Metabolomic analysis of male combat veterans with post traumatic stress disorder

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Abstract

Posttraumatic stress disorder (PTSD) is associated with impaired major domains of psychology and behavior. Individuals with PTSD also have increased co-morbidity with several serious medical conditions, including autoimmune diseases, cardiovascular disease, and diabetes, raising the possibility that systemic pathology associated with PTSD might be identified by metabolomic analysis of blood. We sought to identify metabolites that are altered in male combat veterans with PTSD. In this case-control study, we compared metabolomic profiles from age-matched male combat trauma-exposed veterans from the Iraq and Afghanistan conflicts with PTSD (n = 52) and without PTSD (n = 51) (‘Discovery group’). An additional group of 31 PTSD-positive and 31 PTSD-negative male combat-exposed veterans was used for validation of these findings (‘Test group’). Plasma metabolite profiles were measured in all subjects using ultrahigh performance liquid chromatography/tandem mass spectrometry and gas chromatography/mass spectrometry. We identified key differences between PTSD subjects and controls in pathways related to glycolysis and fatty acid uptake and metabolism in the initial ‘Discovery group’, consistent with mitochondrial alterations or dysfunction, which were also confirmed in the ‘Test group’. Other pathways related to urea cycle and amino acid metabolism were different between PTSD subjects and controls in the ‘Discovery’ but not in the smaller ‘Test’ group. These metabolic differences were not explained by comorbid major depression, body mass index, blood glucose, hemoglobin A1c, smoking, or use of analgesics, antidepressants, statins, or anti-inflammatories. These data show replicable, wide-ranging changes in the metabolic profile of combat-exposed males.
Introduction

Individuals with post-traumatic stress disorder (PTSD) have increased rates of several serious medical diseases, including cardiovascular disease, diabetes, autoimmune diseases and early mortality, suggesting widespread physical concomitants of PTSD [1]. Specific metabolic changes (“metabolic signatures” [2]) have been reported in several central nervous system disorders [3–10], childhood maltreatment [11]; exhaustion disorder [12], as well as in cardiovascular and coronary artery disease [13, 14] and insulin resistance [15–17]. Although analysis of altered metabolic pathways may provide new information about disease pathophysiology and may suggest novel drug targets, a metabolic signature has not yet been identified in PTSD, but a recent small study of non-combat, highly traumatized civilian subjects with PTSD (mean Clinician-Administered PTSD Scale, CAPS, scores >80) suggests there may be metabolomic differences in PTSD [18]. In that study of 20 PTSD and 18 control subjects, 19 metabolites were identified as being different between groups, and included several phospholipids, fatty acid metabolites, nucleosides, and bile acids, and whose abundance correlated with symptom severity [18].

Comorbidities of combat PTSD with cardiovascular disease, metabolic syndrome, and other diseases [19] suggest that there are likely to be metabolic differences between subjects with combat-related PTSD and controls. Therefore, we determined the plasma metabolomic profiles in male United States veterans from the Iraq and Afghanistan conflicts of 2001–2014 (Operation Enduring Freedom/Operation Iraqi Freedom, OEF/OIF) who endured combat-related trauma and developed PTSD, and compared these profiles to those in veterans who also endured combat-related trauma but did not develop PTSD. We also determined if these metabolites are associated with severity of PTSD symptoms. In order to lessen the likelihood of false positive results, which is attendant upon examining a large number of metabolites, we tested two separate cohorts of such subjects, to see which metabolite differences were replicable.

Animal models of PTSD have suggested disrupted brain energy metabolism [20], and studies of prolonged stress in mice have found alterations in mitochondrial pathways that were associated with hippocampal and amygdala apoptosis [21–23]. Further, studies of human blood from the cohort used in the current study [24] and other studies of blood and postmortem brain [25, 26] have shown several differentially methylated or dysregulated genes associated with mitochondrial function in PTSD, of which 20% correlated significantly with the severity of PTSD symptoms [26]. In addition, a study of childhood maltreatment [27] and major depression [28], which may share some behavioral features with PTSD, have also demonstrated significant alterations in mitochondrial function, which may be related to both increased inflammation and increased oxidative stress. Therefore, we hypothesized that subjects with PTSD would have, among other things, differences in metabolites that reflect mitochondrial function. Our data identify a metabolomic profile of combat trauma-exposed veterans with PTSD that is associated with, and may contribute to, the clinical phenotype of this disease.

Materials and methods

Subject recruitment

This study was approved by Institutional Review Boards of the University of California, San Francisco (San Francisco, CA), Ichlan School of Medicine at Mt. Sinai (New York, NY), James
J. Peters Veterans Administration Medical Center (Bronx, NY), and the New York University Langone School of Medicine (New York, NY). All participants gave written informed consent to participate in the study. This study is part of a more global Department of Defense-sponsored systems biology approach to understanding PTSD [29].

Male combat-exposed veterans from OEF/OIF were recruited at The New York University Langone School of Medicine and Mt. Sinai/James J. Peters VA Medical Center through flyers, presentations, newspaper and television advertisement, internet postings, and referral from clinicians. PTSD was diagnosed with structured clinical interviews. Inclusion criteria for PTSD-positive subjects included current war-zone trauma-related PTSD of at least 3 months duration and having Clinician Administered PTSD Scale (CAPS) [30] scores >40, while control (combat trauma-exposed PTSD-negative) veterans had no lifetime history of PTSD and had CAPS scores <20. All subjects were between 20 and 60 years old, and had English as their primary language. Exclusion criteria included: 1) history of alcohol dependence within the past 8 months; 2) history of drug abuse or dependence within the last 12 months; 3) lifetime history of any psychiatric disorder with psychotic features, bipolar or obsessive-compulsive disorder; 4) current exposure to recurrent trauma or a traumatic event within the past 3 months; 5) prominent suicidal or homicidal ideation; 6) neurologic disorder or systemic illness affecting central nervous system function; 7) clinical history of anemia or blood donation within the past 2 months; 8) changes in the past two months of psychotropic medication, anticonvulsants, antihypertensive medication, sympathomimetic medication, medications associated with neurogenesis or systemic steroid medication; 9) diagnosis of moderate or severe traumatic brain injury (TBI) on the Ohio State University TBI Identification Method-Short Form [31]; or 10) classification of mild TBI with a score ≥ 8 for current symptoms on the post-concussive symptom checklist.

