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TITLE: Metabolomics: A Window for Understanding Long-Term Physical Consequences of Disturbed Sleep and Hypothalamic-Pituitary-Adrenal Function in Posttraumatic Stress

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14. ABSTRACT

Post-traumatic stress (PTS) is a common psychiatric condition that may result after combat exposure and can have a profound effect on sleep and physical health conditions, such as metabolic syndrome. Sleep disturbances may lead to alterations in stress response hormones of the hypothalamic-pituitary-adrenal (HPA) axis that may increase metabolic risk. Women may be at particularly high risk for these health concerns, given an increased prevalence of PTS and metabolic conditions in women compared to men. The purpose of this study is to identify biological mechanisms using a broad-based study of metabolomics that may explain differences in PTS, sleep disturbances, and metabolic risk in men and women. This broad approach can reveal circulating small molecules that affect cell and physiological function and will be used to identify biochemical pathways involved in PTS, sleep disturbances, and health. Metabolomic analysis will be performed on pre-collected plasma samples from a study that had a two-group cross-sectional design in which main comparisons were with medically healthy medication-free male and pre-menopausal female subjects with chronic PTS (N= 44) and trauma-exposed, age-matched controls (N= 44). Previously collected measures, including sleep EEG and metabolic markers (e.g., fasting glucose, insulin response to oral glucose tolerance test (OGTT)), fasting lipids, and leptin, will also be examined.

15. SUBJECT TERMS
Adrenocorticotropic hormone; Lipids; Hypothalamic-Pituitary-Adrenal; Kynurenine; Metabolomics; Neurosteroids; Posttraumatic Stress; Polyunsaturated Fatty Acids; Sex Differences; Sleep

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1. INTRODUCTION:

Post-traumatic stress (PTS) results in recurrent nightmares and sleep continuity disturbances. PTS can have a profound effect on physical health and has been associated with increased inflammation, metabolic syndrome, gastrointestinal illness, and even early mortality. Delta sleep disturbances that result in impaired glucocorticoid regulation may increase risk for several of these chronic health conditions, since glucocorticoids regulate metabolism of proteins, carbohydrates, and lipids including processes such as gluconeogenesis (protein to carbohydrate conversion) and redistribution of fat. Furthermore, potential sexual dimorphisms in risk for both PTS and stress-related chronic health conditions could provide clues to sex-specific pathways such as neurosteroids that interact with hypothalamic pituitary adrenal axis (HPA) function that remain unexplored. In addition, specific fatty acid/amino acid metabolites can render PTS patients more susceptible to neuronal excitability, signal transduction, and inflammation. Characterizing the differential expression of metabolites in PTS compared to non-PTS states can reveal the multiple biochemical pathways involved in the pathogenesis and manifestation of symptoms, pointing to a range of molecular changes underlying PTS, sleep disturbances, and health. Our aim was to ascertain the neurosteroid (including glucocorticoid), primary amino acid and lipid metabolite profiles in pre-collected plasma of male and female patients with PTS and healthy controls. We predicted that: 1) There would be a sex-by-PTS status interaction of sex-specific neurosteroid metabolite intermediates which are increased in women versus men that would be the most altered in female PTS patients and would be associated with an exacerbation of HPA axis dysfunction; 2) PTS patients relative to controls would have higher levels of peripheral lipid and amino acid metabolites that are known to traverse the blood-brain barrier and negatively affect sleep and health.

2. KEYWORDS:

Adrenocorticotropic hormone; Lipids; Hypothalamic-Pituitary-Adrenal; Kynurenine; Metabolomics; Neurosteroids; Posttraumatic Stress; Polyunsaturated Fatty Acids; Sex Differences; Sleep

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Study Goals	Timeline (months)	Percentage Complete
1. Study Start Up and Approvals	1-3	100%
2. Coordinate Study Staff for Sample Analysis	1-6	100%
3. Assay Biological Samples	7-30	100%
4. Data Analysis	18-35	100%
5. Finalize study requirements, prepare for future funding, and dissemination of findings	31-42	100%

What was accomplished under these goals?

Major Activities:

The study started on September 30th 2016. Several delays occurred due to equipment malfunction, lab availability and lab closures due to COVID-19. The study obtained a no-cost extension to 3/30/2021 to accommodate these delays. This report describes accomplishments to date, including pre-spending activities. All study start-up activities were completed on schedule, including regulatory paperwork and approvals and hiring and coordination of study staff for sample analysis. Biological samples were organized, procedures for sample shipping and receiving was developed, a tracking system was created, and biological samples were shipped for processing. Three metabolite panels have been assayed, including primary amino acid metabolites, steroids and complex lipids. Initial group analyses and specialized analyses (e.g., enrichment analysis with subsequent regression analyses) were performed to identify the subgroups of amino acid, steroid, and lipid metabolites that significantly differ by group with follow up on metabolic pathway analysis.

Our data analyses using chemical set enrichment analysis found sex-specific alterations in primary metabolites in posttraumatic stress and disturbed sleep (Aim 2). No differences were found in most sex steroids, with the exception of testosterone (Aim 2). This data was accepted for publication in *Clinical and Translational Medicine* (see appendix). Additionally, further analyses indicated sex differences in lipid metabolites were associated with PTSD (Aim 2). This data was presented at the American College of Neuropsychopharmacology 57th Annual Meeting; 2018 December, Hollywood, FL. And December 8-11, 2019, Orlando, FL, The Sex Differences, Dimorphisms, Divergences: Impact on brain and behavior in health and disease meeting, Sicily, Italy, May 2019; and the 75th Annual Meeting for the Society for Biological Psychiatry, April 30 - May 2, 2020, New York, NY. (see appendix). A manuscript on the lipids findings is in preparation.

This project also yielded several secondary manuscripts (see reportable outcomes). These topics examined include 1) alterations in sleep and HPA axis responses to metyrapone in PTS, published in *Psychosomatic Medicine; 2)* alterations in overnight levels of pro-inflammatory cytokines in men and women with posttraumatic stress disorder, published in *Psychoneuroendocrinology.*

Results from this study also formed the basis for a successful Discovery Award funded by the Department of Defense, Peer Reviewed Medical Research Program (PRMRP). This follow up study will examine the link between PTS and cardiovascular disease (CVD) risk, sex differences in this link, and the impact on women in particular. Autonomic, HPA axis, endothelial, and inflammatory alterations that are associated with PTS increase risk for cardiovascular events. A longitudinal assessment over the course of the menstrual cycle would be needed to confirm potential sex differences in lipid metabolism in PTS and the role of sex steroids as they fluctuate across various menstrual phases. Linking putative novel biomarkers with cardiovascular reactivity in response to PTS-specific stress challenges and traditional measures of CVD risk will help establish their utility with relevance to disease states. The identification of alterations in metabolites with a metabolomics approach would allow for inferences to be made regarding the activity of enzymes involved in the synthesis and regulation of lipid metabolism that may account for sex dimorphisms in CVD risk in individuals with PTS.

Specific Objectives:

HYPOTHESES. 1) There will be a sex-by-PTS status interaction of sex-specific neurosteroid metabolite intermediates which are increased in women versus men that will be the most elevated in female PTS patients and will be associated with an exacerbation of HPA axis dysfunction. 2) PTS patients relative to controls will have higher levels of peripheral lipid and amino acid metabolites that are known to traverse the blood-brain barrier and will negatively affect sleep and health.

SPECIFIC AIMS. This proposal examines the metabolomic profile associated with glucocorticoid regulation mediating sleep and metabolic disturbances in pre-collected blood samples drawn from men and women with chronic PTS and trauma-exposed healthy controls. Women with PTS may be at particularly heightened risk for health consequences, given greater prevalence of inflammatory, metabolic, and functional GI disorders that are more likely to occur in women. Sexually dimorphic pathways implicated in PTS include noradrenergic, HPA, serotonergic, and glutamatergic systems. Therapeutics that act on these systems are being investigated in PTS patients, but other physiological changes such as those in metabolites have not been examined. The identification of alterations in glucocorticoid metabolites would allow for inferences to be made regarding the activity of enzymes involved in the synthesis and regulation of glucocorticoid metabolism that may affect PTS, sleep, and metabolic function and account for sex dimorphisms. Furthermore, the roles of steroid metabolites, including sex hormone metabolites that may affect glucocorticoid regulation remain unknown. In the proposed study, metabolomic assays were performed on pre-collected plasma samples addressing two specific aims:

Aim 1. **Ascertain the neurosteroid (including glucocorticoid) metabolite profile in plasma of male and female patients with PTS, and in healthy controls**.

Aim 2. **Ascertain the primary amino acid and lipid metabolite profiles in plasma of male and female PTS patients, and in healthy controls**.
The subset of metabolites that were different between PTS vs. control and male vs. female were the focus of more detailed pathway analyses. Alterations in levels of these PTS and/or sex-specific metabolites were correlated to pre-collected measures of sleep EEG, triglycerides, blood glucose, and body fat content.

Significant Results:

We found that women demonstrate more primary metabolite disturbances than men with similar posttraumatic stress disorder (PTSD) severity; a decrease in tryptophan metabolites indoles might be due to gut microbiota dysbiosis. Chemical Set Enrichment Analysis (11) of primary metabolites identified seven and two metabolite nodes altered in women and men with PTSD, respectively, compared with sex-matched controls (Fig. 1A-B). Each node contained two or more metabolites; men and women with PTSD did not share any primary metabolites (Fig. 1A-B). Since PTSD symptom presentation is highly variable between individuals,(12) and, in our cohort, women had significantly greater PCL-C scores than men (Table S1 in appendix), we reasoned that individual PCL measures may associate differently with metabolites (Fig. 1C and Table S2 (in appendix). Serine levels were lower in women with PTSD,

whereas glycine levels negatively associated with cluster D symptoms on the PCL, suggesting that with more hyperarousal, glycine levels decreased in women, but not men (Fig. 1C).
Serine, a neurotransmitter, serves as a precursor for the synthesis of glycine, cysteine, and 2-aminobutyric acid, a butyrate, and is synthesized directly from glucose (Fig. 2A). In the human myocardium, 2-aminobutyric acid increases glutathione levels via AMPK activation to protect against oxidative stress.(13) An individual's physiological and metabolic state alters glucose metabolism and generation of non-essential amino acids. Serine and glycine shuttle between the glia and neurons where glycine induces release of serine, a coagonist for NMDA receptors; together they regulate long-term potentiation(14) and are critical for the consolidation of extinction of previously conditioned fear memories.(15) Our data suggests that alterations in subsets of metabolites could be protective in PTSD (Table S2 in appendix). Changes in metabolite levels in combat veterans with PTSD are reported,(16, 17) but the contribution of sleep or sex has not been investigated.

Sleep disturbances and quality are associated with overall poor health.(1) Both men and women with PTSD had lower total sleep time (TST) and worse self-reported sleep quality as assessed by the Pittsburgh Sleep Quality Index (PSQI) compared with controls (Fig. 2B). Delta power, a measure of deep sleep activity, decreased in PTSD and showed a significant sex difference; men had lower delta power sleep activity than women (Fig. 2B). Greater PTSD symptoms associated with lower TST in both women and men, and PSQI associated with greater PTSD symptoms in both women and men; delta power was lower in men with PTSD compared to controls, but not in women (Fig. 2C). Humans lack the ability to synthesize eight essential amino acids, including tryptophan, that must be obtained from diet and are mostly absorbed by the gut and metabolized by the resident microbiota. Tryptophan is metabolized to a myriad of biologically active compounds by four different pathways (serotonin, tryptamines, kynurenine, and indoles). TST accounted for alterations in all metabolite nodes in men and two of the six metabolite nodes in women (Fig. 2D, 3A, and Table S2 in appendix). When PSQI was used as a confounder, new, non-overlapping nodes were found to be significant in women and men; indoles, hexose and amino acid nodes were decreased in women (Fig. 2D). Delta power sleep accounted for 50% of the nodes in women (Table S2 in appendix). Interestingly, higher testosterone levels associated with lower delta power in men (Fig. 3C).

Plasma insulin, albumin, and amino acids levels together modulate transport of free tryptophan to the brain.(18) In our cohort, no difference in insulin levels were seen in women; plasma albumin levels did not differ between controls and PTSD in either sex. Insulin levels were significantly elevated in men with PTSD compared with controls (Fig. 3D-E). Tryptophan levels associated negatively with insulin levels in women (Fig. 3F) with concomitant increases in levels of several amino acids that compete with a tryptophan carrier for influx into the brain. Increases in palmitoleic acid levels may be compensatory, more so in women than in men, which can potentially displace albumin from tryptophan in order to generate free tryptophan.

Tryptophan is absorbed in the gut and converted by the actions of gut microbiota such as *Lactobacillus* to indoles (Fig. 4). While the role of serotonin in mood disorders, anxiety, and other disorders is well known, we show here for the first time that the indole metabolite, indole-3-propionic acid, is decreased in women with PTSD. Poorer sleep quality was further associated with decreased levels of two additional indoles, indole-3-lactic and acetic acids; the indoles regulate immune and gut barrier functions (Fig. 4B), and their decreased levels might contribute to altered immune and barrier function

in women. Diets rich in butyrates that support growth of beneficial gut bacteria such as *Lactobacillus* may serve as non-invasive interventions, especially for women.

Surprisingly, sex steroid metabolites (estrogens, testosterone, progesterone) did not differ between controls and PTSD in either men or women (Table S3 in appendix), nor did sex steroids associate with PTSD. Testosterone levels were ~10-fold higher in men (Table S3 in appendix).

Figure 1. Sex differences in primary metabolites in PTSD.

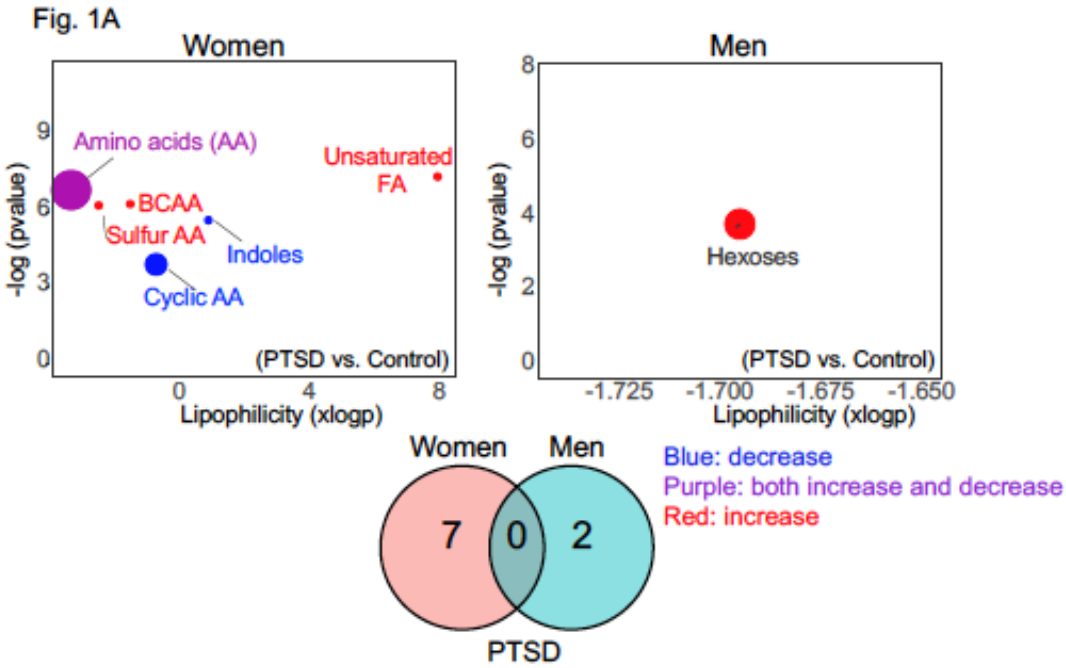


Figure 1. Sex differences in primary metabolites in PTSD. (A) Chemical Set Enrichment (ChemRICH) analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI and age. ChemRICH is a statistical enrichment approach based on chemical structure similarity/chemical ontologies and is an alternative to pathway analysis that relies on limited biochemical knowledge annotations. It yields study-specific, non-overlapping sets of all identified metabolites. Since ChemRICH sets have a self-contained size, thus p-values do not rely on the size of the background database. Blue nodes contain metabolite clusters that were decreased, purple nodes contain metabolites that were both increased or decreased, and red nodes contain metabolites that were increased in PTSD vs. control individuals. Branched-chain and sulfur-containing amino acids, and unsaturated fatty acids were increased, indoles and cyclic amino acids were decreased, whereas the non-polar amino acid node contained metabolites that either increased or decreased in women with PTSD compared to controls. A Venn diagram showing seven nodes were specific to women with PTSD and two nodes were specific to men with PTSD compared with controls.

Figure 1B. Volcano plots of specific metabolites within each node in men and women with PTSD after correcting for type 1 error and adjusting for BMI and age.

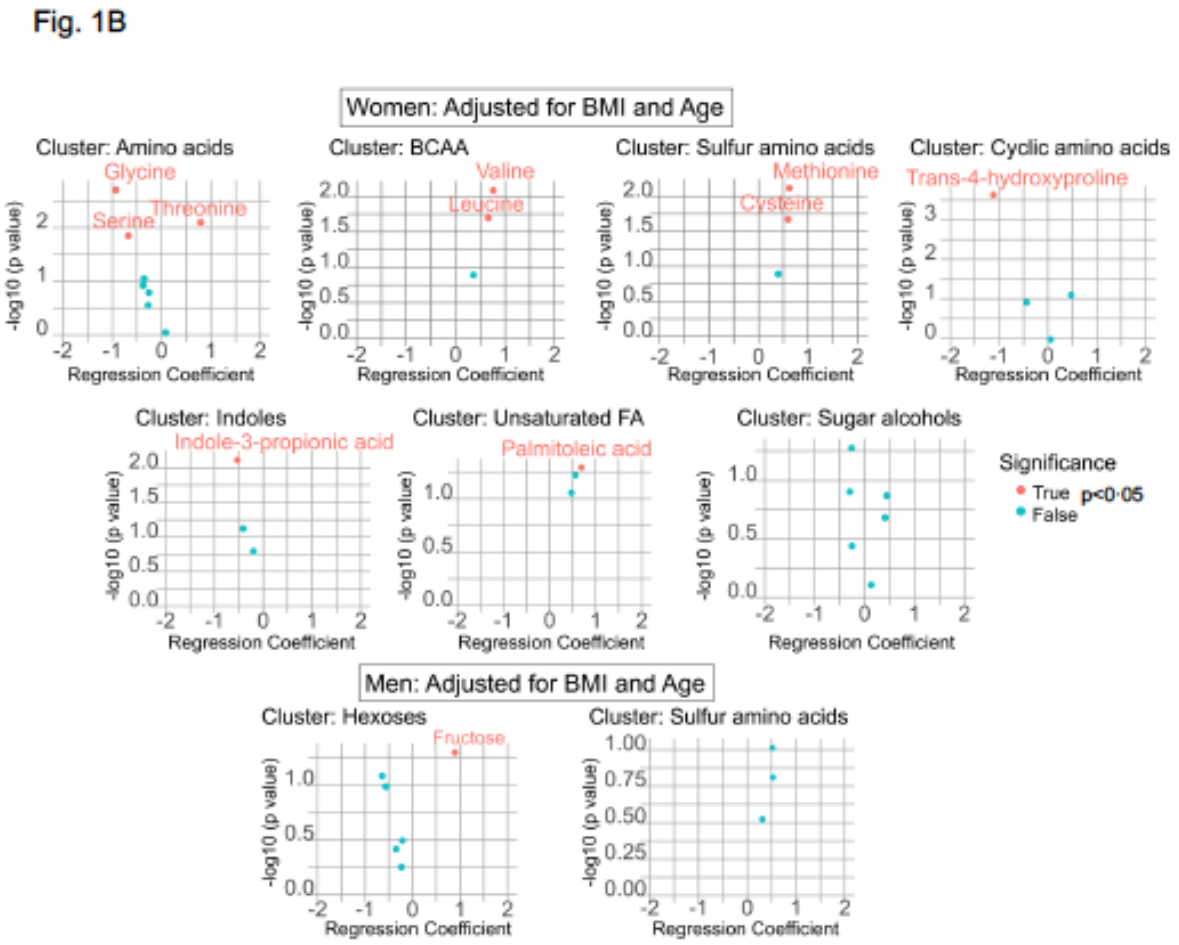


Figure 1C. Box plots of specific amino acids with the seven nodes that differed between women and two nodes in men with PTSD compared to controls shown in Fig. 1A.

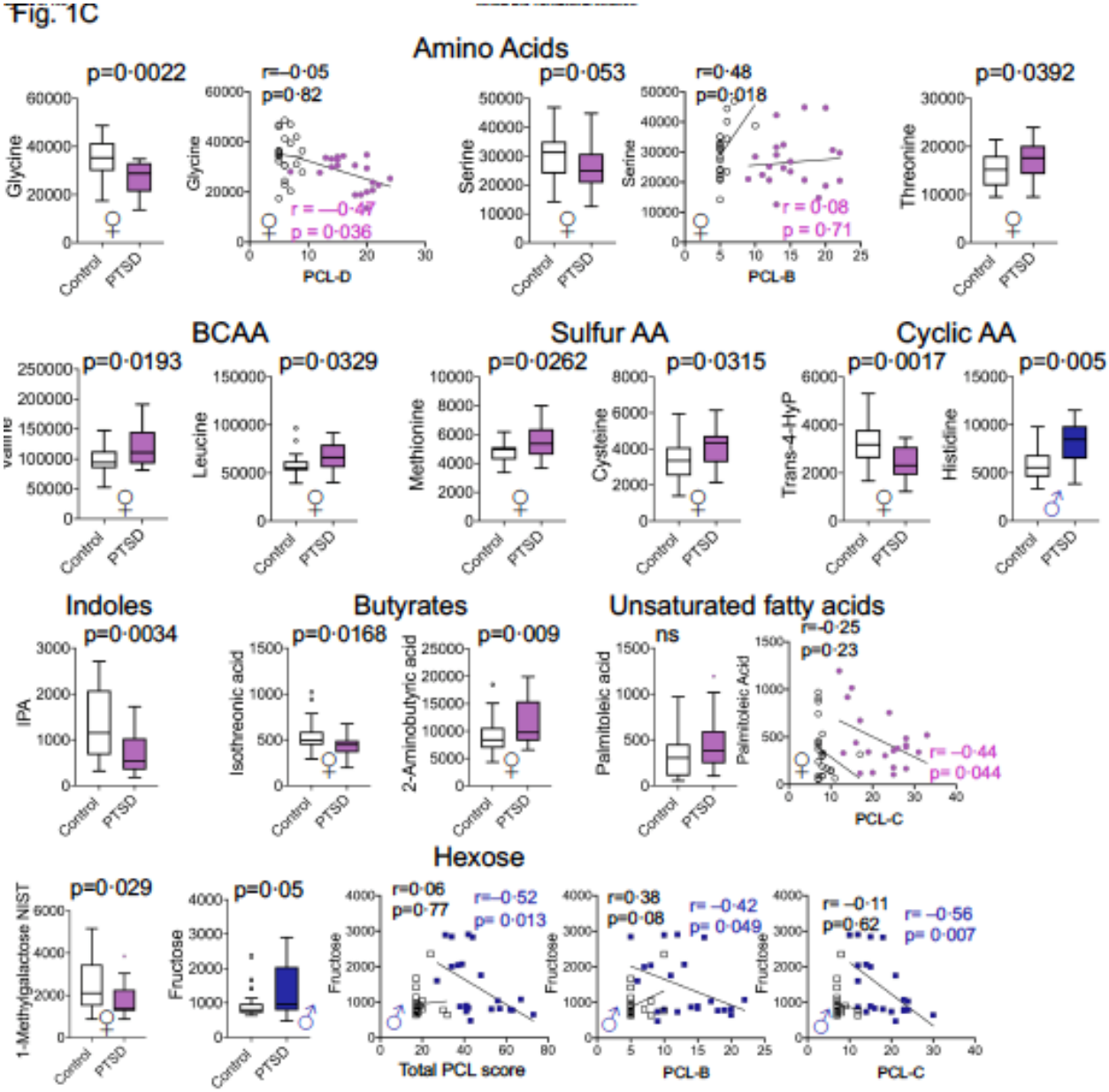


Figure 1C. Box plots of specific amino acids with the seven nodes that differed between women and two nodes in men with PTSD compared to controls shown in Fig. 1A. Glycine levels associated negatively with PCL cluster D in women with PTSD (r = –0·47, p = 0·036), but not controls, whereas association of serine level was lost in women with PTSD.

Cysteine and 2-AB levels were elevated in PTSD compared with controls, but did not show any significant association with specific clusters of PTSD symptoms. Fructose levels increased in men with PTSD vs. controls, but fructose levels associated negatively with PCL scores. Box plot analysis: Mann-Whitney and p<0·05 considered significant.

Figure 2. PTSD-specific alterations in amino acids with respect to their biosynthesis pathway in humans from glucose.

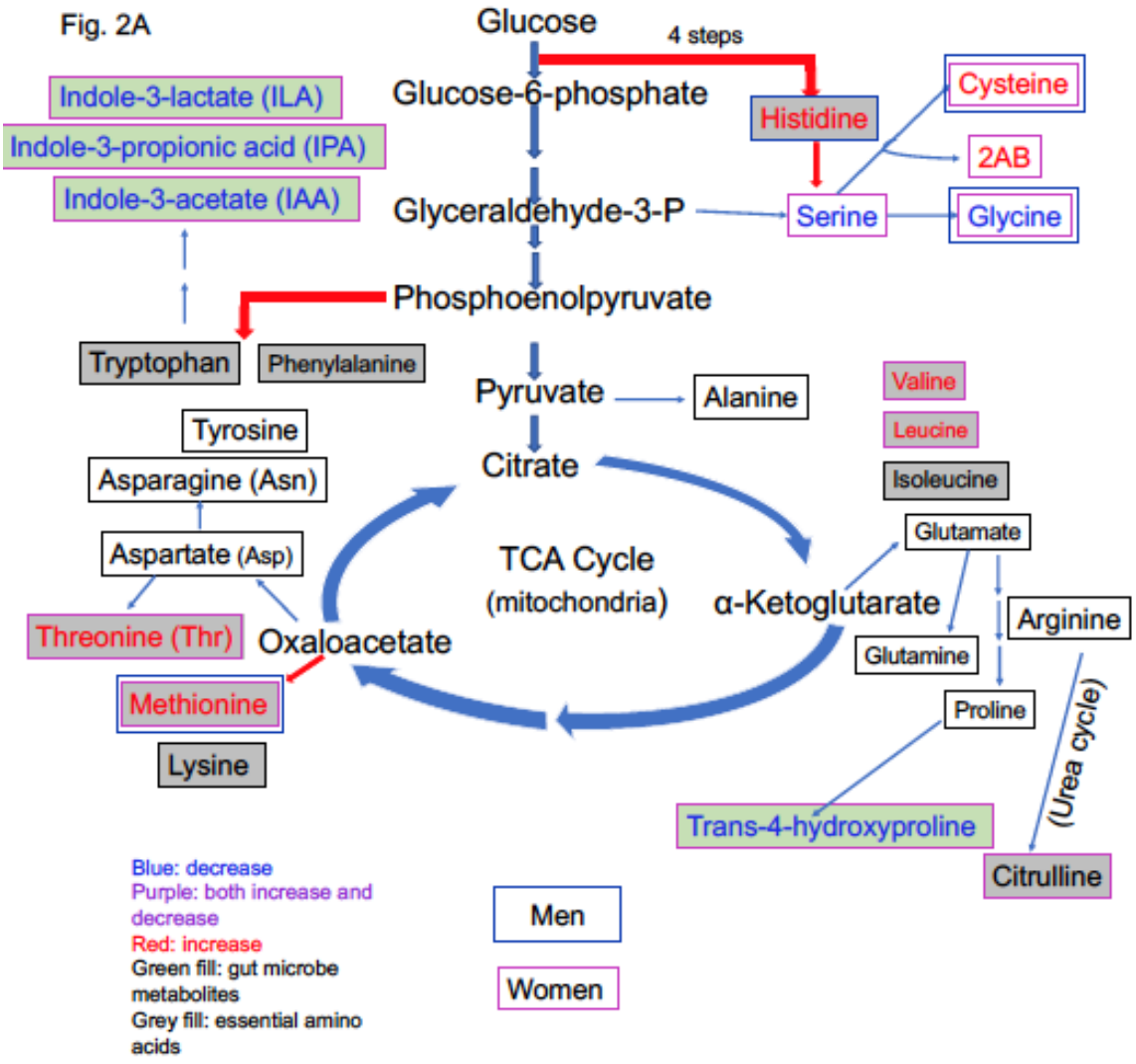


Figure 2. PTSD-specific alterations in amino acids with respect to their biosynthesis pathway in humans from glucose. (Figure 2A) Essential amino acids cannot be synthesized and must be obtained from diet, whereas non-essential amino acids can be synthesized from glucose as it enters the tricarboxylic cycle. Essential amino acids are shown in grey boxes, and those metabolites synthesized as a by-product of gut microbiome are shown in green boxes. Specific amino acids that were increased are shown in red, decreased in blue (women-specific outlined in pink and men-specific in blue).

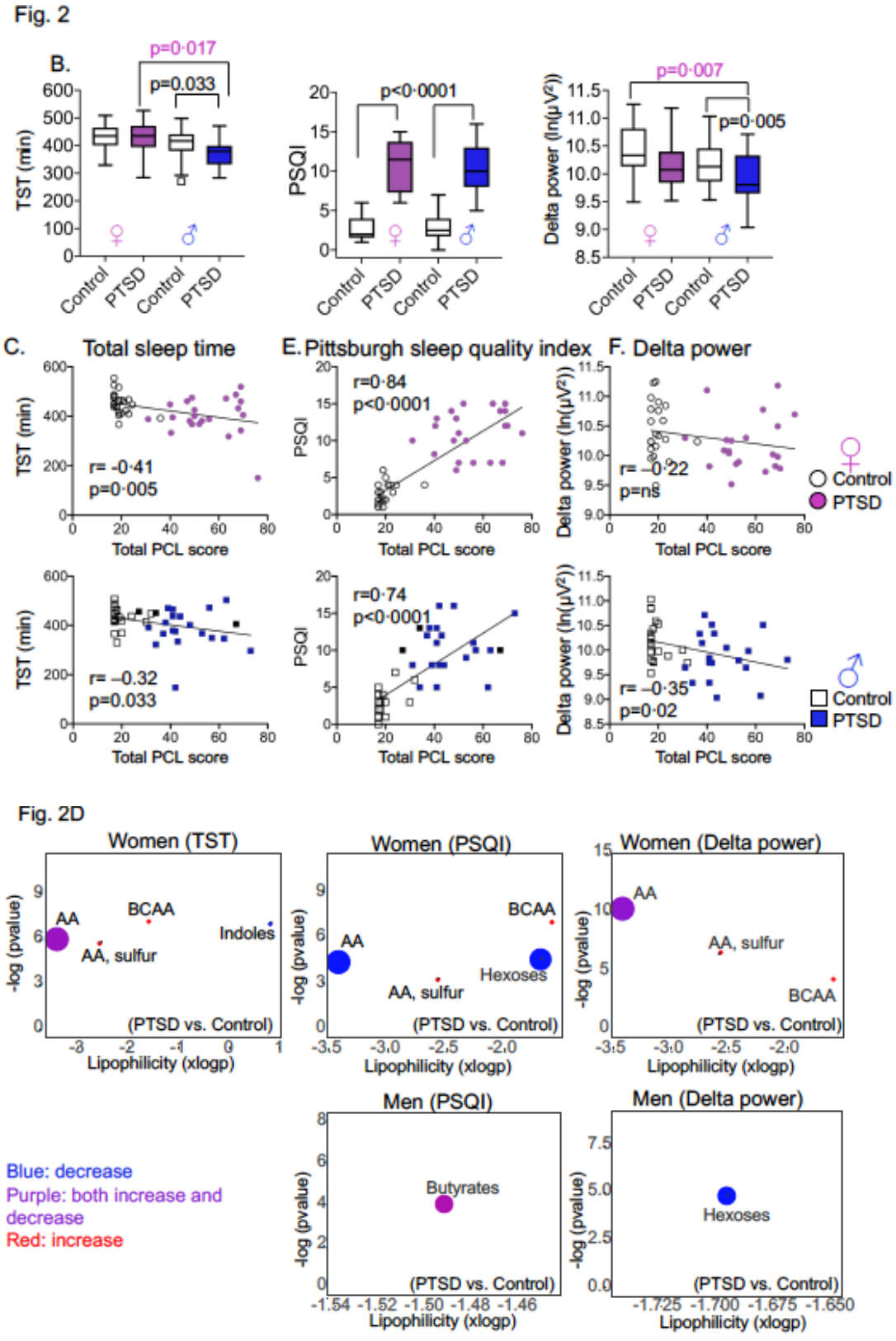
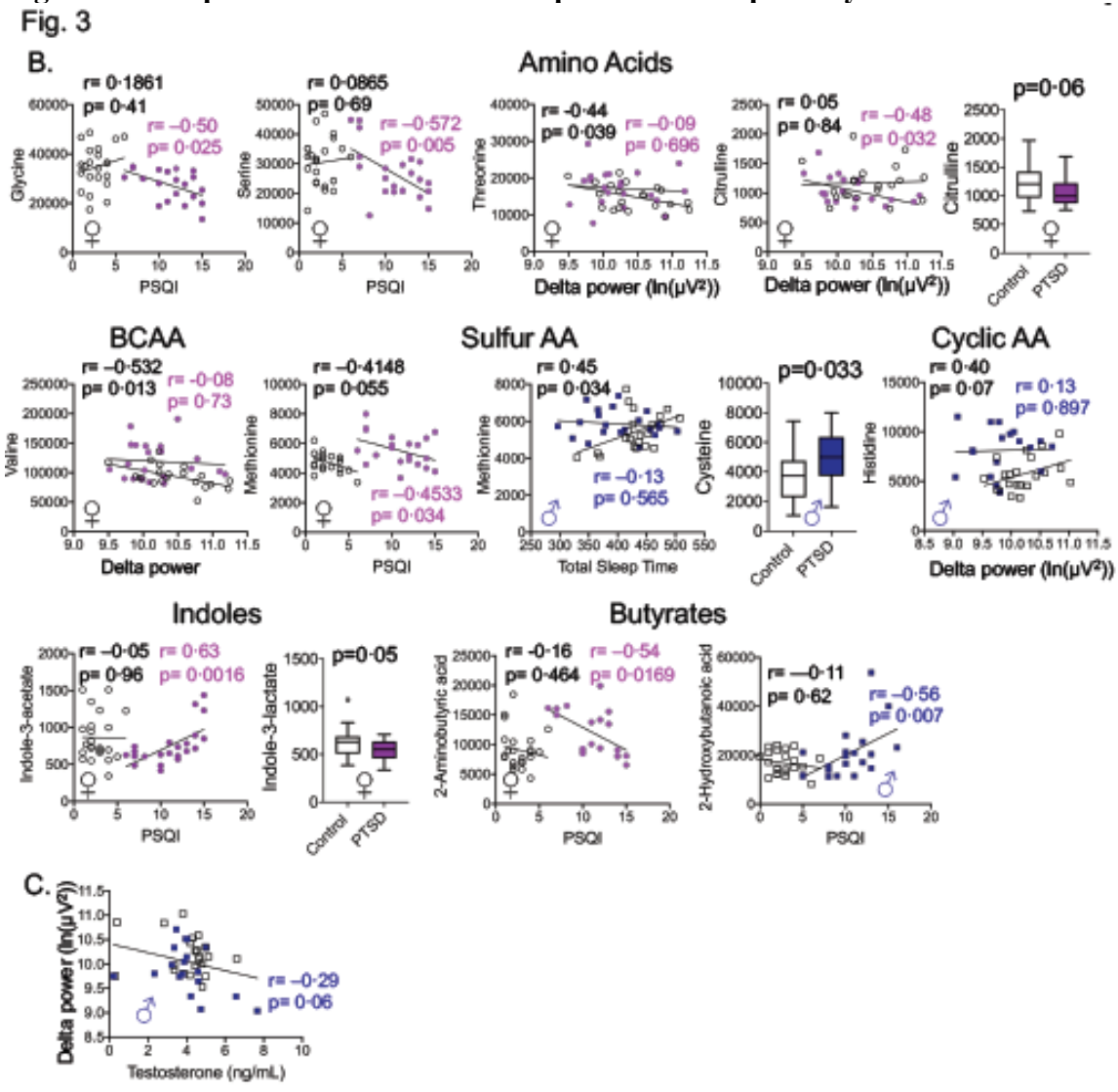


Figure 2 (B). Sex-specific disturbances in sleep measures. Box plots showing sex- and/or PTSD-specific alterations in Total sleep time (in minutes) decreased; Two-way ANOVA: Sex: ns; PTSD: p= 0·004; Sex X PTSD: ns. Sleep quality worsened, Two-way ANOVA: Sex: ns;

PTSD: p< 0·0001; Sex X PTSD: ns, and log-transformed delta power sleep was lower in people with PTSD vs controls, Two-way ANOVA: Sex: p= 0·007; PTSD: p= 0·005; Sex X PTSD: ns. Greater PTSD symptoms (reflected by the total PCL score) negatively associated with TST (r= –0·41, p=0·005 and r= –0·32, p=0·033, women and men, respectively), and PSQI was positively associated with greater PTSD symptoms (r=0·84 and r=0·74, p<0·001, women and men, respectively). Delta power negatively associated with PTSD (r= –0·35, p=0·02) in men alone. (C) Linear regression showing association of PCL scores with three different measures of sleep. (D) Sex differences in primary metabolites after accounting for sleep measures. ChemRich analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI, age, and one of the three sleep measures shown (TST (min), PSQI, or log-transformed delta power (ln(μV2)). Blue nodes contain metabolite clusters that are decreased, purple nodes contain metabolites that are both increased or decreased, and red nodes contain metabolites that are increased in PTSD vs control individuals.

Figure 3. Sex-specific contribution of sleep variables on primary metabolites.



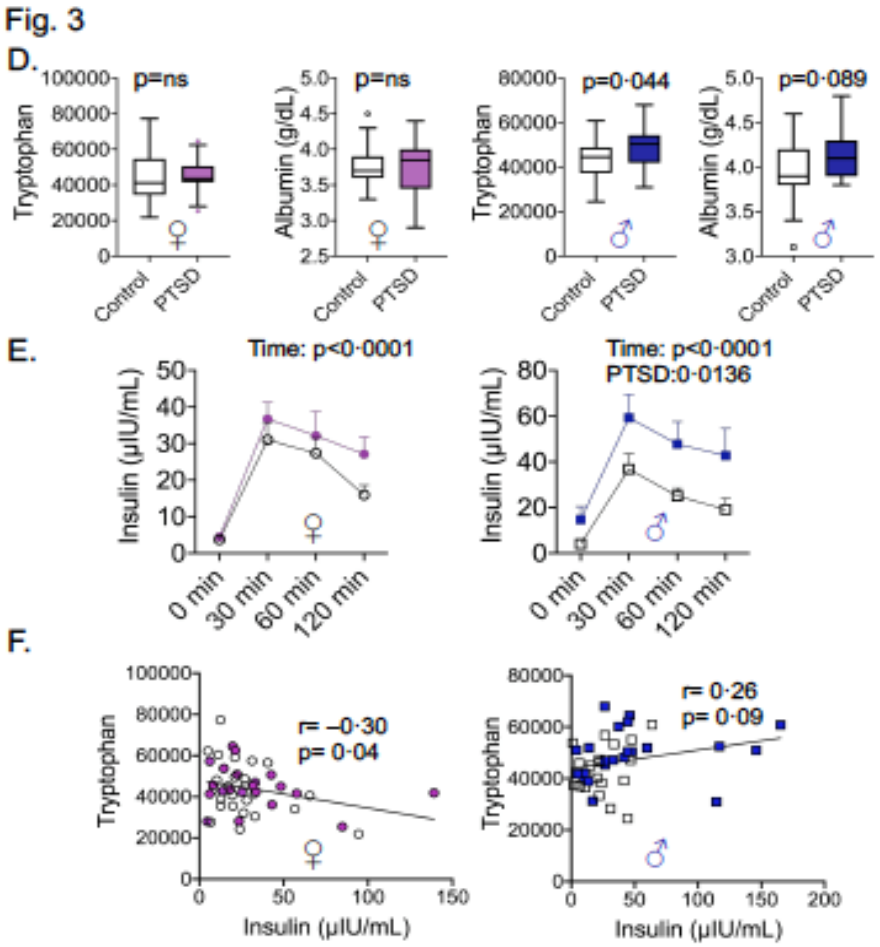


Figure 3. Sex-specific contribution of sleep variables on primary metabolites. (A) Venn diagrams to visualize significant metabolites While adjusting for one sleep variable at a time. (B) Linear regression and box plots of various amino acids with sleep variables. Box plots of specific amino acids with individual nodes that differed between women and men with PTSD compared to controls. (C) Log-transformed delta power (ln(µV2) associated negatively with testosterone in men. (D) Changes in insulin and tryptophan levels in women and men with PTSD. Box plots of tryptophan and albumin levels in women and men. No significant differences were seen in tryptophan and albumin levels in women with PTSD compared with controls, whereas tryptophan levels were significantly elevated in men with PTSD compared with controls ($p = 0{\cdot}044$; Mann-Whitney). (E) Blood insulin levels were determined after an oral glucose challenge at various times shown. Mixed-effect analysis showed that insulin levels changed with time ($p<0{\cdot}0001$) in women and men, but only differed between PTSD and controls in men ($p=0{\cdot}0136$). (F) Tryptophan levels correlated negatively with insulin levels at 60 min in women ($r= –0{\cdot}30$, $p=0{\cdot}04$), but positively in men, although the relationship did not reach statistical significance.

Figure 4. Sex-specific alterations in the tryptophan metabolism pathway in PTSD.

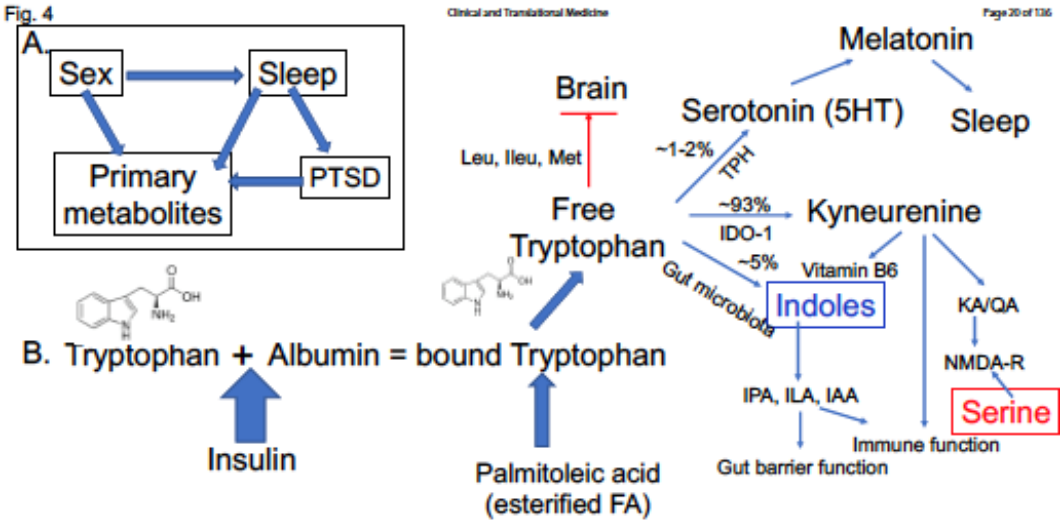


Figure 4. Sex-specific alterations in the tryptophan metabolism pathway in PTSD. (A) Sex, sleep, and PTSD all alter primary metabolites. (B) Albumin-bound tryptophan is present in circulation and dynamic increases in insulin promote binding of albumin to tryptophan, whereas esterified fatty acids can displace tryptophan from albumin. Free tryptophan is then transported to the brain by a transport carrier. Several amino acids, such as leucine, valine etc. compete with tryptophan for binding to the transport carrier, which can decrease influx of free tryptophan into the brain. Reduced free tryptophan levels in the brain can influence production of serotonin and melatonin, affecting brain function and sleep. In the gut, tryptophan is converted to indoles by the action of microbes such as Lactobacillus; these indoles have protective effect on gut barrier and immune functions. Serine can serve as NMDA receptor agonist and alter neuronal function. Thus, disturbances at multiple levels in tryptophan pathway may contribute to pathogenesis of PTSD.

Lipid metabolite findings. The ChemRICH analyses identified 11 lipid nodes that were significantly increased in PTS vs. healthy controls; of these, 4 nodes were specific to women, 2 nodes to men and 5 shared between men and women with PTS (Fig 5). Ten of the 11 lipid ChemRICH plot clusters for PTS were also increased in individuals who reported poorer sleep quality on the PSQI. Saturated phosphatidylcholines (cluster of 7) were only associated with PTS symptoms, whereas unsaturated fatty acids (cluster of 15) were only associated with poorer sleep quality. Interestingly, 4 distinct lipid clusters that were highly enriched in PTS and in those with self-reported poorer sleep quality were highly downregulated in people with greater total sleep time as measured by actigraphy. It is unknown whether alterations in these lipid metabolite clusters are the driver or the consequence of disturbed sleep in PTS. Furthermore, peripheral lipid metabolites that are known to traverse the blood-brain barrier and confer risk for cardiovascular disease (CVD) may place individuals with PTS at heightened risk for these diseases. In dividing the sample by sex, the majority of lipid alterations in PTS and disturbed sleep were found in men, but not in women. While sex specific neurosteroids have previously been shown to affect sex differences in lipid metabolism, we encountered specific challenges in accurately quantifying testosterone, estrogen and progesterone metabolites, but not glucocorticoids, or testosterone itself. Further characterizing sex differences in metabolic changes that are associated with sleep will further our understanding of the pathophysiology of PTS and may ultimately lead to better-targeted, more effective treatment.

Figure 5.

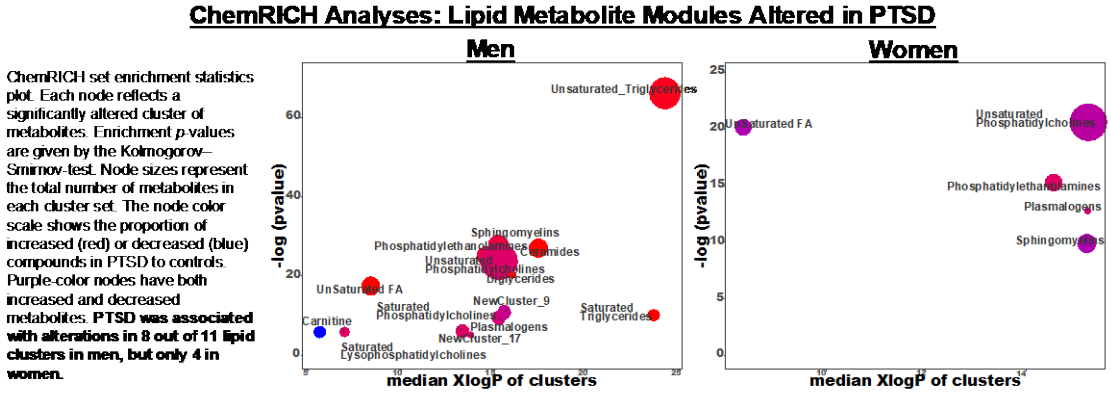
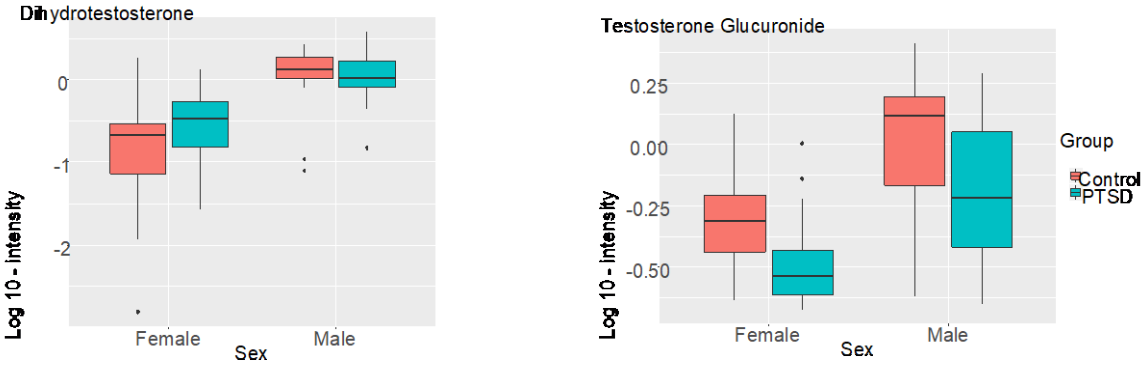


Figure 5. ChemRICH set enrichment statistics plot. Each node reflects a significantly altered cluster of metabolites. Enrichment *p*-values are given by the Kolmogorov–Smirnov-test. Node sizes represent the total number of metabolites in each cluster set. The node color scale shows the proportion of increased (red) or decreased (blue) compounds in PTSD to controls. Purple-color nodes have both increased and decreased metabolites. **PTSD was associated with alterations in 8 out of 11 lipid clusters in men, but only 4 in women.**

Multiple linear regression analyses were performed within each sex controlling for sex steroids that were found to be altered in PTSD to determine their potential influence on lipid alterations. Alteration in testosterone metabolites were associated with PTSD (Figure 6). Among women, 381 lipids (vs. 238) were significantly associated with PTSD when controlling for testosterone glucuronide. Among men, 781 lipid compounds (vs 813) were significantly associated with PTSD, when controlling for testosterone glucuronide. These findings suggest that testosterone glucuronide accounted for the relationship between PTSD and multiple lipid alterations, in women in particular.

Figure 6. Alterations in testosterone metabolites in PTSD



Dihydrotestosterone was increased in women with PTSD compared to controls (Fold Change = 1.91, p<.05).

Testosterone glucuronide was decreased in both women and men with PTSD compared to controls (Fold change = .60, p < .005; Fold change = .46, p<.01).

Major Task 1: Study Start Up and Approvals	**Timeline**
Subtask 1: Prepare regulatory documents and submit for IRB approval	
Develop IRB application and other regulatory documents	Completed
Submit IRB application to UCSF IRB and obtain full committee review	Completed
Review by SFVAMC regulatory personnel	Completed
Review by HRPO	Completed
Prepare IRB reports for continuing review approvals	Annually
Milestone Achieved: IRB approval from UCSF, VA, and HRPO	Complete
Major Task 2: Coordinate Study Staff for Sample Analysis	**Timeline**
Subtask 1: Hiring and Training of Study Staff	
Coordinate with NCIRE to prepare job description and advertisement	Complete
Interview research staff candidates	Complete
Coordinate with SFVAMC for candidate approval and required trainings	Complete
Training of research staff on study procedures and biospecimen storage, shipping, and receiving	Complete
Milestone Achieved: Research staff hired and trained	Complete
Subtask 2: Coordinate with laboratory personnel for sample shipments	
Contact staff at receiving laboratories	Complete
Develop procedures manual for sample shipping and receiving	Complete
Develop sample tracking system	Complete
Schedule batched shipments	Complete
Milestone Achieved: Sample shipment protocol established	Complete
Subtask 3: Build database for incoming data	
Work with Data Manager to establish data extraction protocol and build database	Complete
Establish logistical plan for data quality check	Complete
Milestone Achieved: Database built	Complete
Major Task 3: Assay Biological Samples	**Timeline**
Subtask 1: Ship stored samples to the receiving laboratory and acquire data	
Package and ship stored samples to UC Davis	Complete
Milestone Achieved: 1st batch of samples shipped for assay	Complete
Milestone Achieved: Final batch of samples shipped for assay	Complete
Subtask 2: Receive data from laboratory	
Milestone Achieved: All data acquired	Complete
Milestone Achieved: All Assays complete	Complete

Major Task 4: Data Analysis	**Timeline**
Subtask 1: Enter data and maintain database	
Perform quality checks on incoming data	Complete
Enter all data and maintain database	Complete
Subtask 2: Aim 1: To ascertain the neurosteroid (including glucocorticoid) metabolite profile in plasma of male and female patients with PTSD, and in healthy controls	
Clean and process incoming data and prepare for analysis	Complete
Work with Biostatistician to conduct analyses	Complete
Share output and findings with co-investigators	Complete
Milestone Achieved: Aim 1	Complete
Subtask 3: Aim 2: To ascertain the primary amino acid and lipid metabolite profiles in plasma of male and female PTSD patients, and in healthy controls	
Clean and process incoming data and prepare for analysis	Complete
Work with Biostatistician to conduct analyses	Complete
Share output and findings with co-investigators	Complete
Perform the integration of the metabolite data with the health outcome data that we have collected, including measures of HPA axis function, sleep EEG, triglycerides, blood glucose, and body fat content.	Complete
Milestone Achieved: Aim 2 addressed	Complete
Milestone Achieved: Data analysis complete	Complete
Subtask 4: Share output and findings with co-investigators and with the greater community	
Conduct the background research to interpret the results and the implications of these findings and to write manuscripts to disseminate the findings.	Complete
Dissemination of findings (abstracts, presentation, publications, DOD)	Complete
Milestone Achieved: Report results from data analyses	Complete
Milestone Achieved: Characterize the metabolomic profile associated with glucocorticoid regulation mediating sleep and metabolic disturbances associated with PTS	Complete
Milestone Achieved: Identify specific metabolites associated with PTS for future clinical trial	Complete
Perform the integration of the metabolite data with the health outcome data that we have collected, including measures of HPA axis function, sleep EEG, triglycerides, blood glucose, and body fat content.	Complete
Major Task 5: Finalize study requirements, prepare for future funding, and dissemination of findings	**Timeline**
Subtask 1: Dissemination of findings (abstracts, presentation, publications, DOD)	Complete
Subtask 2: Prepare grant application for DOD or VA Merit Award funding for a clinical trial based on study findings	Complete
Subtask 3: Complete final report.	Complete
Milestone Achieved: Report results from data analyses	Complete
Milestone Achieved: Submit grant proposal for clinical trial to examine changes in specific metabolites and related inflammatory and metabolic processes on limbic responses in an fMRI paradigm.	Complete

Conclusions

Our data analyses on primary metabolites indicated that the there was no overlap in PTSD-related alterations in men and women in any primary metabolite nodes or individual amino acids within those nodes, and associated pathways. A balance between levels of essential amino acids, insulin, and albumin determines availability and transport of free tryptophan to the brain, which might in turn influence production of serotonin and melatonin, and distinct cellular pathway operate within functional clusters in men and women (Fig.4).

Our lipidomics analyses indicated that women with PTSD had fewer lipid alterations than men. Elevated triglycerides found in men replicate previous findings of dyslipidemia and elevated cardiovascular risk factors in PTSD. Elevated sphingomyelin is consistent with previous reports of altered sphingomyelin metabolism in PTSD and depressed samples. Sphingolipids constitute a physical barrier in the brain and provide key functions including cell signaling, has a role in interleukin-1 release from brain astrocytes and hypothalamic-pituitary adrenal axis function and has been associated with cardiovascular disease. As such, alterations in these lipid metabolites may have a role in neuroendocrine and immune alterations in PTSD and associated cardiometabolic health conditions. Testosterone glucuronide, a downstream metabolite of testosterone, accounted for the relationship between PTSD and lipid metabolites, especially among women. These findings are consistent with previous studies reporting that low testosterone was associated with dyslipidemia. Although estrogens were expected to be protective in women, estrogens were not altered in PTSD and were not found to influence the relationship between PTSD and lipids. However, these findings may have been limited by the single blood draw that occurred during the early follicular phase, when estrogen and progesterone levels are low. Future studies should consider studying women at other menstrual phases and after menopause. Testosterone-related compounds may influence the effect of PTSD on lipid function, and potentially result in sex differences in health risks in PTSD. Further characterizing sex differences in these processes will advance our understanding of the pathophysiology of PTSD, and may ultimately lead to better-targeted, more effective treatment.

We plan to continue this line of research in a newly funded DoD grant to examine sex differences in cardiovascular risk factors by examining lipid metabolites that associate with CVD risk and PTS in women (across the menstrual cycle) relative to men and sex steroids that may modulate the relationship between CVD risk, PTS and lipid metabolism in women relative to men. Future grants are planned to examine primary metabolites and lipids metabolites prior to and after insomnia treatment.

What opportunities for training and professional development has the project provided?

The PI has participated in the following scientific meetings:
2016 American College of Neuropsychopharmacology Annual Meeting
2017 American College of Neuropsychopharmacology Annual Meeting
2017 Biological Psychiatry Annual Meeting
2018 VA PTSD Psychopharmacology Initiative
2018 VA NCPTSD Women Veteran's Health Summit
2019 American College of Neuropsychopharmacology Annual Meeting
2019 VA Research Week
2019 Sex Differences, Dimorphisms, Divergences: Impact on brain and behavior in health and disease
2020 Biological Psychiatry

How were the results disseminated to communities of interest?

There were 5 publications and 5 presentations related to this project to reach scientific communities (listed in products section).

Additionally, this material was presented to VA providers with an interest in healthcare of women veterans with PTSD:

Inslicht, S.S. Women Veterans, Traumatic Stress and Post-Military Health: Building Partnerships for Innovation: Biomarkers and Related Treatments; Women Veterans Health Summit sponsored by the Women's Health Sciences Division of the National Center for PTSD. September 25, 2018, Boston, MA.

A presentation was made to the San Francisco Veteran community at the San Francisco Presidio - Live – Dialogue event and promoted by the VA on twitter.

> Twitter: VA San Francisco @SFVAMC: Dr. Sabra Inslicht discussing how war affects our women #Veterans discussing how war affects our women #Veterans and ongoing research to help support their needs. Fear Conditioning and Fear Extinction. Presidio - Live - Dialogue: How War Changes Women, How Women Change War. @PresidioSF 7:40pm 10 May 2018

A panel discussion of sex differences in PTSD and the impact on women Veterans was conducted on Facebook Live with the Heart and Armor Foundation for Veterans Health.

> Facebook Live: Interview with John Mayer. Heart and Armor: Foundation for Veterans Health launch event. How War Affect Women; How Women Affect War. https://www.facebook.com/johnmayer/videos/thank-you-for-watching-the-heart-and-armor-launch-event-and-qa-learn-more-at-hea/1233987773422160/

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The results of this work determined sex differences in primary metabolites and lipid metabolites that were associated with PTSD and differed by sex. Future studies will be required to replicate these findings in order to validate these sex specific biomarkers of PTSD. Presently, we are continuing to pursue the impact of lipidomics findings on cardiovascular risk factors in men and women with PTSD. Specifically, we plan to examine 1) lipid metabolites that associate with CVD risk and PTS in women (across the menstrual cycle) relative to men and 2) which sex steroids modulate the relationship between CVD risk, PTS and lipid metabolism in women relative to men. Exploratory analyses will establish pathways of lipid and sex steroid alterations to stress-related cardiovascular reactivity and traditional markers of CVD risk. Alterations in levels of PTS and/or sex-specific steroid hormones and lipid metabolites will be correlated to pre-collected measures of cardiovascular stress reactivity during laboratory stress challenges incorporating threat processing tasks (e.g., fear conditioning and threat-enhanced acoustic startle tasks). We predict that lipid metabolite intermediates that are associated with PTS will be associated with greater cardiovascular reactivity (HR) and decreased HRV during laboratory stress challenges and that sex steroids will modulate these effects. Altered metabolite levels will be correlated to pre-collected measures of triglycerides, leptin**,** blood glucose levels, and body fat content.

What was the impact on other disciplines?

Our findings of sex differences in lipid metabolites indicated that women with PTSD had fewer lipid alterations than men. This findings suggests that there may be sex differences in alterations in lipids that could have a role in neuroendocrine and immune alterations in PTSD and associated cardiometabolic health conditions. This could affect healthcare delivery and suggests that men and women would benefit from tailored interventions that differ by gender.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Our study adds to research that characterizes sex differences in biological processes underlying PTSD and physical health. This work and others like it will advance our understanding of the pathophysiology of PTSD, and may ultimately lead to better-targeted, more effective treatment. More targeted treatment will benefit the health, well-being, and functioning of military service members and civilians exposed to trauma, their families, and the communities in which they live.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

The study obtained a no-cost extension to 3/30/2021. Due to COVID-19, we experienced laboratory closures at the San Francisco VA Health Care System laboratory and at West Coast Metabolomics Center. We were therefore delayed in organizing and shipping the remaining blood samples to WCMC for the final assays for steroids. WCMC is the only laboratory with the necessary equipment and technical expertise to perform the assays needed to answer the specific research aims: to ascertain the primary amino acid and lipid metabolite profiles in plasma of male and female PTS patients, and in healthy controls. We could not send the final shipment for assay until reopening of the laboratories. We then completed the final statistical analysis and finalized the study results later than expected.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report"

Presentations:

1. Inslicht, S.S. Women Veterans, Traumatic Stress and Post-Military Health: Building Partnerships for Innovation: Biomarkers and Related Treatments; Women Veterans Health Summit sponsored by the Women's Health Sciences Division of the National Center for PTSD. September 25, 2018, Boston, MA.

2. Inslicht, S.S., Roy, R., Olshen, A., Bhargava, A., Neylan, T. Lipidomics of PTSD. Poster presented at the American College of Neuropsychopharmacology 57th Annual Meeting; 2018 December, Hollywood, FL.

3. Bhargava, A., Fan, S., Lujan, C., Feihn, O., Neylan, TC, Inslicht, S.S. Lipidome Analysis in Men and Women with Posttraumatic Stress Disorder. Sex Differences, Dimorphisms, Divergences: Impact on brain and behavior in health and disease; Sicily, Italy, May 2019.

4. Inslicht S.S., Bhargava, A., Olshen A., Lujan, C., Neylan, T.C. Sex Differences in Lipid Metabolism in PTSD. Poster presented at The American College of Neuropsychopharmacology Annual Meeting, December 8-11, 2019, Orlando, FL.

5. Inslicht S.S., Bhargava, A., Olshen A., Lujan, C., Neylan, T.C. Sex Differences in Lipid Metabolism in PTSD. Poster presented at the 75th Annual Meeting for the Society for Biological Psychiatry, April 30 - May 2, 2020, New York, NY.

Publications:

1. Talbot LS, Rao MN, Cohen BE, Richards A, Inslicht SS, O'Donovan A, Maguen S, Metzler TJ, Neylan TC. Metabolic risk factors and posttraumatic stress disorder: the role of sleep in young, healthy adults. Psychosom Med. 2015 May; 77(4):383-91. PMID: 25886830. PMCID: PMC4431908

2. Inslicht, S.S., Rao, M.N., Richards, A., Gibson, C., Metzler, T.J., Neylan, T.C. Sleep and HPA Axis Responses to Metyrapone in Posttraumatic Stress Disorder. Psychoneuroendocrinology. 2017 Dec 7; 88:136-143. PMID: 29268182

3. Inslicht, S.S., Rao, M.N., Richards, A., Gibson, C., Metzler, T.J., Neylan, T.C. Sleep and HPA Axis Responses to Metyrapone in Posttraumatic Stress Disorder. Psychoneuroendocrinology. 2017 Dec 7; 88:136-143. PMID: 29268182

4. Küffer A, Straus LD, Prather AA, Inslicht SS, Richards A, Shigenaga JK, Madden E, Metzler TJ, Neylan TC, O'Donovan A. Altered overnight levels of pro-inflammatory cytokines in men and women with posttraumatic stress disorder. Psychoneuroendocrinology. 2018 Dec 05; 102:114-120. PMID: 30544002

5. Bhargava, A. Fan, S., Lujan, C., Fiehn, O.,Neylan, T., Inslicht, S.S. (accepted). Chemical set enrichment analysis: novel insights into sex-specific alterations in primary metabolites in posttraumatic stress and disturbed sleep. Clinical and Translational Medicine.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name:	**Thomas Neylan**
Project Role:	Co-Investigator
Nearest person month worked:	*1 person month*
Contribution to Project:	Dr. Neylan has extensive expertise in the biology of PTSD, sleep, metabolic function, clinical trials, and laboratory-based psychophysiological research. He provided onsite support to Dr. Inslicht on the conduction of the proposed project, interpretation of sleep and HPA axis data, and was involved in data analysis and manuscript preparation.

Name:	**Sabra Inslicht**
Project Role:	Principal Investigator
Nearest person month worked:	*1 person month*
Contribution to Project:	Dr. Inslicht has expertise in psychophysiology and the neuroendocrinology of PTSD. Dr. Inslicht assumds overall scientific and administrative responsibility for this project, ensuring that research goals are met in a timely manner with scientific integrity. She designed and implemented each phase of the research plan. She worked with the study coordinator to oversee human subjects regulatory documentation and compliance, coordination of personnel involved in this protocol, the coordination of assay completion, as well as the development of a data tracking system to manage participant information, biological samples, and assay data. She also worked with the statistician to conduct data analyses and prepared manuscripts and disseminated findings.

Name:	**Aditi Bhargava**
Project Role:	Co-Investigator
Nearest person month worked:	*1 person month*
Contribution to Project:	Dr. Bhargava is molecular biologist with extensive research experience in the area of neuroendocrinology, including pain, stress, and inflammation.

	Dr. Bhargava was responsible for design, execution, data analysis, and manuscript preparation. She was also responsible for conduction of assays in collaboration with colleagues at the UC Metabolomics Core.

Name:	**Callan Lujan**
Project Role:	*Study Coordinator/Staff Research Associate*
Nearest person month worked:	*6 person months*
Contribution to Project:	Ms. Lujan prepared all regulatory submissions to the IRB and VA Research and Development Committee and oversaw compliance. Ms. Lujan supervised and coordinated study personnel, assisted with sample organization and shipping and the coordination of assay completion. Ms. Lujan worked with the SFVA Stress and Health program data manager to develop a data tracking system to manage participant information, biological samples, and assay data.

Name:	**Olga Mayzel**
Project Role:	Database Manager
Nearest person month worked:	1 person month
Contribution to Project:	Ms. Mayzel has created a database for tracking biological samples, participant information, and metabolomics data collection. The database manager oversaw database operations and maintained all computer equipment including a main data server.

Name:	**Thomas Metzler**
Project Role:	Biostatistician
Nearest person month worked:	1 person month
Contribution to Project:	Mr. Metzler worked closely with the investigators to complete correlate measure analyses.

Name:	**Ritu Roy**
Project Role:	Biostatistician

Nearest person month worked:	1 person month
Contribution to Project:	Dr. Roy has provided bioinformatics support for the metabolomics data and provided statistical support to complete metabolite analyses.

Name:	**Oliver Fiehn, PhD**
Project Role:	Consultant
Nearest person month worked:	1 person month
Contribution to Project:	Dr. Fiehn is the director of the West Coast Metabolomics Center at UC Davis. He reviewed the outputs from the completed assays and determined if additional assays were required.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**
   COLLABORATIVE AWARDS: Nothing to Report

9. **APPENDICES:** Conferences abstracts and publications are attached

2. Inslicht, S.S., Roy, R., Olshen, A., Bhargava, A., Neylan, T. Lipidomics of PTSD. Poster presented at the American College of Neuropsychopharmacology 57th Annual Meeting; 2018 December, Hollywood, FL.

Background: Post-traumatic stress disorder (PTSD) affects approximately 8% of the US population and 22% of US veterans returning from Iraq and Afghanistan. Not only does PTSD impact psychological well-being, it also has been associated with physical health concerns, including greater inflammation, metabolic syndrome, gastrointestinal (GI) illness, and even early mortality. Impaired glucocorticoid regulation associated with PTSD may increase risk for chronic health conditions, since glucocorticoids regulate metabolism of proteins, carbohydrates, and lipids including processes such as gluconeogenesis (protein to carbohydrate conversion) and redistribution of fat. In addition, specific fatty acid/amino acid metabolites can render PTSD patients more susceptible to neuronal excitability, signal transduction, and inflammation. Elevated concentrations of serum lipids were associated with combat-related PTSD. Veterans with combat-related PTSD are at a higher risk for arteriosclerosis, thus elevated or dysregulated serum lipids may contribute to increased immune and cardiovascular responses in PTSD. The aims of this study were two-fold. First, to ascertain if plasma lipid metabolites were associated with PTSD relative to healthy controls. Second, to examine if the plasma lipid profile was associated with sex. We predicted that both PTSD and male sex would be associated with higher levels of peripheral lipid metabolites that are known to traverse the blood-brain barrier and negatively affect health.

Methods: A metabolomics analysis has the potential to explain the regulation of metabolic pathways and networks of physiologically relevant interactions that lead to increased health risks in PTSD. As a global and unbiased approach, metabolomics identifies changes in circulating small molecules that affect cell and physiological function and can provide a more comprehensive examination of the broad range of physiological pathways that may be missed with more traditional, targeted approaches. Metabolomic analyses were performed on plasma samples obtained from fasting male and pre-menopausal follicular phase female subjects with chronic PTSD (N = 44) and trauma-exposed, age-matched controls (N = 44). Participants were between the ages of 20 and 50, primarily civilians (89%), healthy, free of medications, alcohol and drugs, and limited to one cup of caffeine daily. Plasma samples were assayed for lipids, fatty acids, sphingolipids, and short-chain fatty acid metabolites using Time-of-Flight Mass Spectrometer (Agilent Technologies 6220 TOF) coupled with an Ultra HPLC. Statistical analyses were performed using the R/Bioconductor statistical framework. Metabolomic data were transformed to log base 2 scale. Undetected metabolomic values were replaced by 1/10 of minimum value where necessary. Wilcoxon's rank-sum tests were performed for univariate and moderated t-tests from Bioconductor limma package for multivariate analyses. P-values were adjusted for multiple testing.

