PLASMA LIPIDOMICS IN SUBJECTS WITH COMBAT POSTTRAUMATIC STRESS DISORDER

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ABSTRACT

Posttraumatic stress disorder (PTSD) is complex neuropsychiatric disorder triggered by a traumatic event and characterized by the symptoms that represent large burden to patients, as well as to society. Lipidomic approach can be applied as a useful tool for discovery of novel diagnostic, prognostic and therapeutic lipid biomarkers of various disorders, whose etiology is complex and still unknown, including PTSD. Since changes in the levels of lipid metabolites might indicate impairments in various metabolic pathways and cellular processes, the aim of this lipidomic study was to determine altered levels of lipid compounds in PTSD. The study enrolled 235 male patients with combat PTSD and 241 healthy male control subjects. Targeted lipidomic analysis of plasma samples was conducted using reverse-phase liquid chromatography coupled with mass spectrometry. Lipids that have been analyzed belong to the group of ceramides, cholesterol esters, diacylglycerols, lysophosphatidylcholines, lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethanolamines, sphingomyelins and triglycerides. The levels of fifteen lipid compounds were found to be significantly different between PTSD patients and healthy control subjects, including four phosphatidylcholines, two phosphatidylethanolamines, five sphingomyelins, two cholesterol esters and two ceramides. The lipid metabolites whose levels significantly differed between patients with PTSD and control subjects are associated with various biological processes, including impairments of membrane integrity and function, mitochondrial dysfunction, inflammation and oxidative stress. As these processes might be associated with development and progression of PTSD, altered lipid compounds represent potential biomarkers that could facilitate the diagnosis of PTSD, prediction of the disease, as well as identification of novel treatment approaches in PTSD.

Keywords: biomarkers, lipids, lipidomics, PTSD

Abbreviations[1]

[1] ACAT2 - acyl-coenzyme A:cholesterol acyltransferase 2, AUC - area under the curve, BMI - body mass index, CAPS - Clinician Administered PTSD Scale, CE - cholesterol ester, Cer – ceramide, DG – diacylglycerol, DSM-5 - Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, ESI - electrospray ionization, HPA axis - hypothalamic-pituitary-adrenal axis, HPLC – high pressure liquid chromatography, ICD-10 - International Classification of Diseases, LC - liquid chromatography , LCAT - lecithin:cholesterol acyltransferase, LPC – lysophosphatidylcholines, LPE – lysophosphatidylethanolamines, MetOH – methanol, MRM – multiple reaction monitoring, MS – mass spectrometry, OPLS-DA - Orthogonal Partial Least Square - Discriminant analysis, PA - phosphatidic acid, PC – phosphatidylcholine, PE – phosphatidylethanolamine, PG – phosphoglycerol, PI – phosphatidylinositol, PLA2 - phospholipase A2, PLS-DA - Partial Least Square - Discriminant analysis, PS – phosphatidylserine, PTSD - posttraumatic stress disorder, ROC - receiver operating characteristic, RPLC - reversed-phase

INTRODUCTION

Lipids are heterogeneous class of metabolites that take part in various metabolic pathways and different cellular processes [1,2]. According to the hydrophobic group that contains different moieties, they are classified into groups of fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, prenol lipids, saccharolipids, and polyketides [3]. The balance between various metabolites, including lipid metabolites, their cofactors, intermediates, enzymes and substrates is important to maintain the homeostasis [4]. Due to their susceptibility to various environmental factors, such as nutrition, stress and xenobiotics, lipid levels might show large variability [4], and indicate certain health disturbances or illnesses, including cardiovascular diseases, diabetes, neurodegenerative or neuropsychiatric disorders [2]. Lipidomics, the subgroup of rapidly growing field of metabolomics, gives a comprehensive analysis of lipidome, a complete lipid profile present in a certain biological sample [5-7], and represents a powerful tool for the identification and development of potential lipid biomarkers for various disorders [6], including posttraumatic stress disorder (PTSD).

PTSD is a serious and complex, trauma- or stress-related neuropsychiatric disorder, which develops in certain individuals after exposure or witnessing to a traumatic event. It is characterized with symptoms, such as re-experiencing, avoidance, hyperarousal and negative alterations in mood and thinking [8,9], which affect person's everyday functioning and poses a substantial health, social and economic burden [10]. Traumatic experiences exert a major effect on a systemic level of affected individual, causing not only psychological, but also somatic disturbances, including neuroanatomical, immunological, neuroendocrine, neurotransmitter, as well as metabolomic alternations [4,11,12]. So far, only several original investigations [13-16], including our previous study [17], as well as few review articles [4,18,19] assessed metabolic/lipidomic network in PTSD. Among other identified altered metabolites, previous studies enrolling subjects with PTSD, observed changes in the levels of lipid compounds that can be classified as glycerophospholipids, sphingolipids, fatty acids and sterols [13-17]. These lipid classes play an important role as energy source and structural barriers [20], as well as coordinators of cell signaling, apoptosis, differentiation, metabolic reactions, and transportation [13]. Likewise, various studies indicate involvement of oxidative stress in PTSD pathophysiology, which is manifested through altered lipid levels, decreased antioxidant enzyme activity and increased lipid peroxidation products [21].

Therefore, based on our previous and other published results, the aim of this targeted lipidomic study was to determine the lipid compounds that could represent potential biomarkers for better diagnosis of PTSD, prediction of the disease, as well as the identification of novel therapeutic targets for PTSD. The changes in the levels of lipid metabolites in PTSD might indicate impairments in corresponding metabolic pathways and cellular processes, including hypothalamic-pituitary-adrenal (HPA) axis activity, oxidative stress, mitochondrial dysfunction, and inflammation [14,17].

METHODS AND MATERIALS

Subject recruitment

The study was conducted at Ruder Boskovic Institute (Zagreb, Croatia) and the Centre of Metabolomics and Bioanalysis, CEMBIO (Madrid, Spain). Plasma samples of 235 male patients with combat PTSD and 241 healthy male control subjects have been collected from October 2015 to February 2017 at the University Psychiatric Hospital Vrapce (Zagreb, Croatia).

Patients with PTSD were unrelated Caucasian veterans of Croatian origin, who participated in the Croatian War of Independence (1991-1995). They were evaluated using Structured Clinical Interview (SCID) for Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) [9] and Clinician

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liquid chromatography, RT – retention time, SCID - Structured Clinical Interview SM – sphingomyelin, TG – triglyceride, TNF-α - tumor necrosis factor alpha, VIP - variable importance in the projection

Administered PTSD Scale (CAPS) [22]. All subjects were medication free for at least 30 days. Exclusion criteria were somatic diseases, identified according to International Classification of Diseases (ICD-10) [23], fibrosis, sclerosis, cirrhosis and malignant liver disease, use of antidiabetic and antihypertensive medication, as well lipid lowering agents, drug and alcohol abuse, presence of other neuropsychiatric and neurodegenerative disorders, determined using the DSM-5 criteria. Healthy control Caucasian subjects of Croatian origin were assessed with the same diagnostic tools and met the same inclusion and exclusion criteria, with a diagnosis of PTSD as an additional exclusion criterion.

The study was approved by the Bioethics Committee of the Ruder Boskovic Institute (Zagreb, Croatia) and Ethics Committee of the University Psychiatric Hospital Vrapce (Zagreb, Croatia). All participants signed an informed consent for participation, while all experimental procedures were carried out in an accordance with the Helsinki Declaration from 1975 (revised in 2008).

Plasma collection

After overnight fasting, blood samples (8.5 ml) from participants were collected in BD Vacutainer® tubes containing 1.5 mL acid citrate dextrose anticoagulant. Whole blood samples were centrifuged for 3 min at 2900 x g in order to separate the plasma, which was again centrifuged for 15 min at 4800 x g to remove platelets. Aliquots of platelet-poor plasma were stored at -80 ^{o}C.

Targeted compounds

Due to possible involvement of lipid metabolites in the PTSD etiology, targeted lipidomic study of various lipid metabolites has been performed. Analyzed compounds belong to the group of ceramides (Cer), cholesterol esters (CE), diacylglycerols (DG), lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), phosphatidylcholines (PC), phosphatidylethanolamines (PE), sphingomyelins (SM) and triglycerides (TG) (Supplementary table 1).

