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TITLE: Novel Autoantibody Serum and Cerebrospinal Fluid Biomarkers in Veterans with Gulf War Illness

PRINCIPAL INVESTIGATOR: Mohamed Abou Donia

RECIPIENT: Mr. Brett Chaney, Science Officer

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14. ABSTRACT The major goal of this study to develop objective peripheral biomarkers for Gulf War Illness (GWI). The following proposed specific aims have been accomplished. 1) Adapting our autoantibody assay to plasma. 2) Comparative study of Biomarkers for Gulf War Illness (GWI), Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS) has been demonstrated. 3) We only could collect four samples of cerebrospinal fluid during the no-cost extension year that we requested and was approved. During that year, we have been preparing several manuscripts that have now been published in peer-refereed journals (See details in the report). Our results confirm the continuing presence of autoantibodies against neural proteins in GW veterans and are in agreement with recent reports indicating that 30 years after the war, the health of veterans with GWI is not improving and may be getting worse. Such blood-based autoantibody tests have proven to be useful as biomarkers for various conditions of veterans of the 1990-1991 Gulf War such as Gulf War Illness (GWI), Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS). We plan to present our results at scientific meetings and in additional peer-reviewed publications.						
15. SUBJECT TERMS Gulf War Illness, autoantibody, GFAP, Tubulin, tau, myelin basic protein, chronic fatigue syndrome, irritable bowel						
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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The Subject: Assaying of autoantibodies against neuronal and glial proteins in veterans with Gulf War Illness (GWI) using Western blot assays.

PURPOSE: Development of peripheral biomarkers for veterans of the 1990-1991 Gulf War with various conditions i.e., Gulf War Illness (GWI), Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS).

Scope of the Research: Serum and plasma from 250 Gulf War veterans with GWI and 200 controls (100 healthy GW veterans; 50 chronic fatigue syndrome (CFS) and 50 irritable bowel syndrome (IBS) have been investigated. A few samples of cerebrospinal fluid became available for our study. Our results to date confirm the continuing presence of autoantibodies against neural proteins in GW veterans and are in agreement with recent reports indicating that 30 years after the war, the health of veterans with GWI is not improving and may be worsening. Such blood-based autoantibody tests have proven to be useful as biomarker for various conditions of veterans of the 1990-1991 Gulf War such as Gulf War Illness (GWI), Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS). We plan to will continue presenting results in scientific meetings and in new publications this year.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Gulf War Illness (GWI) Microtubule Associated Protein-2 (MAP-2), tubulin, Neurofilament proteins (NFP), tau myelin basic protein (MBP), Myelin Associated Glycoprotein (MAG), CaMKII, alpha-synuclein, GFAP, S100B, Western Blot, chronic fatigue syndrome (CFS), irritable bowel syndrome (IBS).

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

- What were the major goals of the project?
 - The major goals of the project as stated in the approved SOW are listed in the table below. Milestones/target dates for important activities or phases of theses dates and actual completion dates are listed in the table below.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Accomplishments
- What was accomplished under these goals? - The table lists the proposed goals and dates to accomplish and the actual dates that these goals were accomplished during the study period of 36-month period.

Tasks	Timeline	
Task 1: Obtain Regulatory Reviews and Approvals	Planned Months	Actual Months
1a. Obtain necessary IRB approvals or Exempt status	1-3	1-4
1b. Obtain DOD Human Research Protections Office (HRPO) approvals or Exempt Status	1-3	1-4
Milestone(s) Achieved: Regulatory reviews completed and final approval obtained for study	1-3	1-4
Task 2: Obtain Stored Blood plasma and CSF samples from 3 biorepositories for analysis.	Months	
Proposed 2a: 100 GWI and 50 IBS plasma samples shipped from Site 5 to Duke and NIH for analysis.	4-6	
Actual 2 a: 100 GWI and 50 IBS plasma samples shipped from Site 5 to Duke and NIH for analysis		6-8
Actual 2a: Adapting western blot for plasma samples		10-11
Actual 2a: Beginning analysis of all plasma samples from 100 GWI and 50 controls.		10-12
Proposed 2b: 50 GWI and 50 healthy GW veteran controls and 50 CFS control serum samples shipped from site 4 to Duke and NIH for analysis.	4-9	
Actual 2b: Plasma samples received from NOVA University: 1. 50 CFS samples (site 4) 2. 26 healthy GW control samples (site 4) 3. 68 GWI samples from site 1 (Boston GWIC)		12

4. 50 IBS samples from site 5 (BIDMC) 5. Gulf War Illness (GWI)– 100 samples 6. Irritable Bowel Syndrome (IBS) - 50 sample	10	14
Proposed 2c: 100 GWI and 50 healthy GW veteran control serum samples shipped from site 1 to Duke and NIH for analysis.	10-22	
Actual 2c: Plasma samples from Site 4 (NOVA) Received were: Gulf War Illness(GWI) – 68 samples (Total GWI (68 + 100 = 168) Chronic Fatigue Syndrome (CFS) – 50 samples Healthy Control – 26 samples	12	16
Proposed *2d: 25 GWI and 25 healthy GW veteran control CSF samples shipped from site 1 to NIH for analysis	22-24	
Actual 2d: We were only able to obtain 4 CSF samples so this aim was not achieved through NIH. CSF was run for pilot autoantibody results only.		
Milestone(s) Achieved: Site 1, 4 and 5 serum and CSF data collected and set up for laboratory assays (ELISA, western blot). Autoantibody data shipped to analyzing labs from 250 GWI veterans and 200 controls (100 healthy and 100 diseased controls) blood serum samples and 50 CSF (25 GWI, 25 control) samples.	3-24	Samples were shipped to Duke lab for analysis from three sites including 322 samples (235 GWV; 37 IBS; 50 CFS)
Task 3: Perform Serum Assays	Months	
3a: Perform western blot analyses for autoantibodies to CNS proteins in GWI cases and control samples.	4-24	22
3b: Perform ELISA analyses for Neurofascin 155 CNS marker in serum samples from GWI cases and controls.	3-24	
Actual 3b: NIH collaborators declined to analyze samples for NF155 because preliminary ELISA analyses were not promising in their animal studies for GWI.		
Milestone(s) Achieved: Autoantibodies for CNS proteins of myelinogenesis, astroglionogenesis and neurogenesis data analyzed from three biorepository sites.	9-24	322 autoantibodies run for 9 CNS markers. They were merged with demographic datasets, analyzed and published in two manuscripts. (see below).
Task 4: Perform CSF Assays	Months	
4a: Perform ELISA assays of 50 CSF samples for neurofascin 155 biomarker.	22-24	

4a Actual: NIH collaborator declined to analyze CSF samples for NF155 because results were not promising in their animal model data.		
4b: Merge CSF outcome data with clinical neuroimaging, TBI and exposure data.	24-27	
4b Actual: CSF samples were so few that we were not able to merge with other markers in a meaningful way. This was done for autoantibody markers however.		
Milestone(s) Achieved: Antibody for neurofascin 155 marker data analyzed and merged with clinical outcome data from GWIC biorepository site.	24-27	
Task 5: Merge Data and Perform Interim Data analyses	Months	
Proposed 5a: Merge clinical dataset from sites 1, 4, 5 case/control status and demographics with results from laboratory analyses performed at NIH and Duke. Actual 5a: Case/control status merged with autoantibody results for GWI, CFS, IBS groups. Abstracts submitted. Merging with other demographics still being conducted.	10-24	18-24
Proposed 5b: Data analysis of interim ELISA and western blot results of autoantibodies in GWI cases and controls (healthy and diseased groups) with merged clinical datasets. Actual 5b: Merging is completed with clinical datasets and has been published in two manuscripts. See results in sections below.	18-24	
Proposed 5c: Discussion of results and preparation of abstracts for presentations at national meetings and initial manuscript for publication. Actual 5c: In person meeting held in Boston on August 23, 2017 where results, presentations and abstracts were planned. A second meeting happened in August 2019. Three total manuscripts have been published.	18-24	22 A meeting was held in Boston University on August 23, 2017. A second meeting happened in August 2019.
Proposed 5d. Annual reports of progress will be written. Actual 5d. Three yearly progress reports submitted on October 2017 and November 2018 and March 2020.	12-24	12, 24, 36

Milestone(s) Achieved: Preliminary analysis of results and presentation of initial results at scientific meetings and potential publication. Actual: 3 abstracts accepted for scientific meetings, 3 papers published to date, two more in preparation. Possible biomarker selection for GWI and recommendations for treatment development.	18-24	18-24
Milestone Achieved: ○ <i>Determination of autoantibodies against neural proteins in veterans of the Gulf War who have Gulf War Illness (GWI), GW controls, CFS and IBS symptomatic controls.</i>		
Milestone Achieved: <i>Development of ELISA assay for all neural proteins that we assay as biomarkers for nervous system injury.</i>		
Task 6: Perform Final Data analyses and Prepare Manuscripts for Publication	Months	
6a: Merge clinical datasets for neuroimaging, blood and genetic biomarkers, brain injury and exposure history with GWI cases and controls.	25-30	32
6b: Perform Data analysis comparing ELISA and western blot autoantibodies outcomes in GWI cases and controls with merged clinical datasets for neuroimaging, blood and genetic biomarkers, brain injury and exposure history.	25-30	32
6c: Discuss results of data analyses and prepare abstracts for DOD and other scientific meetings.	25-32	32

6d: Preparation of manuscripts 1. ELISA Assay of Autoantibodies Against Neural Proteins (SOT 2019) <i>This manuscript discusses the various steps used and their rational in the analysis of autoantibodies to neural proteins.</i> 2. Comparative study of Biomarkers for Gulf War Illness (GWI), Chronic Fatigue Syndrome (CFS) and Irritable Ball Syndrome (IBS). <i>This manuscript emphasizes the various profiles of autoantibodies of cytoskeletal proteins and their use in the diagnosis of veterans with GWI, CFS and IBS.</i> 3. Alterations in neuronal white matter proteins: MBP, MAG, Neuronal in GWI <i>This manuscript discusses the involvement of white matter in the mechanisms of GWI, CFS, and IBS.</i> 4 Changes in Cytoskeletal Proteins: Neurofilaments, Tubulin, Microtubule Associated Proteins, and Calcium Calmodulin Kinase II in GWI. <i>This manuscript discusses the involvement of Cytoskeletal Proteins in the mechanisms of GWI, CFS and IBS.</i>		
5. Involvement of Glial Proteins, GFAP and S100B in GWI-(SOT-2018) <i>This manuscript discusses the involvement of Glial Proteins in the mechanisms of GWI, CFS, and IBS.</i>	25-36	
6e: Third year report of progress was prepared.	35-36	

Milestone(s) Achieved: 1. Adapting our serum autoantibodies assay to plasma. 2. Confirming the results of our preliminary studies showing increased autoantibodies against neuronal and glial proteins in plasma from veterans with GWI. 3. Assaying plasma from veterans of the Gulf War with Chronic Fatigue Syndrome (CFS) for neural autoantibodies and establishing that it has a profile different from GWI. 4. Similarly, plasma from veterans of the Gulf War with Irritable Bowel Syndrome (IBS) exhibited neural autoantibodies profile that is distinct from both GWI and CFS. 4. Analysis of all study results, presentation of results at scientific meetings, submitted publication and final report in progress. 5. Possible diagnostic biomarker selection for GWI, brain injury and deployment-related exposures and potential recommendation for treatment development.	35-36	
7. No Cost Extension Year		
7.a Proposed: To carry out statistical analysis of the data collected during the past three years. The results of the assay of autoantibodies biomarkers in the total of 322 veterans of the 1991 Gulf War were analyzed for statistical differences.	36-48	Performing final data analyses and obtaining CSF samples for analysis is now complete. CSF was only elevated for MAG at 2.77 higher than controls.
7 b: Analyzing the results and Preparing the final report During the past no-cost extension year, we carried out statistical analysis of the results generated during the past three years on the use of autoantibodies against neural proteins that we generated in our study to distinguish GWI and other related conditions of the Gulf War. The following statistical studies for GWI Autoantibody data were carried out. 1. Analysis by sex for GWI Cases Only 2. Differences between GWI Veterans and All Controls 3. Analysis by Race for GWI Cases Only 4. Differences between GWI Veterans and All Controls Across Race The following statistical analysis analyses were carried out for: 1. Veterans with Gulf War Illness (GWI) 2. Veterans with Chronic Fatigue Syndrome (CFS) 3. Veterans with Irritable Bowel Syndrome (IBS) 4. Veterans Exposed to acetylcholinesterase (AChE) inhibitors	48-60	

Proposed 8. : Discussion of results and preparation of abstracts for presentations at national meetings and initial manuscript for publication. Actual 8: In person meeting held in Boston on August 11, 2019 where results, presentations and abstracts were planned. Final Report was discussed.		
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*** Serum and Cerebrospinal Fluid (CSF) samples are being collected as part of the ongoing Boston GWI consortium study and will be sent to NIH and Duke study sites as the samples are added to the GWIC biorepository. These samples will all be collected by month 24 of the current study.**

- **What was accomplished under these goals?**
 - *Major activities: Sending blood samples from over 300 GWV and symptomatic controls from three study sites to Duke Laboratory for analysis.* It was determined during early discussions with other co-investigators that more plasma than serum was available at the study sites so a large aim of this period was to adapt the Western blot assay of autoantibodies from serum samples to plasma samples so that both could be used in this study and importantly in other validation studies and clinical assessments in the future.
 - *Determination of autoantibodies against neural proteins in veterans of the Gulf War who have had Gulf War Illness (GWI), GW veteran healthy controls, Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS) symptomatic control groups have been completed.*
 - *Development of ELISA assay for all neural proteins that we assay as biomarkers for nervous system injury.*
 - *Specific objectives:*
 - We established Western blot analysis for autoantibodies against neural proteins in plasma samples from GWI subjects and from controls similar to our original method in serum samples from a small pilot study.
 - Quantitative measurement of neuronal autoantibodies by ELISA in the sera of patients with Gulf War Illness (GWI) was worked on but not completed due to staffing difficulties.
 - *Significant results:* These results now allow performing autoantibody assays not only in serum but also in plasma. This is a considerable advantage because many specimens from GW veterans are prepared as plasma not serum and other stored samples could now be used for this purpose and repeated by other laboratories.
 - In addition to GWI, some veterans also have other conditions such as CFS and IBS. We carried out a study to compare biomarkers for GWI, CFS and IBS. The results showed that non-veteran subjects with CFS and IBS had lower levels of autoantibodies (AA) against fewer neural proteins, indicating that the levels of AA against neural proteins in these patients were lower than those seen in veterans with GWI. This suggests that GWI can be distinguished from CFS and IBS patient groups and that GWI has distinct autoantibody patterns. We have now published these results in Abou-donia et al., 2020 (see appendix).