Results: Metabolic analyses indicated that 100/2,390 lipid metabolites were significantly associated with PTSD (97 lipid metabolites were upregulated and 3 were downregulated; adjusted p-value < .05). Lipid metabolites that were altered in PTSD included sphingomyelins, ceramides, and phosphatidylcholines. Two lipids were upregulated (one of which was acylcarnitine) and 1 (sphingomyelin) downregulated in males compared to females. Multivariate analyses revealed no significant interactions between PTSD and sex for any of the lipid metabolites.

Conclusions: Complementing previous findings of an association between hyperlipidemia in PTSD, the metabolomics approach allowed for the identification of lipid metabolites that were associated significantly with PTSD. Our findings are consistent with a previous report of alterations in sphingomyelin metabolism as measured peripherally in combat Veterans with PTSD and in several studies of individuals with major depressive disorders. Sphingolipids constitute a physical barrier in the brain and provide key functions including cell signaling. Sphingomyelin hydrolyses into ceramide and

phosphorylcholine. Acid sphingomyelinase, a lipid metabolizing enzyme responsible for this effect is increased in major depression and is reduced with tricyclic antidepressant intake. Acid sphingomyelinase has a role in interleukin-1 release from brain astrocytes, hypothalamic-pituitary-adrenal axis function and has been associated with cardiovascular disease. As such, sphingolipid metabolism may have a role in neuroendocrine and immune alterations in PTSD and associated cardiometabolic health conditions. While there is some evidence that ceramides in the plasma and brain are correlated and cross blood–brain barrier in rats, whether peripheral lipids are an indirect marker of central lipid metabolism has yet to be determined. Identification of key lipid metabolites in conjunction with steroid hormone biomarkers of PTSD may lead to a more precise and reliable phenotype and a clearer understanding of associations between the brain and glucocorticoid and inflammatory pathways to disease in individuals with PTSD.
Keywords: PTSD, Lipids, Metabolomics **Disclosure:** Nothing to disclose.

Lipidome analysis in men and women with post-traumatic stress disorder

Bhargava A, Fan S, Lujan C, Feihn O, Neylan TC, and Inslicht SS.

Post-traumatic stress disorder (PTSD) is a common psychiatric condition, affecting twice as many women as men. The physiological and mechanistic basis of this sex difference is unknown. PTSD can have a profound effect on sleep and is associated with risk for metabolic syndrome. Specific fatty acid metabolites render PTSD patients more susceptible to inflammation. We tested the hypothesis that subclasses of lipids will be increased in PTSD and sleep measures and may be different between the sexes. Metabolomic analyses were performed on fasting plasma samples collected from a cross-sectional study involving 90 (mean age 30 years, 49% PTSD), non-obese, non-medicated male and follicular phase female adults who participated in a 3-night sleep study in an inpatient sleep laboratory. Lipid metabolites were assayed using QTOF MS/MS. Metabolomic data was transformed to log base 10 scale. Mann-Whitney U tests and logistic regression with sex as variable were performed. P-values were adjusted for multiple testing using Benjamini-Hochberg procedure. Chemical Set Enrichment Analysis (ChemRICH), an alternative to pathway analysis that utilizes structure similarity and chemical ontologies to map known metabolites and name metabolic modules identified 11 clusters that were significantly increased in PTSD vs. healthy controls. Surprisingly, no sex by PTSD differences was found in this study for lipid clusters. Ten of the 11 lipid ChemRICH plot clusters for PTSD were also increased in self-report measure of quality of sleep (Pittsburgh Sleep Quality Index or PSQI). Saturated phosphatidylcholines (cluster of 7) were only associated with CPCL Score, whereas unsaturated fatty acids (cluster of 15) only associated with PSQI. Interestingly, 4 distinct lipid clusters that were highly enriched in PTSD and PQSI were found to be highly downregulated in people with good quality of sleep as measured with polysomnography. Thus, certain classes of lipid metabolites may be responsible for sleep quality in PTSD patients, irrespective of sex.

4. Inslicht S.S., Bhargava, A., Olshen A., Lujan, C., Neylan, T.C. Sex Differences in Lipid Metabolism in PTSD. Poster presented at The American College of Neuropsychopharmacology Annual Meeting, December 8-11, 2019, Orlando, FL.

Background: Post-traumatic stress disorder (PTSD) is associated with increased rates of hyperlipidemia and associated cardiovascular disease (CVD). Compared to men, women are at increased risk of PTSD, yet have decreased risk of CVD at ages pre-menopause. However, since the sex difference in CVD risk diminishes with increasing age, it has been hypothesized that the decline in sex hormones associated with ovarian aging may account for loss of protection from CVD risk factors in older women. While the primary functions of ovarian hormones are to control fertility and reproduction, estrogen, progesterone and their metabolites also provide protection from cardiometabolic risks, including ischemic damage, high-density lipoprotein and triglyceride profiles, waist circumference, glucose levels, and hypertensive status. Glucose and lipid dysregulation have been found during the menopause-related decline in estrogen. Testosterone, the primary reproductive male reproductive hormone promotes reproductive tissue development and secondary sexual characteristics in males, is also decreased as a result of stress and aging, and has been associated with diabetes, hypertension and dyslipidemia, low-density lipoprotein, inflammation, and incidence of atherosclerosis, coronary artery disease, and CVD events. Whether sex steroids confer protection from elevated lipids in individuals with PTSD is unknown. This study examined the effect of sex and PTSD on plasma lipid metabolites and the role of sex steroids in this relationship. We predicted higher lipid levels in PTSD vs. controls and in males compared to females. We also predicted that lipid levels would be inversely related to sex steroids.

Methods: Metabolomic analyses were performed on plasma samples obtained from fasting male and pre-menopausal follicular phase female subjects with chronic PTSD (N = 44) and trauma-exposed, age-matched controls (N = 44). Participants were between the ages of 20 and 50, primarily civilians (89%), healthy, free of medications, alcohol and drugs, and limited to one cup of caffeine daily. Plasma samples were assayed for lipid and steroid metabolites using Time-of-Flight Mass Spectrometer (Agilent Technologies 6220 TOF) coupled with an Ultra HPLC. Lipid and sex hormone metabolites were tested for sex and PTSD effects using ANOVA with FDR correction. Chemical Set Enrichment (ChemRICH) analysis, a technique that utilizes structure similarity and chemical ontologies to map was used to visualize metabolite clusters that differed by PTSD status and sex. Lipid metabolites that were differentially expressed in PTSD vs controls were treated as outcomes in logistic regression models with sex steroids entered as mediators in males and females separately.

Results: Sex differences were found in 67 lipid metabolites. In comparing PTSD vs controls, 217 lipid metabolites were altered (mostly upregulated) in PTSD. The majority of lipid alterations in PTSD occurred in men, but not in women (138 vs 42 lipid metabolites). ChemRICH analyses indicated that PTSD was associated with alterations in 8 out of 11 lipid clusters in men, but only 4 in women. In the steroid panel, testosterone glucuronide, a downstream metabolite of testosterone, was decreased in both men and women with PTSD compared to controls (p's < .05). The testosterone metabolites, dihydrotestosterone and etiocholanolone, were elevated in PTSD in both sexes (p's < .05). Testosterone glucuronide accounted for alterations in lipid metabolites in women only. There were no associations of PTSD with estrogens.

Conclusions: A metabolomics approach was used to identify lipid and sex steroid metabolite alterations in PTSD in men and women. Complementing previous findings of hyperlipidemia in men and in PTSD, this study further identified sex differences in lipid alterations in PTSD. Metabolites in the testosterone pathway accounted for alterations in lipid alterations in women in particular. There were no associations of PTSD with estrogens, which were expected to be protective. However, conclusions about the role of

female sex steroids were limited by the single timepoint blood draw that occurred during the early follicular phase, when estrogen and progesterone are low. Longitudinal assessment over the menstrual cycle would be important to evaluate the role of sex steroids on lipid metabolism in naturally cycling women with PTSD and in older individuals to examine effects of aging. These findings suggest that the testosterone-related compounds may account for sex differences in lipid profiles, potentially leading to differential health risks in PTSD. **Keywords:** PTSD, Dyslipidemia, Sex Steroids, Metabolomic
Disclosure: Nothing to disclose.

Elevated Inflammation Associated With Suicide Related Behavior in Veterans With and Without Post traumatic Stress Disorder Aoife O'Donovan , Amy Byers , Eleanor Woodward , Daniel Bertenthal, Sabra Inslicht , Karen Seal , and Thomas Neylan

University of California, San Francisco and San Francisco VA Healthcare System

Background: Elevated inflammation may play a role in the pathogenesis of posttraumatic stress disorder (PTSD), and specifically increase risk for suicide-related behavior, which is more common in PTSD. However, little is known about associations of inflammatory markers with suicide-related behavior in individuals with PTSD.

Methods: Here, we examine if PTSD, suicidal ideation, and suicide attempts are associated with elevated levels of the inflammatory marker high sensitivity C-reactive protein (hsCRP) in a sample of 16,586 Iraq and Afghanistan veterans (M age ¼ 34.5±8.7 years; 15% women). Data were compiled from VA administrative databases. Generalized linear models were used to ascertain associations of predictors with log-transformed hsCRP. Models were adjusted for demographics, body mass index, Charlson Comorbidity score, and single/multiple deployments.

Results: Participants diagnosed with PTSD (54%) had significantly higher levels of hsCRP compared to those with other (25%; b¼.17, p< .001) and attempts (b ¼ .50, p < .001), independent of PTSD and other psychiatric diagnoses.

Conclusions: Data indicate that inflammation is elevated in PTSD and independently in association with suicide ideation and attempts. Elevated inflammation may be a risk factor and potential mechanism for suicide-related behavior in individuals with and without PTSD.

Supported By: NIMH K01; Sierra-Pacific MIRECC

Keywords: PTSD, Inflammation, Suicide Behavior, Suicidal Ideation, Suicide Attempt

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Metabolic Risk Factors and Posttraumatic Stress Disorder: The Role of Sleep in Young, Healthy Adults

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Abstract

Objective—Posttraumatic stress disorder (PTSD) is associated with indicators of poor physical health and sleep disturbance. This study investigated the relationship between PTSD and metabolic risk factors and examined the role of sleep duration in a sample of medically healthy and medication-free adults.

Methods—Participants with PTSD (n = 44, mean age = 30.6 years) and control participants free of lifetime psychiatric history (n = 50, mean age = 30.3 years) recorded sleep using sleep diary for 10 nights and actigraphy for seven nights. We assessed metabolic risk factors including fasting triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol, as well as abdominal fat using dual-energy X-ray absorptiometry.

Results—PTSD was associated with shorter sleep duration (based on self-report but not actigraphy) and higher metabolic risks (controlling for body fat percentage), including increased triglycerides (p = .03), total cholesterol (p < .001), LDL cholesterol (p = .006), VLDL cholesterol (p = .002), and cholesterol: HDL ratio (p = .024). Additionally, sleep duration was associated with metabolic risks in PTSD (significant correlations ranged from r = −.20 to r = −.40) but did not fully account for the association between PTSD with metabolic measures.

Conclusions—Metabolic risk factors are associated with PTSD even in early adulthood, highlighting the need for early intervention. Future longitudinal research should assess whether sleep disturbance in PTSD is a mechanism that contributes to heightened metabolic risk in order to elucidate the pathway from PTSD to higher rates of medical disorders such as obesity, diabetes, and heart disease.

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Conflicts of interest: Dr. Cohen reported that her spouse is employed by Gilead Sciences, Inc. Dr. Neylan reported receiving study medication from Actelion for a study funded by the Department of Defense and receiving study medication from Glaxo Smith Kline for a study funded by the Department of Veterans Affairs. For the remaining authors none were declared.

Keywords

posttraumatic stress disorder; cholesterol; triglycerides; lipoproteins; visceral adiposity; sleep duration

Accruing evidence suggests that posttraumatic stress disorder (PTSD) is associated with poor metabolic health. For example, several studies have reported dyslipidemia in veteran samples with PTSD (1–5). In a study of over 300,000 young veterans who accessed Department of Veterans Affairs (VA) health care, diagnosis of PTSD was associated with a significantly increased risk for hypertension and dyslipidemia (6). In addition, converging preliminary evidence including a recent meta-analysis of six studies (7) reported an association between PTSD and metabolic syndrome (a cluster of at least three risk factors that are associated with the development of diabetes and cardiovascular disease) (8). For example, Violanti and colleagues observed that police officers with severe PTSD symptoms were three times more likely to have metabolic syndrome, although age attenuated this association (9). Jin and colleagues observed in an outpatient sample of patients requiring ongoing antipsychotic treatment that the risk of metabolic syndrome was higher in PTSD compared to other diagnostic groups (e.g., schizophrenia) (10). In a series of studies, Heppner and colleagues observed that 43% of an older military veteran sample (mean age 52, with 70% of veterans having served in Vietnam) met criteria for metabolic syndrome, that those with more severe PTSD exhibited a higher likelihood of metabolic syndrome, and that antipsychotic use was not uniquely associated with metabolic risk (11, 12). Finally, Weiss and colleagues observed in a sample of 245 civilians of low socioeconomic status recruited from hospital general medical clinics that after controlling for demographics, smoking history, antipsychotic use, depression and exercise, current PTSD was the only significant predictor of metabolic syndrome (13). Body mass index and medical illness were not controlled.

Hence, the majority of the initial evidence suggests that PTSD is associated with a higher prevalence of metabolic risk factors. Despite this evidence, several questions remain in regard to PTSD and metabolic risk factors. First, there is some contradictory evidence. For example, one study did not observe a relationship between PTSD and lipid elevations in a civilian sample (14). Second, there has been a dearth of research on samples of young to middle-aged adults, who are more likely to be free of potentially complicating co-occurring medical issues and medications. Third, the mechanisms through which PTSD disturbs metabolism remain unclear.

Based on these knowledge gaps, we evaluated the association of PTSD and metabolic risk factors in a young, healthy, unmedicated sample. We also evaluated the role of sleep disturbance as a mechanism of increased metabolic risk. In individuals with PTSD, sleep disturbance is the most frequently reported symptom, even when nightmares are excluded (15), and as many as 90% of individuals with PTSD report insomnia and nightmares (16, 17). This sleep disturbance may contribute to metabolic risk, given the substantial evidence in healthy samples from both epidemiological and experimental laboratory studies supporting a critical role of sleep in metabolic regulation (e.g., 18, 19). For example, in a cross-sectional study, self-reported sleep quality was associated with metabolic syndrome,

including measures related to obesity and insulin resistance (20) and another study found that sleep disturbance prospectively predicted the development of metabolic syndrome (21). Moreover, a recent study of over 800 individuals indicated that sleep duration of less than six hours per night was associated with an increase in the odds of having metabolic syndrome after adjusting for possible confounders (22). Finally, Kim and colleagues observed that short sleep was associated with visceral obesity as measured by computed tomography (23).

In the present study, metabolic risk factors and habitual sleep duration as measured by sleep diary and actigraphy were assessed in individuals with PTSD and healthy control participants. First, we assessed group differences in metabolic risk factors including triglycerides, total cholesterol, low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL) cholesterol, high density lipoprotein (HDL) cholesterol, cholesterol: HDL ratio, and truncal fat (a measure of visceral obesity), adjusting for overall body fat percentage. Then, we examined whether sleep duration would be associated with metabolic risk factors in both groups. We predicted that individuals in the PTSD group would exhibit higher metabolic risk factors and that sleep duration would account for metabolic risks in both groups.

Methods

Participants

Participants included 94 adults recruited from newspaper advertisements, web based postings, flyers in community-based outpatient clinics, and from the clinical PTSD Program at the San Francisco VA Medical Center. Participants enrolled between November 2005 and October 2008. Participants were relatively young, healthy, and medication-free in order to examine PTSD without the potential confounds of aging, physical illness, and medication. The majority of participants were civilian (89%). The sample included 44 individuals with current chronic PTSD (50% female) and 50 control subjects without PTSD (54% female), with a mean age of 30.44 (SD = 7.39; range 20 to 50 years).

Chronic PTSD was defined by DSM-IV PTSD criteria (24) or by a Clinician-Administered PTSD Scale (CAPS; 25) score of >40 for at least 3 months. Control participants were negative for lifetime PTSD, had a CAPS score of <20, and were free from lifetime major depressive disorder and panic disorder. Female subjects were studied during the follicular phase of their menstrual cycle. Exclusion criteria for both groups included neurologic disorder or systemic illness affecting CNS function; pregnancy; use of psychiatric, anticonvulsant, antihypertensive, sympathomimetic, estrogen replacement therapy, or steroid medication; lifetime history of any psychiatric disorder with psychotic features; bipolar disorder; obsessive-compulsive disorder; alcohol abuse or dependence within the past two years; and substance abuse or dependence in the past year.

Measures

Clinician-Administered PTSD Scale (CAPS)—Current PTSD was assessed with the CAPS (25). The CAPS has excellent test-retest reliability (r=0.92–0.99) and internal consistency (alpha=0.80–0.90; 26).

Structured Clinical Interview for DSM-IV (SCID)—Diagnoses other than PTSD were assessed with the SCID (27). The SCID has been shown to have good reliability (e.g., 28).

All diagnoses were made by trained clinical interviewers who calibrated their CAPS and SCID assessments at weekly case consensus meetings, supervised by an experienced Ph.D.level clinical psychologist.

Metabolic Risk Factors—Lipids including triglycerides, total cholesterol, VLDL cholesterol, and HDL cholesterol were assessed and then assayed by a clinical laboratory contracted with the University of California, San Francisco. LDL cholesterol was calculated. The sum of LDL cholesterol, VLDL cholesterol, and HDL cholesterol comprised total cholesterol. Truncal fat was measured using dual-energy X-ray absorptiometry (DEXA) scan and truncal fat percentage was computed as DEXA-measured truncal fat divided by DEXA-measured total fat. In addition, body fat percentage was calculated from the DEXA measurements and was used as a covariate in the analyses. Six control participants did not complete the lipids assessment or DEXA body scan, and an additional four participants did not complete the DEXA scan only.

Sleep diary—Average sleep duration was computed from 10 days of sleep diary. Total sleep time (TST) was computed by subtracting reported sleep onset latency and wake after sleep onset from reported time in bed. The sleep diary has been shown to provide a reliable estimate (29) and is considered the gold standard subjective measure of sleep (30). A few participants did not complete all 10 nights of sleep diary. The mean number of nights for the PTSD group was 9.91 (SD = 0.47) and the mean number of nights for the control group was 9.70 (1.13). There was no significant difference between groups in the number of nights completed.

Actigraphy—Participants had their sleep-wake schedule monitored for seven nights with wrist actigraphy (Micro Motionlogger; Ambulatory Monitoring, Inc., Ardsley, NY). Actigraphy is an important objective estimate of sleep (31). Actigraphs were initialized and downloaded with the ActMe program (Ambulatory Monitoring, Inc., Ardsley, NY) using the ZCM sampling mode in one minute epochs. The Cole-Kripke algorithm was used in ActionW Version 2.7 (Ambulatory Monitoring, Inc., Ardsley, NY) software to estimate the sleep parameter of TST. Six participants in the control group did not have actigraphy data due to equipment malfunction (0 in the PTSD group), and a few participants in each group did not complete all seven nights. The mean number of nights for the PTSD group was 6.73 (SD = 0.87) and the mean number of nights for the control group was 6.52 (SD = 1.32). There was no significant difference between groups in the number of nights of actigraphy data.

Procedure

All research was approved by the Committee on Human Research at the University of California, San Francisco and at the San Francisco Veterans Affairs Medical Center. All participants provided written informed consent and appropriate institutional review boards approved the research protocol. Participants who were likely to be eligible after a telephone screen visited the laboratory for administration of the CAPS and SCID. Eligible participants then recorded their sleep at home using sleep diary for 10 nights and actigraphy for seven nights. Participants then visited the hospital for height and weight measurements, measurement of total and abdominal fat by DEXA scan, and venipuncture for measurement of triglycerides, total cholesterol, LDL cholesterol, VLDL cholesterol, and HDL cholesterol. The data in the present study were collected as a part of a larger research project which

examined neuroendocrine factors mediating sleep disturbances in PTSD. The data and analyses presented in this paper do not overlap with previously-published articles from this study (32–34).

Statistical Analysis—We first examined potential demographic differences. Specifically, we examined potential differences in age, years of education, CAPS score, and body fat percentage using t-tests and gender, race, marital status, veteran status, and current depression status using chi-squared tests. We also examined potential group differences in sleep duration using a MANOVA. The MANOVA compared the group with PTSD to the healthy control group on the dependent variables of diary-measured total sleep time and actigraphy-measured total sleep time.

We conducted statistical analyses to test two study hypotheses—that individuals in the PTSD group would exhibit higher metabolic risk factors, and that sleep duration would account for metabolic risks in both the PTSD and healthy control groups. To test the first hypothesis, we conducted a multivariate analysis of covariance (MANCOVA) to examine group differences in metabolic risk factors. To test the second hypothesis, we conducted three sets of analyses to examine associations between the metabolic risk factors and sleep in those with and without PTSD.

For the first analysis, we conducted a multivariate analysis of covariance (MANCOVA) to examine whether there would be group differences in metabolic risk factors including triglycerides, total cholesterol, LDL cholesterol, VLDL cholesterol, HDL cholesterol, cholesterol: HDL ratio, and truncal fat percentage. DEXA-measured body fat percentage was included as a covariate in order to distinguish the effect of body fat on metabolic risk factors from psychiatric group status effects.

The second set of analyses examined associations between these risk factors and sleep in those with and without PTSD. Total sleep time was employed as the sleep outcome measure on the basis of previous research linking sleep duration to metabolic regulation, (e.g., 36, 37). Average total sleep was examined using sleep diary and actigraphy; the correlation between the two measures of sleep was $r = .49$ (95% C.I. .31 – .64, $p < .001$). In the first set of analyses, we examined partial correlations between sleep duration (as measured by sleep diary and actigraphy) and the metabolic risk factors, controlling for body fat percentage. We first examined these correlations in the full group. Next, these correlations were examined in the PTSD group and then in the control group to determine which group was driving any potential relationships. In the final set of analyses, on an exploratory basis (given the crosssectional nature of the data), we tested mediation in path models conducted separately for each outcome, considering both diary and actigraphy TST in each model (see Figure 1 for a schematic diagram).

Results

Participant Characteristics

There were no significant differences between groups in age, gender, or years of education. The PTSD group had fewer Caucasians, more individuals who were divorced or separated, more veterans, and a (marginally significant) higher body fat percentage ($p = .05$) (see Table 1). The mean CAPS score for the PTSD group was 54.79 ($SD = 15.84$) and the mean CAPS score for control participants who experienced a DSM-IV Criterion A event ($n = 12$) was

0.00. Eighteen percent of PTSD participants (n = 8) met criteria for a current Major Depressive Episode.

Sleep Duration

We examined baseline sleep characteristics of participants. A multivariate analysis of variance (MANOVA) was conducted on the sleep variables of total sleep time based on sleep diary and actigraphy with group (PTSD, control) as the between-subjects variable. A significant group effect was observed on the omnibus test ($F(2, 82) = 6.24$, $p = .003$). A significant multivariate effect was also observed on diary-measured TST, with the PTSD group reporting less total sleep time than the control group (see Table 2). No difference was observed on actigraphy-measured TST.

Metabolic Risk Factors

A multivariate analysis of covariance (MANCOVA) was conducted on metabolic risk factors (triglycerides, total cholesterol, LDL cholesterol, VLDL cholesterol, HDL cholesterol, cholesterol: HDL ratio, and truncal fat percentage) with group (PTSD, control) as the between-subjects variable and DEXA-measured body fat percentage as a covariate. A significant group effect was observed on the omnibus test ($F(6,75) = 4.06$, $p < .001$). In addition, significant multivariate effects were observed on the following dependent measures: triglycerides, total cholesterol, LDL cholesterol, VLDL cholesterol, and cholesterol: HDL ratio. No differences were observed on HDL cholesterol or truncal fat percentage, controlling for overall body fat percentage. See Table 2.

Sleep Duration and Metabolic Risk Factors

Partial correlations between sleep duration as measured by sleep diary and actigraphy and the metabolic risk factors were conducted, first in the full sample and then separately in the PTSD and control groups. In the full sample, there were negative correlations between diary-measured sleep duration and triglycerides, cholesterol, VLDL cholesterol, and truncal fat percentage. In the PTSD group, there were negative correlations between diary-measured sleep duration and triglycerides and diary-measured sleep duration and VLDL cholesterol. The five non-significant correlations were all in the predicted direction (see Table 3). The same partial correlation analysis was then conducted in the control group. There was a significant correlation between truncal fat percentage and diary-measured sleep duration, indicating that less sleep was associated with more truncal fat. No other correlations were significant and two of the six non-significant associations were in the predicted direction.

Next, the partial correlations between actigraphy-measured sleep duration and metabolic risk factors were examined in the full sample and then in the PTSD group andcontrol group. In the full sample, there were negative correlations between actigraphy-measured sleep duration and triglycerides, cholesterol, VLDL cholesterol, LDL cholesterol, cholesterol: HDL ration, and trunkal fat percentage, and a positive correlation between actigraphymeasured sleep duration and HDL cholesterol. In the PTSD group, five associations were significant. There were negative correlations between actigraphy-measured sleep duration and triglycerides, VLDL cholesterol, cholesterol: HDL ratio, and truncal fat percentage. There was a positive correlation between actigraphy-measured sleep duration and HDL cholesterol. These significant correlations were all in the predicted direction and suggest that less sleep duration is associated with higher metabolic risk. The two non-significant correlations were also in the predicted direction. Interestingly, there

were more significant sleep-metabolic risk factor correlations based on actigraphy than on diary. Finally, the same partial correlations between actigraphy-measured sleep duration and metabolic risk factors were examined in the control group. Only one significant correlation emerged: sleep duration and truncal fat percentage, such that less sleep was associated with more truncal fat. Four of the six non-significant correlations were in the predicted direction.

Finally, on an exploratory basis, sleep duration (both diary and actigraphy-measured) was examined as a mediator of the effects of PTSD on metabolic risk factors using path models. Results indicated that diary TST partially mediated the effects of PTSD on triglycerides: The standardized mediated effect was .24 ($p < .001$), while the standardized direct effect was .40 ($p = .003$). These results indicate that there was a PTSD vs. control group difference of .24 standard deviation units in triglycerides attributable to differences in sleep duration, plus an additional .40 standard deviation units difference in triglycerides not attributable to sleep duration. Moreover, diary TST completely mediated the effect of PTSD on truncal fat percent (standardized mediated effect = .29, $p < .001$) compared to a standardized direct effect = −.10, $p = .660$). Diary TST did not mediate any of the cholesterol measures. We note that diary TST mediated the effect on truncal fat even though there was no total effect of PTSD on truncal fat. This result is due to the effect of group on TST and the effect of TST on truncal fat, contributing to a significant indirect pathway from PTST to TST to truncal fat (for more on mediation effects in the absence of direct effects, see 38). Actigraphy-based TST did not mediate any relationships.

Discussion

We examined metabolic risk factors and habitual sleep duration in individuals with PTSD compared to a healthy control group. In support of our hypothesis, we observed that individuals with PTSD demonstrated a worse metabolic profile, including higher levels of triglycerides, total cholesterol, LDL cholesterol, VLDL cholesterol, and cholesterol: HDL ratio. We did not observe increased truncal fat or reduced HDL cholesterol in PTSD.

Overall, our findings are consistent with the accruing evidence suggesting higher metabolic risks in PTSD (1, 2, 4–7, 9, 11, 12, 39). However, the differences we observed are particularly notable given the relatively young age of the sample and the lack of cooccurring medical issues and medications. For example, many previous studies have utilized older samples which may present a confound since metabolic syndrome increases with age (e.g., 40). Our data suggest that the poor health risks and outcomes associated with PTSD are present by early adulthood and as such should be assessed early in clinical settings.

Our second hypothesis was that sleep duration would account for the group differences in metabolic risks between individuals with PTSD and healthy control participants. To test this hypothesis, we first examined sleep duration in the sample. We observed that individuals with PTSD reported less total sleep time compared to the healthy control participants based on sleep diary, consistent with substantial research indicating sleep disturbance in PTSD (e.g., 15, 16, 17). Interestingly, we did not observe worse sleep in PTSD as measured by actigraphy, though the means were in the predicted direction. The $r = 0.49$ correlation between the two measures in this study was not surprising. It is common for discrepancies to emerge between objective and subjective measures of sleep, particularly in individuals with psychiatric disorders, e.g., (41, 42). While both measures are useful for longitudinal use as in the current study and present a low participant burden, sleep diaries rely on morning

estimates of the previous night's sleep. Evidence suggests that individuals may have difficulty with such estimates (e.g., 43) and particularly that individuals with insomnia may overestimate their sleep disturbance (for a review see 44). On the other hand, actigraphs, which estimate sleep from wrist accelerometry, may present issues such as incorrectly considering physical inactivity in the bed as sleep or restlessness/movement in sleep was wake time. Finally, age and gender may affect concordance between diary- and actigraphymeasured sleep (45).

As the second step in testing the hypothesis that sleep would account for group differences in metabolic risk factors, we conducted correlation analyses. These analyses demonstrated relationships between sleep duration and metabolic risk factors, such that shorter sleep duration was associated with higher metabolic risk. We then examined the diagnostic groups separately to see if one group drove these differences. In the PTSD group, there were numerous associations between sleep duration and metabolic risk factors, while the same analyses in the healthy control group yielded few associations. It is possible that the habitually longer sleep duration in the control group served as a protective factor. Overall, the data suggest that sleep may contribute to the metabolic risk profile observed in PTSD, although sleep does not fully explain the heightened risk. Treating sleep disturbance in PTSD continues to be important because initial evidence suggests that improving sleep may improve overall functioning (46) and increasing sleep duration could decrease metabolic risks. At the same time, treatment for sleep disturbance is unlikely to serve as a panacea and other interventions that focus on management of chronic stress, diet, and exercise may also be important, given the likelihood that other mechanisms also contribute to these metabolic health risks.

We also note that exploratory mediation analyses demonstrated that diary-measured total sleep time partially mediated the effect of PTSD on triglycerides and completely mediated the effect of PTSD on truncal fat, consistent with previous research, while actigraphymeasured sleep duration did not. These results are consistent with previous research which demonstrated a link between subjectively-measured, but not objectively-measured, sleep and urinary cortisol (another metabolism variable)(47).

Considering other possible contributors to metabolic health risks—beyond sleep—allostatic load may comprise one possible mechanism. Allostatic load refers to the physiologic damage as a result of repeated activation of the body's stress response system (48). For example, chronic glucocorticoid secretion can contribute to abdominal fat deposition directly (49, 50) as well as indirectly via increased intake of so-called "comfort foods" (i.e., foods high in fat and/or sugar; e.g., 51, 52), resulting in visceral obesity. In another example, increased activation of the sympathetic nervous system increases production of lipoprotein lipase, which leads to increased cholesterol and triglycerides (53). Additionally, stress may act through the neuropeptide Y system (54), particularly in the context of a high caloric, fat and sugar diet, which may be more common in PTSD (34, 54). Future research should examine multiple contributory mechanisms to the heightened metabolic risk in PTSD including both sleep disturbance and allostatic load in order to begin delineate causal pathways.

Considering the seven specific metabolic risk factors examined in the present study, no factor in particular emerged as the one most likely to demonstrate heightened risk in this young, healthy sample. All seven of the metabolic risk factors examined demonstrated either

an elevated mean in PTSD compared to the healthy control group or an association with sleep in the PTSD group (and most factors demonstrated both of these). As such, in samples such as this one in which full metabolic syndrome is unlikely to be present in the majority of individuals, it may be important to continue to examine and treat individual risk factors.

We do note, however, that the sleep-truncal fat correlations were the only associations in this sample that were significant in both the individuals with PTSD (according to actigraphy, and marginally significant according to sleep diary) and the healthy control group (significant according to both actigraphy and diary). While we cannot assume directionality from a correlation, the data raise the possibility that sleep duration and truncal fat may be closely connected. The correlations in the PTSD and healthy control groups across both sleep diary and actigraphy with truncal fat ranged from $r = .3$ to $r = .5$, which is noteworthy given that the group means for habitual sleep duration were not extremely low (i.e., the sleep diary and actigraphy means ranged from 6.6–7.0 hours in the PTSD group and 7.3–7.4 hours in the healthy control group). Consistent with our findings, one recent study has observed an association between short sleep and visceral obesity in a community sample of middle-aged Korean adults.(23) The topic has otherwise been understudied but it is an important direction for future research, particularly to establish whether abdominal obesity is an early result of habitually low sleep.

Some limitations of the present study are important to consider. First, the relatively small sample size may have limited the statistical power of the study and therefore reduced the ability to detect group differences. For example, there were some nonsignificant correlations in the PTSD and control groups between sleep and metabolic risk factors. Given the small sample size, it is not clear whether there were truly no relationships in these instances or whether the study was underpowered to detect potential relationships. Future studies should employ larger sample sizes. Second, we used multiple statistical tests to examine the metabolic risk factors, and as such there is an increased possibility of type I errors. We note, however, that all hypotheses were a priori and all significant correlations were in the predicted direction. Also, metabolic risk factors are correlated, and therefore the multiple tests do not represent independent chances for type II error. Third, given the cross-sectional design of the present study, it is not possible to determine causal relationships among PTSD, sleep, and metabolic risk factors. Future research should include longitudinal assessments. Finally, the study used a convenience rather than a representative sample, thus introducing the possibility of bias. Nonetheless, the initial data suggest that PTSD is associated with higher metabolic risks even in a young to middle-aged, medically-healthy, and medicationfree sample. Additionally, sleep duration is associated with these metabolic risks in individuals with PTSD but does not fully account for them. Future longitudinal research should assess whether sleep disturbance in PTSD contributes to heightened metabolic risk as well as explore other potential contributory mechanisms.

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Acronyms

CAPS	Clinician-Administered PTSD Scale
CNS	central nervous system
DSM	Diagnostic and Statistical Manual of Mental Disorders
DEXA	dual-energy X-ray absorptiometry
HDL	high density lipoprotein
LDL	low density lipoprotein
MANCOVA	multivariate analysis of covariance
PTSD	posttraumatic stress disorder
TST	total sleep time
VA	Veterans Affairs
VLDL	very low density lipoprotein

References

1. Kagan BL, Leskin G, Haas B, Wilkins J, Foy D. Elevated lipid levels in Vietnam veterans with chronic posttraumatic stress disorder. Biol Psychiatry. 1999; 45:374–7. [PubMed: 10023518]
2. Karlovic D, Buljan D, Martinac M, Marcinko D. Serum lipid concentrations in Croatian veterans with post-traumatic stress disorder, post-traumatic stress disorder comorbid with major depressive disorder, or major depressive disorder. J Korean Med Sci. 2004; 19:431–6. [PubMed: 15201512]
3. Karlovic D, Martinac M, Buljan D, Zoricic Z. Relationship between serum lipid concentrations and posttraumatic stress disorder symptoms in soldiers with combat experiences. Acta Med Okayama. 2004; 58:23–7. [PubMed: 15157008]
4. Solter V, Thaller V, Karlovic D, Cmkovic D. Elevated serum lipids in veterans with combat-related chronic posttraumatic stress disorder. Croat Med J. 2002; 43:685–9. [PubMed: 12476477]
5. Maia DB, Marmar CR, Mendlowicz MV, Metzler T, Nobrega A, Peres MC, Coutinho ES, Volchan E, Figueira I. Abnormal serum lipid profile in Brazilian police officers with post-traumatic stress disorder. J Affect Disord. 2008; 107:259–63. [PubMed: 17888517]
6. Cohen BE, Marmar C, Ren L, Bertenthal D, Seal KH. Association of cardiovascular risk factors with mental health diagnoses in Iraq and Afghanistan war veterans using VA health care. JAMA. 2009; 302:489–92. [PubMed: 19654382]
7. Bartoli F, Carra G, Crocamo C, Carretta D, Clerici M. Metabolic syndrome in people suffering from posttraumatic stress disorder: a systematic review and meta-analysis. Metab Syndr Relat D. 2013; 11:301–8.
8. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SCJ, Spertus JA, Costa F. Diagnosis and management of the metabolic syndrome.
An American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Executive Summary. Cardiol Rev. 2005; 13:322–27. [PubMed: 16708441]
9. Violanti JM, Andrew M, Burchfiel CM, Hartley TA, Charles LE, Miller DB. Post-traumatic stress symptoms and cortisol patterns among police officers. Policing: an Intl J Police Strategies & Mgmt. 2007; 30:189–202.
10. Jin H, Lanouette NM, Mudaliar S, Henry R, Folsom DP, Khandrika S, Glorioso DK, Jeste DV. Association of posttraumatic stress disorder with increased prevalence of metabolic syndrome. Journal of clinical Psychopharmacology. 2009; 29:210–5. [PubMed: 19440072]
11. Heppner PS, Crawford EF, Haji UA, Afari N, Hauger RL, Dashevsky BA, Horn PS, Nunnink SE, Baker DG. The association of posttraumatic stress disorder and metabolic syndrome: a study of increased health risk in veterans. BMC Medicine. 2009; 7:1. [PubMed: 19134183]

12. Heppner PS, Lohr JB, Kash TB, Jin H, Wang H, Baker DG. Metabolic syndrome: relative risk associated with post-traumatic stress disorder (PTSD) severity and antipsychotic medication use. Psychosomatics. 2012; 53:550–8. [PubMed: 23157993]
13. Weiss T, Skelton K, Phifer J, Javanovic T, Gillespie CF, Smith A, Umpierrez G, Bradley B, Ressler KJ. Posttraumatic stress disorder is a risk factor for metabolic syndrome in an impoverished urban population. Gen Hosp Psychiat. 2011; 33:135–42.
14. Tochigi M, Umekage T, Otani T, Kato T, Iwanami A, Asukai N, Sasaki T, Kato N. Serum cholesterol, uric acid and cholinesterase in victims of the Tokyo subway sarin poisoning: a relation with post-traumatic stress disorder. Neurosci Res. 2002; 44:267–72. [PubMed: 12413655]
15. Roszell DK, McFall ME, Malas KL. Frequency of symptoms and concurrent psychiatric disorder in Vietnam veterans with chronic PTSD. Hosp Community Psych. 1991; 42:293–6.
16. Kilpatrick, DG.; Resnick, HS.; Freedy, JR.; Pelcovitz, D.; Resick, PA.; Roth, S.; van der Kolk, B. Posttraumatic stress disorder field trial: Evaluation of the PTSD construct–Criteria A through E. In: Widiger, TA.; Frances, AJ.; Pincus, HA.; Ross, R.; First, MB.; Davis, W., editors. DSM-IV sourcebook. 4. Washington, DC: American Psychiatric Association; 1998. p. 803-44.
17. Neylan TC, Marmar CR, Metzler TJ, Weiss DS, Zatzick DF, Delucchi KL, Wu RM, Schoenfeld FB. Sleep disturbances in the Vietnam generation: findings from a nationally representative sample of male Vietnam veterans. Am J Psychiatry. 1998; 155:929–33. [PubMed: 9659859]
18. Morselli LL, Guyon A, Spiegel K. Sleep and metabolic function. Pflugers Arch. 2012; 463:139–60. [PubMed: 22101912]
19. Xi B, He D, Zhang M, Xue J, Zhou D. Short sleep duration predicts risk of metabolic syndrome: A systematic review and meta-analysis. Sleep Med Rev. 2013
20. Jennings JR, Muldoon MF, Hall M, Buysse DJ, Manuck SB. Self-reported sleep quality is associated with the metabolic syndrome. SLEEP. 2007; 30:219–23. [PubMed: 17326548]
21. Troxel WM, Buysse DJ, Matthews KA, Kip KE, Strollo PJ, Hall M, Drumheller O, Reis SE. Sleep symptoms predict the development of the metabolic syndrome. SLEEP. 2010; 33:1633–40. [PubMed: 21120125]
22. Chaput JP, McNeil J, Despres JP, Bouchard C, Tremblay A. Seven to eight hours of sleep a night is associated with a lower prevalence of the metabolic syndrome and reduced overall cardiometabolic risk in adults. PLoS. 2013; 8
23. Kim NH, Lee SK, Eun CR, Seo JA, Kim SG, Choi KM, Baik SH, Choi DS, Yun CH, Kim NH, Shin C. Short sleep duration combined with obstructive sleep apnea is associated with visceral obesity in Korean adults. SLEEP. 2013; 36:723–9. [PubMed: 23633755]
24. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. 4. Washington, D.C.: APA; 2000. text revision ed
25. Blake DD, Weathers FW, Nagy LM, Kaloupek DG, Gusman FD, Charney DS, Keane TM. The development of a Clinician-Administered PTSD Scale. J Trauma Stress. 1995; 8:75–90. [PubMed: 7712061]
26. Weathers FW, Keane TM, Davidson JR. Clinician-administered PTSD Scale: a review of the first ten years of research. Depress Anxiety. 2001; 13:132–56. [PubMed: 11387733]
27. First, M.; Spitzer, R.; Williams, J.; Gibbon, M. Structured Clincal Interview for the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV). 4. New York: Biomedics Research Department, New York State Psychiatric Institute; 1996.
28. Williams JB, Gibbon M, First MB, Spitzer RL, Davies M, Borus J, Howes MJ, Kane J, Pope HG Jr, Rounsaville B, Wittchen HU. The Structured Clinical Interview for DSM-III-R (SCID): Multisite test-retest reliability. Arch Gen Psychiatry. 1992; 49:630–6. [PubMed: 1637253]
29. Morin, CM.; Espie, CA. Insomnia: a clinical guide to assessment and treatment. New York: Kluwer Academic/Plenum Publishers; 2003.
30. Buysse DJ, Ancoli-Israel S, Edinger JD, Lichstein KL, Morin CM. Recommendations for a standard research assessment of insomnia. SLEEP. 2006; 29:1155–73. [PubMed: 17040003]
31. Ancoli-Israel S, Cole R, Alessi C, Chambers M, Moorcroft W, Pollack CP. The role of actigraphy in the study of sleep and circadian rhythms. SLEEP. 2003; 26:342–92. [PubMed: 12749557]
32. O'Donovan A, Epel E, Lin J, Wolkowitz O, Cohen B, Maguen S, Metzler T, Lenoci M, Blackburn E, Neylan TC. Childhood trauma associated with short leukocyte telomere length in posttraumatic stress disorder. Biol Psychiatry. 2011; 70:465–71. [PubMed: 21489410]

33. Richards A, Metzler TJ, RUoff LM, Inslicht SS, Rao M, Talbot LS, Neylan TC. Sex differences in objective measures of sleep in post-traumatic stress disorder and healthy control subjects. J Sleep Res. 2013
34. Talbot LS, Maguen S, Epel ES, Metzler TJ, Neylan TC. Posttraumatic stress disorder is associated with emotional eating. J Traum Stress. 2013; 26:521–5.
35. Brown, H.; Prescott, R. Applied Mixed Models in Medicine. Second. England: John Wiley & Sons Ltd; 2006.
36. Hall MH, Muldoon MF, Jennings JR, Buysse DJ, Flory JD, Manuck SB. Self-reported sleep duration is associated with the metabolic syndrome in midlife adults. SLEEP. 2008; 31:635–43. [PubMed: 18517034]
37. Ju SY, Choi WS. Sleep duration and metabolic syndrome in adult populations: a meta-analysis of observational studies. Nutr Diabetes. 2013
38. Kenny DA, Judd CM. Power anomalies in testing mediation. Psychological Science. 2014; 25:334–9. [PubMed: 24311476]
39. Weiss T, Skelton K, Phifer J, Jovanovic T, Gillespie CF, Smith A, Umpierrez G, Bradley B, Ressler KJ. Posttraumatic stress disorder is a risk factor for metabolic syndrome in an impoverished urban population. Gen Hosp Psychiatr. 2011; 33:135–42.
40. Alexander CM, Landsman PB, Teutsch SM, Haffner SM. NCEP-defined metabolic syndrome, diabetes, and prevalence of coronary heart disease among NHANES III participants age 50 years and older. Diabetes. 2003; 52:1210–4. [PubMed: 12716754]
41. Dagan Y, Zinger Y, Lavie P. Actigraphic sleep monitoring in posttraumatic stress disorder (PTSD) patients. J Psychosom Res. 1997; 42:577–81. [PubMed: 9226605]
42. Harvey AG, Schmidt DA, Scarna A, Semler CN, Goodwin GM. Sleep-related functioning in euthymic patients with bipolar disorder, patients with insomnia, and subjects without sleep problems. Am J Psychiatry. 2005; 162:50–7. [PubMed: 15625201]
43. Carskadon MA, Dement WC, Mitler MM, Guilleminault C, Zarcone VP, Spiegel R. Self-reports versus sleep laboratory findings in 122 drug-free subjects with complaints of chronic insomnia. Am J Psychiatry. 1976; 133:1382–8. [PubMed: 185919]
44. Harvey AG, Tang NKY. (Mis)perception of sleep in insomnia: a puzzle and a resolution. Psych Bull. 2012; 138:77–101.
45. Reyner A, Horne JA. Gender- and age-related differences in sleep determined by home-recorded sleep logs and actimetry from 400 adults. SLEEP. 1995; 18:127–34. [PubMed: 7792492]
46. Talbot LS, Maguen S, Metzler TJ, Schmitz M, McCaslin SE, Richards A, Perlis ML, Posner DA, Weiss B, Ruoff L, Varbel J, Neylan TC. Cognitive behavioral therapy for insomnia in posttraumatic stress disorder: a randomized controlled trial. SLEEP. 2014; 37:327–341. [PubMed: 24497661]
47. Rao MN, Blackwell T, Redline S, Punjabi NM, Barrett-Connor E, Neylan TC, Stone KL. Association between sleep duration and 24-hour urine free cortisol in the MrOS Sleep Study. PLOS One. 2013; 8
48. McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. Horm Behav. 2003; 43:2–15. [PubMed: 12614627]
49. Dallman MF, Pecoraro NC, la Fleur SE. Chronic stress and comfort foods: self-medication and abdominal obesity. Brain Behav Immun. 2005; 19:275–80. [PubMed: 15944067]
50. Bjorntorp P, Rosmond R. Neuroendocrine abnormalities in visceral obesity. Int J Obes RelaT Metab Disord. 2000; 24:S80–5. [PubMed: 10997616]
51. Zellner DA, Loaiza S, Gonzalez Z, Pita J, Morales J, Pecora D, Wolf A. Food selection changes under stress. Physiology & Behavior. 2006; 87:789–93. [PubMed: 16519909]
52. Gibson EL. Emotional influences on food choice: sensory, physiological and psychological pathways. Physiology & Behavior. 2006; 89:51–61.
53. Rosmond R. Role of stress in the pathogenesis of the metabolic syndrome. Psychoneuroendocrino. 2005; 30:1–10.
54. Rasmusson AM, Schnurr PP, Zukowska Z, Scioli E, Forman DE. Adaptation to extreme stress: post-traumatic stress disorder, neuropeptide Y and metabolic syndrome. Exp Biol Med (Maywood). 2010; 235:1150–62. [PubMed: 20881319]

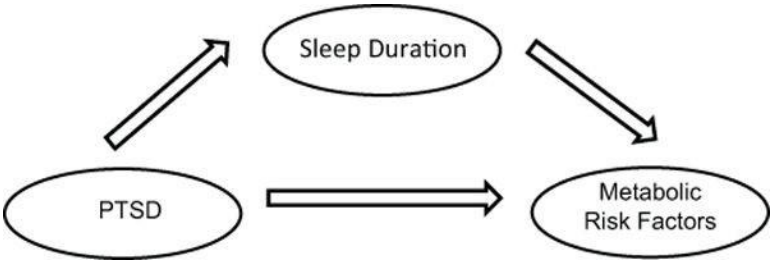

Figure 1.
Diagram of path analyses.

Table 1

Characteristics of Healthy, Unmedicated PTSD Participants and Age-Matched Controls

Variable	Group				Test Statistic	*p* value
	PTSD group (*n* = 44)		Control group (*n* = 50)			
	M* or *n	***SD* or %**	***M* or *n***	***SD* or %**		
Age (*SD*)	30.55	6.57	30.34	8.11	$t(92) = -.13$	$p = 0.89$
Gender (%)					$\chi^2(1) = .15$	$p = 0.70$
Male	22	50	23	46		
Female	22	50	27	54		
Years of education (*SD*)	14.86	2.20	15.40	2.04	$t(91) = 1.22$	$p = 0.23$
Race (%)					$\chi^2(4) = 11.29*$	$p = 0.024$
African American	5	11	1	2		
Asian American	3	7	7	14		
Caucasian	27	61	40	80		
Other	7	16	1	2		
Unknown	2	5	1	2		
Marital Status (%)					$\chi^2(3) = 8.94*$	$p = 0.030$
Single	34	77	42	84		
Married/Partnered	2	5	7	14		
Divorced	5	11	1	2		
Separated	3	7	0	0		
Veteran Status					$\chi^2(1) = 12.72***$	$p < .001$
Civilian	34	77	50	100		
Veteran	10	23	0	0		
Body Fat Percentage	30.77	8.40	26.60	10.63	$t(85) = -2.03*$	$p = .045$
CAPS	54.79	15.84	0.00	0	$t(53) = -11.90***$	$p < .001$
Current Depression					$\chi^2(1) = 9.94**$	$p = .002$
Absent	36	82	50	100		
Present	8	18	0	0		

Note. Mean values presented. CAPS = Clinician-Administered PTSD Scale.

* $p < .05$,

** $p < .01$,

*** $p < .001$. Three participants who described their race as Hispanic comprised the participants in the 'Unknown' race category.

Table 2

Sleep Duration and Metabolic Risk Factor Values

Variable	Group				Test Statistic with body fat percentage as covariate in metabolic analyses)	Test Statistic with body fat percentage and sleep diary TST as covariates)	Test Statistic with body fat percentage and actigraphy TST as covariate)
	PTSD group		Control group				
	M	*SD*	*M*	*SD*			
Diary-measured TST	6.59	1.27	7.43	0.80	F ,83) = 12.25, = 1.32 p = .001		
Actigraphy-measured TST	6.94	1.41	7.32	0.98	(1 F 83)	(	
Triglycerides (mg/dL)	137.51	73.85	90.24	40.34	(F(1 ,80) = 9.53, p = .003	F ,79) = 4.43, p = .038	F ,71) = 5.40, p = .023
Cholesterol (mg/dL)	170.26	33.75	138.89	31.93	(1 F ,80) = 14.32, p < .001	(1 F ,79) = 10.42, p = .002	(1 F ,71) = 12.91, p = .001
VLDL cholesterol (mg/dL)	26.17	11.73	17.96	8.07	(1 F ,80) = 10.01, p = .002	(1 F ,79) = 4.70, p = .033	(1 F ,71) = 5.73, p = .091
LDL cholesterol (mg/dL)	95.19	29.86	74.98	26.48	(1 F ,80) = 7.93, = 0.89 p = .006	(1 F ,79) = 6.24, = 1.11 p = .015	(1 F ,71) = 6.58, = 2.28 p = .012
HDL cholesterol (mg/dL)	47.30	12.63	45.96	11.74	(1 F ,80)	(1 F ,79) = 3.48	(1 F ,71) = 2.60
Cholesterol: HDL ratio	8.1	3.85	3.15	0.88	F(1 ,80) = 5.26, = 0.76 p = .024	(1 F ,79) = .04	(1 F ,71) = .16
Truncal fat percentage	53.00	7.10	52.00	6.16	(1 F ,80)	(1 F ,79)	(1 F ,71)
					(1	(1	(1

Table 3

Partial Correlations between Sleep Duration and Metabolic Risk Factors

	Sleep diary-measuredtotal sleep time		Actigraphy-measured total sleep time	
	r	***P***	***r***	***p***
All participants				
Triglycerides	−.35	.002	−.30	.009
Cholesterol	−.28	.017	−.20	.09
VLDL cholesterol	−.36	.002	−.31	.007
LDL cholesterol	−.18	.12	−.23	.049
HDL cholesterol	−.02	.88	.27	.022
HDL: cholesterol ratio	−.17	.14	−.37	.001
Truncal fat percentage	−.31	.007	−.44	<.001
PTSD group				
Triglycerides	−.40	.011	−.41	.007
Cholesterol	−.17	.28	−.17	.30
VLDL cholesterol	−.40	.010	−.42	.006
LDL cholesterol	−.04	.79	−.19	.23
HDL cholesterol	.02	.91	.40	.009
HDL: cholesterol ratio	−.09	.60	−.40	.009
Truncal fat percentage	−.24	.13	−.42	.006
Control group				
Triglycerides	.06	.74	.05	.77
Cholesterol	−.14	.43	−.13	.48
VLDL cholesterol	.05	.76	.05	.78
LDL cholesterol	−.21	.24	−.23	.19
HDL cholesterol	.04	.85	.11	.53
HDL: cholesterol ratio	−.14	.42	−.22	.22
Truncal fat percentage	−.44	.009	−.48	.004

Note. Partial correlations controlled for body fat percentage. VLDL = very low density lipoprotein. LDL = low density lipoprotein. HDL = high density lipoprotein.

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Sleep and Hypothalamic Pituitary Adrenal Axis Responses to Metyrapone in Posttraumatic Stress Disorder

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Abstract

Disturbed sleep is a core feature of posttraumatic stress disorder (PTSD), characterized in part by decreased delta power sleep that may result from stress-related alterations in corticotropin releasing factor (CRF), hypothalamic pituitary adrenal axis (HPA) regulation and glucocorticoid signaling. Overnight HPA axis response mediating sleep disturbances in men and women with PTSD was examined using a metyrapone challenge. Metyrapone blocks cortisol synthesis, removing negative feedback, and increases the release of hypothalamic CRF and pituitary adrenocorticotropic hormone (ACTH). Laboratory-based polysomnography was used to monitor the sleep of 66 medically healthy, medication-free men and pre-menopausal follicular phase women including 33 with chronic PTSD (16 women and 17 men) and 33 age- and sex-matched controls (14 women and 19 men) over 3 consecutive nights. Participants completed an overnight metyrapone challenge after an adaptation and baseline night of sleep and ACTH was obtained by repeated blood sampling. Metyrapone resulted in a greater increase in ACTH and greater decreases in cortisol and delta spectral power sleep in PTSD subjects compared to controls, and a greater increase in ACTH in women compared to men. There was no sex difference in metyrapone effects on delta power sleep, and no significant metyrapone by PTSD by sex interactions with either ACTH or delta power sleep. Regression analyses indicated that a greater increase in ACTH response was associated with a greater decrease in delta power sleep response in PTSD subjects, but no such relationship was found in controls. The PTSD group difference was similar in men and women. These results suggest that stress-related alterations of the HPA axis in PTSD may

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contribute to sleep difficulties. Therapeutics that target the HPA axis may offer promise as a potential future treatment for PTSD and related sleep difficulties.

Keywords

Posttraumatic Stress Disorder; Sleep; ACTH; Metyrapone; Sex Differences

INTRODUCTION

Posttraumatic stress disorder (PTSD) is a disabling consequence of trauma, manifested by recurrent and distressing re-experiencing of the traumatic event, avoidance of trauma reminders, and hyperarousal symptoms, seen in increased startle reactivity and impaired sleep that result from central and autonomic nervous system alterations (American Psychiatric Association, 2013; Hendrickson and Raskind, 2016). Disturbed sleep is one of the most prevalent PTSD symptoms, with patients reporting difficulties with non-restorative sleep, frequent awakenings and nightmares, and a debilitating impact on many domains of functioning (Hoge et al., 2007). A meta-analysis of objective sleep studies reported alterations in sleep duration and patterns in PTSD, including increased Stage 1 sleep and decreased slow wave sleep (SWS) (Kobayashi et al., 2007). Studies using spectral analysis, a powerful method that examines quantitative sleep microarchitecture, have shown that delta band spectral power, which is most prominent in slow wave NREM sleep, is decreased in PTSD (Neylan et al., 2003; Otte et al., 2007; Richards et al., 2013). Delta power sleep is driven by thalamocortical oscillations and thought to both represent the bioenergetic recovery process of cortical wake activity and replay phenomenon in models of procedural memory consolidation (Genzel et al., 2014). Functions of delta power sleep include homeostatic recovery, glucose metabolism and other fundamental biological processes (Tasali et al., 2008). Deficiencies in delta power sleep associated with PTSD may have important consequences for cognition and health.

Stress-related alterations in corticotropin releasing factor (CRF) and the hypothalamic-pituitary-adrenal (HPA) axis may account for alterations in delta power sleep in PTSD. CRF, which functions as a neurotransmitter in extrahypothalamic sites (e.g., amygdala, locus ceruleus (LC), bed nucleus of the stria terminalis (BNST)), has an arousing effect on the cortex (Reviewed in (Zorrilla and Koob, 2004)). CRF is also the primary hypothalamic neuropeptide involved in the control of adrenal secretion of cortisol. Pulsatile CRF release in the hypothalamus leads to adrenocorticotropic hormone (ACTH) release in the pituitary gland, which then stimulates adrenal cortisol release. CRF, ACTH and cortisol levels are lowest in the first few hours of sleep when delta power sleep activity is maximal (Friess et al., 1995). CRF pulses increase after 4 to 5 hours of sleep and reach peak activity at the beginning of the wake period (Gallagher et al., 1973). The rise in overall activity of the HPA axis prepares the organism for food intake, regulates changes in energy metabolism, and promotes optimal neural activity required for initiating wakeful behavior (McEwen, 1995). Studies have found an inverse relationship between delta power sleep and pulsatile cortisol release (e.g., (Vgontzas et al., 1999)). The apparent relationship between peripheral cortisol levels and delta power sleep is likely to be driven by activity of hypothalamic CRF,

supported by findings that exogenous cortisol infusion, which reduces CRF, increases delta power sleep (Friess et al., 1994).

PTSD is associated with increases in both hypothalamic and extra-hypothalamic CRF activity. CRF mediates anxiety and fear behaviors via signaling with CRF1 receptors located in the amygdala, LC, BNST, and cerebral cortex (See (Zorrilla and Koob, 2004) for review). Several studies have found higher levels of CRF in cerebrospinal fluid (CSF) in PTSD, reflecting mainly extrahypothalamic sources (Baker et al., 1999; Bremner et al., 1997; Sautter et al., 2003). Elevated peripheral CRF measured in plasma was found in veterans with PTSD compared to combat-exposed veterans without PTSD and healthy controls (de Kloet et al., 2008). Increased CRF may explain the increased ACTH and cortisol response to psychological challenge observed in individuals with PTSD (Bremner et al., 2003). However, the findings have been mixed. For example, dexamethasone suppression-CRH stimulation resulted in a blunted ACTH response in PTSD, which was interpreted to indicate possible down-regulation of CRF receptors from chronically elevated hypothalamic CRF release (Strohle et al., 2008).

Metyrapone challenge remains the strongest stimulus for endogenous CRF release that exists in the field. Metyrapone, a medication used for the diagnosis of adrenal insufficiency, blocks cyp11B1 (11ß-hydroxylase) and hence blocks the conversion of 11-deoxycortisol to cortisol. This results in reduced cortisol synthesis and increased levels of 11-deoxycortisol (Fiad et al., 1994). Reduced cortisol concentrations result in removal of glucocorticoid negative feedback to the underlying drive of the paraventricular nucleus of the hypothalamus to release CRF, but has no impact on extrahypothalamic CRF (Kalin et al., 1987). Metyrapone causes a 12-fold amplification of secretory ACTH release and results in increased awakenings and reduced delta power sleep (Jahn et al., 2003).

In two previous studies, we found that the sleep and endocrine responses to metyrapone (i.e., decrease in delta power and increase in ACTH, respectively) were significantly diminished in PTSD compared to controls. Further, in the whole sample, greater decline in delta power sleep activity was significantly correlated with greater increases in 11-deoxycortisol and ACTH measured the morning before and after metyrapone administration (Neylan et al., 2003; Otte et al., 2007), providing initial evidence of alterations in the brain response to increased hypothalamic CRF. However, metyrapone blocks cortisol synthesis for approximately 4 hours, and the endocrine response was assessed 9 hours after metyrapone was administered. Although PTSD subjects had a significantly smaller increase in ACTH compared to controls, it is unclear whether the two groups had the same immediate peak and recovery of the ACTH response proximal to metyrapone administration. In order to better understand the effect of the neuroendocrine challenge on delta power sleep in PTSD, the present study examined the effects of metyrapone on ACTH using repeated sampling of nocturnal hormone activity during sleep recordings in men and women with chronic PTSD compared to healthy controls. We tested the hypothesis that the ACTH response to metyrapone would be associated with a decreased delta power sleep response and that the relationship between ACTH and delta power sleep responses would be moderated by having PTSD. Due to previous findings of sex differences in sleep architecture and in ACTH

responses to metyrapone (Inslicht, 2014; Richards et al., 2013), we also examined sex differences in these relationships.

MATERIALS AND METHODS

The present study used a cross-sectional, 2 × 2 design (PTSD/control × female/male) involving 66 medically healthy, non-medicated adults aged 19–39 years in an inpatient sleep laboratory. The study sample was comprised of 33 individuals with current chronic PTSD (16 women and 17 men; 48% female) and 33 control subjects (14 women and 19 men; 42% female). This sample was drawn from a larger study of 93 participants. We excluded data from 10 participants due to side effects or an inadequate metyrapone dose and from 17 participants due to missing data related to difficulties in blood collection over the night or poor quality sleep EEG recordings. Chronic PTSD was defined by fulfillment of DSM-IV criteria for chronic PTSD on the Clinician-Administered PTSD Scale (CAPS) and a CAPS score >40.

Female participants were premenopausal (having at least one menstrual period in past 12 months) as determined by medical screening interview, and were scheduled during the follicular phase of the menstrual cycle within 10 days following the onset of menses. Exclusion criteria included extreme morning or evening tendencies or irregular sleep wake schedules as documented by actigraphy and sleep diary; a diagnosis of sleep apnea; history of traumatic brain injury, presence of neurologic disorders or systemic illness; use of psychiatric, anticonvulsant, antihypertensive, sympathomimetic, steroidal, statin or other prescription medications; anemia, recent blood donation in the past 2 months, obesity (defined as BMI >30); prominent suicidal or homicidal ideation; alcohol abuse or dependence in the prior 2 years; substance abuse or dependence in the previous year; any psychiatric disorder with psychotic features; bipolar disorder or obsessive compulsive disorder; and pregnancy. Exclusion criteria for control subjects also included a lifetime history of major depressive disorder (MDD) or panic disorder.

Nocturnal blood sampling was obtained as a part of a 3-night polysomnography sleep study (night 1= adaptation, nights 2–3= pre- and post-metyrapone administration) conducted in a Clinical Research Center (CRC). Habitual sleep onset time, calculated from the actigraphy and sleep diary data was used to determine the timing of procedures on the CRC. Subjects were instructed to maintain the same sleep onset time during the week of actigraphy monitoring as well as during the CRC admission. All subjects were alcohol and drug-free and restricted to having one optional cup of caffeinated coffee each morning. Two hours before habitual sleep onset, a catheter was inserted in an antecubital vein for repeated sampling of blood on nights 2 and 3 (5.5 ccs every 15 min providing 32 samples for each night). Assays for ACTH and cortisol were performed on every other sample, resulting in 16 pre- and 16 post-metyrapone measures. Blood was sampled for up to 8 h following the final dose of metyrapone (M = 6.5 h; SD = 1.9 h). A single additional blood sample was obtained in the morning, at habitual wake time, to measure ACTH, cortisol, and 11-deoxycortisol.

Subjects were allowed to walk on the CRC, but were instructed to avoid vigorous activity. They were allowed to watch TV, play games and talk to staff. All subjects were provided

meals at 8:00 AM, 12:00 noon, and 5:30 PM. Prior to the 3rd night of polysomnography subjects were given metyrapone as described below. The timing of the doses were adjusted so that the last dose occurred at the subject's diary and actigraphy determined habitual bedtime. Nocturnal blood sampling provided the opportunity to evaluate the association between acute HPA axis and sleep responses to metyrapone over the course of the night. This research was approved by the Committee on Human Research at the University of California, San Francisco. All participants provided written informed consent before participating in any study procedures.

Measures

Demographics.—Self-report questionnaires were used to gather demographic data.

Psychiatric Diagnoses and Trauma History.—The CAPS was used to assess current and lifetime PTSD (Blake et al., 1995). The CAPS assesses the frequency and intensity of PTSD symptoms corresponding to the re-experiencing, avoidance and hyperarousal symptoms described in the DSM-IV diagnostic criteria. Diagnosis of PTSD was based on symptoms experienced in the previous month, which were associated with the participant's self-identified worst traumatic event.

The **Structured Clinical Interview for DSM-IV, Non-Patient edition (SCID-NP)** was used to diagnose all other psychiatric disorders, including MDD (Spitzer et al., 1992). The type of trauma exposure and age of occurrence was assessed using the **Life Stressor Checklist- Revised** interview (Wolfe et al., 1996).

Metyrapone Challenge and Biological Assays.—On the day after the second night on the GCRC, subjects were given an oral dose of metyrapone of 750mg every 4 hours starting at 12 hours before habitual sleep (HS) onset, for a total of 3 doses (12h, 8h, 4h before HS), and one dose of 2.5g at HS along with 30ccs of an antacid to minimize gastrointestinal upset. The schedule of four doses every 4 hours ending at bedtime adheres to the standard overnight metyrapone test used for evaluating pituitary reactivity. Given our particular focus on slow wave sleep and HPA axis activity, this schedule was selected to ensure that metyrapone was absorbed and had time to inhibit the enzymatic conversion of 11-deoxycortisol to cortisol and for the elimination of cortisol to exert an effect on CRF. The 2.5 mg dose given at bedtime was intended to eliminate any group differences in residual adrenal cortisol release and is similar to doses used in several other studies (Jahn et al., 2003; Yehuda et al., 1996).

Two hours before habitual sleep onset, a catheter was inserted in an antecubital vein for repeated sampling of blood on nights 2 and 3 (5.5 ccs q 15 minutes providing up to 32 samples for ACTH (16 pre- and 16 post-metyrapone)). Blood was sampled for up to 8 hours following the final dose of metyrapone (M = 6.5 hours; SD = 1.9 hours).

Cortisol.—Blood was collected using an EDTA tube and plasma (q 30 minutes) was separated by centrifugation. Plasma levels of cortisol were measured using the Access Chemiluminescent Immunoassay (Beckman Coulter, Fullerton, CA) at the Specialized Assay Core, Brigham and Women's Hospital.

ACTH.—Blood was collected using an EDTA tube and plasma was separated by centrifugation. Plasma levels of ACTH were measured using a commercially available immunoradiometric assay (DiaSorin Inc., Stillwater, Minnesota) at the Specialized Assay Core, Brigham and Women's Hospital. The DiaSorin ACTH immunoradiometric assay is designed to detect whole molecule ACTH and is a more sensitive method for the detection of ACTH in plasma than a radioimmunoassay.

Polysomnographic Measurement.—Polysomnography recordings were obtained with ambulatory polysomnography (Nihon Kohden Trackit Ambulatory Recording System). The parameters recorded included an electroencephalogram (EEG) at leads C3, C4, O1 and O2, left and right electrooculograms (EOG), submental electromyogram (EMG), bilateral anterior tibialis EMGs, and electrocardiogram (EKG) in accordance with standardized guidelines (Rechtschaffen, 1968). Electrode impedance was set at < 5 kohm at the start of the recording. The EEG and EOG leads were referenced to linked mastoids. Raw EEG signals were filtered and amplified, then digitized at 256 Hz and recorded to a removable hard disk in European Data Format (EDF) file format. The low frequency and high frequency hardware filters on the recorder were single pole analog filters with 3 db points at 0.5 Hz and 100 Hz. Pass Plus was utilized for both visual scoring and quantitative EEG analysis of the digitized polysomnography data.

Power Spectral Analysis.—Pass Plus (Delta Software) analytic software was used to measure sleep activity in all frequency bands delta through gamma from the C3 electrode by power spectral analysis (PSA). The C4 electrode was used if there was excessive artifact. A limitation of Pass Plus is that artifact removal is accomplished by removal of whole epochs tagged with artifact. This has the potential to introduce additional confounds given the removal of typically longer bouts of uncontaminated EEG. Therefore, epochs were tagged for slow and fast artifact for additional analyses. Primary analyses were conducted with all epochs and then checked for the impact of removal of epochs with slow and fast artifact. Removal of fast artifact (for bandwidths alpha and above) and slow artifact (for bandwidths delta and theta) did not significantly impact our findings in NREM sleep. All results are therefore reported without removal of epochs containing artifact. Pass Plus applied a 5μV smoothing constant to eliminate spurious waves caused by electrical jitter. PSA was conducted on all epochs of NREM and REM sleep. Epochs visually scored as wake were not included in these analyses. Visual scoring was conducted by one of the authors, a highly experienced registered polysomnography technician who was blind to PTSD status, but not to pre- vs. post- metyrapone status. Pass Plus was used to perform Fast Fourier Transformation analysis on 4.0 second Welch tapered windows with 2 second overlap, yielding 15 windows per 30-second epoch. Power spectra for delta (1–4 Hz) were analyzed to address our primary hypothesis with respect to delta power sleep.

Statistical Analysis

The two groups defined by PTSD status were compared on demographic and clinical characteristics using t-tests for continuous variables and chi-squared tests for categorical variables. ACTH (ng/ml), cortisol (μg/ml), and delta sleep spectral power density (μV^2) were natural log transformed to normalize their distributions. ACTH measures were obtained up

to 16 times per night (30 min apart). Mean delta sleep power was integrated over each 30 min period to provide sleep measures commensurate with the ACTH measurement time points. Separate random-intercepts linear mixed models were used to predict ACTH and mean delta power per epoch, respectively, from metyrapone status, PTSD, sex, and their full interactions.