Sample preparation

The SPLASH® Lipidomix® Mass Spec Standard, an internal standard mixture containing 18:1(d7) LPE, 15:0-18:1(d7) PC, 15:0-18:1(d7) PE, 15:0-18:1(d7)-15:0 TG, 18:1(d7) Chol Ester, 18:1(d7) LPC, 18:1(d9) SM, 18:1(d7) DG, 15:0-18:1(d7)-PA, 15:0-18:1(d7)-PG, 15:0-18:1(d7)-PI, 15:0-18:1(d7)-PS, 18:1(d7)-MG and cholesterol (d7) at different concentrations was obtained from Avanti® Polar Lipids, Inc. (Alabama, U.S.A.), and was 20 times diluted in methanol (MetOH) prior to analysis. The standard working solution was stored at -20 ^{o}C. For lipid extraction, plasma samples were thawed on ice, vortex-mixed and 10 µL of each sample was transferred to the 1.5 mL Eppendorf tubes. Exactly 10 µL of diluted internal standard mixture (SPLASH® LIPIDOMIX® Internal Standard, Avanti, AL, USA) were added to each sample prior to extraction. Protein precipitation and lipid extraction were initiated by adding 800 µL solvent mixture (2:1 ethylacetate:ethanol) to each sample. Samples were vortexed briefly and centrifuged for 10 min at 13700 rpm at 15 ^{o}C. Supernatants were then transferred to chromatographic vials. The use of isotope-labeled standard represents well-grounded approach, since sample matrix can influence the quantitation of metabolites [24]. Blank samples were prepared with water instead of plasma and were analyzed at the beginning and at the end of the sequence for detection of possible contaminations.

Liquid chromatography coupled with mass spectrometry (LC-MS/MS)

The lipidomic analysis included reversed-phase liquid chromatography (RPLC), which is commonly used for non-polar metabolites [25]. It was carried out in an ultra HPLC Agilent 1290 Infinity system (Agilent Technologies, Palo Alto, Calif.) using a 1290 Infinity Binary Pump, coupled to an Agilent 6470 triple quadrupole mass spectrometer (LC-MS/MS), with an electrospray ionization (ESI) interface, working in multiple reaction monitoring (MRM) mode. Samples were injected randomly with an injection volume set to 5 µL. The separation was achieved using a HPLC column Gemini 3u C6-Phenyl 110A (150 x 2.00 mm) (Phenomenex, CA, USA), which has been kept at 60°C during the analysis. The

elution conditions employed a flow rate of 0.6 mL/min with the gradient of the solvent A (30% MetOH, 70% H_2O + 0.1% Formic Acid + 1mM Ammonium Acetate) and the solvent B (MetOH + 0.1% Formic Acid + 1mM Ammonium Acetate). The analysis lasted 12 minutes per sample, and started with 30% of the mobile phase B and increased to the 100% during 12 min (0-12 min), after which it was decreased to the 30% of phase B. All the samples were analyzed in positive ESI mode (full-scan ranging from 50 to 1000 m/z), with a scan rate of 1 scans/s. Samples were kept at 10°C in the autosampler during the analysis. The capillary voltage was set to 4000 V for positive and negative ionization mode. Gas nebulizer was maintained at 30 psi, while drying gas flow rate was 7 L/min at 250°C.

Data treatment and statistical analysis

The MRM signals in the standard curves were used to visually check the homogeneity along the chromatographic runs together with the reproducibility of the pressure curves (Agilent MassHunter Qualitative Analysis, B.09.00, Agilent Technologies). After chromatogram inspection, the software Agilent MassHunter Quantitative Analysis (B.10.00, Agilent Technologies) was used to integrate the peaks and determine area size for each analyzed metabolite. Kolmogorov-Smirnov test was used to check for the normality of the data distribution. For univariate statistical comparisons of obtained data, Student t-test or Mann-Whitney U test were performed (GraphPad Prism version 4.00 for Windows, GraphPad Software). The percentage of change (%) was calculated as follows:

$$\%\Delta = \frac{\text{Average CASES} - \text{Average CONTROLS}}{\text{Average CONTROLS}} x\ 100$$

Positive values indicate increased whereas negative values indicate decreased abundance of a specific lipid compound in the group of PTSD patients compared to the control group. Due to multiple testing, Bonferroni correction was applied, and significant results were considered at $p < 0.000362$. In addition to univariate statistics, supervised Orthogonal Partial Least Square - Discriminant analysis (OPLS-DA) was performed. According to the OPLS-DA models, the volcano plots, plotting variable importance in the projection (VIP) against corrected p-values [p(corr), loading values scaled as correlation coefficients values] were presented. Changes in the levels of metabolites with $p < 0.00036$, VIP > 1.00, absolute p(corr) ≥ 0.30 were considered significant. After the semiquantitation, statistically different concentrations of metabolites between cases with PTSD and healthy control subjects were determined according to the deuterated standard. According to the area under the peak of metabolites in samples and standard, as well as known concentration of the internal standard, the concentration of targeted metabolite was calculated in each sample as following, with *c(S)* indicating the concentration of a certain metabolite in plasma sample, *c(IS)*, the concentration of a certain metabolite in the internal standard, *area (S)*, area under the peak of a corresponding metabolite in plasma sample and *area(IS)*, area under the peak of a corresponding metabolite in the internal standard.

$$\text{c(S)} = (\text{c(IS)} \ \times \ \text{area(S)})/\text{area(IS)}$$

In order to determine the goodness of our model, we performed the receiver operating characteristic (ROC) curve analysis. A high area under the curve (AUC) represents effective model in distinguishing between cases and controls (SIMCA software, version 14.1, Umetrics). ROC curves and AUCs for each compound were determined by using Metaboanalyst 5.0 [26].

Demographic data of two different groups of subjects were compered by Student t-test or Mann-Whitney U test, depending on the normality of the data distribution, while smoking status was assessed by χ^2-test, using GraphPad Prism version 4.00 for Windows (GraphPad Software). To determine whether age and smoking had a significant effect on altered compounds, a multilinear regression model (R Statistics, 3.5.1.) was performed, where the dependent variable was plasma lipid concentration. The first block of multilinear regression included smoking as an independent variable, the second block included smoking and age, while the third block included smoking, age, and diagnosis as independent variables.

RESULTS

Demographic data

All participants involved in this study were male Caucasian subjects of Croatian origin. Patients with PTSD smoked more frequently than control subjects ($p=1.16 \times 10^{-14}$) (Table 1). Out of 235 subjects with PTSD, 64.7% were smokers and 35.3% were non-smokers. On the contrary, only 29.3% of healthy control subjects were smokers, while 70.7% of them were non-smokers. There was no difference in the body mass index (BMI) (p=0.096) between patients with PTSD and healthy control subjects. However, statistically significant difference was found in age ($p=6.91 \times 10^{-20}$) (Table 1), since healthy control subjects (age range: 25-65 years old) were significantly younger than patients with PTSD (age range: 38-77 years old). PTSD subjects had an average CAPS scores that correspond to the moderate PTSD. However, out of 235 PTSD subjects, 18 subjects were diagnosed with severe PTSD.

Table 1. The demographic characteristics of the patients with PTSD and healthy control subjects involved in this study

	PTSD patients N=235	**Control subjects** N=241	**Statistics**
Age (years) [median (25th; 75th)]	55.00 (51; 61)	47.00 (42; 55)	U=14257.5; Z=-9.129; **$p=6.91 \times 10^{-20}$**
Smokers N (%)	152 (64.7)	70 (29.0)	χ^2=59.608; df=1; **$p=1.16 \times 10^{-14}$**
Non-smokers N (%)	83 (35.3)	169 (70.1)	
BMI (kg/m^2) (mean ± SD)	28.23 ± 3.29	28.77 ± 3.80	t=-1.668; p=0.096
CAPS scores [median (25th; 75th)]	86.00 (80; 88)	N/A	N/A

Targeted lipidomic analysis

After the determination of the area under the peak, both OPLS-DA and PLS-DA plots showed clear separation between the cases with PTSD and healthy control subjects (Figure 1).

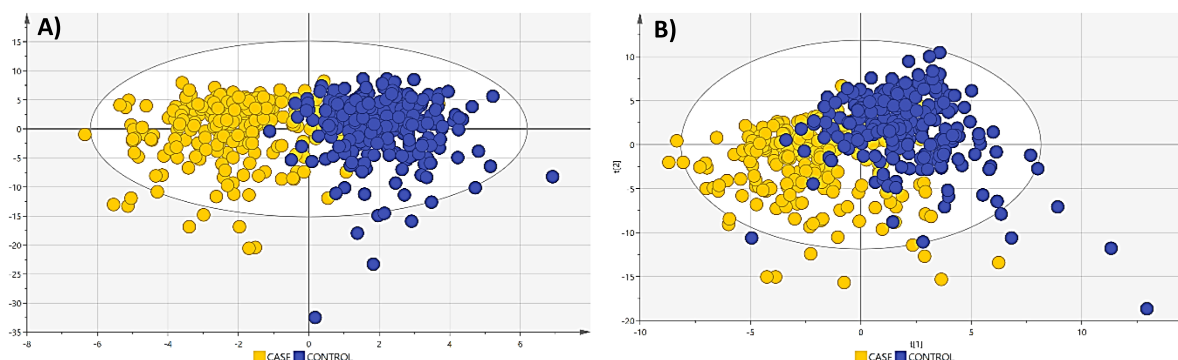

Figure 1. **A)** OPLS-DA [R2X(cum) = 0.612, R2Y(cum) = 0.697, Q2(cum) = 0.621] and **B)** PLS-DA [R2X(cum) = 0.519, R2Y(cum) = 0.644, Q2(cum) = 0.582] plots after targeted LC-MS analysis in the case-control study. Cases (patients with PTSD) are marked in **yellow** and controls (healthy subjects) in **blue**.