- We also analyzed AAs by sex and found that men and women with GWI showed higher AAs than those from the control groups. However, men had higher AA levels overall. These results were published in Abou-Donia et al., 2021 (see appendix).

Summary of Report: Statistical Analysis:

During the past no-cost extension year, we carried out statistical analysis of the results generated during the past three years on the use of autoantibodies against neural proteins to distinguish between GWI and other related conditions of 1991 GW veterans. The following statistical studies for GWI Autoantibody data were carried out and have been published or/are being prepared for publication.

1. Analysis by sex for GWI Cases Only
2. Differences between GWI Veterans and All Controls across sexes
3. Analysis by Race for GWI Cases Only
4. Differences between GWI Veterans and All Controls Across Race

Sample Source:

Task A. Gulf War Subjects and Controls

A total of 322 subjects and controls were investigated for CNS autoantibodies in order to develop a diagnostic marker of GWI and related diseases, i.e., CFS and IBS. Veterans with GWI and controls that were studied, were obtained from Boston GWIC Nova Southeastern University and Beth Israel Deaconness Medical Center (BIDMC) listed in Table 1. The controls included GWI controls, Irritable Bowel Syndrome (IBS), and Chronic Fatigue Syndrome (CFS) cases. These results were reported in Abou-Donia et al., 2020 and Abou-Donia et al., 2021 (see appendix).

Table 1. Sources of GWI Subjects and Controls

Source of Subjects and Controls	Sources	GWI Subjects	GWI Veteran Controls	IBS Controls	CFS Controls
Total N		175	60	37	50
Source of Subjects and Controls	1. Boston GWIC	85 (48.6%)	34 (56.7%)	0 (0.0%)	0 (0.0%)
	2 NOVA Southeastern University/Miami VA Medical Center	1 (0.6%)	26 (43.3%)	0 (0.0%)	50 (100%)
	3. BIDMC	89 (50.9%)	0 (0.0%)	37 (100%)	0 (0.0%)

The Demographic information for GWI and controls are listed in Table 2. The demographic information include age, gender and race of all subjects and controls.

Table 2. Demographic Information of GWI Subjects and Controls

Data	GWI Subjects	GWI Veterans Controls	IBS Controls	CFS Controls
N	171	60	35	50
Age (Mean ±SD)	49 (8)	51 (7)	39 (14)	47 (10)
Gender: Male	138 (80.2%)	56 (93.3%)	3 (8.6%)	5 (10%)
Female	34 (19.8%)	4 (6.7%)	32 (91.4%)	45 (90%)
Race: Caucasian	132 (77.6%)	45 (75%)	30 (85.7)	42 (91.3%)
African American	21 (12.4 %)	12 (20%)	3 (8.6)	4 (8.6%)
Other/Multiracial	17 (10.0%)	3 (5%)	2 (5.7%)	0 (0.0 %)

3.4. CNS Autoantibody (CAI), Neurodegeneration Index (NDI)

We developed a simple, blood-based proxy, the NDI, that is used to determine the combined total of CNS autoantibodies of an individual, based on a plasma assay of autoantibodies for each individual. It is calculated by adding all of the values of autoantibodies for each neural protein, and then, dividing the sum by the number of autoantibodies used.

Neurodegeneration Index (NDI) = (The Sum of Autoantibodies to “n” Proteins/n)

The Neurodegeneration Index score was calculated as described above for each tested group and the results were as follows: GWI = 39.35, GW healthy controls = 21.3, IBS controls = 11.94, and ME/CFS controls = 23.47.

Statistics. The mean NDI score for veterans with GWI was significantly higher than in all controls combined ($p < 0.0001$). In addition, the percentage of participants with NDI > 20 was significantly higher in GWI cases than in all controls combined (94.3% vs. 44.2%; $p < 0.0001$ via the chi-square test). The percentage of participants with NDI > 30 was also significantly higher in GWI cases than in all controls combined (71.8% vs. 14.3%; $p < 0.0001$ via the chi-square test). These results were published in Abou-donia et al., 2020 and Abou-donia et al., 2021 (see Appendix).

Autoantibodies against neural proteins were determined for the following:

- **Neuronal Proteins:** Neurofilament Triplet Proteins (NFP), Tubulin, Microtubule Associated Protein Tau (Tau), Microtubule Associated Protein-2 (MAP-2), Calcium/Calmodulin Kinase 2 (CaMK2), and Alpha Synuclein (SNCA). Autoantibodies B2. glial proteins were also measured:
- **Glial Proteins:**
 - **B1. Oligodendrocytes:** Myelin Basic Protein (MBP) and Myelin Associated Glycoprotein (MAG)

- **B2. Astrocytes:** Glial Fibrillary Associated Protein (GFAP) and Glial S100B (S100B). Autoantibodies were determined for GWI subjects, GWI Veterans Controls, IBS controls and CFS controls.

Autoantibodies against Neuronal and Glial Proteins in GWI Subjects and Controls in descending order as follows were significantly different between groups:

- **GWI Subjects:** MAP-2 9.66 > GFAP 4.27 > MBP 4.28 > GFAP 4.27 > Tubulin 4.13 > (NFP) 3.42 > (Tau) 2.92 > (CaMK2) 2.04 > 2.52 > (S100B) 1.17
- **GWI Veterans Controls:** MAP-2 5.04 > Tubulin 2.36 > GFAP 2.34 > MBP 2.17 > MAG 2.12 > S100B 1.17 NFP > 1.88 > Tau 1.57 > SNCA 1.46 > CaMK2 1.20
- **IBS Controls:** MAG 3.20 > MAG 1.19 Tau 1.14 MAP-2 1.05 > S100B 0.95 NFP 0.86 > SNCA 0.78 CaMK2 0.70 > GFAP 0.84
- **CFS Controls:** GFAP 4.86 > MAP-2 6.97 > MAG 1.58 NFP > MBP 1.52 > NFP 1.18 > CaMK2 1.16 SNCA 1.13 > Tau 1.0
- These results were published in Abou-donia et al., 2020 and for sex specific outcomes in Abou-donia et al., 2021 (see Appendix).

Task B

GWl Autoantibody Data: Differences between GWl veterans and all controls across sexes

This study was carried out on male veterans accounting for 138 (80.2%) obtained from Boston GWIC, 71 (51.5%) obtained from NOVA Southeastern University and 67 (48.6%) from BIDMC and female veterans accounting for 34 (19%), obtained from Boston GWIC, 14 (41%) NOVA Southeastern University and 20 (58%) from and 67 (48.6%) from BIDMC. Mean ages (SD) were 49.18 (7.36) and 20 (9.27) for males and females, respectively. For males, 110 (79.7%) were Caucasian, 19 (13.8%) African Americans and other/multinational were 9 (6.5%).

1. The levels of autoantibodies against all neuronal and glial proteins in male veterans were significantly different than all controls except for glial S-100B. Similarly, female veterans, exhibited significant difference from all controls, except for autoantibodies against Tau.
2. When levels of autoantibodies against male veterans with GWI were compared to healthy GWI controls across sexes, there was highly significant differences, except for levels of glial S100B. Similarly, female veterans, exhibited significant difference from all controls, except for autoantibodies against Tau and SNCA.
3. When levels of autoantibodies against male GWI participants were compared to veterans with Irritable Bowel Syndrome (IBS) controls, across sexes, GWI veterans showed highly significant increases compared to IBS controls, except for S100-B and SNCA. They also showed increased

differences except in levels of glial S100B. Similarly, female veterans, exhibited significant difference from all controls, except for autoantibodies against S100B and MAG.

4. When levels of autoantibodies against male GWI patients were compared to veterans with Chronic Fatigue Syndrome (CFS) controls, across sexes, GWI veterans showed highly significant increase compared to CFS controls, except for GFAP, and tubulin. Similarly, female veterans, exhibited significant difference from all controls, except for autoantibodies against GFAP, MAP-2, and tubulin. These results were published in Abou-donia et al., 2021 (see appendix).

Task C.

GW Autoantibody Data: Differences between GWI veterans and all controls across Races

1. Among Caucasians the levels of autoantibodies against neuronal and glial proteins were significantly higher than all controls except for glial S100B. Similar results were obtained for non-Caucasian GW veterans.
2. When comparing the differences between GWI veterans and healthy GW control across Caucasian Race, the difference for levels of autoantibodies were highly significant, except for the glial; S100-B protein. Similar results were obtained when differences between GWI veterans and healthy GW controls across Non-Caucasian.
3. Also, when comparing the levels of autoantibodies against neural proteins for veterans with GWI and veterans with Irritable Bowel Syndrome (IBS) Controls among Caucasian veterans, no differences were detected except for glial S100B autoantibodies that were not different from controls. In contrast, when comparing the levels of autoantibodies against neural proteins for veterans with GWI and veterans with Irritable Bowel Syndrome (IBS) controls among Non-Caucasian veterans, no statistical differences were found in the levels of autoantibodies against the following neural proteins: S100B, Tau, MAG, Tubulin, CaMKII, and SNCA, GFAP, MAP-2, and neurofilament proteins.
4. When comparing the levels of autoantibodies against neural proteins for veterans with GWI and veterans with Chronic Fatigue Syndrome (CFS) control among Caucasian veterans, significant differences were detected for all proteins except for neuronal tubulin and glial GFAP autoantibodies that were not significantly different from controls. In contrast, when comparing the levels of autoantibodies against neural proteins for GWI Veterans and veterans with CFS Controls among Non-Caucasian veterans, no statistical differences were found in the levels of autoantibodies against the following neural proteins: glial GFAP, and neuronal MAP-2, and Tubulin autoantibodies against GFAP, MAP-2, and neurofilament proteins.

Task D. Gulf War Subjects and Controls in Relation to Acetylcholinesterase (AChE) Exposure Status

a. GWI Veterans: When compared by gender, the levels of autoantibodies against the following plasma neural protein did not show significant sex differences: Tau, alpha-synuclein, CaMKII, myelin basic protein, Glial fibrillary acidic protein, and glial S100B. In contrast, the following autoantibodies were highly significantly less than the male veterans: myelin associated

glycoprotein (MAG) and Tubulin; whereas, MAP-2 and Neurofilament triplet proteins were just significant.

b. All Control Veterans:

In control veterans no sex differences were found in the levels of autoantibodies against all tested neural proteins i.e., neurofilament proteins, MAG, tubulin, MBP, CaMKII, Tau, MAP-2, GFAP and S-100B.

Table 3. Demographic Information of GWI Subjects and Controls in Relation to Acetylcholinesterase (AChE) Exposure Status

Data	GWI Subjects Exposed	GWI Subjects Not Exposed
N	83	88
Age (Mean $\pm$ SD) Range (Years)	50.70 (6.24) 25-75	46.88 (8.67) 24.0-68.0
Gender: Male	70 (84.3%)	68 (76.4%)
Female	13 (15.7%)	21 (23.6%)
Race: Caucasian	66 (79.5%)	73 (79.4%)
African American	14 (16.9 %)	8 (8.7%)
Other/Multiracial	5 (6.0%)	5 (5.4%)

Table 4. Sources of GWI Subjects and Controls in Relation to Acetylcholinesterase (AChE) Exposure Status

Source of Subjects and Controls	Sources	GWI Subjects Exposed	GWI Subjects Not Exposed
Total N (175)		83 (47.4%)	92 (52.6%)
	2 NOVA Southeastern University/Miami VA Medical Center	0 (0%)	1 (1.1%)
	3. BIDMC	(0%)	89 (96.7%)

Table 5. Autoantibodies against Neuronal and Glial Proteins in GWI Subjects and Controls In Relation to Acetylcholinesterase (AChE) Exposure Status

Autoantibodies against		GWI Case Exposed	GWI Case Not Exposed	Comparing those exposed vs. not using ANCOVA adjusting for age, gender, and race (GWI cases only)
N		83	91	p-value

A. Neuronal Proteins				
1. Neurofilament Triplet Proteins (NFP)	Mean ±SD	3.67 ± 2.16	3.19 ±2.94	0.56
2. Tubulin	Mean ±SD	4.45 ± 2.90	3.84 ±3.57	0.56
3. Tau	Mean ±SD	2.90 ± 1.63	9.22 ±5.37	0.63
4. MAP-2	Mean ±SD	10.16 ± 4.60	1.88 ±2.02	0.61
5. Calcium/Calmodulin Kinase 2 (CaMK2)	Mean ±SD	1.92 ± 0.88	2.14 ±1.30	0.07
6. Alpha Synuclein (SNCA)	Mean ±SD	2.26 ± 1.16	1.88 ±2.02	0.20
B. Glial Proteins:				
Oligodendrocytes:				
1. Myelin Basic Protein (MBP)	Mean ±SD	3.86 ± 1.87	4.75 ±2.02	0.61
2. Myelin Associated Glycoprotein (MAG)	Mean ±SD	4.18 ± 2.09	5.64 ±2.02	0.0006
Glial Proteins: Astrocytes:				
3. Glial Fibrillary Associated Protein (GFAP)	Mean ±SD	4.63 ± 2.25	3.95 ±2.95	0.11
4. Fold Change Glial S100B (S100B)	Mean ±SD	1.50 ± 0.40	0.87 ±0.37	< .0001

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

- What opportunities for training and professional development has the project provided?
Professional development. The results of studies that were generated in this project were presented in a national meeting and an international meeting:
 1. The Annual Meeting of the Society of Toxicology (SOT), March 2016, New Orleans: A poster of the results was presented.
 2. The International Neuropsychological Society (INS) annual mid-summer meeting, July 2016, London, England.
 3. An oral presentation was given as part of an invited symposium on Gulf War Illness.
 4. The Annual SOT, March 2017, Baltimore, MD: A poster was presented (see Appendix).
 5. The Annual SOT, March 2018 in San Antonio, Texas: Two posters were presented (see Appendix)
 6. The annual SOT meeting, March 2019 in Baltimore, MD a poster was presented.