To assess the relationship between metyrapone effects on ACTH and on sleep, mean transformed ACTH and delta spectral power values were calculated for each subject averaged over each night, pre- and post- metyrapone administration. A linear regression analysis was conducted on baseline-adjusted change in whole-night delta power sleep predicted from baseline-adjusted change in whole-night ACTH, sex, PTSD status, and their full interactions. The model was parameterized using the delta power sleep difference score (post- minus pre-metyrapone nights) as the outcome, with pre-metyrapone delta power sleep as a covariate. This model is equivalent to using baseline-adjusted post-metyrapone delta power sleep as the outcome, but provides a more interpretable regression coefficient in terms of the effects of predictors on difference scores. Similarly, both ACTH pre-post difference scores and the ACTH pre-treatment scores were included as predictors. Again, this redundancy in parameterization does not affect the model fit, but provides a regression coefficient for the baseline-adjusted ACTH response score to facilitate interpretation.

RESULTS

Demographic Data and Clinical Characteristics.

Sample characteristics are presented in Table 1. There were no significant differences in sex or race/ethnicity distribution between PTSD and control subjects. As expected, PTSD subjects had higher CAPS scores and rates of current MDD. As per the exclusion criteria, no control subjects met criteria for current MDD. Six control subjects (3 men and 3 women) reported a lifetime history of a traumatic criterion A1 event, but all had a current and lifetime CAPS score of zero. Among women, 2 of the 14 controls and 5 of the 16 PTSD participants reported taking hormonal birth control pills, but there were no differences by group, $X^2(1, N = 30) = 1.20$, $p = .273$.

Potential Confounders.

There were no significant associations between BMI, smoking, current MDD, and use of hormonal birth control on baseline-adjusted delta power sleep and ACTH, therefore these variables were not included in further analyses. MDD was associated with the delta power sleep response (lower delta power on night 3 even after adjusting for night 2; $p = .006$), and childhood trauma was associated with ACTH response (higher ACTH on night 3 adjusted for night 2; $p = .006$). However, both of these variables were highly correlated with PTSD status: All 6 people with MDD were in the PTSD+ group, and 14 out of the 17 with childhood trauma were in the PTSD+ group. Since both depression and childhood trauma were highly confounded with PTSD, these variables were not included in further analyses.

Endocrine and Sleep Effects of Metyrapone.

Table 2 shows the mean values and statistical contrasts for the sleep and endocrine measures in each group before and after metyrapone administration. As expected, metyrapone administration led to significant suppression of nocturnal cortisol and enhancement of nocturnal ACTH. Metyrapone also significantly reduced mean delta sleep power. Furthermore, as seen in Table 2, we observed significant interactions of metyrapone with PTSD driven by greater effects of metyrapone on ACTH, cortisol, and delta spectral power sleep in the PTSD subjects. Nocturnal endocrine data before and after metyrapone for PTSD subjects and controls is shown in Figures 1 and 2. Specifically, compared to controls, PTSD subjects had a greater decrease in cortisol (Fig 1), and a greater increase in ACTH (Fig 2.). PTSD subjects also had a greater decrease in delta spectral power in response to metyrapone (Table 2). Examination of other sleep bands indicated that while there was increased beta power on the pre-metyrapone night in the PTSD group, there was no differential group response to metyrapone in any of the sleep bands outside of delta (all p's for interaction terms >.286; supplementary Table 1). Metyrapone caused an increase in power in the Beta1, Beta2, and Gamma frequency bands.

Additional analyses examining sex differences indicated that there was a greater overall nocturnal ACTH response in women (M=1.65, 95% CI [1.54, 1.76], SD=1.08) compared to men (M=1.04, 95% CI [0.94, 1.14], SD = 0.67), leading to a significant metyrapone by sex interaction $F(1, 1636) = 65.39$, $p < .001$. There was no significant difference in delta power sleep response in women (M =−8.1, 95% CI [−7.9, −8.3], SD = 20.5) compared to men (M = −13.1, 95% CI [−13.3, −12.9], SD = 24.9), metyrapone by sex interaction $F(1, 1636) = 1.19$, $p = .275$. There were also no significant 3-way metyrapone X PTSD X sex interactions for either ACTH or delta power, $F(1, 1636) = 2.22$, $p = .137$ for ACTH; $F(1, 1526) = 0.76$, $p = .383$ for delta power.

Examination of single point awakening hormone levels indicated that metyrapone administration resulted in the reduction of awakening morning cortisol from M=4.9, SD=0.31, to M=3.67, SD = 0.36, $F(1, 55) = 412.63$, $p < .001$, while awakening 11-deoxycortisol was enhanced from M=0.76 to M=4.99, $F(1, 55) =3396.57$, $p < .001$. Awakening ACTH increased from M=3.4 to M=5.6, $F(1, 63) = 384.9$, $p < .001$. Furthermore, PTSD subjects compared to controls showed elevated awakening 11-deoxycortisol levels on both days, but there was no differential response to metyrapone, $F(1, 55) = .02$, $p = .899$). There were also no group differences in ACTH or cortisol pre- or post-metyrapone. The awakening ACTH response was marginally greater in women (M=2.37, 95% CI [1.98, 2.76], SD=1.00) than in men (M=2.00, 95% CI [1.63, 2.36], SD=0.90), interaction $F(1, 63) = 2.98$, $p = .089$.

Relationship between ACTH response and Sleep.

Figure 3 shows the relationship between metyrapone-related change in mean delta power and change in ACTH. The linear regression analysis showed that this relationship differs between PTSD and controls, ACTH by group interaction $F (1, 56) = 10.11$, $p = .002$. Within-group contrasts showed that the relationship was negative in PTSD but not in controls, B =

−0.27 (SE = 0.07), p < .001 for PTSD and B = 0.07 (SE. = 0.08), p = .375 for controls. This PTSD group difference was not moderated by sex, interaction F (1, 58) = 0.00, p = .999.

DISCUSSION

Our findings indicate that metyrapone resulted in a greater decrease in nocturnal cortisol, greater increase in ACTH, and greater decrease in delta power in sleep of PTSD subjects compared to controls. Consistent with our hypotheses, we found that PTSD moderated the association between the ACTH and delta power sleep response to metyrapone. Specifically, we found that a greater increase in ACTH was associated with a greater decrease in delta power sleep in PTSD subjects, but no such relationship in controls. These findings highlight alterations of the HPA axis in PTSD that may account for sleep disturbances that are so common in this disorder.

There are several potential explanations that may account for group differences in hormonal response. Metyrapone, by blocking the conversion of 11-deoxycortisol to cortisol, results in decreased cortisol and increased 11-deoxycortisol (Fiad et al., 1994). In healthy individuals, it is expected that CRH and ACTH levels would increase in response to decreasing cortisol levels because of reduced feedback inhibition. Our findings of a greater decrease in cortisol, and a greater increase in ACTH in PTSD subjects suggests even greater responsiveness of underlying HPA axis drive when eliminating naturally circulating cortisol by administration of metyrapone. Our findings of elevated 11-deoxycortisol the morning after both nights, and numerically higher cortisol pre-metyrapone in PTSD suggest some marginal increased adrenal activity in producing cortisol or possibly decreased clearance of cortisol/11-deoxycortisol. The greater effect of metyrapone in PTSD on the HPA axis could also be explained increased sensitivity to glucocorticoid signaling in PTSD which could produce a greater hypothalamic release of corticotropin releasing factor (CRF) and/or pituitary release of ACTH. Our findings are consistent with numerous studies that found enhanced responsiveness of glucocorticoids in PTSD (reviewed in (Daskalakis et al., 2013)). There could also be PTSD effects on sensitivity of CRF signaling at the level of the pituitary. The increased 11-deoxycortisol and the numerically, though non-significantly, increased cortisol pre-metyrapone in PTSD in the setting of virtually identical levels of ACTH suggest that the adrenal gland shows increased responsiveness to ACTH signaling or reduced clearance in the PTSD group.

Polymorphisms of genes that regulate both CRF and glucocorticoid receptor activity have been associated with PTSD, suggesting that individual variability in HPA axis regulation may be associated with risk for PTSD (reviewed in (Castro-Vale et al., 2016)). While the precise mechanism has yet to be determined, overall, our findings suggest that blocking cortisol produced a greater biological response in PTSD. Given this, it is plausible to consider that therapeutics that target the HPA axis, potentially by blocking CRF or by reducing glucocorticoid signaling, may offer promise as a potential future treatment for PTSD and related sleep difficulties. Although, a recently completed clinical trial of a CRF antagonist in PTSD did not produce an overall greater response relative to placebo, a subgroup of subjects who were GG homozygotes for the CRF1 SNP rs110402 who had history of childhood abuse did show greater response to the drug (Dunlop BW, In Press).

While the precise mechanism accounting for the effect of metyrapone on delta power sleep is not entirely delineated, one possible pathway may be through the LC. PVN CRF neurons project to the LC, where CRF type-1 receptors (CRF1) are expressed (reviewed in (Zitnik, 2016)). Metyrapone administration results in c-fos induction in the LC, indicating neuronal activation (Rotllant et al., 2002). Administration of a CRF1-antagonist prevents the effects of CRF on LC neurons (Schulz et al., 1996). Thus, CRF projections from the PVN to the LC may be a point of integration between neurohormonal and neurotransmitter CRF systems (Valentino et al., 1992). The LC is strongly implicated in arousal and waking, with evidence of interactions with suprachiasmatic nucleus (SCN) circuitry that drives circadian rhythms in sleep–wake cycles (Aston-Jones et al., 2007; Schwartz and Kilduff, 2015). It is plausible that stimulation of the LC by CRF is of sufficient magnitude to increase arousal signaling and reduce delta power sleep. While we considered an alternative explanation in which PTSD subjects may have been more arousable, resulting in greater awakenings during blood collection, we did not find any evidence of an effect of PTSD on sleep maintenance in this study or in number of awakenings on the pre-metyrapone night in our previous report of this data (Richards et al., 2013).

Our findings of a greater ACTH response and decreased delta power sleep response to metyrapone in PTSD subjects compared to controls contrasts with our previous studies in which we found that the increase in ACTH and decrease in delta power sleep responses to metyrapone were significantly diminished in individuals with PTSD compared to controls (Neylan et al., 2003; Otte et al., 2007). We considered the possibility that this difference may be related to differences in the timing and measurement of hormonal responses. Our initial studies determined ACTH from blood samples that were drawn at a single time point, delayed to the morning after an overnight metyrapone test (Neylan et al., 2003; Otte et al., 2007). In the present study, we were interested in capturing nocturnal hormonal responses in relation to sleep, and so used values that were based on ACTH values and delta power spectral energy values over the whole night which was a methodological strength over the previous studies. An additional notable difference in methodology is that in the previous studies only 3 grams (4 doses of 750 mg) of metyrapone were administered compared to 4.75 grams in this study (3 doses of 750 mg and 2.5 grams at lights out). In the current and past studies (Neylan et al., 2003; Otte et al., 2007), there was no differential effect of metyrapone in PTSD on the suppression of the single measure of morning cortisol. The differential effect of metyrapone on cortisol suppression was only observable with nocturnal sampling. Jahn previously reported a dose response effect on both ACTH and SWS (Jahn et al., 2003). It is possible that lower doses used in our prior studies resulted in rebound of cortisol, which could have led to some recovery of SWS similar to what has been demonstrated in experimental infusion of cortisol. PTSD subjects may have been more sensitive to this higher dose of metyrapone, resulting in lower nocturnal cortisol than controls arising from greater inhibition of the enzymatic conversion of 11-deoxycortisol to cortisol (Born et al., 1991).

Another potential difference in studies was in the age of participants. Our previous studies consisted of male Vietnam combat veterans who were in their late forties and pre-menopausal female civilians in their mid-thirties (Neylan et al., 2003; Otte et al., 2007). In this study, participants were relatively younger, with a mean age of 30 in both sexes. A large

body of evidence has shown that delta power sleep, measured by visual sleep scoring or by quantitative analysis, diminishes with age (For review see (Landolt and Borbely, 2001)).

While our results indicated a sex difference in ACTH response to metyrapone, sex was not found to moderate PTSD group differences in either ACTH response or delta power sleep response, as indicated by non-significant group by sex by metyrapone interactions. Sex also did not moderate the relationship between ACTH response and delta power sleep response, as indicated by a non-significant group by sex interaction in the regression model predicting delta power sleep response from ACTH response to metyrapone. However, it is important to consider that we had limited power to detect three-way interactions in this study. Moreover, all women in this study participated during the follicular phase of their menstrual cycle when estrogen and progesterone are relatively low and several women in this study were on hormonal birth control (2 controls and 5 PTSD women). More robust sex differences may emerge in studies that examine women at other menstrual phases when estrogen and progesterone levels are higher, and with larger study samples.

There are several additional limitations to consider. Six participants with PTSD also met criteria for depression, and it is possible that depression may impact both CRF and sleep. Fourteen participants with PTSD and three controls reported childhood trauma. Since childhood trauma was also associated with ACTH response, it may account for the effect of PTSD on ACTH. Our relatively small sample size might have reduced our power to detect a significant interaction between PTSD and sex on our outcomes. Further, we are not able to confirm whether our findings are a result of alterations in glucocorticoid, CRF, or ACTH signaling or receptor sensitivity or clearance.

Strengths of this study include nocturnal sampling of hormone activity during sleep across the night, allowing us to confirm that the effect of metyrapone on delta power sleep and hormones during the active phase of metyrapone’s action. Although, limited by power constraints, equally balanced groups of women and age-matched men with and without PTSD allowed us to explore the interaction of PTSD and sex on the relationship between ACTH and sleep responses to metyrapone.

To summarize, our data indicates that PTSD status was associated with a greater increase in ACTH, a greater decrease in cortisol and in delta power sleep in response to metyrapone compared to controls. We also found that PTSD status moderated the association between the ACTH response and delta power sleep response to metyrapone. Our findings may reflect increased hypothalamic CRF, which could result from increased CRF receptor sensitivity and/or increased negative feedback inhibition in PTSD. Implications of these findings are that pharmacological agents that target the HPA axis, such as CRF or GR antagonists, may have therapeutic effects on PTSD-related sleep disturbances. In fact, there may be greater benefits on sleep in individuals experiencing PTSD than in those without any psychiatric conditions. Further studies are needed in the realm of other hormones (i.e., growth hormone, progesterone) that are affected by metyrapone and may modulate sleep.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

American Psychiatric Association, 2013 Diagnostic and statistical manual of mental disorders, (5th ed). Washington, DC.

Aston-Jones G, Gonzalez M, Doran S, 2007 Role of the locus coeruleus-norepinephrine system in arousal and circadian regulation of the sleep–wake cycle in: Ordway GA, Schwartz MA, Frazer A (Eds.), Brain Norepinephrine: Neurobiology and Therapeutics Cambridge University Press, New York, NY, US, pp. 157–195.

Baker DG, West SA, Nicholson WE, Ekhator NN, Kasckow JW, Hill KK, Bruce AB, Orth DN, Geracioti TD, Jr., 1999 Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. Am. J. Psychiatry 156, 585–588. [PubMed: 10200738]

Blake DD, Weathers FW, Nagy LM, Kaloupek DG, Gusman FD, Charney DS, Keane TM, 1995 The development of a clinician-administered PTSD scale. J. Trauma. Stress 8, 75–90. [PubMed: 7712061]

Born J, DeKloet ER, Wenz H, Kern W, Fehm HL, 1991 Gluco-and antimineralocorticoid effects on human sleep: a role of central corticosteroid receptors. Am. J. Physiol 260, E183–188. [PubMed: 1996621]

Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, Southwick SM, Nemeroff CB, Charney DS, 1997 Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. Am. J. Psychiatry 154, 624–629. [PubMed: 9137116]

Bremner JD, Vythilingam M, Vermetten E, Adil J, Khan S, Nazeer A, Afzal N, McGlashan T, Elzinga B, Anderson GM, Heninger G, Southwick SM, Charney DS, 2003 Cortisol response to a cognitive stress challenge in posttraumatic stress disorder (PTSD) related to childhood abuse. Psychoneuroendocrinology 28, 733–750. [PubMed: 12812861]

Castro-Vale I, van Rossum EF, Machado JC, Mota-Cardoso R, Carvalho D, 2016 Genetics of glucocorticoid regulation and posttraumatic stress disorder--What do we know? Neurosci. Biobehav. Rev 63, 143–157. [PubMed: 26872620]

Daskalakis NP, Lehrner A, Yehuda R, 2013 Endocrine aspects of post-traumatic stress disorder and implications for diagnosis and treatment. Endocrinol. Metab. Clin. North Am 42, 503–513. [PubMed: 24011883]

de Kloet CS, Vermetten E, Geuze E, Lentjes EG, Heijnen CJ, Stalla GK, Westenberg HG, 2008 Elevated plasma corticotrophin-releasing hormone levels in veterans with posttraumatic stress disorder. Prog. Brain Res 167, 287–291. [PubMed: 18037027]

Dunlop BW BE, Iosifescu D, Mathew SJ, Neylan TC, Pape JC, Carrillo-Roa T, Green C, Kinkead B, Grigoriadis D, Rothbaum BO, Nemeroff CB, Mayberg HS. , In Press. Corticotropin-Releasing Factor Type 1 Receptor Antagonism is Ineffective for women with Posttraumatic Stress Disorder. Biol. Psychiatry

Fiad TM, Kirby JM, Cunningham SK, McKenna TJ, 1994 The overnight single-dose metyrapone test is a simple and reliable index of the hypothalamic-pituitary-adrenal axis [see comments]. Clin. Endocrinol. (Oxf.) 40, 603–609. [PubMed: 8013141]

Friess E, U VB, Wiedemann K, Lauer CJ, Holsboer F, 1994 Effects of pulsatile cortisol infusion on sleep-EEG and nocturnal growth hormone release in healthy men. J. Sleep Res 3, 73–79. [PubMed: 10607111]

Friess E, Wiedemann K, Steiger A, Holsboer F, 1995 The hypothalamic-pituitary-adrenocortical system and sleep in man. Adv. Neuroimmunol 5, 111–125. [PubMed: 7496607]

Gallagher TF, Yoshida K, Roffwarg HD, Fukushima DK, Weitzman ED, Hellman L, 1973 ACTH and cortisol secretory patterns in man. J. Clin. Endocrinol. Metab 36, 1058–1068. [PubMed: 4350348]

Genzel L, Kroes MC, Dresler M, Battaglia FP, 2014 Light sleep versus slow wave sleep in memory consolidation: a question of global versus local processes? Trends Neurosci 37, 10–19. [PubMed: 24210928]

Hendrickson RC, Raskind MA, 2016 Noradrenergic dysregulation in the pathophysiology of PTSD. Exp Neurol 284, 181–195. [PubMed: 27222130]

Hoge CW, Terhakopian A, Castro CA, Messer SC, Engel CC, 2007 Association of posttraumatic stress disorder with somatic symptoms, health care visits, and absenteeism among Iraq war veterans. Am. J. Psychiatry 164, 150–153. [PubMed: 17202557]

Inslicht SS, Richards A, Madden E, Rao MN, O'Donovan A, Talbot LS, Rucker E, Metzler TJ, Hauger RL, Neylan TC, 2014 Sex Differences in Neurosteroid and Hormonal Responses to Metyrapone in Posttraumatic Stress Disorder. Psychopharmacology (Berl.) 231, 3581–3595. [PubMed: 24952092]

Jahn H, Kiefer F, Schick M, Yassouridis A, Steiger A, Kellner M, Wiedemann K, 2003 Sleep endocrine effects of the 11-beta-hydroxysteroiddehydrogenase inhibitor metyrapone. Sleep 26, 823–829. [PubMed: 14655915]

Kalin NH, Shelton SE, Barksdale CM, Brownfield MS, 1987 A diurnal rhythm in cerebrospinal fluid corticotrophin-releasing hormone different from the rhythm of pituitary-adrenal activity. Brain Res 426, 385–391. [PubMed: 2825918]

Kobayashi I, Boarts JM, Delahanty DL, 2007 Polysomnographically measured sleep abnormalities in PTSD: a meta-analytic review. Psychophysiology 44, 660–669. [PubMed: 17521374]

Landolt HP, Borbely AA, 2001 Age-dependent changes in sleep EEG topography. Clin. Neurophysiol 112, 369–377. [PubMed: 11165543]

McEwen BS, 1995 Neuroendocrine Interactions, in: Bloom FE, Kupfer DJ (Eds.), Psychopharmacology: the fourth generation of progress Raven Press, New York, NY, pp. 705–718.

Neylan TC, Lenoci M, Maglione ML, Rosenlicht NZ, Metzler TJ, Otte C, Schoenfeld FB, Yehuda R, Marmar CR, 2003 Delta sleep response to metyrapone in posttraumatic stress disorder. Neuropsychopharmacology 28, 1666–1676. [PubMed: 12799616]

Otte C, Lenoci M, Metzler T, Yehuda R, Marmar CR, Neylan TC, 2007 Effects of metyrapone on hypothalamic-pituitary-adrenal axis and sleep in women with post-traumatic stress disorder. Biol. Psychiatry 61, 952–956. [PubMed: 17336940]

Richards A, Metzler TJ, Ruoff LM, Inslicht SS, Rao M, Talbot LS, Neylan TC, 2013 Sex differences in objective measures of sleep in post-traumatic stress disorder and healthy control subjects. J. Sleep Res 22, 679–687. [PubMed: 23763708]

Rotllant D, Ons S, Carrasco J, Armario A, 2002 Evidence that metyrapone can act as a stressor: effect on pituitary- adrenal hormones, plasma glucose and brain c-fos induction. Eur. J. Neurosci 16, 693–700. [PubMed: 12270045]

Sautter FJ, Bissette G, Wiley J, Manguno-Mire G, Schoenbachler B, Myers L, Johnson JE, Cerbone A, Malaspina D, 2003 Corticotropin-releasing factor in posttraumatic stress disorder (PTSD) with secondary psychotic symptoms, nonpsychotic PTSD, and healthy control subjects. Biol. Psychiatry 54, 1382–1388. [PubMed: 14675802]

Schwartz MD, Kilduff TS, 2015 The Neurobiology of Sleep and Wakefulness. Psychiatr. Clin. North Am 38, 615–644. [PubMed: 26600100]

Spitzer RL, Williams JB, Gibbon M, First MB, 1992 The Structured Clinical Interview for DSM-III--R (SCID) : I. History, rationale, and description. Arch. Gen. Psychiatry 49, 624–629. [PubMed: 1637252]

Strohle A, Scheel M, Modell S, Holsboer F, 2008 Blunted ACTH response to dexamethasone suppression-CRH stimulation in posttraumatic stress disorder. J. Psychiatr. Res 42, 1185–1188. [PubMed: 18342888]

Tasali E, Leproult R, Ehrmann DA, Van Cauter E, 2008 Slow-wave sleep and the risk of type 2 diabetes in humans. Proc. Natl. Acad. Sci. U. S. A 105, 1044–1049. [PubMed: 18172212]

Valentino RJ, Page M, Van Bockstaele E, Aston-Jones G, 1992 Corticotropin-releasing factor innervation of the locus coeruleus region: distribution of fibers and sources of input. Neuroscience 48, 689–705. [PubMed: 1376457]

Vgontzas AN, Mastorakos G, Bixler EO, Kales A, Gold PW, Chrousos GP, 1999 Sleep deprivation effects on the activity of the hypothalamic-pituitary-adrenal and growth axes: potential clinical implications. Clin. Endocrinol. (Oxf.) 51, 205–215. [PubMed: 10468992]

Wolfe J, Kimerling R, Brown PJ, Chresman KR, Levin K, 1996 Psychometric review of the life stressor checklist-revised Sidran Press, Lutherville, MD.

Yehuda R, Levengood RA, Schmeidler J, Wilson S, Guo LS, Gerber D, 1996 Increased pituitary activation following metyrapone administration in post-traumatic stress disorder. Psychoneuroendocrinology 21, 1–16. [PubMed: 8778898]

Zitnik GA, 2016 Control of arousal through neuropeptide afferents of the locus coeruleus. Brain Res 1641, 338–350. [PubMed: 26688115]

Zorrilla EP, Koob GF, 2004 The therapeutic potential of CRF1 antagonists for anxiety. Expert Opin Investig Drugs 13, 799–828.

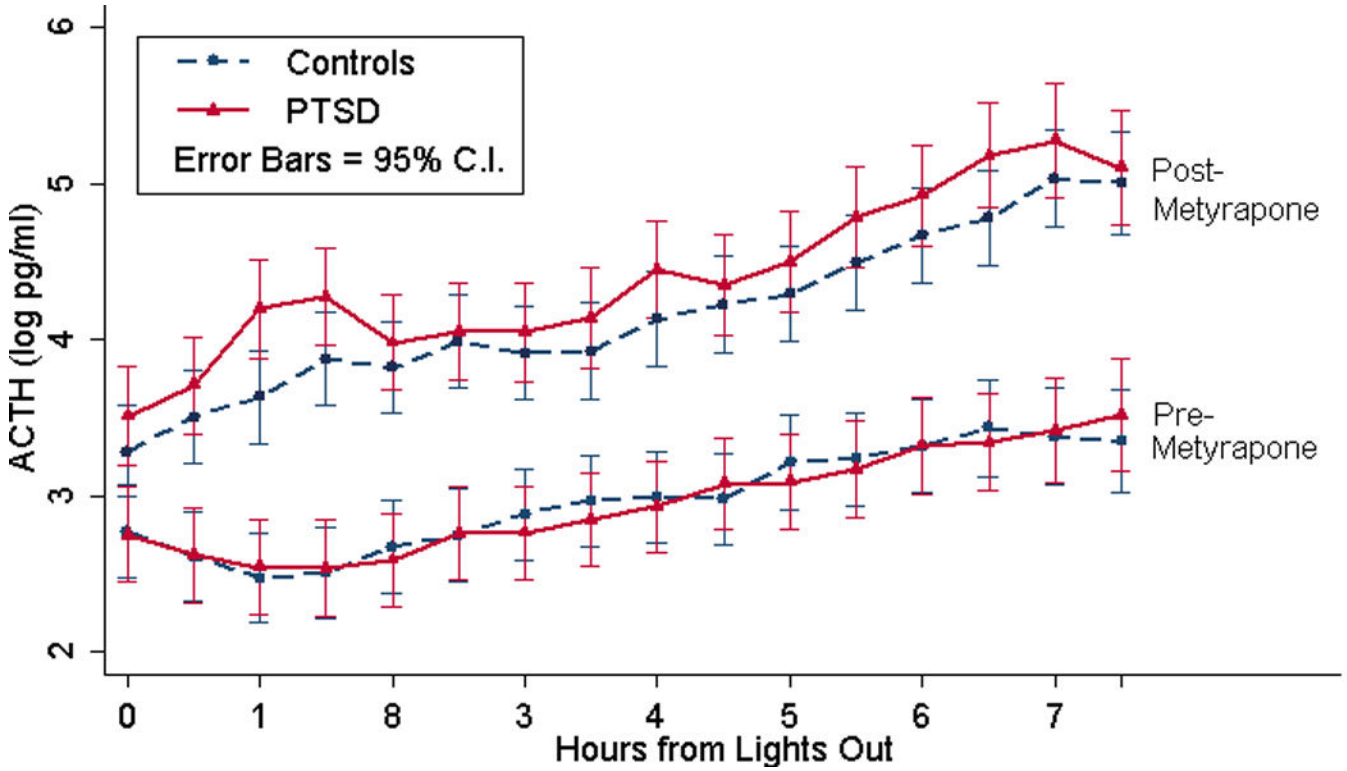

Fig 1.

Time course of mean cortisol level during pre- and post-metyrapone nights, by PTSD status

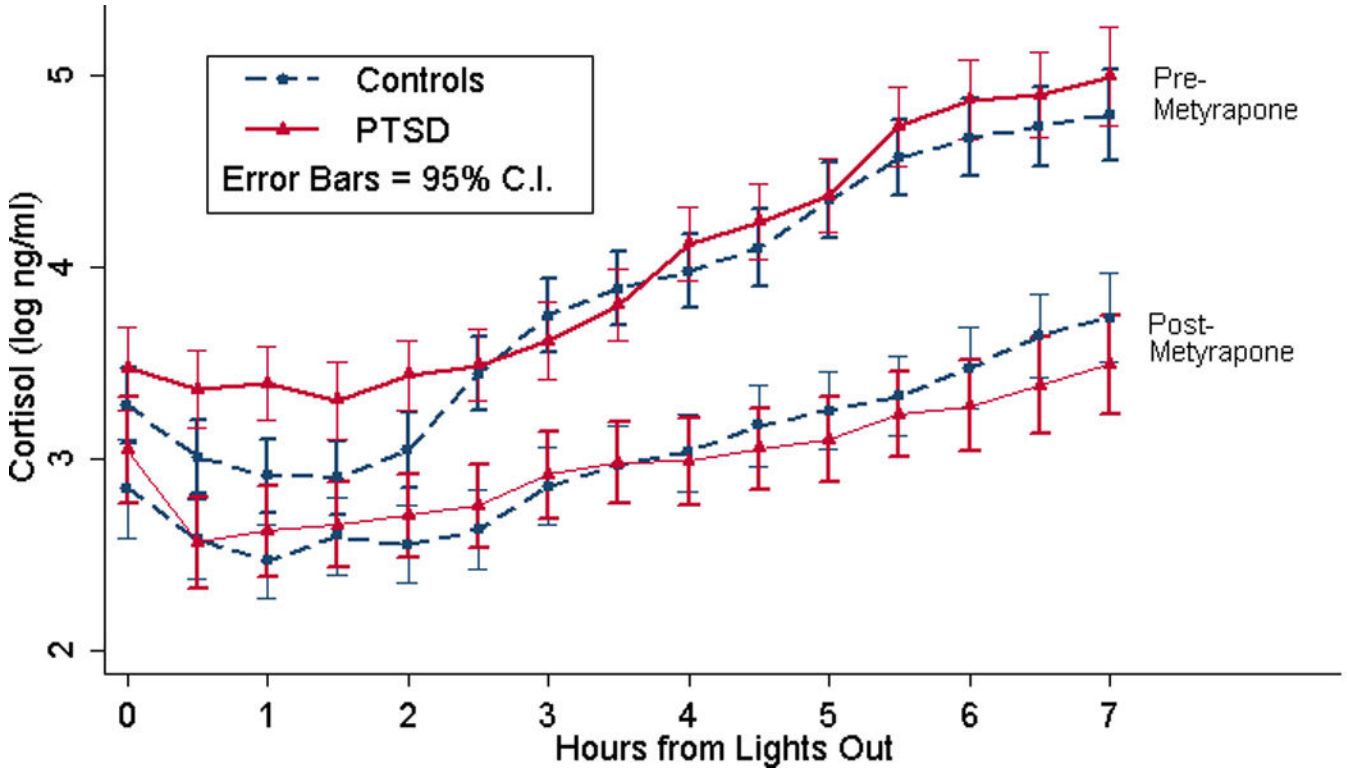

Fig 2.
Time course of mean ACTH level during pre- and post-metyrapone nights, by PTSD status

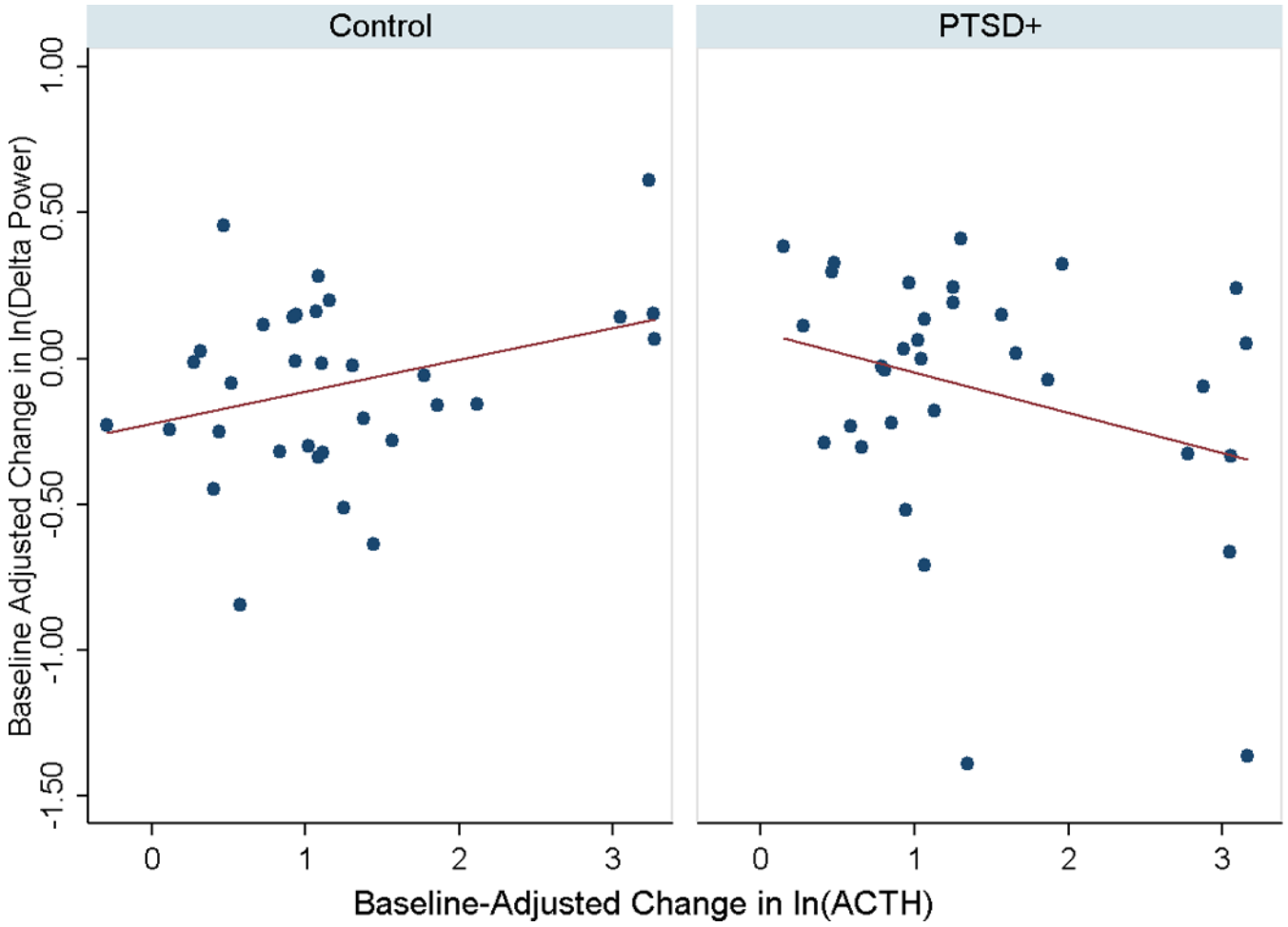

Fig 3.
Relationship of metyrapone-related change in delta power sleep to change in ACTH, by PTSD status

Table 1.

Demographic and clinical characteristics by PTSD status.

	Control (N=33)	PTSD+ (N=33)	P-value
Female Gender	14 (42%)	16 (49%)	.621
Age	30.3 (8.4)	29.6 (5.3)	.715
Education (Years)	15.3 (2.2)	15.3 (2.0)	.931
Minority vs. Caucasian	9 (27%)	15 (45%)	.125
Current CAPS score (Mean ± SD)	0.0 ± 0.0	52.5 ± 13.7	.000
Current MDD	0 (0%)	6 (18%)	.000
BMI	24.3(3.5)	26.5(4.8)	.022
Smoking status	7 (21%)	8 (24%)	.769
Hormonal Birth Control (Among women)	2 (14%)	5 (31%)	.273

CAPS: Clinician Administered PTSD Scale; MDD: Major Depressive Disorder; BMI: Body Mass Index

Table 2.

Endocrine and sleep variables pre- and post-metyrapone.

	Control (N=33)		PTSD+ (N=33)		Pre-Post Metyrapone Contrast[1]			Group Contrast[1]			Metyrapone X Group Interaction[1]		
	Pre-metyrapone Mean (SD)	Post-metyrapone Mean (SD)	Pre-metyrapone Mean (SD)	Post-metyrapone Mean (SD)	F	d.f.	p	F	d.f.	p	F	d.f.	p
Delta spectral power (ln(μV^2))	2.25 (0.39)	2.15 (0.41)	2.15 (0.36)	1.89 (0.54)	7.32	1, 1526	.007	4.18	1, 1526	.041	0.68	1, 1526	.007
ACTH (ln(pg/ml))	2.91 (0.36)	4.13 (0.89)	2.92 (0.51)	4.34 (1.11)	1318.57	1, 1636	.000	0.23	1, 1636	.628	6.14	1, 1636	.013
Cortisol (ln (ng/ml))	3.82 (0.87)	2.99 (0.58)	3.97 (0.85)	2.95 (0.48)	574.78	1, 1446	.000	1.17	1, 1446	.279	5.92	1, 1446	.015

[1]ACTH, delta power, and cortisol contrasts are based on linear mixed models with random subject effects and repeated measures on night (pre/post metyrapone) and timepoint (up to 16 per night). Means and SD's are based are based on raw subject-level data.

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Altered overnight levels of pro-inflammatory cytokines in men and women with posttraumatic stress disorder

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Abstract

Background: Posttraumatic stress disorder (PTSD) is associated with disturbed sleep and elevated levels of pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Studies in animals and healthy humans have also shown that disrupted sleep elevates pro-inflammatory cytokines, including IL-6 and TNF-α. A better understanding of overnight cytokine levels and sleep might shed light on possible mechanisms for elevated inflammation in PTSD. Thus, we investigated overnight levels of IL-6 and TNF-α in individuals with and without PTSD while recording sleep polysomnography (PSG).

Method: Serum samples were collected from otherwise healthy, medication-free participants with chronic PTSD (n=44; 50% female; M age=30.34± 8.11) and matched controls (n=49; 53% female; M age=30.53± 6.57) during laboratory PSG. Levels of IL-6 and TNF-α were measured at hours 0, 2, 4, 6, and 8 after typical sleep onset time using serial serum samples. Plasma IL-6 and TNF-α levels were quantified using enzyme-linked immunosorbent assays.

Results: Growth model analysis indicated a significant group by time interaction for IL-6 (t[247] = −2.92, p = .005) and a significant *group* by *sex* by *time* interaction for TNF-α (t[275] = 2.02, p = .04). PTSD positive men and women initially had higher IL-6 and TNF-α at sleep onset, but not at the end of their sleep cycle. Men with PTSD showed a peak of TNF-α at the end of the sleep

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CRediT author statement

AK: Conceptualization, Formal Analysis, Writing – Original Draft. LDS: Writing – Original Draft. AAP, SSI, and AR: Writing-Reviewing and Editing. JS and EM: Data Curation. TJM: Methodology, Software, Writing – Reviewing and Editing. TCN: Funding Acquisition, Conceptualization, Supervision, Writing – Reviewing and Editing. AO: Conceptualization, Supervision, Writing – Reviewing and Editing.

cycle, whereas male control subjects demonstrated an inverted U-shaped profile. There were no significant differences in TNF-α levels overnight between women with and without PTSD.

Conclusion: To our knowledge, this is the largest study to examine IL-6 overnight in a PTSD sample and the first study to examine overnight TNF-α in PTSD. Overnight IL-6 and TNF-α levels may be altered in individuals with PTSD compared to those without PTSD, and TNF-α trajectories also differed by sex. The current findings highlight the need to consider sex, sleep, time of day, and circadian variation when examining inflammation in PTSD. Additional research in broader study samples will be necessary to clarify associations between disrupted sleep, cytokines, and increased risk for disease in PTSD.

Keywords

Sleep; Cytokines; Inflammation; Posttraumatic stress disorder; Sex differences

Introduction

Posttraumatic Stress Disorder (PTSD) is a debilitating condition with a lifetime prevalence of approximately 8% (Kessler et al., 1995). In addition to severely impairing psychological wellbeing, PTSD is associated with increased risk for cardiovascular, autoimmune and metabolic disorders, and premature mortality (Boscarino, 2006; Cohen et al., 2009, O'Donovan et al., 2015). Inflammation can drive the development of multiple physical diseases and has been proposed as a mechanism linking PTSD with ill health (O'Donovan, et al., 2012). Though studies differ with respect to methodology (Hussein et al., 2017), numerous studies and a meta-analysis have confirmed that individuals with PTSD tend to exhibit elevated levels of inflammatory markers, including levels of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α; Gill, Vythilingam, & Page, 2008; Lindqvist et al., 2014; Passos et al., 2015), as well as other inflammatory markers such as interferongamma (IFN-γ) and high-sensitivity C-reactive protein (Bruenig et al., 2018; Lindqvist et al., 2017). Additionally, certain treatment strategies, such as psychopharmacological agents (Kenis & Maes, 2002) as well as psychotherapy (Himmerich et al., 2016) can lead to changes in levels of inflammatory markers. However, people with PTSD are a highly heterogeneous group and not all individuals with PTSD display elevated inflammation (Söndergaard, Hansson, & Theorell, 2004). Thus, identifying the specific symptoms of PTSD associated with inflammatory activity could be a first step towards designing interventions that reduce inflammation in PTSD.

Most individuals with PTSD exhibit substantial sleep disturbance, in the form of insomnia and frequent nightmares (Neylan et al., 1998). Objective studies of sleep in PTSD have shown decreased sleep continuity (Mellman et al., 1997) decreased slow-wave sleep (Kobayashi, Boarts, & Delahanty, 2007; Richards et al., 2013) and alterations in rapid eye movement (REM) sleep (Breslau et al., 2004). However, objective sleep disturbance in PTSD appears to differ by sex; women with PTSD show more REM sleep than controls (Otte et al., 2007; Richards et al., 2013). Disrupted sleep in the early aftermath of trauma is associated with worse subsequent clinical symptoms (Mellman, et al., 2002), indicating a potential role of sleep as a mechanism of PTSD symptoms. Disrupted sleep is also

associated with poor physical health outcomes in PTSD (Boyko et al., 2013), indicating a potential role for sleep in increasing physical disease risk in this population.

Research in healthy controls and other sleep-disordered groups indicates that sleep and inflammation are closely intertwined. Some studies show sleep disruption leads to increased concentrations of both IL-6 and TNF-α (Irwin et al., 2006), and a large meta-analytic study showed a significant association of short sleep with higher levels of IL-6 when sleep was measured objectively (Irwin, Olmstead, & Carroll, 2016). Circadian release of TNF-α has also been shown to be disrupted in sleep apnea patients, another group with considerable sleep disturbance (Entzian et al., 1996). PTSD-related sleep disturbance, therefore, could plausibly promote the production of pro-inflammatory cytokines during the night and thereby increase risk for disease. Given that elevated levels of pro-inflammatory cytokines may feed back to the nervous system to further interrupt sleep, particularly delta sleep (Redwine, Hauger, Gillin, & Irwin, 2000), the relationship between PTSD-related sleep disturbance and pro-inflammatory cytokines may be bidirectional.

Despite the evidence for an interrelationship between disrupted sleep and elevated cytokines in PTSD, few studies have examined this directly by studying overnight trajectories of pro-inflammatory cytokines in people with PTSD compared to controls. One study (Gill et al., 2010) examined nine participants with PTSD comorbid with major depressive disorder, nine participants with only PTSD, and 14 non-traumatized control subjects, comparing these three groups on IL-6 levels overnight. In this sample, participants with major depressive disorder and PTSD had significantly higher mean levels of IL- 6 than participants in either of the other two groups. Furthermore, peak overnight IL-6 levels correlated significantly with PTSD symptomatology, indicating that people with more severe PTSD showed elevated IL-6 concentrations. These findings support the notion that PTSD is associated with elevated overnight cytokine levels. However, this study did not examine differences in overnight IL-6 by sex and it had a relatively small sample size in each group. Given sex differences in both objective sleep in PTSD (Richards et al., 2013) and associations between sleep and markers of inflammation (Suarez, 2008; Prather et al., 2013), the relationship between disrupted sleep and cytokine production may differ in men and women. To our knowledge, no studies have compared overnight concentrations of TNF-α in people with and without PTSD or examined trajectories of any cytokine overnight.

In the present study, we assessed overnight trajectories for IL-6 and TNF-α in sex- and age-matched adults with and without PTSD. We expected to observe elevated IL-6 and TNF-α concentrations across the night in people with PTSD compared to healthy controls. We also expected that overnight IL-6 and TNF-α would be related to total sleep time such that participants with the shortest sleep duration would show the highest levels of both cytokines.

Methods

Study Participants

Our sample included 85 participants recruited as part of a larger study (N = 93) focused on sleep abnormalities in PTSD. We acknowledge that a mean of 54 on the Clinician Administered PTSD Scale (CAPS) is lower than many studies with treatment seeking

patients, which may be due to our inclusion criteria of being medically healthy and on no medication (see below). As detailed in other studies using this sample (e.g., O'Donovan et al., 2011), participants were recruited through ads and flyers distributed in the community as well as in relevant local clinics for the PTSD sample. Eight participants were excluded from the present study due to difficulties in overnight blood collection. In our sample, 43 participants were positively diagnosed with current, chronic PTSD and 42 were sex- and age-matched medically healthy controls (see Table 1). PTSD diagnosis was established via the CAPS (Blake et al., 1995) conducted by trained clinical interviewers. Eligible participants met DSM-IV criteria for PTSD; see Table 2 for clinical characteristics of the sample. Exclusion criteria included a history of traumatic brain injury; presence of neurologic disorders or systemic illness; use of psychiatric, anticonvulsant, antihypertensive, sympathomimetic, steroidal, statin or other prescription medications; obesity as defined by a body mass index of BMI > 30; alcohol abuse or dependence in the prior 2 years; substance abuse or dependence in the previous year; any psychiatric disorder with psychotic features; bipolar disorder or obsessive compulsive disorder. Subjects with extreme morning tendencies (score < 19) and extreme evening tendencies (score > 47) on the Smith Morningness Scale (Smith, Reilly, & Midkiff, 1989) were excluded. For female participants, pregnancy was an exclusion criterion and all were premenopausal (indicated by having at least one menstrual period in the past 12 months) and scheduled during a follicular phase of the menstrual cycle. Further exclusion criteria for control participants included a history of current or lifetime PTSD and a lifetime history of major depressive disorder (MDD) or panic disorder. All participants provided written informed consent before participating in any study procedures and the project was approved by the Committee on Human Research at the University of California, San Francisco.

Procedure

Participants stayed three consecutive nights at the sleep laboratory in a General Clinical Research Center (GCRC; see Richards et al., 2013 for more details). The current study involved data from the second night, after one habituation night in the sleep laboratory. To account for individual variability of sleep onset, participants were asked to report habitual sleep onset (HSO) via 1-week sleep diary prior to the study. HSO was then used as an individual onset for data recording and acted as the starting point of the experiments, as well as a point of reference for this study. Two hours before HSO, a catheter was inserted into an antecubital vein for repeated blood sampling. Whole blood samples were drawn every 15 minutes starting with HSO until 8 hours after HSO. For this study, blood samples from 0, 2, 4, and 6 hours after HSO were used. Furthermore, a last blood draw was added between 7AM and 10AM after habitual waking time, marking the last timepoint in our analyses.

Measures

PTSD diagnosis: Lifetime and current PTSD were assessed with the Clinician Administered PTSD Scale (CAPS), a structured interview that corresponds to DSM-IV criteria for PTSD (Blake et al., 1995). The CAPS is a 30-item scale that assesses the frequency and intensity of re-experiencing, avoidance, and hyperarousal symptoms of PTSD. Diagnosis of PTSD was based on symptoms experienced in the previous month associated with the subject's self-identified worst traumatic event. Other psychiatric

disorders were assessed by administration of the Structured Clinical Interview for DSM-IV (First et al., 1995).

Cytokines: The human IL-6 Quantikine high sensitivity enzyme-linked immunosorbent assay (hsELISA) and human TNF-α Quantikine hsELISA were used to measure IL- 6 and TNF-α respectively (R&D Systems, USA). The lower limits of detection were 0.17 pg/ml for IL-6 and 0.62 pg/ml for TNF-α. Where duplicates differed more than 20%, samples were repeated in duplicate. Intra-assay coefficients of variation were <10% for both IL-6 and TNF-α. One sample had an IL-6 level below the lowest detectable limit of the assays, and 19 had TNF-α levels below the detectable limit; these samples were all recoded as being one unit below the lowest detectable limit.

Total sleep time: As previously described in (Richards et al. 2013), polysomnography recordings were obtained with ambulatory polysomnography (Nihon Kohden Trackit Ambulatory Recording System) in accordance with standardized guidelines (Rechtschaffen, 1968). Pass Plus was utilized for both visual scoring and quantitative EEG analysis of the digitized polysomnography data. Visual scoring was conducted by a highly experienced registered polysomnography technician, who classified all 30-second epochs in every sleep record as wake; stages 1, 2, 3; REM; or movement using current AASM criteria (AASM, 2007). Sleep onset was defined as the first minute of eight consecutive minutes of stage 2 sleep with no more than 2 intervening minutes of stage 1 sleep or minutes awake. Total sleep time was defined by time spent in epochs scored as NREM stages 1 through 3 and stage REM over a time window of ten hours after HSO.

Covariates: Over and beyond the described variables of interest, biological sex (“female” vs “male”) and age, as well as total hours of sleep, entered the analyses of this study as covariates. Given that there was a strong association between PTSD status and body mass index (BMI), BMI was also added as a covariate.

Statistical analyses

Multilevel modeling was used to assess growth curve analyses on the trajectories of the inflammatory responses for each participant, with two-hour cytokine measurements nested within participants. Growth curve modeling was conducted as implemented in the ‘nlme’ library of R (Pinheiro et al., 2014). In all analyses, time was treated as a within-subject continuous variable, group, sex and the covariates as between-subject variables, and the two inflammatory marker responses of IL-6 and TNF-α served as the outcome variables. For all analyses, we modeled time with a quadratic effect in order to account for the non-linear trajectories of nocturnal cytokine levels previously reported in other studies (Cuesta et al., 2016; Nilsonne et al., 2016). Since time was not centered, the intercept represented HSO for each participant and the beginning of the growth curve.

Backward model selection was performed by contrasting the deviance of a complex model with that of a simple model using a log-likelihood test (Bliese & Ployhart, 2002). Model parameters were estimated using restricted maximum likelihood. If the complex model fitted the data significantly better than the simple model, we retained the complex model. When

indicated by the model-selection procedure, a random participant intercept and/or slope were included in the model. Secondly, we determined whether a linear, quadratic or cubic time effect fitted the data best. As suggested by Bliese and Ployhard (2002), we then added a more complex error structure (i.e. accounting for autocorrelation or heteroscedasticity) to the models, if model comparison indicated significant differences. Post-hoc contrasts were analyzed with the 'LSMEANS' library of R and multiple comparisons were corrected with false discovery rate (FDR; Benjamini & Hochberg, 1995) with a *p*-value of 0.05.

In order to achieve normal distribution for IL-6 and TNF-α, natural log transformations were conducted on both biological measures (see Table 1 for IL-6 and TNF-α levels for both the PTSD and control groups, expressed in log-transformed units). Visual inspection indicated that the two transformed variables were normally distributed. To control for multivariate outliers, we use Mahalanobis distance to identify influenceable individuals (Hadi, 1992). As a consequence, data of three participants were deemed influential and subsequently dismissed from the final analyses.

Results

provides an overview of the demographic information of the N = 85 participants by group. There was a significant group difference in BMI (t[79.80] = −2.71; p = .01), driven by a lower mean BMI of controls compared to PTSD-positive participants. Groups also differed in ethnicity (X^2[4] = 14.62; p = .006), with the control group having a higher frequency of Caucasians compared to the PTSD group. Bivariate Pearson correlations revealed no significant associations among the continuous covariates and mean overnight cytokine levels. However, there was an association between the participants age and BMI, indicating that older participants had significantly higher BMI ($r_{age,BMI}$ = .27; p = .01), and a marginal positive association between the two mean overnight cytokine levels ($r_{IL\text{-}6,TNF\text{-}\alpha}$ = .20; p = .07; Supplementary Table 1).

We conducted independent samples t-tests for each two-hour segment of the experiment where we compared the cytokine levels between the two groups. None of these t-tests indicated a significant group differences between these levels across all timepoints (see Figure 1).

Nocturnal IL-6 Trajectory

Our analyses showed a substantial dependency amongst IL-6 responses within subjects (ICC = .46). To account for within-person change of IL-6, we continued with specifying our growth model. Model specification indicated that the within-subject factor time predicted IL-6 responses best when squared. Furthermore, our model selection process identified a random intercept and slope model for IL-6 responses, while accounting for heteroscedasticity.

To test our first assumption, that nocturnal IL-6 levels would be different between controls and participants with PTSD, we added group as a fixed effect to our above specified model. In this model, *group* interacted significantly with both *time* (B = 0.16, SE = 0.07, p = .02) and *time*^{2} (B = −0.02, SE = 0.01, p = .01). To test our hypothesis that *group* and *sex*

accounted for a significant amount of variability in *IL*-6 *levels* over the course of the sleep cycle, we added *sex* to our specified random intercept and slope model. In this model, none of the interactions involving *sex* were significant (all $p > .21$), leading us to treat *sex* as a covariate in subsequent models. Controlling for *age, sex* and *BMI*, the IL-6 model still indicated a significant 2-way interaction of *group* × *time* (B = 0.19, SE = 0.06, $p = .003$) and *group* × *time*^{2} (B = −0.02, SE = 0.01, $p < .001$). Figure 2 shows that IL-6 levels increased after HSO for both groups and dropped again approximately after 4 hours, following an inverted U-shaped trajectory of IL-6 levels for both groups. This trajectory was distinctively more pronounced in the PTSD group than in the control group, and the control group showed an almost flat slope (see Table 3).

FDR adjusted follow-up analyses examined the differences in predicted IL-6 level at each time point compared to each other time point, based on the fully adjusted model. Within the PTSD group, all pairwise contrasts between time points were statistically significant, showing the robustness of changes over the night (see Table 3). No pairwise comparisons between time points were significant within the control group, indicating the relative stability of IL-6 levels in this group.

In order to assess if our findings were independent from *total sleep time*, we computed a final model that adjusted for the total amount of sleep within a ten-hour window. This model revealed the same *group* × *time* interactions (i.e. linear and quadratic), but no significant effect of *total sleep time* ($p = .14$).

Nocturnal TNF-α Trajectory

Measurements of TNF-α were dependent within subjects (ICC = .66). Subsequent model comparison indicated that a random intercept and slope model fitted the TNF-α responses best. Furthermore, model specification indicated that we did not have to account for autocorrelation, but again for heteroscedasticity.

Following the same process as described for IL-6, we started by adding *group* to the model. In this model, we found a significant *group* × *time*^{2} (B = 0.007, SE = 0.002, $p = .01$) and a significant *group* × *time* interaction (B = −0.04, SE = 0.02, $p = .04$). We then proceeded by adding *sex* to our model. This analysis revealed a 3-way interaction between *group, sex* and *time*^{2} (B = 0.009, SE = 0.004, $p = .05$). The analysis also revealed significant *sex* × *time*^{2} interaction (B = −0.01, SE = 0.003, $p = .02$) and a *sex* × *time* interaction (B = −0.07, SE = 0.03, $p = .01$), while all the other effects were not significant (all $p > .23$).Adding the covariates *age* and *BMI* to the model did not change any of the above-mentioned results.

To better examine our marginal 3-way interaction between *group, sex* and *time*^{2}, we ran our final models stratified by group. The model with only *sex* and *time* of the control sample showed a significant interaction between *sex* and the linear effect of *time* (B = 0.06, SE = 0.03, $p = .02$), as well as with a quadratic effect of *time* (B = −0. 01, SE = 0.003, $p = .01$). For the PTSD group, the interaction effects were not significant (both $p > .55$). But there was a significant quadratic effect of *time* (B = 0.005, SE = 0.002, $p = .02$) and a trend towards a linear effect of *time* (B = −0.04, SE = 0.002, $p = .06$); the effect of *sex* was not significant ($p = .64$). Adding the covariates *age* and *BMI* to both models did not substantially change the

estimates for either group, but BMI was significantly associated with TNF-α levels (B = 0.02, SE = 0.01, p = .03; see Table 3).

The 3-way interaction of *time*^{2}, *group* and *sex* is reflected in an inverted U-shaped trajectory for TNF-α levels of the male control participants over the course of the 8-hour period, peaking around 4 hours after self-reported HSO, while the other three subsamples (control female participants and PTSD positive females and males) followed a U-shaped trajectory (see Figure 3). But while the TNF-α levels of control males followed a pronounced slope and dropped below the initial level (measured at hour 0), TNF-α levels for female controls remained largely unchanged. Both men and women in the PTSD positive group had higher TNF-α levels after 8 hours, while this effect was more pronounced in PTSD positive males and flat in PTSD positive females.

As illustrated by Figure 3, FDR-adjusted follow-up analysis of the predicted means from the fully adjusted model within the control male subsample indicated that the increase of TNF-α levels from hour 0 to 2 was marginally significant, while the drops between hour 4 to 6 and 6 to 8 were significant. The drop from hour 0 to 8 was marginally significant. Within the PTSD positive male subsample, the elevations of hour 4 to 6, the elevation of hour 6 to 8, and the overall increase from hour 0 to hour 8 were significant. All comparisons within the female subsamples were not significant, indicating no significant within-group changes.

Effects of sleep: We finally also assessed if the *TNF*-α was independent from *total sleep time* and computed a sleep-adjusted model. Adding the sleep measure resulted in non-significant 3-way interactions (both ps>.11), albeit without any impact on effect sizes derived from our original models. The *sex* × *time* (B = 0.07, SE = 0.03, p = .02) and *sex* × *time*^{2} (B = −0.01, SE = 0.003, p = .01) interactions were unchanged, while none of the previous main effects were significant (all ps>.16). *Total sleep time* had a significant positive effect (B = 0.001, SE = 0.0005, p = .02), indicating that more sleep was associated with higher peak *TNF*-× levels overnight.

Discussion

The current study examined levels of pro-inflammatory cytokines overnight in a sample of PTSD-positive adults in comparison to age- and sex-matched controls. Broadly, our results showed no significant differences between the PTSD-positive participants and controls at any single timepoint. However, the groups did differ on their overnight trajectories of cytokine levels. With regard to IL-6, the PTSD-positive participants showed more variation overnight, with an exaggerated inverted U-shaped curve consisting of lower IL-6 levels than the controls at the start and end of the night and higher midpoint IL-6 levels four hours after habitual sleep onset. Regarding TNF-α, PTSD-positive participants showed elevated cytokine levels at the end of the night in comparison to controls, though the trajectory of TNF-α levels overnight differed by sex. While the male control participants showed an inverted U-shaped trajectory of TNF-α levels overnight, all three other groups showed flatter U-shaped trajectories of TNF-α overnight.

Although the current study did not support our initial hypothesis that PTSD-positive participants would show elevated cytokines overnight, they did differ from healthy controls when considering the overall shape of the trajectories of cytokine levels over the course of the night. For example, the overnight peak in IL-6 was higher in the PTSD sample in comparison to controls, while TNF-α was elevated in comparison to the controls 8 hours after habitual sleep onset. Additionally, the men without PTSD showed altered TNF-α trajectories overnight in comparison to all other groups. All of these differences suggest alterations in time course of pro-inflammatory activity overnight in PTSD, indicating the importance of considering time of day and trajectories of responses when trying to understand altered inflammatory activity in PTSD.

Based on previous studies showing elevated inflammatory activity in PTSD compared to controls (Passos et al., 2015), disrupted sleep in PTSD (Kobayashi, Boarts, & Delahanty, 2007), and elevated cytokines associated with sleep disruption (Irwin, Olmstead, & Carroll, 2016), we expected overall overnight elevations in both IL-6 and TNF-α in PTSD. There may be a number of reasons why results from the current study were not broadly consistent with this pattern. Importantly, there is a high degree of inter-individual variability in both sleep disruption (Straus et al., 2015) and inflammatory activity in PTSD (O'Donovan et al., 2017), with several studies suggesting that not all PTSD patients show sleep disruption or inflammation. Additionally, previous research has suggested that elevated cytokine levels overnight is associated with severity of PTSD symptoms (Gill et al., 2010). The current study selected for physically healthy, medication-free younger adults with PTSD. Interestingly, PTSD severity in our sample was much lower in comparison to the sample recruited by Gill and colleagues (mean CAPS score = 54 versus 82 in the comorbid PTSD +depression group from Gill et al.). Selection criteria for the current study may have constrained our sample to participants with relatively less severe symptoms.

There are several limitations of the current study worth noting. First, our small sample size limits the ability to draw strong conclusions. Importantly, as stated above, the current study consisted of physically healthy, non-obese, non-medicated younger adults with PTSD, which allowed us to examine associations of PTSD with inflammation independent of some of these potential confounds. However, given that PTSD is associated with increased risk for a number of physical diseases, including cardiovascular, autoimmune, and metabolic disorders (Boscarino, 2006; Cohen et al., 2009, O'Donovan et al., 2015), the current sample may not be generalizable to a more representative sample of PTSD patients. Though the samples were matched based on age and sex, there are a number of ways in which participants with PTSD differed from control participants (trauma exposure, type of trauma, veteran status, ethnicity, smoking), so future studies should attempt to control for these confounding factors. The current study only included analyses of TNF-α and IL-6, though other inflammatory markers such as IFN-γ and high-sensitivity C-reactive protein may also be elevated in PTSD (Breunig et al., 2018; Lindqvist et al., 2017). In addition, both TNF-α production and receptor levels may be modified by treatments such as psychotherapy (Himmerich et al., 2016). Though the current study excluded medication use, no information was collected about IL-6 or TNF-α receptors, and no information is available about prior history of psychotherapy use in the sample. Future studies should more closely examine these associations. Additionally, the current study was constrained to examining a single

night of data collection. Finally, the current study sampled cytokines at only five timepoints overnight, only three of which occurred during the habitual sleep period, limiting the opportunity to examine trajectories at a more fine-grained level of detail, including examining detailed relationships between sleep architecture/sleep cycles and their relationship to cytokine levels during the sleep period. Future studies using more frequent sampling during the sleep period will be able to examine these relationships more thoroughly.

To our knowledge, this is the largest study to examine IL-6 overnight in a PTSD sample and the first study to examine overnight TNF-α in PTSD. The current findings demonstrate altered overnight levels of pro-inflammatory markers in men and women with PTSD in comparison to healthy control participants. Additional research in broader study samples will be necessary to continue to examine relationships between disrupted sleep, cytokines, and increased risk for disease in PTSD. The current findings highlight the need to consider sex, sleep, time of day, and circadian variation when examining inflammation in PTSD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Benjamini Y, & Hochberg Y, 1995 Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Royal Stat. Soc. Series B (Methodological), 289–300.

Blake DD, Weathers FW, Nagy LM, Kaloupek DG, Gusman FD, Charney DS, & Keane TM, 1995 The development of a clinician-administered PTSD scale. J. Trauma. Stress, 8, 75–90. [PubMed: 7712061]

Bliese PD, & Ployhart RE, 2002 Growth modeling using random coefficient models: Model building, testing, and illustrations. Organizational Res. Methods, 5, 362–387.

Boscarino JA, 2006 Posttraumatic stress disorder and mortality among US Army veterans 30 years after military service. Ann. Epidemiology, 16, 248–256.

Boyko EJ, Seelig AD, Jacobson IG, Hooper TI, Smith B, Smith TC, … & Millennium Cohort Study Team, 2013 Sleep characteristics, mental health, and diabetes risk: a prospective study of US military service members in the Millennium Cohort Study. Diabetes Care, 36, 3154–3161. [PubMed: 23835691]

Breslau N, Roth T, Burduvali E, Kapke A, Schultz L, & Roehrs T, 2004 Sleep in lifetime posttraumatic stress disorder: a community-based polysomnographic study. Arch. Gen. Psychiatry, 61, 508–516. [PubMed: 15123496]

Bruenig D, Mehta D, Morris CP, Lawford B, Harvey W, Young RM, & Voisey J (2018). Correlation between interferon γ and interleukin 6 with PTSD and resilience. Psychiatry Res., 260, 193–198. [PubMed: 29202383]

Cohen BE, Marmar C, Ren L, Bertenthal D, & Seal KH, 2009 Association of cardiovascular risk factors with mental health diagnoses in Iraq and Afghanistan war veterans using VA health care. JAMA, 302, 489–492. [PubMed: 19654382]

Cuesta M, Boudreau P, Dubeau-Laramée G, Cermakian N, & Boivin DB, 2016 Simulated night shift disrupts circadian rhythms of immune functions in humans. J. Immunology, 196, 2466–2475. [PubMed: 26873990]

Entzian P, Linnemann K, Schlaak M, & Zabel P, 1996 Obstructive sleep apnea syndrome and circadian rhythms of hormones and cytokines. Am. J. Respiratory Crit. Care Med, 153, 1080–1086.

First MB, Spitzer RL, Gibbon MWJB, & Williams JB, 1995 Structured clinical interview for DSM-IV axis I disorders. New York: New York State Psychiatric Institute.

Gill J, Luckenbaugh D, Charney D, & Vythilingam M, 2010 Sustained elevation of serum interleukin-6 and relative insensitivity to hydrocortisone differentiates posttraumatic stress disorder with and without depression. Biol. Psychiatry, 68, 999–1006. [PubMed: 20951370]

Gill J, Vythilingam M, & Page GG, 2008 Low cortisol, high DHEA, and high levels of stimulated TNF-α, and IL-6 in women with PTSD. J. Trauma. Stress, 21, 530–539. [PubMed: 19107725]

Hadi AS, 1992 Identifying multiple outliers in multivariate data. J. Royal Stat. Soc. Series B (Methodological), 761–771.

Himmerich H, D Willmund G, Zimmermann P, Wolf JE, H Bühler A, C Kirkby K, … & Wesemann U. (2016). Serum concentrations of TNF-a and its soluble receptors during psychotherapy in German soldiers suffering from combat-related PTSD. Psychiatria Danub., 28, 3, 293–298.

Hussein S, Dalton B, D Willmund G, & Ibrahim MA (2017). A systematic review of tumor necrosis factor-a in post-traumatic stress disorder: Evidence from human and animal studies. Psychiatria Danubina, 29, 4, 407–420. [PubMed: 29197197]

Iber C, Ancoli-lsrael S, Chesson A, & Quan SF, 2007 authors; for the American Academy of Sleep Medicine. The AASM manual for the scoring of sleep and associated events: rules, terminology and technical specifications.

Irwin MR, Olmstead R, & Carroll JE, 2016 Sleep disturbance, sleep duration, and inflammation: a systematic review and meta-analysis of cohort studies and experimental sleep deprivation. Biol. Psychiatry, 80, 40–52. [PubMed: 26140821]

Irwin MR, Wang M, Campomayor CO, Collado-Hidalgo A, & Cole S, 2006 Sleep deprivation and activation of morning levels of cellular and genomic markers of inflammation. Arch. Intern. Med, 166, 1756–1762. [PubMed: 16983055]

Kenis G, & Maes M (2002). Effects of antidepressants on the production of cytokines. Internat. J. Neuropsychopharmacology, 5, 4, 401–412.

Kessler RC, Sonnega A, Bromet E, Hughes M, & Nelson CB, 1995 Posttraumatic stress disorder in the National Comorbidity Survey. Arch. Gen. Psychiatry, 52, 1048–1060. [PubMed: 7492257]

Kobayashi I, Boarts JM, & Delahanty DL, 2007 Polysomnographically measured sleep abnormalities in PTSD: a meta-analytic review. Psychophysiology, 44, 660–669. [PubMed: 17521374]

Lindqvist D, Dhabhar FS, Mellon SH, Yehuda R, Grenon SM, Flory JD, … & Reus, VI. (2017). Increased pro-inflammatory milieu in combat related PTSD–a new cohort replication study. Brain Behav. Immun, 59, 260–264. [PubMed: 27638184]

Lindqvist D, Wolkowitz OM, Mellon S, Yehuda R, Flory JD, Henn-Haase C, … & Makotkine I, 2014 Proinflammatory milieu in combat-related PTSD is independent of depression and early life stress. Brain Behav. Immun, 42, 81–88. [PubMed: 24929195]

Mellman TA, Bustamante V, Fins AI, Pigeon WR, & Nolan B, 2002 REM sleep and the early development of posttraumatic stress disorder. Am. J. Psychiatry, 159, 1696–1701. [PubMed: 12359675]

Mellman TA, Nolan B, Hebding J, Kulick-Bell R, & Dominguez R, 1997 A polysomnographic comparison of veterans with combat-related PTSD, depressed men, and non-ill controls. Sleep, 20, 46–51. [PubMed: 9130334]

Neylan TC, Lenoci M, Maglione ML, Rosenlicht NZ, Metzler TJ, Otte C, … & Marmar, CR., 2003 Delta sleep response to metyrapone in post-traumatic stress disorder. Neuropsychopharmacology, 28, 1666. [PubMed: 12799616]

Neylan TC, Marmar CR, Metzler TJ, Weiss DS, Zatzick DF, Delucchi KL, … & Schoenfeld, FB, 1998 Sleep disturbances in the Vietnam generation: findings from a nationally representative sample of male Vietnam veterans. Am. J. Psychiatry, 155, 929–933. [PubMed: 9659859]

Nilsonne G, Lekander M, Åkerstedt T, Axelsson J, & Ingre M, 2016 Diurnal variation of circulating interleukin-6 in humans: a meta-analysis. PloS One, 11, e0165799. [PubMed: 27832117]

O'Donovan A, Ahmadian AJ, Neylan TC, Pacult MA, Edmondson D, & Cohen BE, 2017 Current posttraumatic stress disorder and exaggerated threat sensitivity associated with elevated inflammation in the Mind Your Heart Study. Brain Behav. Immun, 60, 198–205. [PubMed: 27765647]

O'Donovan A, Cohen BE, Seal KH, Bertenthal D, Margaretten M, Nishimi K, & Neylan TC, 2015 Elevated risk for autoimmune disorders in Iraq and Afghanistan veterans with posttraumatic stress disorder. Biol. Psychiatry, 77, 365–374. [PubMed: 25104173]

O'Donovan A, Epel E, Lin J, Wolkowitz O, Cohen B, Maguen S, … & Neylan TC. (2011). Childhood trauma associated with short leukocyte telomere length in posttraumatic stress disorder. Biol. Psychiatry, 70, 5, 465–471. [PubMed: 21489410]

O'Donovan A, Slavich GM, Epel ES, & Neylan TC, 2013 Exaggerated neurobiological sensitivity to threat as a mechanism linking anxiety with increased risk for diseases of aging. Neurosci. Biobehav. Rev, 37, 96–108. [PubMed: 23127296]

Otte C, Lenoci M, Metzler T, Yehuda R, Marmar CR, & Neylan TC, 2007 Effects of metyrapone on hypothalamic-pituitary-adrenal axis and sleep in women with post-traumatic stress disorder. Biol. Psychiatry, 61, 952–956. [PubMed: 17336940]

Passos IC, Vasconcelos-Moreno MP, Costa LG, Kunz M, Brietzke E, Quevedo J, … & Kauer-Sant'Anna M, 2015 Inflammatory markers in post-traumatic stress disorder: a systematic review, meta-analysis, and meta-regression. The Lancet Psychiatry, 2, 1002–1012. [PubMed: 26544749]

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team, 2014 nlme: Linear and Nonlinear Mixed Effects Models_. R package version 3.1-117. Available:http://CRAN.R-project.org/package=nlme

Prather AA, Epel ES, Cohen BE, Neylan TC, & Whooley MA, 2013 Gender differences in the prospective associations of self-reported sleep quality with biomarkers of systemic inflammation and coagulation: Findings from the Heart and Soul Study. J. Psychiatric Res, 47, 1228–1235.

