Investigating Peripheral and Central Inflammation in Individuals with PTSD and Military Sexual Trauma

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Background

Military sexual trauma (MST) is defined as any "sexual harassment that is threatening in character or physical assault of a sexual nature that occurred while the victim was in the military" [15]. A recent meta-analysis demonstrated that 38.4% of female military service members and veterans report experiencing MST [10] and there has been a steady rise of reported cases, with an approximate 70% increase from 2012-2014 [18]. Though women have an estimated 20 times higher likelihood of experiencing MST [9], according to VA intake records, an approximate 40% of veterans who report MST are male [11].

MST is highly associated with post-traumatic stress disorder (PTSD). Veterans who are MST survivors show a 5-8 times increase in PTSD in women and 3-6 times increase in men [9]. Furthermore, MST-related PTSD made up 94% of MST-related claims to the VA from 2010-2013 [18]. Those who reported MST were also twice as likely to report suicidal ideation (SI) than non-MST traumas and when specifically compared with combat trauma, those who experienced MST were four-times more likely to report SI [10]. These increases in SI are especially worrisome when considering how often MST occurs and the observed increasing rates. MST is also a greater predictor of PTSD development than other trauma sources, including sexual trauma not experienced during military service [10], and is predictive of higher rates of mental health visits, which may be an indicator of more resistant PTSD symptoms [18].

There is increasing evidence that trauma type relates to PTSD symptom severity, which can be seen in the differing symptomatology between patients with PTSD from MST (PTSD/MST) and patients with PTSD from other trauma sources differs [10]. Additionally, those with MST have been found to have significantly more severe clinician-rated symptoms compared with any other trauma type [10]. PTSD/MST patients are also known to have

differences in physical health outcomes. While patients with PTSD are at higher risk of developing an autoimmune disorder, those with PTSD from MST show a significantly further increased risk of the same disorders [6]. This elevated autoimmunity risk suggests that there are immune disruptions in conjunction with PTSD development which are heightened by MST.

PTSD is a prevalent psychiatric condition that can develop after a life threatening or life-altering trauma is experienced or witnessed [16]. Lifetime prevalence estimates range anywhere from 4-12% within the United States [16] with an average 15% chance of PTSD development after trauma, though this increases up to 30% in those deployed to combat zones [17]. Lifetime prevalence worldwide is about 3.9% [5]. Possible traumas include domestic abuse, car accidents, fires and other natural disasters, and sexual assault. Military combat trauma (MCT) and MST are two risk factors that military personnel may face for developing PTSD [9]. This disorder is characterized by four main symptom groups: re-experiencing (e.g., flashbacks or nightmares), avoidance (e.g., withdrawing and avoiding places, people, or activities which may be associated with the trauma), negative cognition or mood (e.g., difficulty recalling key features of trauma or depression), and hyperarousal (e.g., anxiety or hypervigilance) [5].

Those in the military have increased chances of being exposed to trauma and therefore may be more likely to develop PTSD [9], though trauma exposure is universal. An estimated 50-60% of the population will experience a trauma that would meet PTSD diagnostic criteria [16]. However, not everyone who experiences a traumatic event will develop PTSD [5]. This has sparked a field of research that looks at risk factors, both biological and environmental, that make people more susceptible to developing PTSD [8]. Biological indications, or biomarkers, can be found using a variety of methods, including the analysis of molecules in biological specimens [8]. These biomarkers have the potential to be a great asset in testing for biological

risk and treatment of PTSD [8]. PTSD biomarker research and discovery are especially important for groups that are faced with increased environmental risk, such as the veteran population and active duty military [8]. Biomarkers which indicate inflammation, such as pro-inflammatory cytokines are one such marker of interest.

Chronic, low-grade inflammation is commonly seen in PTSD [4] and is a prominent candidate for why observed immune disruptions, in PTSD and MST, occur [6]. PTSD is a stress condition marked by physiological changes to the hypothalamic-pituitary adrenal axis (HPA axis). Initial signaling of the pituitary to the adrenal gland leads to increases in cortisol and norepinephrine (NE), which in turn can lead to increased release of pro-inflammatory cytokines [5]. These cytokines can signal back to the hypothalamus, as well as certain CNS glial cells, which can lead to a rise in neural inflammation [5]. When PTSD develops, continued signaling of the pituitary and extended release of adrenocorticotropic hormone (ACTH) can lead to suppressed cortisol levels and a dysfunction of the HPA axis [5], triggering a perpetual cycle, of increase NE and epinephrine which cause extended inflammatory cytokine release, that drives PTSD pathology [5].

Specific inflammatory cytokines, including IL-1β [4,5], IL-6 [4,5], INF-γ [4], and TNF-α [4,5] have been found at higher levels in PTSD patients compared to healthy controls (HC) [4,5]. These pro-inflammatory cytokines are associated with decreased neurogenesis, which can lead to volume reductions throughout the cortex, and specifically within the hippocampus and the prefrontal cortex [4]. Greater inflammation marked by elevated IL-6 levels in the peripheral bloodstream have been found to associate with worse PTSD symptom experience [19]. PTSD is also associated with multiple inflammation-related disorders and these associations are broadly reaching, suggesting that PTSD-associated inflammation affects the whole body [20]. However,

the direct mechanistic link between PTSD symptomology and inflammation has yet to be elucidated.

Inflammation has also been found to lead to interruption in immune cell functioning, immune system maintenance [6], and blood-brain barrier (BBB) permeability [3]. A more permeable BBB plays a role in pro-inflammatory cytokines passing from the peripheral nervous system (PNS) to the central nervous system (CNS) [3]. Furthermore, corticosteroids and pro-inflammatory cytokines themselves can be packaged into cellular vesicles, known as exosomes, that are released from cells, including astrocytes and neurons in the CNS [3].

Exosomes are a form of extracellular vesicle (EVs), which are particles that travel throughout the body, budding off from almost every type of cell in the body, to carry cargo for cell communication [1]. These bodies come in a few sizes, the smallest of which, exosomes, are about 40-200nm, while the largest, apoptotic bodies, are 500-2000nm [3]. They can be released from cells in the CNS and PNS and communicate to widespread areas of the body, even crossing the BBB to carry cargo from the PNS to the CNS and vice versa [3]. These particles have been shown to play a significant role in many biological functions, including basic cell communication, synaptic function, plasticity, and immune responses [3].

