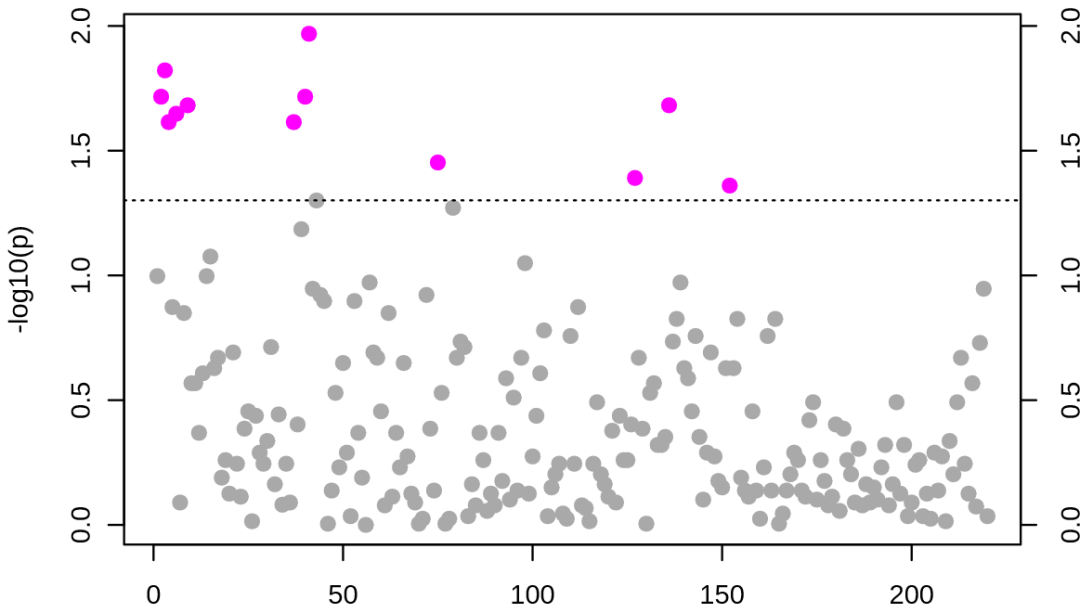


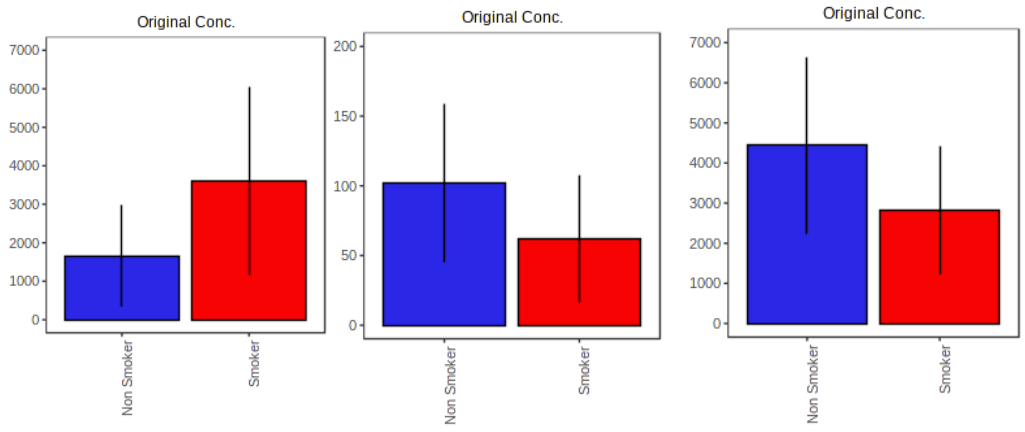
Figure 9. Important features selected by t-tests with threshold 0.05. The purpel circles represent features above the threshold.[27]

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

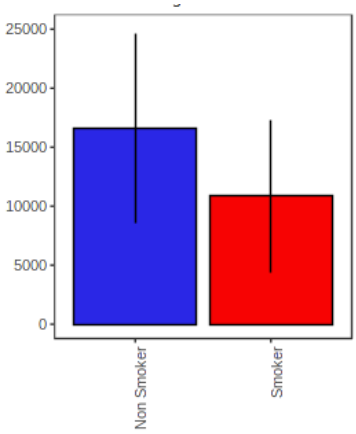
Metabolite name	t.stat	p.value	-log10(p)
CE 18:0	2.5453	0.015218	1.8176
CE 18:1	2.7428	0.009335	2.0299
CE 18:2	2.6418	0.012012	1.9204
CE 20:4	2.7628	0.008874	2.0519
DG 28:2	-2.1246	0.040364	1.394
FA 18:1	-2.9115	0.006061	2.2174
TG 52:7|TG 16:0_18:3_18:4	-2.2582	0.029917	1.5241
TG 54:7|TG 16:0_18:2_20:5.1	-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).

