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TITLE: Glutamate Receptor and Kynurenine Pathway Functioning in the Pathobiology of Gulf War Illness

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14. ABSTRACT

Evidence of possible mechanisms of Gulf War Illness (GWI) development points towards a chronic neuroinflammatory state due to the exposure during the Gulf War (GW) to a plethora of acetylcholinesterase inhibiting and other chemical agents. Preclinical and clinical research have found GWI-associated elevations in pro-inflammatory cytokines and in enhanced astrocyte reactivity. However, the findings in veterans with GWI have come primarily from peripheral measures in blood and not from fluids closer to the brain. In addition, examination of inflammatory pathways that could cause or be caused by changes in cytokines and glia are scarce. In this study we used cerebrospinal fluid (CSF) obtained from veterans with well-characterized GWI and healthy controls to examine the kynurenine pathway, glia functioning, and immune functioning. We obtained CSF from 57 veterans who met CDC and Kansas Gulf War Military and Health Questionnaire (KGWMHQ) GWI criteria (mean age = 48.63; 9 women) and 11 healthy veteran and non-veteran controls (mean age = 47.92; 2 women). **We** used multiple linear regressions to explore group differences in tryptophan, glia-associated kynurenine pathway metabolites (kynurenine; microglia-associated anthranilic acid, 3-hydroxykynurenine, quinolinic acid, picolinic acid, nicotinic acid, nicotinamide; astrocyte-associated kynurenic acid), measures of glia functioning (GFAP, YKL-40, eotaxin-1), and the immune response (IL-4, IL-6, IL-8, IL-10, TNF-alpha; incomplete samples for IL-1 beta, IL-2, IL-12p70, IL-13, IFN-gamma). **Analyses** showed that veterans with GWI compared to healthy controls had significantly (i) enhanced YKL-40 as a markers of astrocyte reactivity, (ii) impaired communication between astrocytes and microglia suggested by enhanced eotaxin-1, (iii) dysregulation of the kynurenine pathway suggested by lower tryptophan, kynurenine, and picolinic acid, and (iv) elevated IL-4 and IL-13 but lower IL-10 suggestive of a dysregulation of immune functioning. **Using** CSF from veterans with GWI and healthy controls appears to confirm a dysregulation of immune functioning and astrocyte reactivity, both indications of a neuroinflammatory state. A more in-depth examination of inflammatory pathways associated with glia functioning revealed a possible change in astrocyte-microglia communication and a change in kynurenine pathway functioning, specifically a reduction in microglia-associated neuroprotective picolinic acid that under normal conditions inhibits neurotoxic and glutamate receptor agonist microglia-associated quinolinic acid.

15. SUBJECT TERMS

Inflammation; kynurenine pathway; microglia; astrocyte; symptoms; Gulf War Illness; ketamine; cerebrospinal fluid.

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1. INTRODUCTION:

Gulf War Illness appears related to neurodegeneration and neuroinflammation but specific mechanisms remain unclear which complicates treatment development. Neurodegenerative and neuroinflammatory disorders share dysregulation of the kynurenine pathways which predisposes to functional and structural changes in neuroimmune pathways, neural over-excitation, and neural tissue damage. The kynurenine pathway metabolizes tryptophan into kynurenine and other neuroactive compounds, some with neuroinflammatory and glutamate N-methyl-D-aspartate (receptor (NMDAR) agonistic properties, and others with neuroprotective and NMDAR antagonist properties. We hypothesize a dysregulation in veterans with Gulf War Illness between neurodegenerative and neuroprotective kynurenine metabolites. The purpose of this project was to examine kynurenine pathway and neuroglia functioning using biomarkers obtained from cerebrospinal fluid (CSF) in 1990-1991 Gulf War veterans with (n=46) and without (n=23) GWI. The second purpose of the project – which was not initiated because of COVID-19 related institutional closure and restrictions on human research – was to examine NMDAR-associated neural excitatory state defined as increased glutamatergic receptor sensitivity by testing the effect of a single infusion of 0.5 mg/kg of NMDAR antagonist ketamine on gamma band EEG (for NMDAR target engagement), other EEG markers, and on symptoms of Gulf War Illness in 19 cases. Outcomes of purpose one provide evidence of an expected neural excitatory and pro-inflammatory state in cases that could predispose to neuronal damage via NMDAR hyperactivation through kynurenine pathway activation, and will provide evidence in humans of possible effects of temporarily blocking NMDAR's with a subanesthetic dose (0.5 mg/kg) of ketamine.

2. KEYWORDS:

Inflammation; kynurenine pathway; microglia; astrocyte; symptoms; Gulf War Illness; ketamine; cerebrospinal fluid.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1 was to determine biomarkers of kynurenine pathway, neuroglia, and central inflammation in cerebrospinal fluid (CSF) of GW veterans with and without GWI, and associate those biomarkers with GWI symptoms.

The goal of sub-task 1 was to obtain approval of the human subject protocol by the Baylor College of Medicine (BCM) IRB, Michael E. DeBakey VA Medical Center (MEDVAMC) R&D, and DoD HRPO.
The goal of sub-task 2 was to start recruitment efforts which continues to the end of the study.
The goal of subtask 3 was to start research procedures in eligible veterans.
The goal of subtask 4 was to assay CSF for the relevant biomarkers.
The goal of subtask 5 was to statistically analyze the data.

Aim 2 is to evaluate the involvement of NMDAR functioning in GWI.

The goal of sub-task 1 is to obtain approval of the human subject protocol by the Baylor College of Medicine (BCM) IRB, Michael E. DeBakey VA Medical Center (MEDVAMC) R&D, and DoD HRPO.
The goal of sub-task 2 is to start recruitment efforts until the end of the study.
The goal of subtask 3 is to start research procedures in eligible veterans.
The goal of subtask 4 was to analyze the EEG data
The goal of subtask 5 was to statistically analyze the data.

What was accomplished under these goals?

AIM 1

We met all milestones and have provided all agreed upon deliverables. Outcomes of the project for aim 1 are provided in Appendix 1.

AIM 2

The project for aim 2 was not initiated because of a year and a half of COVID-19 related institutional closure and restrictions on human research, and the depletion of project funds during that hiatus.

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

Nothing to Report. This project was not intended or designed to provide training and professional development.

How were the results disseminated to communities of interest?

The data were/will be presented at the 2023 annual conference of the Society of Biological Psychiatry in San Diego, and the 2023 annual conference of the International Society of Neuropsychopharmacology (CINP) in Montreal (See Appendix for the two abstracts and the poster). The outcomes will be published in 2023 in a peer-reviewed journal.

What do you plan to do during the next reporting period to accomplish the goals?

This is not applicable for this Final Report. However, we will continue to finalize and submit manuscript(s) and dissemination of the outcomes of this project, possibly with additional analyses for research questions that go beyond the original hypotheses.

4. IMPACT:

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

We cannot as of now report an impact on the field of GWI. We found that cerebrospinal fluid (CSF) from veterans with GWI compared to that of healthy controls had (i) enhanced markers of astrocyte reactivity, (ii) impaired communication between astrocytes and microglia suggested by enhanced eotaxin-1, (iii) dysregulation f the kynurenine pathway that includes lower circulating picolinic acid, and (iv) elevated IL-4 and IL-13 but lower IL-10 suggestive of a neuroinflammatory profile. Our findings support the model of neuroinflammation but through an elevation in brain YKL-40 and eotaxin-1, indicative of reactive astrocytes and microglia, induced perhaps by higher anti-inflammatory cytokines IL-4 and IL-13. Elevated YKL-40 and eotaxin-1 could predispose to neurodegeneration in GWI. The KP seems to have a more limited effect in GWI.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

No impact to report. However, we believe that our outcomes will inform researchers, veterans, and policy makers about (i) neurobiological pathways of GWI, and (ii) neurological outcomes of excessive exposure to acetylcholinesterase inhibitors.

5. CHANGES/PROBLEMS:

We received notification that W81XWH-17-1-0488 was approved for funding on March 7, 2017. The total funding period was from 09/30/2017 to 09/29/2022 which includes two NCEs.

We encountered two major problems:

(1) A low recruitment rate due of the use of a lumbar puncture to obtain CSF which impacted Aim 1
(2) An institution-wide hold of human-subject research because of COVID-19 which impacted Aim 2.

Actual or anticipated problems or delays and actions or plans to resolve them

Aim 1.
We began recruitment for Aim 1 after IRB and HRPO approval of the project. Despite rigorous recruitment efforts, because collection of cerebrospinal fluid requires a spinal tap – a medical procedure with a connotation of being painful – the total number of veterans with and without GWI who wanted to participate was below 10 at the end of 2018. We applied for case and control CSF samples from BBRAIN to complement our samples. Instead of fewer than 10 samples, the final number of CSF samples was 57 for cases and 11 for controls.

Aim 2.
We were unable to finish Aim 2. Approval to begin research procedures associated with Aim 2, coincided with the onset of an institutional holds on all human research because of the COVID-19 pandemic (March 2020). The hold was lifted about one year later at which time we entered our first NCE. By that time our funds were exhausted because of continued staff costs during the hold period. In discussion with the grant analyst, it was decided that Aim 2 could no longer be pursued.