After the semiquantitation, the concentrations of lipid compounds which were found altered in patients with PTSD compared to healthy control subjects were determined. Out of 156 analyzed compounds, 15 lipid metabolites had significantly different concentrations between the patients with PTSD and healthy control subjects. These compounds included 4 phosphatidylcholines (PC(34:2e), PC(36:4)sec, PC(34:2), PC(36:3e)) 2 phosphatidylethanolamines (PE(38:5e), PE(36:3e)), 5 sphingomyelins (SM(d18:1/22:0), SM(d18:0/24:0), SM(d18:1/24:0), SM(d18:2/18:0), SM(d18:2/20:0)), 2 cholesterol esters (CE(16:1), CE(16:0)) and 2 ceramides (Cer(d18:1/24:0),

Cer(d18:0/24:0)). While decreased concentrations of phosphatidylcholines (PC(34:2e), PC(36:4)sec, PC(34:2), PC(36:3e)), phosphatidylethanolamines (PE(38:5e), PE(36:3e)), ceramides (Cer(d18:1/24:0), Cer(d18:0/24:0) and 3 sphingomyelins (SM(d18:1/22:0), SM(d18:0/24:0), SM(d18:1/24:0)) were found, the concentrations of cholesterol esters (CE(16:1), CE(16:0)) and 2 sphingomyelins (SM(d18:2/18:0), SM(d18:2/20:0)) were increased in the plasma of subjects with PTSD (Table 2). In order to determine the goodness of our model, we performed the ROC curve analysis for all compounds and determined the AUC value (Table 2). Three compounds, PC(34:2e), PC(36:4) and SM(d18:1/22:0) had AUC > 0.8 (Table 2), which means that they largely influenced the distinction between PTSD cases and controls [27]. For these three lipid metabolites the ROC curves and the boxplots are presented in Figure 2.

Table 2. The list of compounds whose levels significantly differed between veterans with PTSD and healthy control subjects, obtained after quantification

Compounds	p-value	RT	Precursor ion	Product ion	Concentration (PTSD) (mean ± SD)	Concentration (Control) (mean ± SD)	Direction of change (PTSD vs control)	AUC
PC(34:2e)	2.1946×10^{-42}	5.005	744.590	184.076	2.33 ± 1.07	4.23 ± 1.70	↓	**0.861**
PC(36:4)sec	5.9938×10^{-39}	5.460	769.590	185.081	0.60 ± 0.20	0.91 ± 0.28	↓	**0.846**
SM(d18:1/22:0)	1.4376×10^{-31}	5.600	787.669	184.076	64.62 ± 15.32	81.80 ± 14.21	↓	**0.810**
Cer(d18:1/24:0)	6.0530×10^{-29}	6.403	632.598	264.250	0.61 ± 0.40	1.17 ± 1.74	↓	0.796
SM(d18:0/24:0)	1.3812×10^{-26}	6.154	817.716	184.076	1.14 ± 0.36	1.51 ± 0.34	↓	0.786
PE(38:5e)	1.6981×10^{-20}	5.745	752.559	392.350	0.95 ± 0.48	1.45 ± 0.67	↓	0.746
SM(d18:1/24:0)	3.1622×10^{-20}	6.156	815.700	264.250	0.005 ± 0.003	0.007 ± 0.003	↓	0.744
PC(34:2)	3.3378×10^{-20}	5.187	758.569	496.340	0.62 ± 0.18	0.79 ± 0.24	↓	0.743
PE(36:3e)	5.5039×10^{-19}	5.702	728.559	392.350	0.23 ± 0.14	0.35 ± 0.16	↓	0.736
Cer(d18:0/24:0)	2.7745×10^{-14}	6.576	634.645	266.250	0.05 ± 0.05	0.10 ± 0.17	↓	0.702
PC(36:3e)	4.1061×10^{-14}	5.752	770.606	502.500	0.004 ± 0.003	0.006 ± 0.003	↓	0.699
CE (16:1)	0.0016	9.149	640.602	369.356	15.94 ± 7.03	14.24 ± 6.41	↑	0.584
CE (16:0)	0.0019	9.514	642.618	369.356	3.82 ± 1.02	3.57 ± 1.05	↑	0.582
SM(d18:2/18:0)	0.0042	4.811	729.587	184.076	8.32 ± 2.99	7.41 ± 1.94	↑	0.576
SM(d18:2/20:0)	0.0327	4.999	757.622	184.076	2.78 ± 0.73	2.61 ± 0.57	↑	0.557

RT=retention time in minutes; p-value obtained after Mann-Whitney U test or t-test; m/z of precursor and product ions; ↓ - decreased concentration in PTSD sample, ↑ - increased concentration in PTSD sample; concentration (µg/ml) expressed as mean ± SD; AUC-area under the curve

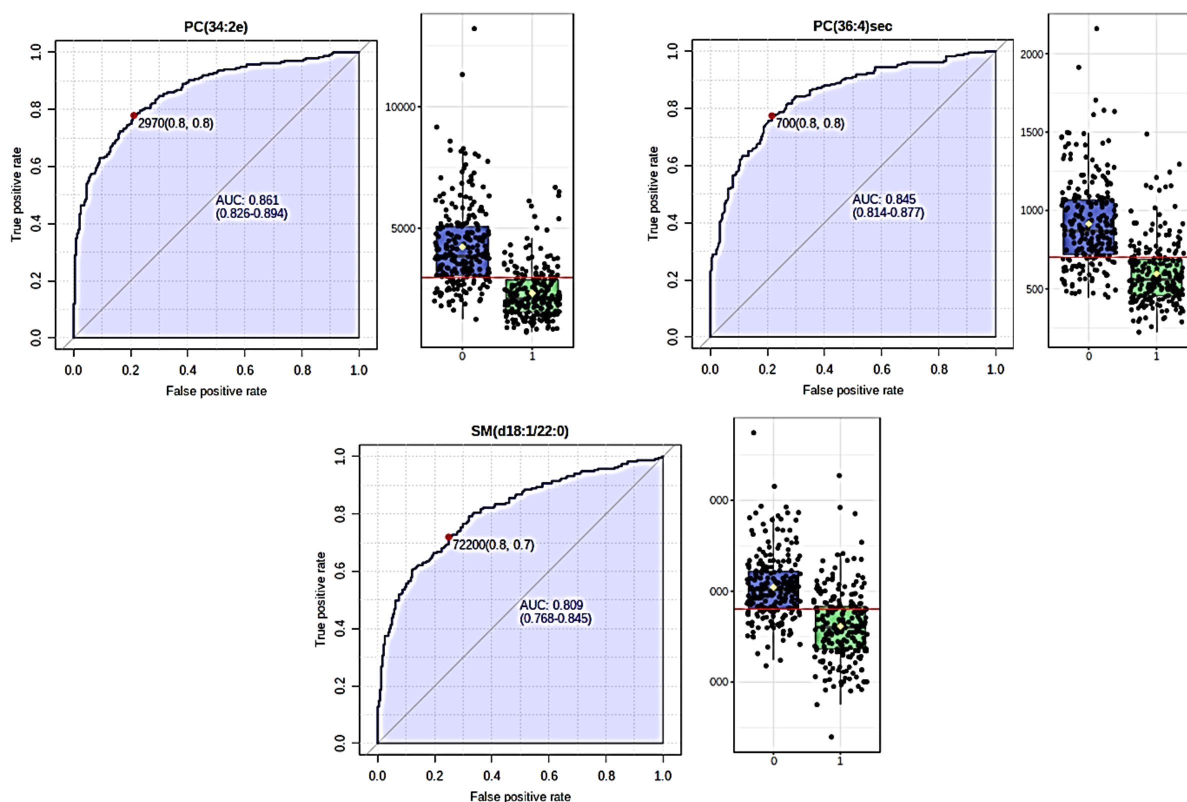

Figure 2. The ROC curve and the boxplot of metabolic features for three lipid metabolites with AUC > 0.8 in the case-control study. Cases (patients with PTSD) are marked in **green** and controls (healthy subjects) in **blue.**

In order to determine whether there was a significant influence of age and smoking on altered lipid compounds, the multilinear regression model was performed (Table 3), where the dependent variable was the plasma lipid concentration of (PC(34:2e), PC(36:4)sec, SM(d18:1/22:0), Cer(d18:1/24:0), SM(d18:0/24:0), PE(38:5e), SM(d18:1/24:0), PC(34:2), PE(36:3e), Cer(d18:0/24:0), PC(36:3e), CE (16:1), CE (16:0), SM(d18:2/18:0) and SM(d18:2/20:0), whereas smoking, age and diagnosis were independent variables. The regression model showed that smoking and age significantly affected the concentration of several lipids. However, with the presence of diagnosis as independent variable, the influence of smoking and age on lipid levels decreased, while the predictive power of the model significantly increased. Therefore, although lipid concentrations might be influenced by the differences in age and smoking status, this model demonstrated that the alternations in the levels of most lipids are mainly due to diagnosis. The changes in CE(16:0) concentration were mostly due to the age differences between patients with PTSD and healthy control subjects, whereas CE(16:1) level was mainly affected by the smoking status of enrolled participants (colored in Table 3).