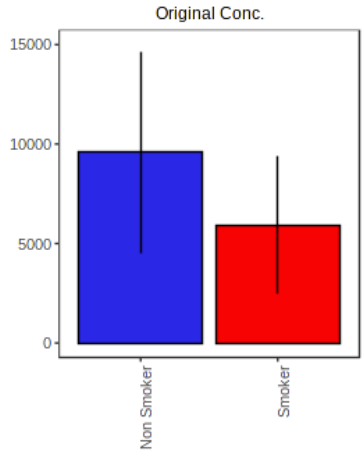
Exact concentrations of significantly different individual lipid are shown in graphs (figure 10). Most of them are decreased in smokers in comparison to non-smokers.



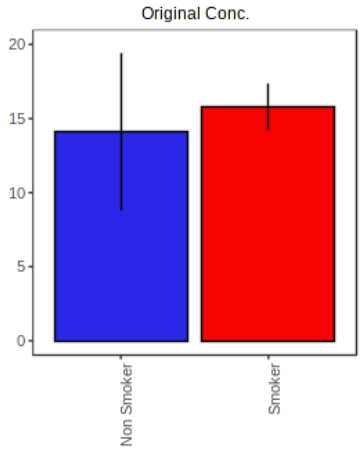
FA 18:1 **CE 18:0** **CE 18:1**



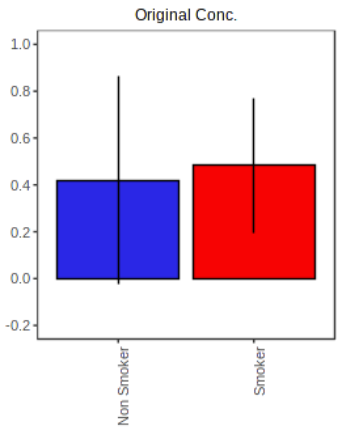
CE 18:2



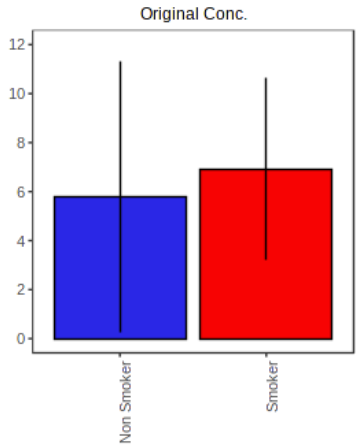
CE 20:4



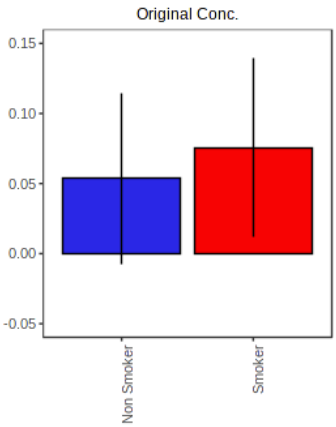
DG 28:2



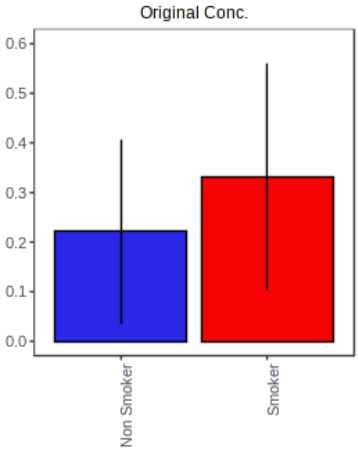
TG 52:7



TG 54:7



TG 56:10



TG 58:11

Figure 10. Lipid content comparison between non- smokers vs smokers Graphs represent the lipid amount (Amount of lipids in µmol/ml, mean ± SD), which indicates the sum of the metabolites intensities within a individual metabolite after normalization by internal standard. [27]