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

- Our results were discussed with many interested scientists during both the Society of Toxicology (SOT) and International Neuropsychological Society (INS) and the GW State of the Science 30th anniversary meetings.
- A symposium on Gulf War Illness took place in London during the International Neuropsychological Society mid-year meeting July 2016, during which the results from this project and several other related projects were presented and stirred interest.
- We published our first manuscript of CNS autoantibodies in the Journal Neurotoxicology and Teratology (see attached and below) we were also publicized in a local media story at <https://www.bu.edu/sph/2017/03/29/identifying-biomarkers-of-gulf-war-illness>
Abou-Donia MB, Conboy LA, Kokkotou E, Jacobson E, Elmasry EM, Elkafrawy P, Neely M, Bass CR, Sullivan K. Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. Neurotoxicol Teratol. 2017 May; 61:36-46. PMID: 28286177.
- We have now published two additional publications in Brain Sciences in Abou-donia et al., 2020 and Abou-donia et al., 2021 (see attached). The first publication was chosen for the cover of the journal and was widely highlighted by the journal. <https://www.mdpi.com/2076-3425/10/9/610>. We also had a media story about the publication at <https://www.bu.edu/sph/news/articles/2020/blood-test-could-identify-gulf-war-illness/> and <https://www.bu.edu/articles/2020/3-discoveries-that-could-impact-diagnosis-treatment-of-ptsd-and-gulf-war-illness/>
- We were also highlighted in a story by the CDMRP https://cdmrp.army.mil/gwirp/research_highlights/20abou_donia_highlight

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

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

1. Currently we are preparing two manuscripts to report the rest of the results of our studies.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

- What was the impact on the development of the principal discipline(s) of the project?
 - Our studies have focused on the development of sensitive, specific, reproducible, and non-invasive blood biomarkers of GWI. Identifying objective biomarkers of GWI helps the veteran and the treating clinicians who must now rely on self-report of symptoms as the primary diagnostic marker. The advantage of a blood-based biomarker is that it can diagnose GWI with greater accuracy and with only a few drops of blood. Our initiative to validate both serum and plasma for these potentially diagnostic autoantibodies will make diagnosing GWI and validating it with other stored blood samples from GWI even easier because it won't be limited by just one type of blood product.
 - The results of the study can be applied immediately to treatment development strategies for the veterans of the Gulf War. Based on the CNS autoantibodies we have found, this will provide the opportunity to develop drugs that treat neuronal injury in those specific pathways (neuronal and glial); such treatment could be directly applicable to Gulf War veterans in the short-term.
 - Our first three publications provide the CNS autoantibodies that we have now validated in our larger study sample of men and women veterans and will be the target for treatment development planning.

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

- What was the impact on other disciplines?
 - A major advantage of our peripheral marker is that it is specific for neural injury irrespective of the cause, thus it can be applied to diagnose or confirm diagnosis of other neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease in addition to GWI. However, the *pattern* of the specific markers elevated can be different with different disease states and thus can also be useful diagnostically once validated in different disorders. For example, we have recently found different patterns of CNS autoantibodies in CFS, IBS and GWI that we have now published which shows that GWI is different than other chronic multi-symptom disorders.
 - Blood-based biomarkers of GWI provide an effective way to enhance its management
 - It can be used as a diagnostic and prognostic tool with the ability to provide information about rate of disease progression.
 - It would help in identification of novel and effective treatments for multiple disorders and environmental exposure groups (i.e. pesticides, nerve agents).
 - It could be used for monitoring therapeutic efficacy for the benefit of patients and caretakers.
 - It could be used to follow-up treatment plans of the patient if used pre-post treatment.
 - It could provide a cost-savings potential for recruitment into clinical trials.

What was the impact on technology transfer? *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*

- *adoption of new practices.*

- Blood-based biomarkers of GWI provide an effective way to enhance its management
 - It can be used as a diagnostic and prognostic tool with the ability to provide information about rate of disease progression.
 - It would help in identification of novel and effective treatments for multiple disorders and environmental exposure groups (i.e. pesticides, nerve agents).
 - It could be used for monitoring therapeutic efficacy for the benefit of patients and caretakers.
 - It could be used to follow-up treatment plans of the patient
 - It could provide a cost-savings potential for recruitment into clinical trials.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

- Our peripheral biomarker should improve the quality of life for the veterans of the GW who have GW illness because:
- Upon their returning from the GW theater in 1991, their subjective complaints could not be diagnosed and they were told that their complaints were “all in their heads”. Our biomarker should confirm CNS injury that is consistent with their complaints. Such consequence should give them a peace of mind.
- Our biomarker should lead studies to develop treatment for brain injury that would provide improvement of their clinical condition.
- The hallmark of Gulf War Illness (GWI) is neuroinflammation, neural cell death in specific regions of the brain and possible progressive neurodegeneration. A challenging aspect of GWI is that it has been difficult to diagnose with objective biomarkers because organophosphate pesticides and nerve agents do not stay in the body and CNS the same way that other exposures do (i.e. agent orange, depleted uranium, lead, mercury). Therefore, researchers have had to develop markers of damage from these chronic exposures rather than markers of the exposure or their bi-products. If successful, this work will impact neurotoxicant exposed individuals including agricultural workers, pesticide applicators and nerve gas exposed groups by providing objective inexpensive markers of chronic damage relating to these exposures that can be conducted virtually anywhere that a simple blood draw can be obtained and analyzed. Other current diagnostic practices including neuroimaging techniques, behavioral history assessments, and neuropsychiatric tests have drawbacks of not always being practical or available in other parts of the world but a simple blood test could provide objective diagnostic markers in the most cost-effective way.

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes.

Remember that significant changes in objectives and scope require prior approval of the agency.

- Changes: serum to plasma samples
Our original studies in determining autoantibodies in blood used serum samples from GWI cases and symptomatic controls. However, it was determined that our co-investigators had more from plasma available than serum. Therefore, we carried out experiments to establish the validity of our assay using plasma, as stated above under 'Accomplishments', we showed that the results from plasma samples were identical to those of serum samples. This was a big accomplishment that either plasma or serum can be used for these analyses because all other major studies of GWI with either serum or plasma samples could potentially validate our findings with their own samples.

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

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

We have been delayed in getting CSF samples for analysis because the GWIC was not able to obtain many samples from our study participants. Therefore, only preliminary CSF analyses were able to be run in our small number of samples which showed increased levels of MAG-2.

In future studies, we will collaborate with other investigators with stored CSF samples to validate these preliminary findings.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

- Changes that had significant impact on expenditures
 - None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:
 - Significant changes in use or care of human subjects: None
 - Significant changes in use or care of vertebrate animals: None
 - Significant changes in use of biohazards, and/or select agents: None

Significant changes in use or care of vertebrate animals.

N/A

N/A

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

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

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

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

- **Journal Publications**

1) Abou-Donia MB, Conboy LA, Kokkotou E, Jacobson E, Elmasry EM, Elkafrawy P, Neely M, Bass CR', Sullivan K. Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. *Neurotoxicol Teratol.* 2017 May;61:36-46. doi: 10.1016/j.ntt.2017.03.002. Epub 2017.

2) Abou-Donia MB, Lapadula ES, Kregel MH, Quinn E, LeClair J, Massaro J, Conboy LA, Kokkotou E, Abreu M, Klimas NG, Nguyen DD, Sullivan K. Using Plasma Autoantibodies of Central Nervous System Proteins to Distinguish Veterans with Gulf War Illness from Healthy and Symptomatic Controls. *Brain Sci.* 2020 Sep 5;10(9):610. doi: 10.3390/brainsci10090610.

3) Abou-Donia MB, Kregel MH, Lapadula ES, Zundel CG, LeClair J, Massaro J, Quinn E, Conboy LA, Kokkotou E, Nguyen DD, Abreu M, Klimas NG, Sullivan K. Sex-Based Differences in Plasma Autoantibodies to Central Nervous System Proteins in Gulf War Veterans versus Healthy and Symptomatic Controls. *Brain Sci.* 2021 Jan 23;11(2):148. doi: 10.3390/brainsci11020148.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

None

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

None

- **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

None

- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

None

- **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

None

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life.

None

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change.”

1. Participants & other Participating Organizations

Site 1: Boston University School of Public Health
715 Albany Street, T4W
Boston, MA 02118
Initiating PI: Dr. Kimberly Sullivan
Co-I: Dr. Joseph Massaro
Co-I: Dr. Maxine Krengel
Tasks 1-6

Site 2: Duke University Medical Center
Durham, North Carolina 27710
Partnering PI: Dr. Mohamed Abou Donia
Co-I: Dr. Cameron R. ‘Dale’ Bass
Tasks 2,3,5,6

Site 3: National Institutes of Health, NICHD
Bldg. 35, Room 2A211, MSC 3713
35 Lincoln Drive
Bethesda, MD 20892

Blood Serum and CSF Biorepository Sites

Site 4: NOVA Southeastern University
Ft. Lauderdale, FL
Co-I: Dr. Nancy Klimas
Tasks 1,2, 5, 6

Site PI: Dr. R. Douglas Fields
Co-I: Dr. Dipankar Dutta
Tasks 2-6

Site 5: Beth Israel Deaconess Medical Ctr.
Boston, MA 02118
Consultant: Dr. Efi Kokkotou
Consultant: Dr. Lisa Conboy
Tasks 1,2, 5,6

2. No Change has taken place

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

3. Participants & other Participating Organizations

Site 1: Boston University School of Public Health 715 Albany Street, T4W Boston, MA 02118 Initiating PI: Dr. Kimberly Sullivan Co-I: Dr. Joseph Massaro Co-I: Dr. Maxine Krengel Tasks 1-6	Site 2: Duke University Medical Center Durham, North Carolina 27710 Partnering PI: Dr. Mohamed Abou Donia Co-I: Dr. Cameron R. 'Dale' Bass Tasks 2,3,5,6
Site 3: National Institutes of Health, NICHD Bldg. 35, Room 2A211, MSC 3713 35 Lincoln Drive Bethesda, MD 20892 Site PI: Dr. R. Douglas Fields Co-I: Dr. Dipankar Dutta Tasks 2-6	<u>Blood Serum and CSF Biorepository Sites</u> Site 4: NOVA Southeastern University Ft. Lauderdale, FL Co-I: Dr. Nancy Klimas Tasks 1,2, 5, 6 Site 5: Beth Israel Deaconess Medical Ctr. Boston, MA 02118 Consultant: Dr. Efi Kokkotou Consultant: Dr. Lisa Conboy Tasks 1,2, 5,6

7. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ebrap.org/> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

- 8. APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Appendix 1: Abou-Donia MB, Conboy LA, Kokkotou E, Jacobson E, Elmasry EM, Elkafrawy P, Neely M, Bass CR', Sullivan K. Screening for novel central nervous system biomarkers in

veterans with Gulf War Illness. *Neurotoxicol Teratol.* 2017 May;61:36-46. doi: 10.1016/j.ntt.2017.03.002. Epub 2017.

Appendix 2: Abou-Donia MB, Lapadula ES, Kregel MH, Quinn E, LeClair J, Massaro J, Conboy LA, Kokkotou E, Abreu M, Klimas NG, Nguyen DD, Sullivan K. Using Plasma Autoantibodies of Central Nervous System Proteins to Distinguish Veterans with Gulf War Illness from Healthy and Symptomatic Controls. *Brain Sci.* 2020 Sep 5;10(9):610. doi: 10.3390/brainsci10090610.

Appendix 3: Abou-Donia MB, Kregel MH, Lapadula ES, Zundel CG, LeClair J, Massaro J, Quinn E, Conboy LA, Kokkotou E, Nguyen DD, Abreu M, Klimas NG, Sullivan K. Sex-Based Differences in Plasma Autoantibodies to Central Nervous System Proteins in Gulf War Veterans versus Healthy and Symptomatic Controls. *Brain Sci.* 2021 Jan 23;11(2):148. doi: 10.3390/brainsci11020148.

Article

Using Plasma Autoantibodies of Central Nervous System Proteins to Distinguish Veterans with Gulf War Illness from Healthy and Symptomatic Controls

Mohamed B. Abou-Donia ^{1,*}, Elizabeth S. Lapadula ¹, Maxine H. Krengel ², Emily Quinn ³, Jessica LeClair ³, Joseph Massaro ³, Lisa A. Conboy ⁴, Efi Kokkotou ⁴, Maria Abreu ^{5,6}, Nancy G. Klimas ^{5,6}, Daniel D. Nguyen ³ and Kimberly Sullivan ^{3,*} 

¹ Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA; elizabeth.suwita@duke.edu

² Department of Neurology, Boston University School of Medicine, Boston, MA 02118, USA; mhk@bu.edu

³ Departments of Biostatistics and Environmental Health, Boston University School of Public Health, Boston, MA 02118, USA; eq@bu.edu (E.Q.); jleclai2@bu.edu (J.L.); jmm@bu.edu (J.M.); ddn@bu.edu (D.D.N.)

⁴ Department of Medicine, Harvard Medical School, Boston, MA 02115, USA; Lisa.Conboy@mcphs.edu (L.A.C.); ekokkoto@bidmc.harvard.edu (E.K.)

⁵ Dr. Kiran C. Patel College of Osteopathic Medicine, Institute for Neuroimmune Medicine, Nova Southeastern University, Fort Lauderdale, FL 33314, USA; mabreu1@nova.edu (M.A.); nklimas@nova.edu (N.G.K.)

⁶ Department of Immunology, Miami VA Medical Center, Miami, FL 33125, USA

* Correspondence: donia@duke.edu (M.B.A.-D.); tty@bu.edu (K.S.); Tel.: +1-919-684-2221 (M.B.A.-D.)