Table 3. The results of multilinear regression model investigating the effect of smoking, age and diagnosis on the altered lipid levels

Compounds	Block 1	Block 2	Block 3	Change statistics
PC(34:2e)	F=41.535; R^2=0.081; p<0.000001	F=46.722; R^2=0.166; p<0.000001	F=75.716; R^2=0.327; p<0.000001	R^2=0.161; p<0.000001
PC(36:4)sec	F=32.244; R^2=0.064; p<0.000001	F=38.426; R^2=0.141; p<0.000001	F=70.098; R^2=0.310; p<0.000001	R^2=0.169; p<0.000001
SM(d18:1/22:0)	F=15.275; R^2=0.032; p=0.000107	F=13.164; R^2=0.053; p=0.000003	F=53.287; R^2=0.255; p<0.000001	R^2=0.202; p<0.000001
Cer(d18:1/24:0)	F=2.658; R^2=0.006; p=0.103713	F=5.053; R^2=0.021; p=0.006746	F=8.090; R^2=0.049; p=0.000029	R^2=0.028; p=0.000218
SM(d18:0/24:0)	F=6.051; R^2=0.013; p=0.014255	F=8.791; R^2=0.036; p=0.000179	F=43.528; R^2=0.219; p<0.000001	R^2=0.182; p<0.000001
PE(38:5e)	F=17.195; R^2=0.035; p=0.000040	F=16.654; R^2=0.066; p<0.000001	F=30.053; R^2=0.162; p<0.000001	R^2=0.095; p<0.000001
SM(d18:1/24:0)	F=3.676; R^2=0.008; p=0.055799	F=6.099; R^2=0.025; p=0.002427	F=30.318; R^2=0.163; p<0.000001	R^2=0.138; p<0.000001
PC(34:2)	F=10.746; R^2=0.022; p=0.001123	F=13.687; R^2=0.055; p=0.000002	F=26.006; R^2=0.143; p<0.000001	R^2=0.088; p<0.000001
PE(36:3e)	F=18.029; R^2=0.037; p=0.000026	F=12.386; R^2=0.050; p=0.000006	F=19.192; R^2=0.110; p<0.000001	R^2=0.059; p<0.000001
Cer(d18:0/24:0)	F=3.450; R^2=0.007; p=0.063878	F=4.802; R^2=0.020; p=0.008624	F=7.061; R^2=0.043; p=0.000119	R^2=0.023; p=0.000810
PC(36:3e)	F=18.029; R^2=0.037; p=0.000026	F=12.386; R^2=0.050; p=0.000006	F=19.192; R^2=0.110; p<0.000001	R^2=0.059; p<0.000001
CE (16:1)	F=4.674; R^2=0.099; p=0.031122	F=3.257; R^2=0.014; p=0.039360	F=3.048; R^2=0.019; p=0.028428	R^2=0.005; p=0.107049
CE (16:0)	F=0.848; R^2=0.002; p=0.357709	F=4.251; R^2=0.018; p=0.014807	F=3.710; R^2=0.023; p=0.011668	R^2=0.005; p=0.107619
SM(d18:2/18:0)	F=0.0003; R^2=0.000001; p=0.986208	F=3.649; R^2=0.015; p=0.026770	F=6.244; R^2=0.039; p=0.000366	R^2=0.023; p=0.000850
SM(d18:2/20:0)	F=0.799; R^2=0.002; p=0.371703	F=0.974; R^2=0.004; p=0.378217	F=2.659; R^2=0.017; p=0.047762	R^2=0.013; p=0.014621

*Block 1 - model with smoking as independent variable; Block 2 - model with smoking and age as independent variables; Block 3 - model with smoking, age and diagnosis as independent variables (F: the ratio of two mean squares; R^2: the proportion of the variance for a dependent variable that's explained by an independent variable or variables in a regression model)

DISCUSSION

PTSD is a complex neuropsychiatric disorder triggered by a traumatic event(s), which develops in only some individuals, and it is characterized by the symptoms that represent large burden for the patients, as well as for the society. Numerous aspects of this disease are still unknown, thus, various studies search for potential predictive, prognostic or diagnostic biomarkers of PTSD, in order to identify underlying molecular, biochemical and metabolomic pathways and to improve current treatment strategies.

The aim of this lipidomic analysis was to determine and compare the concentrations of lipid compounds in plasma between the patients with PTSD and healthy control subjects (Supplementary table 2). Plasma samples have been used in order to find altered peripheral lipids that might represent potential biomarkers of PTSD, due to their advantages such as low-cost, non- invasiveness and easy accessibility. Moreover, lipid changes in the peripheral system might reflect systemic changes, characteristic for PTSD, that affect the whole organism. The use of deuterated corrections allowed more accurate determination of compound concentrations and values to compare differences between groups that are closer to the real values, since the matrix effect is corrected. The LC-MS/MS method was applied for targeted quantitation of the lipid compounds, since altered lipids and their derivatives have been previously identified by the metabolomic studies of patients with PTSD [13-16]. By this method 156 metabolites have been analyzed in a timeframe of 12 minutes per sample, and significantly different levels of fifteen lipid metabolites have been determined between the subjects with PTSD and healthy control subjects, including four phosphatidylcholines, two phosphatidylethanolamines, five sphingomyelins, two cholesterol esters and two ceramides.

Lipid metabolites, which levels were changed in the patients with PTSD in comparison to control group, take part in various biological processes and cell functions, such as such as impairments of membrane integrity and function, mitochondrial alterations, inflammation as well as oxidative stress. Specifically, high levels of free radicals or reactive oxygen species (ROS) are known to induce direct damage to lipids. In addition to polyunsaturated fatty acids (PUFAs), glycolipids, glycerophospholipids, sphingolipids, cholesterol and cholesterol esters are also frequent targets of the Slipid peroxidation [28,29]. Moreover, previous findings suggested that oxidative stress and altered lipid metabolism, reflected by the increase of 4-hydroxynonenal (4-HNE) might be associated with PTSD [21]. For example, increased levels of malondialdehyde (MDA), a reliable marker of lipid peroxidation, as well as decreased activity of serum paraoxonase 1 (PON1) enzyme were measured in PTSD patients [30], indicating an increased oxidative stress in these subjects [31].

Altered glycerophospholipids in PTSD

The largest number of lipid metabolites altered in the PTSD subjects belonged to the group of glycerophospholipids. In this study, decreased concentrations of PC(34:2), PC(36:3), PC(36:4), PE(36:3) and PE(38:5) have been determined in patients with PTSD in comparison to the healthy control subjects. Glycerophospholipids are important building blocks of plasma and neural membranes, which can be transformed into lipid mediators that take part in intercellular communication, as well as transduction and other cellular processes [32]. They play an important role in apoptosis, transportation, and intracellular organization [13]. Depending on the polar group attached to the phosphate group, glycerophospholipids can be divided into PC, phosphatidylserines (PS), PE, phosphatidylinositols (PI), phosphoglycerols (PG) and phosphatidic acid (PA) [33,34]. In addition to PTSD, the alterations in glycerophospholipids levels have also been reported in Alzheimer's disease [35], alcohol abuse [36], metabolic disorders [37], schizophrenia, depression [2] and other neurological disorders [22]. Decreased levels of PC have been found in the patients with schizophrenia, while increased levels of lysophospholipids and glycerophosphoethanolamines have been detected in the subjects with depression [4] and Alzheimer's disease [13].

Among previously published metabolomic studies, the research of Karabatsiakis et al. (2015) [13] determined increased levels of PE in serum of subjects with PTSD, whereas our group reported higher concentrations of LPC and LPE in plasma of patients with PTSD compared to the control group [17]. These discrepancies might be due to the experimental design, the sample size and the differences related to the age of the participants. However, Emmerich and colleagues (2016) [15] observed decreased levels of several phospholipids in patients with PTSD in comparison to the control group [15], which is in line with our findings, where reduced concentrations of PC and PE were also found. In this study, PTSD and control group significantly differed in age, however, in contrast to the other reports, our study enrolled substantially larger number of subjects. Age difference between these two groups is due to the plasma sampling of veterans with PTSD, which were older then healthy control subjects from general population, since they participated in the Croatian War of Independence approximately 30 years ago. Moreover, the finding that the patients with PTSD smoked more frequently than control subjects is not surprising, since various previous studies demonstrated high PTSD-smoking comorbidity [38-40].