This was exemplified in research by Kobayashi et. al., who found that exosomal neprilysin (eNEP) derived from the masseter muscle (MM), a facial muscle which connects from the mandible to the cheekbone and is important in chewing solid foods, was being transported to the hippocampus via the trigeminal nerve, the fifth cranial nerve (responsible for sensation in the face and facial motor movements), via EV transport [2]. Neprilysin (NEP) is known to play a major role in clearing amyloid-β, which builds up in plaques in the brain in Alzheimer's Disease [2]. This provides evidence for how NEP is involved in clearing amyloid-β, without NEP being

made in the brain [2]. Researchers suggest that deteriorating oral health impairs this EV transport method and may play a major role in amyloid-β build up [2].

Moreover, it has also been found that EVs more readily cross into the CNS with increased immune or inflammatory response, as a result of BBB permeability [3]. When lipopolysaccharide, which is found in the outer membrane of certain bacteria and is a driver of the inflammatory response [12], was introduced experimentally into the PNS, the permeability of the BBB and EVs' ability to cross it increased significantly [3]. This poses an interesting question in the role of EVs in psychiatric disorders, especially with inflammation, and their comorbid physical health problems.

The current study aims to investigate inflammatory differences between controls and PTSD patients, as well as between those with PTSD from MST and those with PTSD from all other sources. This will be carried out via the isolation of the smallest class of EVs, exosomes, and the analysis of their inner cytokine cargo via ELISA cytokine assays, looking specifically at pro-inflammatory cytokines (IL-1β, IL-2, IL-6, IFN-ɣ, and TNF-α) and IL-10, a regulatory, or more anti-inflammatory, cytokine. We also seek to identify associations between specific symptoms and cytokine levels, differences in those associations between central and peripheral inflammation, and differences in those associations between groups. This could lead to a better understanding of the symptom differences in PTSD from differing trauma types as well as how these symptoms are promoted biologically.

Methods

Participants

Thirty-two participants were selected from an existing subject pool within the biorepository for CESAMH and separated into three groups: healthy controls (10), PTSD (12),

and PTSD from MST (10). Those in the PTSD/MST group reported MST, as well as sexual assault, and were diagnosed with PTSD. For all participants, we collected peripheral blood plasma samples and psychological symptom data, collected via self-report questionnaires.

Assessments

In order to quantify the mental health status of participants, the Biorepository administers a series of self-report questionnaires at each of three visits, across six to nine months. We used recorded data from first visits only. Among these questionnaires, we utilized the PCL-5 as a measure of PTSD symptoms and severity, the PHQ-9 for depression symptoms and severity, the MASQ to assess anhedonia (the measure of how much pleasure one is getting out of life), the PROMIS for pain data (both intensity and interference with day to day life and ability to concentrate), the AUDIT for alcohol use, and the ISI for insomnia assessment. Military sexual trauma is determined by participant's answers to the LECQ8, where a score of 1 equates to having experienced an MST, which is further specified as "happened to me" or "learned about it."

For the purposes of analysis, the PCL-5 was assessed for correlations in summary score (possible scores range 0-80, where anything equal to or above thirty-three is considered PTSD), as well as four subcluster scores. Subclusters align with symptom categories defined in the DSM-V. These are: re-experiencing (B), avoidance (C), negative cognition or mood (D), and hyperarousal (E). The PHQ-9 was assessed using summary score (possible scores range 0-27, with cutoffs between 5-20 for increasingly severe depression), as well as individual items 1-9. The MASQ was assessed using a summary score (possible scores range 0-110 for level of anhedonic depression). The PROMIS was assessed using two separate summary scores, one for pain intensity (range 3-15) and one for pain interference (range 6-30). The AUDIT was assessed

using a summary score (where a score of 8 or higher is considered hazardous drinking). The ISI was assessed using a summary score (range 0-28), where 22 and up is considered severe insomnia.

Exosome Isolation

The exosome isolation protocol (using System Biosciences (SBI) kit) took place over two days. On the first day, Streptavidin Magnetic Exo-Flow Beads were washed with a Bead Wash Buffer. After washing, one of two antibodies were added to the beads, CD171 (eBioscience) or GLAST(ASCA-1) (Miltenyi Biotec). CD171 was used to isolate neuronally-derived exosomes (NDEs) and GLAST(ASCA-1) was used to isolate astrocyte-derived exosomes (ADEs). 250uL of human plasma was isolated from each sample and 2uL of purified thrombin was added to it. After incubation, this mixture was centrifuged, and the supernatant was collected. 63uL of ExoQuick Exosome Precipitation Solution (SBI) was then added to the exosome enriched supernatant and the new mixture was centrifuged at 1500xg. The pellet that formed was isolated and washed with PBS with protein inhibitor cocktail (PIC; manufacturer) and sonicated. This was then centrifuged once more. The exosome pellet that formed was then isolated, resuspended, and added to Streptavidin beads. This mixture was kept in a cold room on a rotator overnight.

On day two, the supernatant from all samples was removed, with the exosomes attached to the magnetic streptavidin beads. After being washed, 300uL of Exosome Elution Buffer (SBI) was added to the beads and the samples were rotated for 30 minutes. The supernatant (containing all exosomes) was extracted and MSD Lysis buffer was added to extract proteins. This solution was stored at -80°C until protein analysis could be performed.

FACS

Flow cytometry was used to fluorescently label exosomes using Exo-FITC Exosome FACS stain (SBI). This was done for each new exosome kit. This allowed us to measure how many exosomes we were successfully isolating and was performed with each new kit as a validation measure for the exosome isolation method.

Protein Analysis

We used ELISA analysis to measure cytokine levels. We used MSD multiplex cytokine ELISA plates, for this purpose. These plates are 96-well plates, wherein each well has 10 analyte spots. We prepped for 6 cytokine assays. First, a U-PLEX linker-coupled antibody solution was made according to manufacturer's instructions. Thawed exosome samples were lysed 1:5 in MSD lysis buffer. Exosome and plasma samples were diluted 1:2 in MSD sample diluent provided by MSD. MSD ELISAs were then run according to manufacturer protocol and samples were run in duplicate. We specifically looked at IL-1β, IL-2, IL-6, and IL-10, INF-γ, and TNF-α.