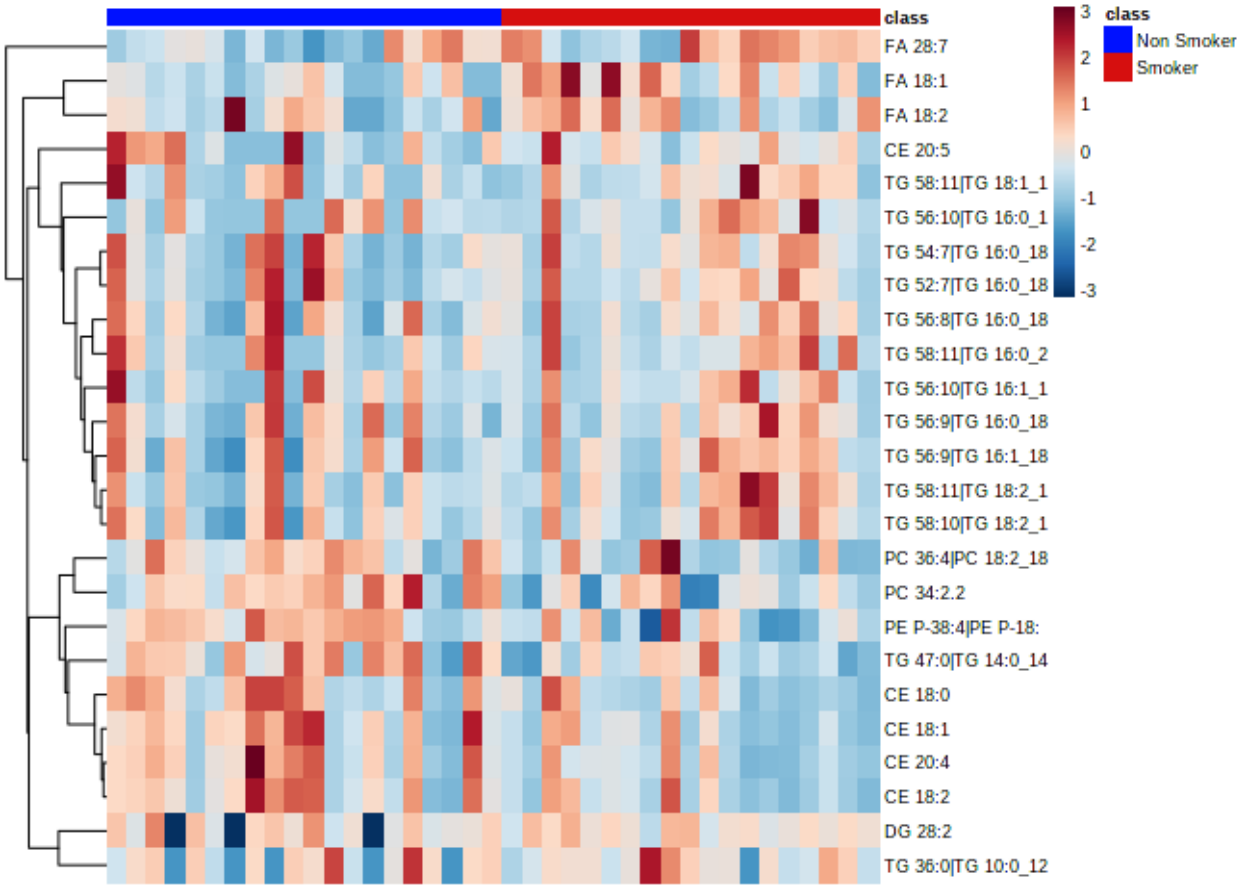


Figure 11: Clustering result shown as heatmap (distance measure using euclidean, and clustering algorithm using ward.D). [27]