Subjects and samples
Combat-exposed male OEF/OIF veterans with PTSD (n = 52) or combat-exposed, male OEF/OIF veterans without PTSD (n = 51) (“Discovery group”) were matched by age. Of the PTSD-positive subjects, 27 were also diagnosed with concurrent Major Depressive Disorder (MDD), as assessed by the Structured Clinical Interview for DSM-IV (SCID) [32]. Depression symptom severity was assessed with the self-rated Beck Depression Inventory-II (BDI-II) [33]. Following urinary toxicology screening for cannabinoids, cocaine, barbiturates, benzodiazepine, opiates, methadone, amphetamines, and phencyclidine, blood was collected for complete blood count, electrolytes, glucose, urea nitrogen, creatinine, glycated hemoglobin (HbA1c) and the liver function tests as specified by a CLIA certified clinical laboratory. Subjects reported to the laboratory at 7:30 AM having fasted overnight. Vital signs, weight, height and waist-to-hip ratio were measured. Following a period of rest, blood was drawn at 8:00 AM. Blood for metabolomics assays was collected into tubes containing EDTA, which were inverted 8–10 times before being placed on ice for up to 30 minutes, centrifuged at 4°C for 15 minutes at 1100 x g, following which plasma was removed and stored in 500 μL aliquots at -80°C until processed.

A second group of combat trauma-exposed male OEF/OIF veterans with PTSD (n = 31) and combat trauma-exposed, male OEF/OIF veterans without PTSD (n = 31) was enrolled as a “Test group” and followed the identical protocol as the initial Discovery group.

Sample preparation and metabolic profiling
Metabolomic profiling of all plasma samples was performed at Metabolon, Inc. (Durham, NC), as described [34–36], with plasma from both PTSD positive and PTSD negative subjects
being run in the same batches. Details of the analytic procedures are provided in S1 File and metabolomic data are found in S1 Dataset.

Statistical analysis
For statistical analyses and data display purposes, values below the limits of detection were replaced with the compound minimum (minimum value imputation). All metabolomic data were transformed in the same manner using the Blom transformation [37]. The Blom transformation is a rank-based normalization transformation, essentially a non-parametric procedure used to lessen deviations from the assumption of normality. As such, it does not distort the underlying data more than any other non-parametric transformation. Statistical analyses of Blom-transformed data were performed using SPSS (IBM, Armonk, NY) and R (http://cran.r-project.org). All tests were 2-tailed with an alpha = 0.05. Significance values between 0.05 and 0.1 are reported as trends. Multiple comparisons were accounted for by estimating the false discovery rate using q-values [38]. Q-values are p-values adjusted for the false discovery rate. They indicate the percent of significant results that will result in false positives, rather than the percent of all tests that will result in false positives; this usually results in smaller numbers of false positives. While a higher q-value indicates diminished confidence, it does not necessarily rule out the significance of a result. Other lines of evidence may be taken into consideration when determining whether a result merits further scrutiny. Such evidence may include a) inclusion in a common pathway that includes a strategically significant compound or b) residing in a similar functional biochemical family with other significant compounds. In addition, bootstrapping based upon random sampling of the subjects was used as an additional statistical method to validate the findings across the Discovery and Test groups. Biochemical differences between groups were assessed by t-tests, analyses of covariance (ANCOVA) and correlation analyses. Independent samples t-test for continuous variables and chi-square test for dichotomous variables were used to examine participants’ baseline between-group differences.

Results
Demographics in the “Discovery group”
There were no significant differences in age, ethnicity between the 52 PTSD subjects and 51 controls in the “Discovery group” (Table 1). There were no significant differences in HbA1c, cholesterol, HDL, LDL, triglycerides, waist-to-hip ratios, sodium, calcium, CO₂, blood urea nitrogen or liver function tests. PTSD-positive subjects had increased body mass index (BMI) (p<0.05), high sensitivity C-reactive protein (p<0.05), glucose (p<0.01), insulin (p<0.01), homeostatic model assessment-estimated insulin resistance (HOMA-IR) (p<0.02), creatinine (p<0.01), pulse rate (p<0.01), hemoglobin (p<0.02), and hematocrit (p<0.03), compared to the PTSD-negative subjects (Table 1). Although the PTSD-positive group had a higher average BMI than the PTSD-negative group, their waist-to-hip ratio, which may be a better predictor of health [39], was not significantly different. In addition to the differences in CAPS scores that were used for inclusion/exclusion, BDI scores were significantly higher in PTSD subjects (p<0.001), among whom 52% (29/52) (chi-square) also met criteria for MDD and 29% (15/52) were receiving antidepressant medications (chi-square). The PTSD group took more medications than did the control group. In the Discovery group, 40.4% (21/52) of the PTSD group and 17.6% (9/51) of the control group took some type of medication (chi-square p<0.01). There were no significant between-group differences in the prevalence of hypertension, heart attacks, stable angina, stroke, or diabetes, or in the number of subjects taking statins, non-steroidal anti-inflammatories, analgesics or oral hypoglycemic agents.
Table 1. Demographic and clinical characteristics of combat veterans with PTSD and controls in Discovery and Test groups.