Statistical Analysis

In our ELISA results, there were a fair amount of non-detectables. In order to perform statistical analysis with cytokine data, we imputed, or substituted, non-detectables with a value equal to the lowest observed value within a cytokine, divided by 2. Ex: for IL-1β (across tissue types), the lowest observed value was .00450218, halved is .00225109, which was then the value used for all non-detectables for IL-1β. This value was unique for each cytokine. These values were imputed this way because each cytokine had its own standard curve. In order to ensure we did not add value to the cytokine that wouldn't have been there, this very small number was used. Sample cytokine concentrations were measured as the optical density of each cytokine analyte within the spot as detected on an MSD plate reader, and compared to the known

concentrations measured in MSD cytokine kit standards, using MSD Discovery Workbench software. ANOVAs and spearman correlations were run on cytokines and psychological symptoms measures using SPSS version 25.

Results

Group Demographics

All groups were majority male (HC: 80%, PTSD: 83%, PTSD/MST: 70%), caucasian (HC: 50%, PTSD: 58.3%, PTSD/MST: 40%), and non-hispanic (HC: 70%, PTSD: 66.7%, PTSD/MST: 70%). See Table 1 for full demographic break down. Average age for all groups was approximately forty years old (HC: 43.2, PTSD: 42.2, PTSD/MST: 38.9).

Table 1. Demographics

	Controls	PTSD	PTSD/MST
Gender	Male: 8 Female: 2	Male: 10 Female: 2	Male: 7 Female: 3
Age	M: 43.2 SD: 13.9	M: 42.2 SD: 10.6	M: 38.9 SD: 12.3
Race	50% Caucasian 20% Black 10% Asian 10% Other 10% Declined	58.3% Caucasian 16.7% Black 16.7% Asian	40% Caucasian 30% Black 20% Other
Ethnicity	70% white/non-Hispanic	66.7% white/non-Hispanic	70% white/non-Hispanic

Group Symptom Severities

All groups were compared against each other for mean scores on all psychological symptom measures using a one-way ANOVA. Participants with either PTSD or PTSD/MST demonstrated higher PCL-5 and PHQ-9 scores, as would be expected. PCL-5 total scores were found to be significantly higher in the PTSD and PTSD/MST groups compared to controls ($F(2,29) = 77.535$, $p < .001$), but not between the PTSD and PTSD/MST groups. A significant difference was also found between the control group with both PTSD and PTSD/MST groups,

but not between the PTSD and PTSD/MST group for PCL-5 subclusters B-D ($F(2,29) = 30.695$, $p < .001$, $F(2,29) = 28.653$, $p < .001$, $F(2,29) = 44.705$, $p <, .001$), re-experiencing (B), avoidance (C), and negative cognition or mood (D) respectively. PCL-5 subcluster E, hyperarousal, showed both significant differences between controls, PTSD, and PTSD/MST groups, and between the PTSD ($M = 15$, $SD = 2.73$) and PTSD/MST ($M = 12.1$, $SD = 2.33$) groups, ($F(2,29) = 59.205$, $p < .001$). See table 2 for all psychological symptom information.

Significant differences were found between groups for PHQ-9 totals, as well as some individual items of the measure. Item 1, which measure anhedonia, ($F(2,29) = 4.27$, $p = .024$), Item 2, which measures feelings of depression, ($F(2,29) = 5.97$, $p = .007$), item 5, which measures appetite, ($F(2,29) = 9.30$, $p = .001$, item 7, which measures concentration, ($F(2,29) = 13.59$, $p < .001$), item 8, psychomotor issues, ($F(2,29) = 5.807$, $p = .008$), and PHQ-9 totals ($F(2,29) = 11.39$, $p < .001$) all showed significant differences between controls and both PTSD and PTSD/MST groups, but not between the PTSD and PTSD/MST groups. Item 6 ($F(2,29) = 6.48$, $p = .005$) showed a significant difference between the control group and PTSD group only. See tables 3 and 4 for information about all sub-measures.

We also found significant differences in the MASQ anhedonic depression scores and ISI insomnia scores. The MASQ scores were found to be significant ($F(2,29) = 4.64$, $p = 0.18$) between the control group and the PTSD group. The ISI scores were found to be significant ($F(2,29) = 6.47$, $p = .005$) between the control group and both PTSD and PTSD/MST groups, but not between PTSD and PTSD/MST groups. No significant differences were found between groups for either PROMIS measure and pain interference, or AUDIT alcohol use scores.

Comparing Plasma and Exosome Cytokine Levels

In order to assess the measured differences in peripheral and central cytokines, we ran spearman correlations between plasma cytokine levels, NDE levels, and ADE levels. This and all other correlation analyses were run using spearman due to non-normal distribution of cytokine data. Analysis of IL-2 had correlations across all tissue types: plasma-NDE ($\rho(32) = .502$, $p = .003$), plasma-ADE ($\rho(32) = .516$, $p = .003$), NDE-ADE ($\rho(32) = .826$, $p < .001$). IL-6 had correlations between exosomes only ($\rho(32) = .595$, $p < .001$). IL-1β ($\rho(32) = .497$, $p = .004$) and the composite cytokine values ($\rho(32) = .789$, $p < .001$) also showed correlations between exosomes only. This composite cytokine value was computed using four of the six cytokines assayed for. IL-10 was excluded because it is more regulatory than inflammatory and IFN-ɣ was excluded due to large variations in measured cytokine between samples, wherein observed values ranged from 1.2 pg/mL to 240.58 pg/mL. These in the upper range were outliers between 60 and 200-fold above the mean, which would have otherwise skewed the mean composite score quite dramatically. The same composite value was used for all data analysis (IL-1β, IL-2, IL-6, TNF-α). Other cytokines showed less consistent results. See table 5 for complete tissue type correlations.