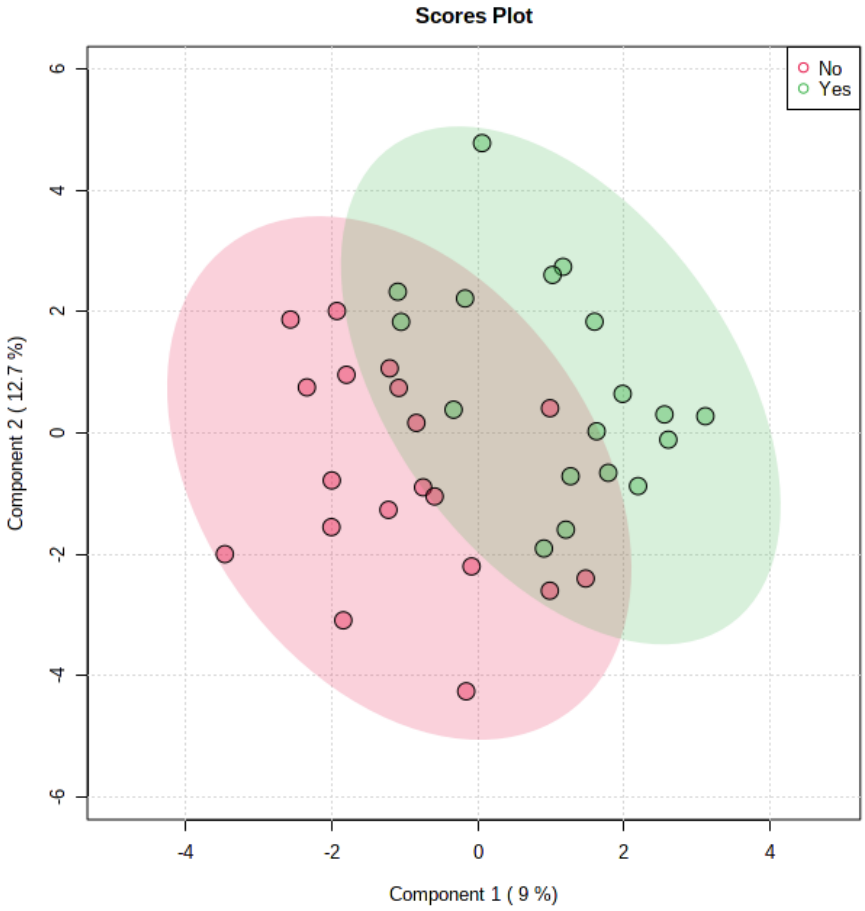


Figure 12- Partial Least Squares Discriminant Analysis (PLS-DA) for the subgroups (red color represents non- smokers, green color represents smokers).[27]

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.

6. REFERENCES

1. Śliwińska-Mossoń, M., Mihułka, E., & Milnerowicz, H. Assessment of lipid profile in non-smoking and smoking young health persons. *Przeglad lekarski*, *71*(11) (2014) 585–587.
2. Mitchell, B, et al., Tobacco Use and Cessation: The Adverse Health Effects of Tobacco and Tobacco-Related Products,Primary Care: Clinics in Office Practice 26(3) (2014) 463-98.
3. Chelland Campbell, S., Moffatt, R. J., & Stamford, B. A. Smoking and smoking cessation -- the relationship between cardiovascular disease and lipoprotein metabolism: a review. *Atherosclerosis*, *201*(2) (2008) 225–235 doi: https://doi.org/10.1016/ j.atherosclerosis.2008.04.046
4. Xu, T., Hu, C., Xuan, Q., & Xu, G. Recent advances in analytical strategies for mass spectrometry-based lipidomics. *Analytica chimica acta*, *1137*, (2020) 156–169. doi: https://doi.org/10.1016/j.aca.2020.09.060
5. Shevchenko A., Simons K. Lipidomics: coming to grips with lipid diversity. *Nat. Rev. Mol. Cell Biol. (*2010) 11(8):593–598.
6. Stephenson, D. J., Hoeferlin, L. A., & Chalfant, C. E. Lipidomics in translational research and the clinical significance of lipid-based biomarkers. *Translational research: the journal of laboratory and clinical medicine 189*, (2017) 13–29. doi:https://doi.org/10.1016/j.trsl.2017.06.006
7. Cajka, T., & Fiehn, O.LC-MS-Based Lipidomics and Automated Identification of Lipids Using the LipidBlast In-Silico MS/MS Library. *Methods in molecular biology (Clifton, N.J.)*, *1609*, (2017) 149–170. doi: https://doi.org/10.1007/978-1-4939-6996-8_14
8. Fahy, E., Cotter, D., Sud, M., & Subramaniam, S. Lipid classification, structures and tools. *Biochimica et biophysica acta*, *1811*(11), (2011) 637–647. doi: https://doi.org/10.1016/j.bbalip.2011.06.009
9. Hinterwirth, H., Stegemann, C., & Mayr, M. Lipidomics: quest for molecular lipid biomarkers in cardiovascular disease. Circulation. Cardiovascular genetics, 7(6), (2014) 941–954. doi: https://doi.org/10.1161 /CIRCGENETICS. 114.000550