Changes that had a significant impact on expenditures

This grant encountered several challenges that significantly affected the timelines of the projects as well as the budget expenditure. For aim 1, the duration to obtained IRB and HRPO approvals to conduct the project to collect CSF, the subsequent recruitment of cases and controls, and the final collaboration with BBRAIN to complement our number of samples was longer than anticipated. The grant began September 30 2017 but the final data was received around Q4 of 2019. For project for aim 2 (which was, as initially planned, submitted for approval after aim 1 outcomes were obtained) was approved by the IRB and HRPO at the end of Q4 2020. In March 2020 we received an institutional hold on all human research because of the COVID-19 pandemic. The hold was lifted about one and a half year later at which time we were nearing the end of our first NCE. By that time our funds were severely depleted because of the generosity of the program that granted us the funds to continue staff salary support. In discussion with the grant analyst, it was decided that not enough funds were available to pursue the project for Aim 2 and that we would use the period until the end of the first NCE and the duration of the second NCE to finalize aim 1 milestones.

Significant changes in use or care of human subjects

The CSF samples and related demographic and symptom information that we obtained through BBRAIN were deidentified before they were send to us. Receipt and deidentifation procedures were approved by the IRB and HRPO.

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

A manuscript with the outcomes of this study will be submitted for publication in a peer-reviewed international journal.

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Data were presented at scientific conferences. See Appendix 2a and 2b for two poster presentations in 2023

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Marijn Lijffijt
Project role	PI
Nearest person month worked	6 per year
Contribution to project	(i) protocol development; (ii) study approval and regulatory management; (iii) composed the DSMB; (vi) data collection and statistical analysis; (v) preparation of reports
Name	Lea Steele
Project role	Co-I
Nearest person month worked	0.5 per year
Contribution to project	(i) advise on protocol development; (iii) advise on access to veteran registries; (iii) interpret data; (iv) preparation of reports
Name	Lena Brundin
Project role	Co-I / vendor; Van Andel Institute, Michigan State University
Nearest person month worked	NA. Although a Co-I Dr. Brundin's lab was our vendor working under a contract
Contribution to project	(i) analysis of CSF samples; (ii) interpretation of data.
Name	Bylinda Vo-Le
Project role	Research Coordinator
Nearest person month worked	12 per year
Contribution to project	(i) Subject recruitment and recruitment efforts; (ii) subject screening; (iii) conducting non-medical research procedures; (iv) manage data integrity, including data obtained from BBRAIN and its contributors; (v) regulatory contact.

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

Dr. Brundin's lab was our vendor and provided time-limited services (analysis of CSF sample for project-relevant markers) under an established contract with established funds. Besides those services and advising on data interpretation Dr. Brundin had limited involvement in the project for the duration of the grant.

What other organizations were involved as partners?

Partner organizations:

(i) Baylor College of Medicine (primary institute and home institution of the PI and the project)
(ii) Michael E. DeBakey VA Medical Center (CSF collection site; recruitment site; collaboration site with GWI-related researcher).
(iii) Van Andel Institute (CSF analysis; contract)
(iv) Boston University (BBRAIN)

8. SPECIAL REPORTING REQUIREMENTS:

Nothing to Report

9. APPENDICES.

APPENDIX 1

Outcomes of Aim 1 of the project

Abstract

Introduction

Gulf War Illness (GWI) is a chronic illness associated with often debilitating pain, intestinal and skin problems, fatigue, and cognitive problems that affects about a third of 1990-1991 Gulf War (GW) veterans. GWI could be related to a chemical-induced chronic neuroinflammatory state that predisposes to neurodegeneration. Animal models of GWI and research in veterans with GWI have found GWI-associated elevations in pro-inflammatory cytokines and glial processes that have been related to neurodegeneration. Neuroinflammation may therefore be a treatment target to slow the progression of GWI. However, evidence in favor of the neuroinflammation model of GWI comes primarily from animal models or, in veterans, from biomarkers in blood. Neuroinflammatory pathways in GWI remain poorly understood but could involve changes microglia-associated toxic and astrocyte-associated protective kynurenine pathway functioning. We used biomarkers in cerebrospinal fluid (CSF) to examine kynurenine pathway, glia and neuroimmune system functioning in veterans with GWI and healthy controls.

Methods

We obtained CSF from 57 1990-1991 GW veterans who met Center of Disease Control and Kansas Gulf War Military and Health Questionnaire (KGWMHQ) GWI criteria (mean age = 48.63; 9 women) and from 11 healthy controls (mean age = 47.92; 2 women). We used multiple linear regressions to explore group differences in CSF biomarkers of (i) kynurenine pathway functioning including tryptophan and kynurenine, microglia-associated anthranilic acid, 3-hydroxykynurenine, quinolinic acid, picolinic acid, nicotinic acid and nicotinamide, and astrocyte-associated kynurenic acid, (ii) glia functioning including GFAP, YKL-40 and eotaxin-1, and (iii) immune system functioning including pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-8, IL-12p70, IFN-γ and TNF-α, and anti-inflammatory cytokines IL-4, IL-10 and IL-13.

Results

Compared to healthy controls, veterans with GWI had significantly (i) lower CSF tryptophan, kynurenine, and picolinic acid, (ii) enhanced CSF YKL-40 and eotaxin-1, and (iii) elevated CSF IL-4 and IL-13 but lower IL-10. Outcomes did not correlate with the severity of GWI in ill veterans.

Conclusion

Our findings support the model of neuroinflammation but through an elevation in brain YKL-40 and eotaxin-1, indicative of reactive astrocytes and microglia, induced perhaps by higher anti-inflammatory cytokines IL-4 and IL-13. Elevated YKL-40 and eotaxin-1 could predispose to neurodegeneration in GWI. The KP seems to have a more limited effect in GWI.

Introduction

An estimated 250,000 of the 700,000 United States 1990-1991 Gulf War veterans suffer from Gulf War illness (GWI)[1], a chronic, often debilitating disease characterized by combinations of muscle or joint pain, fatigue, gastrointestinal complaints, respiratory problems, dermatological symptoms, emotion dysregulation, neurological symptoms, or cognitive difficulties[2,3,4,5]. The currently prevailing model of GWI posits that the illness is sustained by a chronic low-grade proinflammatory immune response of the brain immune system[6,7,8,9] induced by prolonged and simultaneous exposure during the GW to a plethora acetylcholinesterase (AChE) inhibiting and other types of chemicals[2,10,11,12,13,14]. Mechanisms that link low-grade neuroinflammation to neurodegeneration in GWI are understudied but may involve changes in brain kynurenine pathway (KP) and glia functioning.

The KP metabolizes about 95% of dietary tryptophan into a range of biologically neurotoxic or neuroprotective kynurenine metabolites as intermediates towards the synthesis of nicotinamide adenine dinucleotide (NAD^+) [15] which is essential for cellular functioning[16]. The KP metabolizes tryptophan into L-kynurenine through indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO). Kynurenine in turn is metabolized into neuroprotective protein kynurenic acid (KYNA) by enzyme kynurenine transaminase (KAT), and neurotoxic proteins anthranilic acid (AA) by enzyme kynureninase and 3-hydroxykynurenine (3-HK) by enzyme kynurenine 3-monooxygenase (KMO). 3-HK is metabolized further into neuroprotective protein picolinic acid via enzyme α-amino-β-carboxymuconate semialdehyde decarboxylase (ACMSD); when ACMSD is saturated, remaining kynurenine metabolite is metabolized spontaneously into neurotoxic quinolinic acid with NAD+ one of its downstream metabolites[17]. In the brain, KAT is located mostly in astrocytes and neurons and support the production of kynurenic acid[18]. KMO and kynureninase are located mostly in microglia and support the production of neurotoxic kynurenines[19]. Astrocytes and microglia both have TDO and IDO.

Neurotoxic kynurenines metabolized in microglia contribute to neuronal damage and death as a result of excitotoxicity by the agonistic action of quinolinic acid on N-methyl-D-aspartate (NMDA) receptors, and the induction by AA, 3-HK and quinolinic acid of reactive oxygen species[20,21]. Neuroinflammatory and neurodegenerative illnesses such as Alzheimer's disease, multiple sclerosis, amylotropic lateral sclerosis and AIDS related dementia complex have been related to a relative increase in neurotoxic kynurenines AA, 3-HK, and quinolinic acid[20,21]. Kynurenic acid and picolinic acid inhibit the production or resulting effects of elevated AA, 3-HK and quinolinic acid[18,20,22]. Pro-inflammatory cytokines (primarily interferon-gamma, IFN-γ) and reactive oxygen species activate IDO and KMO, shifting kynurenine production into the direction of neurotoxic metabolites produced by microglial processes[16,20]. IDO is suppressed by anti-inflammatory cytokines IL-4 and IL-13[16,20] and could suppress L-kynurenine production and the production of all kynurenines.

Research in veterans with GWI suggest an upregulation of select pro-inflammatory cytokines and chemokines and downregulation of select anti-inflammatory cytokines[8,9,23], possibly combined with an increase in reactive oxygen species that contribute to oxidative stress[24]. The use of extensive biomarker panels showed that veterans with GWI had increased blood concentrations of pro-inflammatory IFN-γ, interleukin-5 (IL-5), IL-17, IL-33, and eotaxin-1, a higher 24-hour circadian variation of blood eotaxin-1, lower pro-inflammatory IL-7, IL-8, IL-25, CCL-5 and tumor-necrosis alpha (TNF-α), and lower anti-inflammatory IL-4 and IL-13[8,9,23]. Pro-inflammatory cytokines and chemokines and reactive oxygen species activates KMO thus favoring metabolism of L-kynurenine into neurotoxic kynurenines.

