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Metabolomics in posttraumatic stress disorder: Untargeted metabolomic analysis of plasma samples from Croatian war veterans

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Abstract

Posttraumatic stress disorder (PTSD) is a severe, multifactorial and debilitating neuropsychiatric disorder, which can develop in a subset of individuals as a result of the exposure to severe stress or trauma. Such traumatic experiences have a major impact on molecular, biochemical and cellular systems, causing psychological and somatic alterations that affect the whole organism. Although the etiology of PTSD is still unclear, it seems to involve complex interaction between various biological genetic and environmental factors. Metabolomics, as one of the rapidly developing "omics" techniques, might be a useful tool for determining altered metabolic pathways and stress-related metabolites as new potential biomarkers of PTSD. The aim of our study was to identify metabolites whose altered levels allow us to differentiate between patients with PTSD and healthy control individuals. The study included two cohorts. The first, exploratory, group included 50 Croatian veterans with PTSD and 50 healthy control subjects, whereas a validation group consisted of 52 veterans with PTSD and 52 control subjects. The metabolomic analysis of plasma samples was conducted using liquid chromatography coupled with mass spectrometry (LC-MS), as well as gas chromatography coupled with mass spectrometry (GC-MS). The LC-MS analysis determined significantly different levels of two glycerophospholipids, PE(18:1/0:0) and PC(18:1/0:0), between control subjects and PTSD patients in both cohorts. The altered metabolites might play a role in multiple cellular processes, including inflammation, mitochondrial dysfunction, membrane breakdown, oxidative stress and neurotoxicity, which could be associated with PTSD pathogenesis.

Keywords: psychiatry, metabolomics, posttraumatic stress disorder

INTRODUCTION

Posttraumatic stress disorder

Posttraumatic stress disorder (PTSD) is a severe, multifactorial and debilitating neuropsychiatric disorder [1] characterized by typical clusters of symptoms: re-experiencing, avoidance, hyperarousal and negative alterations in mood and thinking [2]. However, PTSD develops only in a subset of individuals as a result of experiencing or witnessing violence, injury or death [2]. Such traumatic experiences have a major impact on molecular, biochemical and cellular systems, causing psychological and somatic alterations [3,4]. Although the etiology of PTSD is still unclear, it seems to involve complex interaction between various biological, genetic and environmental factors. Due to the complexity and still unknown mechanisms that contribute to its development and progression, PTSD is a disorder without specific diagnostic biomarkers [5]. Therefore, metabolomics, as an approach encompassing products of all biochemical pathways in one organism, might be a useful tool to determine affected metabolic pathways and stress-related metabolites, as new potential biomarkers of PTSD.

Metabolomics in PTSD

Metabolomics has been used for the determination of altered metabolites in various psychiatric disorders, including PTSD. There are different types of metabolomics approaches, depending on biological problem that needs to be resolved. To the best of our knowledge, so far only two original articles [6,7] assessed the whole metabolome alterations in subjects with PTSD (reviewed in [2,8,9]). One study [6] identified 20 different metabolites whose alternations might be associated with PTSD. Those metabolites included glycerophospholipids, nucleosides, bile acids, sugars, antioxidants, as well as metabolites involved in fatty acid metabolism [6]. Another original study [7] reported altered metabolites related to glycolysis and fatty acid uptake and metabolism, consistent with mitochondrial alterations or dysfunction, suggesting an important role of lipids and fatty acids in the development of PTSD [7]. However, these studies did not detect any common metabolite changes.

Therefore, additional studies are necessary to further investigate and enlighten the possible role of metabolite alternations in the development of PTSD. We hypothesize that individuals with PTSD will demonstrate changes in the levels of certain metabolites, especially lipids and their derivatives. The aim of our study was to determine altered metabolites as possible metabolic biomarkers that would help differentiate patients with PTSD from healthy control individuals.

MATERIALS AND METHODS

Subject recruitment

The study was conducted at Rudjer Boskovic Institute (Zagreb, Croatia) and the Centre of Metabolomics and Bioanalysis (Madrid, Spain). Participants were recruited at the University Psychiatric Hospital Vrapce (Zagreb, Croatia). The study included two cohorts of subjects. The first cohort, used as an exploratory group, included plasma samples of 50 male combat veterans with PTSD and 50 healthy male subjects. The second cohort was used as a validation group, with plasma samples collected from 52 male combat veterans with PTSD and 52 male control subjects. Two cohorts were analyzed under the same conditions, one year apart.