Table 2: Group Symptom Severities – Mean and Standard Deviation

	PCL-5	PHQ-9	PROMIS intensity	PROMIS interference	MASQ	AUDIT	ISI
Control N = 10	M: 11.0** SD: 7.44 *2,3	M: 5.20** SD: 3.55 *2,3	M: 5.50 SD: 2.84	M: 11.4 SD: 6.31	M: 60.2* SD: 13.8 *2	M: 2.33 SD: 3.83	M: 8.70* SD: 5.50 *2,3
PTSD N = 12	M: 49.8** SD: 7.71 *1	M: 15.5** SD: 6.60 *1	M: 7.81 SD: 3.22	M: 8.43 SD: 2.54	M: 76.3* SD: 8.27 *1	M: 6.56 SD: 5.77	M: 17.0* SD: 6.00 *1
PTSD/MST N = 10	M: 44.9** SD: 8.09 *1	M: 14.4** SD: 5.34 *1	M: 7.30 SD: 2.26	M: 14.1 SD: 6.62	M: 70.6 SD: 15.0	M: 9.71 SD: 9.53	M: 15.6* SD: 5.42 *1

*ANOVA significance marked (*0.05, **0.01), groups where significance was found denoted by number markers (1-controls, 2-PTSD, 3-PTSD/MST) [between that row and those listed]

Table 3: Group Symptom Severities – PCL-5 Clusters

	Control	PTSD	PTSD/MST
B: Re-experiencing	M: 2.10** SD: 2.13 *2,3	M: 12.3** SD: 3.70 *1	M: 10.9** SD: 3.48 *1
C: Avoidance	M: 0.90** SD: 1.10 *2,3	M: 5.08** SD: 1.62 *1	M: 5.30** SD: 1.64 *1
D: Negative Cognition or Mood	M: 4.30** SD: 3.62 *2,3	M: 17.4** SD: 3.06 *1	M: 16.6** SD: 3.98 *1
E: Hyperarousal	M: 3.70** SD: 2.31 *2,3	M: 15.0** SD: 2.73 *1,3	M: 12.1** SD: 2.33 *1,2

*ANOVA significance marked (*0.05, **0.01), groups where significance was found denoted by number markers (1-controls, 2-PTSD, 3-PTSD/MST) [between that row and those listed]

Table 4: Group Symptom Severities – PHQ-9 Items

	Control	PTSD	PTSD/MST
PHQ-9: 1	M: 0.50* SD: 0.972 *2,3	M: 1.58* SD: 0.996 *1	M: 1.60* SD: 0.966 *1
PHQ-9: 2	M: 0.70* SD: 0.949 *2,3	M: 1.83* SD: 0.937 *1	M: 1.90* SD: 0.738 *1
PHQ-9: 3	M: 1.30 SD: 1.25	M: 2.25 SD: 1.06	M: 2.20 SD: 1.03
PHQ-9: 4	M: 1.30 SD: 0.823	M: 2.25 SD: 0.965	M: 1.70 SD: 0.949
PHQ-9: 5	M: 0.30* SD: 0.483 *2,3	M: 1.92* SD: 0.996 *1	M: 1.70* SD: 1.16 *1
PHQ-9: 6	M: 0.60* SD: 0.516 *2	M: 2.00* SD: 1.13 *1	M: 1.60 SD: 0.966
PHQ-9: 7	M: 0.50* SD: 0.527 *2,3	M: 2.00* SD: 0.953 *1	M: 2.00* SD: 0.667 *1
PHQ-9: 8	M: 0.00* SD: 0.00 *2,3	M: 1.00* SD: 0.953 *1	M: 1.10* SD: 0.944 *1
PHQ-9: 9	M: 0.00* SD: 0.00 *2,3	M: 0.667* SD: 0.779 *1	M: 0.600* SD: 0.966 *1

*ANOVA significance marked (*0.05, **0.01), groups where significance was found denoted by number markers (1-controls, 2-PTSD, 3-PTSD/MST) [between that row and those listed]

Table 5: Correlations Between Tissue Types (Plasma, ADE, and NDE)

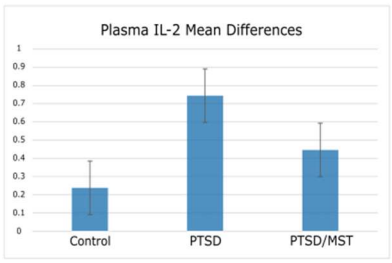
		Plasma	ADE	NDE
IL-1β	Plasma			
	ADE			ρ: 0.497 ; p = .004
	NDE		ρ: 0.497 ; p = .004	
IL-2	Plasma		ρ: 0.516 ; p = .003	ρ: 0.502 ; p = .003
	ADE	ρ: 0.516 ; p = .003		ρ: 0.826 ; p < .001
	NDE	ρ: 0.502 ; p = .003	ρ: 0.826 ; p < .001	
IL-6	Plasma			
	ADE			ρ: 0.595 ; p < .001
	NDE		ρ: 0.595 ; p < .001	
IL-10	Plasma		ρ: 0.502 ; p = .003	
	ADE	ρ: 0.502 ; p = .003		ρ: 0.799 ; p < .001
	NDE		ρ: 0.799 ; p < .001	
IFN- ɣ	Plasma		ρ: 0.364 ; p = .040	
	ADE	ρ: 0.364 ; p = .040		
	NDE			
TNF-α	Plasma		ρ: 0.558 ; p = .001	ρ: 0.368 ; p = .038
	ADE	ρ: 0.558 ; p = .001		
	NDE	ρ: 0.368 ; p = .038		

Table 5: Empty boxes: no relationship found. Spearman's rho correlation co-efficient and significance p-value (at 0.05 two-tailed) provided

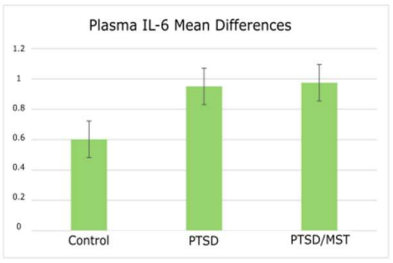
Cytokine Mean Differences

We ran one-way ANOVAs between all groups for each tissue type in each of the cytokines we assayed for. Initial analysis has shown a general main effect where pro-inflammatory cytokine levels are higher in the PTSD groups compared to the control group. This

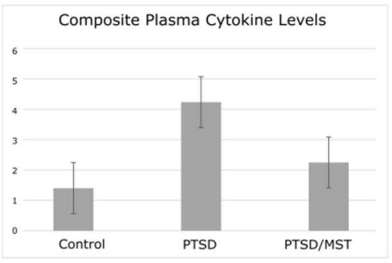
Figure 1: Mean Cytokine Differences Across Plasma and ADEs for IL-2, IL-6, and Composite Cytokine Summary Level