Additional evidence for possible involvement of glia and the kynurenine pathway comes from a finding of a 6.6 times higher concentration of glia fibrillary acidic protein (GFAP) in blood of veterans with GWI compared to controls[23]. Higher GFAP has been found across neurodegenerative illnesses marking reactive astrocytes (astrogliosis) that can be triggered by and release pro-inflammatory cytokines[25] . Although an acute and time-limited increase in reactive astrocytes has been shown to be neuroprotective, chronically reactive astrocytes can be neurotoxic. Veterans with GWI also had increased binding of positron emission tomography (PET) tracer C^{11}-PBR28 to 18 kDa translocator protein (TSPO), a protein upregulated in activated microglia/macrophages and astrocytes[26].

The neuroinflammation model of GWI is supported primarily by preclinical models and, in humans, by biomarkers obtained from blood. The model needs more validation through biofluids that better reflect what happens in the brain. In addition, mechanisms that contribute to neuroinflammation and neurodegeneration in GWI are understudied which makes understanding biological mechanisms of GWI and the selection of possible targets for intervention challenging.

Goal of the Study

We tested the hypothesis that neuroinflammation in GWI involves changes in astrocyte and microglia kynurenine pathway functioning. To test the hypothesis we examined concentrations in cerebrospinal fluid (CSF) of (i) tryptophan, kynurenine, anthranilic acid, 3-hydroxykynurenine, quinolinic acid, picolinic acid, nicotinic acid, nicotinamide and kynurenic acid (kynurenine pathway functioning); (ii) GFAP, YKL-40 and eotaxin-1 (glia functioning); (iii) pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-8, IL-12p70, IFN-γ and TNF-α, and (iv) anti-inflammatory cytokines IL-4, IL-10 and IL-13. The inflammation model of GWI implies that GWI is associated with higher neurotoxic microglia-associated kynurenines, pro-inflammatory states of glia reflected by increased GFAP, YKL-40 and eotaxin-1, increased concentrations of pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-8, IL-12p70, IFN-γ and TNF-α, and diminished neuroprotective kynurenic acid and anti-inflammatory cytokines IL-4, IL-10 and IL-13.

Methods

Participants

CSF samples were obtained from 71 subjects. Fifty-nine subjects were 1990-1991 Gulf War veterans who met CDC and Kansas Gulf War Military and Health Questionnaire (KGWMHQ) case criteria. Twelve subjects were healthy controls. Three subjects were removed from the analyses, leaving 69 CSF samples: 57 from veterans with GWI and 11 from controls. Of the 69 CSF samples, six had been collected at the Michael E. DeBakey VA Medical Center. The remaining samples were obtained from the Boston Biorespository, Recruitment and Integrative Network (BBRAIN) for GWI[27]. BBRAIN provided de-identified CSF samples that had originally been collected at the Boston University School of Public Health (n = 4) and at Georgetown University, Department of Medicine (n = 58).

Research Procedures

All study procedures had been approved by the Baylor College of Medicine (IRB), the Michael E. DeBakey VA Medical Center MEDVAMC) R&D, and the Human Research Protection Office (HRPO) of the Department of Defense (DoD). To recruit 1990-1991 Gulf War veterans, a recruitment letter was sent to veterans who were registered in the Department of Veterans Affairs (VA) Gulf War registry, and information flyers were placed in MEDVAMC public places. Non-veteran control subjects were recruited with information flyers in MEDVAMC public places and with advertisements on social media. The initial contact with subjects was by telephone which allowed the study staff to inform the caller about the study.

Subjects were invited to BCM to undergo the informed consent procedure which consisted of (i) a verbal explanation of the study procedures, risks of the procedures, and the right to stop participating in the study at any time, and (ii) signing of the consent for study participation. After consenting, subjects completed the KGWMHQ to establish case status, underwent a physical examination, and had a blood draw for the assessment of a comprehensive metabolic panel. Veterans with current illnesses that could have been caused by or could cause inflammatory responses (e.g., arthritis), or that used medications that could affect immune functioning were excluded. Eligible veterans were scheduled for a lumbar puncture at the MEDVAMC. The lumbar puncture was performed by a certified anesthesiologist. The subject was seated on a stool. The lower back was cleaned with an antiseptic, and lidocaine was injected into the lower back at the area that was used for the lumbar puncture. A 20-gauge hollow needle was inserted between L4 and L5 to extract five to ten ml of CSF depending on flow rate. After CSF extraction, subjects were placed upright on a hospital bed. They left after a two-hour observation. The CSF was distributed into 0.5 ml tubes and stored at -80 ^{0}C. The de-identified CSF samples obtained from BBRAIN had also been stored at -80 ^{0}C. Samples that were used for assaying were placed in a Styrofoam container containing dry ice and shipped overnight.

CSF Preparation for UPLC Analysis

We measured concentrations of tryptophan, kynurenine, anthranilic acid, 3-hydroxykynurenine, quinolinic acid, picolinic acid, nicotinic acid, nicotinamide and kynurenic acid (KP); GFAP, YKL-40 and eotaxin-1 (glia); and IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IFN-γ and TNF-α (immune system). Sample sizes were smaller for IL-1 beta, IL-2, IL-12p70, IL-13 and IFN-gamma than for the other biomarkers because concentrations were lower than the assays could detect reliably.

CSF samples were thawed on wet ice and combined with equal parts of prepared internal standard stocks and briefly vortexed. Samples were then transferred to COSTAR Spin-X 0.22-um filter tubes and centrifuged for 5 minutes at 12,000 rcf. Once filtered, centrifuged samples were added to Agilent amber vials that contained a glass vial insert. Metabolites of the kynurenine pathway and cytokines were quantified using reverse phase ultra-high performance liquid chromatography (UPLC) coupled to a triple quadrupole mass spectrometer (1290 Infinity II LC System, 6470 Triple quadrupole, Agilent Technologies, Santa Clara, CA). Five microliters of samples were injected

onto a Vanguard HSS T3 Pre-column that was connected to an Acquity HSS T3 analytical column. Elution conditions used a combination of Solvent A (0.1% formic acid in LC/MS grade Water) and Solvent B (0.1% formic acid in 90% LC/MS grade acetonitrile) at a flow rate of 0.4mL/min. GFAP, YKL-40, and eotaxin-1 were assayed with ELISA kits according to instructions. Agilent Masshunter Quantitative Analysis Software (v9.0,Agilent) was used to analyze and export data.

Statistical Analysis

Data were analyzed in three steps using JASP's (v. 0.14.1) multiple linear regression and probit generalized linear models[28].

(Step I) Separate multiple linear regressions were performed for each CSF measure as the dependent variable. Group (case, control), age (in years), and gender (female, male) were included as predictor variables. Other variables such as body-mass index (BMI) were not included because of missing data. Effects of group, age, and gender are expressed as t-values, *p*-values, and Vovk-Sellke Maximum *p*-Ratios (VS-MPR). Regression coefficients were estimated using 5,000 bootstraps. The VS-MPR complements the *p*-value which provides no information about the odds that the alternative hypothesis (H_1) explains the observed data better or worse than the null hypothesis (H_0). That is, the evidence that H_1 is a better model to explain the observed data than H_0 may be weak even when $p < .05$. The VS-MPR calculates the maximum possible likelihood that a given p-value is favored under H_1 relative to the null hypothesis H_0. VS-MPR is based on the premise of a uniform(0,1) distribution of p-values under H_0 (i.e., all p-values are equally probable) but a Beta($^1/_2$,1) distribution of p-values under H1 (i.e., small p-values are more probable). The VS-MPR is interpreted as the maximum odds that the p-value is explained by H_1 relative to H_0. For example, if VS-MPR = 5, the p-value of the linear multiple regression is five times more likely to be explained by H_1 than H_0.

(Step II) Multiple linear regression was used also to test if GWI severity predicts CSF measured. Using separate analyses for each biomarker, one set of analyses were performed with total standardized score on the GWI questionnaire. A second set of analyses was performed that included standardized scores for pain, neurological, fatigue, GI, respiratory, and skin domains as predictor variables. To guard against collinearity and bias in the outcomes of the second set of analyses, we estimated tolerance and VIF for continuous variables. We follow guidelines when interpreting tolerance and VIF, with tolerance < .2 and average VIF > 1 (i.e., VIF averaged across VIFs for individual continuous predictor variables) indicating some evidence of collinearity among predictor variables; tolerance < .1 and VIF > 10 indicate great violation of the non-collinearity assumption. Age and gender were included as predictor variables for all analyses. Regression coefficients were not estimated using bootstraps because bootstraps resulted in models that could not be resolved. Outcomes are expressed in t-values, p-values, and VS-MPR.

(Step III) A Bernoulli-informed probit analysis was performed to test if biomarkers could predict case occurrence (dependent variable). The Bernoulli distribution is a discreet probability distribution in which each subject can be regarded as an independent trial that answers the question if the subject is (success) or is not (failure) a case. The downside of the Bernoulli-informed probit analysis is that the sample is biased towards cases (i.e., any subject has a probability of 83.82% to be a case), and there may not be enough controls to reliably calculate effects of independent measures on the likelihood that a subject is a case or control.

Results

Participants

Seventy-three CSF samples were assayed. Complete data were available for 71 individuals. Fifty-seven individuals were veterans who met criteria for GWI. Eleven individuals were veterans or non-veterans who did not meet criteria for GWI. Table 1 presents information about demographics, GW deployment, PTSD, and nicotine use.