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Abstract: For the past 30 years, there has been a lack of objective tools for diagnosing Gulf War Illness (GWI), which is largely characterized by central nervous system (CNS) symptoms emerging from 1991 Gulf War (GW) veterans. In a recent preliminary study, we reported the presence of autoantibodies against CNS proteins in the blood of veterans with GWI, suggesting a potential objective biomarker for the disorder. Now, we report the results of a larger, confirmatory study of these objective biomarkers in 171 veterans with GWI compared to 60 healthy GW veteran controls and 85 symptomatic civilian controls ($n = 50$ myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and $n = 35$ irritable bowel syndrome (IBS)). Specifically, we compared plasma markers of CNS autoantibodies for diagnostic characteristics of the four groups (GWI, GW controls, ME/CFS, IBS). For veterans with GWI, the results showed statistically increased levels of nine of the ten autoantibodies against neuronal “tubulin, neurofilament protein (NFP), Microtubule Associated Protein-2 (MAP-2), Microtubule Associated Protein-Tau (Tau), alpha synuclein (α -syn), calcium calmodulin kinase II (CaMKII)” and glial proteins “Glial Fibrillary Acidic Protein (GFAP), Myelin Associated Glycoprotein (MAG), Myelin Basic Protein (MBP), S100B” compared to healthy GW controls as well as civilians with ME/CFS and IBS. Next, we summed all of the means of the CNS autoantibodies for each group into a new index score called the Neurodegeneration Index (NDI). The NDI was calculated for each tested group and showed veterans with GWI had statistically significantly higher NDI values than all three control groups. The present study confirmed the utility of the use of plasma autoantibodies for CNS proteins to distinguish among veterans with GWI and other healthy and symptomatic control groups.

Keywords: etiology; Gulf War Illness; CNS autoantibodies; myalgic encephalomyelitis/chronic fatigue syndrome; irritable bowel syndrome

1. Introduction

Although the 1991 Gulf War (GW) only had less than two months of air strikes and less than a week of ground combat, approximately one-third of the 697,000 U.S. veterans developed a combination of health symptom complaints, including debilitating fatigue, chronic headache and body pain, memory and concentration difficulties, gastrointestinal problems, and skin abnormalities, known as Gulf War illness (GWI) [1–3]. In addition, some GW veterans also had increased rates of two other distinct conditions, Irritable Bowel Syndrome (IBS) and Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS), which have overlapping symptoms with GWI [4,5]. Given these overlapping symptoms with other chronic conditions, it has been difficult to confirm the presence or absence of GWI or to differentiate between these disorders [6–8]. Neuroimaging has been found to be useful as a differential diagnostic tool for GW ill versus GW healthy individuals; however, what has been missing is the ability to use a less invasive, more readily available, and less costly tool, such as blood biomarkers to differentiate GWI from GW healthy status and differentiate GWI from another chronic multisymptom disorder [6,8]. There have been some encouraging blood biomarker studies reporting differences between GWI cases and controls on neuroinflammatory markers that require validation in other GWI cohorts [9–11]. In addition, recently, we reported on a pilot study of serum biomarkers, which found seven out of eight markers significantly differed in veterans with GWI versus symptomatic controls with lower back pain, suggesting new potential blood markers for GWI [12]. The current study expanded on these prior findings by adding newly developed cutting-edge blood plasma autoantibodies in GW veteran and civilian cohorts, including those with IBS and ME/CFS, to identify whether veterans with GWI have the signature central nervous system (CNS) damage associated with their deployment that is different from other groups with overlapping chronic symptoms. IBS is a chronic multisymptom illness that affects the gastrointestinal system and results in diarrhea or constipation or both. Although the cause of IBS is not known, it may result from altered gut motility, stress, environmental exposures, and genetic predisposition. It has also been shown to be related to alterations of the gut–brain axis in animal models of GWI and in a pilot study of GW veterans [13–16]. ME/CFS is characterized by extreme fatigue, muscle pain, headaches, multijoint and throat pain, lymph node swelling and soreness, chronic insomnia, and sleep disorders [6]. In addition, it may cause loss of memory and reduced concentration. Contributing factors may include viruses, weakened immune system, stress, or environmental exposures. Although some symptoms among the three disorders (GWI, IBS, ME/CFS) overlap, the etiologies differ, raising the question whether objective blood markers of GWI could be distinguished among these other chronic medical conditions.

GW veterans were exposed to numerous environmental neurotoxicants, including acetylcholinesterase (AChE)-inhibiting organophosphate pesticides and nerve gas agents [17,18]. Early studies investigated the hypothesis that GWI resulted from combined exposures of GW-relevant toxicants including pyridostigmine bromide (PB), *N,N*-diethyl-meta-toluamide (DEET), permethrin, and chlorpyrifos in hens [19,20]. Mixed exposures to multiple toxicants resulted in significantly greater toxic effects than separate exposures. More recent results with GW veterans who were pesticide applicators during the war also showed that combination exposures to PB and pesticides were associated with higher rates of GWI and specifically, with diminished CNS functioning on mood and cognition [18].

Pesticides used during the GW easily enter through the blood–brain barrier (BBB) because they are lipid-soluble [19,20]. These neurotoxicants have been found to be associated with autoantibodies to CNS proteins in the blood in several prior studies and exposed groups [12,20,21]. These exposures have been associated with neurological symptoms associated with CNS cellular functioning. For example, studies showed increased levels of CNS cellular proteins in pesticide-exposed participants with neurological symptoms [22–24]. These results were similar to those found in our pilot study of ill GW veterans [12].

The brain has two types of cells: neurons and supporting glial cells, including astrocytes and oligodendrocytes [21,25–27]. Neurons are characterized by the cell body and two additional parts,

including axons and dendrites. Proteins in the axon include neurofilament triplet proteins (NFP), tubulin, tau, calcium/calmodulin kinase II (CaMKII), and (α -syn) [20,21,26,28]. Proteins in the dendrites include microtubule associate protein (MAP-2) [28]. Microtubules and tau make up the cytoskeleton of neurons.

Glial support cells include oligodendrocytes that myelinate axons using myelin basic protein (MBP) and myelin-associated glycoprotein (MAG). Astrocytes secrete glial fibrillary acidic protein (GFAP) and S100B only in the CNS [21,29,30].

The present study was carried out to use our newly developed biomarker test to differentiate GWI from other chronic conditions and healthy controls and confirm/validate our previous preliminary report of a small number of GW veterans and symptomatic controls showing increased CNS protein autoantibodies in their blood [12]. We hypothesized that as a result of neurotoxicant exposures during the war, autoantibodies to these ten CNS proteins would be increased in veterans with GWI when compared with other healthy and symptomatic control groups. Specifically, we hypothesized that neuronal and glial CNS proteins would differ in veterans with GWI compared with healthy and symptomatic controls with similar multisymptom disorders, including IBS and ME/CFS.

2. Materials and Methods

Study Population: GW illness consortium (GWIC) and the Dynamic Modeling of GWI study participants, two Department of Defense supported studies at Boston University and Nova Southeastern University, provided plasma samples from veterans deployed to the 1991 GW. Additional GWI participant samples were shared from the New England School of Acupuncture. These three established biorepositories of GW veterans were used from veterans who consented to share their blood samples for future studies. Control samples were provided by the Congressionally Directed Medical Research Program (CDMRP) funded studies in Boston and Florida, and samples from patients with Irritable Bowel Syndrome came from the biorepository at Beth Israel Deaconess Medical Center. Institutional Review Boards (IRBs) approvals from these biorepositories were obtained from Boston University, Nova Southeastern University, the Miami VA Medical Center, and Beth Israel Deaconess Medical Center.

The same standard operating procedures for phlebotomy, plasma separation, aliquoting, and storage were followed by all labs for all samples. Plasma samples were obtained from fasting subjects. Samples remained frozen at -80°C until shipped for autoantibody analysis.

Cases and controls were determined by Kansas GWI criteria [31]. This criterion requires GW veterans to self-report symptoms in 3 out of 6 symptom domains (neurologic/mood/cognitive, fatigue, pain, gastrointestinal, respiratory, and skin). Veteran controls were deployed to the GW and did not meet the Kansas GWI or exclusionary criteria. Exclusions included CNS medical conditions and psychiatric illnesses that could account for their symptoms [31]. Plasma samples from symptomatic controls came from prior studies of individuals with ME/CFS and IBS. ME/CFS cases were determined by using 1994 CDC criteria [5]. IBS cases were determined by Rome III criteria [32]. The full cohorts have been described in previous papers (GWIC, ME/CFS, IBS, GWIC subsample) [4,12,14,33,34]. Institutional review boards at Nova Southeastern University/Miami VA Medical Center, New England School of Acupuncture, Beth Israel Deaconess Medical Center, and Boston University provided approval. All participants signed consent to use their plasma for follow-up studies of GWI biomarkers.

Ethical Statement: Approval for the use of stored blood samples for this study was obtained from the Duke University Health System Institutional Review Board for Clinical Investigations on 9 October 2017 and from the Boston University Medical Campus Institutional Review Board on 19 January 2018. The specific protocol components for Duke University were: Protocol ID: Pro00003202, Reference ID: 335940, Principal Investigator: Mohamed Abou Donia, Protocol Title: 'Nervous System Injury'. The specific protocol components for Boston University were Protocol ID: H-34334, Reference ID: 1288716, Principal Investigator: Kimberly Sullivan, Protocol Title: 'Novel Autoantibody Serum and Cerebrospinal Fluid Biomarkers in Veterans with Gulf War Illness'.

2.1. Materials

The proteins used in this study were as follows: Tubulin (human recombinant, Cat. #PRO-982, ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA), Microtubule-Associated Protein 2 (MAP-2, human recombinant, Cat. #TP316775, OriGene, Rockville, MD, USA), Tau-381 (human recombinant, Cat. #AG952, MilliporeSigma, Burlington, MA, USA), Neurofilament Protein (NFP, Cat #PRO-523, ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA), Calmodulin Kinase II (human recombinant, CaMKII, Cat #H000000H15-P01, Novus Biologicals, Littleton, CO, USA), Alpha-synuclein (human recombinant, Cat. #AS-55555, AnaSpec, Fremont, CA, USA), Myelin Basic Protein (MBP, human, Cat. #30R-AM030, Fitzgerald Industries International, Acton, MA, USA), Myelin-Associated Glycoprotein (MAG, human recombinant, Cat. #131-86-H02H, Sino Biological Inc., Wayne, PA, USA), Glial Fibrillary Acidic Protein (GFAP, human, Cat. #345996, CalBiochem, San Diego, CA, USA), and S100B Protein (human, Cat. #30R-AS002, MilliporeSigma, Burlington, MA, USA).

2.2. Procedures

Plasma procedures: All sites used the same written standard operating procedures for venipuncture, blood handling, plasma separation, aliquoting, and storage at -80°C . Blood samples were collected prior to intervention for treatment trials. Samples remained frozen until sent for analyses and were visually inspected to not have hemolysis.

Western blot assay: In this study, a Western blot analysis was used for determination of CNS autoantibodies and antigens from the plasma samples of GWI cases and healthy and symptomatic controls. All plasma samples were analyzed three times for consistency and followed the protocol previously published in [12]. Specifically, each CNS protein was loaded into 10 ng/lanes. Immunoglobulin G (IgG) was loaded into a 100 ng/lane. All proteins were denatured and electrophoresed on SDS-PAGE (gradient 4% to 20% gradient) and a separate gel was used for each plasma sample. Enhanced chemiluminescence was used to determine if proteins were found by using a Typhoon 8600 variable model recorder (GE Lifesciences, Marlborough, MA, USA). The signal intensity was determined by Bio-Rad Quantity One image analysis software (Hercules, CA, USA). Specifically, the protein bands were quantified on digitized images in the mid-dynamic range using Quantity One software (Bio-Rad) and densitometry measurements were normalized to IgG in the same samples. Lab researchers were blinded to the case–control status of the samples.

2.3. Calculations

Measurement of chemiluminescent optical density for cases and controls was obtained by dividing plasma IgG concentrations. This optical density measure was normalized to controls and expressed as fold-change from healthy controls. Therefore, the CNS autoantibody measurements were presented as mean triplicate assay values normalized to healthy control values.

2.4. Neurodegeneration Index (NDI)

This new index was designed to determine the overall neurodegenerative condition of an individual based on the level of autoantibodies in the plasma. It is calculated by adding all of the values of autoantibodies for each neural protein, and then, dividing the sum by the number of autoantibodies used. Finally, this value is multiplied by 10 to produce the NDI.

$$\text{Neurodegeneration Index (NDI)} = (\text{The Sum of Autoantibodies to "n" Proteins}/n) \times 10 \quad (1)$$

The NDI is used here as a simple, blood-based proxy to determine the extent of neurodegeneration of an individual, based on a plasma assay of autoantibodies for an individual.

2.5. Statistical Methodology

Descriptive statistics are presented as mean $\pm$ SE for continuous variables and as number and percent of participants per category of categorical variables. Subjects' demographic values were compared across the four groups using one-way analysis of variance for continuous outcomes and the chi-square test for categorical outcomes. Mean values of the antibodies were compared across groups using analysis of covariance (ANCOVA) adjusting for age, sex, and race. p values were two-sided. To account for multiple comparisons, $p < 0.001$ was accepted as statistically significant for the comparisons between treatments on antibody levels. Analyses were conducted using SAS Version 9.4 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Specificity of Serum Autoantibodies

The specificity of the serum autoantibodies against all tested neural proteins was previously reported by performing protein/peptide competitive assay [12,26]. The specificity of an autoantibody in the sera was assessed by performing a peptide/antigen absorption assay by preabsorbing the serum with the target proteins. The preabsorbed serum was tested by Western blot (Figures 1–3).