| Metabolic measurements | Discovery Group | Test Group | Statistic | X² (p) | Discovery Group | Test Group | Statistic | X² (p) |
|------------------------|-----------------|------------|-----------|--------|-----------------|------------|-----------|--------|
|                        | N: 51           | N: 52      |           |        | N: 31           | N: 31      |           |        |
| BMI                    | Means ± SD      | Means ± SD | T         | P      | Means ± SD      | Means ± SD | T         | P      |
| Total Protein (g/dL)   | 6.85 ± 0.47     | 6.72 ± 0.46 | 4.56     | <0.01* | 7.12 ± 0.48     | 7.20 ± 0.48 | 0.96     | 0.34   |
| Chloride (mEq/L)       | 27.56 ± 2.54    | 27.52 ± 2.54 | 0.11     | 0.91   | 27.02 ± 2.51    | 27.30 ± 2.53 | 0.44     | 0.66   |
| Aspartate transaminase (U/L) | 26.25 ± 14.26 | 30.04 ± 16.61 | 1.24     | 0.22   | 30.97 ± 24.29   | 33.19 ± 17.13 | 0.42     | 0.67   |
| Alanine transaminase (U/L) | 29.55 ± 17.46 | 36.60 ± 23.22 | 1.74     | 0.08   | 34.39 ± 30.31   | 37.13 ± 23.82 | 0.39     | 0.69   |
| Gamma-glutamyl transferase (GGT) (U/L) | 27.17 ± 25.02 | 35.35 ± 24.61 | 1.66     | 0.09   | 53.32 ± 142.58  | 35.72 ± 17.11 | 0.66     | 0.51   |
| Blood Urea Nitrogen (mg/dL) | 15.33 ± 3.85 | 14.00 ± 3.82 | 1.76     | 0.08   | 14.68 ± 2.75    | 15.42 ± 3.20 | 0.98     | 0.33   |
| Creatinine (mg/dL)     | 1.40 ± 0.19     | 0.94 ± 0.17 | 2.89     | <0.01* | 1.03 ± 0.16     | 1.04 ± 0.19 | 0.30     | 0.76   |
| CO2 (mEq/L)            | 27.61 ± 1.88    | 27.56 ± 2.54 | 0.11     | 0.91   | 27.02 ± 2.51    | 27.30 ± 2.53 | 0.44     | 0.66   |
| Pulse (beats/min)      | 64.20 ± 10.65   | 72.35 ± 9.91 | 4.02     | <0.01* | 65.48 ± 12.22   | 73.16 ± 11.15 | 2.58     | 0.01*  |
| Hemoglobin (g/dL)      | 14.22 ± 1.06    | 14.74 ± 1.21 | 2.29     | 0.02*  | 14.58 ± 0.67    | 14.84 ± 1.18 | 1.07     | 0.29   |
| Hematocrit (%)         | 41.98 ± 2.85    | 43.46 ± 3.33 | 2.42     | 0.02*  | 42.88 ± 2.02    | 43.99 ± 3.23 | 1.62     | 0.11   |

(Continued)
Demographics in the “Test group”

In the “Test group” of 31 PTSD-negative and 31 PTSD-positive subjects, demographic and clinical characteristics were similar to the Discovery group, except there were no statistically significant differences in BMI, glucose, insulin, HOMA-IR, creatinine, hemoglobin, or hematocrit between the PTSD-positive and -negative groups. Like the Discovery group, the Test group PTSD-positive subjects had increased high sensitivity C-reactive protein (p < 0.02) and pulse rate (p < 0.01) (Table 1). In addition to the differences in CAPS scores that were used for inclusion/exclusion, BDI scores were significantly higher in PTSD subjects (p < 0.001) in the test group, among whom 64.5% (chi-square) also met criteria for MDD and 16.1% were receiving antidepressant medications (chi-square), and 32.2% of the PTSD subjects and 16.1% of the control subjects took some type of medication (chi-square p = 0.1). There were no significant between-group differences in the prevalence of other comorbid diseases.

Metabolite differences in PTSD-positive vs PTSD-negative subjects in both the Discovery and Test groups

The analysis of metabolites in plasma of the Discovery and Test groups included all detectable compounds of known identity. Of 4400 metabolites and xenobiotics potentially identifiable by our mass spectrometry platforms, 370 named compounds were identified in plasma of our initial Discovery group and 623 named compounds were identified in plasma of our validating Test group. These differences arose because the two metabolomic analyses used slightly different methodologies that resulted in detection and identification of some different compounds in each analysis. Hence, only 244 compounds that were identified in both the Discovery and Test samples were used in the analyses (Table A in S1 File, S1 Dataset, and Tables 2 and 3). Summaries of the numbers of compounds that achieved statistical significance (p ≤ 0.05) are shown in Tables 2 and 3.

All of the significant between-group differences reported below were also confirmed by bootstrap analyses (Figure A in S1 File). The information from the metabolic profiling...
identified significant differences between PTSD-positive and -negative subjects in biochemical pathways involved in glucose metabolism, energy utilization and lipid metabolism in both the Discovery and Test groups.