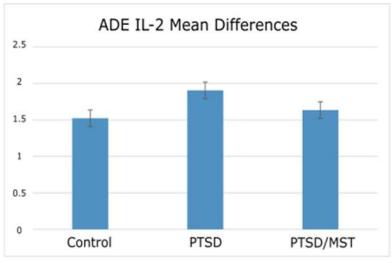
A. Mean levels of IL-2 (pg/mL) in plasma



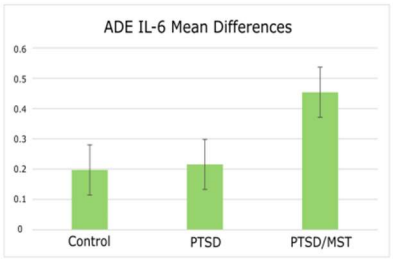
B. Mean levels of IL-6 (pg/mL) in plasma



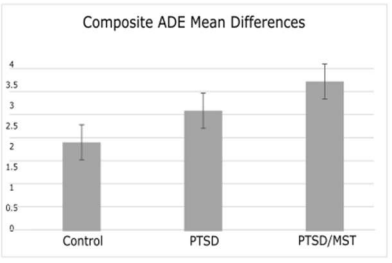
C. Mean levels of cytokines (pg/mL) summated in plasma



D. Mean levels of IL-2 (pg/mL) in astrocyte-derived exosomes (ADEs)



E. Mean levels of IL-6 (pg/mL) in ADEs



F. Mean levels of cytokines (pg/mL) summated in ADEs

effect was not significant from this preliminary data, however full study data with a larger sample size could prove to hold significant differences. Plasma IL-6 showed a steady increase from controls to PTSD to PTSD/MST groups (fig.1B). It should be noted that plasma IL-6 had no non-detectables. Most other plasma measures showed the largest increase in mean for the PTSD group, where PTSD/MST was then less than or about equal to the PTSD group mean. There is a slight trend in ADE cytokine levels. In IL-6, ADE levels increased in the PTSD/MST group (fig.1E). This was also seen in TNF-α, and in the ADE composite (fig.1F). For complete mean and standard deviation information for all cytokines, see Table 6. Effect sizes were

calculated for plasma, ADE, and NDE between control-PTSD, control-PTSD/MST, and PTSD-PTSD/MST for IL-2, IL-6, and the composite values. These cytokines were chosen because they had the lowest percentage of non-detectables. Plasma IL-6 effect size between controls and PTSD groups was found to be .59 and ADE IL-6 effect size between PTSD and PTSD/MST groups was found to be .40. Based on these effect sizes, we are underpowered at this time to find cytokine differences between groups.

Table 6: Mean and Standard Deviation for all Assayed Cytokines and all Tissue Types

		Non-Detectables	Control	PTSD	PTSD/MST
IL-1β	Plasma	34.4%	0.070 ; 0.113	0.193 ; 0.483	0.068 ; 0.081
	NDE	71.8%	0.208 ; 0.436	0.292 ; 0.733	0.027 ; 0.048
	ADE	65.6%	0.079 ; 0.235	0.201 ; 0.418	0.112 ; 0.171
IL-2	Plasma	40.6%	0.238 ; 0.332	0.744 ; 1.77	0.446 ; 0.489
	NDE	18.8%	2.32 ; 3.43	2.24 ; 4.40	1.66 ; 1.78
	ADE	28.1%	1.60 ; 2.36	2.01 ; 2.86	1.72 ; 1.93
IL-6	Plasma	0%	0.603; 0.421	0.951 ; 0.758	0.975 ; 0.959
	NDE	65.6%	0.145 ; 0.262	0.651 ; 2.14	0.127 ; 0.204
	ADE	46.9%	0.197 ; 0.373	0.216 ; 0.316	0.454 ; 0.986
IL-10	Plasma	28.1%	0.099 ; 0.099	0.143 ; 0.244	0.152 ; 0.157
	NDE	84.4%	0.109 ; 0.301	0.119 ; 0.381	3.32E-05 ; 0.000
	ADE	78.1%	0.023 ; 0.066	0.031 ; 0.092	0.030 ; 0.064
IFN- ɣ	Plasma	31.3%	19.6 ; 33.9	26.9 ; 64.2	33.2 ; 64.8
	NDE	93.8%	12.7 ; 39.6	20.2 ; 69.4	0.148 ; .000
	ADE	87.5%	0.148 ; .000	13.4 ; 45.8	5.00 ; 14.9
TNF-α	Plasma	31.3%	0.495 ; 0.443	2.39 ; 6.19	0.765 ; 0.872
	NDE	78.1%	0.087 ; 0.121	1.57 ; 5.33	0.461 ; 0.712
	ADE	75.0%	0.048 ; 0.059	0.190 ; 0.399	0.962 ; 2.57

Table 6: Formatted as, mean ; standard deviation. Non-detectables were calculated by dividing the number of "0"s by the total number of samples (32) multiplied by 100.

Table 7a-c: Correlation Data For IL-2, IL-6, and Composite Value

a. IL-2 Correlations

	Plasma	NDE	ADE
PHQ-9: item 1		ρ: 0.356 ; p = .046	ρ: 0.489 ; p = .005
PHQ-9: item 2			ρ: 0.350 ; p = .049
PHQ-9: item 7			ρ: 0.364 ; p = .041
PROMIS: pain intensity	ρ: 0.357 ; p = .049		

PHQ-9, item 1: little interest or pleasure in doing things, item 2: feeling down, depressed, or hopeless, item 7: trouble concentrating on things, all scored 0-3. Spearman's rho correlation coefficient and significance value (at 0.05 two-tailed) provided. One participant's data was missing for PROMIS data

b. IL-6 Correlations

	Plasma	NDE	ADE
PHQ-9: item 1			ρ: 0.394 ; p = .026
PROMIS: pain interference	ρ: 0.512 ; p = .003		
PROMIS: pain intensity	ρ: 0.405 ; p = .024		
ISI	ρ: 0.377 ; p = .033		