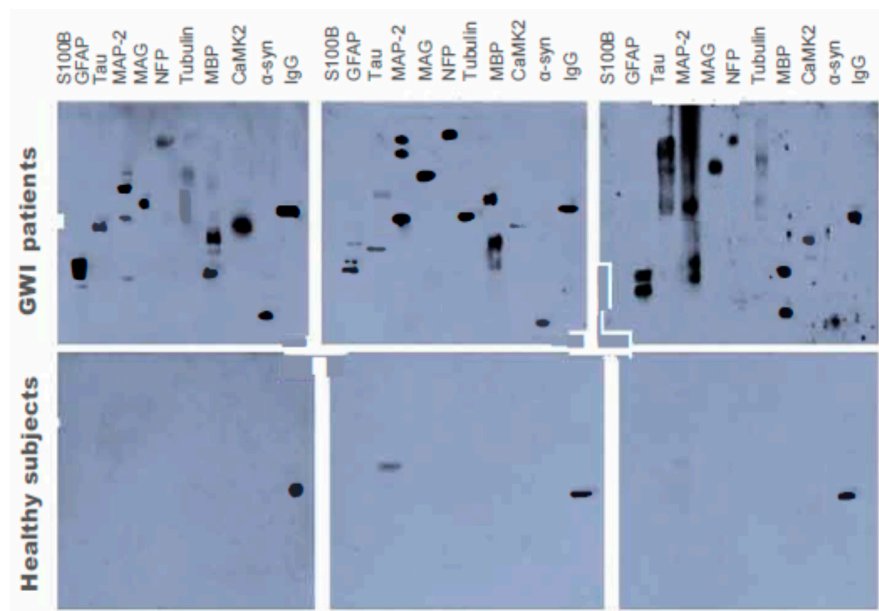


Figure 1. Representative panel of Western blotting from three cases of the GWI patients (upper panels), and healthy controls (lower panels).

3.2. Participant Demographics

Demographics are presented in Table 1. Participants were 175 veterans with GWI, 60 GW veteran healthy controls, 37 IBS controls, and 50 ME/CFS symptomatic controls. Significant differences were seen for age, sex, and race.

This study was carried out to use our newly developed neurodegenerative biomarkers to diagnose veterans of the 1991 Gulf War with GWI compared with healthy and symptomatic controls. The biomarkers consist of circulating autoantibodies of ten neural proteins (six neuronal and four glial) determined in the plasma of GW veterans with GWI, healthy Gulf war veterans, veterans with ME/CFS, and IBS that were used as controls. The NDI was calculated as described in the methods above and was assessed among the groups by chi-square tests.

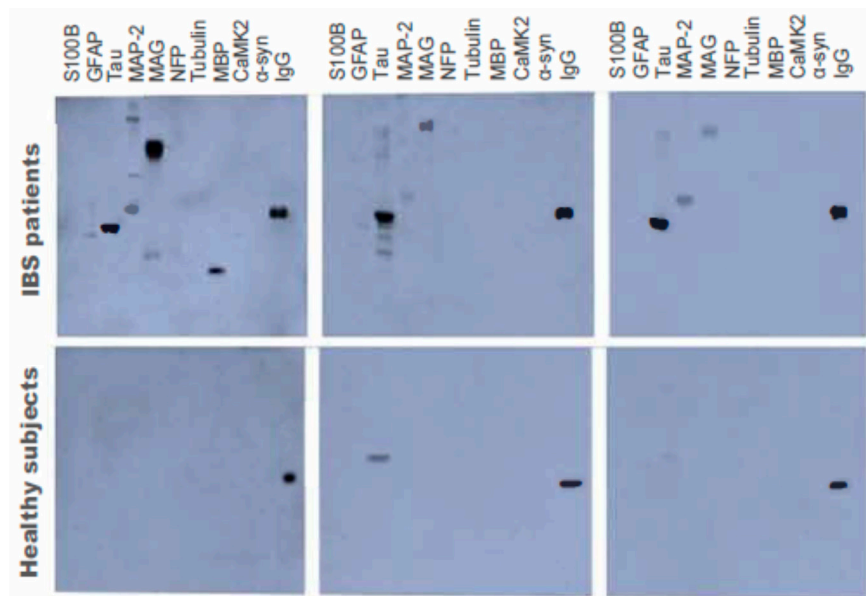


Figure 2. Representative panel of Western blotting from three cases of the IBS patients (upper panels), and healthy controls (lower panels).

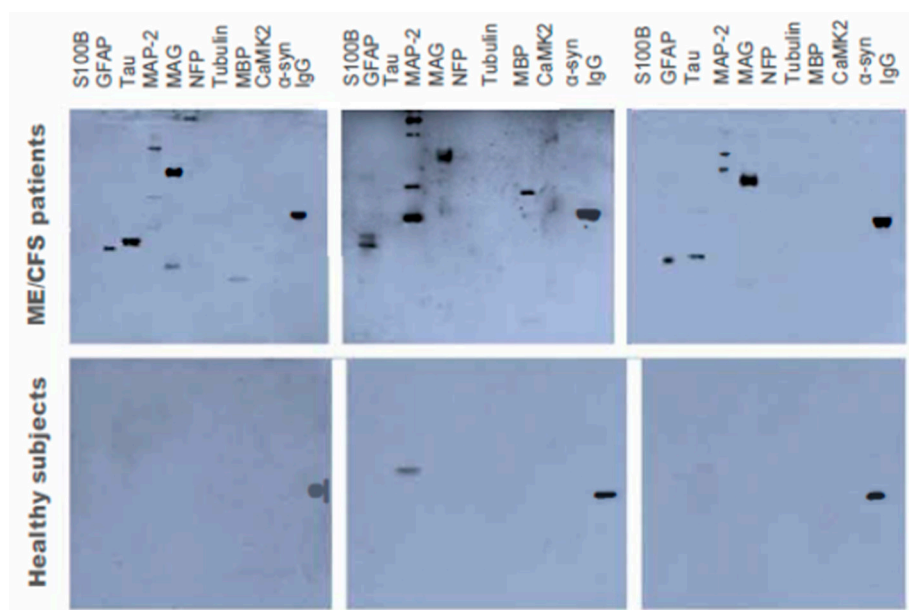


Figure 3. Representative panel of Western blotting from three cases of the ME/CFS patients (upper panels), and healthy controls (lower panels).

3.3. Autoantibody Levels for Neuronal and Glial Proteins using Western Blot

Autoantibodies were determined for GWI cases, GW healthy veteran controls, IBS symptomatic controls, and ME/CFS symptomatic controls for the six neuronal proteins: NFP, tubulin, tau, MAP-2, CaMKII, and α-syn. In addition, four proteins from two types of glial cells were measured, including MBP and MAG from oligodendrocytes and GFAP and S100B from astrocytes.

The first analysis, which compared all three control groups combined (GW controls, IBS and ME/CFS groups), with veterans with GWI showed significantly increased mean levels for veterans GWI for nine out of the ten autoantibodies (Table 2; Figure 4). The only exception was for the glial protein S100B, whose mean level was similar to that of healthy controls (Table 2). In addition, there were no interacting the groups for any of the outcome measures.

Table 1. Demographic information of Gulf War Illness cases and healthy Gulf War and symptomatic controls.

Data	GW Cases	GW Healthy Controls	IBS Controls	ME/CFS Controls
N	175	60	37	50
Age * (Mean $\pm$ SD)	48.7 $\pm$ 7.8	50.92 $\pm$ 7.48	39.40 $\pm$ 13.93	46.74 $\pm$ 10.24
Sex *				
Male (%)	80.2%	93.3%	8.6%	10%
Female (%)	19.8%	6.7%	91.4%	90%
Race *				
Caucasian (%)	81.3%	73.7%	85.7%	91.3%
African American (%)	12.9%	23%	8.6%	8.7%
Other/Multiracial (%)	5.8%	3.3%	5.7%	0.0%

Note: * denotes significant differences $p < 0.01$ across the four groups. We obtained information for age, sex, and race from 171 individuals for GWI veterans, and 35 individuals for IBS controls.

Table 2. Autoantibodies against neural proteins in GWI cases and healthy and symptomatic controls ^a using ANCOVA analysis and adjusting for age, sex, and race.

		GW vs. All Controls	GW vs. GW Controls	GW vs. IBS Controls	GW vs. ME/CFS Controls
A. Neuronal Proteins					
Neurofilament Triplet	Mean (SE)	3.42 (0.19) ***	1.88 (0.26) ***	0.86 (0.02) ***	1.18 (0.05) ***
Proteins (NFP)	Range	2.6–15.15	0.16–9.74	0.64–1.23	0.51–2.25
Tubulin	Mean (SE)	4.13 (0.25) ***	2.36 (0.30) ***	1.14 (0.03) ***	2.71 (0.24) **
	Range	0.32–15.15	0.09–10.76	0.62–15.36	0.63–7.39
Microtubule Associated Protein Tau (Tau)	Mean (SE)	2.92 (0.22) ***	1.57 (0.17) ***	1.24 (0.13) ***	1.02 (0.07) ***
	Range	0.33–10.55	0.34–5.13	0.52–4.11	0.40–3.36
Microtubule Associated Protein-2 (MAP-2)	Mean (SE)	9.66 (0.73) ***	5.04 (0.76) ***	1.05 (0.07) ***	6.97 (0.35) **
	Range	0.88–27.42	0.24–23.00	0.64–2.45	1.40–14.02
Calcium/Calmodulin Kinase 2 (CaMKII)	Mean (SE)	2.04 (0.15) ***	1.20 (0.13) ***	0.70 (0.03) ***	1.16 (0.05) ***
	Range	0.10–5.50	0.15–4.50	0.37–1.43	1.11–1.92
Alpha Synuclein (α -syn)	Mean (SE)	2.52 (0.19) ***	1.46 (0.02) ***	0.78 (0.06) ***	1.13 (0.05) ***
	Range	0.17–11.77	0.37–6.45	0.51–1.93	0.41–1.93
B. Glial Proteins: Oligodendrocytes					
Myelin Basic Protein (MBP)	Mean (SE)	4.28 (0.18) ***	2.17 (0.32) ***	1.19 (0.03) ***	1.52 (0.09) ***
	Range	0.09–17.34	0.42–11.83	0.74–1.7	0.44–4.33
Myelin Associated Glycoprotein (MAG)	Mean (SE)	4.94 (0.28) ***	2.12 (0.25) ***	3.20 (0.21) *	1.58 (0.11) ***
	Range	0.24–20.94	0.38–6.51	1.03–6.51	0.26–4/48
Glial Proteins: Astrocytes					
Glial Fibrillary Associated Protein (GFAP)	Mean (SE)	4.27 (0.18) ***	2.34 (0.30) ***	0.84 (0.03) ***	4.86 (0.26)
	Range	0.39–13.14	0.35–14.98	0.45–1.38	0.66–8.44
Glial S100B (S100B)	Mean (SE)	1.17 (0.04)	1.16 (0.03)	0.95 (0.03)	1.34 (0.06) *
	Range	2.60–2.41	0.37–2.74	0.53–1.34	0.49–2.26

Note: * $p < 0.01$ ** $p < 0.001$ *** $p < 0.0001$. ^a Values reflect fold change relative to control.

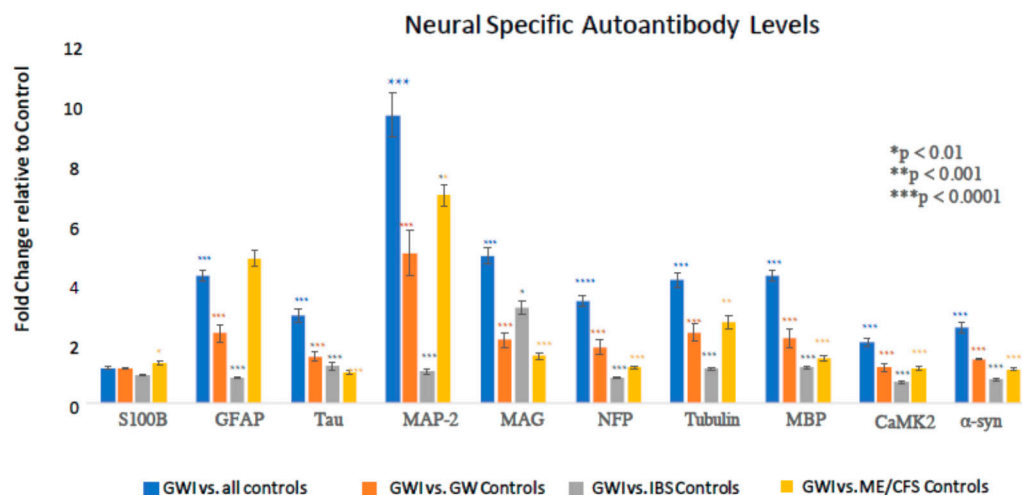


Figure 4. Neural autoantibodies in plasma of GWI cases, GW healthy controls, IBS controls, and ME/CFS controls. *** $p < 0.0001$ (blue) GWI group to all three control groups combined using ANCOVA adjusted for age, sex, and race. *** $p < 0.0001$ (orange) GWI group to GW veteran control group using ANCOVA adjusting for age, sex, and race. *** $p < 0.0001$ (grey) GWI group to IBS group using ANCOVA adjusting for age, sex, and race. *** $p < 0.0001$ (yellow) GWI group to ME/CFS group using ANCOVA adjusting for age, sex, and race.

The next analysis compared only GWI cases to GW veteran controls. The results of this comparison also showed that mean levels of nine out of the ten plasma autoantibodies of GWI cases were significantly higher for veterans with GWI than for healthy GW controls. Again, levels of S100B autoantibodies for GWI cases were not significantly increased from GW veteran controls.

Next, we compared GWI cases to symptomatic non-veteran IBS controls. The results again showed higher mean levels of nine out of ten autoantibodies for GWI cases compared to IBS controls. Again, the only non-significantly different autoantibody was S100B (Table 2).

Finally, we compared GWI cases to symptomatic non-veteran ME/CFS controls. The results showed higher mean levels of nine out of ten autoantibodies for GWI cases compared to ME/CFS controls. The only non-significantly different autoantibody between the two groups was for the GFAP protein (Table 2).