Rechtschaffen A, 1968 A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects. Public health service.

Redwine L, Hauger RL, Gillin JC, & Irwin M, 2000 Effects of sleep and sleep deprivation on interleukin-6, growth hormone, cortisol, and melatonin levels in humans. J. Clin. Endocrinol. & Metabolism, 85, 3597–3603.

Richards A, Metzler TJ, Ruoff LM, Inslicht SS, Rao M, Talbot LS, & Neylan TC, 2013 Sex differences in objective measures of sleep in post-traumatic stress disorder and healthy control subjects. J. Sleep Res, 22, 679–687. [PubMed: 23763708]

Smith CS, Reilly C, & Midkiff K, 1989 Evaluation of three circadian rhythm questionnaires with suggestions for an improved measure of morningness. J. Appl. Psychol, 74, 728. [PubMed: 2793773]

Straus LD, Drummond S, Nappi CM, Jenkins MM, & Norman SB, 2015 Sleep variability in military-related PTSD: A comparison to primary insomnia and healthy controls. J. Trauma. Stress, 28, 8–16. [PubMed: 25630526]

Söndergaard HP, Hansson LO, & Theorell T, 2004 The inflammatory markers C-reactive protein and serum amyloid A in refugees with and without posttraumatic stress disorder. Clinica Chimica Acta, 342, 93–98.

Suarez EC, 2008 Self-reported symptoms of sleep disturbance and inflammation, coagulation, insulin resistance and psychosocial distress: evidence for gender disparity. Brain Behav. Immun, 22, 960–968. [PubMed: 18328671]

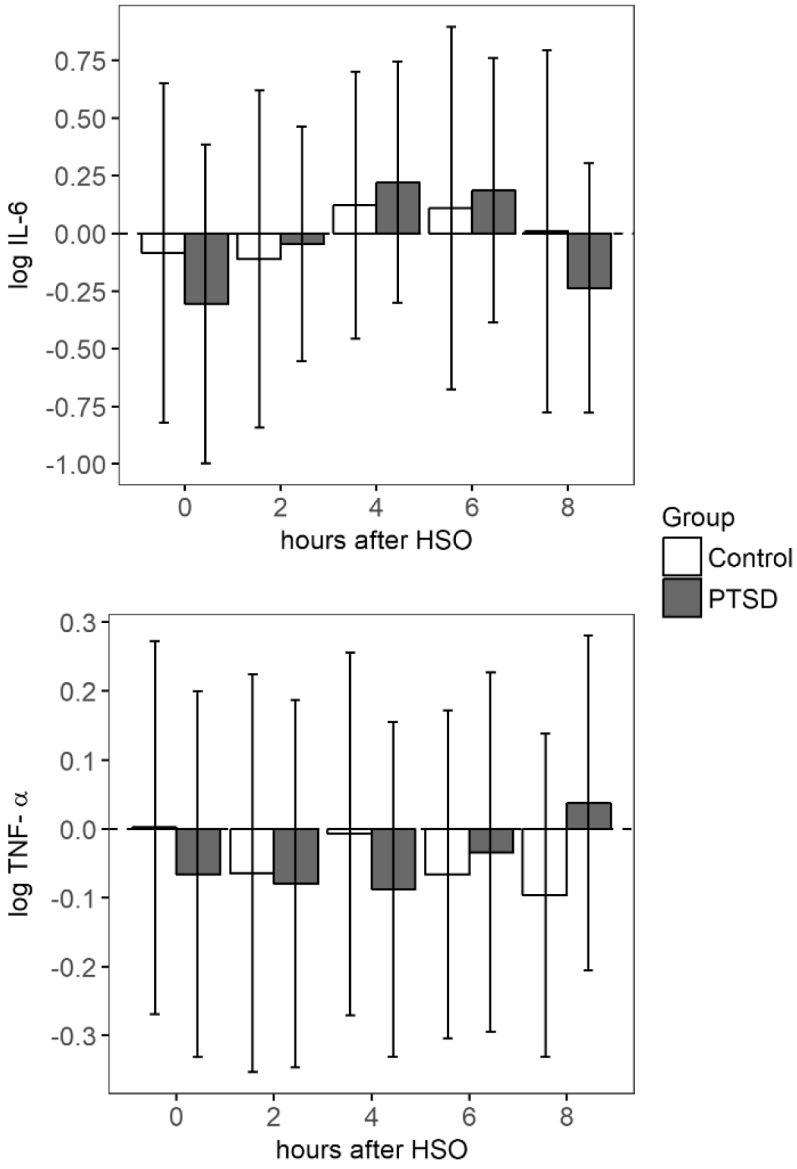

Figure 1.

Barplots of unadjusted, log-transformed cytokine levels over the 5 timepoints by group.

Note. Error bars represent +/− 1 standard errors.

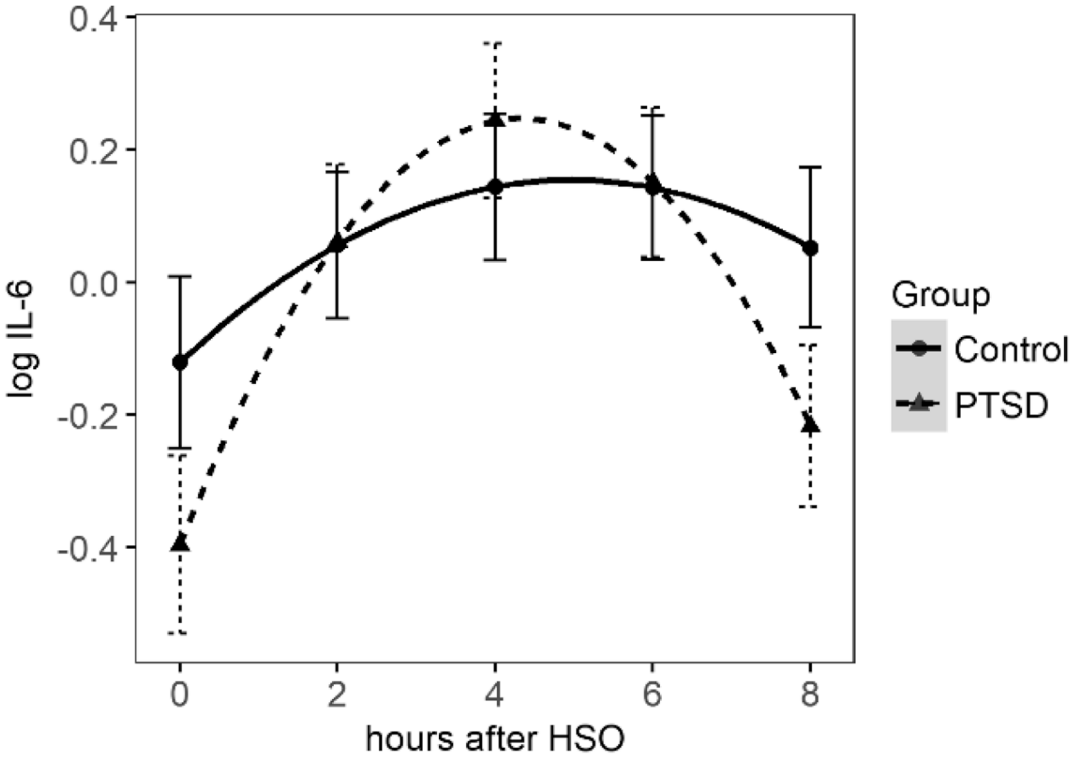

Figure 2.
Predicted IL-6 levels of PTSD positive subjects and healthy controls. *Note.* Error bars represent +/− 1 standard errors.

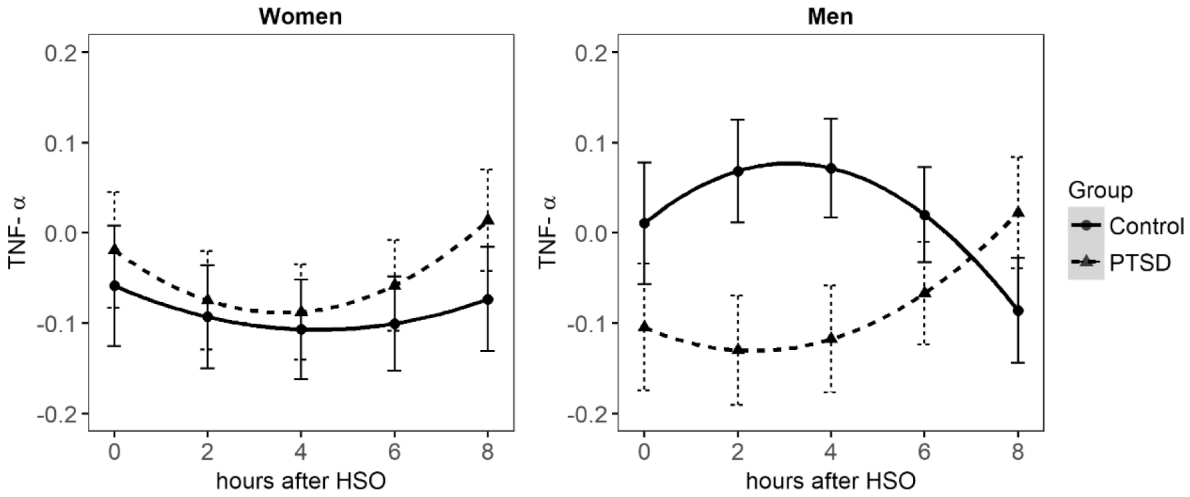

Figure 3.

Predicted TNF-α levels of women and men, by PTSD group.

Note. Error bars represent +/− 1 standard errors.

Table 1.

Demographics of controls and PTSD positive participants.

	Control (*n* = 42)	**PTSD (*n* = 43)**	***d*/φ_c**	**DF**	***t*/X^2**	***P***
Age (M (SD))	30.48 (8.26)	30.63 (6.63)	−0.02	78.48	−0.09	.93
Years of Education (M (SD))	15.38 (2.02)	14.86 (2.23)	0.24	82.55	1.13	.26
BMI (M (SD))	24.45 (3.74)	26.93 (4.69)	−0.59	79.80	−2.71	**.01**
Total sleep time (M (SD))	774.84 (29.16)	748.63 (36.20)	0.20	73.17	0.88	.38
Clock time of HSO (M (SD))	00:07h (69min)	23:48h (76min)	−0.26	82.61	1.21	.23
Average IL-6 (M In pg/ml (SD))	0.10 (0.80)	0.13 (0.97)	−0.02	80.78	−0.11	.86
Average TNF-α (M In pg/ml (SD))	0.03 (0.35)	0.00 (0.33)	0.07	82.33	0.34	.74
Sex (Male %)	21 (50.00)	22 (51.16)	0.00	1	0.00	>.99
Smoking Status (%)	8 (19.05)	11 (25.58)	0.05	1	0.21	.64
Veteran Status (%)	0 (0)	10 (23.26)				
Ethnicity (%)			0.24	4	14.62	**.01**
African American	1 (2.38)	5 (11.63)				
Asian	7 (16.67)	2 (4.65)				
Caucasian	31 (73.81)	23 (53.49)				
Hispanic	3 (7.14)	6 (13.95)				
Others	0 (0.00)	7 (16.28)				
Marital Status (%)			0.21	2	7. 49	**.02**
Divorced/Separated	1 (2.38)	8 (18.60)				
Married	6 (14.29)	2 (4.65)				
Single	35 (83.33)	33 (76.74)				

Note. Welch two-sample *t*-test were applied to compare the groups on continuous measures and X^2-tests were applied to discrete measures. For statistical testing, cytokine measures were log-transformed before entering the analyses to approximate normal distributions. BMI = body mass index; HSO = habitual sleep onset.

Table 2.

Clinical Characteristics.

	Control (*n* = 42)	**PTSD (*n* = 43)**
Trauma-Exposed (n (%))	11 (26.19)	43 (100.00)
Clinician Administered PTSD Scale Total Score (M (SD))	0 (0.00)	54.00 (14.91)
Time Since Trauma (Years (SD))	11.53 (10.20)	9.01 (9.36)
Trauma Type (n (%))		
Combat	0 (0.00)	8 (18.60)
Motor Vehicle Accident	4 (9.52)	0 (0.00)
Physical Violence/Abuse	4 (9.52)	18 (41.86)
Sexual Assault/Abuse	0 (0.00)	16 (37.21)
Sudden/Violent death	2 (4.76)	2 (4.65)
Other	1 (2.38)	5 (11.63)

Note. For trauma type, numbers and percentages overlap because some Criterion A events fit multiple categories. “Other” category included almost drowning (n = 1, PTSD group), stalking (n = 2, PTSD group), and complications from a medical procedure (n = 1, Control group).

Table 3.

Fixed effects of fully adjusted overnight cytokine models by group.

Fully adjusted model for IL-6 overnight levels of control participants						**Fully adjusted model for IL-6 overnight levels of PTSD participants**					
Parameter	**Coefficient (B)**	**SE**	**DF**	***t***	***P***	**Parameter**	**Coefficient (B)**	**SE**	**DF**	***t***	***P***
Intercept	−0.05	0.72	135	−0.72	.47	Intercept	1.01	0.50	119	−2.03	**.04**
Age	0.01	0.01	37	0.68	.50	*Age*	0.01	0.01	37	1.35	.19
BMI	0.01	0.03	37	0.20	.84	*BMI*	0.002	0.02	37	0.11	.92
Sex (Men)	−0.09	0.20	37	−0.42	.68	*Sex (Men)*	0.19	0.16	37	1.19	.24
Time	0.10	0.03	135	3.12	**.01**	*Time*	0.31	0.05	119	6.58	**.001**
$Time^2$	−0.01	0.004	135	−2.35	**.02**	$Time^2$	−0.04	0.01	119	−6.84	**.001**
Fully adjusted model for TNF-α overnight levels of control participants						**Fully adjusted model for TNF-α overnight levels of PTSD participants**					
Parameter	**Coefficient (B)**	**SE**	**DF**	***t***	***P***	**Parameter**	**Coefficient (B)**	**SE**	**DF**	***t***	***P***
Intercept	0.14	0.27	139	0.52	.60	Intercept	−0.36	0.25	136	−1.43	.16
Age	−0.001	0.01	35	−0.11	.92	*Age*	−0.005	0.01	37	−0.87	.39
BMI	−0.01	0.01	35	−0.68	.50	*BMI*	0.02	0.01	37	2.22	**.03**
Sex (Men)	0.04	0.10	35	0.43	.67	*Sex (Men)*	−0.13	0.10	37	−1.37	.18
Time	−0.02	0.02	139	−0.99	.32	*Time*	−0.04	0.02	136	−1.86	.07
$Time^2$	0.002	0.002	139	0.99	.32	$Time^2$	0.005	0.002	136	2.22	**.03**
Sex × Time	0.06	0.03	139	2.36	**.02**	*Sex × Time*	0.01	0.03	136	0.50	.62
Sex × $Time^2$	−0.01	0.00	139	−3.03	**.003**	*Sex* × $Time^2$	0.000	0.003	136	−0.09	.93

Note. The coefficients for *Time* entered the models as hours. All cytokine measures were log-transformed before entering the analyses to approximate normal distributions.

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Chemical set enrichment analysis: novel insights into sexspecific alterations in primary metabolites in posttraumatic stress and disturbed sleep

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Keywords:	Amino acids; delta power sleep; indoles; insulin; mass spectrometry; PSQI; tryptophan.
Themed Topics:	Clinical Trans-omics, Others

Abstract:	Background Primary metabolites serve as substrates for neurotransmitters and are altered in psychiatric disorders. Sleep disturbances are exacerbated in posttraumatic stress disorder (PTSD), but the contribution of sex, sleep and/or PTSD in altering primary metabolites is not known. Methods We used mass spectrometry to ascertain primary metabolites in 90 plasma samples from individuals with chronic PTSD and control subjects. Laboratory-based polysomnography was used to monitor the sleep of participants. PTSD was determined using the Clinician-Administered PTSD Scale (CAPS). Results Men and women with PTSD showed distinct, non-overlapping primary metabolite nodes compared with sex-matched controls as ascertained using Chemical Set Enrichment Analysis (ChemRICH) analysis. Women with PTSD had seven nodes, whereas men with PTSD had just two nodes altered compared with controls; each node contained two or more metabolites. Sex steroids levels did not associate with metabolite nodes. Higher PTSD symptoms were associated with lower total sleep time and decreased Pittsburgh Sleep Quality Index (PSQI) scores in both men and women. Delta power on sleep electroencephalogram was significantly lower in men with PTSD and associated negatively with PTSD symptoms. Sleep measures accounted for nearly 50% of the altered primary metabolite nodes. Tryptophan and insulin levels were significantly increased in men but not women with PTSD compared with controls, whereas tryptophan levels associated inversely with insulin levels in
	women. Conclusions Women demonstrate more metabolic disturbances than men with similar PTSD scores. The presence of sex-specific primary metabolite in PTSD were not affected by sex steroids but were accounted for by sleep measures. Our data provide further evidence for development of sexspecific interventions and disease management.

Chemical set enrichment analysis: novel insights into sex-specific alterations in primary metabolites in posttraumatic stress and disturbed sleep

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Running Title: Sex-specific primary metabolites in PTSD

Key Words: Amino acids; delta power sleep; indoles; insulin; mass spectrometry; PSQI; tryptophan.

Dear Editor,

We found that women demonstrate more primary metabolite disturbances than men with similar posttraumatic stress disorder (PTSD) severity; decrease in tryptophan metabolites indoles might be due to gut microbiota dysbiosis. PTSD may develop after exposure to actual or threatened death, serious injury, or sexual violence; women are at greater risk. The contribution of disordered sleep in PTSD in altering primary metabolites is unknown. Primary metabolites regulate several physiological functions, serve as substrates for neurotransmitters, and are altered in psychiatric disorders. Mass spectrometry was used to ascertain metabolites in 90 plasma samples from individuals with chronic PTSD and control subjects. Clinician-Administered PTSD Scale for DSM-IV was used to diagnose PTSD. Sleep was monitored using laboratory-based polysomnography. We adjusted for BMI and age in our analyses. Men and women with PTSD did not differ in PTSD severity or history of childhood trauma (Table S1).

Sex aggregated data analysis revealed several metabolites that were significantly altered between control and PTSD groups (Fig. S1). Next, sex segregated Chemical Set Enrichment Analysis[1] on primary metabolites identified seven and two metabolite nodes altered in women and men with PTSD, respectively, compared with sex-matched controls (Fig. 1A-B). Each node contained two or more metabolites; men and women with PTSD did not share any primary metabolites (Fig. 1A-B). Since PTSD symptom presentation is highly variable between individuals,[2] and, in our cohort, women had significantly greater PCL-C scores than men (Table S1), we reasoned that individual PCL measures may associate differently with metabolites (Fig. 1C and Table S2). Serine levels were lower in women with PTSD, whereas glycine levels negatively associated with cluster D symptoms on the PCL, suggesting that with more hyperarousal, glycine levels decreased in women, but not men (Fig. 1C).

Serine, a neurotransmitter, serves as a precursor for the synthesis of glycine, cysteine, and 2-aminobutyric acid, a butyrate, and is synthesized directly from glucose (Fig. 2A). In the human myocardium, 2aminobutyric acid increases glutathione levels via AMPK activation to protect against oxidative stress.[3] An

individual's physiological and metabolic state alters glucose metabolism and generation of non-essential amino acids. Serine and glycine shuttle between the glia and neurons where glycine induces release of serine, a coagonist for NMDA receptors; together they regulate long-term potentiation[4] and are critical for the consolidation of extinction of previously conditioned fear memories.[5] Our data suggests that alterations in subsets of metabolites could be protective in PTSD (Table S2). Changes in metabolite levels in combat veterans with PTSD are reported,[6, 7] but the contribution of sleep or sex has not been investigated.

Sleep disturbances and quality are associated with overall poor health.[8] Both men and women with PTSD had lower total sleep time (TST) and worse self-reported sleep quality as assessed by the Pittsburgh Sleep Quality Index (PSQI) compared with controls (Fig. 2B). Delta power, a measure of deep sleep activity,

decreased in PTSD and showed a significant sex difference; men had lower delta power sleep activity than women (Fig. 2B). Greater PTSD symptoms associated with lower TST in both women and men, and PSQI associated with greater PTSD symptoms in both women and men; delta power was lower in men with PTSD compared to controls, but not in women (Fig. 2C). Humans lack the ability to synthesize eight essential amino acids, including tryptophan, that must be obtained from diet; they are mostly absorbed by the gut and metabolized by the resident microbiota. Tryptophan is metabolized to a myriad of biologically active compounds by four different pathways (serotonin, tryptamines, kynurenine, and indoles). TST accounted for alterations in all metabolite nodes in men and two of the six metabolite nodes in women (Fig. 2D, 3A, and Table S2). When PSQI was used as a confounder, new, non-overlapping nodes were found to be significant in women and men; indoles, hexose and amino acid nodes were decreased in women (Fig. 2D). Delta power sleep accounted for 50% of the nodes in women (Table S2). Interestingly, higher testosterone levels associated with lower delta power in men (Fig. 3C).

Plasma insulin, albumin, and amino acids levels together modulate transport of free tryptophan to the brain.[9] In our cohort, no difference in insulin levels were seen in women; plasma albumin levels did not differ between controls and PTSD in either sex. Insulin levels were significantly elevated in men with PTSD

compared with controls (Fig. 3D-E). Tryptophan levels associated negatively with insulin levels in women (Fig. 3F) with concomitant increases in levels of several amino acids that compete with a tryptophan carrier for influx into the brain. Increases in palmitoleic acid levels may be compensatory, more so in women than in men, which can potentially displace albumin from tryptophan in order to generate free tryptophan.

Tryptophan is absorbed in the gut and converted by the actions of gut microbiota such as *Lactobacillus* to indoles (Fig. 4). While the role of serotonin in mood disorders, anxiety, and other disorders is well known,

we show here for the first time that the indole metabolite, indole-3-propionic acid, is decreased in women with PTSD. Poorer sleep quality was further associated with decreased levels of two additional indoles, indole-3-lactic and acetic acids; the indoles regulate immune and gut barrier functions (Fig. 4B), and their decreased levels might contribute to altered immune and barrier function in women. Diets rich in butyrates that support growth of beneficial gut bacteria such as *Lactobacillus* may serve as non-invasive

Surprisingly, sex steroid metabolites did not differ between controls and PTSD in either men or women (Table S3), nor did sex steroids associate with PTSD. Testosterone levels were ~10-fold higher in men

interventions, especially for women.

(Table S3).

In conclusion, there was no overlap in PTSD-related alterations in men and women in any primary metabolite nodes or individual amino acids within those nodes, and associated pathways. A balance between levels of essential amino acids, insulin, and albumin determines availability and transport of free tryptophan to the brain, which might in turn influence production of serotonin and melatonin, and distinct cellular pathway operate within functional clusters in men and women (Fig.4). **Figure Legends**

Figure 1. Sex differences in primary metabolites in PTSD. (A) Chemical Set Enrichment (ChemRICH) analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI and age. ChemRICH is a statistical enrichment approach based on chemical structure similarity/chemical ontologies and is an alternative to pathway analysis that relies on limited biochemical knowledge annotations. It yields study-specific, non-overlapping sets of all identified metabolites. Since ChemRICH sets have a self-contained size, thus p-values do not rely on the size of the background database. Blue nodes contain metabolite clusters that were decreased, purple nodes contain metabolites that were both increased or decreased, and red nodes contain metabolites that were increased in PTSD vs. control individuals. Branched-chain and sulfur-containing amino acids, and unsaturated fatty acids were increased, indoles and cyclic amino acids were decreased, whereas the non-polar amino acid node contained metabolites that either increased or decreased in women with PTSD compared to controls. A Venn diagram showing seven nodes were specific to women with PTSD and two nodes were specific to men with PTSD compared with controls. (B) Volcano plots of specific metabolites within each node in men and women with PTSD after correcting for type 1 error and adjusting for BMI and age. (C) Box plots of specific amino acids with the seven nodes that differed between women and two nodes in men with PTSD compared to controls shown in Fig. 1A. Glycine levels associated negatively with PCL cluster D in women with PTSD ($r = –0{\cdot}47$, $p = 0{\cdot}036$), but not controls, whereas association of serine level was lost in women with PTSD. Cysteine and 2-AB levels were elevated in PTSD compared with controls, but did not show any significant association with specific clusters of PTSD symptoms. Fructose levels increased in men with PTSD vs. controls, but fructose levels associated negatively with PCL scores. Box plot analysis: Mann-Whitney and $p<0{\cdot}05$ considered significant.

Figure 2. PTSD-specific alterations in amino acids with respect to their biosynthesis pathway in humans from glucose. (A) Essential amino acids cannot be synthesized and must be obtained from diet, whereas non-essential amino acids can be synthesized from glucose as it enters the tricarboxylic cycle. Essential amino acids are shown in grey boxes, and those metabolites synthesized as a by-product of gut

microbiome are shown in green boxes. Specific amino acids that were increased are shown in red, decreased in blue (women-specific outlined in pink and men-specific in blue). (B) Sex-specific disturbances in sleep measures. Box plots showing sex- and/or PTSD-specific alterations in Total sleep time (in minutes) decreased; Two-way ANOVA: Sex: ns; PTSD: p= 0·004; Sex X PTSD: ns. Sleep quality worsened, Twoway ANOVA: Sex: ns; PTSD: p< 0·0001; Sex X PTSD: ns, and log-transformed delta power sleep was lower in people with PTSD vs controls, Two-way ANOVA: Sex: p= 0·007; PTSD: p= 0·005; Sex X PTSD: ns. Greater PTSD symptoms (reflected by the total PCL score) negatively associated with TST (r= –0·41, p=0·005 and r= –0·32, p=0·033, women and men, respectively), and PSQI was positively associated with greater PTSD symptoms (r=0·84 and r=0·74, p<0·001, women and men, respectively). Delta power negatively associated with PTSD (*r*= –0·35, *p*=0·02) in men alone. (C) Linear regression showing association of PCL scores with three different measures of sleep. (D) Sex differences in primary metabolites after accounting for sleep measures. ChemRich analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI, age, and one of the three sleep measures shown (TST (min), PSQI, or log-transformed delta power (ln(μV2)). Blue nodes contain metabolite clusters that are decreased, purple nodes contain metabolites that are both increased or decreased, and red nodes contain metabolites that are increased in PTSD vs control individuals.

Figure 3. Sex-specific contribution of sleep variables on primary metabolites. (A) Venn diagrams to visualize significant metabolites While adjusting for one sleep variable at a time. (B) Linear regression and box plots of various amino acids with sleep variables. Box plots of specific amino acids with individual nodes that differed between women and men with PTSD compared to controls. (C) Log-transformed delta power (ln(μV2) associated negatively with testosterone in men. (D) Changes in insulin and tryptophan levels in women and men with PTSD. Box plots of tryptophan and albumin levels in women and men. No significant differences were seen in tryptophan and albumin levels in women with PTSD compared with controls, whereas tryptophan levels were significantly elevated in men with PTSD compared with controls (p = 0·044; Mann-Whitney). (E) Blood insulin levels were determined after an oral glucose challenge at 6

various times shown. Mixed-effect analysis showed that insulin levels changed with time ($p<0{\cdot}0001$) in women and men, but only differed between PTSD and controls in men ($p=0{\cdot}0136$). (F) Tryptophan levels correlated negatively with insulin levels at 60 min in women ($r= -0{\cdot}30$, $p=0{\cdot}04$), but positively in men, although the relationship did not reach statistical significance.

Figure 4. Sex-specific alterations in the tryptophan metabolism pathway in PTSD. (A) Sex, sleep, and PTSD all alter primary metabolites. (B) Albumin-bound tryptophan is present in circulation and dynamic increases in insulin promote binding of albumin to tryptophan, whereas esterified fatty acids can displace tryptophan from albumin. Free tryptophan is then transported to the brain by a transport carrier. Several amino acids, such as leucine, valine etc. compete with tryptophan for binding to the transport carrier, which can decrease influx of free tryptophan into the brain. Reduced free tryptophan levels in the brain can influence production of serotonin and melatonin, affecting brain function and sleep. In the gut, tryptophan is converted to indoles by the action of microbes such as*Lactobacillus*; these indoles have protective effect on gut barrier and immune functions. Serine can serve as NMDA receptor agonist and alter neuronal function. Thus, disturbances at multiple levels in tryptophan pathway may contribute to pathogenesis of PTSD.

References

1. Barupal DK, Fiehn O. Chemical Similarity Enrichment Analysis (ChemRICH) as alternative to biochemical pathway mapping for metabolomic datasets. Sci Rep 2017;7:14567.
2. Galatzer-Levy IR, Bryant RA. 636,120 Ways to Have Posttraumatic Stress Disorder. Perspect Psychol Sci 2013;8:651-62.
3. Irino Y, Toh R, Nagao M, et al. 2-Aminobutyric acid modulates glutathione homeostasis in the myocardium. Sci Rep 2016;6:36749.
4. Neame S, Safory H, Radzishevsky I, et al. The NMDA receptor activation by d-serine and glycine is controlled by an astrocytic Phgdh-dependent serine shuttle. Proc Natl Acad Sci U S A 2019;116:20736-20742.
5. Davis M. NMDA receptors and fear extinction: implications for cognitive behavioral therapy. Dialogues Clin Neurosci;13:463-74.

6. Mellon SH, Bersani FS, Lindqvist D, et al. Metabolomic analysis of male combat veterans with post traumatic stress disorder. PLoS One 2019;14:e0213839.
7. Somvanshi PR, Mellon SH, Flory JD, et al. Mechanistic inferences on metabolic dysfunction in posttraumatic stress disorder from an integrated model and multiomic analysis: role of glucocorticoid receptor sensitivity. Am J Physiol Endocrinol Metab 2019;317:E879-E898.
8. Richards A, Metzler TJ, Ruoff LM, et al. Sex differences in objective measures of sleep in post-traumatic stress disorder and healthy control subjects. J Sleep Res 2013;22:679-87.
9. Daniel PM, Love ER, Moorhouse SR, et al. The effect of insulin upon the influx of tryptophan into the brain of the rabbit. J Physiol 1981;312:551-62.

Contributors

AB, TCN, and SSI contributed to the design of the study. AB wrote the first draft of the manuscript with input from SSI, and AB, TCN, and SSI revised the final manuscript. CL and SSI collected patient data, RR, AO, SF, OF, SSI, and AB extracted and analyzed the data.

All other authors declare no competing interests. The views, opinions and/or findings contained in this research are those of the authors and do not necessarily reflect the views of the Department of Defense, Department of Veteran Affairs, or NIH and should not be construed as an official DoD/Army/VA/NIH position, policy, or decision unless so designated by official documentation. No official endorsement should

Disclosures

be made.

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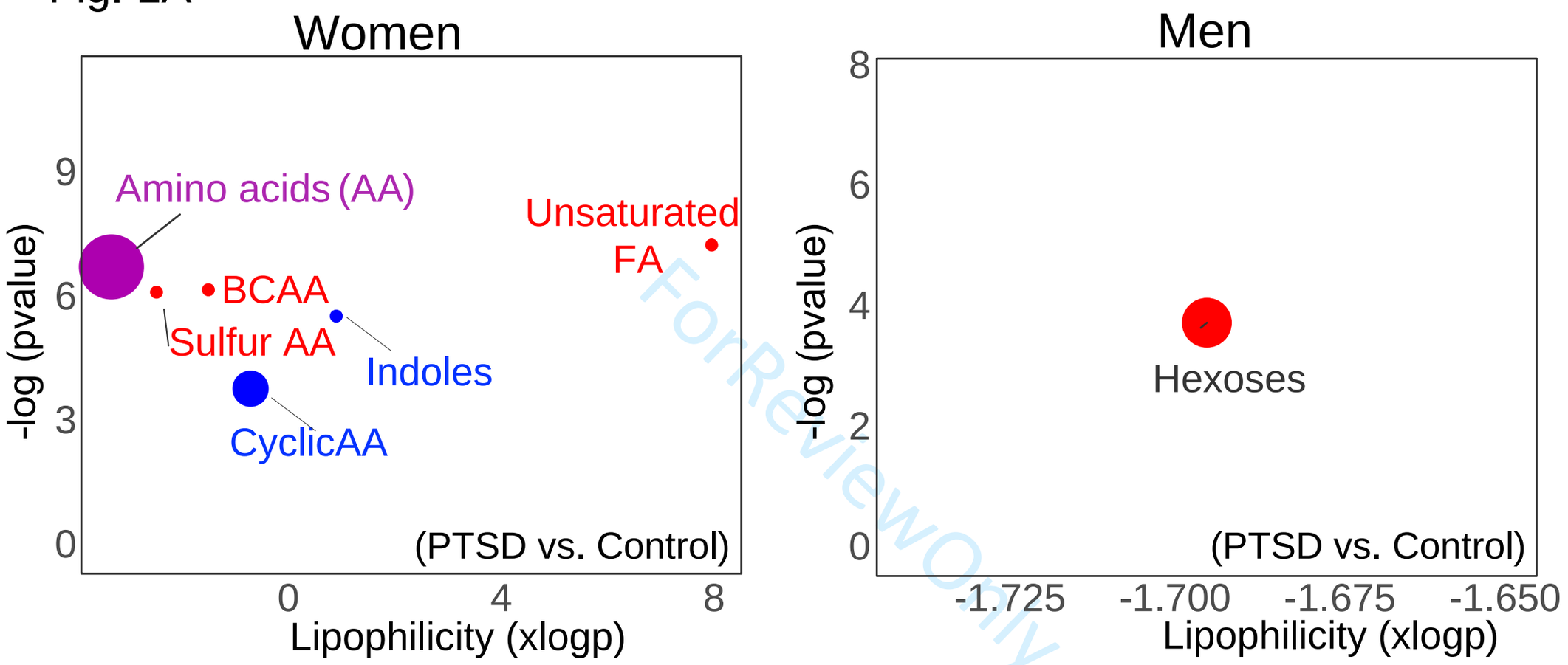
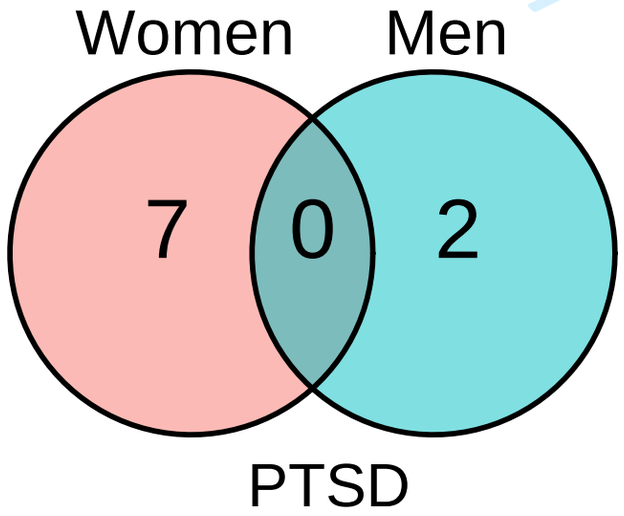
Fig. 1A





Blue: decrease
Purple: both increase and decrease
Red: increase

Fig. 1B

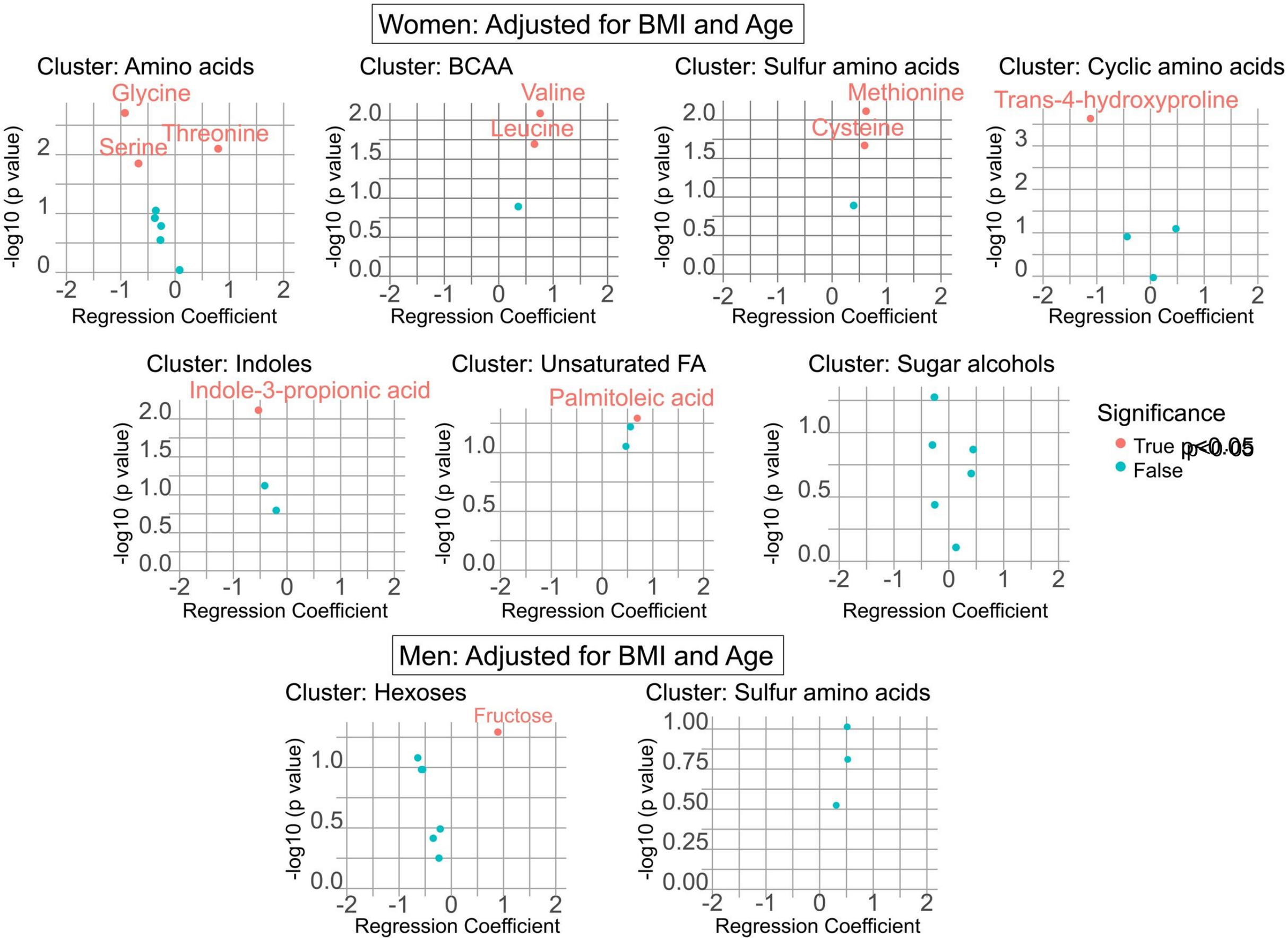
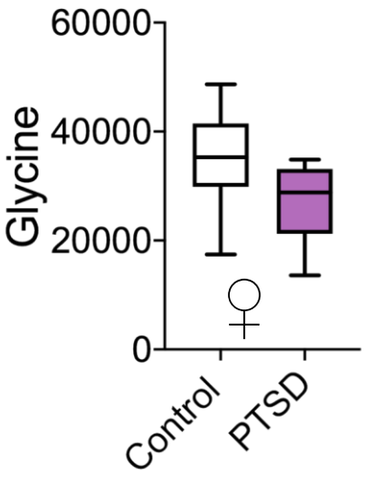
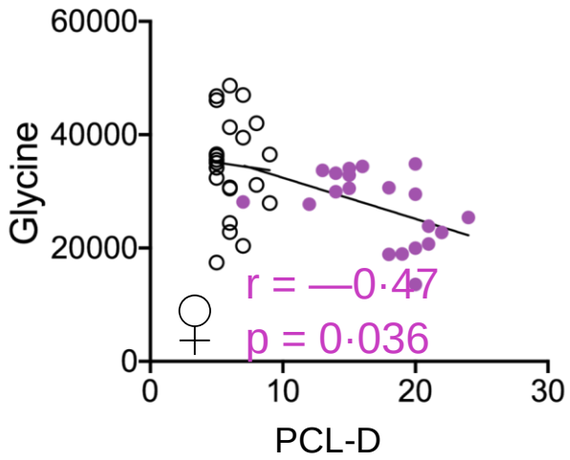

Women: Adjusted for BMI and Age
Cluster: Amino acids
Glycine
Threonine
Serine
Cluster: BCAA
Valine
Leucine
Cluster: Sulfur amino acids
Methionine
Cysteine
Cluster: Cyclic amino acids
Trans-4-hydroxyproline
Cluster: Indoles
Indole-3-propionic acid
Cluster: Unsaturated FA
Palmitoleic acid
Cluster: Sugar alcohols
Significance
True p<0.05
False
-log10 (p value)
Regression Coefficient
Men: Adjusted for BMI and Age
Cluster: Hexoses
Fructose
Cluster: Sulfur amino acids

Fig. 1C

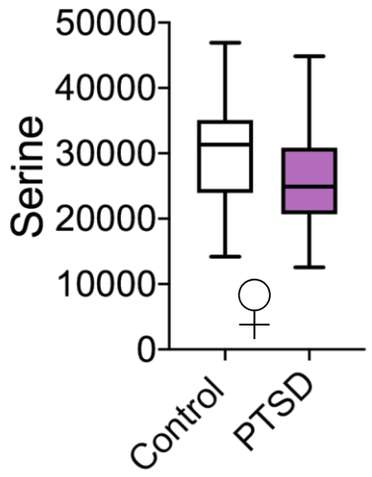
Amino Acids



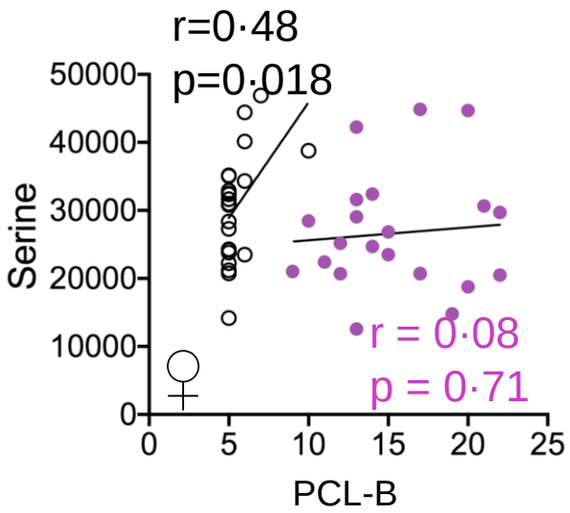
p=0·0022

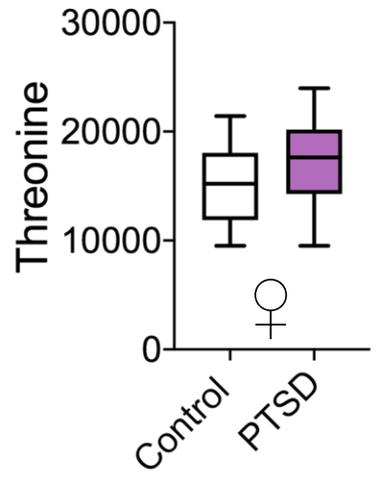


r=–0·05 p=0·053

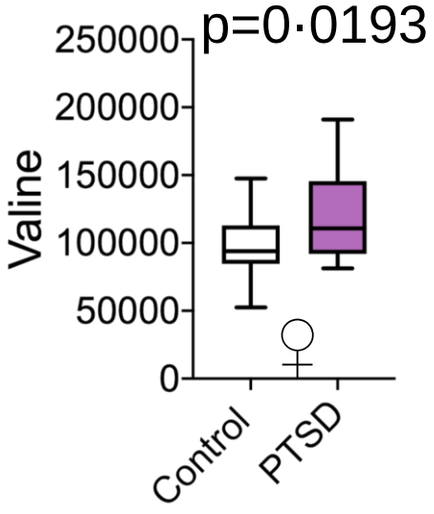


p=0·0392 p=0·82

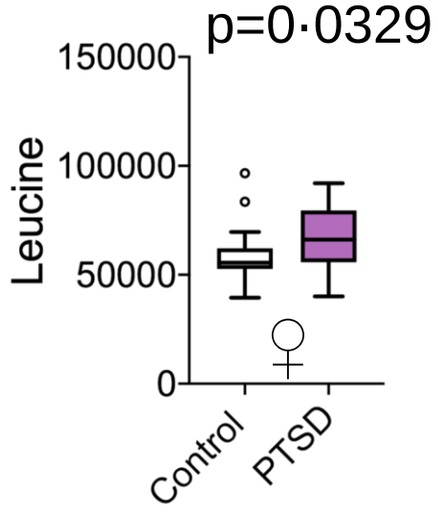




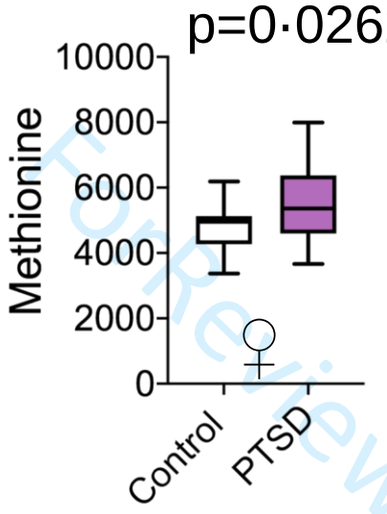
p=0·0193



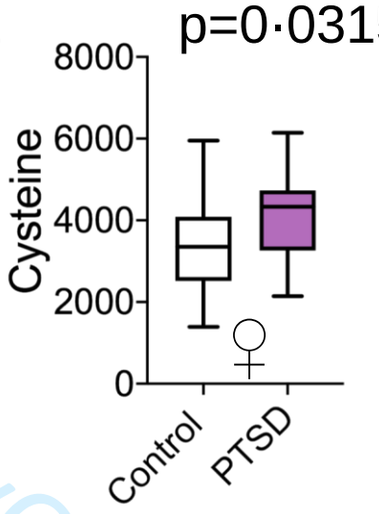
p=0·0329



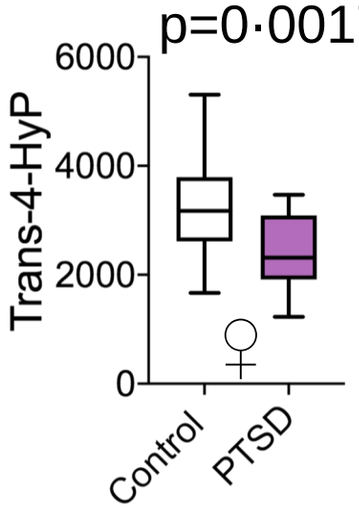
p=0·0262



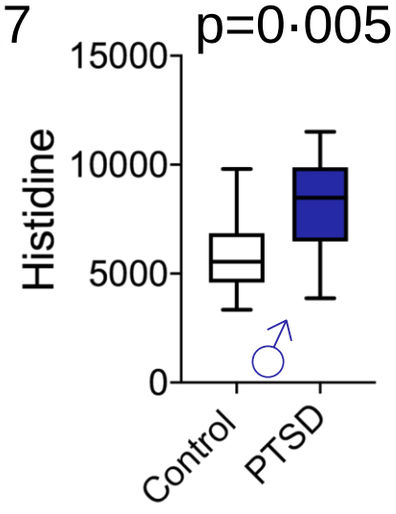
p=0·0315



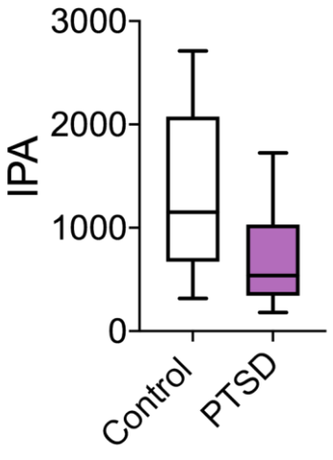
p=0·0017



p=0·005

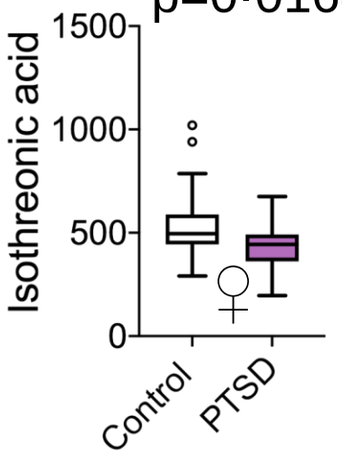


Indoles

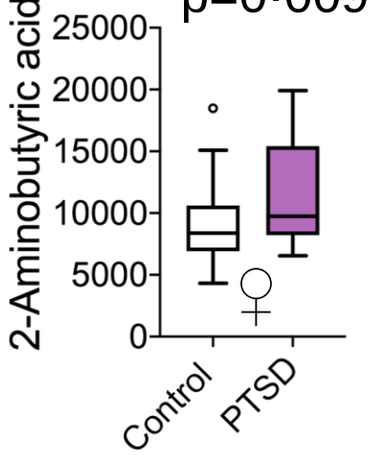


Butyrates

p=0·0168

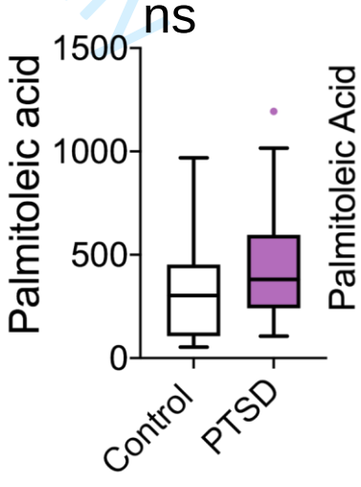


p=0·009

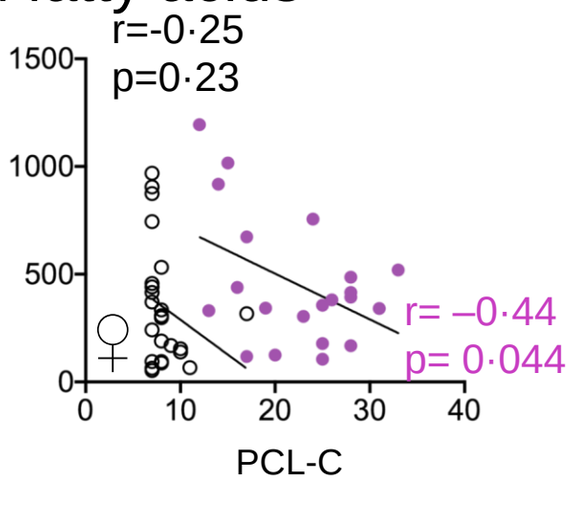


Unsaturated fatty acids

ns



r=-0·25
p=0·23



BCAA

Sulfur AA

Cyclic AA

p=0·0034

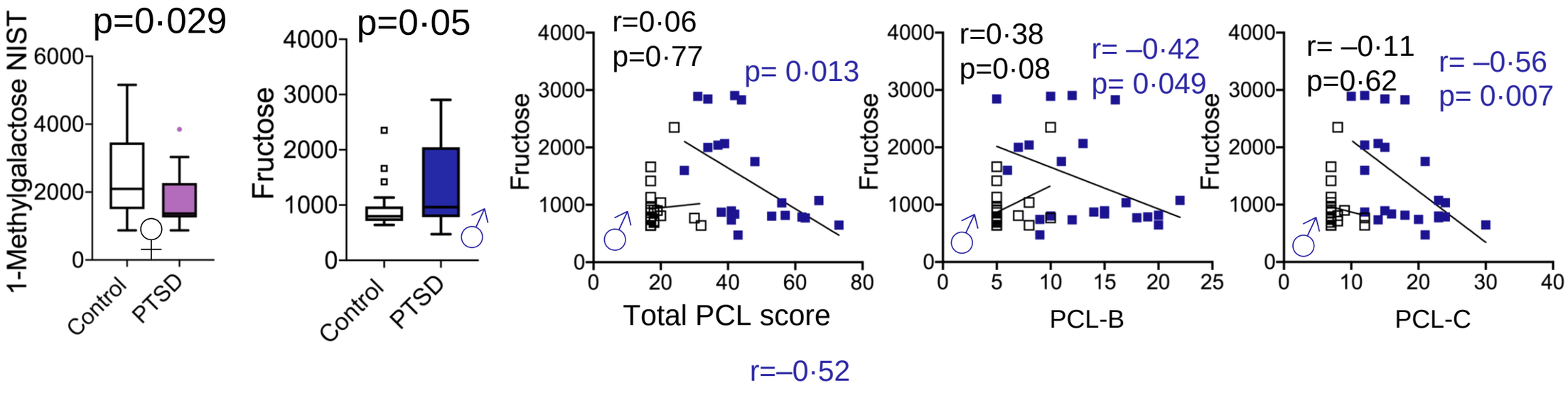

Hexose
p=0·029
1-Methylgalactose NIST
6000
4000
2000
0
Control
PTSD
p=0·05
Fructose
4000
3000
2000
1000
0
Control
PTSD
♂
r=0·06
p=0·77
p= 0·013
Fructose
Total PCL score
r=0·38
p=0·08
r= –0·42
p= 0·049
PCL-B
r= –0·11
p=0·62
r= –0·56
p= 0·007
PCL-C
r=–0·52

Fig. 2A

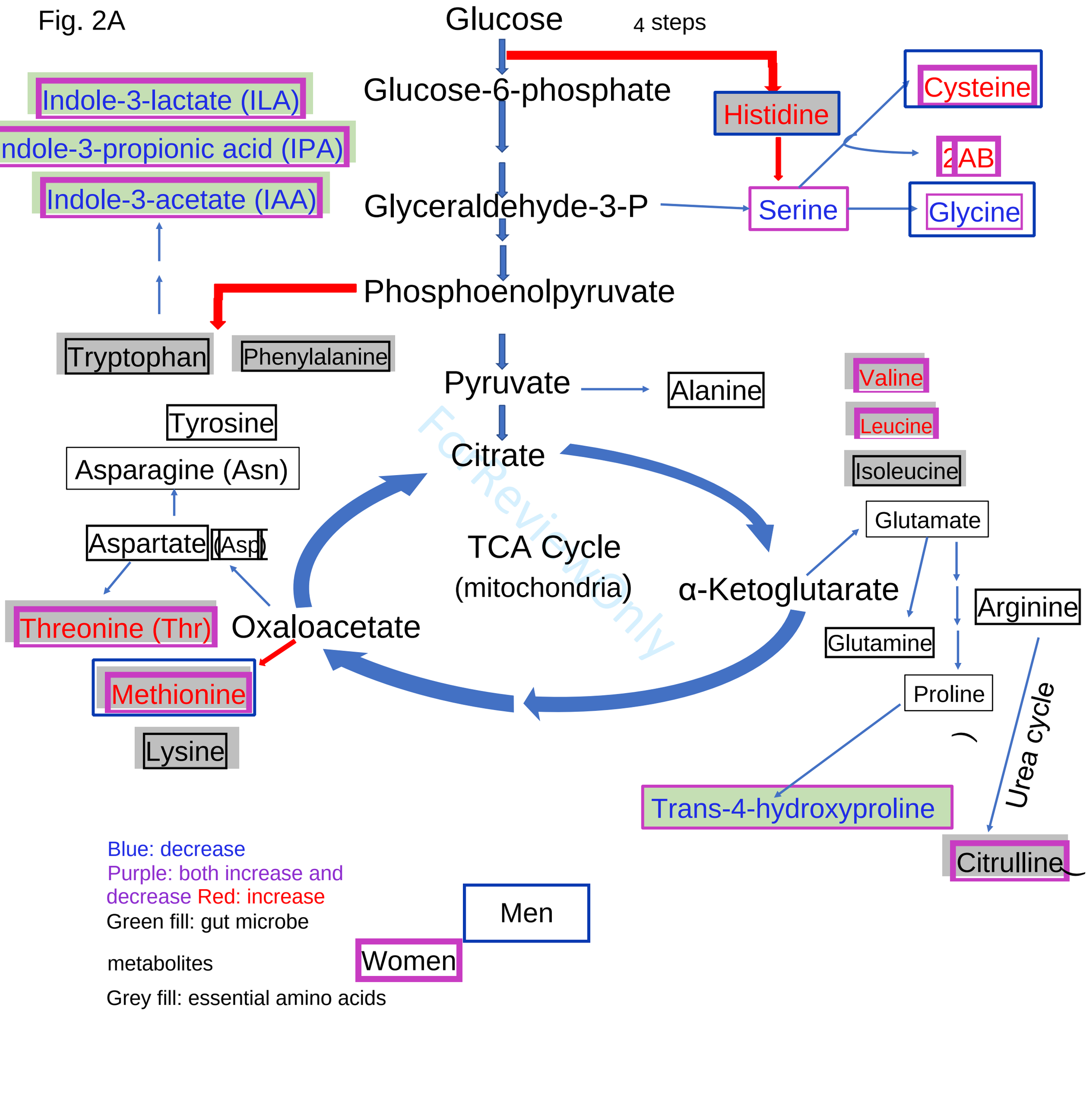
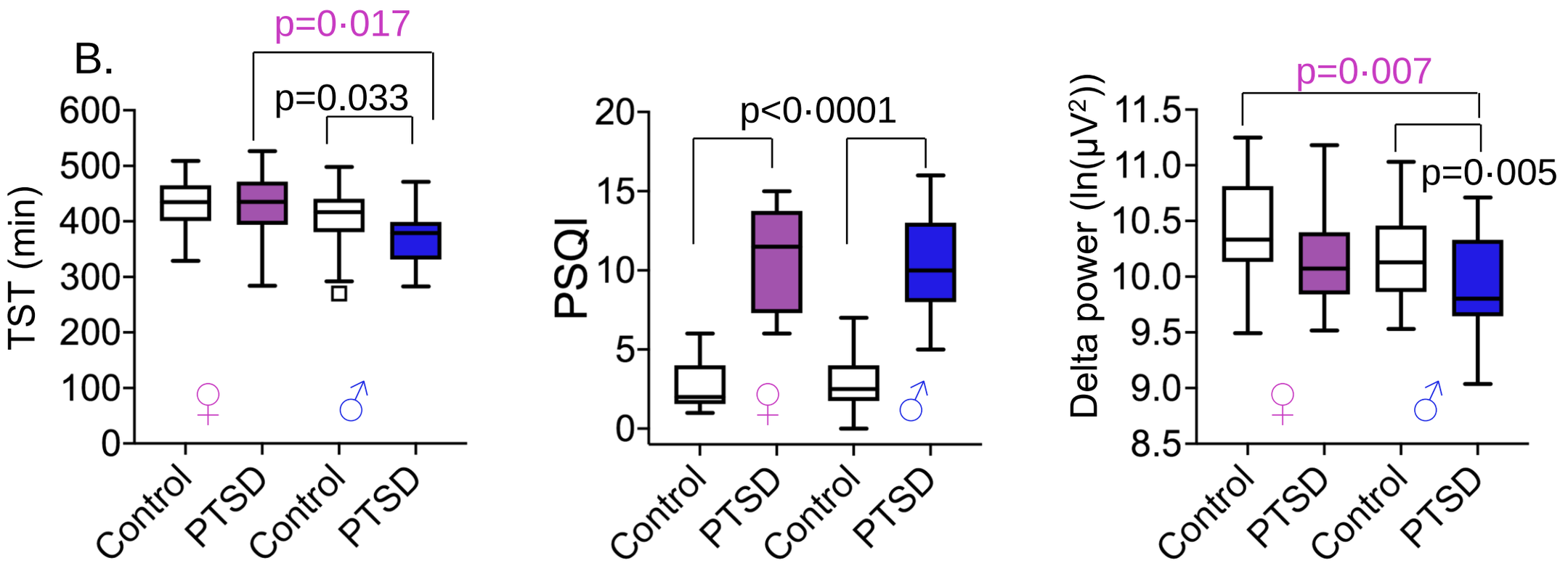


Fig. 2



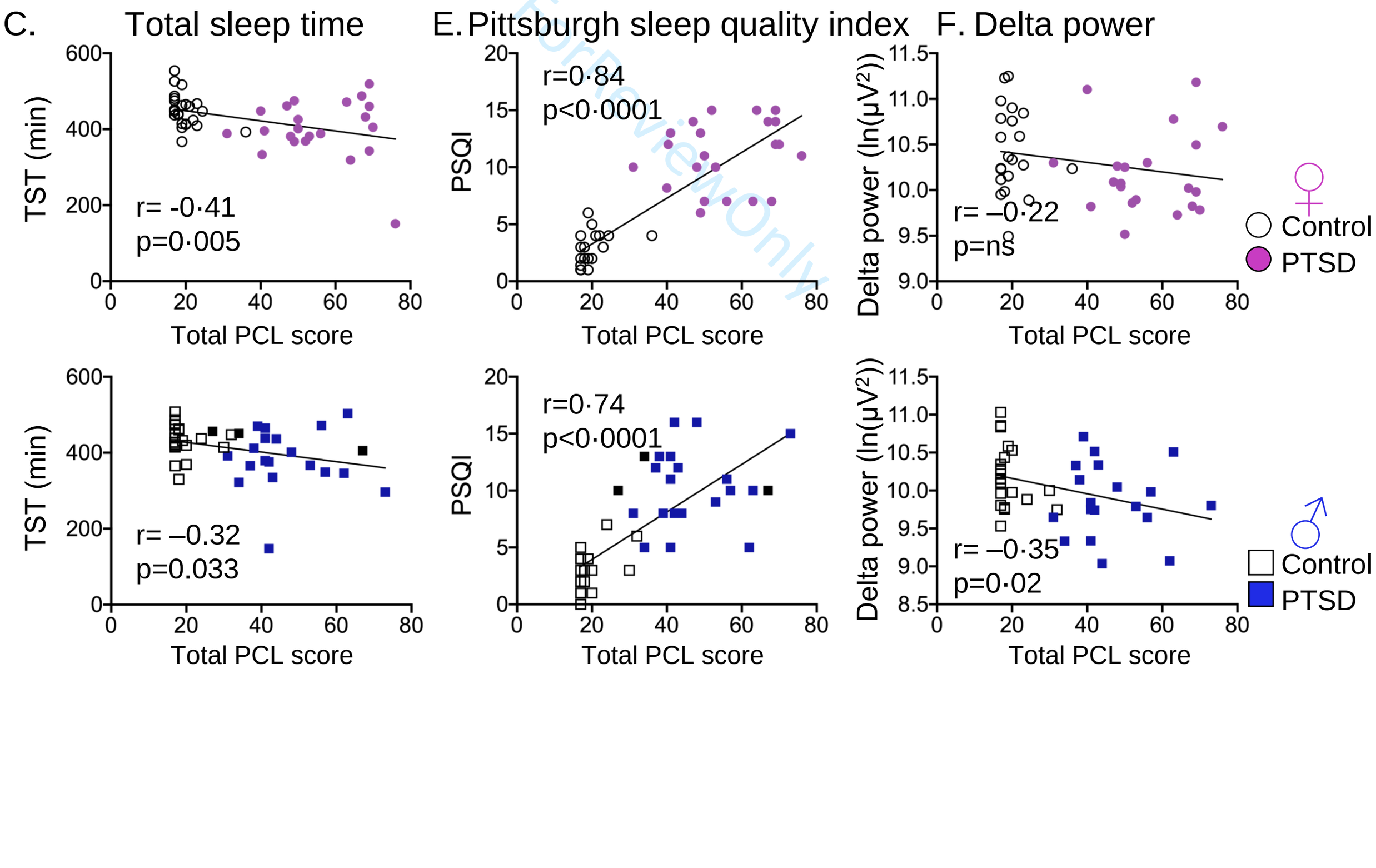
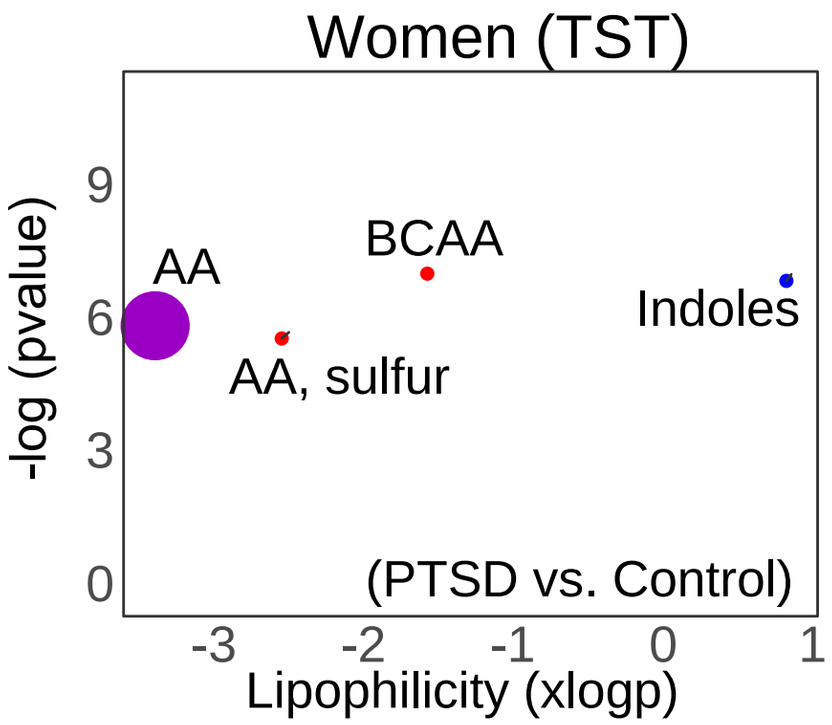
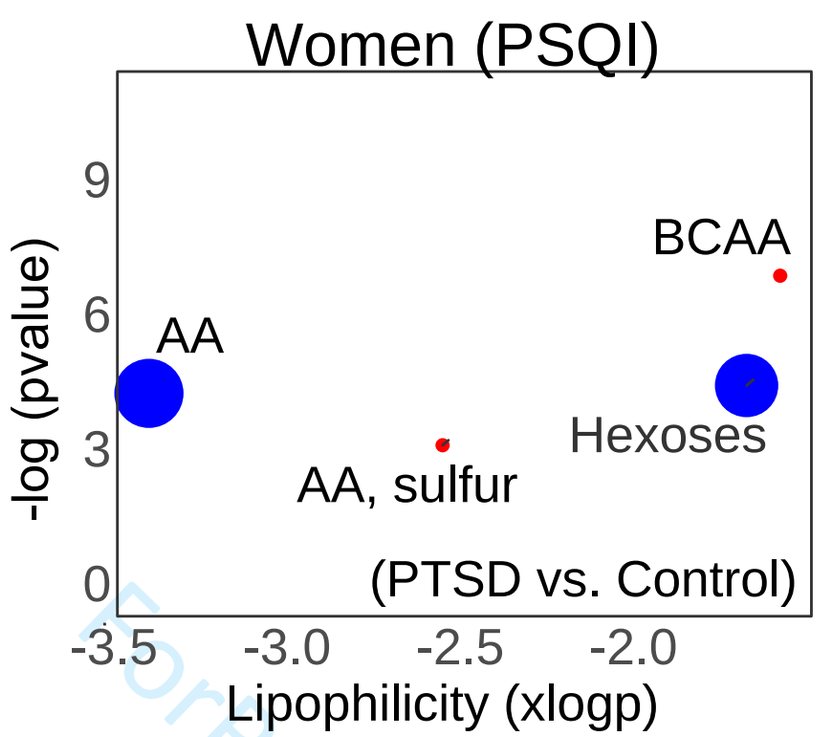
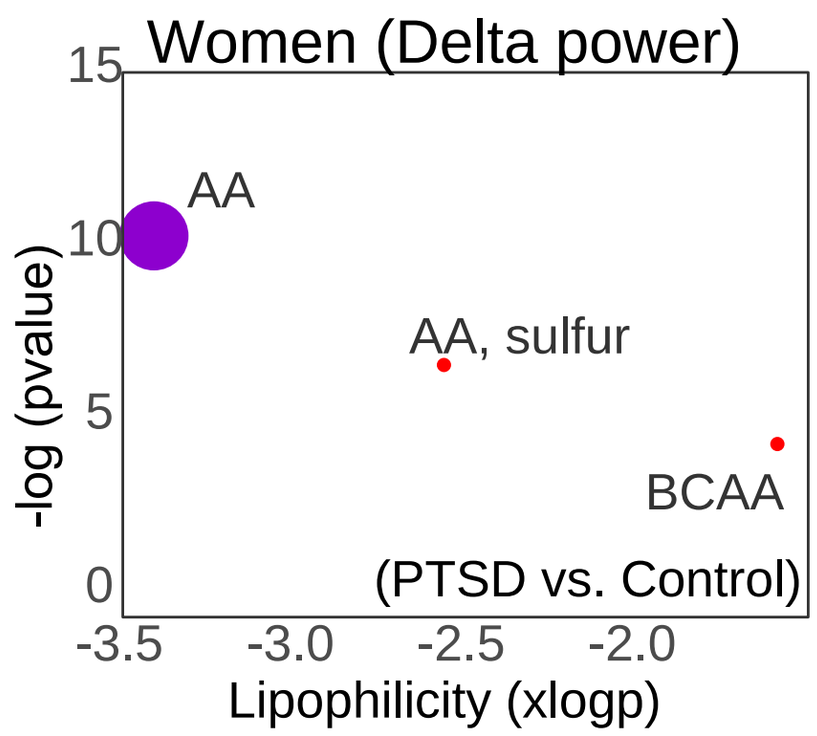