Table 1.
Group Characteristics for Veterans with Gulf War Illness (cases) and Healthy Controls

	Cases (n=57)		Controls (n=11)	
	Mean/n	**SD/%**	**Mean/n**	**SD/%**
Age	49.07	6.49	47.73	10.26
BMI (n=48/10)	30.16	5.24	30.70	4.96
Sex				
Female	8	14.04%	2	18.18%
Male	49	85.96%	9	81.82%
Race				
Black	7	12.28%	2	18.18%
White	46	80.70%	9	81.82%

Other	3	5.26%	0	-
Missing data	1	1.75%	0	-
Ethnicity				
Hispanic	6	10.53%	0	-
Non-Hispanic	51	89.47%	11	100%
Active in GW Theater				
Yes	57	100%	3	27.27%
No	-	-	8	72.73%
PTSD				
Yes	26	45.61%	1	9.09%
No	27	47.37%	10	90.91%
Missing	4	7.02%	0	-
Nicotine use				
Yes	16	28.07%	5	45.46%
No	24	42.11%	4	36.36%
Past	4	7.02%	0	-
Missing	13	22.81%	2	18.18%

Notes: BMI = body-mass index; GW = gulf war; PTSD = post-traumatic stress disorder.

Kynurenine Pathway

Table 2 presents means and standard deviations of CSF tryptophan, kynurenine, and kynurenine metabolite concentrations. Multiple linear regressions with group, age, and gender as predictor variables revealed that group was a significant predictor of tryptophan, kynurenine, and picolinic acid concentrations. Age was a significant predictor of tryptophan concentration, and age and gender were significant predictors of kynurenic acid concentrations.

Group effects

Concentrations

Most CSF KP metabolites were numerically lower in cases compared to controls. Linear regressions showed significant effects of group for CSF tryptophan, kynurenine, and picolinic acid concentrations.

CSF tryptophan was 146.04 pg/mL (95% confidence intervals = -283.36 - -8.72 pg/mL) lower in veterans with GWI than in healthy controls which was significant (t = -2.13, p = .037). The VS-MPR shows that the p-value of the group effect is at most 2.99 times more likely under H_1 than H_0 suggesting that group as a predictor of tryptophan concentration should be interpreted with caution.

CSF kynurenine was 10.67 pg/mL (95% confidence intervals = -18.51 - -2.82 pg/mL) lower in veterans with GWI than in healthy controls which was significant (t = -2.72, p = .008). The VS-MPR indicates that the p-value of the group effect is at most 9.09 times more likely under H_1 than H_0 which suggests that group as a predictor of kynurenine concentration can be interpreted with moderate confidence.

CSF picolinic acid was 16.46 pg/mL (95% confidence intervals = -30.68 - -2.25 pg/mL) lower in veterans with GWI than in healthy controls which was significant (t = -2.31, p = .024). The VS-MPR indicates that the p-value of the group effect is at most 4.12 times more likely under H_1 than H_0 which suggests that group as a predictor of kynurenine concentration can be interpreted with moderate confidence.

We did not find the hypothesized group difference in quinolinic or kynurenic acid.

Ratios

Changes in absolute KP metabolite concentrations provide no information about rate of metabolism or about balances between toxic and protective KP metabolites. In post-hoc analyses, we applied multiple linear regression (age and gender included as covariates) to examine group differences in metabolism rates and balances between metabolites of the variables that we had formulated hypotheses about (kynurenic acid and quinolinic acid) and of variables that showed group differences.

We calculated the kynurenine – tryptophan ratio to examine rate of conversion from tryptophan into kynurenine. Kynurenine – tryptophan ratio was significantly lower (0.009, 95% confidence intervals = -0.018 - -0.001) for cases (mean = 0.018, SD = 0.006) than controls (mean = 0.028, SD = 0.031) (t = -2.16, p = .035, VS-MPR = 3.15). Thus, cases had significantly lower kynurenine, lower tryptophan, and lower conversion rate of tryptophan into kynurenine than controls.

Kynurenic acid can regulate excitotoxic effects of quinolinic acid. We calculated the conversion rate of kynurenine into quinolinic acid and kynurenic acid to examine astrocyte and microglia related KP functioning. The conversion rate of kynurenine into kynurenic acid calculated as kynurenic acid – kynurenine ratio was significantly higher in cases (mean = 0.065, SD = 0.024) than controls (mean = 0.051, SD = 0.019) (group difference = 0.014,

95% confidence intervals = 0.001 - 0.028; t = 2.01, p = .049, VS-MPR = 2.49). The conversion rate of kynurenine into quinolinic acid did not differ significantly between cases and controls. Kynurenic acid – quinolinic acid ratio did not differ significantly between cases (mean = 0.115, SD = 0.063) and controls (mean = 0.105, SD = 0.062). These outcomes suggest a compensatory mechanism in cases that enhance kynurenic acid, including in the context of quinolinic acid concentration, despite lower tryptophan, kynurenine, and tryptophan to kynurenine conversion.

Mean picolinic acid and quinolinic acid are numerically lower in cases than controls. Picolinic acid can regulate excitotoxic effects of quinolinic acid. We calculated picolinic acid – quinolinic acid ratio, showing that cases had a lower picolinic to quinolinic acid ratio (mean = 0.979, SD = 0.520) than controls (mean = 1.727, SD = 2.557) which was significant (group difference = 0.753, 95% confidence intervals = -1.491 - -0.015; t = -2.04, p = .046, VS-MPR = 2.61). This indicates that whereas the concentration of picolinic acid and quinolinic acid are about equal in cases, in controls the former is about twice as high as the latter which suggests a diminished protection in cases against excitotoxic effects of quinolinic acid.

Age and gender effects

Linear regressions showed a significant effect of age on CSF tryptophan concentration (t = 3.16, p = .002). For each additional year in age, CSF tryptophan rises by 11.36 pg/mL (95% confidence interval = 4.17 – 18.55 pg/mL). VS-MPR values reveal that the p-values for the age effect for CSF tryptophan and kynurenic acid concentrations are at most 25.18 and 12.36 times more likely under H_1 than H_0.

Analyses showed a significant effect of age (t = 2.86, p = .006) and gender (t = -4.24, p < .001) on kynurenic acid concentration. For each additional year in age, CSF kynurenic acid rises by 0.030 pg/mL (95% confidence interval = 0.009 – 0.052 pg/mL). In addition, CSF kynurenic acid was 0.90 pg/mL (95% confidence interval = -1.32 – -0.48 pg/mL) lower in men than women. VS-MPR reveals that the p-values for age and gender are at most 12.36 and 527.07, respectively, times more likely under H_1 than H_0. Consistent with these outcomes, men had a lower kynurenic acid – quinolinic acid ratio than women (indicating less kynurenic acid in the context of quinolinic acid).

Table 2.
Group Averages, Standard Deviations, and Group Differences of Cerebrospinal Fluid Tryptophan and Kynurenine Pathway Metabolites Concentrations in Veterans with Gulf War Illness (Cases) and Healthy Controls

	Cases (n=57)		Controls (n=11)	
	Mean	**SD**	**Mean**	**SD**
Tryptophan	**1,128.52**	**202.70**	**1,261.29**	**300.84**
Kynurenine	**20.60**	**7.04**	**31.37**	**25.17**
Microglia related				
Anthranilic acid	2.40	1.35	2.13	0.64
3-hydroxykynurenine	2.36	2.03	2.65	2.76
Quinolinic acid	12.84	5.01	14.79	8.17
Picolinic acid	**11.17**	**4.83**	**27.22**	**53.61**
Nicotinic acid	0.60	1.11	0.87	2.12
nicotinamide	22.02	30.13	35.60	63.45
Astrocyte related				
Kynurenic acid	1.32	0.66	1.39	0.93

Note: Bolded variables showed a group effect at p < .05 for linear regression analysis.

GWI severity effects

Forty-three veterans had complete GWI severity data. Multiple linear regression analyses with KGWMHQ standardized total score, age, and gender as predictor variables revealed no effects of GWI total severity on kynurenine pathway metabolites or ratios that we explored in post-hoc analyses. Outcomes did not change if instead of KGWMHQ total score we tested for differences in biomarkers between ill veterans who fell into the lowest compared to those who fell into the highest tertile of KGWMHQ scores.

Tolerance (> 0.4) and VIF (< 2.5) suggest low collinearity amongst domain scores. The only significant finding for domain score analyses was for nicotinic acid. Nicotinic acid showed an increase by 2.56 pg/mL (95% confidence interval = 0.80 – 4.32 pg/mL) for each one-point increase in respiratory domain score (t = 2.95, p = .006, VS-MPR = 12.43).

Astrocyte functioning

Table 3 presents concentrations of CSF glial fibrillary acidic protein (GFAP, a type III intermediate filament protein responsible for the cytoskeleton structure of astrocytes), chitinase-3 protein 1 (YKL-40, an astrocyte-secreted glycoprotein), and eotaxin-1 (CCL-11, a chemokine). GFAP is a biomarker of astrocyte reactivity and morphology.

Eotaxin-1 is involved in the signaling for an inflammatory response by attracting immune cells to locations of injury.

Group effects

Linear regressions showed significant effects of group for YKL-40 and Eotaxin-1 concentrations. CSF YKL-40 was 28,486.28 pg/mL (95% confidence intervals = 375.40 – 56,597.16 pg/mL) higher in veterans with GWI than in healthy controls which was significant (t = 2.02, p = .047). The VS-MPR shows that the p-value of the group effect is at most 2.56 times more likely under H_1 than H_0 suggesting that group as a predictor of YKL-40 concentration should be interpreted with caution.

CSF eotaxin-1 was 0.493 pg/mL (95% confidence intervals = 0.042 – 0.945 pg/mL) higher in veterans with GWI than in healthy controls which was significant (t = 2.18, p = .033). The VS-MPR indicates that the p-value of the group effect is at most 3.28 times more likely under H_1 than H_0 which suggests that group as a predictor of eotaxin-1 concentration must be interpreted with caution.