3.4. Neurodegeneration Index (NDI)

The Neurodegeneration Index score was calculated as described above for each tested group and the results were as follows (Figure 5): GWI = 39.35, GW healthy controls = 21.3, IBS controls = 11.94, and ME/CFS controls = 23.47. The mean NDI score for veterans with GWI was significantly higher than in all controls combined ($p < 0.0001$). In addition, the percentage of participants with NDI > 20 was significantly higher in GWI cases than in all controls combined (94.3% vs. 44.2%; $p < 0.0001$ via the chi-square test). The percentage of participants with NDI > 30 was also significantly higher in GWI cases than in all controls combined (71.8% vs. 14.3%; $p < 0.0001$ via the chi-square test).

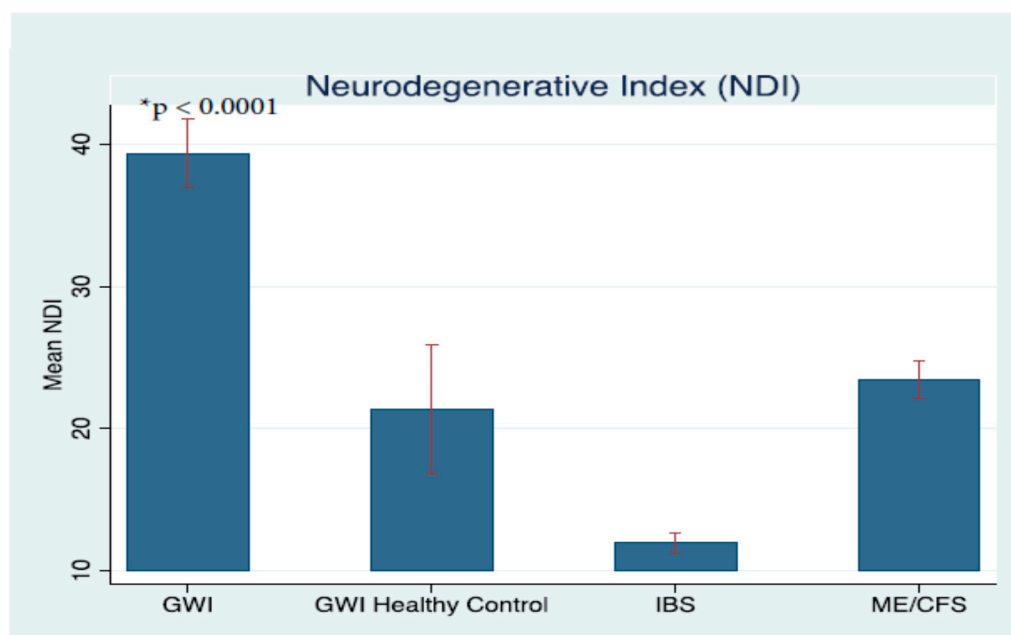


Figure 5. Neurodegenerative index score for GWI cases, GW healthy controls, IBS controls, and ME/CFS controls. Error bars represent the standard error of the mean. The mean NDI for GWI participants is significantly higher than in all controls combined ($p < 0.0001$).

4. Discussion

For the past 30 years, there has not been an objective diagnostic marker for GWI, which has hindered research in the field. Chronic symptoms reported by veterans with GWI have included headache, memory and attention decrements, debilitating fatigue, chronic pain, and gastrointestinal problems [2,3,35]. Many of these symptoms overlap with other comorbid conditions, including IBS and ME/CFS, necessitating the need for an objective marker that can delineate among these diagnostic groups. The present well-powered study confirms and expands the results of our previous descriptive study, where we identified a unique signature of objective biomarkers of CNS proteins in blood samples of 20 GW veterans compared with 10 controls [12]. In this study, we confirmed our prior results in a much larger sample of over 300 participants, including ill and healthy GW veterans and other symptomatic controls with IBS and ME/CFS. Specifically, we compared GWI cases vs. three control groups (GW controls, IBS, ME/CFS) and found that GWI cases had higher autoantibodies than all combined controls in nine out of ten autoantibodies. We then compared GWI cases with the three control groups separately and also found that the GWI cases showed significantly higher levels of nine out of ten autoantibodies than GW controls, IBS, or ME/CFS groups. These results clearly distinguish GWI cases not only from healthy GW counterparts but also other symptomatic controls with chronic multisymptom disorders.

Our results showed significantly elevated CNS autoantibodies in the plasma of veterans with GWI. The presence of low levels of autoantibodies in the plasma of GW healthy controls is consistent with previous findings in healthy individuals [12,36]. These results suggest that GW healthy controls had no lasting CNS effects from their deployment. In contrast, increased levels of CNS autoantibodies are consistent with veterans with GWI's chronic neurological complaints and thus, provides an objective biomarker of the illness. The results revealed large increases in autoantibodies in the GWI cases compared to all controls except for S100B. These increases were significantly higher than controls with autoantibodies against MAP-2, showing the highest overall level in all groups tested. These were followed by autoantibodies against myelin and other glial-related proteins showing the highest levels (MAG > MBP > GFAP) and then, followed by increased neuronal cytoskeletal protein autoantibodies against Tubulin > NFP > Tau.

Our results show that autoantibodies to neural proteins can be used as biomarkers for diagnosis and prognosis of GWI and may also provide insight into the potential mechanisms of GWI. The only consistent risk factors for GWI are environmental exposures, including the use of pyridostigmine bromide pills and pesticides, which are now known to adversely affect the CNS in significant or combined dosages [14,15,17–20,24–28]. Although a total of over 50 pesticide products were used during the Gulf War, less than 20 were designated as “pesticides of concern” by the Department of Defense, including the insecticides permethrin and lindane as well as the repellent, DEET (*N,N*-diethyl-*m*-toluamide) and organophosphate insecticides and nerve gases, sarin and cyclosarin [23]. These same exposures were recently shown by our group to be associated with higher rates of GWI and specifically, with worse mood and cognitive functioning [18].

Furthermore, several studies have shown some organophosphorus (OP) compounds, such as sarin and chlorpyrifos in addition to inhibiting acetylcholinesterase, also cause neurodegeneration of the CNS [37–40]. A recent study reported increased CNS autoantibodies in blood from farm workers exposed to OP pesticides [41]. Investigations into the mechanisms by which these compounds cause neurodegeneration have established that OPs increase the activity and expression of CaMKII, which causes hyperphosphorylation of neural proteins, leading to their aggregation and slowing of axonal transport, resulting in neuronal cell death [19,42–45]. In agreement with this is our prior finding that airline crews who were exposed to OPs developed autoimmune antibodies to neural proteins [46]. Another study using Magnetic Resonance Imaging (MRI) examination of another cohort of aircrews, showed decreased white matter microstructure and blood perfusion are potential causes of cognitive and mood symptoms experienced by the aircrews [46].

These results suggest the involvement of white matter alterations in the development of GWI is consistent with increased autoantibodies against MBP and MAG that are present in myelinated axons [9,37,39,44]. Blood markers of MBP are also elevated in myelin-related CNS disorders. Increased autoantibodies to MBP in the plasma of veterans with GWI correlate with demyelination following axonal degeneration caused by exposure to OPs [12,37,39]. GFAP is a glial protein that is involved in white matter and blood–brain barrier functioning [21]. This finding also correlates with our recent finding that GFAP almost completely distinguished between GWI cases and controls in our prior pilot study [12]. This also correlates with recent findings of increased neuroinflammation seen in imaging the brains of veterans with GWI, as shown by significantly greater glial activation using PET brain imaging [47].

CaMKII is widely distributed in the CNS, constituting up to 2% of the protein in the hippocampus [48]. Exposure to organophosphates, such as di-isopropyl fluorophosphate (DFP), a surrogate compound for sarin, enhanced Ca^{++} release and increased expression and activity of CaMKII, resulting in hyperphosphorylation of several cytoskeletal proteins, i.e., tubulin, MAP-2, Tau, and neurofilament triplet proteins [44,45,49]. Increased phosphorylation of MAP-2, Tubulin, and Tau resulted in their aggregation and slowing of axonal transport [44,45,49]. CaMKII-induced hyperphosphorylation caused significant increase in both c-fos and c-jun expression, leading to apoptosis mediated by cytochrome c released from mitochondria due to the imbalance between the Bax, Bcl-2, and Bcl-xl proteins triggered by the generation of Reactive Oxygen Species [29,30].

The results show that autoantibodies against S100B were not different from controls and were consistent with its neuroprotective action and the chronic nature of GWI. S100B’s half-life is 2 h in blood, supporting the use of its autoantibodies as biomarkers for neuronal conditions [29,30].

When the results of GWI cases were compared to controls with IBS, autoantibodies values were much higher, which is consistent with the fact that IBS is not considered a neurodegenerative disorder. The only elevated autoantibodies in IBS controls were against MAG but even that was less than half that of GWI cases. These results not only confirm the validity of our test as a biomarker for CNS effects, but also establishes its specificity as a marker for chronic GWI. MAG comes from oligodendrocytes in CNS and by Schwann cells in the periphery. The present results suggest that MAG protein was

released from peripheral nerves in the gastrointestinal tract, a major target for IBS, suggesting its potential use for that disorder.

Furthermore, ME/CFS symptomatic controls exhibited levels of autoantibodies against neural proteins that were intermediate between veterans with GWI and controls with IBS. ME/CFS is characterized by body and muscle pains as well as some CNS symptoms, including debilitating fatigue. GWI cases had higher levels of all autoantibodies except for GFAP when compared with ME/CFS controls. The increased GFAP levels in ME/CFS suggest a potential marker and pathobiology for that disorder. Recent studies from other groups have shown increased antibodies against β 2-adrenergic receptors in ME/CFS patients [50,51]. This suggests that ME/CFS is more similar to GWI than IBS based on these autoantibody biomarkers, but GWI still clearly represents a unique disorder based on different autoantibody patterns.

Increased autoantibodies of biomarkers NFP, tau, tubulin, and MBP, and neuronal cytoskeletal disruptions, including microtubule instability, axonal degeneration, and altered axonal transport, have been found in many cell and animal studies of toxicant-induced models of GWI [27,42–45,49,52–54]. We are only aware of the following prior studies, including our prior pilot study, showing increased autoantibodies in much smaller pilot studies of GW veteran blood samples [12,55–58]. To our knowledge, this is the first large, more definitive study to validate these prior animal, cell, and veteran studies in the blood of ill GW veterans compared with combined and separate healthy and symptomatic comparison groups.

We hypothesized that exposures to chemicals present in the GW theater, such as pesticides and nerve gases, can cause CNS damage and release of CNS autoantibodies through the BBB into blood circulation, where B-lymphocytes produce antibodies to proteins and T cells produce cell-mediated immune responses, and IgG autoantibodies are then made [48,59,60]. Theoretically, IgG autoantibodies can enter through the BBB and disrupt CNS functioning, which could lead to symptoms of GWI [59–61]. Further research is needed to confirm this hypothesis. The results of the NDI analyses, showing GWI cases were three times more likely to have an NDI score of 30 or greater, suggest that these individuals may be at increased risk for early onset of age-related neurodegenerative disorders.

Correspondingly, recent studies have reported increased levels of CNS autoantibodies in blood, from neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), suggesting the need for further studies of the potential increased risk of these disorders in GW veterans [62–64]. Having these reports, together with the results of the present study, raises concerns regarding the likelihood for veterans with GWI to develop neurodegenerative diseases such as AD and/or PD as they age. These CNS protein biomarkers may be useful for determining who is at risk for these disorders in addition to using them in clinical trials for treatment efficacy of GWI.

Limitations

Like all studies, our study had limitations. GWI diagnosis was based on veterans' self-reported symptoms, which could have introduced some classification errors. In addition, some veterans could have very early signs of neurodegenerative disorders that were not picked up in the clinical evaluations, which could have increased autoantibody levels in the veterans. However, the Kansas criteria for GWI would have excluded known cases of these disorders, including AD, PD, and other chronic illnesses that could have accounted for their chronic symptoms [31]. There were also sex differences within our groups as might be expected, with more women in the ME/CFS and IBS groups and more men in the GWI groups, and although these sex differences were controlled for in the analyses, future studies should more directly compare these autoantibody outcomes by sex. A major strength of our study included the large sample size and the inclusion of both healthy and symptomatic veteran groups in this objective biomarker study. In addition, the CNS autoantibody analyses were similar chronic multisymptom disorders (IBS, ME/CFS). We confirmed and validated our prior preliminary results of increased autoantibodies in a much larger sample of veterans with GWI compared with healthy GW veterans and with symptomatic non-veteran IBS and ME/CFS controls [12]. This study confirmed

that nine of the ten autoantibodies were significantly increased in veterans with GWI, suggesting considerable CNS differences compared to both healthy and symptomatic controls. This confirms our prior studies, which suggested a strong CNS component to GWI [18,38]. The present study confirmed the utility of the use of plasma autoantibodies for CNS proteins to distinguish among veterans with GWI and other healthy and symptomatic control groups and our newly developed NDI summary score can be further utilized to compare pre and post treatment trial efficacy.

Author Contributions: Conceptualization, M.B.A.-D., K.S., J.M., L.A.C., E.K. and N.G.K.; Data curation, E.Q. and D.D.N.; Formal analysis, E.Q., J.L. and J.M.; Funding acquisition, M.B.A.-D. and K.S.; Investigation, M.H.K.; Methodology, M.B.A.-D., E.S.L., J.M., L.A.C., E.K., M.A., N.G.K., D.D.N. and K.S.; Project administration, E.S.L., M.H.K., J.L., L.A.C., E.K., M.A., D.D.N. and K.S.; Resources, M.A. and N.G.K.; Supervision, M.B.A.-D.; K.S.; Writing—original draft, M.B.A.-D. and K.S.; Writing—review & editing, E.S.L., M.H.K., E.Q., J.M., L.A.C., E.K., M.A., N.G.K., D.D.N. and K.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Article

Sex-Based Differences in Plasma Autoantibodies to Central Nervous System Proteins in Gulf War Veterans versus Healthy and Symptomatic Controls

Mohamed B. Abou-Donia ^{1,*}, Maxine H. Krengel ², Elizabeth S. Lapadula ¹, Clara G. Zundel ² , Jessica LeClair ³, Joseph Massaro ³, Emily Quinn ³, Lisa A. Conboy ⁴, Efi Kokkotou ⁴, Daniel D. Nguyen ⁵, Maria Abreu ^{6,7}, Nancy G. Klimas ^{6,7} and Kimberly Sullivan ^{5,*} 

¹ Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA; elizabeth.lapadula@duke.edu

² Department of Neurology, Boston University School of Medicine, Boston, MA 02118, USA; mnhk@bu.edu (M.H.K.); cgzundel@bu.edu (C.G.Z.)