Patients with PTSD were evaluated using the Structured Clinical Interview (SCID) for Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) [2] and Clinician Administered PTSD Scale (CAPS) [10]. Veterans were unrelated Caucasians of Croatian origin, aged 39 to 76, who participated in Homeland war in Croatia. Exclusion criteria were somatic diseases according to International Classification of Diseases (ICD-10) [11], pathophysiological alterations in liver, use of antidiabetic, antihypertensive and lipid lowering agents, drug and alcohol abuse, presence of other neuropsychiatric and neurodegenerative disorders, determined using DSM-5 criteria.

Healthy control subjects were evaluated using the same diagnostic instruments and had the same inclusion/exclusion criteria as PTSD patients. The diagnosis of PTSD was an additional exclusion criterion. The study was approved by the Bioethics Committee of the Rudjer Boskovic Institute

(Zagreb, Croatia) and Ethics Committee of the University Psychiatric Hospital Vrapce (Zagreb, Croatia). All participants signed an informed consent to participate in the study. All procedures contributing to this work were carried out in accordance with the Helsinki Declaration from 1975, as revised in 2008.

Blood sampling

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

Preparation of samples

Sample preparation for LC-MS

The sample preparation for LC-MS started with deproteinization using a cold mixture of methanol:ethanol (1:1), in the proportion 1:3, followed by centrifugation. Aliquots of plasma samples were used to prepare quality control samples (QCs), which were treated in the same way as all samples and used for the stabilization of the chromatographic system and for detecting possible analytical variabilities. 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.

Sample preparation for GC-MS

The sample preparation started with deproteinization by cold acetonitrile, followed by evaporation to dryness and derivatization, methoximation by O-methoxyamine hydrochloride in pyridine and then silylation using N,O-Bis(trimethylsilyl)trifluoroacetamide as described previously [12]. At the end of the sample treatment, an internal standard (100 µL of 20 ppm tricosane in heptane) was added to each sample in order to monitor sample injection and to correct the intrabatch fluctuation of the retention time (RT). Analytical variability was monitored using QCs as a pool of the same set of

samples. Blank samples, at the beginning and the end of each batch sequence, were used to detect possible contaminations. N-fatty acid methyl ester mixture (n-FAMEs C8-C22; Merck) was used as a retention index marker.

Analytical setup

LC-MS

The LC-MS analysis was completed using high-performance liquid chromatography (HPLC) system (1200 series; Agilent Technologies) coupled with quadrupole time-of-flight mass spectrometer (Q-TOF) 6520 (Agilent Technologies) with electrospray ionization source. Samples were injected randomly with an injection volume set to 10 µL. The autosampler was kept at 4 ^{o}C. The flow was set at 0.6 ml/min. The separation was achieved using a HPLC column Discovery® HS C18 15cm x 2.1 mm, 3 µm (Supelco analytical). The gradient used for separation consisted of two mobile phases: 1) mobile phase A - 0.1% of formic acid in water and 2) mobile phase B - 0.1% of formic acid in acetonitrile. Calibration with the reference masses was performed in order to provide optimum mass accuracy. Two reference masses were used during the analysis per mode: m/z 112.9856 ($C_2O_2F_3(NH_4)$) and m/z 1033.9881 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for negative ionization mode and m/z 121.0509 ($C_5H_4N_4$) and m/z 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for positive ionization mode [13,14].

GC-MS

The GC-MS analysis was conducted using GC instrument 7890A (Agilent Technologies) coupled with single quadropole (Q) mass spectrometer (5975C; Agilent Technologies). Derivatized samples were injected using autosampler (7693; Agilent Technologies) with an injection volume set to 2 µl. The separation was accomplished using GC Column DB-5MS 30m, 0.25mm, 0.25µm (Agilent Technologies). Helium was used as a carrier gas with constant flow of 1 ml/min. The injector temperature was 250 ^{o}C and the split ratio was 1:10. The oven temperature was programed at 60 ^{o}C for 1 minute and then increased at the rate of 10 °C/min to reach a final temperature of 325 ^{o}C.

Before the cool-down, it was held for 10 minutes. The temperatures of the injector, transfer line, filament source and the quadrupole were maintained at 250, 290, 230 and 150 ^{o}C respectively. The system was operated in scan mode (m/z 50-600) at a rate of 2 spectra per second with the electron impact ionization at 70 eV.