PHQ-9, item 1: little interest or pleasure in doing things, scored 0-3. Spearman's rho correlation coefficient and significance value (at 0.05 two-tailed) provided. One participant's data was missing for PROMIS data

c. Composite Correlations

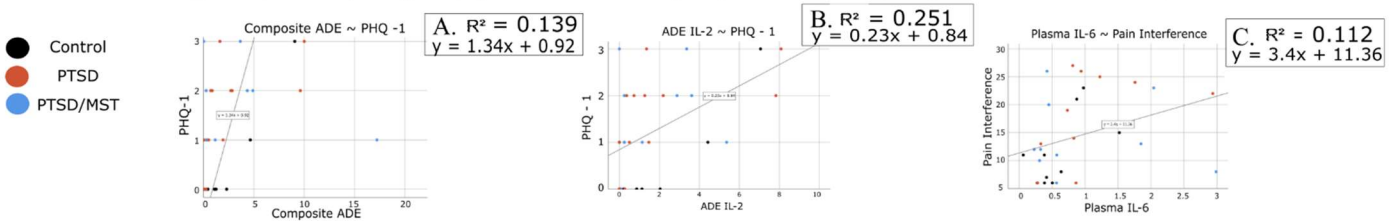
	Plasma	NDE	ADE
PHQ-9: item 1		ρ: 0.463 ; p = .008	ρ: 0.394 ; p = .026
PROMIS: pain interference	ρ: 0.428 ; p = .016		
PROMIS: pain intensity	ρ: 0.464 ; p = .009		
ISI	ρ: 0.357 ; p = .045		

PHQ-9, item 1: little interest or pleasure in doing things, scored 0-3. Spearman's rho correlation coefficient and significance value (at 0.05 two-tailed) provided. One participant's data was missing for PROMIS data

Cytokines and Psychological Symptoms

In order to assess associations between cytokine levels and psychological symptoms, spearman correlations were run between IL-2, IL-6, and composite cytokine values across all tissue types and all psychological symptom measures. Correlations were first run for our total sample (n = 32) and then for individual groups. No significant correlations were found between cytokines and PCL-5 scores using the total sample, however when tested by individual group, a correlation was found between IL-6 NDE and PCL-5 D (negative cognition or mood) subcluster and IL-6 ADE and PCL-5 E (hyperarousal) subcluster for the PTSD group. For PHQ-9 measure

Figure 2. Cytokine-Psychological Symptom Correlation Scatterplots (black dots - controls, orange - PTSD, blue - PTSD/MST)



A. x-axis: composite ADE level (pg/mL), y-axis: PHQ-9, item 1 (little interest or pleasure in doing things) score (1-3)

B. x-axis: IL-2ADE level (pg/mL), y-axis: PHQ-9, item 1 (little interest or pleasure in doing things) score (1-3)

C. x-axis: IL-6 plasma level (pg/mL), y-axis: PROMIS pain interference score, scored 6-30

items and total score, significant correlations were found with item 1 ("I have little interest or pleasure in doing things; 0 = not at all, 3 = nearly every day) and ADE composite levels ($\rho(32) = .463$, $R^2 = 0.139$, $p = .008$; fig.2A), and ADE IL-2 levels ($\rho(32) = .489$, $R^2 = 0.251$, $p = .005$; fig.2B). We also found a correlation between plasma IL-6 levels and PROMIS pain interference scores ($\rho(31) = .512$, $R^2 = .112$, $p = .003$; fig.2C). PROMIS data was missing for one participant. An association was also found between pain interference and composite NDE cytokine levels in the PTSD/MST group. Table 7(a-c) shows correlation data. See supplemental material for all found correlations.

Discussion

The group symptom severities we found follow the current body of literature in PCL-5 and PHQ-9 measure differences. It is expected that the control group would have significantly lower scores for PTSD and depression measures. Along this same line, the differences in MASQ scores follow this expectation as well. Given that sleep disruptions are well documented in PTSD, the ISI differences also follow existing literature. Most of the differences we found were between the control group and both PTSD and PTSD/MST groups, but not between the PTSD and PTSD/MST groups themselves. This tells us that while it is clear these psychological components are affected by PTSD, they may not be affected by MST in particular.

In the case of the PCL-5 E subcluster (hyperarousal), the mean scores of the PTSD group were found to be significantly higher than the mean of those in the PTSD/MST group. The PCL-5 E subcluster was also found to significantly correlate with IL-6 ADE levels in the PTSD group only. Together, these findings could show that one, different traumatic incidents may lead to different symptom patterns and two, the possibility of differential biological responses between trauma types. Considering that central inflammation was found to correlate with this symptom cluster, future research should try to assess the relationship between inflammation and specific brain regions tied to PTSD.

Based on significant differences we found between cytokines measured in plasma versus those in exosomes, it appears that our exosome samples represent true measures of central inflammation. If we were measuring proteins bound from the periphery receptors on the outside of the exosomes and not the cargo inside, we would expect to see consistent correlations with exosomes and plasma. Though not all assayed cytokines showed correlations between exosome types only, IL-1β, IL-6, and composite cytokine values did. With the exception of IL-2, those

that showed correlations with plasma had more undetectables than those that showed exosome only correlations. Due to higher percentages of non-detectables, more of the values were the same imputed value. This means that these cytokines or tissue types had a limited range and variation, which could be part of why these showed correlations with plasma. Further work with exosomes is needed to confirm these results or determine if further work is needed to ensure isolation of exosome cytokine cargo.

At this time, cytokine measures have shown no significant differences between groups. However, we can see pattern trends in these measures, which given further study could prove significant. Plasma IL-6 levels showed a steady increase from controls to the PTSD groups. Given that plasma IL-6 had the best yields, with no undetectables, this may portray what future research could find consistently with more sensitive assay measures. We also found that, generally, plasma showed the highest mean cytokine levels in the PTSD group, while the PTSD/MST group yielded larger levels of cytokines in ADEs. This may indicate a biological difference in the body's response to different traumas. If these results are replicated consistently, this would mean that a biological mechanism is leading to more central inflammation with MST and more peripheral inflammation in other trauma types. Though further research would be required to discover this mechanistic link. Additionally, we have no differences between exosome types. Given that they are derived from different cell types, it would make sense for them to carry different cytokine patterns and this may be found in future studies. We do see trends of different cytokine patterns with our correlation analysis, as IL-6 NDE and ADE correlated with different PCL-5 subclusters. However, at present, there is no significant difference between NDE and ADE for levels of inflammatory cytokine cargo.