³ Department of Biostatistics, Boston University School of Public Health, Boston, MA 02118, USA; jleclair2@bu.edu (J.L.); jmm@bu.edu (J.M.); eq@bu.edu (E.Q.)

⁴ Department of Medicine, Harvard Medical School, Boston, MA 02115, USA; Lisa.Conboy@mcphs.edu (L.A.C.); ekokkoto@bidmc.harvard.edu (E.K.)

⁵ Department of Environmental Health, Boston University School of Public Health, Boston, MA 02118, USA; ddn@bu.edu

⁶ Dr. Kiran C. Patel College of Osteopathic Medicine, Institute for Neuroimmune Medicine, Nova Southeastern University, Fort Lauderdale, FL 33314, USA; mabreu1@nova.edu (M.A.); nklimas@nova.edu (N.G.K.)

⁷ Department of Immunology, Miami VA Medical Center, Miami, FL 33125, USA

* Correspondence: donia@duke.edu (M.B.A.-D.); tty@bu.edu (K.S.); Tel.: +1-919-684-2221 (K.S.)



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Abstract: Veterans from the 1991 Gulf War (GW) have suffered from Gulf War illness (GWI) for nearly 30 years. This illness encompasses multiple body systems, including the central nervous system (CNS). Diagnosis and treatment of GWI is difficult because there has not been an objective diagnostic biomarker. Recently, we reported on a newly developed blood biomarker that discriminates GWI from GW healthy controls, and symptomatic controls with irritable bowel syndrome (IBS) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). The present study was designed to compare levels of these biomarkers between men and women with GWI, as well as sex-specific effects in comparison to healthy GW veterans and symptomatic controls (IBS, ME/CFS). The results showed that men and women with GWI differ in 2 of 10 plasma autoantibodies, with men showing significantly elevated levels. Men and women with GWI showed significantly different levels of autoantibodies in 8 of 10 biomarkers to neuronal and glial proteins in plasma relative to controls. In summary, the present study addressed the utility of the use of plasma autoantibodies for CNS proteins to distinguish among both men and women veterans with GWI and other healthy and symptomatic control groups.

Keywords: CNS autoantibody; Gulf War illness; gender; biomarkers; proteins; myalgic encephalomyelitis/chronic fatigue syndrome; irritable bowel syndrome



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1. Introduction

Gulf War (GW) veterans have suffered from a condition known as Gulf War illness (GWI), which encompasses multiple bodily systems, including the central nervous system (CNS) [1–3]. This chronic condition affects approximately 250,000 GW veterans including a large proportion of the 40,000 women who deployed to the war. GWI has overlapping, but different, symptoms from other disorders that also affect women, including Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS), and Irritable Bowel

Syndrome (IBS). Lack of objective diagnostic markers have minimized the ability to cohesively diagnose and treat the multitude of symptoms of GWI. Recently, we assessed a candidate plasma biomarker for GWI, and we have shown differences in individuals who meet criteria for GWI versus GW healthy, and non-veteran controls with IBS and CFS. Given the differing symptoms of GWI experienced between men and women, it was our next aim to assess possible differences in these plasma biomarkers between sexes [4–8]. Determining whether CNS autoantibodies differ between men and women with GWI and their respective controls will aid in further refining the diagnostic utility of these as plasma biomarkers.

Women who served in the 1991 GW have reported numerous health problems, many of which are gender-specific. Some studies have suggested increased rates of woman's health problems and bladder infections relative to GW-era veteran controls [9]. In the Ft. Devens Cohort (FDC) of GW veterans, GW women relative to non-veteran controls have been found to have a higher risk for diabetes but a lower risk of hypertension when compared to the National Health and Nutrition Examination Survey (NHANES) age and sex-matched cohort [8]. In contrast, GW-deployed men reported increased rates of seven chronic health conditions relative to NHANES men including significantly higher odds of reporting hypertension, high cholesterol, heart attack, diabetes, stroke, arthritis, and chronic bronchitis [8]. Because this study showed that GW men were at increased risk for medical conditions that are known risk factors for later cardiovascular and cerebrovascular disease (i.e., hypertension, high cholesterol, diabetes), GW men may show increased rates of CNS biomarkers relative to their women veteran counterparts.

However, in a recent study utilizing the large GW-era biorepository cohort (GWEBC), GW-deployed women were significantly more likely to report symptoms related to cognitive, neurological, and mood problems than GW-era women [4]. Therefore, GW women may also show increased CNS-related biomarkers compared with other women cohorts. In addition, when comparing deployed GW veteran men versus women, GW women veterans have been found to have more outpatient and inpatient health care use 5 years after deployment [10]. GW women have also been found to have higher rates of severe and moderate cases of GWI relative to their male counterparts [5,11–13].

Several factors may account for the observed sex differences in GWI and individual symptoms including sex-differences in susceptibility to neurotoxicant exposures in theatre and/or higher exposures among male personnel. As early as 1996, we speculated that chemical exposures at the time of the GW was a precipitating factor in GWI [14,15]. We demonstrated in experimental studies that combined exposure to pesticides and pyridostigmine bromide (PB) caused neurotoxicant effects that interfere with neuronal and glial cell function, leading to long-lasting and potentially neurodegenerative effects [14–16]. Neurodegeneration involves neuronal and glial cell death and the release of their contents into circulation through the breached blood brain barrier (BBB), which can then be detected through CNS autoantibodies in plasma [17]. We have previously shown BBB changes in our animal models of GWI exposures [18]. We have also previously shown that these plasma markers can be detected in low levels in individuals without significant illness but increase in those with more symptomatology [16,19].

The CNS has two types of cells: neurons and supporting glial cells. Neuronal cells contain several cytoskeletal proteins, including tau and neurofilaments [20,21]. Along with microtubules made from tubulin, they form the backbone of the axonal cytoskeleton. These proteins are involved in axonal transport of essential nutrients, amino acids, and organelles down the axon [22]. The Microtubule-Associated Proteins, (MAP)-2 and tau, promote polymerization and stabilization of microtubules in axons, cross-bridge neurofilaments, and microtubules connecting themselves to each other; this helps to maintain the cytoskeleton for axonal transport [23]. Tau proteins are localized primarily in the axon in neuronal cells. The supporting glial cells, oligodendrocytes in CNS and Schwann cells in the peripheral nervous system (PNS), produce myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) that are membrane proteolipids [24]. Glial astrocytic proteins pro-

duce glial fibrillary acidic protein (GFAP), a component of astroglia cytoskeleton, which is highly specific to the CNS and is released from the astrocyte into the extracellular space after CNS injury [25]. Calcium-binding S100B protein is also released by astrocytic glial cells (Figure 1). Many of these neuronal cytoskeletal and especially glial proteins are only found in the CNS and their autoantibodies are found in the peripheral blood, if these proteins have at some point leaked through the BBB and the immune system mounts an autoantibody response to them [18,26]. The temporal course of this BBB breach could have occurred sometime in the past in these individuals, resulting in leakage of these proteins from neurons and/or glia. So, although S100B, a marker of current BBB compromise, did not differ in our prior study in veterans with GWI, this does not mean that a BBB breach did not occur sometime in the past causing lasting detectable autoantibodies to these CNS proteins in the blood.

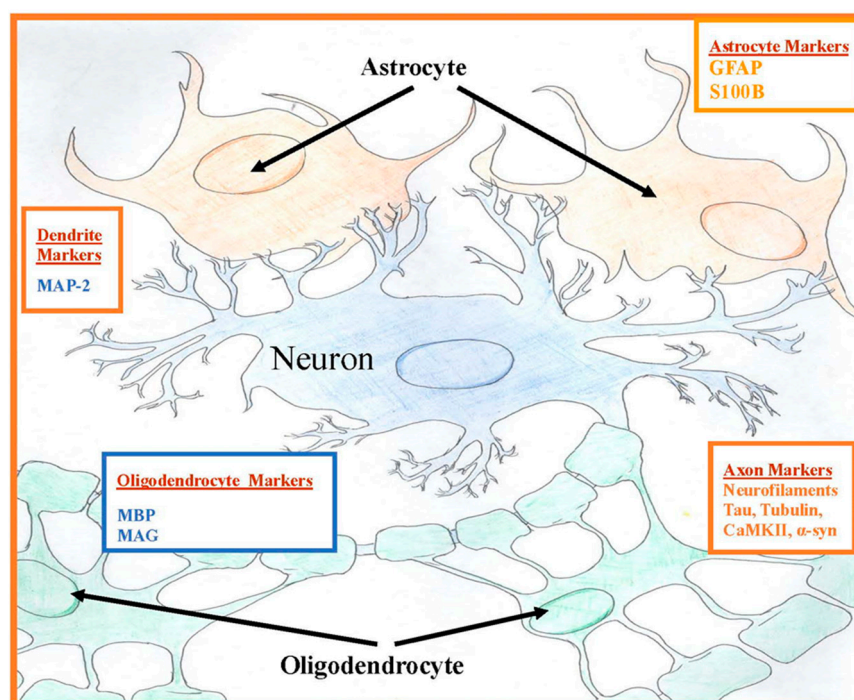


Figure 1. Central nervous system protein locations of plasma autoantibodies. Oligodendrocytes myelinate inside the central nervous system (CNS) and Schwann cells myelinate outside of the CNS.

The present study investigates the effects of gender on the presence of immunoglobulin G (IgG) class circulating autoantibodies against CNS-specific neuronal and glial proteins in the plasma of GW veterans with and without GWI as well as in symptomatic non-veteran controls (IBS, and ME/CFS). Specifically, we hypothesized that men and women veterans with GWI would differ from each other on the distribution of CNS autoantibody proteins and that GW men and women would have higher levels of CNS autoantibody proteins than other symptomatic groups with chronic illnesses including IBS and ME/CFS. Due to exposure during the war, all GW veterans could have had some level of BBB compromise as evidenced by CNS autoantibodies in plasma, but we hypothesized that those with GWI would have higher levels of these blood markers.

2. Materials and Methods

2.1. Study Population

Methods are the same as our recently published study [19]. Briefly, plasma from GW veterans were provided by the Gulf War Illness Research Consortium (GWIC), the Dynamic Modeling of GWI study from Nova Southeastern University, the Congressionally Directed Medical Research Program (CDMRP) funded study to the South Florida Veterans Affairs

Foundation for Research and Education, Inc., the Acupuncture treatment trial, and the irritable bowel study biorepository at Harvard/BIDMC. Institutional Review Board (IRB) approval was obtained from Boston University, Nova Southeastern University and the Miami VAMC, and Harvard University.

The same standard operating procedures were used for each study site for phlebotomy, plasma separation, and aliquoting. Plasma samples were obtained from fasting subjects and stored at -80°C . They remained frozen until analyses. Participants were consented for their respective studies using the International Conference of Harmonisation Good Clinical Practice (ICH GCP) guidelines.

The Kansas GWI criteria were used to determine cases of GWI and controls [27]. The Kansas GWI criteria require that GW veterans endorse symptoms in at least 3 of 6 symptom domains (fatigue, pain, cognitive/neurological/mood, gastrointestinal, respiratory, and skin). Veteran controls included deployed veterans from the 1991 GW who did not meet the Kansas GWI or exclusionary criteria. Kansas GWI exclusion criteria excludes individuals who report being diagnosed with another medical condition that could explain their chronic medical symptoms, including veterans with a history of prior central nervous system or major psychiatric disorders that may affect cognitive function (e.g., epilepsy, stroke, brain tumor, multiple sclerosis, Parkinson's Disease, Alzheimer's disease, schizophrenia).

Plasma samples from symptomatic controls came from prior studies of individuals with ME/CFS and IBS [28,29]. ME/CFS cases were determined by using 1994 Centers for Disease Control and Prevention (CDC) criteria [28]. IBS participants met Rome III criteria [29]. The full cohorts have been described in previous papers (GWIC, CFS, IBS, GWIC subsample [16,30–33]).

2.2. Materials

The sources of protein were also described previously [19]: Tubulin (human recombinant, Prospec Cat. # PRO-982), Microtubule-Associated Protein 2 (MAP-2, human recombinant, Origene, Cat. #TP316775, human recombinant), Tau-381 (human recombinant, Millipore Cat. #AG952), Neurofilament Protein (NFP, Prospec, Cat #PRO-523), Calmodulin Kinase II (human recombinant, CaMKII, Novus Biologicals, Cat #H000000H15-P01), Alpha-synuclein (human recombinant, AnaSpec Cat. #AS-55555), Myelin Basic Protein (MBP, human, Fitzgerald Cat. #30R-AM030), Myelin-Associated Glycoprotein (MAG, human recombinant, Sinobiological Cat. 131-86-H02H), Glial Fibrillary Acidic Protein (GFAP, human, CalBiochem Cat. #345996), S100B Protein (human, Millipore Cat. #30R-AS002). Goat anti-human IgG conjugated to horseradish peroxidase and the improved chemiluminescence reagent was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Sodium Dodecyl Sulfate (SDS) gels, 2–20% gradient (8×8), and 15 mM tris-glycine were obtained from Invitrogen (Carlsbad, CA, USA). All other materials were purchased from Amersham.

2.3. Ethics Statement

Approval for the use of stored blood samples from all study sites for this study was obtained from the Duke University Health System Institutional Review Board for Clinical Investigations on 10/9/2017 and from the Boston University Medical Campus Institutional Review Board on 1/19/2018. The specific protocol components for Duke University were: Protocol ID: Pro00003202, Reference ID: 335940, Principal Investigator: Mohamed Abou Donia, Protocol Title: 'Nervous System Injury'. The specific protocol components for Boston University were Protocol ID: H-34334, Reference ID: 1288716, Principal Investigator: Kimberly Sullivan, Protocol Title: 'Novel Autoantibody Serum and Cerebrospinal Fluid Biomarkers in Veterans with Gulf War Illness.' Plasma samples were shared from IRB-approved repositories from studies at Boston University (IRB # H-32768), NOVA Southeastern University/Miami VA Medical Center (IRB # 4987.76 and IRB # 4987.75), Beth Israel Hospital/Harvard University (IRB # 2011P-000124), and the New England School of Acupuncture (IRB # 09-204).