10. Antonny B. Mechanisms of membrane curvature sensing. In: Kornberg R.D., Raetz C.R.H., Rothman J.E., Thorner J.W., editors. vol. 80. Annual Reviews; Palo Alto: (2011) 101–123.
11. Hinterwirth, H., Stegemann, C., & Mayr, M. Lipidomics: quest for molecular lipid biomarkers in cardiovascular disease. *Circulation. Cardiovascular genetics*, *7*(6), (2014) 941–954. doi: https://doi.org/10.1161/CIRCGENET ICS.114.000550
12. van Meer, G., Voelker, D. R., & Feigenson, G. W. Membrane lipids: where they are and how they behave. *Nature reviews. Molecular cell biology*, *9*(2) (2008) 112–124. doi:https://doi.org/10.1038/nrm2330
13. Tao, B. Y., Industrial Applications for Plant Oils and Lipids Bioprocessing for Value-Added Products from Renewable Resources, Elsevier, (2007), Pages 611-627, doi: https://doi.org/10.1016/B978-044452114-9/50025-6
14. Alfred H. MerrillJr., Sphingolipids, Biochemistry of Lipids, Lipoproteins and Membranes (Fifth Edition), Elsevier, (2008) Pages 363-397, doi: https://doi.org/10.1016/B978-044453219-0.50015-5.2008
15. Roger Hull, Plant Virus Viromics: Involvement of Genomes of Three Organisms—Virus, Host, and Vector,Academic Press, (2014) Pages 929-971, doi: https://doi.org/10.1016/B978-0-12-384871-0.00016-9.
16. Paola Donato, Paola Dugo, Luigi Mondello, Separation of lipids,Liquid Chromatography (Second Edition),Elsevier, (2017) Pages 201-243, doi: https://doi.org/10.1016/B978-0-12-805392-8.00008-6
17. Amitav Bhattacharya, Lipid Metabolism in Plants Under High Temperature, Effect of High Temperature on Crop Productivity and Metabolism of Macro Molecules, Academic Press, (2019), Pages 311-389, doi: https://doi.org/10.1016/B978-0-12-817562-0.00004-5.
18. C.P. Ridley, C. Khosla, Polyketides, Encyclopedia of Microbiology (Third Edition), Academic Press, (2009), Pages 472-481, doi: https://doi.org/ 10.1016/B978-012373944-5.00158-9
19. Francesco Vinale, Krishnapillai Sivasithamparam, Susanne Zeilinger, Santiago Gutiérrez, Fungal Secondary Metabolism, Encyclopedia of Mycology, Elsevier, (2021)Pages 54-63, doi: https://doi.org/10.1016/B978-0-12-819990-9.00031-7
20. Dei Cas, M., Zulueta, A., Mingione, A., Caretti, A., Ghidoni, R., Signorelli, P., & Paroni, R. An Innovative Lipidomic Workflow to Investigate the Lipid Profile in

a Cystic Fibrosis Cell Line. Cells, 9(5), (2020) 1197. doi: https:// doi . org/ 10.3390/cells9051197

21. URL: https://avantilipids.com/product/330707 (18.10.2021.)
22. URL: https://avantilipids.com/assets/products/attachment_s/33_0707-_Mixture-Components-and-Concentrations.pdf (18.10.2021.)
23. Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A and Schwudke D Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lip Res (2008) 49: 1137-1146
24. Tomas Cajka, Jennifer T. Smilowitz, and Oliver Fiehn Validating Quantitative Untargeted Lipidomics Across Nine Liquid Chromatography–High-Resolution Mass Spectrometry Platforms, Analytical Chemistry (2017) 89 (22), 12360-12368
25. Dei Cas, M., Zulueta, A., Mingione, A., Caretti, A., Ghidoni, R., Signorelli, P., & Paroni, R. An Innovative Lipidomic Workflow to Investigate the Lipid Profile in a Cystic Fibrosis Cell Line. *Cells*, *9*(5), (2020) 1197. doi: https://doi.org/ 10 .3390/ ce lls9051197
26. Hu C., Zhou Y., Feng J., Zhou S., Li C., Zhao S., Shen Y., Hong L., Xuan Q., Liu X., et al. Untargeted Lipidomics Reveals Specific Lipid Abnormalities in Nonfunctioning Human Pituitary Adenomas. *J. Proteome Res. (*2019); 19:455–463.
27. Xia J., Wishart D.S. Using metaboanalyst 3.0 for comprehensive metabolomics data analysis. Curr. Protoc. Bioinforma. (2016) 55:14.10.1–14.10.91.
28. Chong J., Soufan O., Li C., Caraus I., Li S., Bourque G., Wishart D.S., Xia J. MetaboAnalyst 4.0: Towards more transparent and integrative metabolomics analysis. Nucleic Acids Res. (2018); 46:486–494. doi: 10.1093/nar/gky310
29. (WHO) World Health Organization (1983). Chronicle 37: 86-90.
30. Lillian M, Muula AS Tobacco Use among High School Students in Kampala, Uganda: Questionnaire Study 45 (2004): 80-83.
31. Bhatt J Impact of tobacco smoking on coronary risk factor profile. J. Appl. Basic Med. Sci. 5 (2003) 105-108.
32. Buring JE, O'Connor GT, SZ Goldhaber SZ Decreased HDL2 and HDL3 cholesterol, apo A-I and apo A-II, and increased risk of myocardial infarction. Circulation 85 (1992) 22-29.