Given our small n per group, we are best able to form conclusions from those correlations found with our whole sample. The correlation between ADE and NDE composites, and ADE and NDE IL-2 with PHQ-9 item 1, which pertains to anhedonia, falls in line with existing literature which shows a relationship between anhedonia and inflammation. Research by Eisenberg, et al. (2010) showed that when pro-inflammatory cytokine levels increased, anhedonic symptoms did as well [13]. This study did not, however, separate central and peripheral inflammation. Given that ADE and NDE levels in particular were found to correlate, further research should look at central inflammation and anhedonia. Since both the composite levels and individual IL-2 ADE and NDE levels correlated, this suggests that IL-2 is driving the composite, but IL-6 ADE also correlated with this PHQ-9 item, and there may be other cytokines that might prove to be associated as well. However, at this time, only IL-2 and IL-6 were used for psychological measure correlations because they had the lowest percentages of non-detectables.

ADE IL-2 levels were found to correlate with item 2 and 7 of the PHQ-9 as well. These items do not relate to anhedonia, but more general depression symptoms such as feeling down (2) and concentration (7). The literature connecting depression and inflammation has grown considerably in the past years, so these associations are not surprising. However, it is interesting that these measures correlate with central inflammation and not peripheral inflammation, and furthermore that they correlate specifically with ADE. As previously stated, we have no significant data showing a difference between ADEs and NDEs, but these differential patterns in correlations could hint at how mechanistic differences in the brain may promote symptomatology.

Plasma IL-2 was found to be associated with pain intensity, and plasma IL-6 and composite levels were found to be associated with both PROMIS measures (intensity and

interference). These findings also complement existing literature which show a relationship between inflammation and pain, both in acute and chronic conditions [14]. It is intriguing that while PHQ-9 depression symptoms correlated with central inflammation, pain correlated with peripheral inflammation This could possibly be due to different biological mechanisms involved in psychological versus physical symptoms. However, it is also known that pain can be both exacerbated and quelled by top-down signaling and can be affected by psychological state. This duality may be further exemplified by the fact that pain intensity and interference were found to be associated with ADE IL-2 levels in the PTSD group. For these reasons, further research is needed to investigate different kinds of pain in conjunction with peripheral versus central inflammation, while controlling for psychological state.

Insomnia ISI scores were found to correlate plasma IL-6 and composite plasma cytokine levels. There is also a healthy body of literature which associates insomnia and sleep disturbances with inflammation. Sleep disturbances are also associated with higher risk of inflammatory disease [15]. Research by Irwin, et al. found that sleep disturbance was associated with IL-6 cytokine levels [15]. Our findings fit with this existing literature in that we found associations between inflammation and insomnia, and IL-6 in particular. It is interesting that these associations were only found in plasma. This could imply that insomnia or sleep disturbance leads to a build up of inflammation in the peripheral nervous system. Given the associations of PTSD with inflammation and PTSD with sleep disturbance, this may paint a troubling picture of further inflammatory cycles occurring in perpetuity. Insomnia was not found to correlate with any group individually.

We also ran correlations between all tested cytokines and psychological measure with gender to check for any sex-based effects. No significant differences, nor correlations were

found based on gender, however it should be noted that all groups were largely male. At this time, we have not matched for alcohol use or BMI, which are possible confounds for inflammation level. Although there were no significant differences found between groups for alcohol use, given the disparity in means between groups, this may still be an important confound which should be accounted for with larger sample sizes. In regards to the associations found between cytokines and PTSD symptoms, it is important to note that while these associations help tell a more complete story, at this time our n per group is small and thus these correlations can't be stated to be conclusive. Future research should replicate these analyses with a greater n for this reason. Should these findings be replicated at sufficient statistical power, we could see many cases of symptoms relating to inflammation differentially based on traumatic incidents. This would suggest that the immune system does respond in different mechanistic ways to different traumas.

Conclusion

PTSD patients, and in particular those with MST [6], may be particularly susceptible to chronic low-grade inflammation due to the initial stress response, causing a perpetuating cycle of pro-inflammatory cytokines and immune response [5]. These data indicate that immune disruption could contribute to PTSD risk and symptoms development, particularly after MST. Further investigation, though, is required to elucidate more of the mechanism between PTSD symptomatology and specific types of inflammation. Currently, inflammatory biomarkers are an active area of investigation given that immune disruption and inflammation are associated with PTSD [4-6]. Extracellular vesicles, including exosomes, are known to carry such inflammatory biomarkers, including cytokines, and CNS exosomes can cross the BBB, especially as the BBB becomes significantly more permeable with inflammation [3]. Thus, by isolating CNS-derived

exosomes from peripheral blood samples, we were able to quantify inflammatory cytokines within these exosomes to directly measure CNS inflammation resulting from PTSD. By doing so, we have found that different cytokines associate with different symptoms, as well as that central versus peripheral inflammation associate with different symptoms.

We also found evidence that different traumatic incidents may lead to different biological responses, marked by these inflammatory cytokines, in that different cytokine-symptom associations were found in different groups. However, future work is needed to replicate and verify these results. While we did not find significant differences in cytokine levels between groups, we did observe patterns in the ADE cytokine levels of the PTSD/MST group that could prove significant with increased power. This study has allowed us to determine that utilizing cytokines from exosomes as a biomarker for PTSD is feasible, though further optimization measures should be taken. At present, we are considering centrifuging with larger volume, giving us the opportunity to have a larger quantity of exosomes to isolate. In tandem with this, ultracentrifugation is being looked at as a means of procuring a richer exosome sample. We are also looking at more sensitive cytokine assays which should reduce the percentage of non-detectables. Future studies should continue using these methods to determine the relationship between peripheral and central inflammation resulting from MST and PTSD and to develop biomarkers for PTSD diagnosis, prognosis, and potential treatment targets.

Acknowledgements

I would thank my P.I. Dr. Victoria Risbrough (UCSD, VA) for accepting into her labs for the past year and Dr. Samantha Friend (UCSD, VA) for allowing me to assist her in collecting and processing data for her CDA grant. This past year has been an incredible experience in

learning about the research process, in both clinical and wet-lab settings. I have gained invaluable experience and knowledge from these opportunities

I want to also extend my gratitude to Dr.Andrea Chiba (UCSD) and Pai-Kai Huang (UCSD) for facilitating my entry into the Cognitive Science Honor's Program, and for the advice and kindness and that they have given me. I truly do appreciate their willingness to perform these roles on my honor's committee.