Fig. 2D



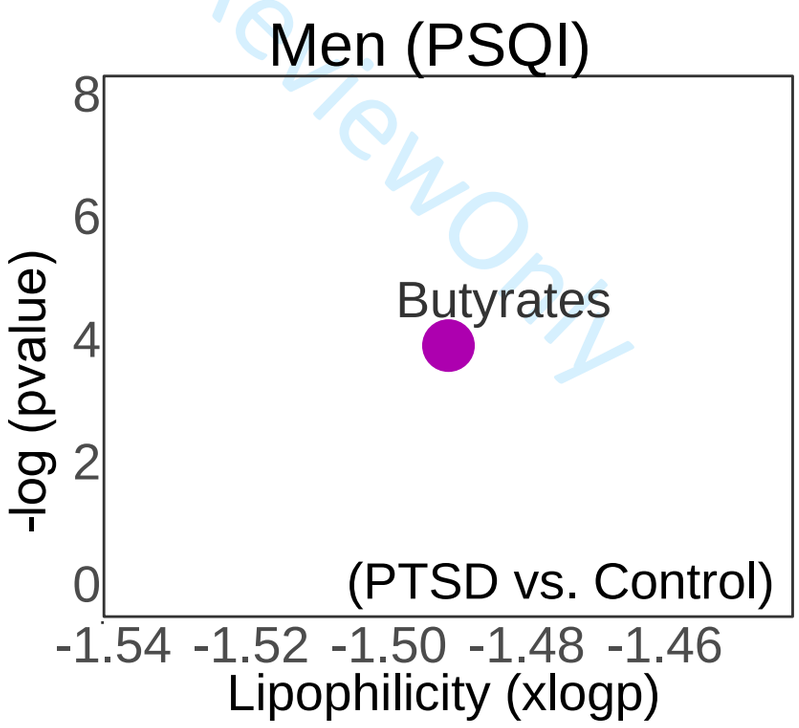




Blue: decrease

Purple: both increase and decrease

Red: increase



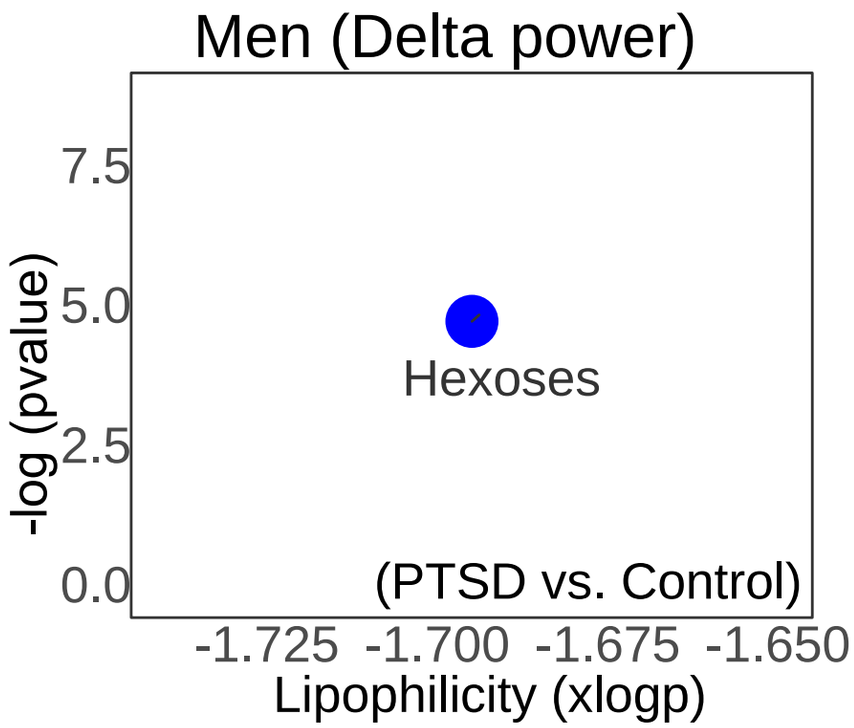


Fig. 3A

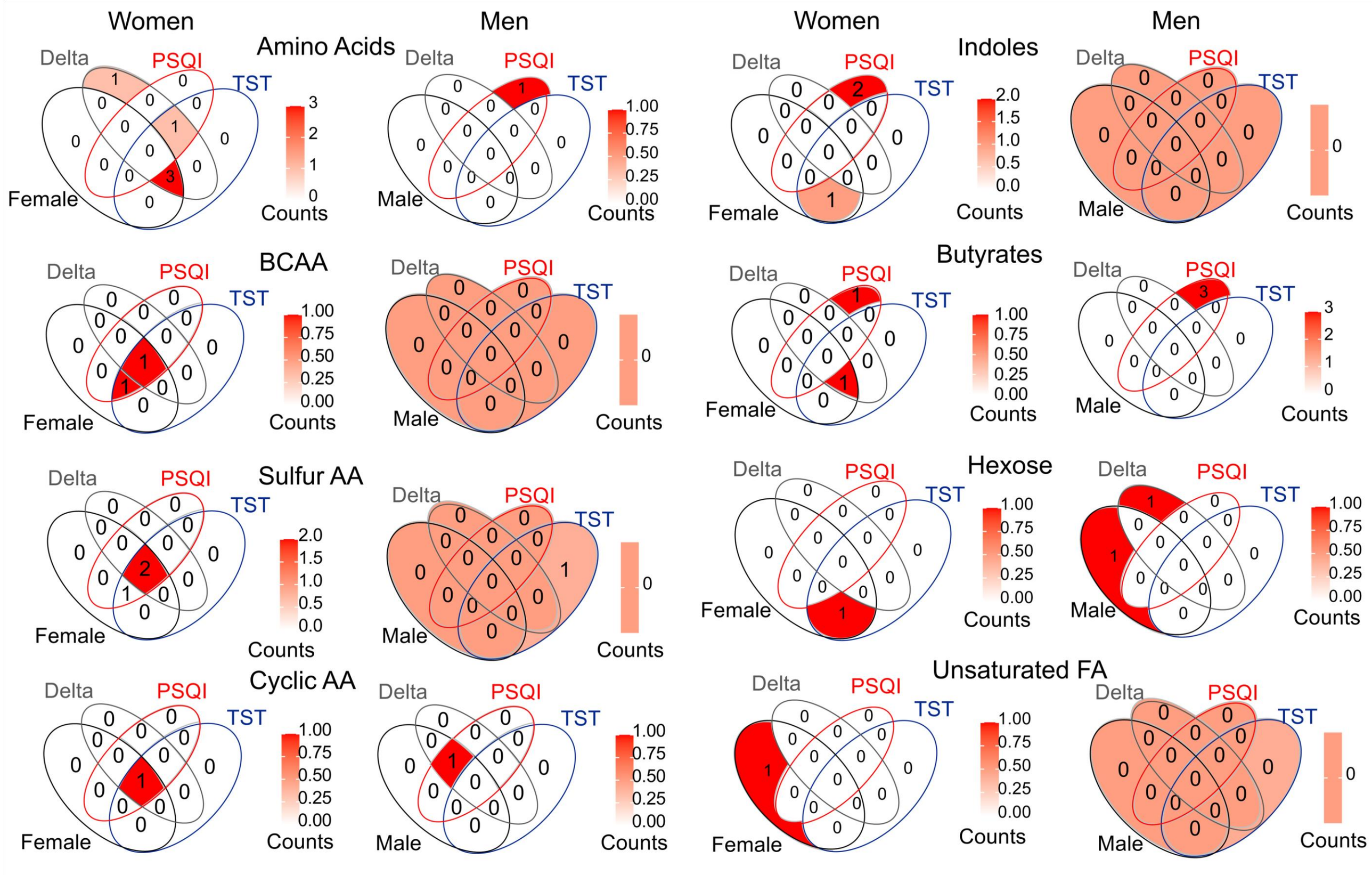
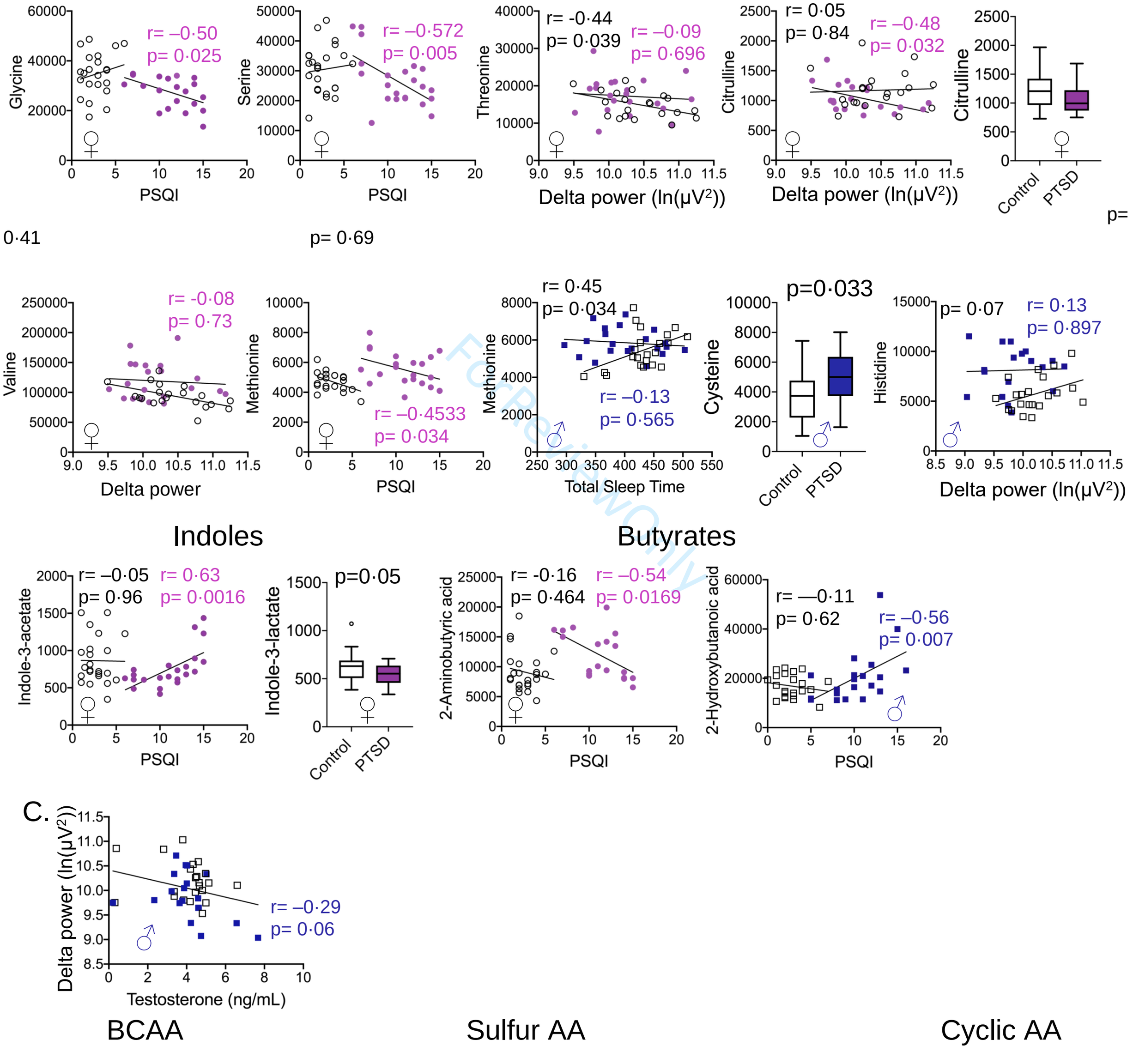

Women
Men
Amino Acids
Delta
PSQI
TST
Female
Male
Counts
Indoles
BCAA
Butyrates
Sulfur AA
Hexose
Cyclic AA
Unsaturated FA

Fig. 3

B. Amino Acids



Indoles

Butyrates

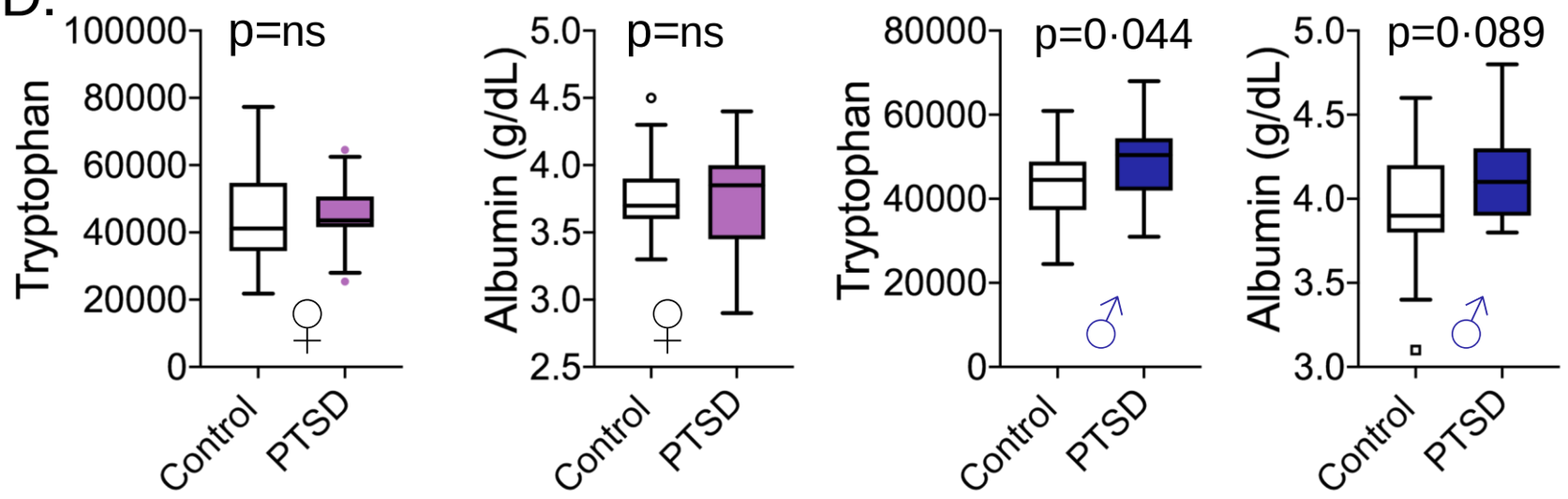
C.

BCAA Sulfur AA Cyclic AA

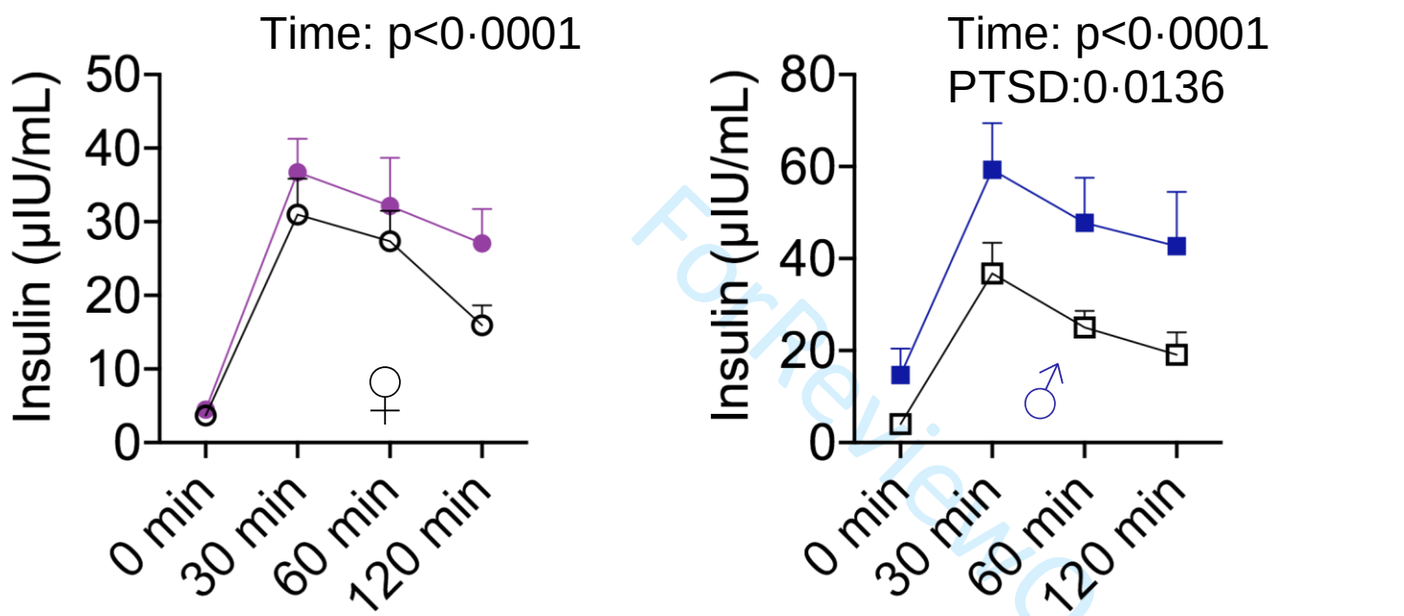
r= –0·532 r= –0·4148 r= 0·40 p= 0·013 p= 0·055

Fig. 3

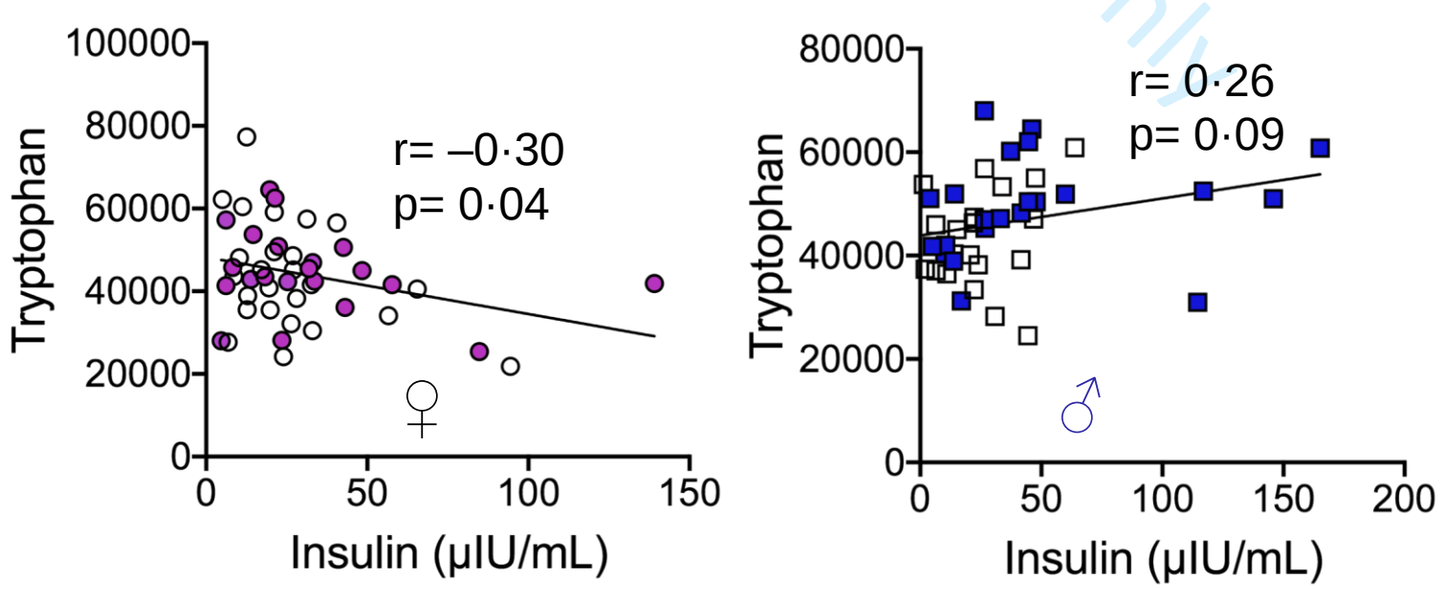
D.

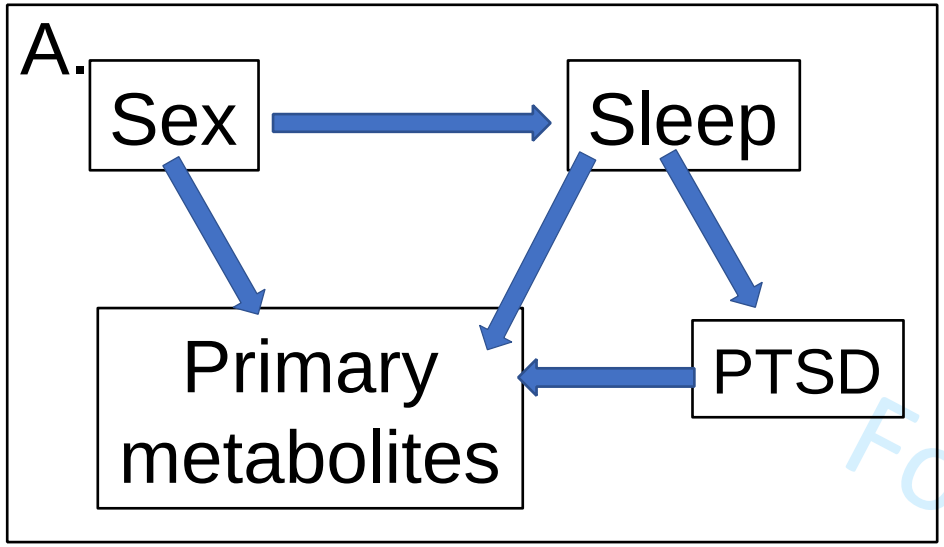


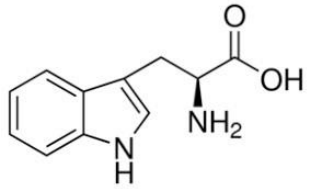
E.



F.



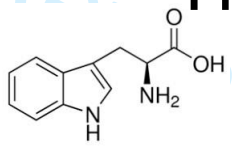




B. Tryptophan + Albumin = bound Tryptophan

Insulin

Palmitoleic acid (esterified FA)



Brain

Leu, Ileu, Met

Free Tryptophan

~1-2%

TPH

Melatonin

Serotonin (5HT)

Sleep

~93%

IDO-1

Kyneurenine

~5%

Gut microbiota

Vitamin B6

Indoles

KA/QA

NMDA-R

Serine

IPA, ILA, IAA

Immune function

Gut barrier function

Chemical set enrichment analysis: novel insights into sex-specific alterations in primary metabolites in posttraumatic stress and disturbed sleep

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Running Title: Sex-specific primary metabolites in PTSD

Key Words: Amino acids; ChemRich plot; Delta power sleep; insulin; mass spectrometry; PSQI.

List of Abbreviations

AAs: amino acids
BMI: body mass index
ChemRICH: Chemical Set Enrichment Analysis
CAPS:Clinician-Administered PTSD Scale
COVID-19: Coronavirus disease-19
DSM: Diagnostic and Statistical Manual of Mental Disorders
EEG: electroencephalogram
EOG: electrooculograms
GCRC: General Clinical Research Center
GC-TOF-MS: gas chromatography /time of flight mass spectrometry (GC-TOF MS)
HPLC: High pressure liquid chromatography
HPA: hypothalamic-pituitary-adrenal
ICU: Intensive care unit
KYN: Kynurenine
NREM: non-rapid eye movement
NMDA: *N*-methyl-D-aspartate receptors
OGTT: Oral Glucose Tolerance Test
PTSD: posttraumatic stress disorder
PSQI: Pittsburgh Sleep Quality Index
TST: Total sleep time

Supplemental materials and methods

Human subjects

This study was a 2 x 2 cross-sectional design with 4 groups (PTSD/control × women/men) of 44 individuals with current chronic PTSD and 46 control subjects. Participant's age ranged from 19-39 years. Data from 4 participants were excluded due to difficulties in blood collection. Sleep measures were recorded in an inpatient sleep laboratory at the General Clinical Research Center (GCRC) at the University of California, San Francisco. The Committee on Human Research at the University of California, San Francisco approved this study. Written informed consent was provided to all participants before enrollment and start of any study procedures.

PTSD subjects met DSM-IV criteria as ascertained using the Clinician-Administered PTSD Scale (CAPS) or score >40. Control subjects had no lifetime or current history of a PTSD diagnosis. Women participants were premenopausal, and were scheduled during the follicular phase of the menstrual cycle. All study procedures were timed according to habitual sleep onset, determined by actigraphy and sleep diary in the week prior to the GCRC study. Study participants were limited to one cup of caffeine daily, maintained regular bed and waking times, did not consume alcohol, and were drug-free. Exclusion criteria: history of traumatic brain injury, presence of neurologic disorders or systemic illness; use of psychiatric, anticonvulsant, antihypertensive, sympathomimetic, steroidal, statin or other prescription medications; obesity (defined as body mass index (BMI) >30); alcohol abuse or dependence in the prior two years; substance abuse or dependence in the previous year; any psychiatric disorder with psychotic features; bipolar disorder or obsessive-compulsive disorder; and pregnancy. Exclusion criteria for control subjects also included a lifetime history of major depressive disorder or panic disorder.

Psychiatric diagnoses and trauma history

The Life Stressor Checklist-Revised interview was used to determine trauma exposure and age of occurrence.[1] The PTSD Checklist (civilian version) for DSM-IV (PCL-C)[2] was used as a self-reported

measure of severity of chronic PTSD symptoms.[3] The PCL-C consists of 17 items that correspond to the DSM-IV criteria and include intrusive thoughts and re-experiencing symptoms (cluster B), avoidance (cluster C), and hyperarousal (cluster D). The structured clinical interview for DSM-IV, non-patient edition (SCID-NP) was used to diagnose all other psychiatric disorders, including major depressive disorder.[4]

Sleep clinic and measures

The Pittsburgh Sleep Quality Index[5] was used to provide a subjective assessment of sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbances (including nightmares), use of sleep medication, and daytime dysfunction over the previous month as described elsewhere.[6]

Polysomnographic measurements

Ambulatory polysomnography (Nihon Kohden Trackit Ambulatory Recording System) was used to record electroencephalogram (EEG) at leads C3, C4, O1 and O2, left and right electrooculograms (EOG), submental electromyogram, bilateral anterior tibialis EMGs, and electrocardiogram in accordance with standardized guidelines by Rechtschaffen.[7] Electrode impedance was set at < 5K Ohm at the start of the recording. The EEG and EOG leads were referenced to linked mastoids. Raw EEG signals were filtered and amplified, then digitized at 256 Hz and recorded to a removable hard disk in European Data Format file format. The low frequency and high frequency hardware filters on the recorder were single pole analog filters with 3 db points at 0·5 Hz and 100 Hz. Pass Plus was utilized for both visual scoring and quantitative EEG analysis of the digitized polysomnography data.

Power spectral analysis for polysomnographic measures

Pass Plus (Delta Software) analytic software was used to measure sleep activity in all frequency bands delta through gamma from the C3 electrode by power spectral analysis. The C4 electrode was used if there was excessive artifact. A limitation of Pass Plus is that artifact removal is accomplished by removal of whole epochs tagged with artifact. This has the potential to introduce additional confounds given the removal of

typically longer bouts of uncontaminated EEG. Therefore, epochs were tagged for slow and fast artifact for additional analyses. Primary analyses were conducted with all epochs and then checked for the impact of removal of epochs with slow and fast artifact. Removal of fast artifact (for bandwidths alpha and above) and slow artifact (for bandwidths delta and theta) did not significantly impact our findings in non-rapid eye movement (NREM) sleep. All results are therefore reported without removal of epochs containing artifact. Pass Plus applied a 5μV smoothing constant to eliminate spurious waves caused by electrical jitter. Power spectral analysis was conducted on all epochs of NREM and REM sleep. Epochs visually scored as wake

were not included in these analyses. Visual scoring was conducted by a highly experienced registered polysomnography technician, blind to PTSD status, classified all 30-second epochs in every sleep record as wake; stages 1, 2, 3; REM; or movement using current American Academy of Sleep Medicine criteria. Total sleep time was defined by time spent in epochs scored as NREM stages 1 through 3 and stage REM. Pass Plus was used to perform Fast Fourier Transformation analysis on 4·0 second Welch tapered windows with 2 second overlap, yielding 15 windows per 30-second epoch. Power spectra for delta (1-4 Hz) were analyzed to address our hypothesis with respect to delta spectral power. Delta sleep spectral power density (μV2) was natural log transformed to normalize its distributions.

Blood collection and clinical laboratory measures

Blood was collected at habitual wake-up time on the morning after the second night on the GCRC, while the subject was fasting, from an indwelling catheter inserted the night before. Blood (10mL) was drawn into a chilled EDTA tube and processed for plasma separation for mass spectrometry analysis.

Oral glucose tolerance test (OGTT)

Oral Glucose Tolerance Test (OGTT) was performed on the morning of day two after a 10-hour overnight fast. Subjects were given 75g dextrose in 250 cc of water, and blood samples were taken at baseline and 30, 60 and 120 minutes for measurement of glucose and insulin levels. Glucose was measured by the hexokinase method (Roche Applied Sciences, Indianapolis, IN) on the Cobas C501, at the Washington

University Core Laboratory. Insulin measurement was performed by EMD Millipore Corporation (St. Charles, MO, USA) by radioimmunoassay.

Analysis of primary metabolites in human plasma using gas chromatography /time of flight mass spectrometry (GC-TOF MS)

Primary metabolites that included sugar phosphates, amino acids, hydroxyl acids, and free fatty acids, were measured using GC-TOF MS. Briefly, metabolites from plasma were extracted using 1 milliliter of degassed, −20°C cold solvent mixture of acetonitrile (ACN):isopropanol (IPA):water (H_2O) (3:3:2, v/v/v) per 20 μL sample aliquot. Samples were vortexed for 10 seconds, shaken for 5 min and then centrifuged for

2 min at 12,800 x g. Two 450 µL supernatant aliquots were transferred to new tubes. To remove any excess protein and triacylglycerides, the supernatant evaporated and resuspended in 500 µL 1:1 acetonitrile:water and vortexed for 10 seconds, centrifuged for 2 min at 12,800 x g. The supernatant was transferred to a clean tube and then dried down in a CentriVap concentrator. Samples were derivatized with 10 µL of methoxyamine hydrochloride (20mg/mL) in pyridine and subsequently by 90 µL of MSTFA for trimethylsilylation of acidic protons, including a mixture of C8–C30 fatty acid methyl esters as internal standards for retention index correction.[8] Internal standards in GC-MS included retention index markers for database annotations[9]. These internal standards are not used for quantification. Instead, we use mixtures of 30 external standards (Quality Control (QC) mix) in dilution curves before and after each batch of samples, in addition the highest-concentrated QC mix sample after each set of 10 biological samples, again, as described elsewhere.[9]

Data acquisition, extraction, and processing of metabolites

Data were acquired using the following chromatographic parameters[10]: Column: Restek corporation Rtx5Sil MS (30 m length x 0.25 mm internal diameter with 0·25µm film made of 95% dimethyl/5%diphenylpolysiloxane); Mobile phase: Helium; Column temperature: 50-330°C Flow-rate: 1 mL min-1; Injection volume: 0·5µL; Injection: 25 splitless time into a multi-baffled glass liner Injection

temperature: 50°C ramped to 250°C by 12°C s-1; Oven temperature program: 50°C for 1 min, then ramped at 20°C min-1 to 330°C, held constant for 5 min. Data processing. Raw data files were preprocessed directly after data acquisition and stored as ChromaTOF-specific *.peg files, as generic *.txt result files and additionally as generic ANDI MS *.cdf files. ChromaTOF vs. 4·0 was used for data preprocessing without smoothing, 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram. Apex masses are reported for use in the BinBase algorithm. Result *.txt files were exported to a data server with absolute spectra intensities and further processed by a filtering algorithm implemented in the metabolomics BinBase database. Given compound was identified and cross-referenced with external database identifiers such as InChI key, PubChem ID, and KEGG ID. Following equation was then used for normalizations for metabolite *i* of sample *j*:

$$metabilite\ ij\ normalized = \frac{metaboliteij\ raw}{mTICj}.mTICaverage$$

The normalized data are shown as peak heights for the quantification ion (mz value) at the specific retention index. Missing values were imputed using half of the minimum detected value for each compound. Autoscaling was performed to make compounds the same scale.[11]

All metabolomic data were investigated using pooled quality control samples and blank samples. Metabolites that had more than 30% relative standard deviation in pooled QC samples were removed, and metabolites that showed less than 3-fold higher intensity compared to blank samples were removed as well. We do not count such signals as genuine metabolites; hence, the final data sheet is what was used for statistical analyses. Briefly, the number of reported signals from raw processed data to final reported data went from 313 metabolites to 266 metabolites. Technical errors reduce biological power, so findings reported in this work were observed at statistical power despite possible technical variance. **Statistical analysis**

We first analyzed data in a sex aggregated manner and found several metabolites to be significantly altered between control and PTSD groups (Fig. S1). Men with PTSD were more likely to be obese than other groups. Therefore, we adjusted for BMI and age in our analyses. We next performed our more detailed analyses in a sex segregated manner. We conducted robust linear regression for each sex (i.e. male and female, respectively) to examine the association between compound intensity, PTSD status, sleeping factors, and to examine the interaction effect between sex and PTSD status for each metabolite. Specifically, robust linear regression was conducted with compound intensity as response and PTSD status as predictor, controlling for body mass index and age. The effect size was determined by the coefficient of the regression model, with a positive value indicating compound intensity being higher in PTSD compared with control and a negative value indicating the opposite. We used the Benjamini Hochberg procedure to correct for multiple comparisons. The p-values and effect sizes from robust linear regression were used as input for the ChemRICH analysis[12] to study the enrichment effect on metabolite clustered defined according to chemical

similarity. Kolmogorov–Smirnov test was used to calculate the p-value for each of the metabolite clusters. We further used Venn-diagrams to highlight the sex difference from the ChemRICH analyses. Volcano plots were used to visualize the significance and effect sizes of each metabolite in each of the significant clusters for each sex after correcting for type 1 errors. We also performed the same analysis but with each of the three sleeping factors (total sleep time (min), PSQI, and log-transformed delta energy (μV2sec)) as confounding variables. We used box plot and Venn diagrams to visualize the significance effect while adjusting for sleep variables. We used the statistical computing language R version 3.6.3.

Robust linear regression was performed in MASS library.

References

1. Wolfe J, Kimerling R, Brown PJ, Chresman KR, Levin K. Psychometric review of the life stressor checklist-revised. Lutherville, MD: Sidran Press; 1996.
2. Blanchard EB, Jones-Alexander J, Buckley TC, Forneris CA. Psychometric properties of the PTSD Checklist (PCL). *Behaviour Research & Therapy* 1996; **34**(8).
3. Blake DD, Weathers FW, Nagy LM, et al. The development of a Clinician-Administered PTSD Scale. *J Trauma Stress* 1995; **8**(1): 75-90.

4. Spitzer RL, Williams JB, Gibbon M, First MB. The Structured Clinical Interview for DSMIII--R (SCID) : I. History, rationale, and description. *Archives of general psychiatry* 1992; **49**(8): 624-9.
5. Buysse DJ, Reynolds CF, 3rd, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry Res* 1989; **28**(2): 193-213.
6. Richards A, Metzler TJ, Ruoff LM, et al. Sex differences in objective measures of sleep in post-traumatic stress disorder and healthy control subjects. *Journal of sleep research* 2013; **22**(6): 679-87.
7. Kales A, Rechtschaffen A. A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects. : Washington, DC : United States Government Printing Office, 1968.; 1968.
8. La Frano MR, Carmichael SL, Ma C, et al. Impact of post-collection freezing delay on the reliability of serum metabolomics in samples reflecting the California mid-term pregnancy biobank. *Metabolomics* 2018; **14**(11): 151.
9. Fiehn O. Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling. *Curr Protoc Mol Biol* 2016; **114**: 30 4 1- 4 2.
10. Fiehn O, Wohlgemuth G, Scholz M, et al. Quality control for plant metabolomics: reporting MSI-compliant studies. *Plant J* 2008; **53**(4): 691-704.
11. Wanichthanarak K, Fan S, Grapov D, Barupal DK, Fiehn O. Metabox: A Toolbox for Metabolomic Data Analysis, Interpretation and Integrative Exploration. *PLoS One* 2017; **12**(1): e0171046.
12. Barupal DK, Fiehn O. Chemical Similarity Enrichment Analysis (ChemRICH) as alternative to biochemical pathway mapping for metabolomic datasets. *Sci Rep* 2017; **7**(1): 14567.

Table S1. Demographics data and clinical characteristics of PTSD and control subjects.

	Men		Women		
Variable	Control (N=22)	PTSD+ (N=22)	Control (N=24)	PTSD+ (N=22)	Total (N=90)
Age (Mean ± SD) [a]	30·2 ± 8·76	30·6 ± 7·61	30·0 ± 7·47	30·2 ± 6·82	30·3 ± 7·31
Education (Years) (Mean ± SD) [a]	15·5 ± 2·08	14·4 ± 2·32	15·5 ± 1·92	15·3 ± 2·03	15·2 ± 2·11
Race [b]					
African American	1 (4·5%)	3 (13·6%)	0 (0·0%)	2 (9·1%)	6 (6·7%)
Asian/Hawaiian/Pacific Islander	4 (18·2%)	1 (4·5%)	3 (12·5%)	2 (9·1%)	10 (11·1%)
Caucasian	17 (77·3%)	13 (59·1%)	19 (79·2%)	14 (63·6%)	54 (63·5%)
Other/Unknown	0 (0·0%)	5 (22·7%)	2 (8·3%)	4 (18·2%)	11 (12·2%)
Hispanic Ethnicity [b,c]	0 (0·0%)	5 (22·7%)	3 (12·5%)	1 (4·5%)	9 (10·0%)
Current CAPS score [d] (Mean ± SD)	0·0 ± 0·0	51·9 ± 13·03	0·0 ± 0·0	55·2 ± 21·58	53·5 ± 17·70
PTSD Symptom Checklist (PCL; Mean ± SD) [e]	19·09 ± 4·21	46·0 ± 12·29*	19·9 ± 4·08	55·6 ± 12·27*	34·8 ± 18·39
Current MDD [f]	0 (0·0%)	5 (22·7%)	0 (0·0%)	3 (13·6%)	8 (8·9%)
Childhood trauma ≤14 years of age [g,h]	0 (0·0%)	8 (36·4%)	0 (0·0%)	11 (50·0%)	19 (21·1%)
Hormonal birth control [b,i]	NA	NA	2 (8·3%)	6 (27·3%)	8 (17·4%)
BMI [b]					
Underweight = <18·5	2 (9·1%)	0 (0·0%)	1 (4·2%)	0 (0·0%)	3 (3·3%)
Normal weight = 18·5–24·9	10 (45·5%)	4 (18·2%)	12 (50·0%)	13 (59·1%)	39 (43·3%)
Overweight = 25–29·9	10 (45·5%)	5 (22·7%)	8 (33·3%)	7 (31·8%)	30 (33·3%)
Obesity = BMI of 30+	0 (0·0%)	13 (59·1%)	3 (12·5%)	2 (9·1%)	18 (20·0%)
Smoker [b, j]	3 (13·6%)	4 (18·2%)	6 (25·0%)	6 (27·3%)	19 (21·1%)

a based on F-test b based on Chi-square test

c Three subjects endorsed Hispanic ethnicity but did not select a racial descriptor. Six additional subjects endorsed Hispanic ethnicity, in addition to a racial category of Caucasian or African-American race yielding a total of 9 subjects self-identifying as Hispanic in this sample. Comparison of Hispanic ethnicity, p=·063.

d Control subjects had CAPS scores of zero or had an absence of criterion A events. Comparison of male and female PTSD subjects on current CAPS score, p=0·54.

e PTSD group by gender interaction on current PCL score, p<.05. *:Comparison of PTSD groups vs controls, p<.001. Comparison of male vs female PTSD groups vs controls, p<0·01.

f Absence of current MDD was required for inclusion into the control group. Comparison of male and female PTSD subjects on rate of current MDD, p=0·43.

g Childhood trauma exposure was defined, based on findings from our prior research, by exposure to 2 or more categories of childhood trauma under the age of 14. Three (6·5%) control subjects reported a history of 1 category of childhood trauma.

h Chi-square test compared frequency of childhood trauma between male and female PTSD subjects only, p=·36.

i Chi-square test compared use of hormonal birth control female PTSD and control subjects only, p=·09 j based on diary

Table S2. Sex steroid metabolite concentrations in plasma of men and women.

Metabolite	LOD (ng/mL)	Control □ SD (ng/mL) Women	PTSD □ SD (ng/mL) Women	Control □ SD (ng/mL) Men	PTSD □ SD (ng/mL) Men
Progesterone (P4)	0.393	0·59 □ 1·19	<LOD (<0·28)	<LOD (<0·09)	<LOD (<0·09)
Testosterone*	0.144	0·55 □ 0·90	0·44 □ 0·68	4·03 □ 1·39	4·17 □ 1·61
Estrone (E1)	2.702	<LOD (<0·11)	<LOD (<0·24)	<LOD (<0·16)	<LOD (<0·11)
Estradiol (E2)	0.680	<LOD (<0·03)	<LOD (<0·06)	<LOD (<0·02)	<LOD (<0·03)
Estriol (E3)	2.882	5·7 □ 8·93	3·23 □ 2·26	3·39 □ 3·96	4·68 □ 9·69

Steroid metabolite values plasma in ng/mL. Limit of detection (LOD) for each analyte is shown. Values in red text were below LOD. *: p<0·0001 Men vs. Women (sex); PTSD: ns; PTSD x Sex: ns

Table S3. Altered primary metabolite clusters in PTSD with sleep confounders

Men PTSD	Men Delta Power	Men PSQI	Men TST	Women PTSD	Women Delta Power	Women PSQI	Women TST
Amino Acids (AA)							
.	.	Gly	.	Glycine (Gly)	Gly	.	Gly
.	.	.	.	Threonine (Thr)	Thr	.	Thr
.	.	.	.	Serine (Ser)	Ser	.	Ser
.	.	.	.	.	Citrulline	.	.
.	.	.	.	.	□-Ala	□-Alanine	□-Ala
Branched-chain amino acids (BCAA)							
.	.	.	.	Valine (Val)	Val	Val	Val
.	.	.	.	Leucine (Leu)	.	Leu	Leu
Sulfur amino acids							
.	.	.	Met	Methionine (Met)	Met	Met	.
.	.	.	Cys	Cysteine (Cys)	Cys	Cyst	.
Cyclic amino acids							
Histidine (His)	His	.	.	.	.	.	.
.	.	.	4-Hyp	Trans-4hydroxyproline (4-Hyp)	4-Hyp	4-Hyp	.
Indoles (Tryptophan metabolites)							

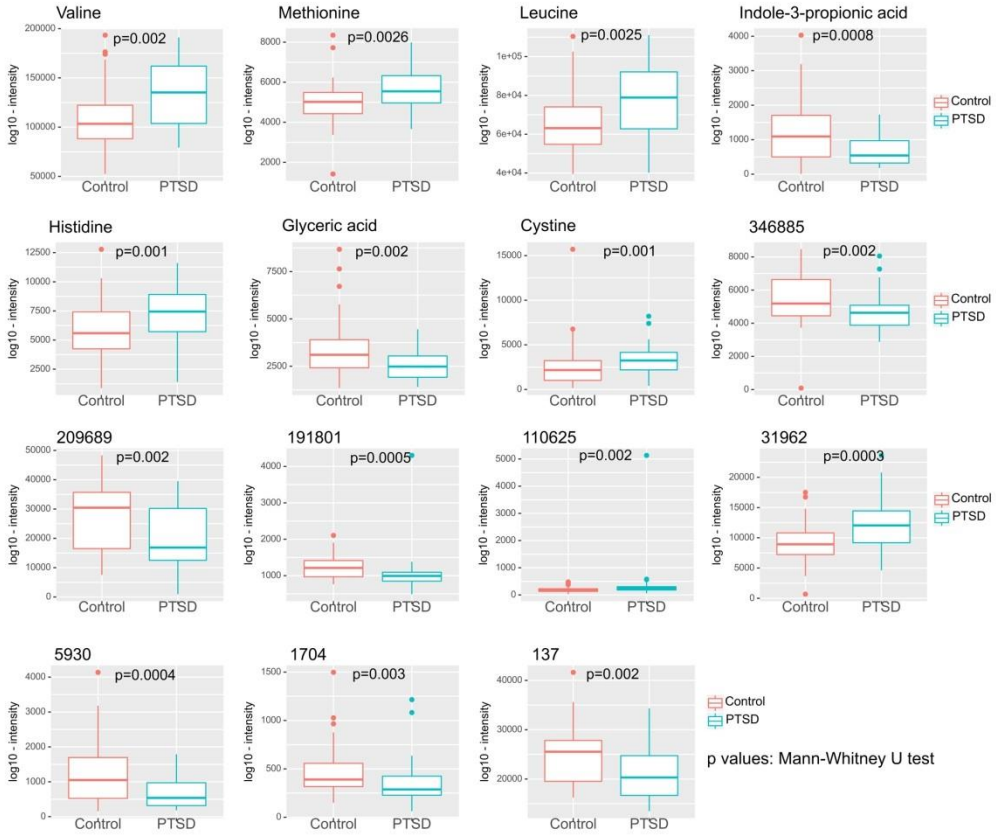
.	.	.	.	Indole-3-propionic acid (IPA)	.	.	IPA
.	.	.	.	.	.	Indole-3-lactate (ILA)	.
.	.	.	.	.	.	Indole-3acetate (IAA)	.
Butyrates (Gut microbes)							
.	.	Threonic acid	.	.	.	.	.
.	.	3-Hydroxybutyric acid	.	.	.	.	.
.	.	2-Hydroxybutanoic acid	.	.	.	.	.
.	.	.	.	Isothreonic acid (ITA)	ITA	.	ITA
.	.	.	.	.	.	2-Aminobutyric acid (2AB)	.

Hexose							
Fructose	.	.	.	.	.	.	.
.	Sucrose	.	.	.	.	.	.
.	.	.	.	1-Methylgalactose NIST	.	.	1-MG NIST
Unsaturated fatty acids (UFA)							
.	.	.	.	Palmitoleic acid	.	.	.

Supplementary Figure Legend

P values as shown using Mann-Whitney U-test.

Fig. S1. Sex aggregated analysis of primary metabolites . There were seven primary metabolites that were significantly different between PTSD and control subjects when data were analyzed in a sex aggregated manner. Eight metabolites that have not been thoroughly characterized yet, were also identified.



Sex-specific alterations in primary metabolites and tryptophan pathways in posttraumatic stress and disturbed sleep

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Abstract

Background

Primary metabolites serve as substrates for neurotransmitters and are altered in psychiatric disorders. Sleep disturbances are exacerbated in posttraumatic stress disorder (PTSD), but the contribution of sex, sleep and/or PTSD in altering primary metabolites is not known.

Methods

We used mass spectrometry to ascertain primary metabolites in 90 plasma samples from individuals with chronic PTSD and control subjects. Laboratory-based polysomnography was used to monitor the sleep of participants. PTSD was determined using the Clinician-Administered PTSD Scale (CAPS).

Results

Men and women with PTSD showed distinct, non-overlapping primary metabolite nodes compared with sex-matched controls as ascertained using Chemical Set Enrichment Analysis (ChemRICH) analysis. Women with PTSD had seven nodes, whereas men with PTSD had just two nodes altered compared with controls; each node contained two or more metabolites. Sex steroids levels did not associate with metabolite nodes. Higher PTSD symptoms were associated with lower total sleep time and decreased Pittsburgh Sleep Quality Index (PSQI) scores in both men and women. Delta power on sleep electroencephalogram was significantly lower in men with PTSD and associated negatively with PTSD symptoms. Sleep measures accounted for nearly 50% of the altered primary metabolite nodes. Tryptophan and insulin levels were significantly increased in men but not women with PTSD compared with controls, whereas tryptophan levels associated inversely with insulin levels in women.

Conclusions

Women demonstrate more metabolic disturbances than men with similar PTSD scores. The presence of sex-specific primary metabolite in PTSD were not affected by sex steroids but were accounted for by sleep measures. Our data provide further evidence for development of sex-specific interventions and disease management.

Introduction

Posttraumatic stress disorder (PTSD) is a common psychiatric condition. PTSD may develop after exposure to an actual or threatened death, serious injury, trauma, or sexual violence. PTSD is currently characterized on diagnostic and statistical manual of mental disorders (DSM-5) by: (i) reexperiencing (e.g., intrusive thoughts, nightmares, flashbacks); (ii) avoidance; (iii) negative changes in cognition and mood (hopelessness, lack of emotions), and (iv) hyperarousal (trouble sleeping, self-destructive behavior, angry outbursts). While it is widely recognized that PTSD is a common consequence of trauma exposure, women are at particularly high risk, with some studies finding that women develop PTSD at twice the rate of men, despite greater trauma exposure in men.[1, 2]

One of the most common complaints among individuals with PTSD is sleep disturbance. PTSD is associated with lower slow wave sleep duration and delta power sleep, which is more pronounced in men than women. In contrast, greater rapid eye movement sleep is found in women with PTSD compared to healthy controls, a difference not seen in men.[3] Sleep disturbance is a key risk factor for health consequences as it alters hypothalamic-pituitary-adrenal (HPA) axis function, resulting in impaired glucose and lipid metabolism. The HPA and somatotropic axes activities are temporally associated with delta power sleep and promote insulin sensitivity and metabolic syndrome. Additionally, sleep duration correlates with metabolic risk in PTSD but does not fully account for the association between PTSD with known metabolic disturbances such as in blood insulin or glucose levels.[4] While prior studies have found alterations in neuroendocrine, immune and aging processes in PTSD, the role of metabolite disturbances is PTSD is limited and can help elucidate new discovery of yet unknown biological mechanisms of disease.

More recent technological developments such as mass spectrometry allow for the discovery of novel pathways using an unbiased method to examine multiple analytes simultaneously. Metabolomics is a global

and unbiased approach to understanding regulation of metabolic pathways and networks of physiologically relevant interactions. The metabolome is regulated by gene-environment interactions and reflects the

intermediary state between genotype and phenotype. Gene mutations, single nucleotide polymorphisms, and mutations in proteins are associated with PTSD, but none of these alone explains the complex manifestation of PTSD and comorbid health conditions. A multi-omics approach has been used to identify potential biomarkers that range from DNA methylation, proteins, miRNA, lipids, and other metabolites in warzone male veterans with PTSD.[5] Metabolomic profiling has also led to identification of key differences in glycolysis and fatty acid pathways that were associated with mitochondrial dysfunction in men with PTSD. [6]

Essential and non-essential amino acids (AAs) as well as biogenic amines are building blocks of proteins and peptide hormones involved in a plethora of functions that include membrane stabilization, neurotransmission, and neuroimmune modulation. AAs act as neurotransmitters (e.g. glutamate, glycine, serine). Kynurenine (KYN), a metabolite of AA tryptophan, is correlated with changes in hippocampal and amygdalar volumes in depressed patients.[7] KYN is associated with stress exposure, neuronal cell death, glutamate transmission, and neuroinflammation. Alterations in amino acid metabolites that are more bloodbrain barrier permeable, such as KYN, may account for some of these differences. We are not aware of any study that has systematically ascertained sex differences in the status of primary metabolites in serum samples of participants with PTSD and matched-controls. In this study, we aimed to examine alterations of primary amino acids and metabolites in PTSD and the contribution of sleep measures in both men and women.

Methods

Human subjects

In this study, we used a cross-sectional, 2 × 2 design (PTSD/control × women/men) involving 90 medically healthy, non-medicated adults aged 19-39 years in an inpatient sleep laboratory at the General Clinical Research Center (GCRC) at the University of California, San Francisco. The study sample was comprised of 44 individuals with current chronic PTSD (50% women) and 46 control subjects (52% women). This sample was drawn from a larger study of 94 participants. Data from 4 participants were excluded due to difficulties in blood collection. This research was approved by the Committee on Human Research at the University of California, San Francisco. All participants provided written informed consent before participating in any study procedures.

Psychiatric diagnoses and trauma history

The type of trauma exposure and age of occurrence was assessed using the Life Stressor Checklist- Revised interview. The PTSD Checklist (civilian version) for DSM-IV (PCL-C) [9] was used to assess severity of chronic PTSD symptoms and on the Clinician-Administered PTSD Scale (CAPS) and a CAPS score >40. This self-report measure consists of 17 items that correspond to the DSM-IV criteria and include intrusive thoughts and re-experiencing symptoms (cluster B), avoidance (cluster C), and hyperarousal (cluster D). The structured clinical interview for DSM-IV, non-patient edition (SCID-NP) was used to diagnose all other psychiatric disorders, including major depressive disorder.[11]

Control subjects had no lifetime or current history of a PTSD diagnosis. Women participants were premenopausal, and were scheduled during the follicular phase of the menstrual cycle. All study procedures were timed according to habitual sleep onset, determined by actigraphy and sleep diary in the week prior to the GCRC study. Study participants were alcohol and drug-free, limited to one cup of caffeine daily, and maintained regular bed and waking times. Exclusion criteria included a history of traumatic brain injury, presence of neurologic disorders or systemic illness; use of psychiatric, anticonvulsant, antihypertensive, sympathomimetic, steroidal, statin or other prescription medications; obesity (defined as body mass index (BMI) >30); alcohol abuse or dependence in the prior two years; substance abuse or dependence in the

previous year; any psychiatric disorder with psychotic features; bipolar disorder or obsessive-compulsive disorder; and pregnancy. Exclusion criteria for control subjects also included a lifetime history of major depressive disorder or panic disorder.

Sleep clinic and measures

The Pittsburgh Sleep Quality Index[12] was used to provide a subjective assessment of sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbances (including nightmares), use of sleep medication, and daytime dysfunction over the previous month. Participants rated items on a four-point Likert scale within each domain with higher scores indicating poorer sleep. A total score of > 5 is indicative of poor

Polysomnography recordings were obtained with ambulatory polysomnography (Nihon Kohden Trackit Ambulatory Recording System). The parameters recorded included an electroencephalogram (EEG) at leads C3, C4, O1 and O2, left and right electrooculograms (EOG), submental electromyogram, bilateral anterior tibialis EMGs, and electrocardiogram in accordance with standardized guidelines by Rechtschaffen. Electrode impedance was set at < 5 kohm at the start of the recording. The EEG and EOG leads were referenced to linked mastoids. Raw EEG signals were filtered and amplified, then digitized at sleep quality.

Polysomnographic measurements

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256 Hz and recorded to a removable hard disk in European Data Format file format. The low frequency and high frequency hardware filters on the recorder were single pole analog filters with 3 db points at 0·5 Hz and 100 Hz. Pass Plus was utilized for both visual scoring and quantitative EEG analysis of the digitized polysomnography data.

Power spectral analysis for polysomnographic measures

Pass Plus (Delta Software) analytic software was used to measure sleep activity in all frequency bands delta through gamma from the C3 electrode by power spectral analysis. The C4 electrode was used if there was

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excessive artifact. A limitation of Pass Plus is that artifact removal is accomplished by removal of whole epochs tagged with artifact. This has the potential to introduce additional confounds given the removal of typically longer bouts of uncontaminated EEG. Therefore, epochs were tagged for slow and fast artifact for additional analyses. Primary analyses were conducted with all epochs and then checked for the impact of removal of epochs with slow and fast artifact. Removal of fast artifact (for bandwidths alpha and above) and slow artifact (for bandwidths delta and theta) did not significantly impact our findings in non-rapid eye movement (NREM) sleep. All results are therefore reported without removal of epochs containing artifact. Pass Plus applied a 5μV smoothing constant to eliminate spurious waves caused by electrical jitter. Power spectral analysis was conducted on all epochs of NREM and REM sleep. Epochs visually scored as wake were not included in these analyses. Visual scoring was conducted by a highly experienced registered polysomnography technician, blind to PTSD status, classified all 30-second epochs in every sleep record as wake; stages 1, 2, 3; REM; or movement using current American Academy of Sleep Medicine criteria. Total sleep time was defined by time spent in epochs scored as NREM stages 1 through 3 and stage REM. Pass

Plus was used to perform Fast Fourier Transformation analysis on 4·0 second Welch tapered windows with 2 second overlap, yielding 15 windows per 30-second epoch. Power spectra for delta (1-4 Hz) were analyzed to address our hypothesis with respect to delta spectral power. Delta sleep spectral power density (μV2) was natural log transformed to normalize its distributions.

Blood collection and clinical laboratory measures

Blood was collected at habitual wake-up time on the morning after the second night on the GCRC, while the subject was fasting, from an indwelling catheter inserted the night before. Blood (10mL) was drawn into a chilled EDTA purple-topped tube and processed for plasma separation for mass spectrometry analysis.

Oral glucose tolerance test (OGTT)

Oral Glucose Tolerance Test (OGTT) was performed on the morning of day two after a 10-hour overnight fast. Subjects were given 75g dextrose in 250 cc of water, and blood samples were taken at baseline and 30, 60 and 120 minutes for measurement of glucose and insulin levels. Glucose was measured by the hexokinase method (Roche Applied Sciences, Indianapolis, IN) on the Cobas C501, at Washington University Core Laboratory. Insulin measurement was performed by EMD Millipore Corporation (St. Charles, MO, USA) by radioimmunoassay.

Analysis of primary metabolites in human plasma using gas chromatography /time of flight mass spectrometry (GC-TOF MS)

Primary metabolites that included sugar phosphates, amino acids, hydroxyl acids, and free fatty acids, were measured using GC-TOF MS. Briefly, metabolites from plasma were extracted using 1 milliliter of degassed, −20°C cold solvent mixture of acetonitrile (ACN):isopropanol (IPA):water (H_2O) (3:3:2, v/v/v) per 20 μL sample aliquot. Samples were vortexed for 10 seconds, shaken for 5 min and then centrifuged for 2 min at 12,800 x g. Two 450 μL supernatant aliquots were transferred to new tubes. To remove any excess protein and triacylglycerides, the supernatant evaporated and resuspended in 500 μL 1:1 acetonitrile:water

and vortexed for 10 seconds, centrifuged for 2 min at 12,800 x g. The supernatant was transferred to a clean tube and then dried down in a CentriVap concentrator. Samples were derivatized with 10 µL of methoxyamine hydrochloride (20mg/mL) in pyridine and subsequently by 90 µL of MSTFA for trimethylsilylation of acidic protons, including a mixture of C8–C30 fatty acid methyl esters as internal standards for retention index correction.[14] Internal standards in GC-MS included retention index markers for database annotations[15]. These internal standards are not used for quantification. Instead, we use mixtures of 30 external standards (Quality Control (QC) mix) in dilution curves before and after each batch of samples, in addition the highest-concentrated QC mix sample after each set of 10 biological samples, again, as described elsewhere.[15]

Data acquisition, extraction, and processing of metabolites

Data were acquired using the following chromatographic parameters[16]: Column: Restek corporation Rtx5Sil MS (30 m length x 0.25 mm internal diameter with 0·25µm film made of 95% dimethyl/5%diphenylpolysiloxane); Mobile phase: Helium; Column temperature: 50-330°C Flow-rate: 1 mL min-1; Injection volume: 0·5µL; Injection: 25 splitless time into a multi-baffled glass liner Injection temperature: 50°C ramped to 250°C by 12°C s-1; Oven temperature program: 50°C for 1 min, then ramped at 20°C min-1 to 330°C, held constant for 5 min. Data processing. Raw data files were preprocessed directly after data acquisition and stored as ChromaTOF-specific *.peg files, as generic *.txt result files and additionally as generic ANDI MS *.cdf files. ChromaTOF vs. 4·0 was used for data preprocessing without smoothing, 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram. Apex masses are reported for use in the BinBase algorithm. Result *.txt files were exported to a data server with absolute spectra intensities and further processed by a filtering algorithm implemented in the metabolomics BinBase database. Given compound was identified and cross-referenced with external database identifiers such as InChI key, PubChem ID, and KEGG ID. Following equation was then used for normalizations for metabolite *i* of sample *j*:

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went from 313 metabolites to 266 metabolites. Technical errors reduce biological power, so findings reported in this work were observed at statistical power despite possible technical variance.

Statistical analysis

We first analyzed data in a sex aggregated manner and found several metabolites to be significantly altered between control and PTSD groups (Fig. S1). Men with PTSD were more likely to be obese than other groups. Therefore, we adjusted for BMI and age in our analyses. We next performed our more detailed analyses in a sex segregated manner. We conducted robust linear regression for each sex (i.e. male and female, respectively) to examine the association between compound intensity, PTSD status, sleeping factors, and to examine the interaction effect between sex and PTSD status for each metabolite. Specifically, robust linear regression was conducted with compound intensity as response and PTSD status as predictor, controlling for body mass index and age. The effect size was determined by the coefficient of the regression model, with a positive value indicating compound intensity being higher in PTSD compared with control and a negative value indicating the opposite. We used the Benjamini Hochberg procedure to correct for multiple comparisons. The p-values and effect sizes from robust linear regression were used as input for the ChemRICH analysis[18] to study the enrichment effect on metabolite clustered defined according to chemical similarity. Kolmogorov–Smirnov test was used to calculate the p-value for each of the metabolite clusters. We further used Venn-diagrams to highlight the sex difference from the ChemRICH analyses. Volcano

plots were used to visualize the significance and effect sizes of each metabolite in each of the significant clusters for each sex after correcting for type 1 errors. We also performed the same analysis but with each of the three sleeping factors (total sleep time (min), PSQI, and log-transformed delta energy (μV2sec)) as confounding variables. We used box plot and Venn diagrams to visualize the significance effect while adjusting for sleep variables. We used the statistical computing language R version 3.6.3.

Robust linear regression was performed in MASS library.

Results

Demographic Data and Clinical Characteristics

By design, there were no significant differences in sex distribution between PTSD and control subjects, nor were there significant differences in age, education, or race/ethnicity across all four groups. Sample characteristics are presented in Table 1. Men with PTSD were more likely to be obese and male controls by the presence of two or more categories of childhood trauma as compared to one or none). Eleven control subjects reported a lifetime history of a traumatic criterion A1 event, but all had current CAPS scores of zero and none had a lifetime history of PTSD. However, the PTSD groups scored higher on the PCL-C (avoidance) than controls; women with PTSD had higher PCL-C scores than men with PTSD (Table 1). As per the exclusion criteria, no control subjects met criteria for current MDD. Additionally, none of the control subjects reported a history of two or more categories of childhood trauma. There were no differences between PTSD and control women in use of hormonal birth control or group differences in smoking of

Chemical Set Enrichment Analysis (ChemRICH) identified sex differences in primary and steroid

were less likely to be obese. Therefore, we adjusted for BMI in all our analyses. Men and women with PTSD did not differ in terms of CAPS scores, rates of current MDD, or history of childhood trauma (defined tobacco. **metabolites**

We performed ChemRICH analysis on primary metabolites to identify chemical classes that were significantly altered in PTSD compared to controls. ChemRICH utilizes structure similarity and chemical ontologies to map all known metabolites and name metabolic modules.[18] This is a statistical enrichment approach based on chemical similarity and alternative to pathway analysis that relies on limited biochemical knowledge annotations.[18] It yields study-specific, non-overlapping sets of all identified metabolites. Since ChemRICH sets have a self-contained size, thus p-values do not rely on the size of the background database. ChemRICH analysis identified seven primary metabolite nodes altered in women with PTSD compared

with control women (Fig. 1A) after adjusting for age and BMI as confounding factors. Each node reflects a significantly altered cluster of metabolites and the node size represents the total number metabolites within a cluster set. Branched-chain and sulfur-containing amino acids, and unsaturated fatty acids were increased, indoles and cyclic amino acids were decreased, whereas the non-polar amino acid node contained metabolites that either increased or decreased in women with PTSD compared to controls. In contrast, men with PTSD had only two altered nodes compared with controls (Fig. 1A-C). Surprisingly, men and women with PTSD did not share any overlapping primary metabolite clusters (Fig. 1B). Although, when data was analyzed in a sex aggregated manner, a number of metabolites were significantly different between control and PTSD groups (Fig. S1). Seven metabolites that included both essential and non-essential amino acids as well as eight uncharacterized metabolites were significantly different between PTSD and controls (Fig. S1). Further analysis of the metabolites in a sex segregated manner within each node revealed presence of several metabolites that remained statistically significant after adjusting for false discovery rate and effect

size (Fig. 1C). Surprisingly, sex steroid metabolites (estrogens, testosterone, progesterone or their metabolites) did not differ between controls and PTSD in either men or women (Table 2), nor did sex steroids associate with PTSD. Progesterone was below the level of detection in men and women with PTSD, whereas estrone and estradiol were also below LOD in both men and women. Testosterone, but not estriol levels differed between men and women, with levels nearly ~10-fold higher in men (Table 2).

Essential, non-essential amino acids, and butyrates were altered in women with PTSD

Since PTSD symptom presentation is highly variable between individuals,[19] and in our cohort, women had significantly greater PCL-C scores than men, we reasoned that individual PCL measures may associate differently with individual metabolites. To better understand which of the PTSD symptom clusters contributed to alterations in specific metabolites after adjusting for type 1 errors, linear regression analysis for each PCL symptom cluster with significant individual metabolites was performed (Fig. 2A-H and Table 3). Serine, a neurotransmitter and precursor for the synthesis of glycine and cysteine as well as for 2-

aminobutyric acid, a butyrate (2-AB), is a byproduct of cysteine biosynthesis pathway, which are all in the glucose metabolism pathway (Fig. 3). While serine levels trended to be lower in women with PTSD compared to controls, no significant relationship with PTSD measures were found (Fig. 2A). Glycine levels were negatively associated with cluster D symptoms on the PCL, suggesting that with more hyperarousal, glycine levels were decreased in women, but not men. Cysteine and 2-AB levels were elevated in PTSD compared with controls (Fig. 2C and 2F) but did not show any significant association with specific clusters of PTSD symptoms.

Hexose metabolites were altered in men, but not women with PTSD

Alterations in glucose metabolism are decreased within specific brain regions of people with schizophrenia and mood disorders as ascertained with functional imaging. [20, 21]

Sucrose is metabolized to glucose and fructose, and fructose can only be metabolized in the liver via the tricarboxylic acid cycle. We found that whilst overall levels of fructose were increased in men with PTSD compared with controls (Fig. 2H), its levels were negatively correlated with both B and C symptom clusters, suggesting that more reexperiencing and avoidance symptoms contribute to altered hexose metabolism in men, but not women.

Sleep disturbances and quality are associated with overall poor health[3]. We found that both men and women with PTSD had lower total sleep time (TST), and worse self-reported sleep quality as assessed by the PSQI compared with controls in (Fig. 4A-B). Delta power, a measure of deep sleep activity, was also significantly decreased in PTSD and showed a significant sex difference with men having lower delta power sleep activity than women (Fig. 4C). Specifically, greater PTSD symptoms (as reflected by total PCL score) was associated with lower TST in both women and men ($r = –0{\cdot}41$, $p=0{\cdot}005$ and $r = –0{\cdot}32$, $p=0{\cdot}033$, respectively, Fig. 4D), and poor sleep quality (PSQI) was highly associated with greater PTSD symptoms in both women and men ($r=0{\cdot}84$ and $r=0{\cdot}74$, $p<0{\cdot}001$, respectively, Fig. 4E). In contrast, delta power was lower in men

Sleep quality was worse in PTSD in both sexes

with PTSD (r= –0·35, p=0·02) compared to controls, but there were no differences in PTSD status on delta activity in women (Fig. 4F).