Data treatment

Data treatment after LC-MS analysis

Raw data obtained by the LC-MS was cleaned of background noise and unrelated ions using Molecular Feature Extraction (MFE) and Batch Recursive Feature Extraction (RFE) as part of Agilent MassHunter Profinder software (B.08.00, Agilent Technologies). As an extraction parameter, expected ions H^+, Na^+, K^+ for positive ionization and $HCOO^-$ for negative ionization were selected. Dehydration neutral loss was selected for both ionization modes [15]. Alignment and filtering were performed using Mass Profiler Professional v.B.12.01 Software (Agilent Technologies). The quality of the collected data was assured as described by Godzien et al. [16], while data normalization was performed according to the injection order of the QC samples [17]. Tandem mass spectrometry (MS/MS) was performed for the statistically significant features in positive and negative ionization, in order to facilitate the annotation of significant metabolites. MS/MS analysis was performed using the LC (Agilent 1200)-QTOF-MS (Agilent 6520) platform with the same chromatographic conditions as applied for the primary analysis. Identification of compounds was performed according to Naz et al. [15], using CEU Mass Mediator [18], METLIN [19], KEGG [20], LIPID MAPS [21] and HMDB [22] databases.

Data treatment after GC-MS analysis

Total Ion Chromatograms (TIC) obtained with the GC-MS were visually examined. After chromatogram inspection, the software Agilent MassHunter Unknowns Analysis (B.09.00, Agilent Technologies) was used to perform a deconvolution of the signals. Further, Agilent Mass Profiler

Professional software (version 13.0, Agilent Technologies) was used for signal alignment. After obtaining the list of compounds, NIST 14 (National Institute of Standards and Technology Mass Spectral Library v.2.2, 2014) and Fiehn RTL library (version 2013) [23] were used for metabolite identification comparing their mass fragmentation patterns [15]. Due to the quality assurance procedure, only metabolites present in more than 75% of samples and with CV less than 20% were kept. Data was normalized according to the internal standard, 20 ppm tricosane in heptane.

Statistical analysis

Clinical differences between control subjects and patients with PTSD in both cohorts were evaluated by independent samples t-test, while smoking status was assessed by χ^2-test, using GraphPad Prism version 4.00 for Windows (GraphPad Software). PTSD cases and control subjects were age-matched to avoid the effects associated with aging. Normality of the distribution was assessed with the Shapiro-Wilk test (MATLAB, version 14.9.1). For chromatographic peaks that had normal distribution, Levene's test was performed (MATLAB, version 14.9.1) to check the equality of variance, in order to choose proper t-test (Microsoft® Office Excel). Most of the chromatographic peaks were not normally distributed, therefore Mann-Whitney U test was used in such cases (MATLAB, version 14.9.1). Due to multiple testing, Benjamini-Hochberg (FDR, false discovery rate) correction (q) was applied. In addition to univariate, multivariate statistics were also performed. Unsupervised Principal Component Analysis (PCA), and supervised Orthogonal Partial Least Square - Discriminant analysis (OPLS-DA) were performed in this study. Based on OPLS-DA models, volcano plots plotting variable importance in the projection (VIP) against corrected p-values [p(corr), loading values scaled as correlation coefficients values] were created. Variables with VIP > 1.00, $q \leq 0.050$ and absolute p(corr) ≥ 0.30 were considered significant, and only data that fulfilled all these criteria were presented. The percentage of change (%) was calculated as follows: [(average value in the PTSD group − average value in control group)/(average value in control group)] × 100, with positive

values indicating increased and negative values decreased abundance of specific metabolite in the PTSD group compared to healthy control group.

RESULTS

Demographic data

All participants were age-matched Caucasian male subjects of Croatian origin. In order to exclude subjects with somatic diseases causing potential alteration in the metabolite profile, the levels of glucose, lipids and liver enzymes were determined in all participants. In both cohorts, there were no significant differences between patients with PTSD and control subjects in the level of blood glucose, cholesterol, high-density and low-density lipoprotein, triglycerides, aspartate aminotransferase, alanine transaminase and gamma-glutamyltransferase. In the second cohort, control subjects had significantly higher average body mass index (BMI) ($p<0.001$). Patients with PTSD smoked more frequently than control subjects in the first cohort ($p=0.020$) (Table 1).