We did not find the expected group difference for GFAP.

Age and gender effects

Linear regressions showed a significant effect of age on CSF YKL-40 concentration (t = 3.36, p = .001). For each additional year in age, CSF YKL-40 rises by 2,470.90 pg/mL (95% confidence interval = 999.59 – 3,942.22 pg/mL). VS-MPR values reveal that the p-values for the age effect is at most 41.55 more likely under H_1 than H_0 meaning that the effect of age on YKL-40 can be interpreted with high confidence.

Table 3.
Group Averages, Standard Deviations, and group differences of Cerebrospinal Fluid Concentrations of Astrocyte Related Proteins in Veterans with Gulf War Illness (Cases) and Healthy Controls

	Cases			Controls		
	n	**Mean**	**SD**	**n**	**Mean**	**SD**
GFAP (ng/mL)	55	1.44	1.03	11	1.25	0.43
YKL-40 (pg/mL)	**57**	**113,692.57**	**48,199.82**	**11**	**81.853.57**	**26,362.98**
Eotaxin-1 (pg/mL)	**57**	**1.91**	**0.72**	**11**	**1.43**	**0.35**

Note: Bolded variables showed a group effect at $p < .05$ for linear regression analysis.

GWI severity effects

Forty-three veterans had complete GWI severity data. Multiple linear regression analyses with KGWMHQ standardized total score, age, and gender as predictor variables revealed no effects of GWI total severity on GFAP, YKL-40, and eotaxin-1 concentrations. Outcomes did not change if instead of KGWMHQ total score we tested for differences in biomarkers between ill veterans who fell into the lowest compared to those who fell into the highest tertile of KGWMHQ scores.

Tolerance (> 0.4) and VIF (< 2.5) suggest low collinearity amongst domain scores. Domains did not predict any of the three proteins.

Cytokines

Group effects

Linear regressions showed significant effects of group on CSF IL-10, IL-4, IL-13. CSF IL-10 was 0.119 pg/mL (95% confidence intervals = -0.17 - -0.73 pg/mL) lower in veterans with GWI than in healthy controls which was significant (t = -2.31, p = .024). The VS-MPR shows that the p-value of the group effect is at most 4.13 times more likely under H_1 than H_0 suggesting that group as a predictor of IL-10 concentration can be interpreted only with low confidence and thus with caution.

CSF IL-4 was 0.008 pg/mL (95% confidence intervals = 0.00072 – 0.015 pg/mL) higher in veterans with GWI than in healthy controls which was significant (t = 2.19, p = .032). The VS-MPR indicates that the p-value of the group effect is at most 3.34 times more likely under H_1 than H_0 which suggests that group as a predictor of IL-4 concentration can be interpreted with low confidence.

CSF IL-13 was 0.67 pg/mL (95% confidence intervals = 0.18 – 1.15 pg/mL) higher in veterans with GWI than in healthy controls which was significant (t = 2.82, p = .008). The VS-MPR indicates that the p-value of the group effect is at most 9.18 times more likely under H_1 than H_0 which suggests that group as a predictor of IL-13 concentration can be interpreted with moderate confidence. However, the control group contained only 4 observations which offset the implied confidence in the outcome by the VS-MPR. As such, the difference should be interpreted with extreme caution. No group differences were found for pro-inflammatory cytokines.

Age and gender effects

Linear regressions showed significant effects of age on CSF IL-10 concentration (t = -2.14, p = .036). For each additional year in age, CSF IL-10 dropped by 0.006 pg/mL (95% confidence interval = -0.011 – -0.0004 pg/mL). VS-MPR values reveal that the p-values for the age effect for CSF IL-10 are at most 3.05 times more likely under H_1 than H_0.

GWI severity effects

Forty-three veterans had complete GWI severity data for the first 5 cytokines presented in Table 4. The sample sizes were considerably smaller for the last 5 cytokines in Table 4 because exact concentrations for many subjects exceeded the lowest concentrations that the essays could detect IFN-gamma n=26; IL-12p70 n = 8; IL-13 n = 22; IL-1 beta n = 35; IL-2 n=8).

Linear regressions analyses with KGWMHQ standardized total score, age, and gender as predictor variables revealed no significant effects of GWI total severity on any of the 10 cytokines. Outcomes of the first 5 cytokines did not change if instead of KGWMHQ total score we tested for differences between ill veterans who fell into the lowest compared to those who fell into the highest tertile of KGWMHQ scores. Models for the last 5 cytokines could not be resolved because of low sample sizes in the two tertiles.

Tolerance (> 0.4) and VIF (< 2.5) suggest low collinearity amongst the six domain scores. The analyses included the six domain scores, age, and gender. We found effects for IL-10 and IL-4 concentrations. CSF IL-10 diminished by 0.007 pg/mL (95% confidence interval = -0.014 – -0.0005 pg/mL) for each one-point increase in fatigue domain score (t = -2.19, p = .036, VS-MPR = 3.10). CSF IL-4 diminished by 0.0007 pg/mL (95% confidence interval = -0.001 – -0.0.000007 pg/mL) for each one-point increase in skin domain score (t = -2.22, p = 0.033, VS-MPR = 3.27). No other effects were found, although the models for IL-12p70 and IL-2 could not be resolved because small numbers of observations.

Table 4.
Group Averages, Standard Deviations, and group differences of Cerebrospinal Fluid Concentrations of Cytokines in Veterans with Gulf War Illness (Cases) and Healthy Controls

	Cases			Controls		
	n	**Mean**	**SD**	**n**	**Mean**	**SD**
IL-4 (pg/mL)	**57**	**0.023**	**0.012**	**11**	**0.015**	**0.008**
IL-6 (pg/mL)	57	2.031	2.058	11	1.970	1.751
IL-8 (pg/mL)	57	48.403	17.711	11	52.021	23.222
IL-10 (pg/mL)	**57**	**0.095**	**0.040**	**11**	**0.220**	**0.400**
TNF-α (pg/mL)	57	0.173	0.047	11	0.182	0.077
IL-1β (pg/mL)	44	0.206	0.077	4	0.223	0.098
IL-2 (pg/mL)	21	0.488	0.313	5	0.320	0.240
IL-12p70 (pg/mL)	16	0.058	0.020	2	0.037	0.010
IL-13 (pg/mL)	**31**	**0.842**	**0.438**	**4**	**0.263**	**0.028**
IFN-γ (pg/mL)	33	0.524	0.154	5	0.521	0.099

Note: Bolded variables showed a group effect at p < .05 for linear regression analysis.

Discussion

GWI has been hypothesized to be sustained by a chronic neuroinflammatory state[6,7,8,9] that predisposes to veterans with GWI to neurodegeneration[29,30,31,32,33]. Understanding biological mechanisms related to GWI and the development of treatments requires deep insights into pathways that are related or contribute to neuroinflammation and neurodegeneration. The evidence for the neuroinflammation model of GWI is based primarily on research with animals and with blood. We used CSF to examine biomarkers of the brain kynurenine pathway, glia, and cytokines in veterans with GWI compared to healthy controls. Table 5 summarizes our findings. Compared to healthy controls, veterans with GWI had lower CSF concentrations of tryptophan, kynurenine and picolinic acid, higher glia markers YKL-40 and eotaxin-1, higher anti-inflammatory cytokines IL-4 and IL-13, and lower anti-inflammatory cytokine IL-10. Other biomarkers did not differ between the two groups, and no biomarker related to GWI severity. These are partially inconsistent with the neuroinflammation model of GWI and with findings in blood.

Lower CSF picolinic acid, higher YKL-40 and eotaxin-1, and lower IL-10 support the inflammation model of GWI. This model is not complete, however, because it does not account for elevated IL-4 and IL-13, or the absence of group differences in neurotoxic kynurenines or pro-inflammatory cytokines.

Table 5
Summary of Significant Outcomes

		Cases < controls	Cases > controls	Interpretation
Kynurenine pathway				
	TRP	↓		Low CSF TRP in cases
	KYN	↓		Low CSF KYN in cases
	PIC	↓		Low CSF PIC in cases
	KYN – TRP ratio	↓		Low TRP to KYN conversion in cases
	KYNA – KYN ratio		↑	Compensatory KYN to KYNA conversion in cases
	PIC – QA ratio	↓		Low PIC protection against QA's excitotoxic effects
Glia				
	YKL-40		↑	Perhaps changes in astrocyte internal functions
	Eotaxin-1		↑	
Cytokines				
	IL-4		↑	High anti-inflammatory IL-4
	IL-13		↑	High anti-inflammatory IL-13
	IL-10	↓		Low anti-inflammatory L-10

Note: CSF = cerebrospinal fluid; TRP = tryptophan; KYN = kynurenine; PIC = picolinic acid; KYNA = kynurenic acid; QA = quinolinic acid; IL = interleukin

Kynurenine Pathway

The KP metabolizes about 95% of dietary tryptophan into a range of biologically neurotoxic or neuroprotective kynurenine metabolites[15]. Pro-inflammatory cytokines (primarily IFN-γ) and reactive oxygen species activate KP enzymes IDO and KMO, shifting kynurenine production into the direction of neurotoxic metabolites produced by microglial processes[16,20]. IDO is suppressed by anti-inflammatory cytokines IL-4 and IL-13[16,20]. Prior findings of higher blood IFN-γ, and lower blood IL-4 and IL-13 in veterans with GWI[8,9,23] had us hypothesize activation of KMO and increased concentrations of neurotoxic kynurenines AA, 3-HK or quinolinic acid in that population.