The alterations in glycerophospholipids levels might be associated with activity of different enzymes, including lysophospholipase and phospholipase A2 (PLA2). Specifically, changes in the enzyme activity might influence metabolite levels through processes of degradation, bond cleavage or synthesis. For instance, decreased activity of lysophospholipase results in increased LPC levels, while changes in the phospholipase A2 might affect the cleavage of fatty acids on position 2 in glycerophospholipids and production of LPC, which, together with arachidonic acid, act as lipid mediators [32]. Together with PC, PE represent the most abundant phospholipids in mammals. Since PE are synthesized in the endoplasmic reticulum or mitochondria [41], alterations in PE levels might suggest mitochondrial dysfunction in PTSD. Likewise, several studies indicated the association between altered PE and PC levels and oxidative stress [21], while these modified phospholipids might exert proinflammatory activity [42], which is characteristic for PTSD. Glycerophospholipids are characterized by PUFAs, and therefore more suitable for oxidation [43]. Oxidized glycerophospholipids represent pathogenic factors for various pathophysiological conditions, indicating the association between oxidative stress and inflammation [29]. However, study by Sparvero et al. (2010) reported that PC and PE were not preferred substrates for oxidation, unlike other less common glycerophospholipids [44].

Therefore, according to the already published results, changes in the concentrations of glycerophospholipids might be associated with altered activity of corresponding enzymes, modifications in mitochondrial function and membrane integrity, as well as oxidative stress and inflammation, suggesting their role in various metabolic disorders, including obesity, insulin resistance and atherosclerosis [21,35,41].

Altered sphingolipids in PTSD

The sphingolipids are class of lipids, which make up the part of plasma and neural membranes with several physiological functions in signaling, glucose metabolism, inflammation, cell growth and adhesion, differentiation, apoptosis and stress response [45]. Sphingolipids are constituted from hydrophobic backbone, called ceramide and polar head group. The polar head group can be either a sugar chain (glycosphingolipids) or phosphocholine (sphingomyelin, SM). The hydrophobic backbone or ceramide is comprised of long-chain base with amide-linked fatty acid, while sphingosine is one of the most important long-chain bases in mammals [45]. In this study, different concentrations of five sphingomyelins, (SM(d18:1/22:0), SM(d18:0/24:0), SM(d18:1/24:0), SM(d18:2/18:0) and SM(d18:2/20:0)) and two ceramides, (Cer(d18:1/24:0), Cer(d18:0/24:0)) discriminated between the patients with PTSD and healthy subjects. While increased levels of SM(d18:2/18:0) and SM(d18:2/20:0) have been observed in plasma of the subjects with PTSD in comparison to the control group, other significantly different sphingolipids demonstrated decreased concentrations in PTSD. SM, which are found decreased in PTSD in this study are characterized by a longer chain and zero or one double bond, compared to SM that showed increased concentrations in the PTSD group.

The sphingolipids and glycerophospholipids are interconnected through sphingosine-1-phosphate lyase and production of hexadecenal and hexadecenoic acid. Therefore, observed alterations in the sphingomyelins in PTSD might be reflected in glycerophospholipids changes, including PE [37]. Likewise, ceramides might influence PC production through the inhibition of their synthesis and release of LPC. Together with complex sphingomyelins, they might regulate the activity of cytosolic PLA2, whose alternations might be associated with changed concentrations of plasma glycerophospholipids in the subjects with PTSD. Figure 3 shows the association between glycerophospholipids and sphingolipids metabolism. It is assumed that the activation of PLA2 plays an important role in the metabolism of sphingolipids and phospholipids, and promotes ceramide production through activation of other enzymes, associated with various mitochondrial alterations and dysfunction [37], and observed in subjects with PTSD in our study. Moreover, it is assumed that oxidative stress affects SM-ceramide signaling, including synthesis of ceramides whose changes might be directly associated with the oxidative stress in mitochondria [46]. However, in nature, SM are usually saturated and therefore do not represent a target for oxidation [29]. Furthermore, another study has also reported changes in plasma sphingolipids [47]. Specifically, Cyr and colleagues (2021) [47] reported altered sphingolipid levels after traumatic injury at different time points for different types of sphingolipids, while some of them, including sphingosine 1-phosphate, regulate the inflammatory processes. Observed connection between sphingolipid alterations, inflammatory mediators and clinical outcomes imply on the possible involvement of the sphingolipids in the immune system [47].

Elevated levels of interferon-γ, tumor necrosis factor alpha (TNF-α), interleukin 6 and interleukin 10, as well as elevated levels of the sphingomyelins and increased activity of corresponding enzymes, have been found in the subjects with PTSD compared to the control group [48]. However, that study included small number of participants (8 PTSD subjects and 5 healthy control subjects), opposed to 235 PTSD subjects and 241 healthy controls in our study, which might explain the different results compared to our findings. A common factor linking PTSD and the sphingolipids is inflammation. The sphingolipids are involved in the production, differentiation and migration of several pro-inflammatory cytokines, immune cells and eicosanoids [49]. A pro-inflammatory cytokine, TNF-α promotes sphingolipid metabolism through activation of enzymes, such as ceramidase, which converts ceramide into the sphingosine, and sphingomyelinases, which is responsible for catalyzing the reaction of ceramide synthesis [49,50]. Therefore, the changes in the sphingolipid levels, including ceramide levels, might influence *protein-protein*, *protein-lipid* and *lipid-lipid* interactions [48], which might be associated with protein misfolding observed in neurodegenerative disorders. Altered sphingolipids have been reported in the metabolic disorders, diabetes, obesity, inflammatory diseases and several neurodegenerative disorders, including Alzheimer's, Parkinson's and Huntington's disease [49-51].

Altered cholesterol esters in PTSD

In this study, increased concentrations of cholesteryl palmitic acid – CE(16:0) and cholesteryl palmitoleic acid - CE(16:1) have been observed in the patients with PTSD, when compared to the healthy subjects. The formation of cholesterol esters is one way to maintain the cholesterol homeostasis and regulate cholesterol accumulation, transportation, secretion and absorption [52]. Study by Maia et al. (2008) [53] showed that patients with PTSD had increased levels of cholesterol and triglycerides, compared to the control group, which might affect concentration of cholesterol esters in PTSD. Likewise, increased concentration of 8-Oxoguanine, which is an indicator of oxidative DNA damage has been associated with increased concentration of fatty acids in CE [54], indicating the association of altered CE with the oxidative stress. Although, in this study, multilinear regression analysis showed that cholesterol esters were altered due to differences in the age or smoking status between PTSD cases and healthy controls, further studies are necessary for elucidating the role of the cholesterol metabolism in the etiology of PTSD.

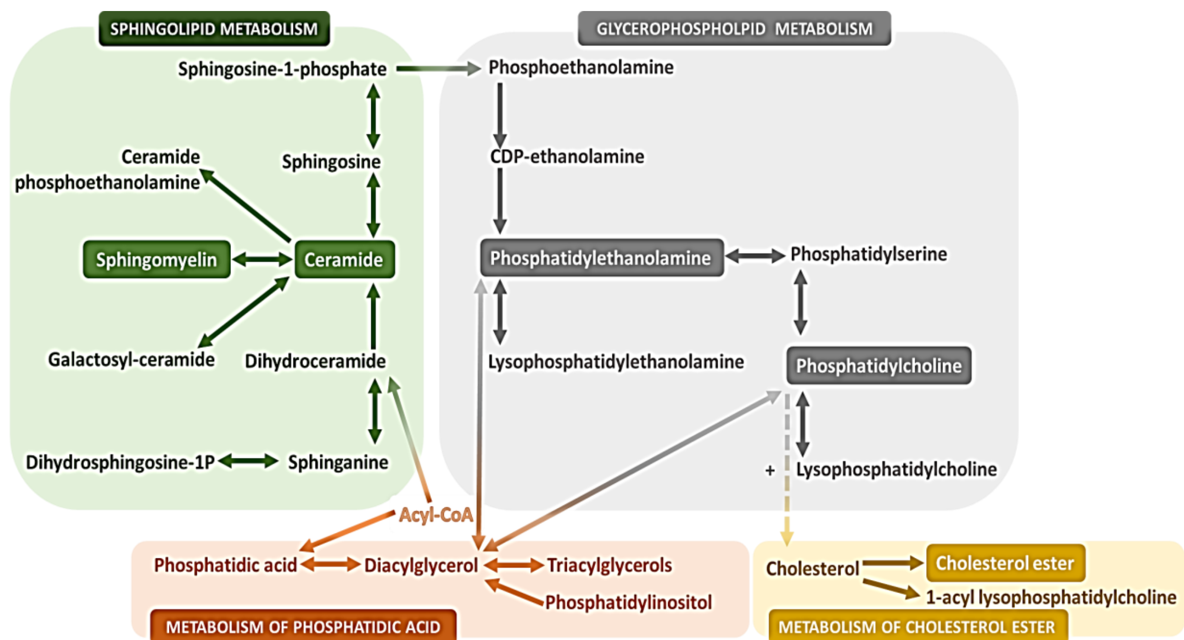

Figure 3. The simplified summary of altered lipid metabolites and their corresponding metabolic pathways.