Decreased levels of essential amino acids in both sexes and indoles in women associated with sleep parameters and PTSD symptoms

Humans lack the ability to synthesize eight essential amino acids which must be obtained from diet and are mostly absorbed by the gut and metabolized by the resident microbiota. For example, tryptophan is metabolized to a myriad of biologically active compounds by four different pathways (serotonin, tryptamines, kynurenine, indoles, and NAD+). When we accounted for TST, two of the six metabolite nodes that included cyclic amino acids and unsaturated fatty acids, were no longer significant in women with PTSD, whereas no node remained significant in men with PTSD (Fig. 5 and Fig. 6) and Table 3. In contrast, when PSQI was used as a confounder, new, albeit non-overlapping nodes were found to be significant in women and men; indoles, hexose and amino acid nodes were decreased in women, whereas the butyrate node was found to harbor significantly increased as well as decreased metabolites in men with PTSD (Fig. 5). Alterations in delta power sleep accounted for 50% of the nodes in women (Table 3), whereas delta power was not associated with alterations in any nodes in men with PTSD (Fig. 5 vs Fig. 1A). Further analysis revealed that decreased sleep quality and delta power contributed to a decrease in levels of several essential amino acid as well as metabolites in the glucose metabolism pathway for which they serve as substrates (Fig. 7). Interestingly, we found a nonsignificant trend with higher testosterone levels associated with lower delta power in men (Fig. 7G, r= –0·29, p=0·06).

Plasma insulin levels associated negatively with tryptophan levels in women, but not men

While tryptophan levels were not associated with either sex, PTSD or sleep at the node level, when examined as sex segregated, tryptophan was significantly increased in men with PTSD compared to controls, but not in women (Fig. 8A). As influx of free tryptophan into the brain by its transport carrier is regulated by several factors including levels of plasma albumin and insulin,[22] we next examined the levels

of plasma albumin and insulin levels 30, 60, and 120 min after an oral glucose challenge. While plasma albumin levels did not differ between controls and individuals with PTSD in either sex, insulin levels were

significantly elevated in men with PTSD compared with controls (Fig. 8B). Interestingly, tryptophan levels associated negatively with insulin levels at 60 min in women (r= –0·30, p=0·04), whereas insulin levels exhibited a positive association with free tryptophan levels in men, although the relationship did not reach statistical significance (Fig. 8C).

Discussion

To our knowledge, this is the first systematic study to report sex-specific alterations in primary metabolites in men and women with PTSD with several novel findings. First, both sex and sleep measures altered distinct amino acids in individuals with PTSD compared with controls. Second, levels of sex steroids such as estriol, progesterone, and testosterone did not differ between controls and PTSD. Third, sex steroids did not associate with PTSD or primary metabolites in our cohort. Fourth, delta power and perceived sleep quality account for nearly half the changes in metabolite clusters in women with PTSD compared with control women (Fig. 9A). In women, perceived sleep quality was associated with increases in 2hydroxybutonic acid, a butyrate and decrease in indoles, generated by the gut microbes. Finally, there was no overlap in PTSD-related alterations in men and women in any primary metabolite nodes or individual amino acids within those nodes, and associated pathways.

An individual's physiological and metabolic state shifts glucose metabolism and generation of non-essential amino acids. Serine and glycine, both non-essential amino acids are directly synthesized from glucose in several cells including the glia, astrocytes, and hippocampal neurons. Both serine and glycine shuttle between the glia and neurons where glycine induces release of serine, which is a coagonist for *N*-methylD-aspartate (NMDA) receptors.[23] NMDA receptor function in the amygdala and prefrontal cortex have been found to be critical for the consolidation of extinction of previously conditioned fear memories.[24]

Enhanced fear conditioning and a deficit in the extinction of conditioned fear that are reliant on NMDA activity have been proposed to underlie the development and maintenance of PTSD.[25, 26]

We find that both serine and glycine levels were decreased in women with PTSD, and sleep measures such as delta power and total sleep time account for changes in these two amino acids in women (Table 3). In contrast, neither serine nor glycine levels were altered in men with PTSD and whereas sleep quality decreased glycine levels in men, after correcting for type 1 errors, its levels were no longer significant (Table 3). Other studies have shown that kynurenine pathway metabolites, kynurenic acid and quinolinic acid, associated with neuroprotective and neurotoxic functions, respectively, were altered in patients with major depressive disorders.[7] Like serine, quinolinic acid is also an NMDA receptor agonist (Fig. 9B), and increase in levels of serine may alter neuronal function in PTSD and other psychiatric disorders.

Valine, lysine, methionine, tryptophan, and histidine are all essential amino acids that cannot be synthesized by human cells. Tryptophan is absorbed in the gut and converted by the actions of gut microbiota such as *Lactobacillus* to indoles (Fig. 9B). While the role of serotonin in mood disorders, anxiety, and other disorders is well known, we show here for the first time that indole metabolite, indole-3-propionic acid, is decreased in women with PTSD. Poorer sleep quality was further associated with decreased levels of two additional indoles, indole-3-lactic and acetic acids; the indoles regulate immune and gut barrier functions (Fig. 9B), and their decreased levels might contribute to altered immune and barrier function in women. In agreement with these data, altered immune and gut barrier function is seen in patients with other psychiatric disorders.[27, 28] Diets rich in butyrates that support growth of beneficial gut bacteria such as *Lactobacillus* may serve as non-invasive interventions, especially for women. While diet is hard to control (self-report is not reliable) and we acknowledge this as a limitation of our study; although it should be noted that all participants were in the sleep clinic for 3 nights and had to choose from the menu provided at the clinic.

Increased blood insulin levels affect transport of free tryptophan to the brain in two ways: first, insulin enhances binding of tryptophan to the albumin, thereby decreasing levels of free tryptophan in the circulation by half, and decreasing influx into the brain.[22] Second, simultaneous reduction in levels of six or more amino acids such as leucine, serine, cysteine, histidine, methionine, valine, which would otherwise compete with tryptophan for binding to the transport carrier into the brain to increase its influx.[22] Ethnicity

along with many factors predispose individuals to a higher risk of insulin resistance; however, our data did not examine ethnicity as a contributing factor. In our cohort, we did not find any difference in insulin levels in women with PTSD and controls. However, we did find an increase in levels of several amino acids in women with PTSD that are known to compete with tryptophan carrier for influx into the brain (Fig. 9B and Table 3). This could result in decrease in substrate for conversion to serotonin and subsequently to melatonin, thereby affecting sleep. Although sex differences in insulin resistance and glucose clearance are reported in people with diabetes[29] as well as in animal models of diabetes,[30] we did not find any sex differences in insulin levels after an oral glucose challenge in our PTSD cohort. Non-esterified fatty acids can displace albumin from tryptophan[31, 32] and this free tryptophan can be converted in to serotonin or degraded in cells. We found an increase in palmitoleic acid levels, a monounsaturated non-esterified fatty acid in women with PTSD, which can potentially displace albumin from tryptophan in order to generate free tryptophan. Concomitant increase in palmitoleic acid levels may compensate for increases in amino acids that prevent influx of tryptophan into the brain and conversion to serotonin, more so in women than in men. In contrast, men with PTSD had significantly increased insulin levels and no increases in amino acid levels that would compete with transport of tryptophan to the brain; men also did not show changes in levels of palmitoleic acid levels that have beneficial function.

In the human myocardium, 2-aminobutyric acid, a metabolite of the serine to cysteine conversion pathway (Fig. 3) is known to increase glutathione levels via AMPK activation to protect against oxidative stress.[33] Thus, our data on differential levels of amino acids and their metabolites in women with PTSD suggests that alterations in subsets of metabolites is protective. Other studies have reported changes in metabolite

levels in male combat veterans with PTSD,[6, 34] but did not include women, nor did they investigate contribution of sleep. In agreement with our data, body mass index, and smoking did not explain differences in metabolite levels.

Conclusions

PTSD symptoms are myriad, vary from person-to-person, but typically include flashbacks and/or nightmares, emotional numbing, avoidance of thoughts that might remind them of traumatic events, and

hyperarousal. Symptoms usually develop within a month in most cases, but some may experience delayed onset. Nearly 30% of individuals who experience distressing events develop PTSD. While war trauma, sexual assault, or accidents are most associated with PTSD, nearly 25% of all ICU patients[35] and 32% of ICU survivors of pandemics, developed PTSD.[36] The psychological burden from the ongoing COVID-19 pandemic is yet to be fully determined. Sleep disorders are also high in patients that recovered from COVID-19-related ICU stays. Thus, while genetics predisposes one towards psychiatric disorders, other factors such as the environment, and individual's metabolic state together influence disease progression and outcomes; together these provide one explanation for nuanced and variable symptoms between individuals diagnosed with the same psychiatric disorders. Our study suggests that men and women recruit different metabolic pathways and mechanisms along with several pathways that are shared between the sexes, to reach the same outcomes. Evidence of sex-specific metabolite clusters in PTSD suggest the need for more tailored interventions to address the unique needs that may differ between men and women exposed to trauma.

List of Abbreviations

AAs: amino acids
BMI: body mass index
ChemRICH: Chemical Set Enrichment Analysis
CAPS:Clinician-Administered PTSD Scale
COVID-19: Coronavirus disease-19
DSM: Diagnostic and Statistical Manual of Mental Disorders
EEG: electroencephalogram

EOG: electrooculograms
GCRC: General Clinical Research Center
GC-TOF-MS: gas chromatography /time of flight mass spectrometry (GC-TOF MS)
HPLC: High pressure liquid chromatography
HPA: hypothalamic-pituitary-adrenal
ICU: Intensive care unit
KYN: Kynurenine
NREM: non-rapid eye movement
NMDA: *N*-methyl-D-aspartate receptors
OGTT: Oral Glucose Tolerance Test
PTSD: posttraumatic stress disorder
PSQI: Pittsburgh Sleep Quality Index TST:
Total sleep time

AB, TCN, and SSI contributed to the design of the study. AB wrote the first draft of the manuscript with input from SSI, and AB, TCN, and SSI revised the final manuscript. CL and SSI collected patient data, RR, AO, SF, OF, SSI, and AB extracted and analyzed the data.

All other authors declare no competing interests. The views, opinions and/or findings contained in this research are those of the authors and do not necessarily reflect the views of the Department of Defense, Department of Veteran Affairs, or NIH and should not be construed as an official DoD/Army/VA/NIH position, policy, or decision unless so designated by official documentation. No official endorsement should be made.

Contributors

Disclosures

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References

1. Breslau N, Kessler RC, Chilcoat HD, et al. Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma. Arch Gen Psychiatry 1998;55:62632.
2. Tanielian T, Jaycox L. Invisible Wounds of War: Psychological and Cognitive Injuries, Their Consequences, and Services to Assist Recovery: RAND Center for Military Health Policy Research, RandCorporation, 2008.
3. Richards A, Metzler TJ, Ruoff LM, et al. Sex differences in objective measures of sleep in post-traumatic stress disorder and healthy control subjects. J Sleep Res 2013;22:679-87.
4. Rao MN, Chau A, Madden E, et al. Hyperinsulinemic response to oral glucose challenge in individuals with posttraumatic stress disorder. Psychoneuroendocrinology 2014;49:17181.
5. Dean KR, Hammamieh R, Mellon SH, et al. Multi-omic biomarker identification and validation for diagnosing warzone-related post-traumatic stress disorder. Mol Psychiatry 2020;25:3337-3349.
6. Mellon SH, Bersani FS, Lindqvist D, et al. Metabolomic analysis of male combat veterans with post traumatic stress disorder. PLoS One 2019;14:e0213839.
7. Savitz J, Drevets WC, Smith CM, et al. Putative neuroprotective and neurotoxic kynurenine pathway metabolites are associated with hippocampal and amygdalar volumes in subjects with major depressive disorder. Neuropsychopharmacology 2015;40:463-71.
8. Wolfe J, Kimerling R, Brown PJ, et al. Psychometric review of the life stressor checklistrevised. Lutherville, MD: Sidran Press, 1996.

9. Blanchard EB, Jones-Alexander J, Buckley TC, et al. Psychometric properties of the PTSD Checklist (PCL). Behaviour Research & Therapy 1996;34.
10. Blake DD, Weathers FW, Nagy LM, et al. The development of a Clinician-Administered PTSD Scale. J Trauma Stress 1995;8:75-90.
11. Spitzer RL, Williams JB, Gibbon M, et al. The Structured Clinical Interview for DSM-III-R (SCID) : I. History, rationale, and description. Archives of General Psychiatry 1992;49:624-629.

12. Buysse DJ, Reynolds CF, 3rd, Monk TH, et al. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry Res 1989;28:193-213.
13. Kales A, Rechtschaffen A. A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects. : Washington, DC : United States Government Printing Office, 1968., 1968.
14. La Frano MR, Carmichael SL, Ma C, et al. Impact of post-collection freezing delay on the reliability of serum metabolomics in samples reflecting the California mid-term pregnancy biobank. Metabolomics 2018;14:151.
15. Fiehn O. Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling. Curr Protoc Mol Biol 2016;114:30 4 1-30 4 32.
16. Fiehn O, Wohlgemuth G, Scholz M, et al. Quality control for plant metabolomics: reporting MSI-compliant studies. Plant J 2008;53:691-704.
17. Wanichthanarak K, Fan S, Grapov D, et al. Metabox: A Toolbox for Metabolomic Data Analysis, Interpretation and Integrative Exploration. PLoS One 2017;12:e0171046.
18. Barupal DK, Fiehn O. Chemical Similarity Enrichment Analysis (ChemRICH) as alternative to biochemical pathway mapping for metabolomic datasets. Sci Rep 2017;7:14567.
19. Galatzer-Levy IR, Bryant RA. 636,120 Ways to Have Posttraumatic Stress Disorder. Perspect Psychol Sci 2013;8:651-62.
20. Haznedar MM, Buchsbaum MS, Hazlett EA, et al. Cingulate gyrus volume and metabolism in the schizophrenia spectrum. Schizophr Res 2004;71:249-62.
21. Zuccoli GS, Saia-Cereda VM, Nascimento JM, et al. The Energy Metabolism Dysfunction in Psychiatric Disorders Postmortem Brains: Focus on Proteomic Evidence. Front Neurosci 2017;11:493.
22. Daniel PM, Love ER, Moorhouse SR, et al. The effect of insulin upon the influx of tryptophan into the brain of the rabbit. J Physiol 1981;312:551-62.
23. Neame S, Safory H, Radzishevsky I, et al. The NMDA receptor activation by d-serine and glycine is controlled by an astrocytic Phgdh-dependent serine shuttle. Proc Natl Acad Sci U S A 2019;116:20736-20742.
24. Davis M. NMDA receptors and fear extinction: implications for cognitive behavioral therapy. Dialogues Clin Neurosci;13:463-74.
25. Orr SP, Metzger LJ, Lasko NB, et al. De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. J Abnorm Psychol 2000;109:290-8.
26. Santini E, Muller RU, Quirk GJ. Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. J Neurosci 2001;21:9009-17.
27. Rieder R, Wisniewski PJ, Alderman BL, et al. Microbes and mental health: A review. Brain Behav Immun 2017;66:9-17.
28. Rogers GB, Keating DJ, Young RL, et al. From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. Mol Psychiatry 2016;21:738-48.

29. Kautzky-Willer A, Harreiter J, Pacini G. Sex and Gender Differences in Risk, Pathophysiology and Complications of Type 2 Diabetes Mellitus. Endocr Rev 2016;37:278-316.
30. Paruthiyil S, Hagiwara SI, Kundassery K, et al. Sexually dimorphic metabolic responses mediated by CRF2 receptor during nutritional stress in mice. Biol Sex Differ 2018;9:49.
31. Coppen A, Wood K. Tryptophan and depressive illness. Psychol Med 1978;8:49-57.

32. Smith SA, Pogson CI. The metabolism of L-tryptophan by isolated rat liver cells. Effect of albumin binding and amino acid competition on oxidatin of tryptophan by tryptophan 2,3dioxygenase. Biochem J 1980;186:977-86.
33. Irino Y, Toh R, Nagao M, et al. 2-Aminobutyric acid modulates glutathione homeostasis in the myocardium. Sci Rep 2016;6:36749.
34. Somvanshi PR, Mellon SH, Flory JD, et al. Mechanistic inferences on metabolic dysfunction in posttraumatic stress disorder from an integrated model and multiomic analysis: role of glucocorticoid receptor sensitivity. Am J Physiol Endocrinol Metab 2019;317:E879-E898.
35. Burki TK. Post-traumatic stress in the intensive care unit. Lancet Respir Med 2019;7:843844.
36. Rogers JP, Chesney E, Oliver D, et al. Psychiatric and neuropsychiatric presentations associated with severe coronavirus infections: a systematic review and meta-analysis with comparison to the COVID-19 pandemic. Lancet Psychiatry 2020;7:611-627.

Table 1. Demographics data and clinical characteristics of PTSD and control subjects.

	Men		Women		
Variable	Control (N=22)	PTSD+ (N=22)	Control (N=24)	PTSD+ (N=22)	Total (N=90)
Age (Mean ± SD) [a]	30·2 ± 8·76	30·6 ± 7·61	30·0 ± 7·47	30·2 ± 6·82	30·3 ± 7·31
Education (Years) (Mean ± SD) [a]	15·5 ± 2·08	14·4 ± 2·32	15·5 ± 1·92	15·3 ± 2·03	15·2 ± 2·11
Race [b]					
African American	1 (4·5%)	3 (13·6%)	0 (0·0%)	2 (9·1%)	6 (6·7%)
Asian/Hawaiian/Pacific Islander	4 (18·2%)	1 (4·5%)	3 (12·5%)	2 (9·1%)	10 (11·1%)
Caucasian	17 (77·3%)	13 (59·1%)	19 (79·2%)	14 (63·6%)	54 (63·5%)
Other/Unknown	0 (0·0%)	5 (22·7%)	2 (8·3%)	4 (18·2%)	11 (12·2%)
Hispanic Ethnicity [b,c]	0 (0·0%)	5 (22·7%)	3 (12·5%)	1 (4·5%)	9 (10·0%)
Current CAPS score [d] (Mean ± SD)	0·0 ± 0·0	51·9 ± 13·03	0·0 ± 0·0	55·2 ± 21·58	53·5 ± 17·70
PTSD Symptom Checklist (PCL; Mean ± SD) [e]	19·09 ± 4·21	46·0 ± 12·29*	19·9 ± 4·08	55·6 ± 12·27*	34·8 ± 18·39
Current MDD [f]	0 (0·0%)	5 (22·7%)	0 (0·0%)	3 (13·6%)	8 (8·9%)
Childhood trauma ≤14 years of age [g,h]	0 (0·0%)	8 (36·4%)	0 (0·0%)	11 (50·0%)	19 (21·1%)
Hormonal birth control [b,i]	NA	NA	2 (8·3%)	6 (27·3%)	8 (17·4%)
BMI [b]					
Underweight = <18·5	2 (9·1%)	0 (0·0%)	1 (4·2%)	0 (0·0%)	3 (3·3%)
Normal weight = 18·5–24·9	10 (45·5%)	4 (18·2%)	12 (50·0%)	13 (59·1%)	39 (43·3%)
Overweight = 25–29·9	10 (45·5%)	5 (22·7%)	8 (33·3%)	7 (31·8%)	30 (33·3%)
Obesity = BMI of 30+	0 (0·0%)	13 (59·1%)	3 (12·5%)	2 (9·1%)	18 (20·0%)
Smoker [b, j]	3 (13·6%)	4 (18·2%)	6 (25·0%)	6 (27·3%)	19 (21·1%)

a based on F-test b based on Chi-square test

c Three subjects endorsed Hispanic ethnicity but did not select a racial descriptor. Six additional subjects endorsed Hispanic ethnicity, in addition to a racial category of Caucasian or African-American race yielding a total of 9 subjects self-identifying as Hispanic in this sample. Comparison of Hispanic ethnicity, p=·063.

d Control subjects had CAPS scores of zero or had an absence of criterion A events. Comparison of male and female PTSD subjects on current CAPS score, p=0·54.

e PTSD group by gender interaction on current PCL score, p<.05. *:Comparison of PTSD groups vs controls, p<.001. Comparison of male vs female PTSD groups vs controls, p<0·01.

f Absence of current MDD was required for inclusion into the control group. Comparison of male and female PTSD subjects on rate of current MDD, p=0·43.

g Childhood trauma exposure was defined, based on findings from our prior research, by exposure to 2 or more categories of childhood trauma under the age of 14. Three (6·5%) control subjects reported a history of 1 category of childhood trauma. h Chi-square test compared frequency of childhood trauma between male and female PTSD subjects only, p=·36.

i Chi-square test compared use of hormonal birth control female PTSD and control subjects only, p=·09

j based on diary

Table 2. Sex steroid metabolite concentrations in plasma of men and women.

Metabolite	LOD (ng/mL)	Control □ SD (ng/mL) Women	PTSD □ SD (ng/mL) Women	Control □ SD (ng/mL) Men	PTSD □ SD (ng/mL) Men
Progesterone (P4)	0.393	0·59 □ 1·19	<LOD (<0·28)	<LOD (<0·09)	<LOD (<0·09)
Testosterone*	0.144	0·55 □ 0·90	0·44 □ 0·68	4·03 □ 1·39	4·17 □ 1·61
Estrone (E1)	2.702	<LOD (<0·11)	<LOD (<0·24)	<LOD (<0·16)	<LOD (<0·11)
Estradiol (E2)	0.680	<LOD (<0·03)	<LOD (<0·06)	<LOD (<0·02)	<LOD (<0·03)
Estriol (E3)	2.882	5·7 □ 8·93	3·23 □ 2·26	3·39 □ 3·96	4·68 □ 9·69

Steroid metabolite values plasma in ng/mL. Limit of detection (LOD) for each analyte is shown. Values in red text were below LOD. *: p<0·0001 Men vs. Women (sex); PTSD: ns; PTSD x Sex: ns

Table 3. Altered primary metabolite clusters in PTSD with sleep confounders

Men PTSD	Men Delta Power	Men PSQI	Men TST	Women PTSD	Women Delta Power	Women PSQI	Women TST
Amino Acids (AA)							
.	.	Gly	.	Glycine (Gly)	Gly	.	Gly
.	.	.	.	Threonine (Thr)	Thr	.	Thr
.	.	.	.	Serine (Ser)	Ser	.	Ser
.	.	.	.	.	Citrulline	.	.
.	.	.	.	.	□-Ala	□-Alanine	□-Ala
Branched-chain amino acids (BCAA)							
.	.	.	.	Valine (Val)	Val	Val	Val
.	.	.	.	Leucine (Leu)	.	Leu	Leu
Sulfur amino acids							
.	.	.	Met	Methionine (Met)	Met	Met	.
.	.	.	Cys	Cysteine (Cys)	Cys	Cyst	.
Cyclic amino acids							
Histidine (His)	His	.	.	.	.	.	.
.	.	.	4-Hyp	Trans-4-hydroxyproline (4-Hyp)	4-Hyp	4-Hyp	.
Indoles (Tryptophan metabolites)							
.	.	.	.	Indole-3-propionic acid (IPA)	.	.	IPA
.	.	.	.	.	.	Indole-3-lactate (ILA)	.

.	.	.	.	.	.	Indole-3-acetate (IAA)	.
Butyrates (Gut microbes)							
.	.	Threonic acid	.	.	.	.	.
.	.	3-Hydroxybutyric acid	.	.	.	.	.
.	.	2-Hydroxybutanoic acid	.	.	.	.	.
.	.	.	.	Isothreonic acid (ITA)	ITA	.	ITA
.	.	.	.	.	.	2-Aminobutyric acid (2AB)	.
Hexose							
Fructose	.	.	.	.	.	.	.
.	Sucrose	.	.	.	.	.	.
.	.	.	.	1-Methylgalactose NIST	.	.	1-MG NIST
Unsaturated fatty acids (UFA)							
.	.	.	.	Palmitoleic acid	.	.	.

Figure Legends

Figure 1. Sex differences in primary metabolites in PTSD. (**A**) ChemRich analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI and age. Blue nodes contain metabolite clusters that were decreased, purple nodes contain metabolites that were both increased or decreased, and red nodes contain metabolites that were increased in PTSD vs. control individuals. (**B**) Venn diagram showing seven nodes were specific to women with PTSD and two nodes

significant.

were specific to men with PTSD compared with controls. (**C**) Volcano plots of specific metabolites within each node in men and women with PTSD after correcting for type 1 error and adjusting for BMI and age.

Figure 2. Alterations in specific amino acids within a metabolite cluster/node with PTSD symptoms. (**A-G**) Box plots of specific amino acids with the seven nodes that differed between women and two nodes in men with PTSD compared to controls shown in Fig. 1A. Glycine levels associated negatively with PCL cluster D in women with PTSD ($r = –0{\cdot}47$, $p = 0{\cdot}036$), but not controls, whereas association of serine level was lost in women with PTSD. (**H**) Fructose levels increased in men with PTSD vs. controls, but fructose levels associated negatively with PCL scores. Box plot analysis: Mann-Whitney and $p<0{\cdot}05$ considered

Figure 3. PTSD-specific alterations in amino acids with respect to their biosynthesis pathway in humans from glucose. Essential amino acids cannot be synthesized and must be obtained from diet, whereas non-essential amino acids can be synthesized from glucose as it enters the tricarboxylic cycle. Essential amino acids are shown in grey boxes, and those metabolites synthesized as a by-product of gut microbiome are shown in green boxes. Specific amino acids that were increased are shown in red, decreased in blue (women-specific outlined in pink and men-specific in blue).

Figure 4. Sex-specific disturbances in sleep measures. Box plots showing sex- and/or PTSD-specific alterations in (**A**) Total sleep time (in minutes) decreased; Two-way ANOVA: Sex: ns; PTSD: $p= 0{\cdot}004$; Sex X PTSD: ns. (**B**) sleep quality worsened, Two-way ANOVA: Sex: ns; PTSD: $p< 0{\cdot}0001$; Sex X PTSD: ns. and (**C**) log-transformed delta power sleep was lower in people with PTSD vs controls, Two-way ANOVA: Sex: $p= 0{\cdot}007$; PTSD: $p= 0{\cdot}005$; Sex X PTSD: ns. (**D**) Linear regression showing association of PCL scores with three different measures of sleep. individuals.

Figure 5. Sex differences in primary metabolites after accounting for sleep measures . ChemRich analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI, age, and one of the three sleep measures shown (TST (min), PSQI, or log-transformed delta power (ln(μV2)). Blue nodes contain metabolite clusters that are decreased, purple nodes contain metabolites that are both increased or decreased, and red nodes contain metabolites that are increased in PTSD vs control

Figure 6. Sex-specific contribution of sleep variables on primary metabolites. Venn diagrams to visualize significant metabolites whilst adjusting for one sleep variable at a time.

Figure 7. Linear regression and box plots of various amino acids with sleep variables (**A-F**) Box plots of specific amino acids with individual nodes that differed between women and men with PTSD compared to controls shown in Fig. 5 and 6. (**G**) Log-transformed delta power (ln(μV2) associated negatively with testosterone in men.

Figure 8. Changes in insulin and tryptophan levels in women and men with PTSD. Box plots of tryptophan and albumin levels in women and men. (**A**) No significant differences were seen in tryptophan and albumin levels in women with PTSD compared with controls, whereas tryptophan levels were significantly elevated in men with PTSD compared with controls (p = 0·044; Mann-Whitney). (**B**) Blood insulin levels were determined after oral glucose challenge at various times shown. Mixed-effect analysis showed that insulin levels changed with time (p<0·0001) in women and men, but only differed between PTSD and controls in men (p=0·0136). (**C**) Tryptophan levels correlated negatively with insulin levels in women, but positively in men.

Figure 9. Sex-specific alterations in tryptophan metabolism pathway in PTSD. (**A**) Sex, sleep, and PTSD all alter primary metabolites. (**B**) Albumin-bound tryptophan is present in circulation and dynamic

increases in insulin promote binding of albumin to tryptophan, whereas esterified fatty acids can displace tryptophan from albumin. Free tryptophan is then transported to the brain by a transport carrier. Several amino acids, such as leucine, valine etc. compete with tryptophan for binding to the transport carrier, which can decrease influx of free tryptophan into the brain. Reduced free tryptophan levels in the brain can influence production of serotonin and melatonin, affecting brain function and sleep. In the gut, tryptophan is converted to indoles by the action of microbes such as*Lactobacillus*; these indoles have protective effect on gut barrier and immune functions. Serine can serve as NMDA receptor agonist and alter neuronal function. Thus, disturbances at multiple levels in tryptophan pathway may contribute to pathogenesis of PTSD.

Supplementary Figure Legend

Fig. S1. Sex aggregated analysis of primary metabolites. There were seven primary metabolites that were significantly different between PTSD and control subjects when data were analyzed in a sex aggregated manner. Eight metabolites that have not been thoroughly characterized yet, were also identified. P values as shown using Mann-Whitney U-test.

Sex-specific alterations in primary metabolites and tryptophan pathways in posttraumatic stress and disturbed sleep

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Abstract

Background

Primary metabolites serve as substrates for neurotransmitters and are altered in psychiatric disorders. Sleep disturbances are exacerbated in posttraumatic stress disorder (PTSD), but the contribution of sex, sleep and/or PTSD in altering primary metabolites is not known.

Methods

We used mass spectrometry to ascertain primary metabolites in 90 plasma samples from individuals with chronic PTSD and control subjects. Laboratory-based polysomnography was used to monitor the sleep of participants. PTSD was determined using the Clinician-Administered PTSD Scale (CAPS).

Results

Men and women with PTSD showed distinct, non-overlapping primary metabolite nodes compared with sex-matched controls as ascertained using Chemical Set Enrichment Analysis (ChemRICH) analysis. Women with PTSD had seven nodes, whereas men with PTSD had just two nodes altered compared with controls; each node contained two or more metabolites. Sex steroids levels did not associate with metabolite nodes. Higher PTSD symptoms were associated with lower total sleep time and decreased Pittsburgh Sleep Quality Index (PSQI) scores in both men and women. Delta power on sleep electroencephalogram was significantly lower in men with PTSD and associated negatively with PTSD symptoms. Sleep measures accounted for nearly 50% of the altered primary metabolite nodes. Tryptophan and insulin levels were significantly increased in men but not women with PTSD compared with controls, whereas tryptophan levels associated inversely with insulin levels in women.

Conclusions

Women demonstrate more metabolic disturbances than men with similar PTSD scores. The presence of sex-specific primary metabolite in PTSD were not affected by sex steroids but were accounted for by sleep measures. Our data provide further evidence for development of sex-specific interventions and disease management.

Introduction

Posttraumatic stress disorder (PTSD) is a common psychiatric condition. PTSD may develop after exposure to an actual or threatened death, serious injury, trauma, or sexual violence. PTSD is currently characterized on diagnostic and statistical manual of mental disorders (DSM-5) by: (i) reexperiencing (e.g., intrusive thoughts, nightmares, flashbacks); (ii) avoidance; (iii) negative changes in cognition and mood (hopelessness, lack of emotions), and (iv) hyperarousal (trouble sleeping, self-destructive behavior, angry outbursts). While it is widely recognized that PTSD is a common consequence of trauma exposure, women are at particularly high risk, with some studies finding that women develop PTSD at twice the rate of men, despite greater trauma exposure in men.[1, 2]

One of the most common complaints among individuals with PTSD is sleep disturbance. PTSD is associated with lower slow wave sleep duration and delta power sleep, which is more pronounced in men than women. In contrast, greater rapid eye movement sleep is found in women with PTSD compared to healthy controls, a difference not seen in men.[3] Sleep disturbance is a key risk factor for health consequences as it alters hypothalamic-pituitary-adrenal (HPA) axis function, resulting in impaired glucose and lipid metabolism. The HPA and somatotropic axes activities are temporally associated with delta power sleep and promote insulin sensitivity and metabolic syndrome. Additionally, sleep duration correlates with metabolic risk in PTSD but does not fully account for the association between PTSD with known metabolic disturbances such as in blood insulin or glucose levels.[4] While prior studies have found alterations in neuroendocrine, immune and aging processes in PTSD, the role of metabolite disturbances is PTSD is limited and can help elucidate new discovery of yet unknown biological mechanisms of disease.

More recent technological developments such as mass spectrometry allow for the discovery of novel pathways using an unbiased method to examine multiple analytes simultaneously. Metabolomics is a global

and unbiased approach to understanding regulation of metabolic pathways and networks of physiologically relevant interactions. The metabolome is regulated by gene-environment interactions and reflects the

intermediary state between genotype and phenotype. Gene mutations, single nucleotide polymorphisms, and mutations in proteins are associated with PTSD, but none of these alone explains the complex manifestation of PTSD and comorbid health conditions. A multi-omics approach has been used to identify potential biomarkers that range from DNA methylation, proteins, miRNA, lipids, and other metabolites in warzone male veterans with PTSD.[5] Metabolomic profiling has also led to identification of key differences in glycolysis and fatty acid pathways that were associated with mitochondrial dysfunction in men with PTSD. [6]

Essential and non-essential amino acids (AAs) as well as biogenic amines are building blocks of proteins and peptide hormones involved in a plethora of functions that include membrane stabilization, neurotransmission, and neuroimmune modulation. AAs act as neurotransmitters (e.g. glutamate, glycine, serine). Kynurenine (KYN), a metabolite of AA tryptophan, is correlated with changes in hippocampal and amygdalar volumes in depressed patients.[7] KYN is associated with stress exposure, neuronal cell death, glutamate transmission, and neuroinflammation. Alterations in amino acid metabolites that are more bloodbrain barrier permeable, such as KYN, may account for some of these differences. We are not aware of any study that has systematically ascertained sex differences in the status of primary metabolites in serum samples of participants with PTSD and matched-controls. In this study, we aimed to examine alterations of primary amino acids and metabolites in PTSD and the contribution of sleep measures in both men and women.

Methods

Human subjects

In this study, we used a cross-sectional, 2 × 2 design (PTSD/control × women/men) involving 90 medically healthy, non-medicated adults aged 19-39 years in an inpatient sleep laboratory at the General Clinical Research Center (GCRC) at the University of California, San Francisco. The study sample was comprised of 44 individuals with current chronic PTSD (50% women) and 46 control subjects (52% women). This sample was drawn from a larger study of 94 participants. Data from 4 participants were excluded due to difficulties in blood collection. This research was approved by the Committee on Human Research at the University of California, San Francisco. All participants provided written informed consent before participating in any study procedures.

Psychiatric diagnoses and trauma history

The type of trauma exposure and age of occurrence was assessed using the Life Stressor Checklist- Revised interview. The PTSD Checklist (civilian version) for DSM-IV (PCL-C) [9] was used to assess severity of chronic PTSD symptoms and on the Clinician-Administered PTSD Scale (CAPS) and a CAPS score >40. This self-report measure consists of 17 items that correspond to the DSM-IV criteria and include intrusive thoughts and re-experiencing symptoms (cluster B), avoidance (cluster C), and hyperarousal (cluster D). The structured clinical interview for DSM-IV, non-patient edition (SCID-NP) was used to diagnose all other psychiatric disorders, including major depressive disorder.[11]

Control subjects had no lifetime or current history of a PTSD diagnosis. Women participants were premenopausal, and were scheduled during the follicular phase of the menstrual cycle. All study procedures were timed according to habitual sleep onset, determined by actigraphy and sleep diary in the week prior to the GCRC study. Study participants were alcohol and drug-free, limited to one cup of caffeine daily, and maintained regular bed and waking times. Exclusion criteria included a history of traumatic brain injury, presence of neurologic disorders or systemic illness; use of psychiatric, anticonvulsant, antihypertensive, sympathomimetic, steroidal, statin or other prescription medications; obesity (defined as body mass index (BMI) >30); alcohol abuse or dependence in the prior two years; substance abuse or dependence in the

previous year; any psychiatric disorder with psychotic features; bipolar disorder or obsessive-compulsive disorder; and pregnancy. Exclusion criteria for control subjects also included a lifetime history of major depressive disorder or panic disorder.

Sleep clinic and measures

The Pittsburgh Sleep Quality Index[12] was used to provide a subjective assessment of sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbances (including nightmares), use of sleep medication, and daytime dysfunction over the previous month. Participants rated items on a four-point Likert scale within each domain with higher scores indicating poorer sleep. A total score of > 5 is indicative of poor

Polysomnography recordings were obtained with ambulatory polysomnography (Nihon Kohden Trackit Ambulatory Recording System). The parameters recorded included an electroencephalogram (EEG) at leads C3, C4, O1 and O2, left and right electrooculograms (EOG), submental electromyogram, bilateral anterior tibialis EMGs, and electrocardiogram in accordance with standardized guidelines by Rechtschaffen. Electrode impedance was set at < 5 kohm at the start of the recording. The EEG and EOG leads were referenced to linked mastoids. Raw EEG signals were filtered and amplified, then digitized at sleep quality.

Polysomnographic measurements

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256 Hz and recorded to a removable hard disk in European Data Format file format. The low frequency and high frequency hardware filters on the recorder were single pole analog filters with 3 db points at 0·5 Hz and 100 Hz. Pass Plus was utilized for both visual scoring and quantitative EEG analysis of the digitized polysomnography data.

Power spectral analysis for polysomnographic measures

Pass Plus (Delta Software) analytic software was used to measure sleep activity in all frequency bands delta through gamma from the C3 electrode by power spectral analysis. The C4 electrode was used if there was

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excessive artifact. A limitation of Pass Plus is that artifact removal is accomplished by removal of whole epochs tagged with artifact. This has the potential to introduce additional confounds given the removal of typically longer bouts of uncontaminated EEG. Therefore, epochs were tagged for slow and fast artifact for additional analyses. Primary analyses were conducted with all epochs and then checked for the impact of removal of epochs with slow and fast artifact. Removal of fast artifact (for bandwidths alpha and above) and slow artifact (for bandwidths delta and theta) did not significantly impact our findings in non-rapid eye movement (NREM) sleep. All results are therefore reported without removal of epochs containing artifact. Pass Plus applied a 5μV smoothing constant to eliminate spurious waves caused by electrical jitter. Power spectral analysis was conducted on all epochs of NREM and REM sleep. Epochs visually scored as wake were not included in these analyses. Visual scoring was conducted by a highly experienced registered polysomnography technician, blind to PTSD status, classified all 30-second epochs in every sleep record as wake; stages 1, 2, 3; REM; or movement using current American Academy of Sleep Medicine criteria. Total sleep time was defined by time spent in epochs scored as NREM stages 1 through 3 and stage REM. Pass

Plus was used to perform Fast Fourier Transformation analysis on 4·0 second Welch tapered windows with 2 second overlap, yielding 15 windows per 30-second epoch. Power spectra for delta (1-4 Hz) were analyzed to address our hypothesis with respect to delta spectral power. Delta sleep spectral power density (μV2) was natural log transformed to normalize its distributions.

Blood collection and clinical laboratory measures

Blood was collected at habitual wake-up time on the morning after the second night on the GCRC, while the subject was fasting, from an indwelling catheter inserted the night before. Blood (10mL) was drawn into a chilled EDTA purple-topped tube and processed for plasma separation for mass spectrometry analysis.

Oral glucose tolerance test (OGTT)

Oral Glucose Tolerance Test (OGTT) was performed on the morning of day two after a 10-hour overnight fast. Subjects were given 75g dextrose in 250 cc of water, and blood samples were taken at baseline and 30, 60 and 120 minutes for measurement of glucose and insulin levels. Glucose was measured by the hexokinase method (Roche Applied Sciences, Indianapolis, IN) on the Cobas C501, at Washington University Core Laboratory. Insulin measurement was performed by EMD Millipore Corporation (St. Charles, MO, USA) by radioimmunoassay.

Analysis of primary metabolites in human plasma using gas chromatography /time of flight mass spectrometry (GC-TOF MS)

Primary metabolites that included sugar phosphates, amino acids, hydroxyl acids, and free fatty acids, were measured using GC-TOF MS. Briefly, metabolites from plasma were extracted using 1 milliliter of degassed, −20°C cold solvent mixture of acetonitrile (ACN):isopropanol (IPA):water (H_2O) (3:3:2, v/v/v) per 20 µL sample aliquot. Samples were vortexed for 10 seconds, shaken for 5 min and then centrifuged for 2 min at 12,800 x g. Two 450 µL supernatant aliquots were transferred to new tubes. To remove any excess protein and triacylglycerides, the supernatant evaporated and resuspended in 500 µL 1:1 acetonitrile:water

and vortexed for 10 seconds, centrifuged for 2 min at 12,800 x g. The supernatant was transferred to a clean tube and then dried down in a CentriVap concentrator. Samples were derivatized with 10 µL of methoxyamine hydrochloride (20mg/mL) in pyridine and subsequently by 90 µL of MSTFA for trimethylsilylation of acidic protons, including a mixture of C8–C30 fatty acid methyl esters as internal standards for retention index correction.[14] Internal standards in GC-MS included retention index markers for database annotations[15]. These internal standards are not used for quantification. Instead, we use mixtures of 30 external standards (Quality Control (QC) mix) in dilution curves before and after each batch of samples, in addition the highest-concentrated QC mix sample after each set of 10 biological samples, again, as described elsewhere.[15]

Data acquisition, extraction, and processing of metabolites

Data were acquired using the following chromatographic parameters[16]: Column: Restek corporation Rtx5Sil MS (30 m length x 0.25 mm internal diameter with 0·25µm film made of 95% dimethyl/5%diphenylpolysiloxane); Mobile phase: Helium; Column temperature: 50-330°C Flow-rate: 1 mL min-1; Injection volume: 0·5µL; Injection: 25 splitless time into a multi-baffled glass liner Injection temperature: 50°C ramped to 250°C by 12°C s-1; Oven temperature program: 50°C for 1 min, then ramped at 20°C min-1 to 330°C, held constant for 5 min. Data processing. Raw data files were preprocessed directly after data acquisition and stored as ChromaTOF-specific *.peg files, as generic *.txt result files and additionally as generic ANDI MS *.cdf files. ChromaTOF vs. 4·0 was used for data preprocessing without smoothing, 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram. Apex masses are reported for use in the BinBase algorithm. Result *.txt files were exported to a data server with absolute spectra intensities and further processed by a filtering algorithm implemented in the metabolomics BinBase database. Given compound was identified and cross-referenced with external database identifiers such as InChI key, PubChem ID, and KEGG ID. Following equation was then used for normalizations for metabolite *i* of sample *j*:

$$metabolite_{ij\ raw}$$

$$metabilite\ ij\ normalized = \frac{}{mTICj}.mTICaverage$$

The normalized data are shown as peak heights for the quantification ion (mz value) at the specific retention index. Missing values were imputed using half of the minimum detected value for each compound. Autoscaling was performed to make compounds the same scale.[17]

All metabolomic data were investigated using pooled quality control samples and blank samples. Metabolites that had more than 30% relative standard deviation in pooled QC samples were removed, and metabolites that showed less than 3-fold higher intensity compared to blank samples were removed as well. We do not count such signals as genuine metabolites; hence, the final data sheet is what was used for statistical analyses. Briefly, the number of reported signals from raw processed data to final reported data

went from 313 metabolites to 266 metabolites. Technical errors reduce biological power, so findings reported in this work were observed at statistical power despite possible technical variance.

Statistical analysis

We first analyzed data in a sex aggregated manner and found several metabolites to be significantly altered between control and PTSD groups (Fig. S1). Men with PTSD were more likely to be obese than other groups. Therefore, we adjusted for BMI and age in our analyses. We next performed our more detailed analyses in a sex segregated manner. We conducted robust linear regression for each sex (i.e. male and female, respectively) to examine the association between compound intensity, PTSD status, sleeping factors, and to examine the interaction effect between sex and PTSD status for each metabolite. Specifically, robust linear regression was conducted with compound intensity as response and PTSD status as predictor, controlling for body mass index and age. The effect size was determined by the coefficient of the regression model, with a positive value indicating compound intensity being higher in PTSD compared with control and a negative value indicating the opposite. We used the Benjamini Hochberg procedure to correct for multiple comparisons. The p-values and effect sizes from robust linear regression were used as input for the ChemRICH analysis[18] to study the enrichment effect on metabolite clustered defined according to chemical similarity. Kolmogorov–Smirnov test was used to calculate the p-value for each of the metabolite clusters. We further used Venn-diagrams to highlight the sex difference from the ChemRICH analyses. Volcano

plots were used to visualize the significance and effect sizes of each metabolite in each of the significant clusters for each sex after correcting for type 1 errors. We also performed the same analysis but with each of the three sleeping factors (total sleep time (min), PSQI, and log-transformed delta energy (μV2sec)) as confounding variables. We used box plot and Venn diagrams to visualize the significance effect while adjusting for sleep variables. We used the statistical computing language R version 3.6.3.

Robust linear regression was performed in MASS library.

Results

Demographic Data and Clinical Characteristics

By design, there were no significant differences in sex distribution between PTSD and control subjects, nor were there significant differences in age, education, or race/ethnicity across all four groups. Sample characteristics are presented in Table 1. Men with PTSD were more likely to be obese and male controls by the presence of two or more categories of childhood trauma as compared to one or none). Eleven control subjects reported a lifetime history of a traumatic criterion A1 event, but all had current CAPS scores of zero and none had a lifetime history of PTSD. However, the PTSD groups scored higher on the PCL-C (avoidance) than controls; women with PTSD had higher PCL-C scores than men with PTSD (Table 1). As per the exclusion criteria, no control subjects met criteria for current MDD. Additionally, none of the control subjects reported a history of two or more categories of childhood trauma. There were no differences between PTSD and control women in use of hormonal birth control or group differences in smoking of

Chemical Set Enrichment Analysis (ChemRICH) identified sex differences in primary and steroid

were less likely to be obese. Therefore, we adjusted for BMI in all our analyses. Men and women with

PTSD did not differ in terms of CAPS scores, rates of current MDD, or history of childhood trauma (defined

tobacco. **metabolites**

We performed ChemRICH analysis on primary metabolites to identify chemical classes that were significantly altered in PTSD compared to controls. ChemRICH utilizes structure similarity and chemical ontologies to map all known metabolites and name metabolic modules.[18] This is a statistical enrichment approach based on chemical similarity and alternative to pathway analysis that relies on limited biochemical knowledge annotations.[18] It yields study-specific, non-overlapping sets of all identified metabolites. Since ChemRICH sets have a self-contained size, thus p-values do not rely on the size of the background database. ChemRICH analysis identified seven primary metabolite nodes altered in women with PTSD compared

with control women (Fig. 1A) after adjusting for age and BMI as confounding factors. Each node reflects a significantly altered cluster of metabolites and the node size represents the total number metabolites within a cluster set. Branched-chain and sulfur-containing amino acids, and unsaturated fatty acids were increased, indoles and cyclic amino acids were decreased, whereas the non-polar amino acid node contained metabolites that either increased or decreased in women with PTSD compared to controls. In contrast, men with PTSD had only two altered nodes compared with controls (Fig. 1A-C). Surprisingly, men and women with PTSD did not share any overlapping primary metabolite clusters (Fig. 1B). Although, when data was analyzed in a sex aggregated manner, a number of metabolites were significantly different between control and PTSD groups (Fig. S1). Seven metabolites that included both essential and non-essential amino acids as well as eight uncharacterized metabolites were significantly different between PTSD and controls (Fig. S1). Further analysis of the metabolites in a sex segregated manner within each node revealed presence of several metabolites that remained statistically significant after adjusting for false discovery rate and effect

size (Fig. 1C). Surprisingly, sex steroid metabolites (estrogens, testosterone, progesterone or their metabolites) did not differ between controls and PTSD in either men or women (Table 2), nor did sex steroids associate with PTSD. Progesterone was below the level of detection in men and women with PTSD, whereas estrone and estradiol were also below LOD in both men and women. Testosterone, but not estriol levels differed between men and women, with levels nearly ~10-fold higher in men (Table 2).

Essential, non-essential amino acids, and butyrates were altered in women with PTSD

Since PTSD symptom presentation is highly variable between individuals,[19] and in our cohort, women had significantly greater PCL-C scores than men, we reasoned that individual PCL measures may associate differently with individual metabolites. To better understand which of the PTSD symptom clusters contributed to alterations in specific metabolites after adjusting for type 1 errors, linear regression analysis for each PCL symptom cluster with significant individual metabolites was performed (Fig. 2A-H and Table 3). Serine, a neurotransmitter and precursor for the synthesis of glycine and cysteine as well as for 2-

aminobutyric acid, a butyrate (2-AB), is a byproduct of cysteine biosynthesis pathway, which are all in the glucose metabolism pathway (Fig. 3). While serine levels trended to be lower in women with PTSD compared to controls, no significant relationship with PTSD measures were found (Fig. 2A). Glycine levels were negatively associated with cluster D symptoms on the PCL, suggesting that with more hyperarousal, glycine levels were decreased in women, but not men. Cysteine and 2-AB levels were elevated in PTSD compared with controls (Fig. 2C and 2F) but did not show any significant association with specific clusters of PTSD symptoms.

Hexose metabolites were altered in men, but not women with PTSD

Alterations in glucose metabolism are decreased within specific brain regions of people with schizophrenia and mood disorders as ascertained with functional imaging. [20, 21]

Sucrose is metabolized to glucose and fructose, and fructose can only be metabolized in the liver via the tricarboxylic acid cycle. We found that whilst overall levels of fructose were increased in men with PTSD compared with controls (Fig. 2H), its levels were negatively correlated with both B and C symptom clusters, suggesting that more reexperiencing and avoidance symptoms contribute to altered hexose metabolism in men, but not women.

Sleep disturbances and quality are associated with overall poor health[3]. We found that both men and women

Sleep quality was worse in PTSD in both sexes

with PTSD had lower total sleep time (TST), and worse self-reported sleep quality as assessed by the PSQI compared with controls in (Fig. 4A-B). Delta power, a measure of deep sleep activity, was also significantly decreased in PTSD and showed a significant sex difference with men having lower delta power sleep activity than women (Fig. 4C). Specifically, greater PTSD symptoms (as reflected by total PCL score) was associated with lower TST in both women and men ($r = –0{\cdot}41$, $p=0{\cdot}005$ and $r = –0{\cdot}32$, $p=0{\cdot}033$, respectively, Fig. 4D), and poor sleep quality (PSQI) was highly associated with greater PTSD symptoms in both women and men ($r=0{\cdot}84$ and $r=0{\cdot}74$, $p<0{\cdot}001$, respectively, Fig. 4E). In contrast, delta power was lower in men

with PTSD (r= –0·35, p=0·02) compared to controls, but there were no differences in PTSD status on delta activity in women (Fig. 4F).

Decreased levels of essential amino acids in both sexes and indoles in women associated with sleep parameters and PTSD symptoms

Humans lack the ability to synthesize eight essential amino acids which must be obtained from diet and are mostly absorbed by the gut and metabolized by the resident microbiota. For example, tryptophan is metabolized to a myriad of biologically active compounds by four different pathways (serotonin, tryptamines, kynurenine, indoles, and NAD+). When we accounted for TST, two of the six metabolite nodes that included cyclic amino acids and unsaturated fatty acids, were no longer significant in women with PTSD, whereas no node remained significant in men with PTSD (Fig. 5 and Fig. 6) and Table 3. In contrast, when PSQI was used as a confounder, new, albeit non-overlapping nodes were found to be significant in women and men; indoles, hexose and amino acid nodes were decreased in women, whereas the butyrate node was found to harbor significantly increased as well as decreased metabolites in men with PTSD (Fig. 5). Alterations in delta power sleep accounted for 50% of the nodes in women (Table 3), whereas delta power was not associated with alterations in any nodes in men with PTSD (Fig. 5 vs Fig. 1A). Further analysis revealed that decreased sleep quality and delta power contributed to a decrease in levels of several essential amino acid as well as metabolites in the glucose metabolism pathway for which they serve as substrates (Fig. 7). Interestingly, we found a nonsignificant trend with higher testosterone levels associated with lower delta power in men (Fig. 7G, r= –0·29, p=0·06).

Plasma insulin levels associated negatively with tryptophan levels in women, but not men

While tryptophan levels were not associated with either sex, PTSD or sleep at the node level, when examined as sex segregated, tryptophan was significantly increased in men with PTSD compared to controls, but not in women (Fig. 8A). As influx of free tryptophan into the brain by its transport carrier is regulated by several factors including levels of plasma albumin and insulin,[22] we next examined the levels

of plasma albumin and insulin levels 30, 60, and 120 min after an oral glucose challenge. While plasma albumin levels did not differ between controls and individuals with PTSD in either sex, insulin levels were

significantly elevated in men with PTSD compared with controls (Fig. 8B). Interestingly, tryptophan levels associated negatively with insulin levels at 60 min in women (r= –0·30, p=0·04), whereas insulin levels exhibited a positive association with free tryptophan levels in men, although the relationship did not reach statistical significance (Fig. 8C).

Discussion

To our knowledge, this is the first systematic study to report sex-specific alterations in primary metabolites in men and women with PTSD with several novel findings. First, both sex and sleep measures altered distinct amino acids in individuals with PTSD compared with controls. Second, levels of sex steroids such as estriol, progesterone, and testosterone did not differ between controls and PTSD. Third, sex steroids did not associate with PTSD or primary metabolites in our cohort. Fourth, delta power and perceived sleep quality account for nearly half the changes in metabolite clusters in women with PTSD compared with control women (Fig. 9A). In women, perceived sleep quality was associated with increases in 2hydroxybutonic acid, a butyrate and decrease in indoles, generated by the gut microbes. Finally, there was no overlap in PTSD-related alterations in men and women in any primary metabolite nodes or individual amino acids within those nodes, and associated pathways.

An individual's physiological and metabolic state shifts glucose metabolism and generation of non-essential amino acids. Serine and glycine, both non-essential amino acids are directly synthesized from glucose in several cells including the glia, astrocytes, and hippocampal neurons. Both serine and glycine shuttle between the glia and neurons where glycine induces release of serine, which is a coagonist for *N*-methylD-aspartate (NMDA) receptors.[23] NMDA receptor function in the amygdala and prefrontal cortex have been found to be critical for the consolidation of extinction of previously conditioned fear memories.[24]

Enhanced fear conditioning and a deficit in the extinction of conditioned fear that are reliant on NMDA activity have been proposed to underlie the development and maintenance of PTSD.[25, 26]

We find that both serine and glycine levels were decreased in women with PTSD, and sleep measures such as delta power and total sleep time account for changes in these two amino acids in women (Table 3). In contrast, neither serine nor glycine levels were altered in men with PTSD and whereas sleep quality decreased glycine levels in men, after correcting for type 1 errors, its levels were no longer significant (Table 3). Other studies have shown that kynurenine pathway metabolites, kynurenic acid and quinolinic acid, associated with neuroprotective and neurotoxic functions, respectively, were altered in patients with major depressive disorders.[7] Like serine, quinolinic acid is also an NMDA receptor agonist (Fig. 9B), and increase in levels of serine may alter neuronal function in PTSD and other psychiatric disorders.

Valine, lysine, methionine, tryptophan, and histidine are all essential amino acids that cannot be synthesized by human cells. Tryptophan is absorbed in the gut and converted by the actions of gut microbiota such as *Lactobacillus* to indoles (Fig. 9B). While the role of serotonin in mood disorders, anxiety, and other disorders is well known, we show here for the first time that indole metabolite, indole-3-propionic acid, is decreased in women with PTSD. Poorer sleep quality was further associated with decreased levels of two additional indoles, indole-3-lactic and acetic acids; the indoles regulate immune and gut barrier functions (Fig. 9B), and their decreased levels might contribute to altered immune and barrier function in women. In agreement with these data, altered immune and gut barrier function is seen in patients with other psychiatric disorders.[27, 28] Diets rich in butyrates that support growth of beneficial gut bacteria such as *Lactobacillus* may serve as non-invasive interventions, especially for women. While diet is hard to control (self-report is not reliable) and we acknowledge this as a limitation of our study; although it should be noted that all participants were in the sleep clinic for 3 nights and had to choose from the menu provided at the clinic.

Increased blood insulin levels affect transport of free tryptophan to the brain in two ways: first, insulin enhances binding of tryptophan to the albumin, thereby decreasing levels of free tryptophan in the circulation by half, and decreasing influx into the brain.[22] Second, simultaneous reduction in levels of six or more amino acids such as leucine, serine, cysteine, histidine, methionine, valine, which would otherwise compete with tryptophan for binding to the transport carrier into the brain to increase its influx.[22] Ethnicity

along with many factors predispose individuals to a higher risk of insulin resistance; however, our data did not examine ethnicity as a contributing factor. In our cohort, we did not find any difference in insulin levels in women with PTSD and controls. However, we did find an increase in levels of several amino acids in women with PTSD that are known to compete with tryptophan carrier for influx into the brain (Fig. 9B and Table 3). This could result in decrease in substrate for conversion to serotonin and subsequently to melatonin, thereby affecting sleep. Although sex differences in insulin resistance and glucose clearance are reported in people with diabetes[29] as well as in animal models of diabetes,[30] we did not find any sex differences in insulin levels after an oral glucose challenge in our PTSD cohort. Non-esterified fatty acids can displace albumin from tryptophan[31, 32] and this free tryptophan can be converted in to serotonin or degraded in cells. We found an increase in palmitoleic acid levels, a monounsaturated non-esterified fatty acid in women with PTSD, which can potentially displace albumin from tryptophan in order to generate free tryptophan. Concomitant increase in palmitoleic acid levels may compensate for increases in amino acids that prevent influx of tryptophan into the brain and conversion to serotonin, more so in women than in men. In contrast, men with PTSD had significantly increased insulin levels and no increases in amino acid levels that would compete with transport of tryptophan to the brain; men also did not show changes in levels of palmitoleic acid levels that have beneficial function.

In the human myocardium, 2-aminobutyric acid, a metabolite of the serine to cysteine conversion pathway (Fig. 3) is known to increase glutathione levels via AMPK activation to protect against oxidative stress.[33] Thus, our data on differential levels of amino acids and their metabolites in women with PTSD suggests that alterations in subsets of metabolites is protective. Other studies have reported changes in metabolite

levels in male combat veterans with PTSD,[6, 34] but did not include women, nor did they investigate contribution of sleep. In agreement with our data, body mass index, and smoking did not explain differences in metabolite levels.

Conclusions

PTSD symptoms are myriad, vary from person-to-person, but typically include flashbacks and/or nightmares, emotional numbing, avoidance of thoughts that might remind them of traumatic events, and

hyperarousal. Symptoms usually develop within a month in most cases, but some may experience delayed onset. Nearly 30% of individuals who experience distressing events develop PTSD. While war trauma, sexual assault, or accidents are most associated with PTSD, nearly 25% of all ICU patients[35] and 32% of ICU survivors of pandemics, developed PTSD.[36] The psychological burden from the ongoing COVID-19 pandemic is yet to be fully determined. Sleep disorders are also high in patients that recovered from COVID-19-related ICU stays. Thus, while genetics predisposes one towards psychiatric disorders, other factors such as the environment, and individual's metabolic state together influence disease progression and outcomes; together these provide one explanation for nuanced and variable symptoms between individuals diagnosed with the same psychiatric disorders. Our study suggests that men and women recruit different metabolic pathways and mechanisms along with several pathways that are shared between the sexes, to reach the same outcomes. Evidence of sex-specific metabolite clusters in PTSD suggest the need for more tailored interventions to address the unique needs that may differ between men and women exposed to trauma.

List of Abbreviations

AAs: amino acids
BMI: body mass index
ChemRICH: Chemical Set Enrichment Analysis
CAPS:Clinician-Administered PTSD Scale
COVID-19: Coronavirus disease-19
DSM: Diagnostic and Statistical Manual of Mental Disorders
EEG: electroencephalogram

EOG: electrooculograms
GCRC: General Clinical Research Center
GC-TOF-MS: gas chromatography /time of flight mass spectrometry (GC-TOF MS)
HPLC: High pressure liquid chromatography
HPA: hypothalamic-pituitary-adrenal
ICU: Intensive care unit
KYN: Kynurenine
NREM: non-rapid eye movement
NMDA: *N*-methyl-D-aspartate receptors
OGTT: Oral Glucose Tolerance Test
PTSD: posttraumatic stress disorder
PSQI: Pittsburgh Sleep Quality Index TST:
Total sleep time

AB, TCN, and SSI contributed to the design of the study. AB wrote the first draft of the manuscript with input from SSI, and AB, TCN, and SSI revised the final manuscript. CL and SSI collected patient data, RR, AO, SF, OF, SSI, and AB extracted and analyzed the data.

All other authors declare no competing interests. The views, opinions and/or findings contained in this research are those of the authors and do not necessarily reflect the views of the Department of Defense, Department of Veteran Affairs, or NIH and should not be construed as an official DoD/Army/VA/NIH position, policy, or decision unless so designated by official documentation. No official endorsement should be made.

Contributors

Disclosures

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References

1. Breslau N, Kessler RC, Chilcoat HD, et al. Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma. Arch Gen Psychiatry 1998;55:62632.
2. Tanielian T, Jaycox L. Invisible Wounds of War: Psychological and Cognitive Injuries, Their Consequences, and Services to Assist Recovery: RAND Center for Military Health Policy Research, RandCorporation, 2008.
3. Richards A, Metzler TJ, Ruoff LM, et al. Sex differences in objective measures of sleep in post-traumatic stress disorder and healthy control subjects. J Sleep Res 2013;22:679-87.
4. Rao MN, Chau A, Madden E, et al. Hyperinsulinemic response to oral glucose challenge in individuals with posttraumatic stress disorder. Psychoneuroendocrinology 2014;49:17181.
5. Dean KR, Hammamieh R, Mellon SH, et al. Multi-omic biomarker identification and validation for diagnosing warzone-related post-traumatic stress disorder. Mol Psychiatry 2020;25:3337-3349.
6. Mellon SH, Bersani FS, Lindqvist D, et al. Metabolomic analysis of male combat veterans with post traumatic stress disorder. PLoS One 2019;14:e0213839.
7. Savitz J, Drevets WC, Smith CM, et al. Putative neuroprotective and neurotoxic kynurenine pathway metabolites are associated with hippocampal and amygdalar volumes in subjects with major depressive disorder. Neuropsychopharmacology 2015;40:463-71.
8. Wolfe J, Kimerling R, Brown PJ, et al. Psychometric review of the life stressor checklistrevised. Lutherville, MD: Sidran Press, 1996.

9. Blanchard EB, Jones-Alexander J, Buckley TC, et al. Psychometric properties of the PTSD Checklist (PCL). Behaviour Research & Therapy 1996;34.
10. Blake DD, Weathers FW, Nagy LM, et al. The development of a Clinician-Administered PTSD Scale. J Trauma Stress 1995;8:75-90.
11. Spitzer RL, Williams JB, Gibbon M, et al. The Structured Clinical Interview for DSM-III-R (SCID) : I. History, rationale, and description. Archives of General Psychiatry 1992;49:624-629.

12. Buysse DJ, Reynolds CF, 3rd, Monk TH, et al. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry Res 1989;28:193-213.
13. Kales A, Rechtschaffen A. A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects. : Washington, DC : United States Government Printing Office, 1968., 1968.
14. La Frano MR, Carmichael SL, Ma C, et al. Impact of post-collection freezing delay on the reliability of serum metabolomics in samples reflecting the California mid-term pregnancy biobank. Metabolomics 2018;14:151.
15. Fiehn O. Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling. Curr Protoc Mol Biol 2016;114:30 4 1-30 4 32.
16. Fiehn O, Wohlgemuth G, Scholz M, et al. Quality control for plant metabolomics: reporting MSI-compliant studies. Plant J 2008;53:691-704.
17. Wanichthanarak K, Fan S, Grapov D, et al. Metabox: A Toolbox for Metabolomic Data Analysis, Interpretation and Integrative Exploration. PLoS One 2017;12:e0171046.
18. Barupal DK, Fiehn O. Chemical Similarity Enrichment Analysis (ChemRICH) as alternative to biochemical pathway mapping for metabolomic datasets. Sci Rep 2017;7:14567.
19. Galatzer-Levy IR, Bryant RA. 636,120 Ways to Have Posttraumatic Stress Disorder. Perspect Psychol Sci 2013;8:651-62.
20. Haznedar MM, Buchsbaum MS, Hazlett EA, et al. Cingulate gyrus volume and metabolism in the schizophrenia spectrum. Schizophr Res 2004;71:249-62.
21. Zuccoli GS, Saia-Cereda VM, Nascimento JM, et al. The Energy Metabolism Dysfunction in Psychiatric Disorders Postmortem Brains: Focus on Proteomic Evidence. Front Neurosci 2017;11:493.
22. Daniel PM, Love ER, Moorhouse SR, et al. The effect of insulin upon the influx of tryptophan into the brain of the rabbit. J Physiol 1981;312:551-62.
23. Neame S, Safory H, Radzishevsky I, et al. The NMDA receptor activation by d-serine and glycine is controlled by an astrocytic Phgdh-dependent serine shuttle. Proc Natl Acad Sci U S A 2019;116:20736-20742.
24. Davis M. NMDA receptors and fear extinction: implications for cognitive behavioral therapy. Dialogues Clin Neurosci;13:463-74.
25. Orr SP, Metzger LJ, Lasko NB, et al. De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. J Abnorm Psychol 2000;109:290-8.
26. Santini E, Muller RU, Quirk GJ. Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. J Neurosci 2001;21:9009-17.
27. Rieder R, Wisniewski PJ, Alderman BL, et al. Microbes and mental health: A review. Brain Behav Immun 2017;66:9-17.
28. Rogers GB, Keating DJ, Young RL, et al. From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. Mol Psychiatry 2016;21:738-48.

29. Kautzky-Willer A, Harreiter J, Pacini G. Sex and Gender Differences in Risk, Pathophysiology and Complications of Type 2 Diabetes Mellitus. Endocr Rev 2016;37:278-316.
30. Paruthiyil S, Hagiwara SI, Kundassery K, et al. Sexually dimorphic metabolic responses mediated by CRF2 receptor during nutritional stress in mice. Biol Sex Differ 2018;9:49.
31. Coppen A, Wood K. Tryptophan and depressive illness. Psychol Med 1978;8:49-57.

32. Smith SA, Pogson CI. The metabolism of L-tryptophan by isolated rat liver cells. Effect of albumin binding and amino acid competition on oxidatin of tryptophan by tryptophan 2,3dioxygenase. Biochem J 1980;186:977-86.
33. Irino Y, Toh R, Nagao M, et al. 2-Aminobutyric acid modulates glutathione homeostasis in the myocardium. Sci Rep 2016;6:36749.
34. Somvanshi PR, Mellon SH, Flory JD, et al. Mechanistic inferences on metabolic dysfunction in posttraumatic stress disorder from an integrated model and multiomic analysis: role of glucocorticoid receptor sensitivity. Am J Physiol Endocrinol Metab 2019;317:E879-E898.
35. Burki TK. Post-traumatic stress in the intensive care unit. Lancet Respir Med 2019;7:843844.
36. Rogers JP, Chesney E, Oliver D, et al. Psychiatric and neuropsychiatric presentations associated with severe coronavirus infections: a systematic review and meta-analysis with comparison to the COVID-19 pandemic. Lancet Psychiatry 2020;7:611-627.

Table 1. Demographics data and clinical characteristics of PTSD and control subjects.

	Men		Women		
Variable	Control (N=22)	PTSD+ (N=22)	Control (N=24)	PTSD+ (N=22)	Total (N=90)
Age (Mean ± SD) [a]	30·2 ± 8·76	30·6 ± 7·61	30·0 ± 7·47	30·2 ± 6·82	30·3 ± 7·31
Education (Years) (Mean ± SD) [a]	15·5 ± 2·08	14·4 ± 2·32	15·5 ± 1·92	15·3 ± 2·03	15·2 ± 2·11
Race [b]					
African American	1 (4·5%)	3 (13·6%)	0 (0·0%)	2 (9·1%)	6 (6·7%)
Asian/Hawaiian/Pacific Islander	4 (18·2%)	1 (4·5%)	3 (12·5%)	2 (9·1%)	10 (11·1%)
Caucasian	17 (77·3%)	13 (59·1%)	19 (79·2%)	14 (63·6%)	54 (63·5%)
Other/Unknown	0 (0·0%)	5 (22·7%)	2 (8·3%)	4 (18·2%)	11 (12·2%)
Hispanic Ethnicity [b,c]	0 (0·0%)	5 (22·7%)	3 (12·5%)	1 (4·5%)	9 (10·0%)
Current CAPS score [d] (Mean ± SD)	0·0 ± 0·0	51·9 ± 13·03	0·0 ± 0·0	55·2 ± 21·58	53·5 ± 17·70
PTSD Symptom Checklist (PCL; Mean ± SD) [e]	19·09 ± 4·21	46·0 ± 12·29*	19·9 ± 4·08	55·6 ± 12·27*	34·8 ± 18·39
Current MDD [f]	0 (0·0%)	5 (22·7%)	0 (0·0%)	3 (13·6%)	8 (8·9%)
Childhood trauma ≤14 years of age [g,h]	0 (0·0%)	8 (36·4%)	0 (0·0%)	11 (50·0%)	19 (21·1%)
Hormonal birth control [b,i]	NA	NA	2 (8·3%)	6 (27·3%)	8 (17·4%)
BMI [b]					
Underweight = <18·5	2 (9·1%)	0 (0·0%)	1 (4·2%)	0 (0·0%)	3 (3·3%)
Normal weight = 18·5–24·9	10 (45·5%)	4 (18·2%)	12 (50·0%)	13 (59·1%)	39 (43·3%)
Overweight = 25–29·9	10 (45·5%)	5 (22·7%)	8 (33·3%)	7 (31·8%)	30 (33·3%)
Obesity = BMI of 30+	0 (0·0%)	13 (59·1%)	3 (12·5%)	2 (9·1%)	18 (20·0%)
Smoker [b, j]	3 (13·6%)	4 (18·2%)	6 (25·0%)	6 (27·3%)	19 (21·1%)

a based on F-test b based on Chi-square test

c Three subjects endorsed Hispanic ethnicity but did not select a racial descriptor. Six additional subjects endorsed Hispanic ethnicity, in addition to a racial category of Caucasian or African-American race yielding a total of 9 subjects self-identifying as Hispanic in this sample. Comparison of Hispanic ethnicity, p=·063.

d Control subjects had CAPS scores of zero or had an absence of criterion A events. Comparison of male and female PTSD subjects on current CAPS score, p=0·54.

e PTSD group by gender interaction on current PCL score, p<.05. *:Comparison of PTSD groups vs controls, p<.001. Comparison of male vs female PTSD groups vs controls, p<0·01.

f Absence of current MDD was required for inclusion into the control group. Comparison of male and female PTSD subjects on rate of current MDD, p=0·43.

g Childhood trauma exposure was defined, based on findings from our prior research, by exposure to 2 or more categories of childhood trauma under the age of 14. Three (6·5%) control subjects reported a history of 1 category of childhood trauma. h Chi-square test compared frequency of childhood trauma between male and female PTSD subjects only, p=·36.

i Chi-square test compared use of hormonal birth control female PTSD and control subjects only, p=·09

j based on diary

Table 2. Sex steroid metabolite concentrations in plasma of men and women.