Table 1. Demographic and clinical characteristics of healthy control subjects and PTSD patients in both cohorts

	Exploratory group			*Validation group*		
	PTSD patients N=50	**Control subjects N=50**	**p-value**	**PTSD patients N=52**	**Control subjects N=52**	**p-value**
Age (years) (mean ± SD)	58.40 ± 8.76	58.04 ± 9.03	p=0.8401	63.56 ± 7.28	63.75 ± 7.36	p=0.8936
Smokers N (%)	35 (70.0)	23 (46.9)	p=0.0199	38 (73.1)	30 (57.7)	p=0.0992
Non-smokers N (%)	15 (30.0)	26 (53.1)		14 (26.9)	22 (42.3)	
Glucose (mmol/L) (mean ± SD)	4.96 ± 0.77	4.78 ± 0.82	p=0.2748	5.56 ± 1.33	5.20 ± 1.32	p=0.1624
Cholesterol (mmol/L) (mean ± SD)	5.18 ± 1.17	5.36 ± 1.30	p=0.4778	4.95 ± 0.86	4.93 ± 1.13	p=0.9301
HDL (mmol/L) (mean ± SD)	1.28 ± 0.45	1.17 ± 0.27	p=0.1291	1.17 ± 0.35	1.20 ± 0.32	p=0.6427
LDL (mmol/L) (mean ± SD)	3.12 ± 0.99	3.05 ±0.93	p=0.7170	2.84 ± 0.64	2.92 ± 0.87	p=0.5647
TG (mmol/L) (mean ± SD)	1.86 ± 0.82	1.77 ± 0.88	p=0.6236	1.89 ± 0.92	1.91 ± 1.11	p=0.9161
AST (U/L) (mean ± SD)	17.54 ± 6.73	17.28 ± 6.36	p=0.8429	17.98 ± 6.57	17.42 ± 5.79	p=0.6471
ALT (U/L) (mean ± SD)	19.92 ± 11.79	18.54 ± 6.12	p=0.4643	20.46 ± 7.77	19.04 ± 6.28	p=0.3068
GGT (U/L) (mean ± SD)	27.30 ± 42.24	20.08 ± 8.91	p=0.2398	28.87 ± 43.27	20.13 ±8.42	p=0.1563
BMI (kg/m2) (mean ± SD)	26.73 ± 2.48	27.33 ± 2.52	p=0.2329	28.13 ± 3.10	30.48 ± 3.15	p<0.0001

HDL = high density lipoprotein; LDL = low density lipoprotein; TG = triglycerides; AST = aspartate transaminase; ALT = alanine transaminase; GGT = gamma-glutamyltransferase; BMI = body mass index

Liquid chromatography coupled with the mass spectrometry

The LC-MS analysis of plasma metabolites in both cohorts contained large spectra of metabolites. An OPLS-DA model for the LC-MS analysis showed well-defined distribution between PTSD cases and control subjects (Supplementary Fig 1 and 2). After combined multi- and univariate statistics, the LC-MS/MS analysis provided 3 compounds in positive and 6 compounds in negative ionization mode that had significantly different abundance between control subjects and PTSD patients. Compounds that we were not able to identify according to MS/MS spectra were excluded. The majority of metabolites from the first cohort belonged to the group of glycerophospholipids and carnitines. In the second cohort, 12 compounds in positive and 7 in negative ionization mode had significantly different abundance in PTSD individuals compared to healthy controls, whereas non-identified compounds were excluded. Most of the identified compounds belonged to the group of glycerophospholipids. The levels of phosphatidylcholines, phosphatidylethanolamines and phosphatidylinositol were significantly increased among PTSD patients compared to the control group (Table 2). Carnitines were decreased, while fatty acids were more abundant in PTSD than in control group (Table 3). Significant differences were replicated in the second cohort only for lysophospholipids PE (18:1/0:0) and PC (18:1/0:0). The levels of those compounds were significantly increased in PTSD subjects compared to control group in both cohorts (Table 2).