CONCLUSION

The study represents the targeted lipidomic analysis in plasma of the patients with combat PTSD and healthy control subjects. As far as we know, this lipidomic study has the largest sample size published so far (N=476), and could offer new potential biomarkers of PTSD, which could be useful in facilitating the PTSD diagnosis and/or identification of novel treatment approaches. The obtained results revealed large number of altered lipid metabolites involved in PTSD pathogenesis, indicating changes in several metabolic pathways, which might enlighten still unknown etiology of PTSD. In addition, we developed a short method for targeted analysis, which can be used for determination of large number of lipid metabolites in the plasma samples of PTSD patients, as well as individuals diagnosed with other disorders or conditions. This is of particular importance if the use of such biomarkers is desired in the clinical practice.

However, several limitations of the study should be acknowledged. One limitation of our lipidomic study is that it is focused on changes of unoxidized lipids in PTSD. However, the findings of our study could be useful for determination of lipids, whose levels are significantly altered in PTSD, such as sphingolipids, glycerophospholipids and cholesterol esters, which have been previously shown to be affected by the oxidative stress and lipid peroxidation [43]. Furthermore, the study enrolled only male subjects with combat-related PTSD. Specifically, in our research, females and subjects with civilian or acute PTSD have not been included. In addition, samples were not age-matched, and significant difference in the age has been found between PTSD and control group. Literature search revealed small number of articles that have been published in this area of research, which reported inconsistent findings. The contradictions in obtained results might be due to the differences in the method of analysis, PTSD type, sample size, as well as in the differences between groups in age and other clinical and demographic parameters. Likewise, in order to offer diagnostic, prognostic or predictive biomarkers, additional validation study should be performed for analyzed lipid metabolites, in order to control for the analytical performance, including repeatability, intermediate precision, detection limit, linearity and coefficient of variation.

Nevertheless, altered lipids linked to PTSD in this study, as well as the metabolites assessed in previous research [13-16], are associated to the common processes, such as impairments of membrane integrity and function, mitochondrial alterations, inflammation and oxidative stress, which all might

be associated with PTSD. In order to continue search for the potential biomarkers of PTSD, further metabolomic/lipidomic studies, which would include oxidized lipids, combat as well as civilian PTSD, female subjects and both untargeted and targeted metabolomic approaches, are required.

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Conflict of interest

The authors declare no conflict of interest.

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PLASMA LIPIDOMICS IN SUBJECTS WITH COMBAT POSTTRAUMATIC STRESS DISORDER

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Supplementary table 1. List of the targeted metabolites and corresponding analytical properties

Metabolites	Precursor ion	Product ion	RT (min)	Collision Energy	Polarity
18:1 (d7) LPE	487.650	346.650	3.709	15	Positive
15:0-18:1(d7) DG 1	605.580	299.2673	5.861	20	Positive
15:0-18:1(d7) DG 2	605.5844	570.5473	7.000	20	Positive
15:0-18:1(d7) DG 3	605.5844	299.2473	7.000	20	Positive
15:0-18:1(d7) DG 4	605.5844	346.3325	7.000	20	Positive
15:0-18:1(d7) PC 1	753.620	184.076	5.193	35	Positive
15:0-18:1(d7) PC 2	753.620	482.330	5.193	35	Positive
15:0-18:1(d7) PE	711.570	570.550	5.155	25	Positive
15:0-18:1(d7)-15:0 TG 1	829.800	523.500	9.348	35	Positive
15:0-18:1(d7)-15:0 TG 2	829.800	272.300	9.348	35	Positive
15:0-18:1(d7)-15:0 TG 3	829.800	570.700	9.348	35	Positive
15:0-18:1(d7)-PA	684.600	341.000	4.888	35	Positive
15:0-18:1(d7)-PG	758.600	281.100	5.220	30	Positive
15:0-18:1(d7)-PS	754.500	183.800	5.187	35	Positive
18:1(d7) Chol Ester	675.670	369.3521	9.780	10	Positive
18:1(d7) LPC	529.390	184.076	3.717	30	Positive
18:1(d7)-MG	364.3478	272.310	3.869	10	Positive
18:1(d9) SM	738.650	184.076	4.803	30	Positive
CE (16:0)	642.618	369.356	9.514	10	Positive
CE (16:1)	640.602	369.356	9.149	10	Positive
CE (18:2)	666.618	369.356	9.369	10	Positive
CE (18:3)	664.602	369.356	9.115	10	Positive
CE (20:4)	690.618	369.356	9.327	10	Positive
CE (22:5)	716.634	369.356	9.572	10	Positive
CE (22:6)	714.618	369.356	9.310	10	Positive
CE(14:0)	614.587	369.356	8.972	10	Positive
CE(15:0)	628.602	369.356	9.200	10	Positive
CE(16:2)	638.587	369.356	8.929	10	Positive
CE18:1	668.633	369.356	9.708	10	Positive
Cer(d18:0/24:0)	634.645	266.250	6.576	40	Positive
Cer(d18:1/22:0)	604.598	264.250	6.032	40	Positive
Cer(d18:1/24:0)	632.598	264.250	6.403	40	Positive
Cer(d18:1/24:1)	630.614	264.250	6.161	40	Positive
DG(18:1/18:1)	638.571	339.289	6.221	20	Positive
DG(32:0)	586.540	313.280	5.894	20	Positive
DG(34:0)	614.571	341.305	6.282	20	Positive
DG(34:1)	612.556	313.273	6.058	20	Positive

Metabolites	Precursor ion	Product ion	RT (min)	Collision Energy	Polarity
DG(36:1)	640.586	341.304	6.429	20	Positive
DG(36:3)	636.556	337.273	6.023	20	Positive
DG(36:4)	634.540	337.273	5.825	20	Positive
DG(38:5)	660.556	339.289	6.066	20	Positive
LPC(14:0)sn-1	468.308	184.076	3.488	30	Positive
LPC(15:0)	482.324	184.076	3.557	30	Positive
LPC(16:0)sn-1	496.340	184.076	3.642	30	Positive
LPC(16:0e)	482.360	184.076	3.557	30	Positive
LPC(16:1)sn-1	494.324	184.076	3.548	30	Positive
LPC(16:1e)	480.345	184.076	3.686	30	Positive
LPC(17:0)	510.355	184.076	3.702	30	Positive
LPC(17:1)	508.340	184.076	3.608	30	Positive
LPC(18:0)sn-1	524.371	184.076	3.788	30	Positive
LPC(18:0e)	510.390	184.076	3.702	30	Positive
LPC(18:0p)	508.376	184.076	3.608	30	Positive
LPC(18:1)sn-1	522.355	184.076	3.694	30	Positive
LPC(18:1e)	508.376	184.076	3.608	30	Positive
LPC(18:2)sn-1	520.340	184.076	3.607	30	Positive
LPC(18:2e)	506.361	184.076	3.548	30	Positive
LPC(18:3)	518.324	184.076	3.547	30	Positive
LPC(20:3)	546.356	184.076	3.685	30	Positive
LPC(20:4)sn-1	544.340	184.076	3.624	30	Positive
LPC(20:5)	542.330	184.076	3.556	30	Positive
LPC(22:5)	570.356	184.076	3.693	30	Positive
LPC(22:6)sn-1	568.340	184.076	3.641	30	Positive
LPE(14:0)	426.270	285.270	7.000	15	Positive
LPE(16:0)sn-1	454.293	313.274	3.626	15	Positive
LPE(18:1)sn-1	480.308	339.290	3.686	15	Positive
LPE(18:2)sn-1	478.293	337.274	3.600	15	Positive
LPE(20:4)sn-1	502.293	361.275	3.616	15	Positive
PC(30:0)	706.538	184.076	4.863	35	Positive
PC(32:0)	734.569	184.076	5.231	35	Positive
PC(32:1)	732.554	494.330	5.025	35	Positive
PC(34:0)	762.601	184.076	5.417	35	Positive
PC(34:1)	760.585	496.340	5.409	35	Positive
PC(34:1e)	746.606	184.076	5.564	35	Positive
PC(34:2)	758.569	496.340	5.187	35	Positive
PC(34:2e)	744.590	184.076	5.005	35	Positive
PC(34:3)	756.554	494.330	4.981	35	Positive
PC(36:0)	792.574	608.494	7.000	35	Positive
PC(36:0)	790.632	524.500	7.000	35	Positive
PC(36:1)	788.616	524.380	5.794	35	Positive
PC(36:2)	786.601	522.360	5.562	35	Positive
PC(36:2e)	772.620	184.076	5.340	35	Positive
PC(36:3)	785.588	185.081	5.357	35	Positive
PC(36:3e)	770.606	502.500	5.752	35	Positive
PC(36:4)	783.572	184.076	5.280	35	Positive
PC(36:4)sec	769.590	185.081	5.460	35	Positive
PC(36:5)	780.564	184.076	5.071	35	Positive
PC(38:2)	814.632	548.375	7.000	35	Positive
PC(38:3)	812.616	546.360	5.793	35	Positive
PC(38:4)	810.604	524.500	5.655	35	Positive
PC(38:4e)	796.625	528.352	7.000	35	Positive