Instead, we found lower CSF tryptophan, L-kynurenine and picolinic acid. Glia and neurons use tryptophan to produce L-kynurenine via IDO. Tryptophan enters the brain freely through the blood-brain barrier as do L-kynurenine and 3-HK. Lower CSF tryptophan amongst cases may signify diminished entry of tryptophan into the brain or lower concentrations in the periphery resulting in the conversion of less L-kynurenines. That mechanism does not square with the absence of a group difference in other downstream kynurenines (except picolinic acid), however.

Instead, our results could signify that glia and neurons of veterans with GWI take up more tryptophan and L-kynurenine than glia and neurons of controls. Tryptophan and L-kynurenine in CSF is what remains after binding and uptake. Increased uptake result in lower circulating tryptophan and L-kynurenines. This mechanism would also explain the absence of changes in other kynurenines. This in turn suggests that the KP (at least in terms of absolute concentrations of kynurenines) may not be involved in sustaining a neuroinflammatory state in GWI or contribute to neurodegeneration.

However, picolinic acid was lower in cases. Picolinic acid can suppress neurotoxic effects of quinolinic acid, a kynurenine that induced the production of reactive oxygen species and pro-inflammatory cytokines, and have excitotoxic properties by acting as an NMDA receptor agonist. Lower picolinic acid implies, therefore, an inflammatory state of the brain because of an enhanced influence of quinolinic acid.

Neuroprotective protein picolinic acid is actively metabolized from a precursor by enzyme α-amino-β-carboxymuconate semialdehyde decarboxylase; when ACMSD is saturated, remaining kynurenine metabolite is metabolized spontaneously into neurotoxic quinolinic acid[17]. Lower picolinic acid suggests diminished ACMSD activity or availability in GWI. This interpretation is inconsistent with the hypothesis postulated in the last paragraph of enhanced uptake of tryptophan and L-kynurenine because lower ACMSD activity must result into elevated quinolinic acid. This opens questions of why tryptophan and L-kynurenine are lower in GWI, and how it affects the functioning or astrocytes, microglia and neurons.

Neuroglia

We expected a robust increase in GFAP in veterans with GWI based on the finding of a 6.6-times higher GFAP concentration in blood of ill veterans. Instead, we found no evidence of changes in GFAP in CSF suggesting that GWO is not associated with reactive astrocytes/astrogliosis. Instead, we did find higher concentrations of CSF YKL-40 and eotaxin-1. Higher eotaxin-1 appears in line with higher concentrations of and increased diurnal variation in eotaxin-1 in blood of ill veterans[8,9,23].

YKL-40 and eotaxin-1 are elevated in many neurodegenerative disorders[34,35] where they could exert neurotoxic effects on neurons resulting in their damage or death[36,37]. Elevated CSF YKL-40 could be a biomarker of reactive, inflammatory, astrocytes and of infiltrated macrophages[38].

Two sources of CSF eotaxin-1 are peripheral eotaxin-1 transported into the brain through the blood-brain barrier[39], and eotaxin-1 produced by activated astrocytes[37] perhaps mediated by anti-inflammatory cytokines IL-4, IL-10, and IL-13, and/or pro-inflammatory cytokines IL-1β and TNF-α[40]. Our finding that IL-4 and IL-13 concentrations are elevated in veterans with GWI compared to healthy controls suggest that although considered classically as anti-inflammatory, these two cytokines could contribute to increased eotaxin-1 and therefore to a pro-inflammatory environment in the brain and to neuronal damage and death. Elevated eotaxin-1 could be a biomarker of reactive microglia[41] and production by reactive microglia or reactive oxygen species[40]. Elevated eotaxin-1 may reflect or underlie a state of accelerated aging of the brain[41].

Cytokines

We studied the neuroimmune system using concentrations in CSF of pro-inflammatory cytokines and chemokines IL-1beta, IL-2, IL-6, IL-8, IL-12p70, IFN-gamma and TNF-alpha, and of anti-inflammatory cytokines IL-4, IL-10, and IL-13. Pro-inflammatory cytokines and chemokines recruit and activate immune cells that lead to a further increase in pro-inflammatory proteins and induces or sustains inflammation; anti-inflammatory cytokines downregulate pro-inflammatory proteins and suppress inflammation[42]. Concentrations of pro-inflammatory cytokines did not differ between groups which contrasts higher concentration of anti-inflammatory cytokines IL-4 and IL-13, and lower concentrations of anti-inflammatory cytokine IL-10 in veterans with GWI compared to healthy controls.

The absence of a significant difference between veterans with GWI and healthy controls in pro-inflammatory cytokines suggest an absence of a pro-inflammatory environment at least as far as these pro-inflammatory cytokines are concerned. This absence could explain why we did not find the expected increase in microglia-associated kynurenine metabolites: inflammatory cytokines upregulate activity of KMO, the enzyme that is found primarily in microglia and that metabolizes kynurenine into the neurotoxic 3-HK, the precursor of neurotoxic protein quinolinic acid.

Higher IL-4 and IL-13 suggest that GWI is associated with an overexpression in the brain of anti-inflammatory proteins [43] which is inconsistent with the inflammatory model of GWI and our finding of a lower concentration of CSF IL-10. In addition, higher IL-4 and IL-13 in CSF contradicts lower IL-4 and IL-13 concentrations in serum of veterans with GWI compared to healthy controls[9] which may reflect differences between peripheral and central immune system functioning. However, IL-4 and IL-13 could contribute to increased eotaxin-1 and therefore to a pro-inflammatory environment in the brain and to neuronal damage and death.

Conclusion

Our findings support the model of neuroinflammation but through an elevation in brain YKL-40 and eotaxin-1, indicative of reactive astrocytes and microglia, induced perhaps by higher anti-inflammatory cytokines IL-4 and IL-13. Elevated YKL-40 and eotaxin-1 could predispose to neurodegeneration in GWI. The KP seems to have a more limited effect in GWI.

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Supplemental Material

Table S1
Outcomes of Multiple Linear Regression for Cerebrospinal Fluid Tryptophan and Kynurenine Pathway Metabolites Concentrations with Group, Age, and Gender as Predictor Variables.

	Group (case vs control)				**Age**				**Gender (male vs female)**			
	Beta (B)	**t**	**p**	**VS-MPR**	**Beta (B)**	**t**	**p**	**VS-MPR**	**beta**	**t**	**p**	**VS-MPR**
Tryptophan	**-146.04**	**-2.13**	**.037**	**2.99**	**11.36**	**3.16**	**.002**	**25.18**	-47.82	-0.66	.509	1
Kynurenine	**-10.67**	**-2.72**	**.008**	**9.09**	.01	0.05	.964	1	-2.76	-0.67	.505	1
Microglia related												
Anthranilic acid	0.26	0.60	.548	1	0.01	0.55	.583	1	-0.05	-0.11	.913	1
3-hydroxykynurenine	-0.31	-0.43	.667	1	-0.03	-0.67	.505	1	1.10	1.47	.146	1.31
Quinolinic acid	-2.08	-1.11	.269	1.04	0.06	0.62	.535	1	1.06	0.54	.590	1
Picolinic acid	**-16.46**	**-2.31**	**.024**	**4.12**	0.15	0.39	.696	1	5.37	0.72	.474	1
Nicotinic acid	-0.27	-0.61	.544	1	0.01	0.61	.544	1	-0.62	-1.36	.178	1.20
nicotinamide	-13.91	-1.13	.264	1.05	-0.19	-0.30	.768	1	14.24	1.10	.275	1.04
Astrocyte related												
Kynurenic acid	-0.07	-0.37	0.716	1	**0.03**	**2.86**	**.006**	**12.36**	**-0.90**	**-4.24**	**<.001**	**527.07**

Note: VS-MPR = Vovk-Sellke Maximum p-ratio. Significant effects ($p < .05$) are in **bold**. Metabolites were included in the multiple regression analysis as dependent variable. Group, age, and gender were included as predictor variables.

Table S2

Outcomes of Multiple Linear Regression for Cerebrospinal Fluid Glia Biomarker Concentrations with Group, Age, and Gender as Predictor Variables.

	Group (case vs control)				**Age**				**Gender (male vs female)**			
	Beta (B)	**t**	**p**	**VS-MPR**	**Beta (B)**	**t**	**p**	**VS-MPR**	**beta**	**t**	**p**	**VS-MPR**
GFAP (ng/mL)	0.162	0.51	.615	1	0.018	1.06	.292	1	0.05	0.14	.890	1
YKL-40 (pg/mL)	**28,486.28**	**2.02**	**.047**	**2.56**	**2,470.91**	**3.36**	**.001**	**41.55**	821.25	0.06	.956	1
Eotaxin-1 (pg/mL)	**0.49**	**2.18**	**.033**	**3.28**	-0.01	-0.93	.358	1	0.06	.25	.803	1

Note: VS-MPR = Vovk-Sellke Maximum p-ratio. Significant effects ($p < .05$) are in bold. Metabolites were included in the multiple regression analysis as dependent variable. Group, age, and gender were included as predictor variable.