Table 2. List of glycerophospholipids, identified by the LC-MS/MS, found to be significantly different between veterans with PTSD and healthy control subjects in both cohorts

Compound class	Ionization mode	m/z (Da)	RT (min)	Metabolite	VIP	Absolute p(corr)	q	%	p
Exploratory cohort									
Glycerophosphoethanolamines	−	479.3018	20.41	PE(18:1/0:0)	1.93	0.41	0.0081	44.65	0.0002
Glycerophosphocholines	+	493.3172	17.28	PC(16:1/0:0)	2.97	0.66	0.0003	51.62	<0.0001
	−	539.3217	17.28	PC(16:1/0:0)	2.43	0.51	0.0001	62.58	<0.0001
	−	635.3402	20.59	PC(18:1/0:0)	2.68	0.57	0.0001	67.57	<0.0001
Validation cohort									
Quaternary ammonium salts	+	103.0996	20.21	Choline	1.54	0.39	0.0045	30.69	0.0001
Glycerophosphocholines	−	541.3382	19.22	PC(16:0/0:0)	1.44	0.46	0.0090	50.46	0.0009
	−	555.3529	21.29	PC(17:0/0:0)	1.43	0.53	0.0090	35.24	0.0009
	−	567.3528	19.48	PC(18:1/0:0)	1.56	0.53	0.0082	147.66	0.0006
	−	581.3684	22.02	PC(19:0/0:0)	1.44	0.54	0.0185	47.56	0.0037
	+	523.3643	22.64	PC(18:0/0:0)	1.35	0.40	0.0215	-77.00	0.0032
	+	521.3484	20.21	PC(18:1/0:0)	1.53	0.43	0.0043	35.34	0.0001
	+	477.3212	18.45	PC(16:2/0:0)	1.50	0.35	0.0045	40.18	0.0001
	+	495.5285	19.23	PC(16:0/0:0)	1.58	0.30	0.0048	55.37	0.0002
	+	543.3330	17.79	PC(20:4/0:0)	1.26	0.80	0.0233	30.10	0.0038
	+	571.3611	20.12	PC(22:4/0:0)	1.19	0.30	0.0378	37.35	0.0076
	+	479.3378	20.34	PC(O-16:1/0:0)	1.28	0.57	0.0294	31.43	0.0052
	+	549.3764	23.32	PC(P-18:0/2:0)	1.22	0.61	0.0184	65.89	0.0026
	+	505.3533	23.46	PC(P-18:1/0:0)	1.36	0.41	0.0163	62.61	0.0020
Glycerophosphoethanolamines	−	453.2852	18.26	PE(0:0/16:0)	1.22	0.43	0.0366	37.84	0.0098
	−	479.3008	16.34	PE(18:1/0:0)	1.23	0.44	0.0239	26.47	0.0050
Glycerophosphoinositols	−	598.3097	23.79	PI(18:1/0:0)	1.20	0.66	0.0400	86.62	0.0110

grey box = replicated compounds

RT = retention time; VIP = variable importance in the projection in OPLS-DA model; p(corr) = loading values scaled as correlation coefficients values in OPLS-DA model; q = Benjamini-Hochberg (FDR, false discovery rate) correction; % = percentage of change calculated as follows: [(average value in the PTSD group − average value in control group)/(average value in control group)] × 100; p = level of significance obtained with t-test or Mann-Whitney U test

Table 3. List of other metabolites, identified by the LC-MS/MS, found to be significantly different between veterans with PTSD and healthy control subjects in both cohorts

Compound class	Ionization mode	m/z (Da)	RT (min)	Metabolite	VIP	Absolute p(corr)	q	%	p
Exploratory cohort									
Acyl carnitines	+	423.3350	16.73	Linoelaidyl carnitine	2.29	0.43	0.0355	-30.81	0.0005
	+	427.3650	21.18	Stearoylcarnitine	1.62	0.49	0.0045	-23.09	<0.0001
Phosphosphingolipids	−	379.2484	14.78	Sphingosine 1-phosphate	1.28	0.80	0.0033	-13.69	<0.0001
Long-chain fatty acids	−	282.2556	31.82	Hydroxy stearic acid	1.67	0.35	0.0420	30.39	0.0012
Steroidal glycosides	−	510.2455	7.47	Testosterone like glucuronide (tentatively annotated)	2.42	0.35	0.0349	-40.15	0.0051
Validation cohort									
Fatty acid esters	+	318.2571	32.87	Arachidonic acid methyl ester	1.34	0.72	0.0413	25.11	0.0087
Dicarboxylic acids	+	208.1097	4.62	3,7-Dimethyl-2E,6E-decadien-1,10-dioic acid	1.28	0.49	0.0028	21.18	<0.0001