Metabolites	Precursor ion	Product ion	RT (min)	Collision Energy	Polarity
PC(38:5)	808.588	522.362	5.467	35	Positive
PC(38:5e)	794.609	526.500	5.785	35	Positive
PC(38:6)	807.573	185.081	5.287	35	Positive
PC(40:0)	847.698	185.079	6.051	35	Positive
PC(40:4)	838.635	184.076	5.947	35	Positive
PC(40:6)	835.604	185.081	5.680	35	Positive
PC(40:7)	832.585	184.076	5.457	35	Positive
PC(40:8)	830.569	184.076	6.276	35	Positive
PE(34:1)	718.538	577.520	5.351	25	Positive
PE(34:2)	716.522	575.504	5.137	25	Positive
PE(36:2)	744.554	603.535	5.539	25	Positive
PE(36:3)	742.538	601.520	5.324	25	Positive
PE(36:3e)	728.559	392.350	5.702	25	Positive
PE(36:4)	740.522	599.504	5.205	25	Positive
PE(38:4)	768.554	627.535	5.606	25	Positive
PE(38:5e)	752.559	392.350	5.745	25	Positive
PE(38:6)	764.522	623.504	5.263	25	Positive
PE(40:6)	792.554	651.535	5.622	25	Positive
PE(40:7e)	776.559	392.350	5.778	25	Positive
SM(d16:1/17:0)	689.560	184.076	4.510	30	Positive
SM(d18:0/14:0)	677.560	184.076	4.389	30	Positive
SM(d18:0/16:0)	705.591	184.076	4.760	30	Positive
SM(d18:0/18:0)	733.653	184.076	5.017	30	Positive
SM(d18:0/24:0)	817.716	184.076	6.154	30	Positive
SM(d18:1/14:0)	675.544	184.076	4.389	30	Positive
SM(d18:1/16:0)	704.578	185.081	4.665	30	Positive
SM(d18:1/17:0)	717.591	184.076	4.820	30	Positive
SM(d18:1/18:0)	731.606	184.076	4.991	30	Positive
SM(d18:1/18:2)	727.567	184.076	4.613	30	Positive
SM(d18:1/20:0)	759.637	264.250	7.000	30	Positive
SM(d18:1/21:0)	773.653	184.076	5.588	30	Positive
SM(d18:1/22:0)	787.669	184.076	5.600	30	Positive
SM(d18:1/23:0)	801.684	184.076	5.974	30	Positive
SM(d18:1/24:0)	815.700	264.250	6.156	30	Positive
SM(d18:1/25:1)	827.700	184.076	6.017	30	Positive
SM(d18:2/16:0)	701.559	184.076	4.501	30	Positive
SM(d18:2/18:0)	729.587	184.076	4.811	30	Positive
SM(d18:2/20:0)	757.622	184.076	4.999	30	Positive
SM(d18:2/22:0)	785.653	262.250	5.536	30	Positive
SM(d18:2/23:0)	799.669	184.076	5.761	30	Positive
SM(d18:2/24:0)	814.687	185.081	5.888	30	Positive
SM(d18:2/24:1)	811.669	262.250	5.707	30	Positive
SM(d18:2/24:2)	809.653	262.250	5.467	30	Positive
SM(d19:0/24:1)	829.716	184.076	6.267	30	Positive
TG(44:0)	768.705	523.470	8.614	35	Positive
TG(46:0)	796.738	523.472	9.071	35	Positive
TG(46:1)	794.721	521.460	8.834	35	Positive
TG(46:2)	792.705	521.454	8.571	35	Positive
TG(48:0)	824.770	551.503	9.511	35	Positive
TG(48:1)	822.754	549.487	9.265	35	Positive
TG(48:2)	820.738	575.503	9.037	35	Positive
TG(48:3)	818.723	521.500	8.799	35	Positive
TG(50:0)	852.801	579.534	9.714	35	Positive

Metabolites	Precursor ion	Product ion	RT (min)	Collision Energy	Polarity
TG(50:1)	850.785	577.490	9.721	35	Positive
TG(50:2)	848.770	575.503	9.468	35	Positive
TG(50:3)	846.754	549.487	9.222	35	Positive
TG(50:4)	844.738	547.738	8.993	35	Positive
TG(51:0)	867.819	580.550	10.169	35	Positive
TG(52:1)	878.817	605.550	10.181	35	Positive
TG(52:2)	876.801	575.503	9.916	35	Positive
TG(52:3)	874.785	575.503	9.662	35	Positive
TG(52:4)	872.770	575.503	9.416	35	Positive
TG(52:5)	870.754	575.503	9.213	35	Positive
TG(52:6)	868.738	547.472	9.052	35	Positive
TG(54:1)	906.848	605.550	10.679	35	Positive
TG(54:2)	904.832	605.550	10.403	35	Positive
TG(54:3)	902.817	603.354	10.143	35	Positive
TG(54:4)	900.801	601.519	9.856	35	Positive
TG(54:5)	898.785	599.503	9.617	35	Positive
TG(54:6)	896.770	599.503	9.390	35	Positive
TG(54:7)	894.754	597.487	9.169	35	Positive
TG(56:3)	930.848	631.570	10.637	35	Positive
TG(56:4)	928.832	629.550	10.36	35	Positive
TG(56:5)	926.817	627.534	10.108	35	Positive
TG(56:6)	924.801	603.534	9.965	35	Positive
TG(56:7)	922.785	601.519	9.720	35	Positive
TG(56:8)	920.770	599.503	9.516	35	Positive
TG(58:10)	944.770	623.503	9.533	35	Positive
TG(58:6)	952.832	952.832	7.000	35	Positive
TG(58:8)	948.801	601.519	9.947	35	Positive
TG(58:9)	946.785	601.519	9.728	35	Positive
TG(60:10)	972.801	651.534	10.006	35	Positive
TG(60:12)	968.770	647.550	9.540	35	Positive
TG(60:8)	976.832	655.550	7.000	35	Positive

Supplementary table 2. Final list of the lipid metabolites and their concentrations in plasma of patients with PTSD and healthy control subjects

Metabolites	**Concentration (µg/ml) – PTSD** (mean ± SD)	**Concentration (µg/ml) – Control** (mean ± SD)
CE (16:0)	3.82 ± 1.02	3.57 ± 1.05
CE (16:1)	15.94 ± 7.03	14.24 ± 6.41
CE (18:2)	971.06 ± 210.85	1160.60 ± 324.51
CE (18:3)	81.37 ± 55.03	90.15 ± 40.34
CE (20:4)	523.21 ± 177.35	535.45 ± 180.68
CE (22:5)	8.16 ± 2.80	7.75 ± 2.77
CE (22:6)	51.03 ± 19.30	51.71 ± 23.12
CE(14:0)	0.40 ± 0.27	0.45 ± 0.23
CE(15:0)	0.09 ± 0.05	0.09 ± 0.05
CE(16:2)	0.33 ± 0.25	0.36 ± 0.20
CE18:1	75.15 ± 21.31	80.41 ± 28.93
Cer(d18:0/24:0)	0.05 ± 0.05	0.10 ± 0.17
Cer(d18:1/22:0)	0.17 ± 0.12	0.32 ± 0.71
Cer(d18:1/24:0)	0.61 ± 0.40	1.17 ± 1.74
Cer(d18:1/24:1)	0.27 ± 0.14	0.36 ± 0.47
DG(18:1/18:1)	6.34 ± 4.08	8.15 ± 10.33
DG(32:0)	0.92 ± 1.27	1.62 ± 3.12