Metabolite	LOD (ng/mL)	Control □ SD (ng/mL) Women	PTSD □ SD (ng/mL) Women	Control □ SD (ng/mL) Men	PTSD □ SD (ng/mL) Men
Progesterone (P4)	0.393	0·59 □ 1·19	<LOD (<0·28)	<LOD (<0·09)	<LOD (<0·09)
Testosterone*	0.144	0·55 □ 0·90	0·44 □ 0·68	4·03 □ 1·39	4·17 □ 1·61
Estrone (E1)	2.702	<LOD (<0·11)	<LOD (<0·24)	<LOD (<0·16)	<LOD (<0·11)
Estradiol (E2)	0.680	<LOD (<0·03)	<LOD (<0·06)	<LOD (<0·02)	<LOD (<0·03)
Estriol (E3)	2.882	5·7 □ 8·93	3·23 □ 2·26	3·39 □ 3·96	4·68 □ 9·69

Steroid metabolite values plasma in ng/mL. Limit of detection (LOD) for each analyte is shown. Values in red text were below LOD. *: p<0·0001 Men vs. Women (sex); PTSD: ns; PTSD x Sex: ns

Table 3. Altered primary metabolite clusters in PTSD with sleep confounders

Men PTSD	Men Delta Power	Men PSQI	Men TST	Women PTSD	Women Delta Power	Women PSQI	Women TST
Amino Acids (AA)							
.	.	Gly	.	Glycine (Gly)	Gly	.	Gly
.	.	.	.	Threonine (Thr)	Thr	.	Thr
.	.	.	.	Serine (Ser)	Ser	.	Ser
.	.	.	.	.	Citrulline	.	.
.	.	.	.	.	□-Ala	□-Alanine	□-Ala
Branched-chain amino acids (BCAA)							
.	.	.	.	Valine (Val)	Val	Val	Val
.	.	.	.	Leucine (Leu)	.	Leu	Leu
Sulfur amino acids							
.	.	.	Met	Methionine (Met)	Met	Met	.
.	.	.	Cys	Cysteine (Cys)	Cys	Cyst	.
Cyclic amino acids							
Histidine (His)	His	.	.	.	.	.	.
.	.	.	4-Hyp	Trans-4-hydroxyproline (4-Hyp)	4-Hyp	4-Hyp	.
Indoles (Tryptophan metabolites)							
.	.	.	.	Indole-3-propionic acid (IPA)	.	.	IPA
.	.	.	.	.	.	Indole-3-lactate (ILA)	.

.	.	.	.	.	.	Indole-3-acetate (IAA)	.
Butyrates (Gut microbes)							
.	.	Threonic acid	.	.	.	.	.
.	.	3-Hydroxybutyric acid	.	.	.	.	.
.	.	2-Hydroxybutanoic acid	.	.	.	.	.
.	.	.	.	Isothreonic acid (ITA)	ITA	.	ITA
.	.	.	.	.	.	2-Aminobutyric acid (2AB)	.
Hexose							
Fructose	.	.	.	.	.	.	.
.	Sucrose	.	.	.	.	.	.
.	.	.	.	1-Methylgalactose NIST	.	.	1-MG NIST
Unsaturated fatty acids (UFA)							
.	.	.	.	Palmitoleic acid	.	.	.

Figure Legends

Figure 1. Sex differences in primary metabolites in PTSD. (**A**) ChemRich analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI and age. Blue nodes contain metabolite clusters that were decreased, purple nodes contain metabolites that were both increased or decreased, and red nodes contain metabolites that were increased in PTSD vs. control individuals. (**B**) Venn diagram showing seven nodes were specific to women with PTSD and two nodes

significant.

were specific to men with PTSD compared with controls. (**C**) Volcano plots of specific metabolites within each node in men and women with PTSD after correcting for type 1 error and adjusting for BMI and age.

Figure 2. Alterations in specific amino acids within a metabolite cluster/node with PTSD symptoms. (**A-G**) Box plots of specific amino acids with the seven nodes that differed between women and two nodes in men with PTSD compared to controls shown in Fig. 1A. Glycine levels associated negatively with PCL cluster D in women with PTSD ($r = –0{\cdot}47$, $p = 0{\cdot}036$), but not controls, whereas association of serine level was lost in women with PTSD. (**H**) Fructose levels increased in men with PTSD vs. controls, but fructose levels associated negatively with PCL scores. Box plot analysis: Mann-Whitney and $p<0{\cdot}05$ considered

Figure 3. PTSD-specific alterations in amino acids with respect to their biosynthesis pathway in humans from glucose. Essential amino acids cannot be synthesized and must be obtained from diet, whereas non-essential amino acids can be synthesized from glucose as it enters the tricarboxylic cycle. Essential amino acids are shown in grey boxes, and those metabolites synthesized as a by-product of gut microbiome are shown in green boxes. Specific amino acids that were increased are shown in red, decreased in blue (women-specific outlined in pink and men-specific in blue).

Figure 4. Sex-specific disturbances in sleep measures. Box plots showing sex- and/or PTSD-specific alterations in (**A**) Total sleep time (in minutes) decreased; Two-way ANOVA: Sex: ns; PTSD: $p= 0{\cdot}004$; Sex X PTSD: ns. (**B**) sleep quality worsened, Two-way ANOVA: Sex: ns; PTSD: $p< 0{\cdot}0001$; Sex X PTSD: ns. and (**C**) log-transformed delta power sleep was lower in people with PTSD vs controls, Two-way ANOVA: Sex: $p= 0{\cdot}007$; PTSD: $p= 0{\cdot}005$; Sex X PTSD: ns. (**D**) Linear regression showing association of PCL scores with three different measures of sleep. individuals.

Figure 5. Sex differences in primary metabolites after accounting for sleep measures . ChemRich analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI, age, and one of the three sleep measures shown (TST (min), PSQI, or log-transformed delta power (ln(μV2)). Blue nodes contain metabolite clusters that are decreased, purple nodes contain metabolites that are both increased or decreased, and red nodes contain metabolites that are increased in PTSD vs control

Figure 6. Sex-specific contribution of sleep variables on primary metabolites. Venn diagrams to visualize significant metabolites whilst adjusting for one sleep variable at a time.

Figure 7. Linear regression and box plots of various amino acids with sleep variables (**A-F**) Box plots of specific amino acids with individual nodes that differed between women and men with PTSD compared to controls shown in Fig. 5 and 6. (**G**) Log-transformed delta power (ln(μV2) associated negatively with testosterone in men.

Figure 8. Changes in insulin and tryptophan levels in women and men with PTSD. Box plots of tryptophan and albumin levels in women and men. (**A**) No significant differences were seen in tryptophan and albumin levels in women with PTSD compared with controls, whereas tryptophan levels were significantly elevated in men with PTSD compared with controls (p = 0·044; Mann-Whitney). (**B**) Blood insulin levels were determined after oral glucose challenge at various times shown. Mixed-effect analysis showed that insulin levels changed with time (p<0·0001) in women and men, but only differed between PTSD and controls in men (p=0·0136). (**C**) Tryptophan levels correlated negatively with insulin levels in women, but positively in men.

Figure 9. Sex-specific alterations in tryptophan metabolism pathway in PTSD. (**A**) Sex, sleep, and PTSD all alter primary metabolites. (**B**) Albumin-bound tryptophan is present in circulation and dynamic

increases in insulin promote binding of albumin to tryptophan, whereas esterified fatty acids can displace tryptophan from albumin. Free tryptophan is then transported to the brain by a transport carrier. Several amino acids, such as leucine, valine etc. compete with tryptophan for binding to the transport carrier, which can decrease influx of free tryptophan into the brain. Reduced free tryptophan levels in the brain can influence production of serotonin and melatonin, affecting brain function and sleep. In the gut, tryptophan is converted to indoles by the action of microbes such as*Lactobacillus*; these indoles have protective effect on gut barrier and immune functions. Serine can serve as NMDA receptor agonist and alter neuronal function. Thus, disturbances at multiple levels in tryptophan pathway may contribute to pathogenesis of PTSD.

Supplementary Figure Legend

Fig. S1. Sex aggregated analysis of primary metabolites. There were seven primary metabolites that were significantly different between PTSD and control subjects when data were analyzed in a sex aggregated manner. Eight metabolites that have not been thoroughly characterized yet, were also identified. P values as shown using Mann-Whitney U-test.

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Abstract:	Background Primary metabolites serve as substrates for neurotransmitters and are altered in psychiatric disorders. Sleep disturbances are exacerbated in posttraumatic stress disorder (PTSD), but the contribution of sex, sleep and/or PTSD in altering primary metabolites is not known. Methods We used mass spectrometry to ascertain primary metabolites in 90 plasma samples from individuals with chronic PTSD and control subjects. Laboratory-based polysomnography was used to monitor the sleep of participants. PTSD was determined using the Clinician-Administered PTSD Scale (CAPS). Results Men and women with PTSD showed distinct, non-overlapping primary metabolite nodes compared with sex-matched controls as ascertained using Chemical Set Enrichment Analysis (ChemRICH) analysis. Women with PTSD had seven nodes, whereas men with PTSD had just two nodes altered compared with controls; each node contained two or more metabolites. Sex steroids levels did not associate with metabolite nodes. Higher PTSD symptoms were associated with lower total sleep time and decreased Pittsburgh Sleep Quality Index (PSQI) scores in both men and women. Delta power on sleep electroencephalogram was significantly lower in men with PTSD and associated negatively with PTSD symptoms. Sleep measures accounted for nearly 50% of the altered primary metabolite nodes. Tryptophan and insulin levels were significantly

	increased in men but not women with PTSD compared with controls, whereas tryptophan levels associated inversely with insulin levels in

	women. Conclusions Women demonstrate more metabolic disturbances than men with similar PTSD scores. The presence of sex-specific primary metabolite in PTSD were not affected by sex steroids but were accounted for by sleep measures. Our data provide further evidence for development of sexspecific interventions and disease management.

Sex-specific alterations in primary metabolites and tryptophan pathways in posttraumatic stress and disturbed sleep

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Abstract

Background

Primary metabolites serve as substrates for neurotransmitters and are altered in psychiatric disorders. Sleep disturbances are exacerbated in posttraumatic stress disorder (PTSD), but the contribution of sex, sleep and/or PTSD in altering primary metabolites is not known.

Methods

We used mass spectrometry to ascertain primary metabolites in 90 plasma samples from individuals with chronic PTSD and control subjects. Laboratory-based polysomnography was used to monitor the sleep of participants. PTSD was determined using the Clinician-Administered PTSD Scale (CAPS).

Results

Men and women with PTSD showed distinct, non-overlapping primary metabolite nodes compared with sex-matched controls as ascertained using Chemical Set Enrichment Analysis (ChemRICH) analysis. Women with PTSD had seven nodes, whereas men with PTSD had just two nodes altered compared with controls; each node contained two or more metabolites. Sex steroids levels did not associate with metabolite nodes. Higher PTSD symptoms were associated with lower total sleep time and decreased Pittsburgh Sleep Quality Index (PSQI) scores in both men and women. Delta power on sleep electroencephalogram was significantly lower in men with PTSD and associated negatively with PTSD symptoms. Sleep measures accounted for nearly 50% of the altered primary metabolite nodes. Tryptophan and insulin levels were significantly increased in men but not women with PTSD compared with controls, whereas tryptophan levels associated inversely with insulin levels in women.

Conclusions

Women demonstrate more metabolic disturbances than men with similar PTSD scores. The presence of sex-specific primary metabolite in PTSD were not affected by sex steroids but were accounted for by sleep measures. Our data provide further evidence for development of sex-specific interventions and disease management.

Introduction

Posttraumatic stress disorder (PTSD) is a common psychiatric condition. PTSD may develop after exposure to an actual or threatened death, serious injury, trauma, or sexual violence. PTSD is currently characterized on diagnostic and statistical manual of mental disorders (DSM-5) by: (i) reexperiencing (e.g., intrusive thoughts, nightmares, flashbacks); (ii) avoidance; (iii) negative changes in cognition and mood (hopelessness, lack of emotions), and (iv) hyperarousal (trouble sleeping, self-destructive behavior, angry outbursts). While it is widely recognized that PTSD is a common consequence of trauma exposure, women are at particularly high risk, with some studies finding that women develop PTSD at twice the rate of men, despite greater trauma exposure in men.[1, 2]

One of the most common complaints among individuals with PTSD is sleep disturbance. PTSD is associated with lower slow wave sleep duration and delta power sleep, which is more pronounced in men than women. In contrast, greater rapid eye movement sleep is found in women with PTSD compared to healthy controls, a difference not seen in men.[3] Sleep disturbance is a key risk factor for health consequences as it alters hypothalamic-pituitary-adrenal (HPA) axis function, resulting in impaired glucose and lipid metabolism. The HPA and somatotropic axes activities are temporally associated with delta power sleep and promote insulin sensitivity and metabolic syndrome. Additionally, sleep duration correlates with metabolic risk in PTSD but does not fully account for the association between PTSD with known metabolic disturbances such as in blood insulin or glucose levels.[4] While prior studies have found alterations in neuroendocrine, immune and aging processes in PTSD, the role of metabolite disturbances is PTSD is limited and can help elucidate new discovery of yet unknown biological mechanisms of disease.

More recent technological developments such as mass spectrometry allow for the discovery of novel pathways using an unbiased method to examine multiple analytes simultaneously. Metabolomics is a global

and unbiased approach to understanding regulation of metabolic pathways and networks of physiologically relevant interactions. The metabolome is regulated by gene-environment interactions and reflects the intermediary state between genotype and phenotype. Gene mutations, single nucleotide polymorphisms, and mutations in proteins are associated with PTSD, but none of these alone explains the complex manifestation of PTSD and comorbid health conditions. A multi-omics approach has been used to identify potential biomarkers that range from DNA methylation, proteins, miRNA, lipids, and other metabolites in warzone male veterans with PTSD.[5] Metabolomic profiling has also led to identification of key differences in glycolysis and fatty acid pathways that were associated with mitochondrial dysfunction in men with PTSD. [6]

Essential and non-essential amino acids (AAs) as well as biogenic amines are building blocks of proteins and peptide hormones involved in a plethora of functions that include membrane stabilization, neurotransmission, and neuroimmune modulation. AAs act as neurotransmitters (e.g. glutamate, glycine, serine). Kynurenine (KYN), a metabolite of AA tryptophan, is correlated with changes in hippocampal and amygdalar volumes in depressed patients.[7] KYN is associated with stress exposure, neuronal cell death, glutamate transmission, and neuroinflammation. Alterations in amino acid metabolites that are more bloodbrain barrier permeable, such as KYN, may account for some of these differences. We are not aware of any study that has systematically ascertained sex differences in the status of primary metabolites in serum samples of participants with PTSD and matched-controls. In this study, we aimed to examine alterations of primary amino acids and metabolites in PTSD and the contribution of sleep measures in both men and women.

Methods

Human subjects

In this study, we used a cross-sectional, 2 × 2 design (PTSD/control × women/men) involving 90 medically healthy, non-medicated adults aged 19-39 years in an inpatient sleep laboratory at the General Clinical Research Center (GCRC) at the University of California, San Francisco. The study sample was comprised of 44 individuals with current chronic PTSD (50% women) and 46 control subjects (52% women). This sample was drawn from a larger study of 94 participants. Data from 4 participants were excluded due to difficulties in blood collection. This research was approved by the Committee on Human Research at the University of California, San Francisco. All participants provided written informed consent before participating in any study procedures.

Psychiatric diagnoses and trauma history

The type of trauma exposure and age of occurrence was assessed using the Life Stressor Checklist- Revised interview. The PTSD Checklist (civilian version) for DSM-IV (PCL-C) [9] was used to assess severity of chronic PTSD symptoms and on the Clinician-Administered PTSD Scale (CAPS) and a CAPS score >40. This self-report measure consists of 17 items that correspond to the DSM-IV criteria and include intrusive thoughts and re-experiencing symptoms (cluster B), avoidance (cluster C), and hyperarousal (cluster D). The structured clinical interview for DSM-IV, non-patient edition (SCID-NP) was used to diagnose all other psychiatric disorders, including major depressive disorder.[11]

Control subjects had no lifetime or current history of a PTSD diagnosis. Women participants were premenopausal, and were scheduled during the follicular phase of the menstrual cycle. All study procedures were timed according to habitual sleep onset, determined by actigraphy and sleep diary in the week prior to the GCRC study. Study participants were alcohol and drug-free, limited to one cup of caffeine daily, and maintained regular bed and waking times. Exclusion criteria included a history of traumatic brain injury, presence of neurologic disorders or systemic illness; use of psychiatric, anticonvulsant, antihypertensive, sympathomimetic, steroidal, statin or other prescription medications; obesity (defined as body mass index (BMI) >30); alcohol abuse or dependence in the prior two years; substance abuse or dependence in the

previous year; any psychiatric disorder with psychotic features; bipolar disorder or obsessive-compulsive disorder; and pregnancy. Exclusion criteria for control subjects also included a lifetime history of major depressive disorder or panic disorder.

Sleep clinic and measures

The Pittsburgh Sleep Quality Index[12] was used to provide a subjective assessment of sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbances (including nightmares), use of sleep medication,

and daytime dysfunction over the previous month. Participants rated items on a four-point Likert scale within each domain with higher scores indicating poorer sleep. A total score of > 5 is indicative of poor

Polysomnography recordings were obtained with ambulatory polysomnography (Nihon Kohden Trackit Ambulatory Recording System). The parameters recorded included an electroencephalogram (EEG) at leads C3, C4, O1 and O2, left and right electrooculograms (EOG), submental electromyogram, bilateral anterior tibialis EMGs, and electrocardiogram in accordance with standardized guidelines by Rechtschaffen. Electrode impedance was set at < 5 kohm at the start of the recording. The EEG and EOG leads were referenced to linked mastoids. Raw EEG signals were filtered and amplified, then digitized at sleep quality.

Polysomnographic measurements

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256 Hz and recorded to a removable hard disk in European Data Format file format. The low frequency and high frequency hardware filters on the recorder were single pole analog filters with 3 db points at 0·5 Hz and 100 Hz. Pass Plus was utilized for both visual scoring and quantitative EEG analysis of the digitized polysomnography data.

Power spectral analysis for polysomnographic measures

Pass Plus (Delta Software) analytic software was used to measure sleep activity in all frequency bands delta through gamma from the C3 electrode by power spectral analysis. The C4 electrode was used if there was

excessive artifact. A limitation of Pass Plus is that artifact removal is accomplished by removal of whole epochs tagged with artifact. This has the potential to introduce additional confounds given the removal of typically longer bouts of uncontaminated EEG. Therefore, epochs were tagged for slow and fast artifact for additional analyses. Primary analyses were conducted with all epochs and then checked for the impact of removal of epochs with slow and fast artifact. Removal of fast artifact (for bandwidths alpha and above) and slow artifact (for bandwidths delta and theta) did not significantly impact our findings in non-rapid eye movement (NREM) sleep. All results are therefore reported without removal of epochs containing artifact. Pass Plus applied a 5μV smoothing constant to eliminate spurious waves caused by electrical jitter. Power spectral analysis was conducted on all epochs of NREM and REM sleep. Epochs visually scored as wake were not included in these analyses. Visual scoring was conducted by a highly experienced registered polysomnography technician, blind to PTSD status, classified all 30-second epochs in every sleep record as wake; stages 1, 2, 3; REM; or movement using current American Academy of Sleep Medicine criteria. Total sleep time was defined by time spent in epochs scored as NREM stages 1 through 3 and stage REM. Pass Plus was used to perform Fast Fourier Transformation analysis on 4·0 second Welch tapered windows with 2 second overlap, yielding 15 windows per 30-second epoch. Power spectra for delta (1-4 Hz) were analyzed to address our hypothesis with respect to delta spectral power. Delta sleep spectral power density (μV2) was natural log transformed to normalize its distributions.

Blood collection and clinical laboratory measures

Blood was collected at habitual wake-up time on the morning after the second night on the GCRC, while the subject was fasting, from an indwelling catheter inserted the night before. Blood (10mL) was drawn into a chilled EDTA purple-topped tube and processed for plasma separation for mass spectrometry analysis.

Oral glucose tolerance test (OGTT)

Oral Glucose Tolerance Test (OGTT) was performed on the morning of day two after a 10-hour overnight fast. Subjects were given 75g dextrose in 250 cc of water, and blood samples were taken at baseline and 30, 60 and 120 minutes for measurement of glucose and insulin levels. Glucose was measured by the hexokinase method (Roche Applied Sciences, Indianapolis, IN) on the Cobas C501, at Washington University Core Laboratory. Insulin measurement was performed by EMD Millipore Corporation (St. Charles, MO, USA) by radioimmunoassay.

Analysis of primary metabolites in human plasma using gas chromatography /time of flight mass spectrometry (GC-TOF MS)

Primary metabolites that included sugar phosphates, amino acids, hydroxyl acids, and free fatty acids, were measured using GC-TOF MS. Briefly, metabolites from plasma were extracted in anti-oxidant solution (0·2 mg/ml Butylated hydroxytoluene /EDTA solution in 1:1 methanol:water) by homogenizing in GenoGrinder 2x30 seconds, centrifuged, followed by washes in appropriate solvents,[14] and lyophilized. Samples were reconstituted for GC-MS in 100µL of 1µM 1-Phenyl 3-Hexadecanoic Acid Urea/ 1-cyclohexyluriedo-3dodecanoic acid in methanol/acetonitrile (50:50) solvent, mixed, then sonicated for 5 min, followed by centrifugation for 5 min in spin filters, and supernatant transferred to glass inserts in HPLC vial. A Leco Pegasus IV mass spectrometer was used with unit mass resolution at 17 spectra s-1 from 80-500 Da at -70 eV ionization energy and 1800 V detector voltage with a 230°C transfer line and a 250°C ion source. Use of automatic liner exchanges after each set of 10 injections ensured reduce sample carryover for highly lipophilic compounds such as free fatty acids. This chromatography method yields excellent retention and separation of primary metabolite classes (amino acids, hydroxyl acids, carbohydrates, sugar acids, sterols, aromatics, nucleosides, amines and miscellaneous compounds) with narrow peak widths of 2–3 s and very

good within-series retention time reproducibility of better than 0·2 s absolute deviation of retention times.

Data acquisition, extraction, and processing of metabolites

Data were acquired using the following chromatographic parameters[15]: Column: Restek corporation Rtx5Sil MS (30 m length x 0.25 mm internal diameter with 0·25µm film made of 95% dimethyl/5%diphenylpolysiloxane); Mobile phase: Helium; Column temperature: 50-330°C Flow-rate: 1 mL min-1; Injection volume: 0·5µL; Injection: 25 splitless time into a multi-baffled glass liner Injection temperature: 50°C ramped to 250°C by 12°C s-1; Oven temperature program: 50°C for 1 min, then ramped at 20°C min-1 to 330°C, held constant for 5 min. Data processing. Raw data files were preprocessed directly after data acquisition and stored as ChromaTOF-specific *.peg files, as generic *.txt result files and additionally as generic ANDI MS *.cdf files. ChromaTOF vs. 4·0 was used for data preprocessing without smoothing, 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram. Apex masses are reported for use in the BinBase algorithm. Result *.txt files were exported to a data server with absolute spectra intensities and further processed by a filtering algorithm implemented in the metabolomics BinBase database. Given compound was identified and cross-referenced with external database identifiers such as InChI key, PubChem ID, and KEGG ID. Following equation was then used for normalizations for metabolite *i* of sample *j*:

$$metabilite\ ij\ normalized = \frac{metaboliteij\ raw}{mTICj}.mTICaverage$$

The normalized data are shown as peak heights for the quantification ion (mz value) at the specific retention index. Missing values were imputed using half of the minimum detected value for each compound. Autoscaling was performed to make compounds the same scale.[16]

Statistical analysis

We first conducted robust linear regression for each sex (i.e. male and female, respectively) to examine the association between compound intensity, PTSD status, sleeping factors, and to examine the interaction effect between sex and PTSD status for each metabolite. Specifically, robust linear regression was conducted with compound intensity as response and PTSD status as predictor, controlling for body mass

index and age. The effect size was determined by the coefficient of the regression model, with a positive value indicating compound intensity being higher in PTSD compared with control and a negative value indicating the opposite. We used the Benjamini Hochberg procedure to correct for multiple comparisons. The p-values and effect sizes from robust linear regression were used as input for the ChemRICH analysis[17] to visualize the significance and effect sizes of each metabolite in each of the significant clusters for each sex after correcting for type 1 errors. We also performed the same analysis but with each of the three sleeping factors (total sleep time (min), PSQI, and log-transformed delta energy (μV2sec)) as confounding variables. We used box plot and Venn diagrams to visualize the significance effect while adjusting for sleep variables. We used the statistical computing language R version 3.6.3. Robust linear regression was

Demographic Data and Clinical Characteristics

By design, there were no significant differences in sex distribution between PTSD and control subjects, nor to study the enrichment effect on metabolite clustered defined according to chemical similarity.

Kolmogorov–Smirnov test was used to calculate the p-value for each of the metabolite clusters. We further used Venn-diagrams to highlight the sex difference from the ChemRICH analyses. Volcano plots were used performed in MASS library. **Results**

were there significant differences in age, education, or race/ethnicity across all four groups. Sample characteristics are presented in Table 1. Men with PTSD were more likely to be obese and male controls were less likely to be obese. Therefore, we adjusted for BMI in all our analyses. Men and women with PTSD did not differ in terms of CAPS scores, rates of current MDD, or history of childhood trauma (defined by the presence of two or more categories of childhood trauma as compared to one or none). Eleven control subjects reported a lifetime history of a traumatic criterion A1 event, but all had current CAPS scores of zero and none had a lifetime history of PTSD. However, the PTSD groups scored higher on the PCL-C (avoidance) than controls; women with PTSD had higher PCL-C scores than men with PTSD (Table 1).

As per the exclusion criteria, no control subjects met criteria for current MDD. Additionally, none of the control subjects reported a history of two or more categories of childhood trauma. There were no differences between PTSD and control women in use of hormonal birth control or group differences in smoking of tobacco.

Chemical Set Enrichment Analysis (ChemRICH) identified sex differences in primary and steroid metabolites

We performed ChemRICH analysis on primary metabolites to identify chemical classes that were significantly altered in PTSD compared to controls. ChemRICH utilizes structure similarity and chemical ontologies to map all known metabolites and name metabolic modules.[17] This is a statistical enrichment

approach based on chemical similarity and alternative to pathway analysis that relies on limited biochemical knowledge annotations.[17] It yields study-specific, non-overlapping sets of all identified metabolites. Since ChemRICH sets have a self-contained size, thus p-values do not rely on the size of the background database. ChemRICH analysis identified seven primary metabolite nodes altered in women with PTSD compared with control women (Fig. 1A) after adjusting for age and BMI as confounding factors. Each node reflects a significantly altered cluster of metabolites and the node size represents the total number metabolites within a cluster set. Branched-chain and sulfur-containing amino acids, and unsaturated fatty acids were increased, indoles and cyclic amino acids were decreased, whereas the non-polar amino acid node contained metabolites that either increased or decreased in women with PTSD compared to controls. In contrast, men with PTSD had only two altered nodes compared with controls (Fig. 1A-C). Surprisingly, men and women with PTSD did not share any overlapping primary metabolite clusters (Fig. 1B). Further analysis of the metabolites within each node revealed presence of several metabolites that remained statistically significant after adjusting for false discovery rate and effect size (Fig. 1C). Surprisingly, sex steroid metabolites (estrogens, testosterone, progesterone or their metabolites) did not differ between controls and PTSD in either men or women (Table 2), nor did sex steroids associate with PTSD. Progesterone was below the level

of detection in men and women with PTSD, whereas estrone and estradiol were also below LOD in both men and women. Testosterone, but not estriol levels differed between men and women, with levels nearly ~10-fold higher in men (Table 2).

Essential, non-essential amino acids, and butyrates were altered in women with PTSD

Since PTSD symptom presentation is highly variable between individuals,[18] and in our cohort, women had significantly greater PCL-C scores than men, we reasoned that individual PCL measures may associate differently with individual metabolites. To better understand which of the PTSD symptom clusters contributed to alterations in specific metabolites after adjusting for type 1 errors, linear regression analysis

for each PCL symptom cluster with significant individual metabolites was performed (Fig. 2A-H and Table 3). Serine, a neurotransmitter and precursor for the synthesis of glycine and cysteine as well as for 2aminobutyric acid, a butyrate (2-AB), is a byproduct of cysteine biosynthesis pathway, which are all in the glucose metabolism pathway (Fig. 3). While serine levels trended to be lower in women with PTSD compared to controls, no significant relationship with PTSD measures were found (Fig. 2A). Glycine levels were negatively associated with cluster D symptoms on the PCL, suggesting that with more hyperarousal, glycine levels were decreased in women, but not men. Cysteine and 2-AB levels were elevated in PTSD compared with controls (Fig. 2C and 2F) but did not show any significant association with specific clusters of PTSD symptoms.

Hexose metabolites were altered in men, but not women with PTSD

Alterations in glucose metabolism are decreased within specific brain regions of people with schizophrenia and mood disorders as ascertained with functional imaging.[19, 20] Sucrose is metabolized to glucose and fructose, and fructose can only be metabolized in the liver via the tricarboxylic acid cycle. We found that whilst overall levels of fructose were increased in men with PTSD compared with controls (Fig. 2H), its

levels were negatively correlated with both B and C symptom clusters, suggesting that more reexperiencing and avoidance symptoms contribute to altered hexose metabolism in men, but not women.

Sleep quality was worse in PTSD in both sexes

Sleep disturbances and quality are associated with overall poor health.[3] We found that both men and women decreased in PTSD and showed a significant sex difference with men having lower delta power sleep activity than women (Fig. 4C). Specifically, greater PTSD symptoms (as reflected by total PCL score) was associated with lower TST in both women and men (r= –0·41, p=0·005 and r= –0·32, p=0·033, respectively, Fig. 4D), and poor sleep quality (PSQI) was highly associated with greater PTSD symptoms in both women and men (r=0·84 and r=0·74, p<0·001, respectively, Fig. 4E). In contrast, delta power was lower in men with PTSD (*r*= –0·35, *p*=0·02) compared to controls, but there were no differences in PTSD status on delta

Decreased levels of essential amino acids in both sexes and indoles in women associated with sleep

Humans lack the ability to synthesize eight essential amino acids which must be obtained from diet and are with PTSD had lower total sleep time (TST), and worse self-reported sleep quality as assessed by the PSQI compared with controls in (Fig. 4A-B). Delta power, a measure of deep sleep activity, was also significantly activity in women (Fig. 4F).

parameters and PTSD symptoms

mostly absorbed by the gut and metabolized by the resident microbiota. For example, tryptophan is metabolized to a myriad of biologically active compounds by four different pathways (serotonin, tryptamines, kynurenine, indoles, and NAD+). When we accounted for TST, two of the six metabolite nodes

that included cyclic amino acids and unsaturated fatty acids, were no longer significant in women with PTSD, whereas no node remained significant in men with PTSD (Fig. 5 and Fig. 6) and Table 3. In contrast, when PSQI was used as a confounder, new, albeit non-overlapping nodes were found to be significant in women and men; indoles, hexose and amino acid nodes were decreased in women, whereas the butyrate node was found to harbor significantly increased as well as decreased metabolites in men with PTSD (Fig.

5). Alterations in delta power sleep accounted for 50% of the nodes in women (Table 3), whereas delta power was not associated with alterations in any nodes in men with PTSD (Fig. 5 vs Fig. 1A). Further analysis revealed that decreased sleep quality and delta power contributed to a decrease in levels of several essential amino acid as well as metabolites in the glucose metabolism pathway for which they serve as substrates (Fig. 7). Interestingly, we found a nonsignificant trend with higher testosterone levels associated with lower delta power in men (Fig. 7G, r= –0·29, p=0·06).

Plasma insulin levels associated negatively with tryptophan levels in women, but not men

While tryptophan levels were not associated with either sex, PTSD or sleep at the node level, when examined as sex segregated, tryptophan was significantly increased in men with PTSD compared to controls, but not in women (Fig. 8A). As influx of free tryptophan into the brain by its transport carrier is regulated by several factors including levels of plasma albumin and insulin,[21] we next examined the levels of plasma albumin and insulin levels 30, 60, and 120 min after an oral glucose challenge. While plasma albumin levels did not differ between controls and individuals with PTSD in either sex, insulin levels were significantly elevated in men with PTSD compared with controls (Fig. 8B). Interestingly, tryptophan levels associated negatively with insulin levels at 60 min in women (r= –0·30, p=0·04), whereas insulin levels exhibited a positive association with free tryptophan levels in men, although the relationship did not reach statistical significance (Fig. 8C).

Discussion

To our knowledge, this is the first systematic study to report sex-specific alterations in primary metabolites in men and women with PTSD with several novel findings. First, both sex and sleep measures altered distinct amino acids in individuals with PTSD compared with controls. Second, levels of sex steroids such as estriol, progesterone, and testosterone did not differ between controls and PTSD. Third, sex steroids did not associate with PTSD or primary metabolites in our cohort. Fourth, delta power and perceived sleep quality account for nearly half the changes in metabolite clusters in women with PTSD compared with

control women (Fig. 9A). In women, perceived sleep quality was associated with increases in 2hydroxybutonic acid, a butyrate and decrease in indoles, generated by the gut microbes. Finally, there was no overlap in PTSD-related alterations in men and women in any primary metabolite nodes or individual amino acids within those nodes, and associated pathways.

An individual's physiological and metabolic state shifts glucose metabolism and generation of non-essential amino acids. Serine and glycine, both non-essential amino acids are directly synthesized from glucose in several cells including the glia, astrocytes, and hippocampal neurons. Both serine and glycine shuttle between the glia and neurons where glycine induces release of serine, which is a coagonist for *N*-methylD-aspartate (NMDA) receptors.[22] Enzymatic removal of extracellular L-serine also impairs long-term potentiation in hippocampal neurons and the visual cortex.[22, 23] NMDA receptor function in the amygdala and prefrontal cortex have been found to be critical for the consolidation of extinction of previously conditioned fear memories.[24] Enhanced fear conditioning and a deficit in the extinction of conditioned fear that are reliant on NMDA activity have been proposed to underlie the development and maintenance of PTSD.[25, 26]

We find that both serine and glycine levels were decreased in women with PTSD, and sleep measures such as delta power and total sleep time account for changes in these two amino acids in women (Table 3). In

contrast, neither serine nor glycine levels were altered in men with PTSD and whereas sleep quality decreased glycine levels in men, after correcting for type 1 errors, its levels were no longer significant (Table 3). Other studies have shown that kynurenine pathway metabolites, kynurenic acid and quinolinic acid, associated with neuroprotective and neurotoxic functions, respectively, were altered in patients with major depressive disorders.[7] Like serine, quinolinic acid is also an NMDA receptor agonist (Fig. 9B), and increase in levels of serine may alter neuronal function in PTSD and other psychiatric disorders.

Valine, lysine, methionine, tryptophan, and histidine are all essential amino acids that cannot be synthesized by human cells. Tryptophan is absorbed in the gut and converted by the actions of gut microbiota such as *Lactobacillus* to indoles (Fig. 9B). While the role of serotonin in mood disorders, anxiety, and other disorders is well known, we show here for the first time that indole metabolite, indole-3-propionic acid, is decreased in women with PTSD. Poorer sleep quality was further associated with decreased levels of two additional indoles, indole-3-lactic and acetic acids; the indoles regulate immune and gut barrier functions (Fig. 9B), and their decreased levels might contribute to altered immune and barrier function in women. In agreement with these data, altered immune and gut barrier function is seen in patients with other psychiatric disorders.[27, 28] Diets rich in butyrates that support growth of beneficial gut bacteria such as *Lactobacillus* may serve as non-invasive interventions, especially for women.

Increased blood insulin levels affect transport of free tryptophan to the brain in two ways: first, insulin enhances binding of tryptophan to the albumin, thereby decreasing levels of free tryptophan in the circulation by half, and decreasing influx into the brain.[21] Second, simultaneous reduction in levels of six or more amino acids such as leucine, serine, cysteine, histidine, methionine, valine, which would otherwise compete with tryptophan for binding to the transport carrier into the brain to increase its influx.[21] In our cohort, we did not find any difference in insulin levels in women with PTSD and controls. However, we

did find an increase in levels of several amino acids in women with PTSD that are known to compete with tryptophan carrier for influx into the brain (Fig. 9B and Table 3). This could result in decrease in substrate for conversion to serotonin and subsequently to melatonin, thereby affecting sleep. Although sex differences in insulin resistance and glucose clearance are reported in people with diabetes[29] as well as in animal models of diabetes,[30] we did not find any sex differences in insulin levels after an oral glucose challenge in our PTSD cohort. Non-esterified fatty acids can displace albumin from tryptophan[31, 32] and this free tryptophan can be converted in to serotonin or degraded in cells. We found an increase in palmitoleic acid levels, a monounsaturated non-esterified fatty acid in women with PTSD, which can potentially displace albumin from tryptophan in order to generate free tryptophan. Concomitant increase in palmitoleic

acid levels may compensate for increases in amino acids that prevent influx of tryptophan into the brain and conversion to serotonin, more so in women than in men. In contrast, men with PTSD had significantly increased insulin levels and no increases in amino acid levels that would compete with transport of tryptophan to the brain; men also did not show changes in levels of palmitoleic acid levels that have beneficial function.

In the human myocardium, 2-aminobutyric acid, a metabolite of the serine to cysteine conversion pathway

(Fig. 3) is known to increase glutathione levels via AMPK activation to protect against oxidative stress. Thus, our data on differential levels of amino acids and their metabolites in women with PTSD suggests that alterations in subsets of metabolites is protective. Other studies have reported changes in metabolite [6, 34] but did not include women, nor did they investigate contribution of sleep. In agreement with our data, body mass index, and smoking did not explain differences

PTSD symptoms are myriad, vary from person-to-person, but typically include flashbacks and/or nightmares, emotional numbing, avoidance of thoughts that might remind them of traumatic events, and hyperarousal. Symptoms usually develop within a month in most cases, but some may experience delayed levels in male combat veterans with PTSD,

in metabolite levels. **Conclusions** onset. Nearly 30% of individuals who experience distressing events develop PTSD. While war trauma, sexual assault, or accidents are most associated with PTSD, nearly 25% of all ICU patients[35] and 32% of ICU survivors of pandemics, developed PTSD.[36] The psychological burden from the ongoing COVID-19 pandemic is yet to be fully determined. Sleep disorders are also high in patients that recovered from COVID-19-related ICU stays. Thus, while genetics predisposes one towards psychiatric disorders, other factors such as the environment, and individual's metabolic state together influence disease progression and outcomes; together these provide one explanation for nuanced and variable symptoms between individuals diagnosed with the same psychiatric disorders. Our study suggests that men and women recruit

different metabolic pathways and mechanisms along with several pathways that are shared between the sexes, to reach the same outcomes. Evidence of sex-specific metabolite clusters in PTSD suggest the need for more tailored interventions to address the unique needs that may differ between men and women exposed to trauma.

List of Abbreviations

AAs: amino acids
BMI: body mass index
ChemRICH: Chemical Set Enrichment Analysis
CAPS:Clinician-Administered PTSD Scale
COVID-19: Coronavirus disease-19
DSM: Diagnostic and Statistical Manual of Mental Disorders
EEG: electroencephalogram
EOG: electrooculograms
GCRC: General Clinical Research Center
GC-TOF-MS: gas chromatography /time of flight mass spectrometry (GC-TOF MS)
HPLC: High pressure liquid chromatography
HPA: hypothalamic-pituitary-adrenal
ICU: Intensive care unit
KYN: Kynurenine
NREM: non-rapid eye movement
NMDA: *N*-methyl-D-aspartate receptors
OGTT: Oral Glucose Tolerance Test
PTSD: posttraumatic stress disorder
PSQI: Pittsburgh Sleep Quality Index TST:
Total sleep time

Contributors

AB, TCN, and SSI contributed to the design of the study. AB wrote the first draft of the manuscript with input from SSI, and AB, TCN, and SSI revised the final manuscript. CL and SSI collected patient data, RR, AO, SF, OF, SSI, and AB extracted and analyzed the data.

Disclosures

All other authors declare no competing interests. The views, opinions and/or findings contained in this research are those of the authors and do not necessarily reflect the views of the Department of Defense, Department of Veteran Affairs, or NIH and should not be construed as an official DoD/Army/VA/NIH

position, policy, or decision unless so designated by official documentation. No official endorsement should be made.

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References

1. Breslau N, Kessler RC, Chilcoat HD, et al. Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma. Arch Gen Psychiatry 1998;55:626-32.
2. Tanielian T, Jaycox L. Invisible Wounds of War: Psychological and Cognitive Injuries, Their Consequences, and Services to Assist Recovery: RAND Center for Military Health Policy Research, RandCorporation, 2008.
3. Richards A, Metzler TJ, Ruoff LM, et al. Sex differences in objective measures of sleep in posttraumatic stress disorder and healthy control subjects. J Sleep Res 2013;22:679-87.
4. Rao MN, Chau A, Madden E, et al. Hyperinsulinemic response to oral glucose challenge in individuals with posttraumatic stress disorder. Psychoneuroendocrinology 2014;49:171-81.
5. Dean KR, Hammamieh R, Mellon SH, et al. Multi-omic biomarker identification and validation for diagnosing warzone-related post-traumatic stress disorder. Mol Psychiatry 2020;25:3337-3349.
6. Mellon SH, Bersani FS, Lindqvist D, et al. Metabolomic analysis of male combat veterans with post traumatic stress disorder. PLoS One 2019;14:e0213839.

7. Savitz J, Drevets WC, Smith CM, et al. Putative neuroprotective and neurotoxic kynurenine pathway metabolites are associated with hippocampal and amygdalar volumes in subjects with major depressive disorder. Neuropsychopharmacology 2015;40:463-71.
8. Wolfe J, Kimerling R, Brown PJ, et al. Psychometric review of the life stressor checklist-revised. Lutherville, MD: Sidran Press, 1996.
9. Blanchard EB, Jones-Alexander J, Buckley TC, et al. Psychometric properties of the PTSD Checklist (PCL). Behaviour Research & Therapy 1996;34.
10. Blake DD, Weathers FW, Nagy LM, et al. The development of a Clinician-Administered PTSD Scale. J Trauma Stress 1995;8:75-90.
11. Spitzer RL, Williams JB, Gibbon M, et al. The Structured Clinical Interview for DSM-III--R (SCID) : I. History, rationale, and description. Archives of General Psychiatry 1992;49:624-629.
12. Buysse DJ, Reynolds CF, 3rd, Monk TH, et al. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry Res 1989;28:193-213.
13. Kales A, Rechtschaffen A. A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects. : Washington, DC : United States Government Printing Office, 1968., 1968.
14. La Frano MR, Carmichael SL, Ma C, et al. Impact of post-collection freezing delay on the reliability of serum metabolomics in samples reflecting the California mid-term pregnancy biobank. Metabolomics 2018;14:151.
15. Fiehn O, Wohlgemuth G, Scholz M, et al. Quality control for plant metabolomics: reporting MSIcompliant studies. Plant J 2008;53:691-704.
16. Wanichthanarak K, Fan S, Grapov D, et al. Metabox: A Toolbox for Metabolomic Data Analysis, Interpretation and Integrative Exploration. PLoS One 2017;12:e0171046.
17. Barupal DK, Fiehn O. Chemical Similarity Enrichment Analysis (ChemRICH) as alternative to biochemical pathway mapping for metabolomic datasets. Sci Rep 2017;7:14567.
18. Galatzer-Levy IR, Bryant RA. 636,120 Ways to Have Posttraumatic Stress Disorder. Perspect Psychol Sci 2013;8:651-62.
19. Haznedar MM, Buchsbaum MS, Hazlett EA, et al. Cingulate gyrus volume and metabolism in the schizophrenia spectrum. Schizophr Res 2004;71:249-62.
20. Zuccoli GS, Saia-Cereda VM, Nascimento JM, et al. The Energy Metabolism Dysfunction in Psychiatric Disorders Postmortem Brains: Focus on Proteomic Evidence. Front Neurosci 2017;11:493.
21. Daniel PM, Love ER, Moorhouse SR, et al. The effect of insulin upon the influx of tryptophan into the brain of the rabbit. J Physiol 1981;312:551-62.
22. Neame S, Safory H, Radzishevsky I, et al. The NMDA receptor activation by d-serine and glycine is controlled by an astrocytic Phgdh-dependent serine shuttle. Proc Natl Acad Sci U S A 2019;116:20736-20742.
23. Meunier CN, Dallerac G, Le Roux N, et al. D-Serine and Glycine Differentially Control Neurotransmission during Visual Cortex Critical Period. PLoS One 2016;11:e0151233.
24. Davis M. NMDA receptors and fear extinction: implications for cognitive behavioral therapy. Dialogues Clin Neurosci;13:463-74.
25. Orr SP, Metzger LJ, Lasko NB, et al. De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. J Abnorm Psychol 2000;109:290-8.
26. Santini E, Muller RU, Quirk GJ. Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. J Neurosci 2001;21:9009-17.
27. Rieder R, Wisniewski PJ, Alderman BL, et al. Microbes and mental health: A review. Brain Behav Immun 2017;66:9-17.
28. Rogers GB, Keating DJ, Young RL, et al. From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. Mol Psychiatry 2016;21:738-48.

29. Kautzky-Willer A, Harreiter J, Pacini G. Sex and Gender Differences in Risk, Pathophysiology and Complications of Type 2 Diabetes Mellitus. Endocr Rev 2016;37:278-316.

30. Paruthiyil S, Hagiwara SI, Kundassery K, et al. Sexually dimorphic metabolic responses mediated by CRF2 receptor during nutritional stress in mice. Biol Sex Differ 2018;9:49.
31. Coppen A, Wood K. Tryptophan and depressive illness. Psychol Med 1978;8:49-57.
32. Smith SA, Pogson CI. The metabolism of L-tryptophan by isolated rat liver cells. Effect of albumin binding and amino acid competition on oxidatin of tryptophan by tryptophan 2,3-dioxygenase. Biochem J 1980;186:977-86.
33. Irino Y, Toh R, Nagao M, et al. 2-Aminobutyric acid modulates glutathione homeostasis in the myocardium. Sci Rep 2016;6:36749.
34. Somvanshi PR, Mellon SH, Flory JD, et al. Mechanistic inferences on metabolic dysfunction in posttraumatic stress disorder from an integrated model and multiomic analysis: role of glucocorticoid receptor sensitivity. Am J Physiol Endocrinol Metab 2019;317:E879-E898.
35. Burki TK. Post-traumatic stress in the intensive care unit. Lancet Respir Med 2019;7:843-844.
36. Rogers JP, Chesney E, Oliver D, et al. Psychiatric and neuropsychiatric presentations associated with severe coronavirus infections: a systematic review and meta-analysis with comparison to the COVID-19 pandemic. Lancet Psychiatry 2020;7:611-627.

Table 1. Demographics data and clinical characteristics of PTSD and control subjects.

	Men		Women		
Variable	Control (N=22)	PTSD+ (N=22)	Control (N=24)	PTSD+ (N=22)	Total (N=90)
Age (Mean ± SD) [a]	30·2 ± 8·76	30·6 ± 7·61	30·0 ± 7·47	30·2 ± 6·82	30·3 ± 7·31
Education (Years) (Mean ± SD) [a]	15·5 ± 2·08	14·4 ± 2·32	15·5 ± 1·92	15·3 ± 2·03	15·2 ± 2·11
Race [b]					
African American	1 (4·5%)	3 (13·6%)	0 (0·0%)	2 (9·1%)	6 (6·7%)
Asian/Hawaiian/Pacific Islander	4 (18·2%)	1 (4·5%)	3 (12·5%)	2 (9·1%)	10 (11·1%)
Caucasian	17 (77·3%)	13 (59·1%)	19 (79·2%)	14 (63·6%)	54 (63·5%)
Other/Unknown	0 (0·0%)	5 (22·7%)	2 (8·3%)	4 (18·2%)	11 (12·2%)
Hispanic Ethnicity [b,c]	0 (0·0%)	5 (22·7%)	3 (12·5%)	1 (4·5%)	9 (10·0%)
Current CAPS score [d] (Mean ± SD)	0·0 ± 0·0	51·9 ± 13·03	0·0 ± 0·0	55·2 ± 21·58	53·5 ± 17·70
PTSD Symptom Checklist (PCL; Mean ± SD) [e]	19·09 ± 4·21	46·0 ± 12·29*	19·9 ± 4·08	55·6 ± 12·27*	34·8 ± 18·39
Current MDD [f]	0 (0·0%)	5 (22·7%)	0 (0·0%)	3 (13·6%)	8 (8·9%)
Childhood trauma ≤14 years of age [g,h]	0 (0·0%)	8 (36·4%)	0 (0·0%)	11 (50·0%)	19 (21·1%)
Hormonal birth control [b,i]	NA	NA	2 (8·3%)	6 (27·3%)	8 (17·4%)
BMI [b]					
Underweight = <18·5	2 (9·1%)	0 (0·0%)	1 (4·2%)	0 (0·0%)	3 (3·3%)
Normal weight = 18·5–24·9	10 (45·5%)	4 (18·2%)	12 (50·0%)	13 (59·1%)	39 (43·3%)
Overweight = 25–29·9	10 (45·5%)	5 (22·7%)	8 (33·3%)	7 (31·8%)	30 (33·3%)
Obesity = BMI of 30+	0 (0·0%)	13 (59·1%)	3 (12·5%)	2 (9·1%)	18 (20·0%)
Smoker [b, j]	3 (13·6%)	4 (18·2%)	6 (25·0%)	6 (27·3%)	19 (21·1%)

a based on F-test b based on Chi-square test

c Three subjects endorsed Hispanic ethnicity but did not select a racial descriptor. Six additional subjects endorsed Hispanic ethnicity, in addition to a racial category of Caucasian or African-American race yielding a total of 9 subjects self-identifying as Hispanic in this sample. Comparison of Hispanic ethnicity, p=·063.

d Control subjects had CAPS scores of zero or had an absence of criterion A events. Comparison of male and female PTSD subjects on current CAPS score, p=0·54.

e PTSD group by gender interaction on current PCL score, p<.05. *:Comparison of PTSD groups vs controls, p<.001. Comparison of male vs female PTSD groups vs controls, p<0·01.

f Absence of current MDD was required for inclusion into the control group. Comparison of male and female PTSD subjects on rate of current MDD, p=0·43.

g Childhood trauma exposure was defined, based on findings from our prior research, by exposure to 2 or more categories of childhood trauma under the age of 14. Three (6·5%) control subjects reported a history of 1 category of childhood trauma. h Chi-square test compared frequency of childhood trauma between male and female PTSD subjects only, p=·36.

i Chi-square test compared use of hormonal birth control female PTSD and control subjects only, p=·09

j based on diary

Table 2. Sex steroid metabolite concentrations in plasma of men and women.

Metabolite	LOD (ng/mL)	Control □ SD (ng/mL) Women	PTSD □ SD (ng/mL) Women	Control □ SD (ng/mL) Men	PTSD □ SD (ng/mL) Men
Progesterone (P4)	0.393	0·59 □ 1·19	<LOD (<0·28)	<LOD (<0·09)	<LOD (<0·09)
Testosterone*	0.144	0·55 □ 0·90	0·44 □ 0·68	4·03 □ 1·39	4·17 □ 1·61
Estrone (E1)	2.702	<LOD (<0·11)	<LOD (<0·24)	<LOD (<0·16)	<LOD (<0·11)
Estradiol (E2)	0.680	<LOD (<0·03)	<LOD (<0·06)	<LOD (<0·02)	<LOD (<0·03)
Estriol (E3)	2.882	5·7 □ 8·93	3·23 □ 2·26	3·39 □ 3·96	4·68 □ 9·69

Steroid metabolite values plasma in ng/mL. Limit of detection (LOD) for each analyte is shown. Values in red text were below LOD. *: p<0·0001 Men vs. Women (sex); PTSD: ns; PTSD x Sex: ns

Table 3. Altered primary metabolite clusters in PTSD with sleep confounders

Men PTSD	Men Delta Power	Men PSQI	Men TST	Women PTSD	Women Delta Power	Women PSQI	Women TST
Amino Acids (AA)							
.	.	Gly	.	Glycine (Gly)	Gly	.	Gly
.	.	.	.	Threonine (Thr)	Thr	.	Thr
.	.	.	.	Serine (Ser)	Ser	.	Ser
.	.	.	.	.	Citrulline	.	.
.	.	.	.	.	□-Ala	□-Alanine	□-Ala
Branched-chain amino acids (BCAA)							
.	.	.	.	Valine (Val)	Val	Val	Val
.	.	.	.	Leucine (Leu)	.	Leu	Leu
Sulfur amino acids							
.	.	.	Met	Methionine (Met)	Met	Met	.
.	.	.	Cys	Cysteine (Cys)	Cys	Cyst	.
Cyclic amino acids							
Histidine (His)	His	.	.	.	.	.	.
.	.	.	4-Hyp	Trans-4-hydroxyproline (4-Hyp)	4-Hyp	4-Hyp	.
Indoles (Tryptophan metabolites)							
.	.	.	.	Indole-3-propionic acid (IPA)	.	.	IPA
.	.	.	.	.	.	Indole-3-lactate (ILA)	.

.	.	.	.	.	.	Indole-3-acetate (IAA)	.
Butyrates (Gut microbes)							
.	.	Threonic acid	.	.	.	.	.
.	.	3-Hydroxybutyric acid	.	.	.	.	.
.	.	2-Hydroxybutanoic acid	.	.	.	.	.
.	.	.	.	Isothreonic acid (ITA)	ITA	.	ITA
.	.	.	.	.	.	2-Aminobutyric acid (2AB)	.
Hexose							
Fructose	.	.	.	.	.	.	.
.	Sucrose	.	.	.	.	.	.
.	.	.	.	1-Methylgalactose NIST	.	.	1-MG NIST
Unsaturated fatty acids (UFA)							
.	.	.	.	Palmitoleic acid	.	.	.

Figure Legends

Figure 1. Sex differences in primary metabolites in PTSD. (**A**) ChemRich analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI and age. Blue nodes contain metabolite clusters that were decreased, purple nodes contain metabolites that were both increased or decreased, and red nodes contain metabolites that were increased in PTSD vs. control individuals. (**B**) Venn diagram showing seven nodes were specific to women with PTSD and two nodes

significant.

were specific to men with PTSD compared with controls. (**C**) Volcano plots of specific metabolites within each node in men and women with PTSD after correcting for type 1 error and adjusting for BMI and age.

Figure 2. Alterations in specific amino acids within a metabolite cluster/node with PTSD symptoms. (**A-G**) Box plots of specific amino acids with the seven nodes that differed between women and two nodes in men with PTSD compared to controls shown in Fig. 1A. Glycine levels associated negatively with PCL cluster D in women with PTSD ($r = –0{\cdot}47$, $p = 0{\cdot}036$), but not controls, whereas association of serine level was lost in women with PTSD. (**H**) Fructose levels increased in men with PTSD vs. controls, but fructose levels associated negatively with PCL scores. Box plot analysis: Mann-Whitney and $p<0{\cdot}05$ considered

Figure 3. PTSD-specific alterations in amino acids with respect to their biosynthesis pathway in humans from glucose. Essential amino acids cannot be synthesized and must be obtained from diet, whereas non-essential amino acids can be synthesized from glucose as it enters the tricarboxylic cycle. Essential amino acids are shown in grey boxes, and those metabolites synthesized as a by-product of gut microbiome are shown in green boxes. Specific amino acids that were increased are shown in red, decreased in blue (women-specific outlined in pink and men-specific in blue).

Figure 4. Sex-specific disturbances in sleep measures. Box plots showing sex- and/or PTSD-specific alterations in (**A**) Total sleep time (in minutes) decreased; Two-way ANOVA: Sex: ns; PTSD: $p= 0{\cdot}004$; Sex X PTSD: ns. (**B**) sleep quality worsened, Two-way ANOVA: Sex: ns; PTSD: $p< 0{\cdot}0001$; Sex X PTSD: ns. and (**C**) log-transformed delta power sleep was lower in people with PTSD vs controls, Two-way ANOVA: Sex: $p= 0{\cdot}007$; PTSD: $p= 0{\cdot}005$; Sex X PTSD: ns. (**D**) Linear regression showing association of

PCL scores with three different measures of sleep. individuals.

Figure 5. Sex differences in primary metabolites after accounting for sleep measures . ChemRich analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI, age, and one of the three sleep measures shown (TST (min), PSQI, or log-transformed delta power (ln(μV2)). Blue nodes contain metabolite clusters that are decreased, purple nodes contain metabolites that are both increased or decreased, and red nodes contain metabolites that are increased in PTSD vs control

Figure 6. Sex-specific contribution of sleep variables on primary metabolites. Venn diagrams to visualize significant metabolites whilst adjusting for one sleep variable at a time.

Figure 7. Linear regression and box plots of various amino acids with sleep variables (**A-F**) Box plots of specific amino acids with individual nodes that differed between women and men with PTSD compared to controls shown in Fig. 5 and 6. (**G**) Log-transformed delta power (ln(μV2) associated negatively with testosterone in men.

Figure 8. Changes in insulin and tryptophan levels in women and men with PTSD. Box plots of tryptophan and albumin levels in women and men. (**A**) No significant differences were seen in tryptophan and albumin levels in women with PTSD compared with controls, whereas tryptophan levels were significantly elevated in men with PTSD compared with controls (p = 0·044; Mann-Whitney). (**B**) Blood

insulin levels were determined after oral glucose challenge at various times shown. Mixed-effect analysis showed that insulin levels changed with time (p<0·0001) in women and men, but only differed between PTSD and controls in men (p=0·0136). (**C**) Tryptophan levels correlated negatively with insulin levels in women, but positively in men.

Figure 9. Sex-specific alterations in tryptophan metabolism pathway in PTSD. (**A**) Sex, sleep, and PTSD all alter primary metabolites. (**B**) Albumin-bound tryptophan is present in circulation and dynamic

increases in insulin promote binding of albumin to tryptophan, whereas esterified fatty acids can displace tryptophan from albumin. Free tryptophan is then transported to the brain by a transport carrier. Several amino acids, such as leucine, valine etc. compete with tryptophan for binding to the transport carrier, which can decrease influx of free tryptophan into the brain. Reduced free tryptophan levels in the brain can influence production of serotonin and melatonin, affecting brain function and sleep. In the gut, tryptophan is converted to indoles by the action of microbes such as*Lactobacillus*; these indoles have protective effect on gut barrier and immune functions. Serine can serve as NMDA receptor agonist and alter neuronal function. Thus, disturbances at multiple levels in tryptophan pathway may contribute to pathogenesis of

Fig. 1A

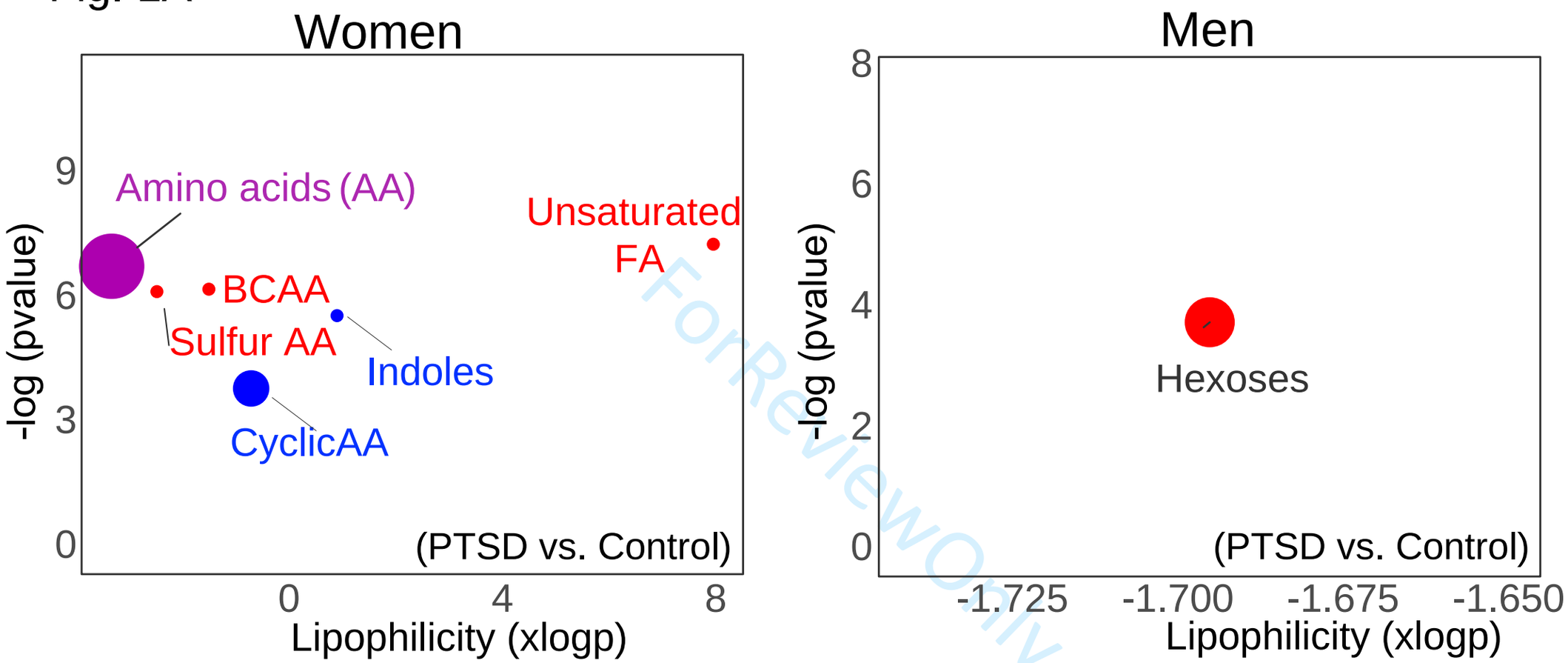
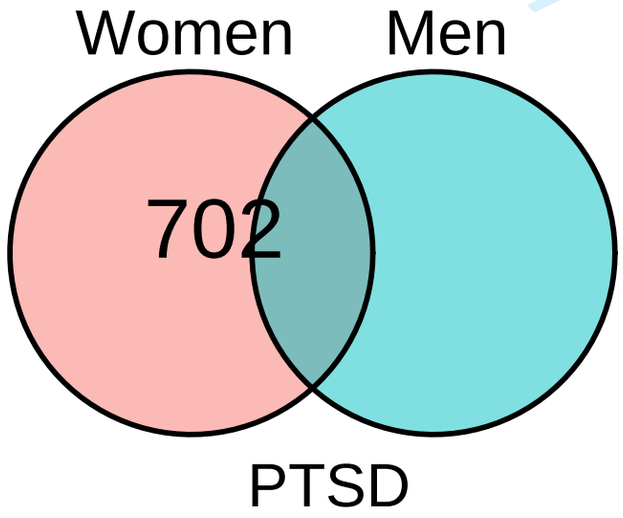


Fig. 1B



Blue: decrease

Purple: both increase and decrease

Red: increase

Fig. 1C

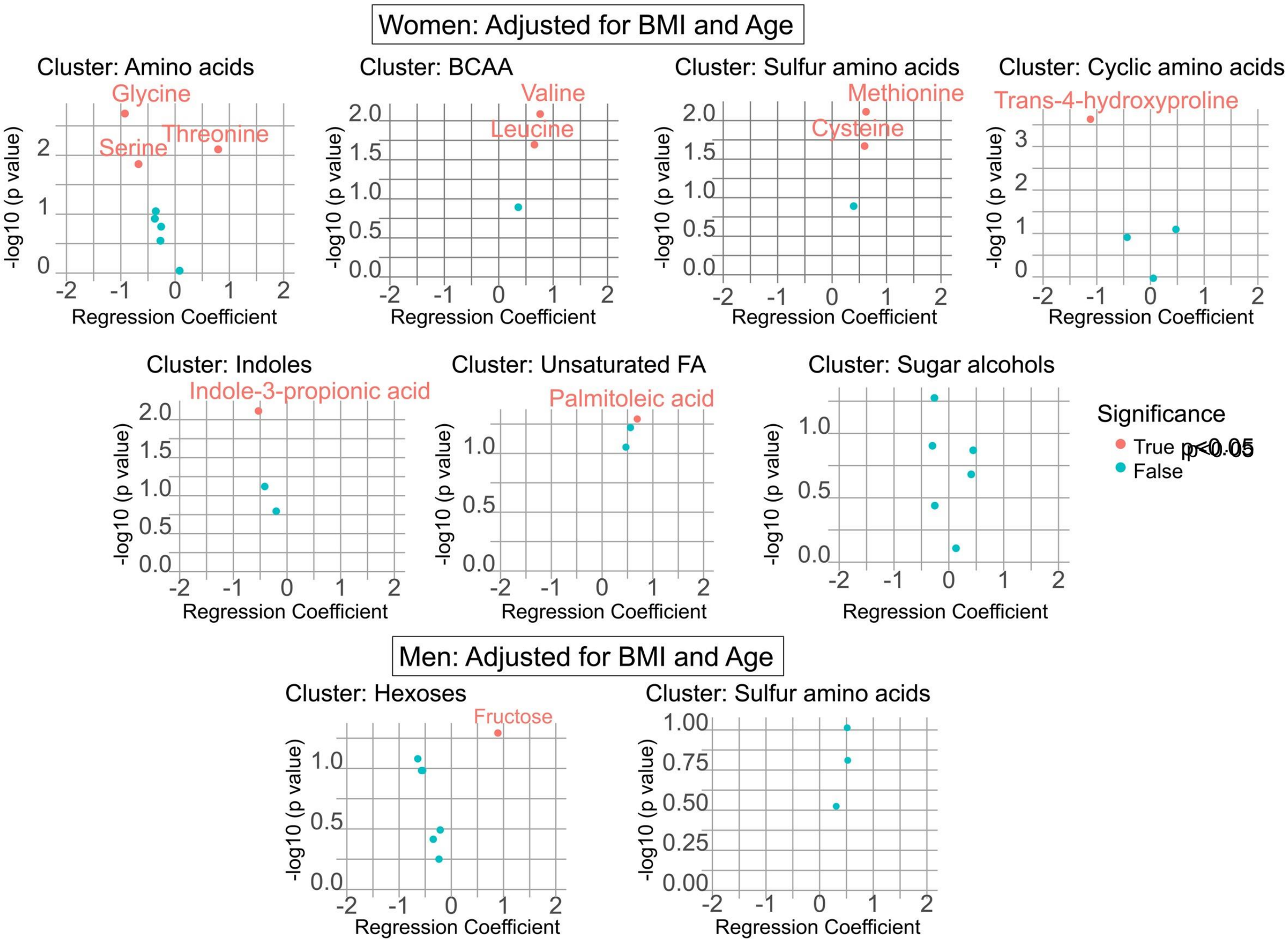
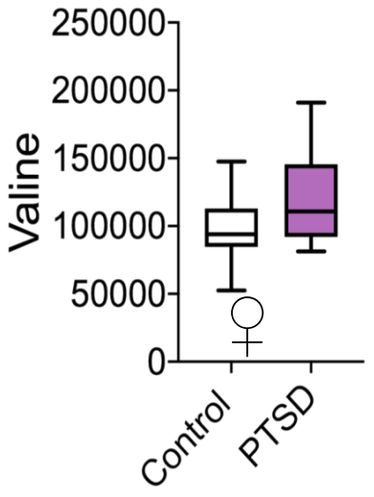