**Carbohydrates.** Carbohydrates, amino acids and fats can be used to generate reducing equivalents (NADH) and ATP. The main, highly significant finding of our study was that lactate (Discovery group: \( p < 1.6 \times 10^{-6}, q = 0.00 \); Test group: \( p = 0.003; q = 0.047 \)) and pyruvate (Discovery group: \( p = 0.017, q = 0.27 \); Test group: \( p = 0.010, q = 0.10 \)), two products of anaerobic respiration in glycolysis, were significantly elevated in the PTSD-positive subjects compared to the PTSD negative subjects. Among the intermediates in the TCA cycle (aerobic respiration), citrate was decreased in PTSD subjects in the Discovery group (\( p = 0.017, q = 0.27 \)), although this finding was not replicated in the Test group (\( p = 0.669, q = 0.62 \)). To assess the relative contribution of metabolites from anaerobic and aerobic respiration in our subjects, we defined a ratio indicative of anaerobic relative to aerobic respiration, and calculated a ratio from the relative amounts of pyruvate, lactate and citrate in each subject’s plasma ([Pyruvate + Lactate]/Citrate), and called this the “glycolytic ratio”. The glycolytic ratio was significantly higher in veterans with PTSD than in veterans without PTSD in both the Discovery (\( p < 1 \times 10^{-4}, q = 0.00 \)) and Test (\( p = 0.005; q = 0.07 \)) groups, suggesting increased anaerobic and decreased aerobic respiration in these subjects, which may indicate mitochondrial (TCA cycle) alterations or dysfunction.

**Lipids.** Many fatty acids were lower in PTSD-positive subjects. The long chain fatty acids eicosanoate (20:1n9 or 11) (Discovery group: \( p = 0.019, q = 0.28 \); Test group: \( p = 0.014, q = 0.12 \)) and 10-nonadecenoate (19:1n9) (Discovery group: \( p = 0.047, q = 0.38 \); Test group: \( p = 0.091, q = 0.27 \)) were less abundant in PTSD than in the controls. These differences could result from decreased breakdown of storage lipids, increased fatty acid catabolism via β-oxidation, and/or decreased uptake of dietary fat. Several long-chain essential fatty acids that must be absorbed from the diet, including linolenate (18:3n3 or 6) (Discovery group: \( p = 0.039, q = 0.38 \); Test group: \( p = 0.026, q = 0.15 \)), dihomo-linoleate (20:2n6) (Discovery group: \( p = 0.042, q = 0.38 \); Test group: \( p = 0.066, q = 0.24 \)), and dihomo-linolenate (20:3n3 or n6) (Discovery group: \( p = 0.039, q = 0.38 \); Test group: \( p = 0.001, q = 0.02 \)), and docosahexaenoate (DHA, 22:6n3) (Discovery group: \( p = 0.047, q = 0.38 \); Test group: \( p < 0.001, q = 0.00 \)) were also decreased in PTSD versus controls. These data suggest that decreased dietary fat uptake, mitochondrial alterations or dysfunction, or differences in the gut microbiome may contribute to the observed decrease in free fatty acids in PTSD.

**Hypoxanthine.** Levels of hypoxanthine were higher in PTSD compared to controls (Discovery group: \( p = 0.013, q = 0.25 \); Test group: \( p = 0.016, q = 0.13 \)). Hypoxanthine is a naturally occurring purine derivative that is involved in ATP catabolism and the salvage pathway for purine synthesis. However, we did not see elevated levels of xanthine and uric acid, other products of the purine catabolism pathway. Increased levels of hypoxanthine, a substrate for xanthine oxidase, may result in generating reactive oxygen species (ROS) [40].

**Other metabolites.** Information about metabolites that were significantly different only in the Discovery group, but not the smaller Test group, can be found in Tables 2 and 3 and in Supplementary Material (Table A in S1 File and S1 Dataset).