Metabolites	Concentration (µg/ml) – PTSD (mean ± SD)	Concentration (µg/ml) – Control (mean ± SD)
DG(34:0)	0.14 ± 0.21	0.31 ± 0.81
DG(34:1)	3.00 ± 2.50	4.37 ± 6.97
DG(36:1)	0.38 ± 0.37	0.65 ± 1.35
DG(36:3)	3.28 ± 2.21	4.33 ± 5.50
DG(36:4)	1.52 ± 1.22	2.13 ± 2.58
DG(38:5)	0.65 ± 0.48	0.83 ± 0.79
LPC(14:0)sn-1	0.50 ± 0.27	0.64 ± 0.25
LPC(15:0)	0.10 ± 0.03	0.13 ± 0.03
LPC(16:0)sn-1	51.08 ± 12.95	59.49 ± 11.07
LPC(16:0e)	0.10 ± 0.03	0.13 ± 0.04
LPC(16:1)sn-1	1.21 ± 0.51	1.16 ± 0.43
LPC(16:1e)	0.06 ± 0.02	0.07 ± 0.02
LPC(17:0)	0.74 ± 0.28	0.79 ± 0.26
LPC(17:1)	0.07 ± 0.02	0.08 ± 0.02
LPC(18:0)sn-1	19.82 ± 7.42	24.03 ± 6.38
LPC(18:0e)	0.77 ± 0.29	0.80 ± 0.26
LPC(18:0p)	0.07 ± 0.02	0.08 ± 0.02
LPC(18:1)sn-1	10.11 ± 3.53	11.36 ± 3.64
LPC(18:1e)	0.05 ± 0.02	0.06 ± 0.01
LPC(18:2)sn-1	11.70 ± 5.48	16.37 ± 5.35
LPC(18:3)	0.13 ± 0.09	0.15 ± 0.07
LPC(20:3)	1.15 ± 0.48	1.32 ± 0.42
LPC(20:4)sn-1	3.17 ± 1.06	3.63 ± 1.05
LPC(20:5)	0.10 ± 0.10	0.13 ± 0.09
LPC(22:5)	0.19 ± 0.06	0.20 ± 0.07
LPC(22:6)sn-1	0.46 ± 0.18	0.51 ± 0.21
PC(30:0)	4.71 ± 2.85	5.79 ± 3.71
PC(32:0)	7.68 ± 2.10	7.95 ± 2.48
PC(32:1)	0.005 ± 0.004	0.004 ± 0.004
PC(34:0)	2.78 ± 0.88	3.26 ± 1.14
PC(34:1)	0.34 ± 0.12	0.38 ± 0.14
PC(34:1e)	3.27 ± 0.95	3.52 ± 0.99
PC(34:2)	0.62 ± 0.18	0.79 ± 0.24
PC(34:2e)	2.33 ± 1.07	4.23 ± 1.70
PC(34:3)	0.01 ± 0.01	0.02 ± 0.01
PC(36:1)	0.07 ± 0.04	0.08 ± 0.04
PC(36:2)	0.03 ± 0.01	0.03 ± 0.02
PC(36:2e)	2.06 ± 0.73	2.71 ± 0.87
PC(36:3)	5.27 ± 1.72	6.35 ± 2.10
PC(36:3e)	0.004 ± 0.003	0.006 ± 0.003
PC(36:4)	58.46 ± 18.41	69.05 ± 19.73
PC(36:4)sec	0.60 ± 0.20	0.91 ± 0.28
PC(36:5)	10.03 ± 7.08	12.79 ± 7.47
PC(38:4)	0.19 ± 0.07	0.22 ± 0.07
PC(38:5)	0.03 ± 0.01	0.03 ± 0.01
PC(38:5e)	0.01 ± 0.01	0.02 ± 0.01
PC(38:6)	1.80 ± 0.60	2.06 ± 0.75
PC(40:4)	5.16 ± 2.43	5.57 ± 2.61
PC(40:6)	0.73 ± 0.28	0.77 ± 0.32
PC(40:7)	2.08 ± 0.66	2.22 ± 0.84
PE(34:1)	0.82 ± 0.61	0.84 ± 0.55
PE(34:2)	1.73 ± 1.26	1.82 ± 1.14

Metabolites	Concentration (µg/ml) – PTSD (mean ± SD)	Concentration (µg/ml) – Control (mean ± SD)
PE(36:2)	4.73 ± 3.46	6.77 ± 4.48
PE(36:3)	1.36 ± 0.99	1.74 ± 1.11
PE(36:3e)	0.23 ± 0.14	0.35 ± 0.16
PE(36:4)	2.06 ± 1.15	1.90 ± 0.88
PE(38:4)	7.04 ± 3.77	7.47 ± 3.67
PE(38:5e)	0.95 ± 0.48	1.45 ± 0.67
PE(38:6)	2.52 ± 1.50	2.21 ± 1.21
PE(40:6)	1.92 ± 1.34	1.93 ± 1.27
PE(40:7e)	0.24 ± 0.12	0.34 ± 0.16
SM(d16:1/17:0)	2.66 ± 0.91	2.94 ± 0.98
SM(d18:0/14:0)	0.20 ± 0.08	0.25 ± 0.10
SM(d18:0/16:0)	7.69 ± 1.96	8.30 ± 1.72
SM(d18:0/18:0)	7.44 ± 3.26	7.19 ± 3.05
SM(d18:0/24:0)	1.14 ± 0.36	1.51 ± 0.34
SM(d18:1/14:0)	4.57 ± 1.61	5.59 ± 1.96
SM(d18:1/16:0)	3.84 ± 0.90	3.99 ± 0.75
SM(d18:1/17:0)	2.06 ± 0.67	1.99 ± 0.58
SM(d18:1/18:0)	23.18 ± 7.86	22.27 ± 5.24
SM(d18:1/18:2)	0.33 ± 0.13	0.30 ± 0.08
SM(d18:1/21:0)	3.31 ± 0.82	3.98 ± 1.07
SM(d18:1/22:0)	64.62 ± 15.32	81.80 ± 14.21
SM(d18:1/23:0)	5.45 ± 1.62	6.83 ± 1.70
SM(d18:1/24:0)	0.005 ± 0.003	0.007 ± 0.003
SM(d18:1/25:1)	1.11 ± 0.41	1.08 ± 0.34
SM(d18:2/16:0)	9.42 ± 2.73	9.70 ± 2.26
SM(d18:2/18:0)	8.32 ± 2.99	7.41 ± 1.94
SM(d18:2/20:0)	2.78 ± 0.73	2.61 ± 0.57
SM(d18:2/23:0)	3.79 ± 0.91	3.99 ± 0.98
SM(d19:0/24:1)	0.44 ± 0.16	0.58 ± 0.19
TG(46:0)	14.47 ± 34.07	41.96 ± 184.52
TG(46:1)	7.17 ± 15.45	19.27 ± 91.11
TG(46:2)	1.21 ± 2.10	2.25 ± 9.49
TG(48:0)	57.03 ± 106.27	106.41 ± 162.15
TG(48:1)	74.94 ± 104.28	128.27 ± 224.50
TG(48:2)	18.80 ± 26.56	37.77 ± 106.60
TG(48:3)	4.93 ± 7.41	11.16 ± 42.34
TG(50:0)	24.80 ± 39.47	48.30 ± 75.62
TG(50:1)	299.66 ± 272.31	459.91 ± 468.51
TG(50:2)	236.66 ± 171.26	324.05 ± 335.35
TG(50:3)	77.56 ± 60.58	109.11 ± 146.24
TG(50:4)	10.44 ± 12.04	18.96 ± 48.76
TG(52:1)	68.00 ± 83.88	118.66 ± 202.99
TG(52:2)	28.25 ± 23.05	46.62 ± 52.91
TG(52:3)	498.65 ± 231.79	645.74 ± 440.30
TG(52:4)	245.16 ± 153.04	336.36 ± 298.72
TG(52:5)	15.84 ± 13.80	22.48 ± 23.38
TG(52:6)	0.93 ± 1.17	1.40 ± 2.11
TG(54:1)	9.58 ± 19.51	41.68 ± 261.61
TG(54:2)	93.60 ± 109.40	191.80 ± 454.91
TG(54:3)	318.74 ± 201.25	474.41 ± 535.05
TG(54:4)	203.38 ± 110.54	304.52 ± 294.97
TG(54:5)	56.95 ± 34.04	90.51 ± 89.38

Metabolites	**Concentration (µg/ml) – PTSD** (mean ± SD)	**Concentration (µg/ml) – Control** (mean ± SD)
TG(54:6)	26.21 ± 25.37	52.13 ± 83.34
TG(54:7)	3.37 ± 5.79	5.54 ± 9.41
TG(56:3)	4.09 ± 4.54	9.84 ± 24.79
TG(56:4)	7.98 ± 6.30	12.39 ± 16.43
TG(56:5)	12.11 ± 7.30	15.53 ± 11.68
TG(56:6)	14.69 ± 7.07	18.80 ± 10.06
TG(56:7)	8.33 ± 4.11	10.33 ± 5.66
TG(56:8)	2.83 ± 1.72	3.66 ± 2.40
TG(58:10)	0.67 ± 0.47	0.82 ± 0.55
TG(58:8)	1.21 ± 0.61	1.36 ± 0.80
TG(58:9)	1.49 ± 1.38	2.00 ± 1.61
TG(60:10)	0.23 ± 0.15	0.25 ± 0.16
TG(60:12)	0.07 ± 0.09	0.08 ± 0.07