Fig. 2 31 of 37

A. Amino Acids



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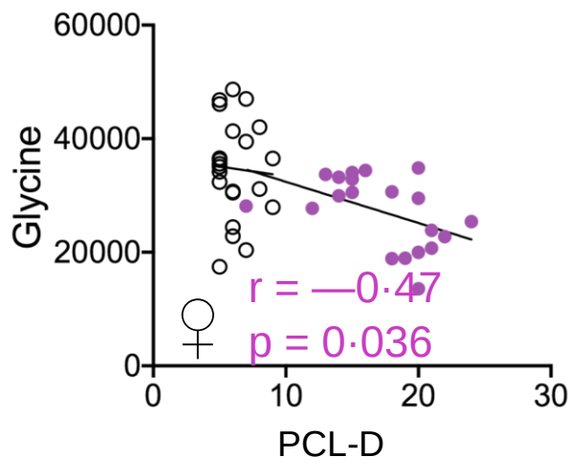
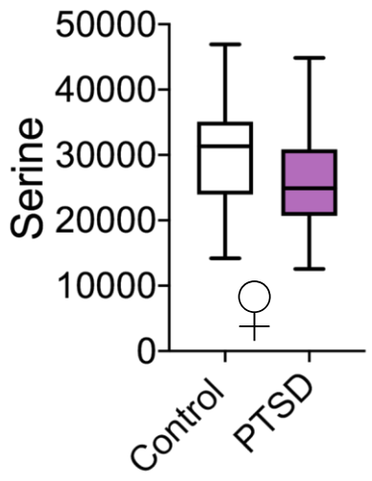
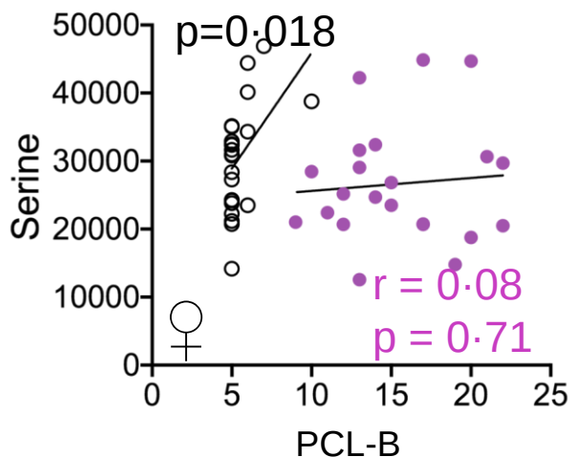


r=–0·05 p=0·053



p=0·0392

r=0·48
p=0·018



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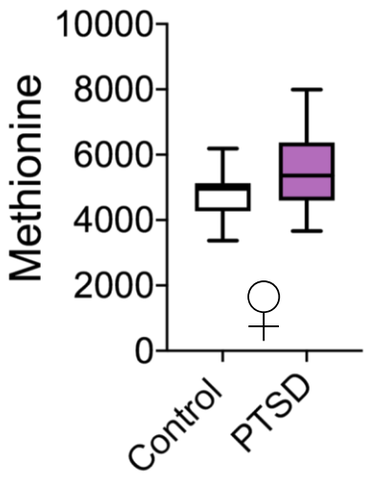

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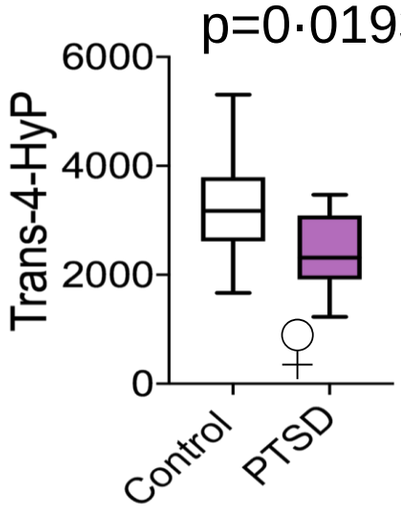

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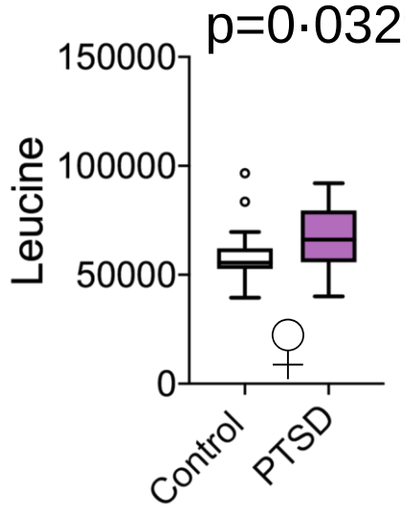

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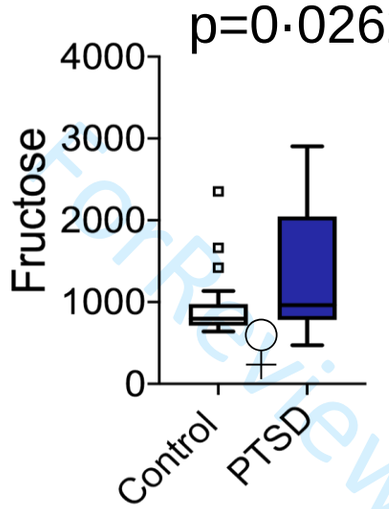

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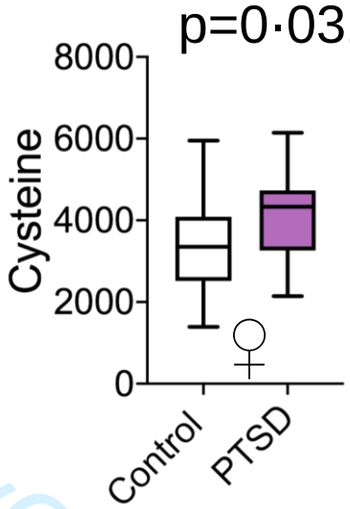

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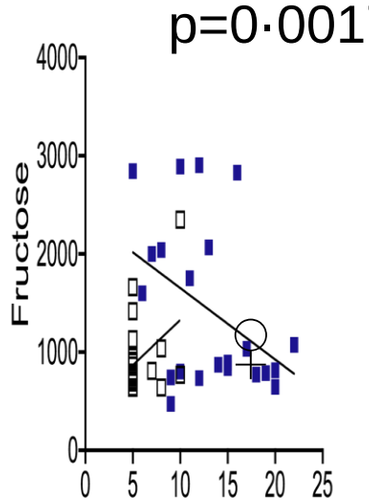

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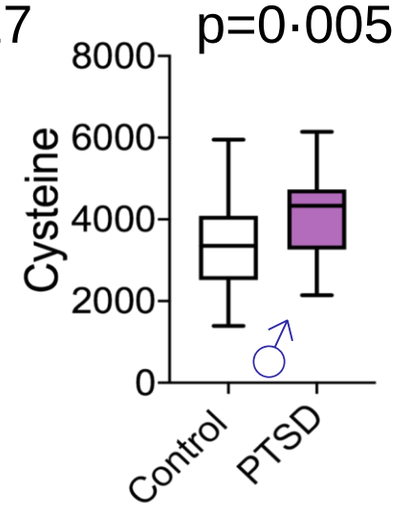
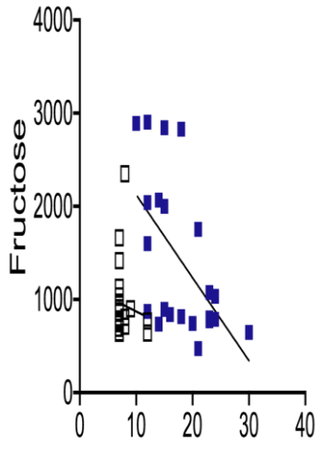
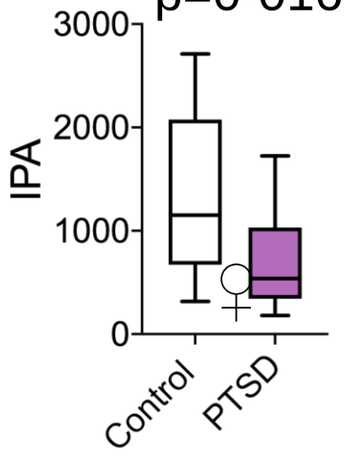


E. Indoles



F. Butyrates

p=0·0168



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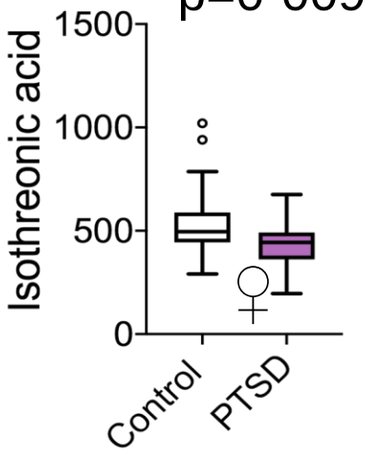
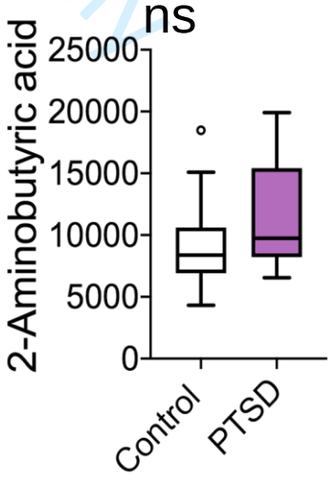
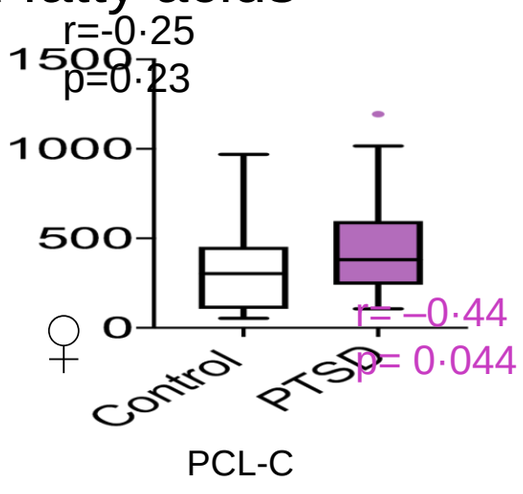


G. Unsaturated fatty acids

ns



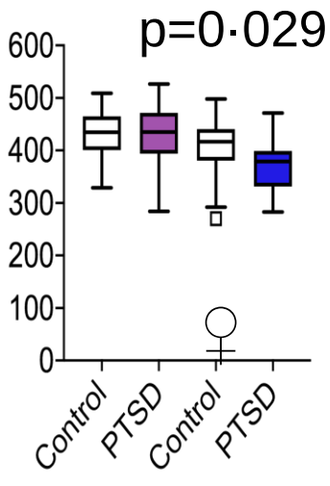
r=-0·25
p=0·23

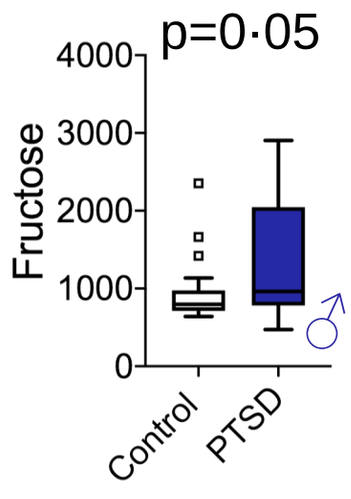


B. BCAA C. Sulfur AAD. Cyclic AA

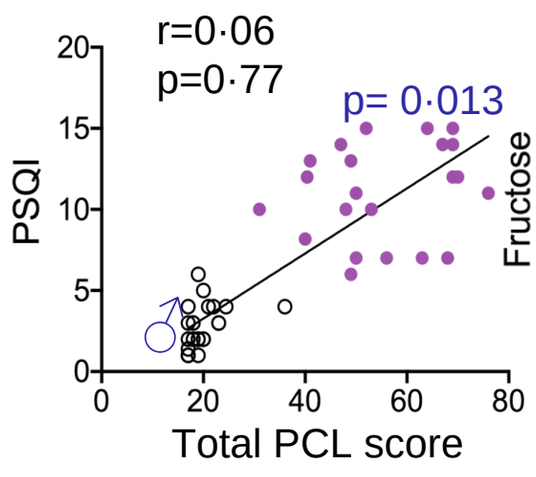
p=0·0034

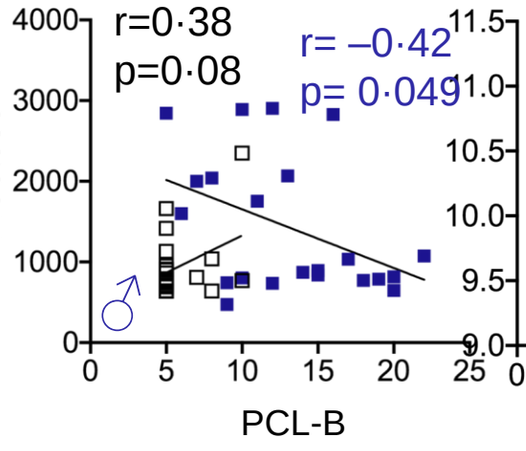
H.

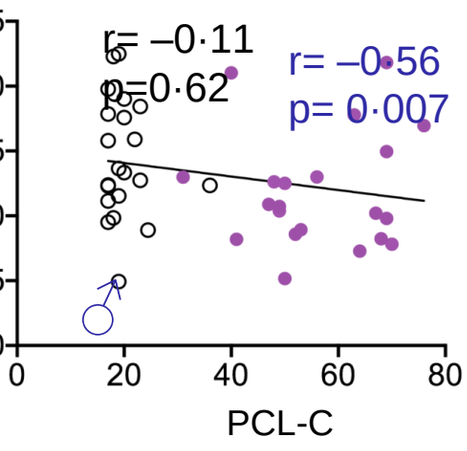




Hexose







r=–0·52

Fig. 3

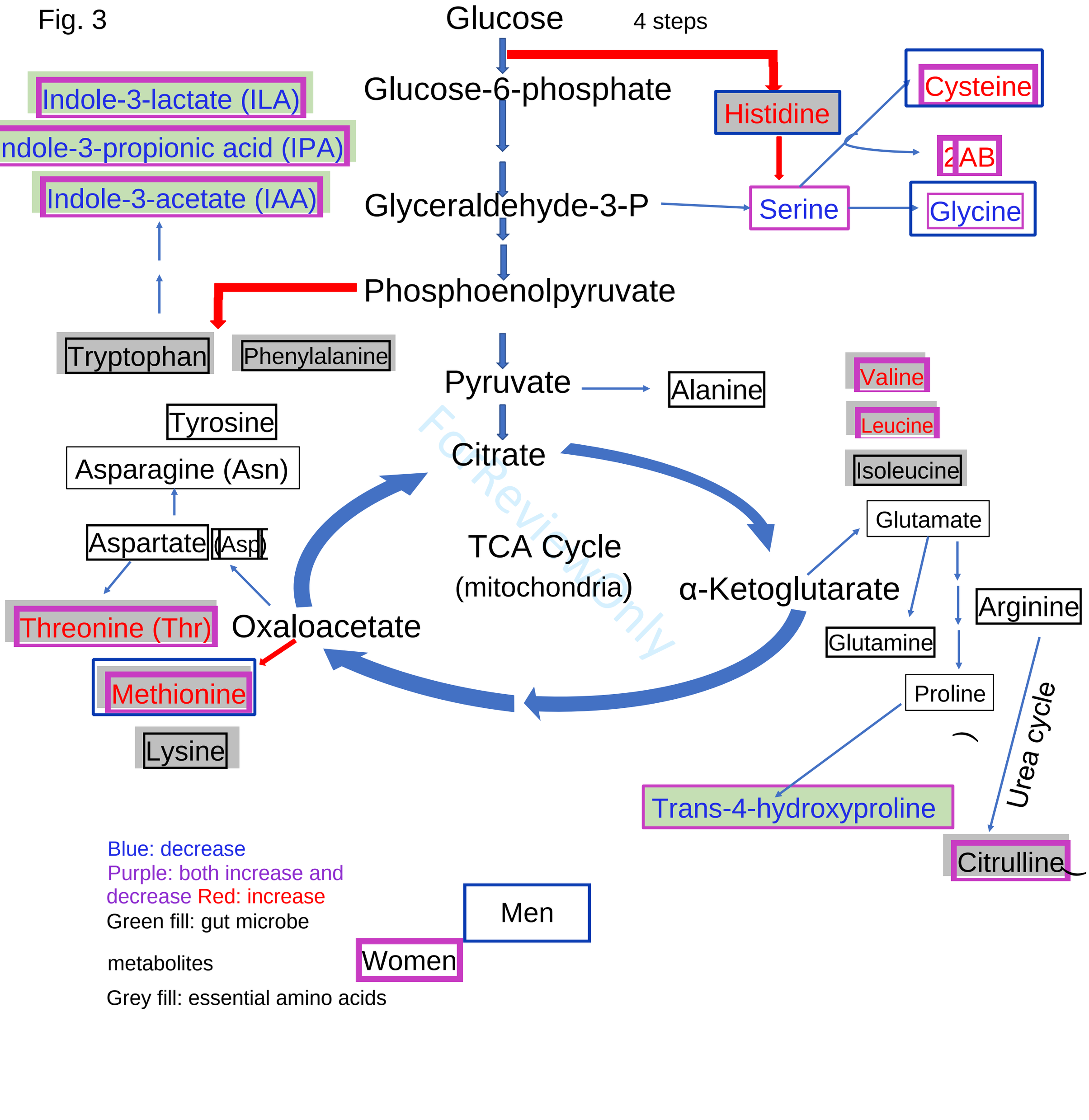
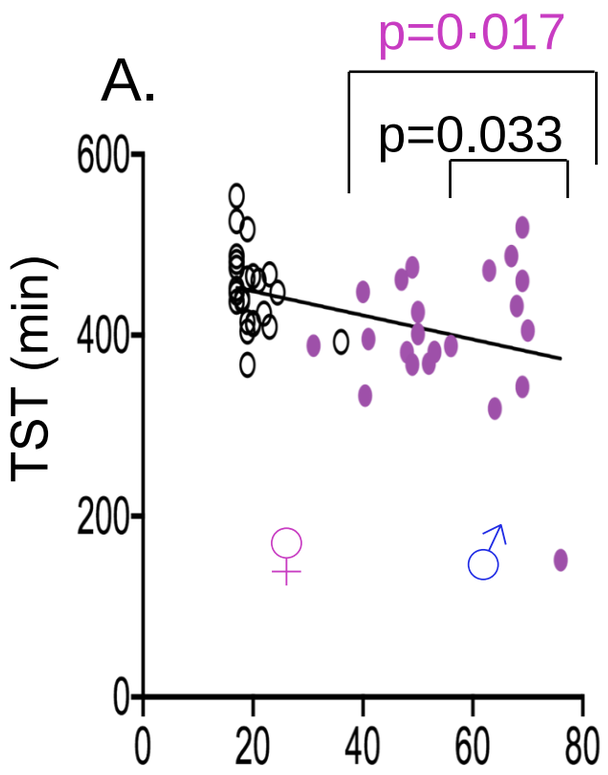
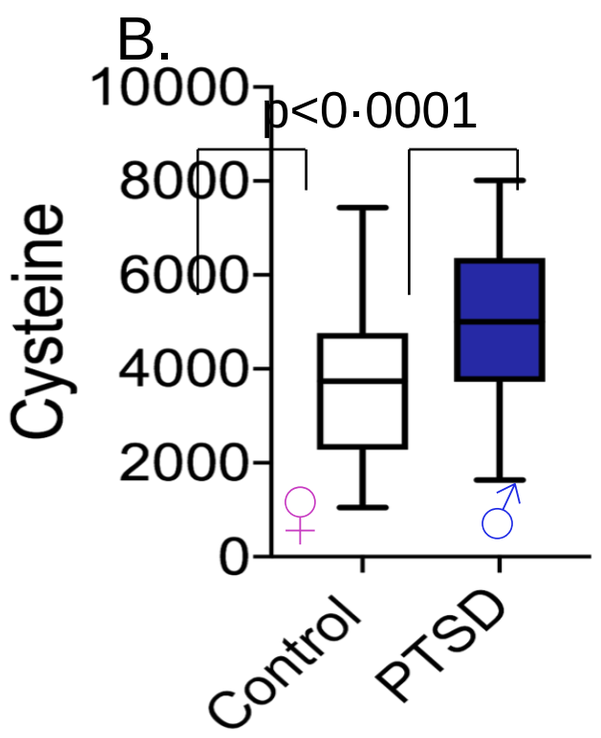
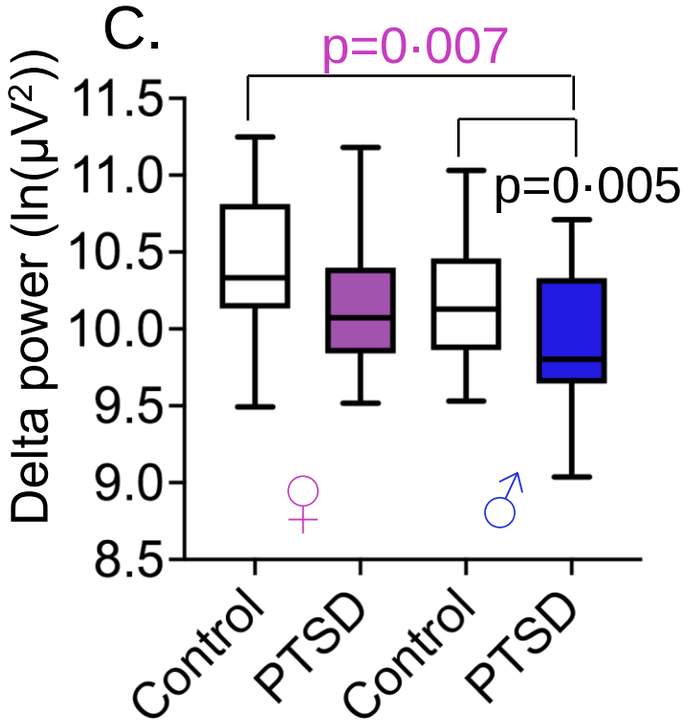
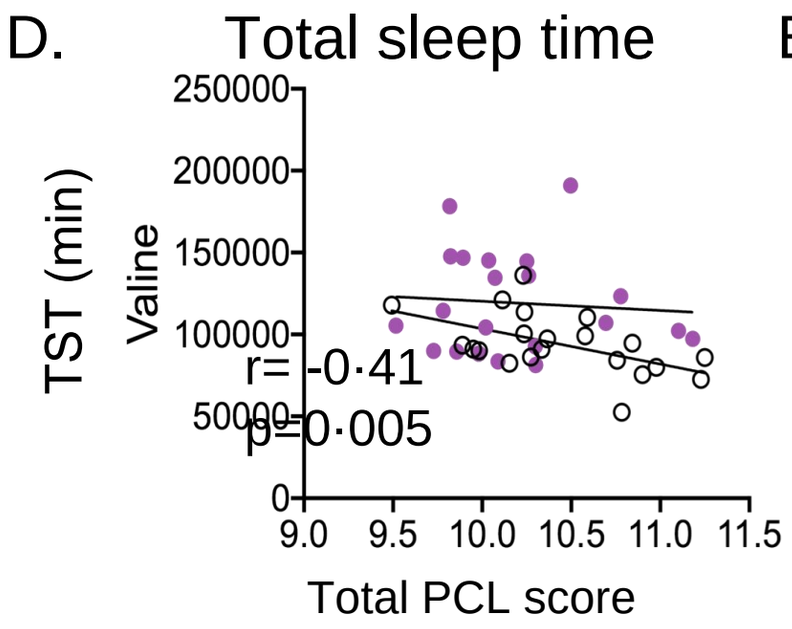


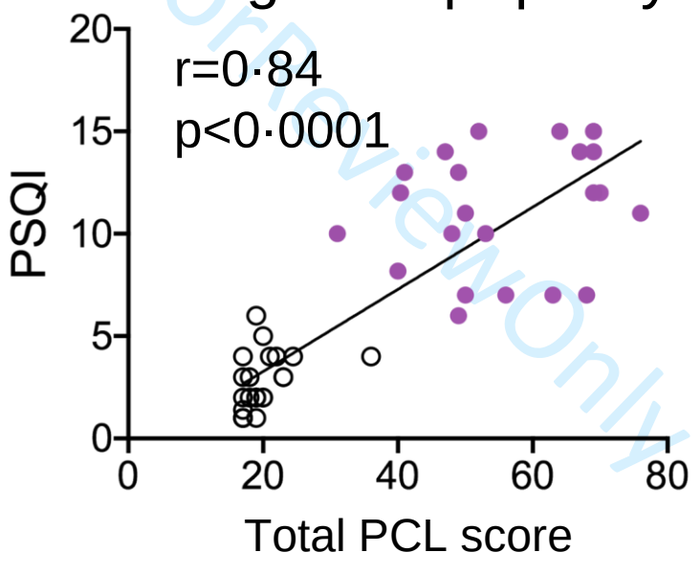
Fig. 4

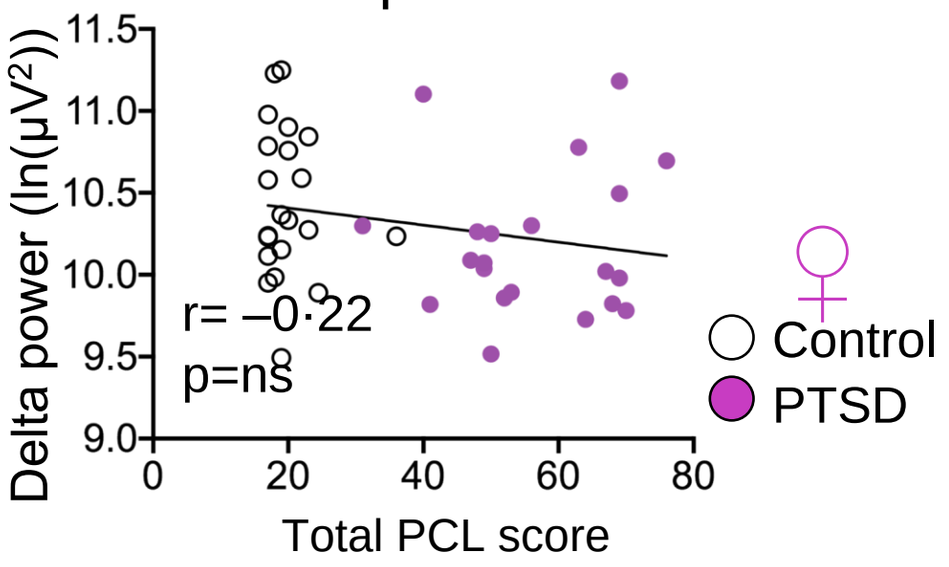


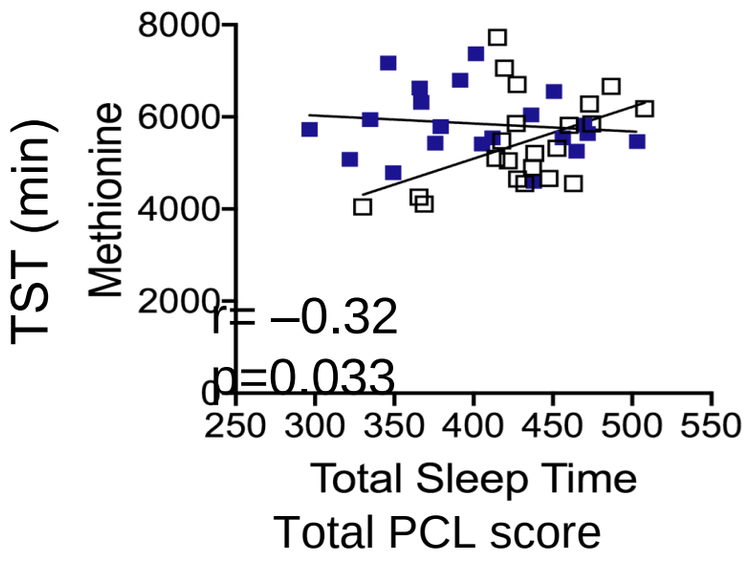


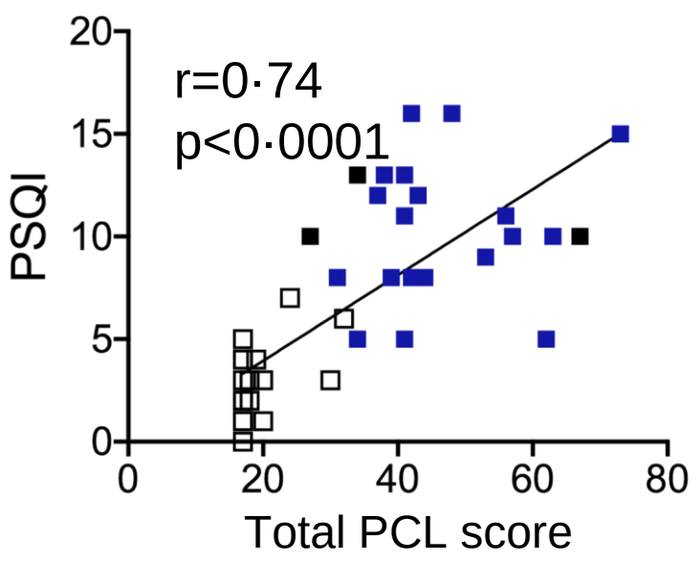












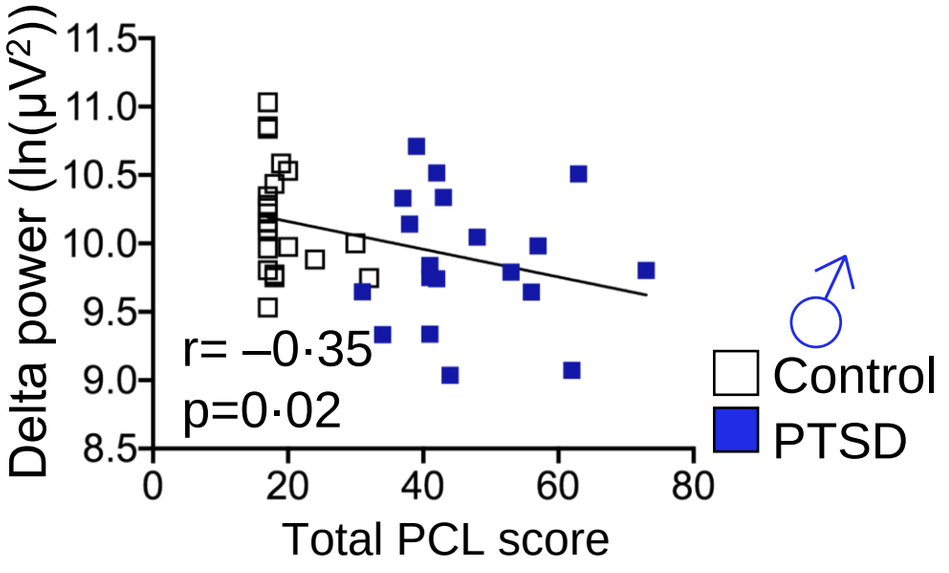
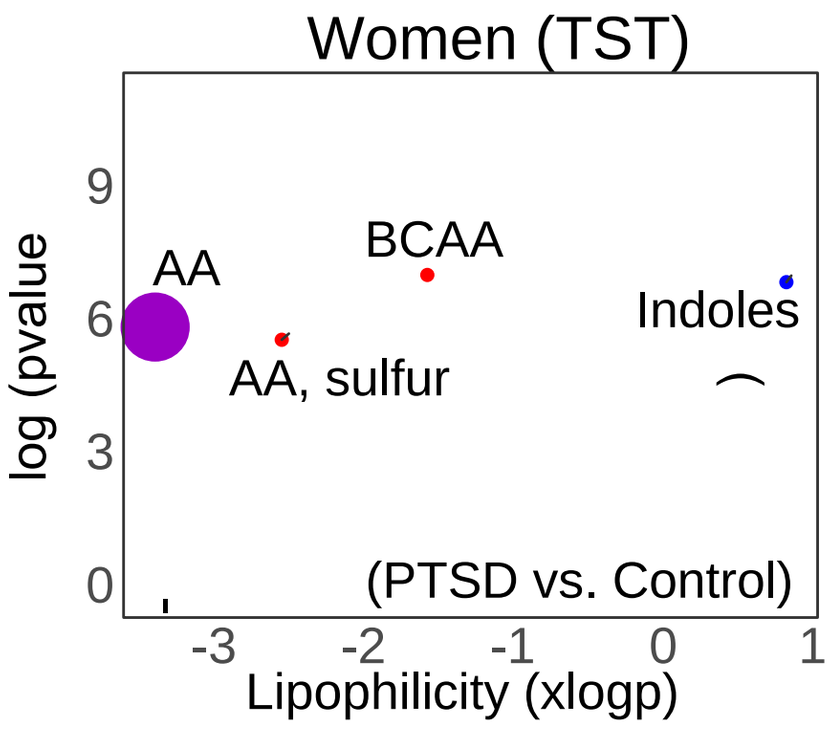
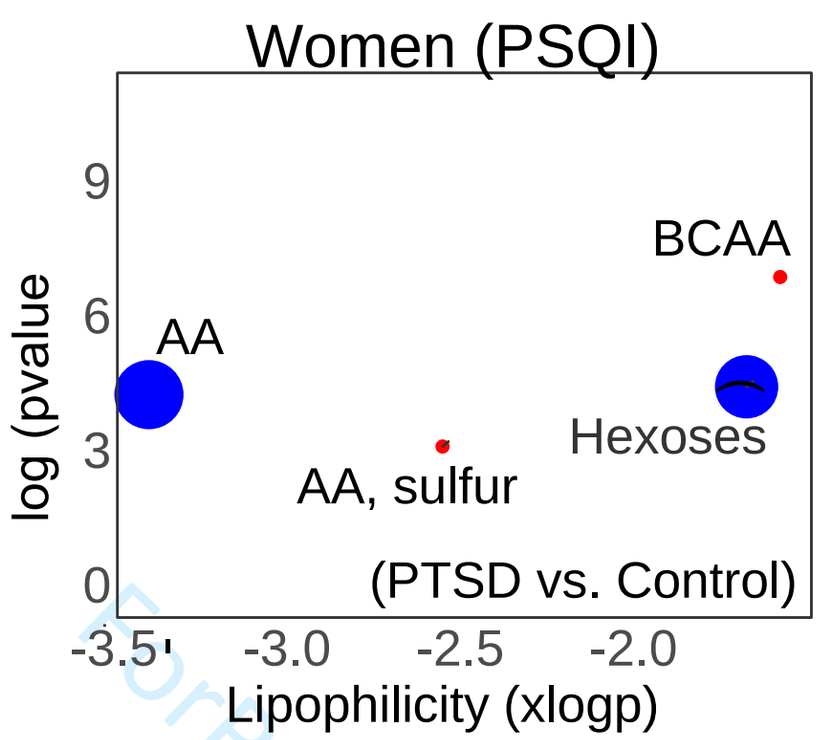
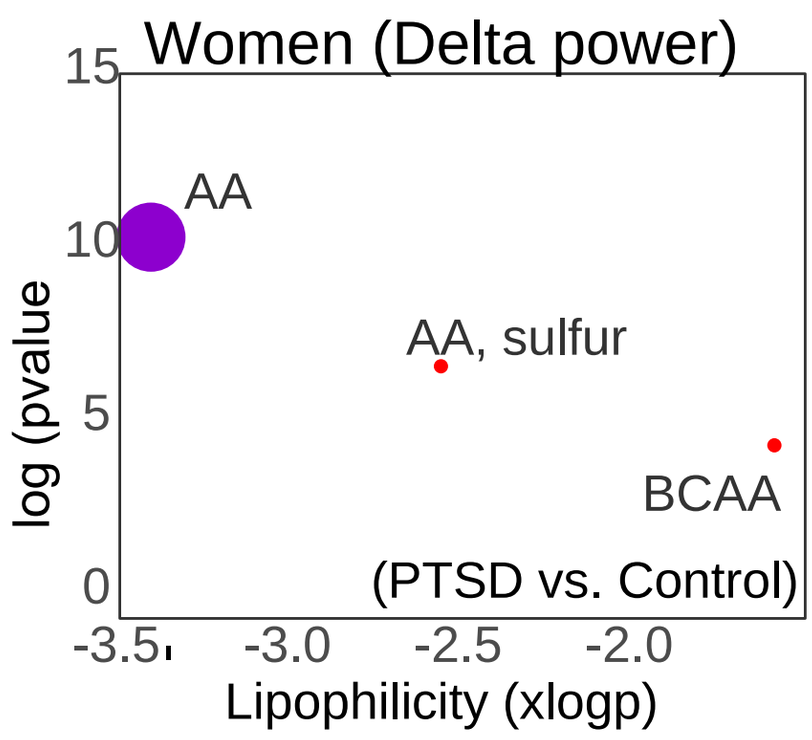


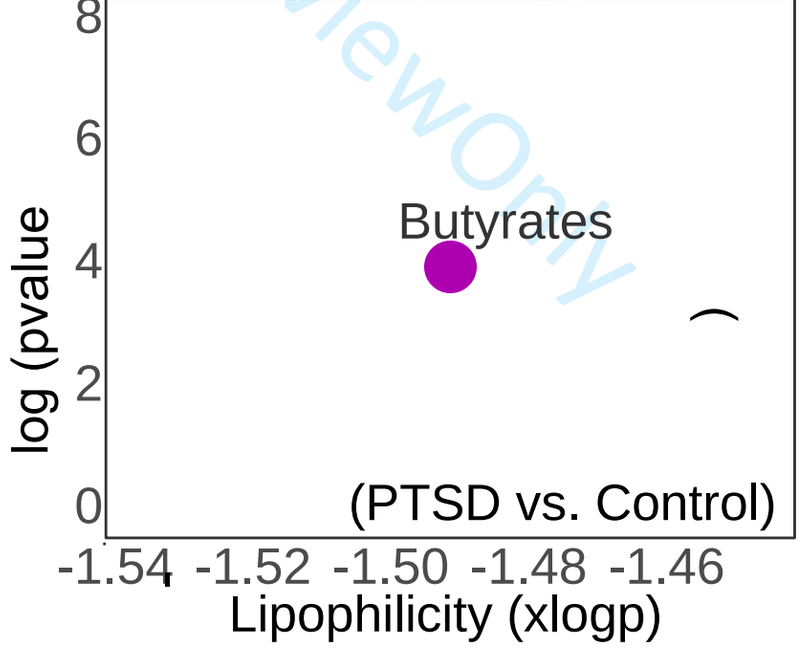
Fig. 5







Blue: decrease
Purple: both increase and decrease
Red: increase



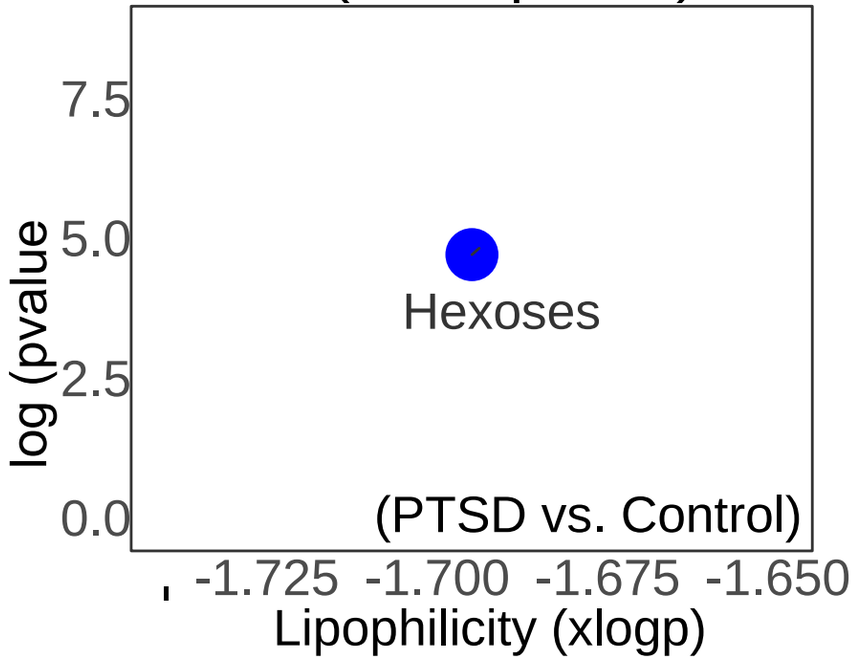


Fig. 6

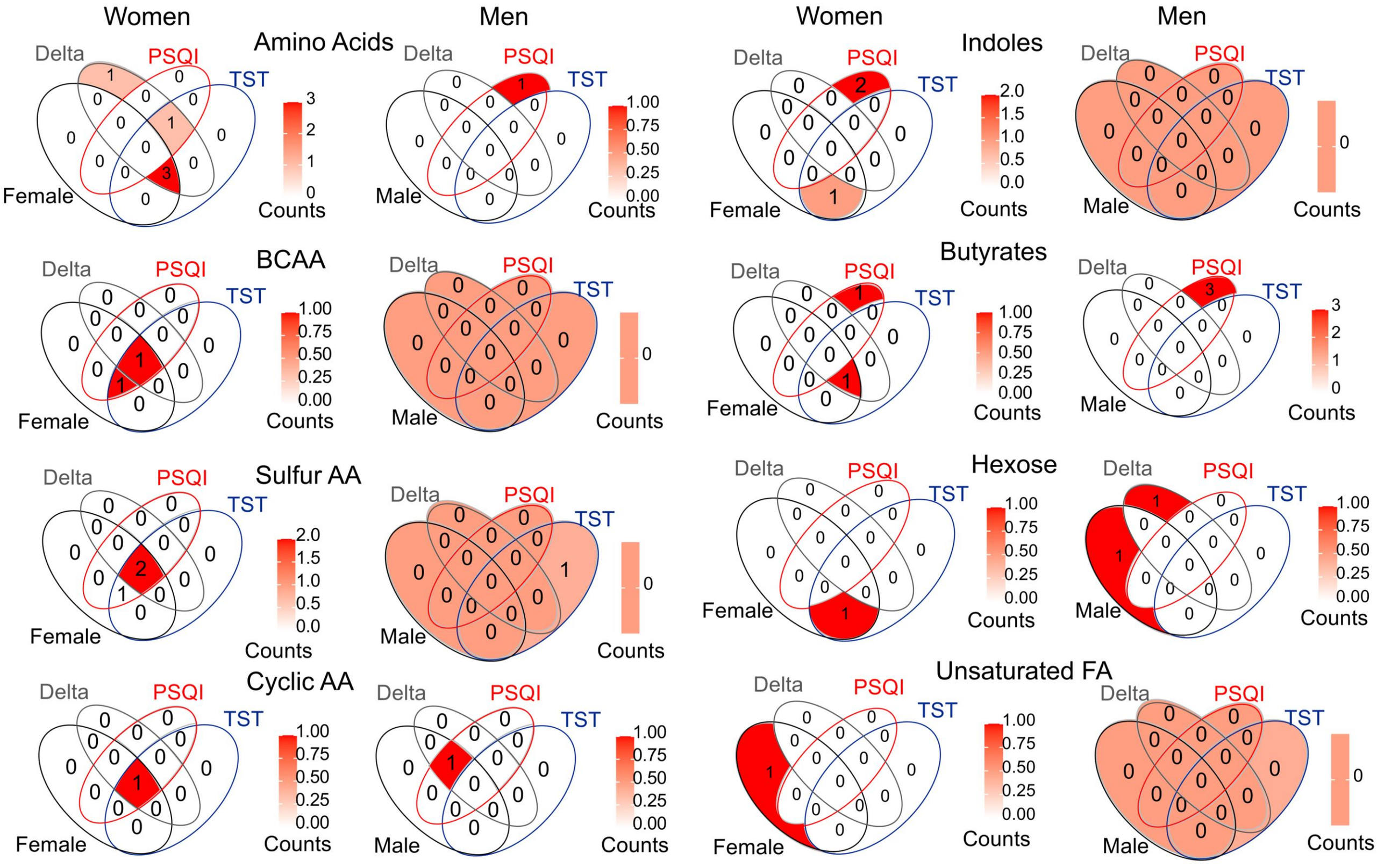
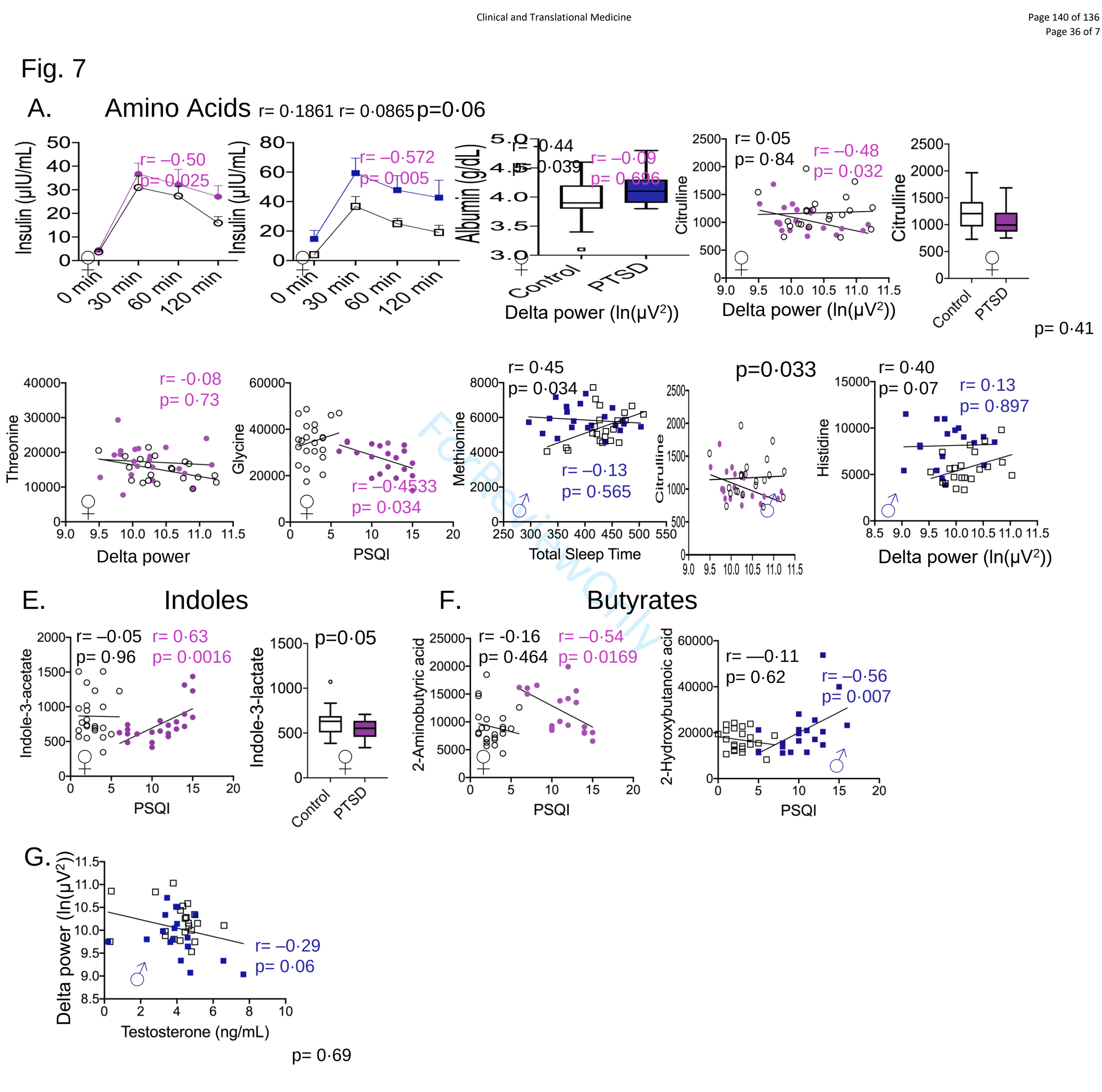
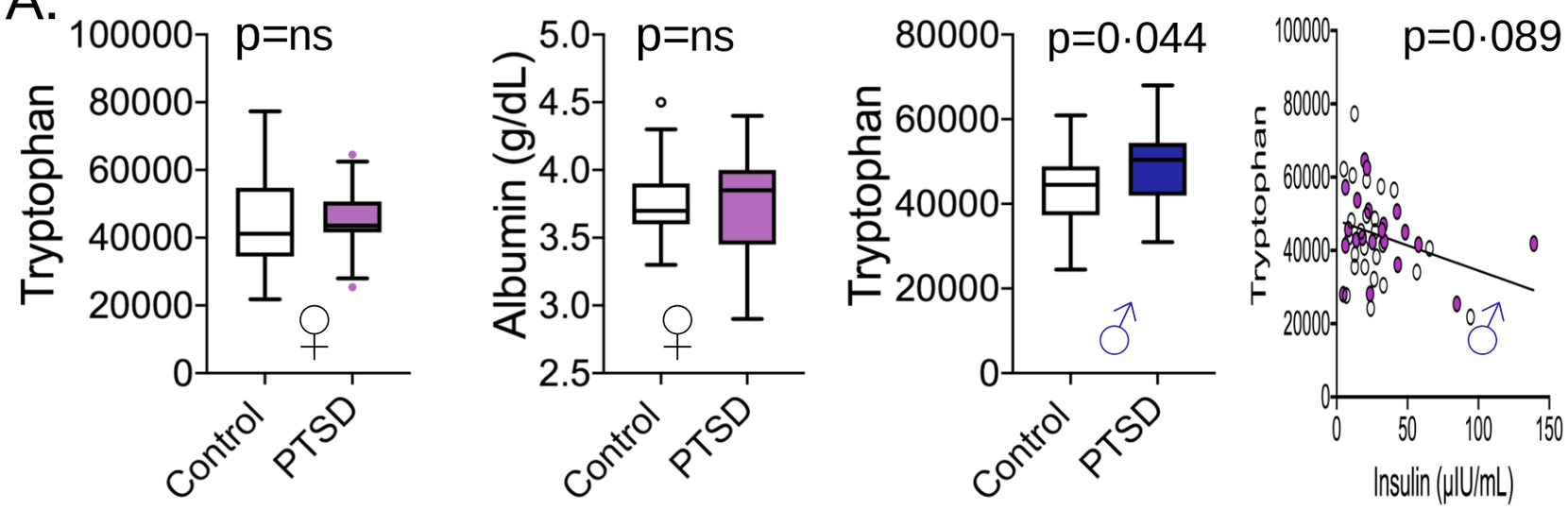

Women
Men
Amino Acids
Delta
PSQI
TST
Female
Male
Counts
Indoles
BCAA
Butyrates
Sulfur AA
Hexose
Cyclic AA
Unsaturated FA

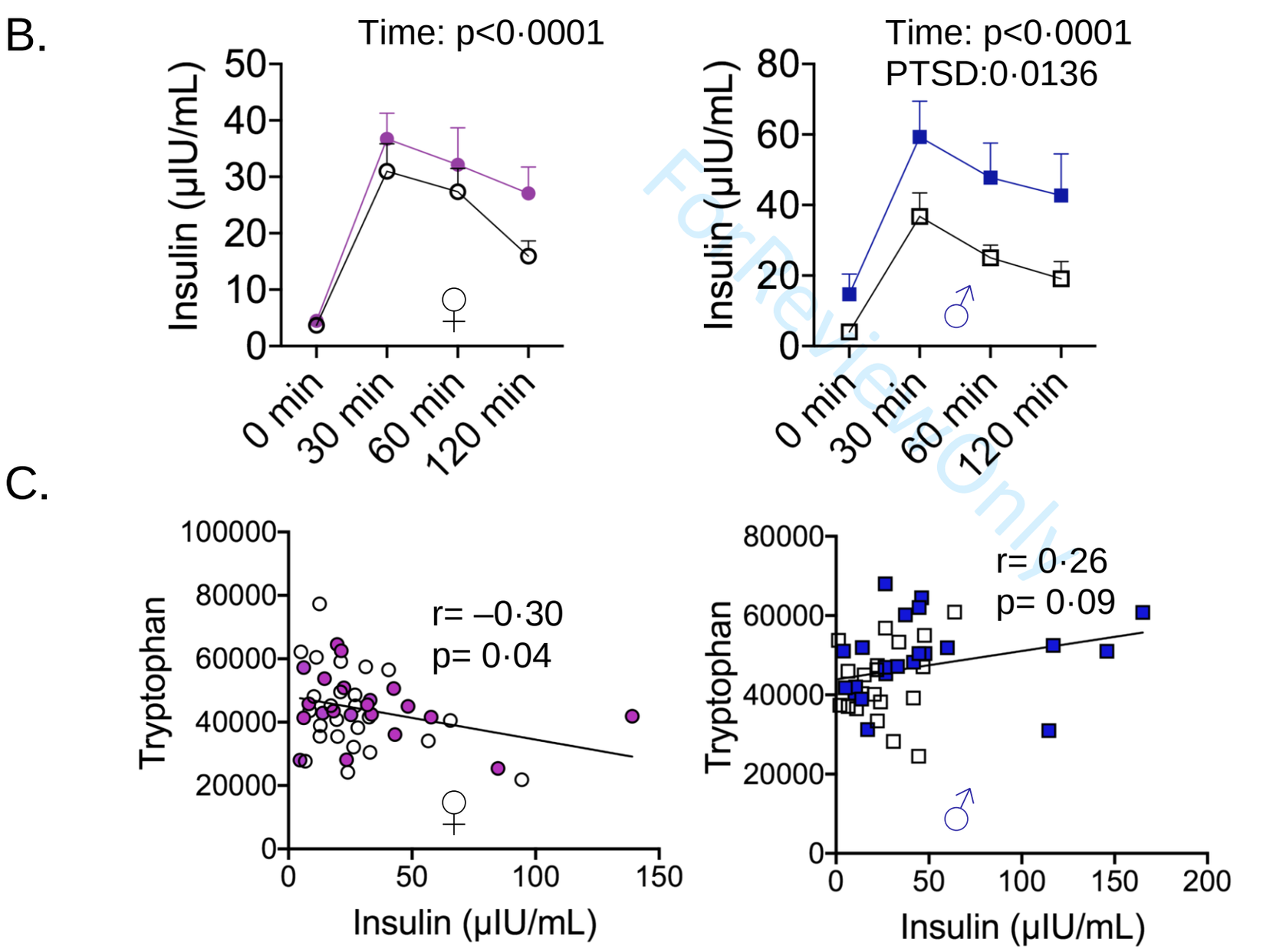
Fig. 7

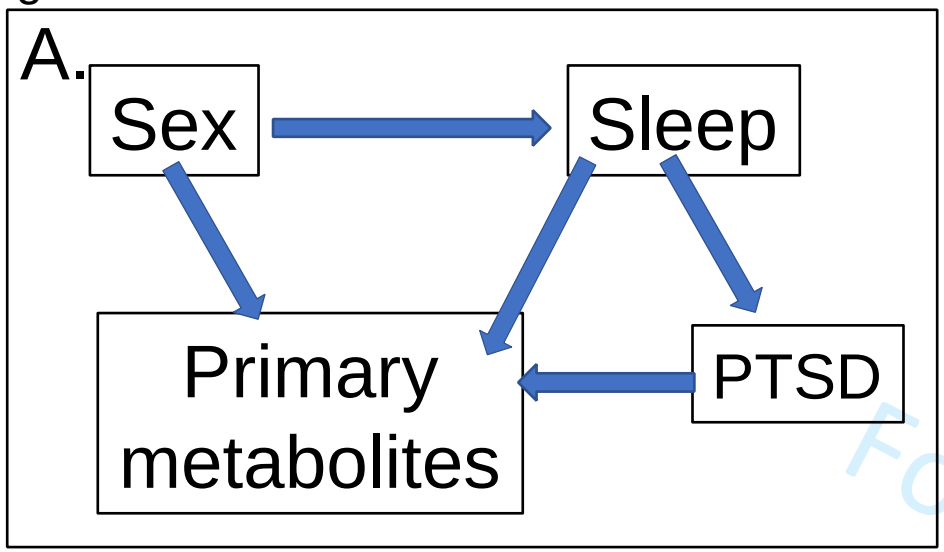


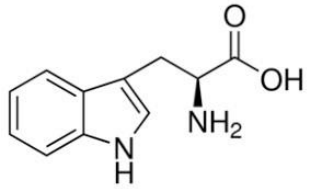
B. BCAA r= –0·532 C. r= –0·4148 p= 0·013 Sulfur AA p= 0·055 D. Cyclic AA

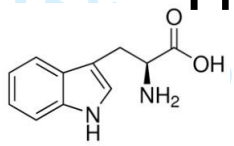
Fig. 8











B. Tryptophan + Albumin = bound Tryptophan

Insulin

Palmitoleic acid (esterified FA)

Brain

Leu, Ileu, Met

Free Tryptophan

~1-2%

TPH

Melatonin

Serotonin (5HT)

Sleep

~93%

IDO-1

Kyneurenine

~5%

Gut microbiota

Vitamin B6

Indoles

KA/QA

NMDA-R

Serine

IPA, ILA, IAA

Immune function

Gut barrier function

Chemical set enrichment analysis: novel insights into sex-specific alterations in primary metabolites in posttraumatic stress and disturbed sleep

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Running Title: Sex-specific primary metabolites in PTSD

Key Words: Amino acids; delta power sleep; indoles; insulin; mass spectrometry; PSQI; tryptophan.

Dear Editor,

We found that women demonstrate more primary metabolite disturbances than men with similar posttraumatic stress disorder (PTSD) severity; decrease in tryptophan metabolites indoles might be due to gut microbiota dysbiosis. PTSD may develop after exposure to actual or threatened death, serious injury, or sexual violence; women are at greater risk. The contribution of disordered sleep in PTSD in altering primary metabolites is unknown. Primary metabolites regulate several physiological functions, serve as substrates for neurotransmitters, and are altered in psychiatric disorders. Mass spectrometry was used to ascertain metabolites in 90 plasma samples from individuals with chronic PTSD and control subjects. Clinician-Administered PTSD Scale for DSM-IV was used to diagnose PTSD. Sleep was monitored using laboratory-based polysomnography. We adjusted for BMI and age in our analyses. Men and women with PTSD did not differ in PTSD severity or history of childhood trauma (Table S1).

Sex aggregated data analysis revealed several metabolites that were significantly altered between control and PTSD groups (Fig. S1). Next, sex segregated Chemical Set Enrichment Analysis[1] on primary metabolites identified seven and two metabolite nodes altered in women and men with PTSD, respectively, compared with sex-matched controls (Fig. 1A-B). Each node contained two or more metabolites; men and women with PTSD did not share any primary metabolites (Fig. 1A-B). Since PTSD symptom presentation is highly variable between individuals,[2] and, in our cohort, women had significantly greater PCL-C scores than men (Table S1), we reasoned that individual PCL measures may associate differently with metabolites (Fig. 1C and Table S2). Serine levels were lower in women with PTSD, whereas glycine levels negatively associated with cluster D symptoms on the PCL, suggesting that with more hyperarousal, glycine levels decreased in women, but not men (Fig. 1C).

Serine, a neurotransmitter, serves as a precursor for the synthesis of glycine, cysteine, and 2-aminobutyric acid, a butyrate, and is synthesized directly from glucose (Fig. 2A). In the human myocardium,

2aminobutyric acid increases glutathione levels via AMPK activation to protect against oxidative stress.[3] An

individual's physiological and metabolic state alters glucose metabolism and generation of non-essential amino acids. Serine and glycine shuttle between the glia and neurons where glycine induces release of serine, a coagonist for NMDA receptors; together they regulate long-term potentiation[4] and are critical for the consolidation of extinction of previously conditioned fear memories.[5] Our data suggests that alterations in subsets of metabolites could be protective in PTSD (Table S2). Changes in metabolite levels in combat veterans with PTSD are reported,[6, 7] but the contribution of sleep or sex has not been investigated.

Sleep disturbances and quality are associated with overall poor health.[8] Both men and women with PTSD had lower total sleep time (TST) and worse self-reported sleep quality as assessed by the Pittsburgh Sleep Quality Index (PSQI) compared with controls (Fig. 2B). Delta power, a measure of deep sleep activity, decreased in PTSD and showed a significant sex difference; men had lower delta power sleep activity than women (Fig. 2B). Greater PTSD symptoms associated with lower TST in both women and men, and PSQI associated with greater PTSD symptoms in both women and men; delta power was lower in men with PTSD compared to controls, but not in women (Fig. 2C). Humans lack the ability to synthesize eight essential amino acids, including tryptophan, that must be obtained from diet; they are mostly absorbed by the gut and metabolized by the resident microbiota. Tryptophan is metabolized to a myriad of biologically active compounds by four different pathways (serotonin, tryptamines, kynurenine, and indoles). TST accounted for alterations in all metabolite nodes in men and two of the six metabolite nodes in women (Fig. 2D, 3A, and Table S2). When PSQI was used as a confounder, new, non-overlapping nodes were found to be significant in women and men; indoles, hexose and amino acid nodes were decreased in women (Fig. 2D). Delta power sleep accounted for 50% of the nodes in women (Table S2). Interestingly, higher testosterone levels associated with lower delta power in men (Fig. 3C).

Plasma insulin, albumin, and amino acids levels together modulate transport of free tryptophan to the brain.[9] In our cohort, no difference in insulin levels were seen in women; plasma albumin levels did not differ between controls and PTSD in either sex. Insulin levels were significantly elevated in men with PTSD

compared with controls (Fig. 3D-E). Tryptophan levels associated negatively with insulin levels in women (Fig. 3F) with concomitant increases in levels of several amino acids that compete with a tryptophan carrier for influx into the brain. Increases in palmitoleic acid levels may be compensatory, more so in women than in men, which can potentially displace albumin from tryptophan in order to generate free tryptophan.

Tryptophan is absorbed in the gut and converted by the actions of gut microbiota such as *Lactobacillus* to indoles (Fig. 4). While the role of serotonin in mood disorders, anxiety, and other disorders is well known, we show here for the first time that the indole metabolite, indole-3-propionic acid, is decreased in women with PTSD. Poorer sleep quality was further associated with decreased levels of two additional indoles, indole-3-lactic and acetic acids; the indoles regulate immune and gut barrier functions (Fig. 4B), and their decreased levels might contribute to altered immune and barrier function in women. Diets rich in butyrates that support growth of beneficial gut bacteria such as *Lactobacillus* may serve as non-invasive interventions, especially for women.

Surprisingly, sex steroid metabolites did not differ between controls and PTSD in either men or women (Table S3), nor did sex steroids associate with PTSD. Testosterone levels were ~10-fold higher in men (Table S3).

In conclusion, there was no overlap in PTSD-related alterations in men and women in any primary metabolite nodes or individual amino acids within those nodes, and associated pathways. A balance between levels of essential amino acids, insulin, and albumin determines availability and transport of free tryptophan to the brain, which might in turn influence production of serotonin and melatonin, and distinct cellular pathway operate within functional clusters in men and women (Fig.4).

Figure Legends

4

Figure 1. Sex differences in primary metabolites in PTSD. (A) Chemical Set Enrichment (ChemRICH) analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI and age. ChemRICH is a statistical enrichment approach based on chemical structure similarity/chemical ontologies and is an alternative to pathway analysis that relies on limited biochemical knowledge annotations. It yields study-specific, non-overlapping sets of all identified metabolites. Since ChemRICH sets have a self-contained size, thus p-values do not rely on the size of the background database. Blue nodes contain metabolite clusters that were decreased, purple nodes contain metabolites that were both increased or decreased, and red nodes contain metabolites that were increased in PTSD vs. control individuals. Branched-chain and sulfur-containing amino acids, and unsaturated fatty acids were increased, indoles and cyclic amino acids were decreased, whereas the non-polar amino acid node contained metabolites that either increased or decreased in women with PTSD compared to controls. A Venn diagram showing seven nodes were specific to women with PTSD and two nodes were specific to men with PTSD compared with controls. (B) Volcano plots of specific metabolites within each node in men and women with PTSD after correcting for type 1 error and adjusting for BMI and age. (C) Box plots of specific amino acids with the seven nodes that differed between women and two nodes in men with PTSD compared to controls shown in Fig. 1A. Glycine levels associated negatively with PCL cluster D in women with PTSD ($r = –0{\cdot}47$, $p = 0{\cdot}036$), but not controls, whereas association of serine level was lost in women with PTSD. Cysteine and 2-AB levels were elevated in PTSD compared with controls, but did not show any significant association with specific clusters of PTSD symptoms. Fructose levels increased in men with PTSD vs. controls, but fructose levels associated negatively with PCL scores. Box plot analysis: Mann-Whitney and $p<0{\cdot}05$ considered significant.

Figure 2. PTSD-specific alterations in amino acids with respect to their biosynthesis pathway in humans from glucose. (A) Essential amino acids cannot be synthesized and must be obtained from diet, whereas non-essential amino acids can be synthesized from glucose as it enters the tricarboxylic cycle. Essential amino acids are shown in grey boxes, and those metabolites synthesized as a by-product of gut

microbiome are shown in green boxes. Specific amino acids that were increased are shown in red, decreased in blue (women-specific outlined in pink and men-specific in blue). (B) Sex-specific disturbances in sleep measures. Box plots showing sex- and/or PTSD-specific alterations in Total sleep time (in minutes) decreased; Two-way ANOVA: Sex: ns; PTSD: p= 0·004; Sex X PTSD: ns. Sleep quality worsened, Twoway ANOVA: Sex: ns; PTSD: p< 0·0001; Sex X PTSD: ns, and log-transformed delta power sleep was lower in people with PTSD vs controls, Two-way ANOVA: Sex: p= 0·007; PTSD: p= 0·005; Sex X PTSD: ns. Greater PTSD symptoms (reflected by the total PCL score) negatively associated with TST (r= –0·41, p=0·005 and r= –0·32, p=0·033, women and men, respectively), and PSQI was positively associated with greater PTSD symptoms (r=0·84 and r=0·74, p<0·001, women and men, respectively). Delta power negatively associated with PTSD (*r*= –0·35, *p*=0·02) in men alone. (C) Linear regression showing association of PCL scores with three different measures of sleep. (D) Sex differences in primary metabolites after accounting for sleep measures. ChemRich analysis of primary metabolites in men and women with PTSD compared with controls after adjusting for BMI, age, and one of the three sleep measures shown (TST (min), PSQI, or log-transformed delta power (ln(μV2)). Blue nodes contain metabolite clusters that are decreased, purple nodes contain metabolites that are both increased or decreased, and red nodes contain metabolites that are increased in PTSD vs control individuals.

Figure 3. Sex-specific contribution of sleep variables on primary metabolites. (A) Venn diagrams to visualize significant metabolites While adjusting for one sleep variable at a time. (B) Linear regression and box plots of various amino acids with sleep variables. Box plots of specific amino acids with individual nodes that differed between women and men with PTSD compared to controls. (C) Log-transformed delta power (ln(μV2) associated negatively with testosterone in men. (D) Changes in insulin and tryptophan

levels in women and men with PTSD. Box plots of tryptophan and albumin levels in women and men. No significant differences were seen in tryptophan and albumin levels in women with PTSD compared with controls, whereas tryptophan levels were significantly elevated in men with PTSD compared with controls ($p = 0{\cdot}044$; Mann-Whitney). (E) Blood insulin levels were determined after an oral glucose challenge at 6 various times shown. Mixed-effect analysis showed that insulin levels changed with time ($p<0{\cdot}0001$) in women and men, but only differed between PTSD and controls in men ($p=0{\cdot}0136$). (F) Tryptophan levels correlated negatively with insulin levels at 60 min in women ($r= -0{\cdot}30$, $p=0{\cdot}04$), but positively in men, although the relationship did not reach statistical significance.

Figure 4. Sex-specific alterations in the tryptophan metabolism pathway in PTSD. (A) Sex, sleep, and PTSD all alter primary metabolites. (B) Albumin-bound tryptophan is present in circulation and dynamic increases in insulin promote binding of albumin to tryptophan, whereas esterified fatty acids can displace tryptophan from albumin. Free tryptophan is then transported to the brain by a transport carrier. Several amino acids, such as leucine, valine etc. compete with tryptophan for binding to the transport carrier, which can decrease influx of free tryptophan into the brain. Reduced free tryptophan levels in the brain can influence production of serotonin and melatonin, affecting brain function and sleep. In the gut, tryptophan is converted to indoles by the action of microbes such as *Lactobacillus*; these indoles have protective effect on gut barrier and immune functions. Serine can serve as NMDA receptor agonist and alter neuronal function. Thus, disturbances at multiple levels in tryptophan pathway may contribute to pathogenesis of PTSD.

References

1. Barupal DK, Fiehn O. Chemical Similarity Enrichment Analysis (ChemRICH) as alternative to biochemical pathway mapping for metabolomic datasets. Sci Rep 2017;7:14567.
2. Galatzer-Levy IR, Bryant RA. 636,120 Ways to Have Posttraumatic Stress Disorder. Perspect Psychol Sci 2013;8:651-62.
3. Irino Y, Toh R, Nagao M, et al. 2-Aminobutyric acid modulates glutathione homeostasis in the myocardium. Sci Rep 2016;6:36749.

4. Neame S, Safory H, Radzishevsky I, et al. The NMDA receptor activation by d-serine and glycine is controlled by an astrocytic Phgdh-dependent serine shuttle. Proc Natl Acad Sci U S A 2019;116:20736-20742.
5. Davis M. NMDA receptors and fear extinction: implications for cognitive behavioral therapy. Dialogues Clin Neurosci;13:463-74.

6. Mellon SH, Bersani FS, Lindqvist D, et al. Metabolomic analysis of male combat veterans with post traumatic stress disorder. PLoS One 2019;14:e0213839.
7. Somvanshi PR, Mellon SH, Flory JD, et al. Mechanistic inferences on metabolic dysfunction in posttraumatic stress disorder from an integrated model and multiomic analysis: role of glucocorticoid receptor sensitivity. Am J Physiol Endocrinol Metab 2019;317:E879-E898.
8. Richards A, Metzler TJ, Ruoff LM, et al. Sex differences in objective measures of sleep in post-traumatic stress disorder and healthy control subjects. J Sleep Res 2013;22:679-87.
9. Daniel PM, Love ER, Moorhouse SR, et al. The effect of insulin upon the influx of tryptophan into the brain of the rabbit. J Physiol 1981;312:551-62.

Contributors

AB, TCN, and SSI contributed to the design of the study. AB wrote the first draft of the manuscript with input from SSI, and AB, TCN, and SSI revised the final manuscript. CL and SSI collected patient data, RR, AO, SF, OF, SSI, and AB extracted and analyzed the data.

All other authors declare no competing interests. The views, opinions and/or findings contained in this research are those of the authors and do not necessarily reflect the views of the Department of Defense, Department of Veteran Affairs, or NIH and should not be construed as an official DoD/Army/VA/NIH position, policy, or decision unless so designated by official documentation. No official endorsement should

Disclosures be

made.

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