RT = retention time; VIP = variable importance in the projection in OPLS-DA model; p(corr) = loading values scaled as correlation coefficients values in OPLS-DA model; q = Benjamini-Hochberg (FDR, false discovery rate) correction; % = percentage of change calculated as follows: [(average value in the PTSD group − average value in control group)/(average value in control group)] × 100; p = level of significance obtained with t-test or Mann-Whitney U test

Gas chromatography coupled with the mass spectrometry

The GC-MS analysis detected mostly metabolites that belong to the group of sugars, organic acids, amino acids and their derivatives. An OPLS-DA model for the GC-MS analysis did not show a clear distribution between control subjects and PTSD patients (Supplementary Fig. 3). However, VIP generated a list of significantly different metabolites that contribute the most to the distribution between PTSD cases and control individuals. The combination of multi- and univariate statistics did not provide any metabolites with significantly different abundances between PTSD and healthy control subjects, although there were some compounds with VIP greater than 1. Among them was aminomalonic acid with VIP=1.17, p(corr)=0.52, %=35.06 and p=0.016, but this compound was rejected due to the FDR correction (q=0.412). In the second, validation, cohort there were 5 metabolites differently represented between healthy controls and subjects with PTSD. Aminomalonic acid showed a trend towards association with PTSD diagnosis in both cohorts and all significant metabolites, detected in the validation group, were increased in plasma of PTSD individuals (Table 4).

Table 4. List of metabolites, identified by the GC-MS, found to be significantly different between veterans with PTSD and healthy control subjects in validation cohort

Compound class	RT average (min)	Metabolite	VIP	Absolute p(corr)	q	%	p
D-alpha-amino acids	9.44	Proline	1.36	0.93	0.0235	34.49	0.0067
Proline and derivatives	13.15	4-hydroxyproline	1.38	0.67	0.0007	60.10	0.0001
Beta hydroxyl acids	10.17	Malic acid	1.29	0.85	0.0487	27.39	0.0153
Alpha amino acids	12.45	Aminomalonic acid	1.30	0.52	0.0029	58.72	0.0005
Carbohydrates	16.88	Fructose	2.27	0.46	0.0000	43.61	<0.0001

RT = retention time; VIP = variable importance in the projection in OPLS-DA model; p(corr) = loading values scaled as correlation coefficients values in OPLS-DA model; q = Benjamini-Hochberg (FDR, false discovery rate) correction; % = percentage of change calculated as follows: [(average value in the PTSD group − average value in control group)/(average value in control group)] × 100; p = level of significance obtained with t-test or Mann-Whitney U test

DISCUSSION

Systemic inflammation and metabolic disorders represent major hallmarks in the development and progression of PTSD [24]. In addition, impairments such as mitochondrial dysfunction, increased DNA damage and aging, hypersensitivity of sympathetic system, alterations in neurotransmitter system and dysregulation of HPA axis might be reflected in the altered metabolomic profile [7,9].

Glycerophospholipids

Glycerophospholipids are polar lipids, the main components of biological membranes, with a role in transportation, metabolic reactions, development, apoptosis, signal induction and transmission [25]. The group of glycerophospholipids is divided into subgroups of phosphatidylcholines (PC), phosphatidylserines (PS), phosphatidylethanolamines (PE) and phosphatidylinositols (PI). Our results showed significantly altered PC, PE and PI levels among patients with PTSD in comparison to healthy subjects. Most of the altered phospholipids were found to be increased in PTSD subjects in both cohorts, with the exception of PC(18:0/0:0). In plasma, PC(18:0/0:0) is produced by an enzyme lecithin-cholesterol acyltransferase (LCAT) and it plays an important role in signaling by binding to the G-protein-coupled receptors (GPCRs) [22]. Impairments in the GPCRs or LCAT activity might affect the PC(18:0/0:0) metabolism [26]. Our study also observed increased levels of choline, constituent of PC. Various other studies observed the same changes in the choline levels with assumption that its altered levels are associated with symptoms of depression or bipolar disorder [27]. However, in our sample, depression, anxiety, bipolar disorder and other neuropsychiatric comorbidities were exclusion criteria. Second most abundant phospholipids are phosphatidylethanolamines, which are present in the mitochondria and internal membranes, while phosphatidylinositols are involved in the pathophysiological processes, such as inflammation [25,28]. Chronic systemic inflammation is a hallmark of PTSD and it is reflected through the increased levels of inflammatory cytokines [29].