**ANCOVA analyses with meta-data in the Discovery group**

**Hemoglobin A1c (HbA1c)** reflects average blood glucose levels over the previous three months. Because our data suggest that metabolite differences between PTSD and control subjects may reflect differences in use of energy sources (glucose vs fatty acids), we evaluated status of glucose dysregulation as a covariate. Only 2 subjects (1 PTSD, 1 control) in the
| Super Pathway | Sub Pathway | Biochemical Name | Control | PTSD | PTSD/Control | p-value\(^a\) | Cohen’s d | q-value\(^b\) | ANCOVA, covarying for: | ANCOVA, covarying for: |
|--------------|-------------|------------------|--------|-----|-------------|-----------|----------|-----------|-------------------------|-------------------------|
|              |             |                  | Mean   | SD  | Mean        | SD        |          |           | BMI                     | HbA1C                   |
| Amino acid   | Glutamate metabolism | glutamine | 1.050  | 0.145 | 0.978       | 0.133    | 0.932    | 0.010     | 0.592                   | 0.241                   |
|              | Histidine metabolism | trans-urocanate | 1.051 | 0.539 | 0.834       | 0.427    | 0.794    | 0.033     | 0.460                   | 0.378                   |
|              | Phenylalanine & tyrosine metabolism | phenyllactate (PLA) | 0.781  | 0.327 | 1.033       | 0.639    | 1.323    | 0.026     | 0.491                   | 0.359                   |
|              | Urea cycle; arginine-, proline-, metabolism | arginine | 1.142 | 0.297 | 1.013       | 0.374    | 0.887    | 0.031     | 0.386                   | 0.378                   |
|              | Leucine, Isoleucine and Valine Metabolism | 3-hydroxyisobutyrate | 1.094 | 0.301 | 0.987       | 0.296    | 0.902    | 0.040     | 0.333                   | 0.378                   |
|              | Glutathione metabolism | 5-oxoproline | 0.943 | 0.149 | 1.073       | 0.187    | 1.138    | <0.001    | 0.759                   | 0.000                   |
| Carbohydrate | Glycolysis, gluconeogenesis, pyruvate metabolism | pyruvate | 1.056 | 0.736 | 1.272       | 0.649    | 1.205    | 0.017     | 0.302                   | 0.274                   |
|              | Energy       | citrate          | 1.071 | 0.267 | 0.948       | 0.231    | 0.885    | 0.017     | 0.478                   | 0.274                   |
| Lipid        | Essential fatty acid | linolenate [alpha or gamma; (18:3n3 or 6)] | 1.198 | 0.533 | 1.013       | 0.577    | 0.846    | 0.039     | 0.342                   | 0.378                   |
|              |              | dihomo-linoleate (20:2n6) | 1.196 | 0.598 | 0.979       | 0.510    | 0.819    | 0.042     | 0.395                   | 0.378                   |
|              |              | dihomo-linolenate (20:3n3 or n6) | 1.096 | 0.407 | 0.951       | 0.393    | 0.868    | 0.039     | 0.375                   | 0.378                   |
|              |              | docosahexaenoate (DHA; 22:6n3) | 1.117 | 0.568 | 0.938       | 0.501    | 0.840    | 0.047     | 0.317                   | 0.378                   |
|              | Long chain fatty acid | 10-nonadecenoate (19:1n9) | 1.212 | 0.525 | 1.041       | 0.631    | 0.859    | 0.047     | 0.275                   | 0.378                   |
|              |              | eicosanoate (20:1n9 or 11) | 1.282 | 0.646 | 1.028       | 0.625    | 0.802    | 0.019     | 0.391                   | 0.282                   |
|              | Sphingolipid | sphingosine-1-phosphate | 0.947 | 0.386 | 1.122       | 0.459    | 1.185    | 0.046     | 0.399                   | 0.378                   |
|              | Carnitine metabolism | octanoylcarnitine | 0.936 | 0.406 | 1.241       | 0.690    | 1.326    | 0.004     | 0.529                   | 0.129                   |
|              |              | hexanoylcarnitine | 1.003 | 0.288 | 1.174       | 0.439    | 1.171    | 0.047     | 0.456                   | 0.378                   |
|              |              | decanoylcarnitine | 0.970 | 0.432 | 1.272       | 0.643    | 1.311    | 0.003     | 0.550                   | 0.116                   |
|              | Sterol/Steroid | cortisol | 0.912 | 0.398 | 1.085       | 0.339    | 1.190    | 0.012     | 0.485                   | 0.251                   |
| Nucleotide   | Purine metabolism, (hypo)xanthine/inosine containing | hypoxanthine | 0.943 | 0.384 | 1.286       | 0.687    | 1.364    | 0.013     | 0.628                   | 0.251                   |
|              |              | GABR | 0.575 | 0.208 | 0.475       | 0.170    | 0.826    | 0.009     | 0.523                   | 0.241                   |
|              |              | GLYCOLITIC RATIO | 1.977  | 1.154 | 2.787       | 1.145    | 1.410    | <0.001    | 0.619                   | 0.000                   |

\(^a\) uncorrected p values
\(^b\), q values are p values adjusted for the false discovery rate

Green boxes indicate metabolites are lower in the PTSD positive group vs PTSD negative group. Red boxes indicate metabolites are higher in the PTSD positive group vs PTSD negative group.

https://doi.org/10.1371/journal.pone.0213839.t002
Discovery group carried pre-existing diabes of diabetes, and HbA1c values were not different between groups (Table 1). Using Hb1Ac as a covariate had little effect on metabolite differences between PTSD-positive and–negative groups (Table 2): 14 of 21 metabolites that were significantly different between PTSD-positive and–negative subjects remained significant, while the other 7 metabolites were now trends (p < 0.09). Similarly, using glucose as a covariate had little effect on differences in metabolite concentrations between PTSD-positive and–negative subjects.

BMI. Because we identified potential differences in energy metabolism between PTSD and control subjects, we tested BMI as covariate. Although BMI was significantly greater in PTSD-positive than in PTSD-negative subjects in the Discovery group (Table 1 and [41]), using BMI as a covariate had little effect on differences in metabolite concentrations between PTSD-positive and–negative subjects (Table 2). After covarying for BMI, 17 of the 21
metabolites that were significantly different between groups remained significantly different, and the other 4 now trended toward significance (p<0.083).

Because high BMI may be co-morbid with PTSD in the Discovery group, and because there were no differences in BMI between PTSD and control subjects in the Test group, we also covaried for BMI in the Test group to see if any of the metabolites were influenced by BMI. The metabolites identified as statistically significant between PTSD and control subjects remained significant after covarying for BMI (all p<0.035).

In addition, we also covaried for waist-to-hip ratio instead of BMI, since it may be a better predictor of health [39], and we found that none of our main findings were altered. These analyses suggest that our findings are not driven by anthropometric variables.

**Cotinine.** To test the possible impact of tobacco use on the findings, we evaluated plasma cotinine levels as a covariate, since cotinine is the predominant metabolite of nicotine. Using cotinine as a covariate had little effect on metabolite differences between PTSD-positive and negative groups (Table 2): 17 of 21 metabolites that were significantly different between PTSD-positive and -negative subjects remained significant, while the other 4 metabolites were now trends (p<0.09).

**Sensitivity analysis with meta-data.** Many of the subjects were receiving medications that might affect metabolic profiles. As expected, the PTSD group took more medication than did the control group across all medications (Table 1). In the Discovery Group, 40.4% of the PTSD group and 17.6% of the control group took some type of medication (chi-square, p<0.01), and in the Test Group, 32.2% of the PTSD group and 16.1% of the control group took some type of medication (chi-square, p = 0.1).