Finally, I would like to thank the CogSci advisors for answering all my many questions without complaint and Dr. Marta Kutas for committing her time weekly to advising me, and the rest of the cogsci honor's students, on our presentations.

References

[**1**] Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., … Beckham, C. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles*, *7*(1), 1. https://doi.org/10.1080/20013078.2018.1535750

[**2**] Kobayashi, Takuya & Nagai, Masazumi & Dasilva, John & Galaburda, Albert & Rosenberg, Sara & Hatakeyama, Wataru & Kuwajima, Yukinori & Kondo, Hisatomo & Ishikawa-Nagai, Shigemi. (2019). Retrograde transport of masseter muscle-derived neprilysin to hippocampus. Neuroscience Letters. 698. 10.1016/j.neulet.2019.01.021.

[**3**] Saeedi, S., Israel, S., Nagy, C. *et al.* The emerging role of exosomes in mental disorders. *Transl Psychiatry* 9, 122 (2019) doi:10.1038/s41398-019-0459-9

[**4**] Passos IC, Vasconcelos-Moreno MP, Costa LG, Kunz M, Brietzke E, Quevedo J, et al. (2015). Inflammatory markers in post-traumatic stress disorder: a systematic review, meta-

analysis, and meta-regression. Lancet Psychiatry, 2(11):1002-12. https://doi.org/10.1016/S2215-0366(15)00309-0

[**5**] Hori, H., & Kim, Y. (2019). Inflammation and post-traumatic stress disorder. Psychiatry and Clinical Neurosciences, 73(4), 143–153. https://doi.org/10.1111/pcn.12820

[**6**] O, D. A., Cohen, B. E., Seal, K. H., Bertenthal, D., Margaretten, M., Nishimi, K., & Neylan, T. C. (2015). Elevated Risk for Autoimmune Disorders in Iraq and Afghanistan Veterans with Posttraumatic Stress Disorder. *Biological Psychiatry*, *77*(4), 365–374. https://doi.org/10.1016/j.biopsych.2014.06.015

[**7**] Renna, M. E., O'Toole, M. S., Spaeth, P. E., Lekander, M., & Mennin, D. S. (2018). The association between anxiety, traumatic stress, and obsessive-compulsive disorders and chronic inflammation: A systematic review and meta-analysis. *Depression & Anxiety (1091-4269)*, *35*(11), 1081–1094. https://doi.org/10.1002/da.22790

[**8**] Bassil, K. C., Rutten, B. P. F., & Horstkötter, D. (2019). Biomarkers for PTSD Susceptibility and Resilience, Ethical Issues. *AJOB Neuroscience*, *10*(3), 122–124. https://doi.org/10.1080/21507740.2019.1632964

[**9**] Sexton, M. B., Raggio, G. A., McSweeney, L. B., Authier, C. C., & Rauch, S. A. M. (2017). Contrasting Gender and Combat Versus Military Sexual Traumas: Psychiatric Symptom Severity and Morbidities in Treatment-Seeking Veterans. *Journal of Women's Health (15409996)*, *26*(9), 933–940. https://doi.org/10.1089/jwh.2016.6080

[**10**] Blais, R. K., & Monteith, L. L. (2019). Suicide Ideation in Female Survivors of Military Sexual Trauma: The Trauma Source Matters. *Suicide & Life-Threatening Behavior*, *49*(3), 643. https://doi.org/10.1111/sltb.12464

[**11**] DAV: Disabled American Veterans. (2019). Military Sexual Trauma - MST. [online] Available at: https://www.dav.org/veterans/resources/military-sexual-trauma-mst/ [Accessed 29 Nov. 2019].

[**12**] Asami, K., Kondo, A., Suda, Y., Shimoyamada, M., & Kanauchi, M. (2017). Neutralization of Lipopolysaccharide by Heat Shock Protein in Pediococcus pentosaceus AK-23. *Journal of Food Science (John Wiley & Sons, Inc.)*, *82*(7), 1657–1663. https://doi.org/10.1111/1750-3841.13679

[**13**] Eisenberger, N. I., Berkman, E. T., Inagaki, T. K., Rameson, L. T., Mashal, N. M., & Irwin, M. R. (2010). Inflammation-induced anhedonia: endotoxin reduces ventral striatum responses to reward. *Biological psychiatry*, *68*(8), 748–754. https://doi.org/10.1016/j.biopsych.2010.06.010

[**14**] Matsuda, M., Huh, Y., & Ji, R. R. (2019). Roles of inflammation, neurogenic inflammation, and neuroinflammation in pain. *Journal of anesthesia*, *33*(1), 131–139. https://doi.org/10.1007/s00540-018-2579-4

[**15**] US. Department of Veterans Affairs, Veteran Health Administration. Military sexual trauma. [Accessed May 2020]. https://www.publichealth.va.gov/docs/vhi/military_sexual_trauma.pdf. Published January 2004.

[**16**] "VA.Gov | Veterans Affairs". *Ptsd.Va.Gov*, https://www.ptsd.va.gov/professional/treat/essentials/epidemiology.asp#two.

[**17**] Girgenti, M. J., Hare, B. D., Ghosal, S., & Duman, R. S. (2017). Molecular and Cellular Effects of Traumatic Stress: Implications for PTSD. *Current psychiatry reports*, *19*(11), 85. https://doi.org/10.1007/s11920-017-0841-3

[**18**] Parnell, D., Ram, V., Cazares, P., Webb-Murphy, J., Roberson, M., & Ghaed, S. (2018). Sexual Assault and Disabling PTSD in Active Duty Service Women. *Military medicine*, *183*(9-10), e481–e488. https://doi.org/10.1093/milmed/usy048

[**19**] Gill, J., Mustapic, M., Diaz-Arrastia, R., Lange, R., Gulyani, S., Diehl, T., Motamedi, V., Osier, N., Stern, R. A., & Kapogiannis, D. (2018). Higher exosomal tau, amyloid-beta 42 and IL-10 are associated with mild TBIs and chronic symptoms in military personnel. *Brain injury*, *32*(10), 1277–1284. https://doi.org/10.1080/02699052.2018.1471738

[**20**] Tsai, J., & Shen, J. (2017). Exploring the Link Between Posttraumatic Stress Disorder and inflammation-Related Medical Conditions: An Epidemiological Examination. *The Psychiatric quarterly*, *88*(4), 909–916. https://doi.org/10.1007/s11126-017-9508-9