APPENDIX 2a

Poster presented at the April 27-29, 2023, meeting of the Society of Biological Psychiatry in San Diego

Title: Biomarkers in Cerebrospinal Fluid Show Inflammation-Associated Changes in Glia and Kynurenine Pathway Functioning in Gulf War Illness

Presenter: Marijn Lijffijt, PhD

Affiliation: Baylor College of Medicine, Houston, Texas, USA; Michael E. DeBakey VA Medical Center, Houston, Texas, USA

Abstract

Background

Gulf War Illness (GWI), a neuropsychiatric disease affecting 250,000 1990-1991 Gulf War (GW) veterans, is associated with chronic neuroinflammation, including microglia and astrocyte reactivity, following simultaneous and excessive exposure to a plethora of acetylcholinesterase inhibiting and other chemicals in the GW theater. The neuroinflammatory model of GWI is based primarily on blood biomarkers from a limited number of inflammatory mechanisms. Using cerebrospinal fluid (CSF) from veterans with GWI and healthy controls we examined glia and tryptophan-kynurenine pathway (KP) functioning, including microglia-associated NMDAR agonist and pro-inflammatory quinolinic acid, and astrocyte-associated NMDAR antagonist and anti-inflammatory kynurenic acid.

Methods

Fifty-seven GW veterans met CDC and Kansas Gulf War Military and Health Questionnaire (KGWMHQ) GWI criteria (mean age=48.63; 9 women). Eleven were healthy (non-)veteran controls (mean age=47.92; 2 women). Multiple linear regressions controlling for age and sex tested for group differences in concentrations of CSF (i) tryptophan and kynurenine (precursors of KP metabolites), (ii) microglia-associated KP metabolites (anthranilic acid, 3-hydroxykynurenine, quinolinic acid, picolinic acid, nicotinic acid, nicotinamide), (iii) astrocyte-associated KP metabolites (kynurenic acid), and (iv) glia functioning (GFAP, YKL-40, eotaxin-1). Data have not been presented elsewhere.

Results

GWI veterans had significantly (i) higher YKL-40 ($t=2.02$, $p=.047$), (ii) higher eotaxin-1 ($t=2.18$, $p=.033$), and (iii) lower tryptophan, kynurenine, and picolinic acid ($t=2.13–2.72$, $p>.037$) than controls.

Conclusions

Astrocyte reactivity (higher YKL-40), dysregulated astrocyte-microglia communication (higher eotaxin-1), and impaired microglia KP functioning and quinolinic acid control (lower picolinic acid) confirm GWI-associated neuroinflammation. Outcomes suggest a GWI-associated neurotoxic environment that predisposes to or protect against neurodegeneration.

Disclosures:

1 This study was funded by a grant from the U.S. Army Medical Research and Development Command (W81XWH-16-GWIRP-NIA)

2 Marijn Lijffijt is currently an employee at Sage Therapeutics. The data presented in this presentation was gathered, analyzed, and interpreted during Dr. Lijffijt's employment at BCM and MEDVAMC and has not been influenced in any way by his current employment.

Biomarkers in Cerebrospinal Fluid Show Inflammation-Associated Changes in Glia and Kynurenine Pathway Functioning in Gulf War Illness

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ABSTRACT

Background

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Results

GWI veterans had significantly (i) higher YKL-40 (t=2.02, p=.047), (ii) higher eotaxin-1 (t=2.18, p=.033), and (iii) lower tryptophan, kynurenine, and picolinic acid (t=2.13–2.72, p>.037) than controls.

Conclusions

Astrocyte reactivity (higher YKL-40), dysregulated astrocyte-microglia communication (higher eotaxin-1), and impaired microglia KP functioning and quinolinic acid control (lower picolinic acid) confirm GWI-associated neuroinflammation. Outcomes suggest a GWI-associated neurotoxic environment that predisposes to or protect against neurodegeneration.

BACKGROUND

250,000 of the 70,000 1990-1991 Gulf War (GW) United States military veterans suffer from GWI, a disease marked by debilitating muscle or joint pain, fatigue, gastrointestinal complaints, respiratory problems, dermatological symptoms, emotion dysregulation, neurological symptoms, and/or cognitive difficulties [1] associated with excessive and simultaneous exposure in the 1990-1991 GW theater to a plethora of acetylcholinesterase inhibiting and other chemicals, perhaps in combination with combat stress, head injury, and genetic predisposition [2].

GWI could be a neurodegenerative illness perpetuated by chronic neuroinflammation [2]. Veterans with GWI had a 6.60 times higher concentration of serum glial fibrillary acidic protein (GFAP) than non-veteran healthy controls indicative of reactive astrocytes (astrogliosis) and of brain immune activation [3]. Acute and time-limited increase in reactive astrocytes can be neuroprotective, whereas chronically reactive astrocytes in veterans with GWI could be neurotoxic. Positron emission tomography (PET) with tracer [^{11}C]PBR28 showed upregulation in veterans with GWI of 18kDA translocator protein (TSPO) indicative of reactive microglia or astrocytes, or infiltration into the brain of macrophages that can have neurotoxic effects [4]. It is unclear how reactive astrocytes and microglia contribute to neurodegeneration or a neuroinflammatory state in GWI. Dysregulation of the kynurenine pathway (KP) may be one mechanism.

The KP metabolizes tryptophan into kynurenine. Kynurenine in turn is metabolized into neuroprotective protein kynurenic acid (KYNA) by enzyme kynurenine transaminase (KAT), and neurotoxic proteins anthranilic acid (AA) by enzyme kynureninase and 3-hydroxykynurenine (3-HK) by enzyme kynurenine 3-monooxygenase (KMO). 3-HK is metabolized further into neuroprotective protein picolinic acid via enzyme α-amino-β-carboxymuconate semialdehyde decarboxylase (ACMSD); when ACMSD is saturated, remaining kynurenine metabolite is metabolized spontaneously into neurotoxic quinolinic acid (QA). Peripheral tryptophan, kynurenine and 3-HK cross the blood brain barrier. In the brain, KAT is located mostly in astrocytes and neurons, whereas KMO and kynureninase are located mostly in microglia. **Astrocytes therefore produce neuroprotective KYNA whereas microglia produce neurotoxic AA, 3-HK and QA, as well as neuroprotector PIC.** Reactive astrocytes and microglia may point towards KP dysregulation as a basis of neuroinflammation or neurodegeneration.

Whereas prior studies used blood biomarkers of glia activation and neuroinflammation, we explored KP dysregulation as well as neurodegenerative biomarkers of astrocyte functioning (GFAP, YKL-40) and astrocyte-microglia communication (eotaxin-1) in cerebrospinal fluid.

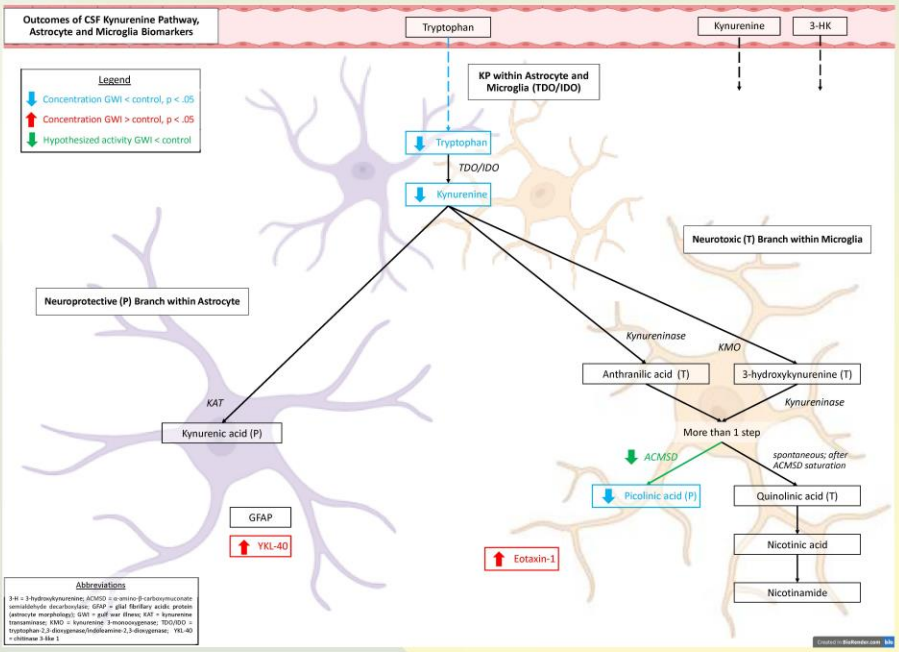
Aims

1. Explore astrocyte and microglia internal processes GWI using cerebrospinal fluid (CSF) metabolites of the KP.
2. Show elevated serum GFAP in CSF.
3. Beyond GFAP, explore astrocyte and microglia functioning with CSF YKL-40 and eotaxin-1 which could be precursors of progression of neurodegenerative disorders such as Alzheimer's disease.

METHODS

Study procedures, including informed consent procedures, had been approved by the Baylor College of Medicine (IRB), the Michael E. DeBakey VA Medical Center MEDVAMC) R&D, and the Human Research Protection Office (HRPO) of the Department of Defense (DoD). CSF samples were obtained from 12 healthy controls and 59 1990-1991 Gulf War veterans who met CDC and Kansas Gulf War Military and Health Questionnaire (KGWMHQ) case criteria. Removal of 3 subjects left 57 case and 11 control samples. Sixty-two of the 68 CSF samples had been requested from the Boston Biorepository, Recruitment and Integrative Network (BBRAIN) for GWI (Keating, 2021, doi:10.1016/j.lfs.2021.119903). Requested samples had been de-identified. Metabolites of the kynurenine pathway were quantified using reverse phase ultra-high performance liquid chromatography (UPLC) coupled to a triple quadrupole mass spectrometer (1290 Infinity II LC System, 6470 Triple quadrupole, Agilent Technologies, Santa Clara, CA). GFAP, YKL-40, and eotaxin-1 were assayed with ELISA kits according to instructions. Data were analyzed with multiple linear regression implemented in JASP (v. 0.14.1). Separate multiple linear regressions were performed for each CSF measure as the dependent variable. Group (case, control), age (in years), and gender (female, male) were included as predictor variables. Outcomes with $p < .05$, uncorrected for multiple comparisons, were considered significant.