It is assumed that mitochondrial dysfunction is involved in the development of various diseases through phospholipid degradation and membrane breakdown. Mitochondrial lipids regulate the activity of membrane proteins and determine the physical characteristics of the mitochondrial membranes, which might affect the oxidative phosphorylation [28]. The role of phospholipids still remains unclear. However, alterations in the fatty acid metabolism, enzymatic activity, as well as impairments in the signal transduction through GPCRs, might be associated with disrupted phospholipid metabolism in neuropsychiatric disorders [26]. Furthermore, it has been assumed that oxidative stress and consequent increase in free radical levels might trigger lipid peroxidation, which is followed by the reduction of glycerophospholipids [26]. However, further research should be carried out to confirm and validate these findings that might help in future understanding of the involvement of these metabolomic alterations in the development of PTSD.

Proline and hydoxyproline

Following GC-MS analysis, most compounds altered in subjects with PTSD, relative to control subjects, belong to the group of amino acids or their derivatives. Proline and hydroxyproline play an important role in the immune system, healing, homeostasis, survival, as well as in antioxidative processes [30]. Hydroxyproline is produced endogenously or can be taken through diet, however, its molecular, cellular and systemic role is still unclear [31]. Proline takes part in the arginine, glutamate and polyamine synthesis, and it is most required among amino acid [32]. Therefore, alterations in proline and 4-hydroxyproline levels might indicate certain changes in the metabolic processes associated with the proline cycle. Proline and 4-hydroxyproline are susceptible to different cytosolic enzymatic reactions [33]. Prolidase exopeptidase is the enzyme that breaks C-terminal peptide bond in proline or hydroxyproline [33,34]. It has been assumed that the alterations in different proline peptidases and proline metabolism are involved in the pathology of various diseases, including Parkinson's and Alzheimer's disease, autism spectrum disorder, schizophrenia, depression, and altered behaviors [35]. In agreement, our findings have demonstrated an increase of proline and 4-

hydroxyproline among patients with PTSD. In contrast to our results, previous study [34] showed decreased prolidase activity, which corresponded to decreased levels of proline and 4-hydroxyproline in patients with PTSD compared to healthy control subjects. However, it is known that hydroxyproline levels are increased during oxidative stress or hypoxia [31], in order to maintain homeostasis. This might explain increased levels of hydroxyproline in patients with PTSD observed in our study, which might be due to increased chronic systemic inflammation and oxidative stress. Proline affects glutamate levels through induction of glutamatergic signaling and inhibition of glutamate release that is involved in the etiology of depression [36]. Therefore, the prolidase activity might be affected by alterations in proline-glutamate metabolism or influenced indirectly by oxidative stress triggered by proline [36]. However, the results obtained so far are inconsistent. This might mean that different metabolic routes, alone or in the combination with the ones related to proline and 4-hydroxyproline levels, might be involved in the development of PTSD.

CONCLUSION

Our study has some advantages and limitations. Although it was conducted on the largest sample so far, it did not include both genders. Additionally, it was impossible to exclude the impact of medication in the group of patients with PTSD, since they all had chronic and current PTSD and were on stable medication regimen. However, to minimize the impact of drug administration, all metabolites related to drug metabolism were not taken into consideration as differentiation factors between PTSD and healthy control groups. In addition, it was confirmed [7] that medication had little or no effect on the metabolomic profile. Although we managed to match our group of PTSD subjects with the respective control group according to most metabolic indices, we failed to match them according to smoking status in exploratory or BMI in the validation cohort. The findings gathered from the literature demonstrate contradictions among reported data, as well as compared to our results. These discrepancies might be explained with the low number of metabolomics studies in PTSD, differences in the sample size, in combat vs. civilian PTSD, gender and age-related

differences, comorbid disorders etc. However, common metabolites found to be different between PTSD cases and control subjects were related to several processes, such as inflammation, mitochondrial dysfunction, membrane breakdown, oxidative stress or neurotoxicity. Additionally, the source of discrepancies can be in the selection of significant compounds. Our parameters were quite strict, including VIP and p(corr) as well as FDR corrected level of significance, making our results even more reliable. In order to provide valid information regarding metabolites as potential biomarkers of PTSD, as well as to elucidate involved metabolic pathways, further untargeted and targeted metabolomic studies, involving civilian PTSD or female subjects with PTSD, are required.

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Conflict of interest

The Authors declare no conflict of interest.

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