33. Komiya H, Mori Y, Yokose T, et al. Smoking as a risk factor for visceral fat accumulation in Japanese men. Tohoku J Exp Med (2006); 208(2): 123–132.
34. Tie, F., Ding, J., Hu, N., Dong, Q., Chen, Z., & Wang, H. Kaempferol and Kaempferide Attenuate Oleic Acid-Induced Lipid Accumulation and Oxidative Stress in HepG2 Cells. *International journal of molecular sciences*, *22*(16), (2021) 8847. doi: https://doi.org/10.3390/ijms22168847
35. Steffen, B. T., Duprez, D., Szklo, M., Guan, W., & Tsai, M. Y. Circulating oleic acid levels are related to greater risks of cardiovascular events and all-cause mortality: The Multi-Ethnic Study of Atherosclerosis. *Journal of clinical lipidology*, *12*(6), (2018) 1404–1412. doi: https://doi.org/ 10.1016/j.jacl. 2018 . 0 8.004
36. Goettel M., Niessner R., Pluym N., Scherer G., and Scherer M.: "A Fully Validated GC-TOF-MS Method for the Quantification of Fatty Acids Revealed Alterations in the Metabolic Profile of Fatty Acids after Smoking Cessation" J Chromatogr B, (2017.) Vol. 1041, pp. 141-50
37. Rudel, L.L.; Shelness, G.S. Cholesterol esters and atherosclerosis-a game of ACAT and mouse. Nat. Med. (2000), 6, 1313–1314
38. Yetukuri, L., Söderlund, S., Koivuniemi, A., Seppänen-Laakso, T., Niemelä, P. S., Hyvönen, M., Taskinen, M. R., Vattulainen, I., Jauhiainen, M., & Oresic, M. Composition and lipid spatial distribution of HDL particles in subjects with low and high HDL-cholesterol. *Journal of lipid research*, *51*(8), (2010) 2341–2351. doi: https://doi.org/10.1194/jlr.M006494
39. Gepner, A. D., Piper, M. E., Johnson, H. M., Fiore, M. C., Baker, T. B., & Stein, J. H. Effects of smoking and smoking cessation on lipids and lipoproteins: outcomes from a randomized clinical trial. *American heart journal*, *161*(1),(2011) 145–151. doi: https://doi.org/10.1016/j.ahj.2010.09.023
40. Mjøs O. D. Lipid effects of smoking. *American heart journal*, *115*(1 Pt 2), (1988) 272–275. doi: https://doi.org/10.1016/0002-8703(88)90649-7
41. Komiya H, Mori Y, Yokose T, et al. Smoking as a risk factor for visceral fat accumulation in Japanese men. Tohoku J Exp Med (2006); 208(2): 123–132.
42. Devaranavadgi BB, Aski BS, Kashinath RT, et al. Effect of cigarette smoking on blood lipids–a study in Belgaum, Northern Karnataka, India. *Global J Med Res* (2012); 6: 57–61.

43. Jain, R. B., & Ducatman, A. Associations between smoking and lipid/lipoprotein concentrations among US adults aged ≥20 years. *Journal of circulating biomarkers*,(2018), *7*.
44. Titz, B., Luettich, K., Leroy, P., Boue, S., Vuillaume, G., Vihervaara, T., Ekroos, K., Martin, F., Peitsch, M. C., & Hoeng, J. Alterations in Serum Polyunsaturated Fatty Acids and Eicosanoids in Patients with Mild to Moderate Chronic Obstructive Pulmonary Disease (COPD). *International journal of molecular sciences*, *17*(9), (2016) 1583. doi:https://doi.org/10.3390/ijms17091583
45. Djekic D, Pinto R , Repsilber D, Hyotylainen T, Henein M,(2019):15 Pages 123—135 doi: https://doi.org/10.2147/VHRM.S202344