Because the number of subjects taking each medication was too underpowered for ANCOVA analysis, medication effects were assessed in a sensitivity analysis in which t-tests were conducted to compare the PTSD-positive and -negative groups, with subjects taking particular medications excluded. When medications were grouped by type, the number of subjects regularly taking each was: anti-inflammatories (9), anti-depressants (17), statins (3), sedatives (7), anticonvulsants (3), antidiabetic agents (2), antibiotics (2) and beta-blockers (2) (Table 1). The reported metabolite differences between PTSD-positive and -negative subjects remained significant even in the subgroup of subjects not taking each type of medication (not shown). We also performed ANOVAs in both the Discovery and Test groups, excluding subjects taking any medication (Table 1). In the Discovery group, docosahexaenoate (DHA22:6n3) lost significance (p = 0.16), dimhomolinoleate 20:2n6, 10nonadecenoate 19:1n9, and eicosenoate 20:1 became trends (p<0.055), and the other metabolites remained significant. In the Test group, dihomolinoleate 20:2n6 (p = 0.19) and 10nonadecenoate 19:1n9 (p = 0.20) lost significance, while the other metabolites remained significant. In the Discovery group, we also compared metabolites from PTSD subjects taking any type of medication with PTSD subjects not taking any medication, and found that the PTSD positive and negative subjects do not show any significant difference (all p>0.3).

Among the PTSD subjects in the Discovery group, 27 were diagnosed with concurrent MDD, while no control subject was diagnosed with MDD. Nevertheless, using MDD status as a covariate did not affect the differences in metabolites seen between PTSD-positive and -negative subjects; furthermore, the reported metabolite differences between PTSD-positive and -negative subjects remained significant even in the subgroup of subjects without MDD. There were also no significant metabolite differences between PTSD subjects who did or did not also have MDD (all p > 0.3, not shown). These analyses suggest that MDD status did not play a significant role in explaining the between-group differences observed in PTSD-positive and PTSD-negative groups.
Correlations with current CAPS scores

Using the current CAPS score as a continuous variable, none of the metabolites that were identified as significantly different between groups correlated significantly with the current CAPS score within the PTSD group.

Discussion

In addition to traditional symptoms of PTSD (re-experiencing, avoidance, hyperarousal, negative thoughts or moods associated with the traumatic event [42]), individuals with PTSD also have a significantly increased medical burden, including higher rates of cardiovascular disease, metabolic syndrome, diabetes, autoimmune disease, and early mortality [1, 41, 43, 44], suggesting that PTSD is both a behavioral and somatic disease. Certain processes have been proposed as contributing to the risk for these somatic diseases, including accelerated biological aging [45–56], sympathetic and glucocorticoid dysregulation [43, 57–61], metabolic changes [41, 45, 62, 63], inflammation [43, 57, 58, 64–71] and others. Some of these processes may involve changes in energy balance and mitochondrial function [72–77] that may be revealed by changes in metabolomic profiles.

Studies in mice [21] and humans [24–26] found dysregulation of genes affecting mitochondrial function in PTSD, hence we initially hypothesized that combat veterans with PTSD would have metabolite signatures indicating impaired mitochondrial function. In our Discovery group, we found significant differences between the metabolite profiles of male combat veterans who developed PTSD and those who did not, and these profiles were largely, but not uniformly, confirmed in our smaller Test group. The metabolite profiles are consistent with significant differences in mitochondrial function, energy utilization, and nutrient absorption or gut microbiota between these two groups of combat veterans. Alternatively, increases in inflammation and oxidative stress may lead to mitochondrial alteration or dysfunction [78]. In turn, these metabolite differences may contribute to increased inflammation, oxidative stress, anxiety, panic, obesity, metabolic syndrome, and cardiovascular disease, which are strongly associated with PTSD (Fig 1) [1, 19, 71, 79–81]. There are several mechanisms by which the altered metabolites, or the dysfunctional or compensatory pathways these metabolites reflect, may associate with PTSD.

Lactate and pyruvate

The most robust metabolic between-group difference was the elevated concentrations of pyruvate and lactate, indicating enhanced anaerobic glycolysis in the PTSD-positive group. Decreased metabolites in the TCA cycle have been seen in brains of animal models of PTSD [20]. The pathophysiological significance of increased lactate in PTSD is unknown, but to the extent that lactate contributes to PTSD symptoms [82–92], several potential mechanisms are proposed, as described below.

GPR81, a cell-surface receptor coupled to Gi proteins, is activated by lactate and decreases intracellular cAMP [93–97]. GPR81 is highly expressed in adipocytes, suggesting a role in regulating lipolysis, and is also expressed in liver, kidney, skeletal muscle, spleen, and testis, where it may play a role in lipid metabolism or have other functions [97]. The elevated concentrations of lactate in PTSD may have synergistic anti-lipolytic effects, reducing the availability of long chain fatty acids for energy metabolism, which may also lead to the increased adiposity and BMI seen in these subjects. Elevated basal lactate concentrations are also associated with the development of insulin resistance [98, 99], which has been suggested to be due to decreased glucose utilization [100–102].
GPR81 is also found in neurons of the cerebral cortex, hippocampus (pyramidal and granule cells) and cerebellum (granule cells), where it can be activated by physiological concentrations (low mM) of lactate \[103, 104\]. In the cortex, GPR81 is found mainly on synaptic membranes of excitatory synapses, and is predominantly expressed on postsynaptic membranes. GPR81 is also enriched at the blood-brain barrier, further suggesting that lactate may play a role in signaling in the brain. Lactate, via GPR81, may act as a volume transmitter, linking neuronal activity, cerebral energy metabolism, and energy substrate availability \[103, 104\]. This occurs through regulating formation of cAMP and by adjusting the NADH/NAD ratio. Thus, lactate is a mediator of metabolic information in addition to being a metabolic substrate \[105\].