RESULTS



		Cases (n=57)		Controls (n=11)	
		Mean/n	SD/%	Mean/n	SD/%
	Age	49.07	6.49	47.73	10.26
	BMI (n=48/10)	30.16	5.24	30.70	4.96
	Sex				
	Female	8	14.04%	2	18.18%
	Male	49	85.96%	9	81.82%
	Race				
	Black	7	12.28%	2	18.18%
	White	46	80.70%	9	81.82%
	Other	3	5.26%	0	-
	Missing data	1	1.75%	0	-
	Ethnicity				
	Hispanic	6	10.53%	0	-
	Non-Hispanic	51	89.47%	11	100%
	Active in GW Theater				
	Yes	57	100%	3	27.27%
	No	-	-	8	72.73%
	PTSD				
	Yes	26	45.61%	1	9.09%
	No	27	47.37%	10	90.91%
	Missing	4	7.02%	0	-
	Nicotine use				
	Yes	16	28.07%	5	45.46%
	No	24	42.11%	4	36.36%
	Past	4	7.02%	0	-
	Missing	13	22.81%	2	18.18%

CONCLUSIONS

Outcomes from CSF appear to confirm the neuroinflammatory model of GWI, suggesting that GWI is associated with a neurotoxic environment that may predispose to neurodegeneration.

1) **Lower PIC in cases compared to controls.**
   - I. IMPLICATION: diminished protection from neurotoxic effects of QA.
   - II. QUESTION: Is lower PIC related to lower ACMSD enzyme activity and does lower PIC relate to neurodegeneration in cases?
2) **Lower CSF tryptophan and kynurenine in cases did not result in case – control differences in KYNA, AA or 3-HK.**
   - I. IMPLICATION: no apparent contribution of AA and 3-HT to a neuroinflammatory state in GWI.
   - II. QUESTION: Is lower CSF tryptophan and kynurenine a reflection of a change in peripheral KP functioning or are astrocyte KAT and microglia kynureninase and KMO upregulated in cases?
3) **No change in CSF GFAP which is inconsistent with findings of higher serum GFAP in cases and higher GFAP in animal models of GWI.**
   - I. IMPLICATION: (a) no change in astrocyte morphology and no reactive astrocytes in GWI, (b) poor translation between brain GFAP and case serum or GWI animal model GFAP.
   - II. QUESTION: (a) How is elevated serum GFAP explained, (b) how valid is the GWI animal model?
4) **Higher CSF YKL-40 in cases compared to controls.**
   - I. IMPLICATIONS: Dysregulated astrocyte functioning in GWI that has been related also to neurodegeneration in other neuropsychiatric disorders.
   - II. QUESTION: Instead of contributing to neurodegeneration, may higher eotaxin-1 reflect processes that try to contain neuronal damage and neurodegeneration?
5) **Higher CSF eotaxin-1 in cases compared to controls.**
   - I. IMPLICATIONS: Dysregulated astrocyte-microglia communication that has been related also to neurodegeneration in other neuropsychiatric disorders.
   - II. QUESTION: Instead of contributing to neurodegeneration, may higher eotaxin-1 reflect processes that try to contain neuronal damage and neurodegeneration?

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2. CSF was made available through **the Boston Biorepository, Recruitment and Integrative Network (BBRAIN) for GWI** (W81XWH-18-1-0549)

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3 Marijn Lijffijt is currently an employee at Sage Therapeutics. The data presented in this presentation was gathered, analyzed, and interpreted during Dr. Lijffijt's employment at BCM and MEDVAMC and has not been influenced in any way by his current employment.

APPENDIX 2b

Oral presentation at the May 7-10, 2023, meeting of the CINP World Congress on Neuropsychopharmacology

Title: Biomarkers in Cerebrospinal Fluid Show Changes in Brain Kynurenine Pathway Functioning in 1990-1991 Gulf War Veterans with Gulf War Illness

Presenter: Marijn Lijffijt, PhD

Affiliation: Baylor College of Medicine, Houston, Texas, USA; Michael E. DeBakey VA Medical Center, Houston, Texas, USA

Abstract

Background

An estimated 250,000 1990-1991 Gulf War (GW) veterans suffer from Gulf War Illness (GWI), a neurodegenerative disorder associated with chronic neuroinflammation and activated microglia and astrocytes, two types of brain cells that react and contribute to neuroinflammation and neurodegeneration through changes in kynurenine pathway (KP) functioning. The KP is an enzymatic pathway that metabolizes tryptophan into a variety of neurotoxic and neuroprotective kynurenine intermediates before converting into nicotinamide adenine dinucleotide (NAD+) that aids cellular energy generation, DNA repair, and transcriptional regulation. Microglia and astrocytes contain different KP enzymes and convert tryptophan into different KP metabolites. Microglia-associated KP enzymes metabolize tryptophan into quinolinic acid and picolinic acid. Quinolinic acid contributes to excitotoxicity and cell damage by producing reactive oxygen species, damaging fatty acid membranes, disrupting astrocyte functioning, contributing to higher extracellular glutamate concentrations, and agonizing glutamate receptors. Picolinic acid downregulates neurotoxic effects of quinolinic acid. Astrocyte-associated KP enzymes metabolize tryptophan into kynurenic acid, an antioxidant and glutamate and acetylcholine receptor antagonist that could downregulate excitotoxic effects of quinolinic acid. KP functioning is disrupted across neuroinflammatory and neurodegenerative disorders. The neuroinflammatory model of GWI predicts elevated quinolinic acid and lower kynurenic and picolinic acid in ill veterans compared to healthy controls.

Aims & Objectives

Using cerebrospinal fluid (CSF) from veterans with GWI and healthy controls, we explored brain KP functioning.

Methods

Fifty-seven GW veterans met CDC and Kansas Gulf War Military and Health Questionnaire (KGWMHQ) GWI criteria (age=48.6; 9 women). Eleven subjects were healthy controls (mean age=47.9; 2 women). A first set of multiple linear regressions tested for group differences in CSF concentrations of tryptophan and kynurenine, (ii) microglia-associated KP metabolites 3-anthranilic acid, 3-hydroxykynurenine, quinolinic acid, picolinic acid, NAD+, and nicotinic acid, (iii) astrocyte-associated KP metabolite kynurenic acid. Ratios between kynurenine–tryptophan, quinolinic acid–kynurenine, and kynurenic acid–kynurenine served as proxies of enzymatic metabolism rates. Ratios between kynurenic acid–quinolinic acid and picolinic acid–quinolinic acid served as proxies of balanced between neuroprotective and neurotoxic kynurenines. A second set of set of multiple linear regressions tested among ill veterans tested for relationships between biomarkers and KGWMHQ severity score. Outcomes were controlled for age and sex. Outcomes were not controlled for multiple comparisons. Data have not been presented elsewhere.

Results

Veterans with GWI had significantly (i) lower CSF tryptophan, kynurenine, and picolinic acid ($t=2.13–2.72$, $p>.037$), (ii) lower kynurenine–tryptophan ratio ($t=2.16$, $p=.035$), (iii) higher kynurenic acid–kynurenine ratio ($t=2.01$, $p=.049$), (iv) lower picolinic acid–quinolinic acid ratio ($t=2.04$, $p=.046$). Biomarkers were not related to KGWMHQ score.

Despite lower CSF tryptophan and kynurenine, quinolinic acid and kynurenic acid did not differ between cases and controls; kynurenic acid–kynurenine ratio was enhanced. These outcomes suggest enhanced microglia and astrocyte enzyme activity to convert kynurenine into quinolinic acid and kynurenic acid. By contrast, picolinic acid and picolinic acid–quinolinic acid ratio were lower in cases, suggesting diminished enzymatic conversion into neuroprotective picolinic acid in favor of conversion into quinolinic acid. Outcomes are in line with the neuroinflammation model of GWI.

Discussion & Conclusion

Despite lower CSF tryptophan and kynurenine, quinolinic acid and kynurenic acid did not differ between cases and controls; kynurenic acid–kynurenine ratio was enhanced. These outcomes suggest enhanced microglia and astrocyte enzyme activity to convert kynurenine into quinolinic acid and kynurenic acid. By contrast, picolinic acid and picolinic acid–quinolinic acid ratio were lower in cases, suggesting diminished enzymatic conversion into neuroprotective picolinic acid in favor of conversion into quinolinic acid. Outcomes are in line with the neuroinflammation model of GWI.

Keywords

Gulf War Illness; Neuroinflammation; Neurodegeneration; Metabolic pathway; Kynurenine pathway.

Disclosures:

1 This study was funded by a grant from the U.S. Army Medical Research and Development Command (W81XWH-17-1-0488).

2 CSF samples were obtained through Boston Biorepository, Recruitment & Integrated Network (BBRAIN), an initiative funded by a grant from the U.S. Army Medical Research and Development Command (W81XWH-18-1-0549).

3 Marijn Lijffijt is currently an employee at Sage Therapeutics. The data presented in this presentation were gathered, analyzed, and interpreted during Dr. Lijffijt's employment at BCM and MEDVAMC, and has not been influenced in any way by his current employment.