While the molecular mechanism is unknown, lactate can predispose to panic attacks in susceptible subjects \[82–91\], and patients with a panic disorder may have elevated brain lactate responses to metabolic challenges \[92\]. Lactate exerts excitatory effects on neuronal activity \[106, 107\]; thus, increased hippocampal firing, a direct action of lactate on the CNS, may contribute to lactate-induced panic.
Lactate also stimulates neuronal expression of genes related to synaptic plasticity (e.g. Arc, c-Fos, and Zif268), via NMDA receptors and their downstream signaling cascade to Erk1/2 in mouse primary neuronal cultures and in vivo [108]. Lactate can potentiate currents mediated by NMDA receptors via increased intracellular calcium or increased intraneuronal concentrations of NADH. By contrast, lactate binding to GPR81 may also directly inhibit both glutamatergic and GABA-ergic neuronal function by inhibiting the frequency of calcium transients, thus reducing the frequency of neuronal firing [109]. Elevated lactate and pyruvate decrease the cellular NADH/NAD ratio, which in turn regulates various clock genes [110], which may contribute to the disordered sleep [111] and glucocorticoid sensitivity [112] associated with PTSD. Our peripheral measurements of plasma lactate, however, may not completely reflect intracerebral lactate concentrations.

**Fatty acids**

Fatty acids can be both pro- and anti-inflammatory [113]. Saturated free fatty acids are involved in the inflammatory response via toll-like receptors [114]. Because we found no differences in plasma concentrations of saturated fatty acids in PTSD versus controls, it is unlikely that the increased inflammation, previously reported in our PTSD subjects [69, 70] is due to increased saturated fatty acids stimulating toll-like receptors. However, there was a significant decrease in several unsaturated fatty acids in PTSD subjects. Dietary long chain polyunsaturated fatty acids are ligands of the nuclear peroxisome proliferator-activated receptors (PPAR). Binding to PPAR-α, -γ, and -δ suppresses expression of sterol regulatory element-binding proteins, nuclear transcription factor NFκB, and other transcription factors that regulate expression of genes involved in intermediary metabolism, thermoregulation, energy partitioning, growth, differentiation, and inflammatory responses [115–119]. Hence, reduced concentrations of unsaturated fatty acids could lead to enhanced inflammation and a variety of other effects in PTSD.

In addition to fatty acids being ligands for PPAR receptors, the omega-3 fatty acids are ligands for the G-protein coupled receptor GPR120 [120, 121], and are protective and anti-inflammatory [120, 121]. In obese mice, stimulation of GPR120 by omega-3 fatty acids inhibits inflammatory signaling and improves insulin sensitivity [121, 122]. We found low concentrations of the omega-3 unsaturated fatty acid docosahexaenoic acid (DHA) in PTSD subjects, raising the possibility that its reduced plasma concentration may contribute to inflammation in these PTSD subjects [69, 70]. Although not assessed directly, reduced concentrations of DHA may also contribute to reduced insulin sensitivity seen in our subjects [41].

Studies in humans [123–127] and rodents [128–134] indicate that omega-3 fatty acid deficiency may be associated with a variety of neuropsychiatric illnesses, including attention deficit hyperactivity disorder, depression, schizophrenia, autism spectrum disorders, and anxiety. Our data are consistent with animal studies showing that chronic social defeat stress [135] and variable, intermittent social defeat stress [136], models of human PTSD, disrupts regulation of lipid synthesis, including reduced levels of non-esterified fatty acids, increased levels of cholesterol and LDL cholesterol, and reduced fatty acid oxidation. Reduced abundance of omega-3 fatty acids in the CNS may reduce neurotransmission, especially by the dopaminergic and serotonergic systems, by affecting membrane fluidity and related receptor functions, thereby ultimately affecting brain structure and function [131, 137]. Based on promising pilot results [138], a clinical trial of omega-3 fatty acids has been started in patients with PTSD [139].

**Hypoxanthine**

Hypoxanthine, a naturally occurring purine derivative that is involved in the salvage pathway for purine synthesis, has been shown to stimulate oxidative stress [140, 141], and elevated
concentrations have been implicated in fear in dogs [142]. Hypoxanthine is also high in fecal samples in a chronic variable stress rat model of depression [143]. Smoking [144] and heavy drinking [145] are also associated with elevated levels of hypoxanthine. In our subjects, however, the increases in hypoxanthine in the PTSD group were not accounted for by smoking, as indicated by cotinine levels. In apolipoprotein E-deficient mice and cells, hypoxanthine also induces cholesterol accumulation and stimulates atherosclerosis through alterations in lipid transport enzymes, independent of conversion to xanthine and uric acid [146], a known risk for cardiovascular disease. Indeed, elevated hypoxanthine levels have been reported in human myocardial infarction [147], a condition with higher prevalence in individuals with PTSD.

Limitations and strengths

There are three significant limitations to our study. First, the study utilized modest sample sizes, although this is the largest human PTSD metabolomics study published to date. Second, this study utilized only male combat trauma-exposed subjects. Thus, our findings should not be extrapolated uncritically to females with PTSD or to individuals of either sex with non-combat-related PTSD. Third, since this was a cross-sectional study, based on single time-point for blood and behavioral measurements, we cannot assess any causal relationship or variability in the measures over time. Being an exploratory study, we did not correct for multiple hypothesis testing. However, the q values (which assess the significance of the false discovery rate), the identification of different metabolites that exist within specific pathways, and the replication of our strongest results with a second group of subjects suggest that the metabolites identified are indeed significantly different between groups. Finally, it is possible that other genetic and epigenetic risks may contribute to the metabolomic differences between PTSD positive and negative subjects.

Among the strengths of the study, first, we used well-characterized, young, combat-exposed veterans and excluded subjects with significant traumatic brain injury or current uncontrolled medical illness. Due to the deep phenotyping of our subjects, we were able to account for metabolomics effects secondary to several health (e.g. fasting blood sugar, HbA1c, tobacco use) medication, comorbidity, and anthropometric (e.g. BMI and waist-to-hip ratio) issues. Although many of our subjects were receiving various medications or had concomitant controlled medical illnesses, sensitivity analyses showed that medication and concomitant medical illnesses had little effect on the metabolic profiles between groups of subjects, and analysis of only subjects who were taking no medications gave similar results. Many of our PTSD subjects also had MDD; however, covarying for MDD did not change the results, and more importantly, the metabolite differences remained significant even when comparing only subjects without comorbid MDD. Nonetheless, we cannot rule out the possibility that comorbid MDD influenced some of the observed metabolic differences. Second, we used combat trauma-exposed veterans who did not develop PTSD as our control group, thus eliminating the contribution of prior combat trauma exposure per se to our findings. However, use of this sample as a control group may have resulted in selecting a particularly resilient control sample. Total and subcategory scores of early trauma (ETI) showed no differences between our PTSD positive and negative subjects, suggesting that the metabolomic differences we identified between groups was not due to early life trauma prior to combat. However, we did not have an assessment of prior adulthood traumas, which may have contributed to a different lifetime “trauma load” between groups. Third, and most importantly, we confirmed and validated the initial findings of our Discovery group in a wholly separate Test group, greatly limiting the Type I errors that frequently compromise metabolomics studies [148, 149].
Conclusions

We have identified several metabolites and metabolic pathways that may distinguish male combat-exposed PTSD-positive and -negative subjects. These metabolites and metabolic pathways were different from those seen in a study of male and female civilian PTSD [18], which identified seven phospholipids (four of which were phosphatidyl ethanolamines and were elevated in PTSD) two fatty acid metabolites (reduced in PTSD, and different from those identified in the current study), two nucleosides (reduced in PTSD), three bile acids and derivatives (two reduced, one increased in PTSD), one monosaccharide (reduced in PTSD) and one antioxidant (reduced in PTSD) [18], suggesting that sex and the type of trauma, and perhaps comorbid medical illnesses (other than autoimmune disease and infection) and medications, may influence metabolic features. Alternatively, the metabolomic methodologies used by these two studies differed, and may not have had overlapping analyte identification, rather than necessarily having found different metabolites. Even though the Discovery study was designed to be exploratory and hypothesis-generating, many of the strongest findings were replicated in our smaller Test group. Nevertheless, our results must be replicated in other studies with larger and more diverse samples. Similar studies in female combat trauma-exposed veterans are critically needed, as sex may moderate metabolic function [150]. PTSD appears to share some metabolomic features with cardiovascular disease [151], Alzheimer’s disease [7, 8, 152], diabetes [17], multiple sclerosis [153], and depression [2, 4]. However, the overall metabolomic profiles of these diseases differ from those in our study, so it remains to be determined whether the metabolomic changes identified in our subjects are unique or specific to PTSD. In accordance with recent Research Domain Criteria (RDoC) for neuropsychiatric illnesses [154], it is possible that the metabolomic abnormalities identified here are trans-diagnostic and may map onto specific symptoms or disease characteristics more so than to specific DSM diagnoses [155]. Therapies targeting some of these apparently dysregulated metabolic pathways or perhaps targeting mitochondrial function [71, 156] may provide treatment for some pathologic aspects of PTSD, both behavioral and somatic. The fact that the abnormalities we observed were seen in young, somatically healthy individuals with PTSD raises the possibility that they precede and presage later somatic illness, suggesting the possibility of early identification and prophylactic treatment. Indeed, it is unknown whether these abnormalities are sequelae of PTSD or, rather, are pre-existing risk factors for developing PTSD. Therapies targeting mitochondrial dysfunction have been used in animals and humans with Parkinson’s, Huntington’s and Alzheimer’s diseases [157–160]. Conversely, effective PTSD treatments such as SSRI antidepressants [161] may have beneficial effects on these dysregulated biochemical pathways, in addition to the observed behavioral manifestations of PTSD, as noted in an animal study where fluoxetine pre-treatment averted some physiological sequelae of stress (energy metabolism) [20]. Our findings raise the possibility that metabolomic differences, or the processes they reflect, underlie some of the somatic illnesses seen more commonly in PTSD, and that they may contribute to biomarker-based personalized ways of tracking and treating underlying pathophysiology in PTSD.

Supporting information

S1 File. Supplementary methods and results metabolomics in PTSD. Table A. Analytes identified in the discovery group that were not significantly different between PTSD positive and PTSD negative subjects Figure A. Barplot of metabolite validation success fraction over 1000 permutations of discovery and test groups. (DOCX)
S1 Dataset. Metabolomics PTSD database PLoS One. This file contains the primary demographic and metabolomic data reported in this manuscript. (XLSX)

Acknowledgments

We thank other members of The PTSD Systems Biology Consortium (Kerry Ressler, McLean Hospital, Harvard University, Cambridge, MA; Leroy Hood, Kai Wang and Inyoul Lee, Institute for Systems Biology, Seattle; Michael Weiner, Susanne Mueller, and Norbert Shuff, University of California, San Francisco, San Francisco, CA for ongoing discussions, and thank Elizabeth Morin-Kensicki and Janice Jones of Metabolon, Inc. for performing the initial analyses.

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