2.4. Procedures

2.4.1. Plasma Procedures

All sites followed the same protocol: for venipuncture, blood handling, plasma separation, aliquoting, and storage at -80°C . The same phlebotomy and sample protocol were distributed in writing to all sites and included fasting before blood draw in the morning. All samples analyzed were baseline blood samples collected pre-intervention therapy. Samples used in this study were not previously thawed and were free of hemolysis by visual inspection.

2.4.2. Western Blot Assay

In this study, Western blot analysis was used to determine autoantibodies against specific proteins in the plasma sample of GWI cases and healthy and symptomatic controls by gender. This assay allowed the determination of the autoantibodies and associated isoforms of the antigen. As previously described, each plasma sample was analyzed in triplicate [19]. All proteins were loaded at 10 ng/lane except for IgG, which was loaded as 100 ng/lane. The proteins were denatured and electrophoresed on SDS-PAGE (gradient 4% to 20%) purchased from Invitrogen (Carlsbad, CA, USA). One gel was used for each serum sample. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham). Nonspecific binding sites were blocked with Tris-buffered Saline-Tween (TBST) (40 mM Tris (pH 7.6), 300 mM NaCl, and 0.1% Tween 20) containing 5% fat-free milk powder for 1 h at 22°C . Membranes were incubated with serum samples at 1:100 dilutions in TBST with 3% nonfat milk powder overnight at 4°C . After five washes in TBST, the membranes were incubated with a 1:2000 dilution of goat anti-human IgG conjugated to horseradish peroxidase (Amersham). Membranes were developed by enhanced chemiluminescence using the manufacturer's protocol (Amersham) and a Typhoon 8600 variable model recorder (GE Lifesciences, Marlborough, MA, USA). The signal intensity was quantified using Bio-Rad image analysis software version 4.5 (Hercules, CA, USA). All tests were performed with the researchers blinded to case-control and gender status of the samples.

2.4.3. Specificity of Plasma Autoantibodies

Previously, we have checked the specificity of the plasma and serum autoantibody by performing a peptide/antigen competition assay, in which the serum and plasma were spiked with the target protein or peptide [34]. The serum from random healthy controls was mixed with or without tau, MAP2, or MBP. The protein mix was centrifuged at 15,000 rpm to deplete any immune complexes. The supernatants were then carefully removed and used in Western blotting. Specificities of autoantibodies against all tested proteins were confirmed in a follow-up study [35].

2.5. Statistics

The pooled data are presented as mean $\pm$ SD for continuous variables and the number and percent of participants in each category for categorical variables. Subjects' demographic values were compared to the control groups using Students t-test continuous and chi-square for categorical variables. Mean values of autoantibodies of the GWI men and women were compared using analysis of covariance (ANCOVA), adjusting for age and race. Race was used dichotomously (Caucasian or not). Next, mean values of the GWI women were compared with GW healthy women and then repeated for GWI men and GW healthy men. Lastly, mean values of the GWI women were compared with the combined 3 control groups (GW controls, IBS, ME/CFS) using ANCOVA, adjusting for age and race; this analysis was then repeated for men. A two-sided p value < 0.05 was accepted as statistically significant for all analyses and analyses were not adjusted for multiple comparisons. Analyses were carried out using SAS version 9.4 (SAS Institute Inc. 2013. Cary, NC, USA).

2.6. Calculations

Optical density measurement for cases and controls was divided by the concentration of serum IgG; this value for each subject was normalized to controls and expressed as change from healthy controls. Therefore, the results are expressed as mean triplicate assay values of arbitrary optical density units normalized to IgG optical density as compared to healthy controls.

2.7. CNS Autoantibody Index (CAI)

This index determines the overall neurodegenerative condition of an individual based on the level of autoantibodies in the plasma. It is calculated by adding all of the values of autoantibodies for each neural protein, and then dividing the sum by the number of the autoantibodies used. Finally, the value is multiplied by 10 to produce an easy CAI score as previously reported under the former name neurodegeneration index [19].

3. Results

3.1. Participant Demographics

The study sample included a total of 171 veterans with GWI (137 men, 34 women), a total of 56 GW male healthy controls and 4 GW women veteran healthy controls, a total of 3 male and 32 women IBS controls, and a total of 5 male and 45 women CFS symptomatic controls (Table 1). Some of the groups (Men GWI versus Women GWI, and GWI versus all combined controls) were significantly different in age and race (Caucasian or not), so all further analyses controlled for these demographic variables.

Table 1. Demographics of study sample.

Demographics	Men		Women	
	GW Cases (N = 137)	All Controls (N = 64)	GW Cases (N = 34)	All Controls (N = 81)
Age-Mean + SD	49.18 + 7.36	49.69 + 8.36	46.91 + 9.27	44.33 + 12.52
Race-N (%)				
Caucasian	110 (79.7)	46 (71.9)	29 (85.3)	71 (87.7)
African American	19 (13.8)	13 (20.3)	3 (8.8)	8 (9.9)
Other/Multiracial	9 (6.5)	2 (3.1)	1 (2.9)	2 (2.5)

This study reports the results of the use of our newly developed plasma neurodegenerative biomarkers to differentiate men versus women service personnel who served in the 1990/1991 GW and developed symptoms of GWI or remained healthy. In addition, analyses were conducted to detect differences between women with GWI versus the GW women and then the combined women control group (healthy GW veterans, IBS, ME/CFS) and between men with GWI vs. GW healthy men and then with the combined control men group. The levels of 10 circulating autoantibodies against neural proteins were analyzed in the plasma of men and women veterans with GWI, and with healthy GW men and women and with symptomatic men and women with CFS and IBS, which were used as controls.

3.2. Effects of Gender on the Levels of Autoantibodies against Plasma Neural Proteins

3.2.1. Autoantibody Results Analyzed by Sex for GWI Cases and GW Healthy Only

This study comprised 171 plasma samples from veterans with GWI, including 137 (80.2%) men (mean age of 49.2-years) and 34 women (19.9%) (with mean age of 46.9 years) (Table 1). Differences between men and women with respect to autoantibodies in plasma within the GWI groups were found such that males with GWI showed significantly higher levels of tubulin and MAG compared with women veterans with GWI (Table 2, Figure 2). There were no antibodies where women had higher means than the men. We also compared

56 GW healthy males (mean age of 50.5 years) with 4 GW healthy females (mean age of 56.5 years). Although we lacked statistical power, no significant differences were found between the male and female GW healthy controls on any of the autoantibodies (data not shown).

Table 2. Autoantibody ANCOVA analysis comparing men and women with Gulf War illness (GWI).

Neural Proteins	Men (N = 137) Mean + SD	Women (N = 34) Mean + SD	p-Value
A. Neuronal Proteins			
Neurofilament triplet proteins (NFP)	3.70 + 2.75	2.40 + 1.69	0.0120
Tubulin	4.51 + 3.52	2.79 + 1.46	0.0071
Microtubule-associated tau proteins (tau)	2.99 + 1.86	2.50 + 1.53	0.1647
Microtubule-associated protein-2 (MAP)	10.11 + 4.96	8.19 + 5.18	0.0629
Calmodulin-dependent protein kinase II (CaMKII)	2.09 + 1.08	1.85 + 1.27	0.2879
Alpha-synuclein (α -syn)	2.58 + 1.62	2.28 + 1.68	0.3906
B. Glial Proteins Oligodendrocytes			
Myelin-associated glycoprotein (MAG)	5.26 + 3.44	3.57 + 2.70	0.0097
Myelin basic protein (MBP)	4.34 + 2.24	3.82 + 2.82	0.2900
Astrocytes			
Glial fibrillary acidic protein (GFAP)	4.30 + 2.37	4.01 + 2.67	0.6437
Glial S100B protein (S100B)	1.21 + 0.47	1.04 + 0.58	0.1015
CNS autoantibody Index (CAI)	41.1 + 15.7	32.4 + 16.9	0.0076

ANCOVA analyses were adjusted for age and race. Values reflect fold change relative to control.

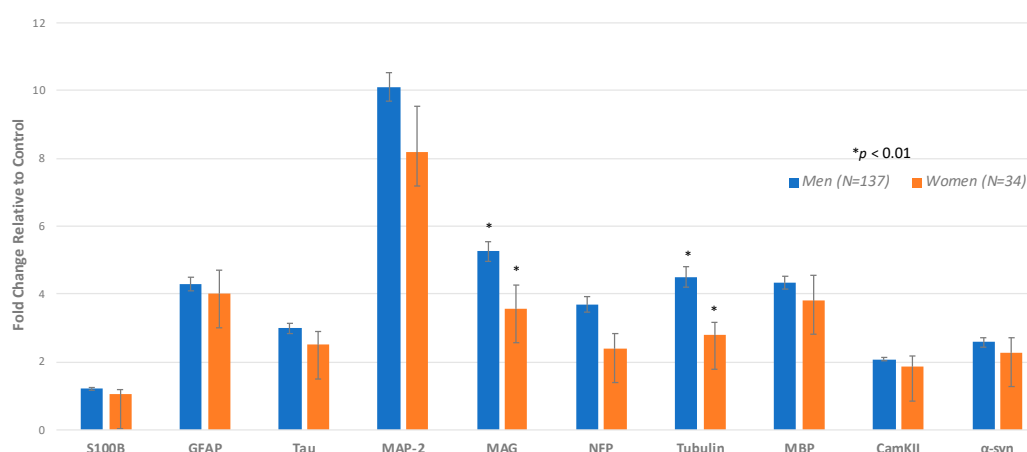


Figure 2. Analysis by sex for GWI cases only. Comparing men and women using ANCOVA adjusting for age and race. The height of the bar denotes the mean of increase in individual autoantibody biomarker +/− standard error.

3.2.2. Autoantibody Results in Plasma of Veteran Men with GWI versus Healthy Veteran Men and then Women with GWI versus Healthy Veteran Women

The second analyses compared male veterans with GWI ($N = 137$) with GW healthy men ($N = 56$) and GWI women ($N = 34$) with GW healthy women ($N = 4$). When compared to the healthy men, GWI men had significantly higher autoantibody values for 9 out of the 10 autoantibodies with the exception of S100B. When compared with healthy women, GWI women had significantly higher autoantibody values for 2 of the 10 autoantibodies, including GFAP and Tubulin (Table 3).

Table 3. Autoantibody ANCOVA analysis of GWI men and women compared to healthy Gulf War (GW) control men and women.

Neural Proteins	Male			Female		
	GWI Cases ($N = 137$) Mean + SD	Healthy GW Controls ($N = 56$) Mean + SD	p -Value	GWI Cases ($N = 34$) Mean + SD	Healthy GW Controls ($N = 4$) Mean + SD	p -Value
A. Neuronal Proteins						
Neurofilament triplet proteins (NFP)	3.70 + 2.75	1.91 + 2.08	<0.0001	2.40 + 1.69	1.51 + 1.05	0.0492
Tubulin	4.51 + 3.52	2.43 + 2.40	<0.0001	2.79 + 1.46	1.36 + 0.86	0.0081
Microtubule-associated tau proteins (tau)	2.99 + 1.86	1.57 + 1.32	<0.0001	2.50 + 1.53	1.61 + 1.43	0.1561
Microtubule-associated protein-2 (MAP)	10.11 + 4.96	5.03 + 5.80	<0.0001	8.19 + 5.18	5.19 + 8.22	0.1022
Calmodulin-dependent protein kinase II (CaMkII)	2.09 + 1.08	1.21 + 1.02	<0.0001	1.85 + 1.27	1.12 + 0.42	0.0555
Alpha-synuclein (SNCA)	2.58 + 1.62	1.47 + 1.33	<0.0001	2.28 + 1.68	1.37 + 1.48	0.1391
B. Glial Proteins Oligodendrocytes						
Myelin basic protein (MBP)	4.34 + 2.24	2.20 + 2.50	<0.0001	3.82 + 2.82	1.80 + 1.74	0.0724
Myelin-associated glycoprotein (MAG)	5.26 + 3.44	2.18 + 2.02	<0.0001	3.57 + 2.70	1.33 + 1.01	0.0531
Astrocytes						
Glial fibrillary acidic protein (GFAP)	4.30 + 2.37	2.38 + 2.39	<0.0001	4.01 + 2.67	1.84 + 1.12	0.0377
Glial S100B protein (S100B)	1.21 + 0.47	1.16 + 0.43	0.4174	1.04 + 0.58	1.19 + 0.36	0.6037
CNS Autoantibody Index (CAI)	41.1 + 15.7	21.5 + 17.7	<0.0001	32.4 + 16.9	18.3 + 17.6	0.0212

ANCOVA analyses were adjusted for age and race. Values reflect fold change relative to control.

3.2.3. Autoantibody Results in Plasma of Men GWI Veterans Versus All Men Controls and Women GWI Veterans versus All Women Controls

The third analysis compared male veterans with GWI ($n = 137$) and combined IBS, ME/CFS, and healthy control GW veteran males ($n = 64$), and female veterans with GWI ($n = 34$) and combined IBS, ME/CFS, and health control GW veteran females ($n = 81$) (Table 4). When compared to all male controls, men with GWI showed significantly higher

($p < 0.01$) mean levels of 9 out of 10 autoantibodies against neural proteins (tubulin, tau, MAP-2, MBP, NFP, MAG, CamKII, α -syn, and GFAP). There was no significant difference observed for S100B. Women veterans with GWI exhibited significantly increased ($p < 0.01$) mean levels of 8 out of 10 autoantibodies when compared with the combined female control group (IBS, ME/CFS, healthy control GW veterans) including tubulin, tau, MAP-2, MBP, MAG, NFP, CamKII, and α -syn (Table 4).

Table 4. ANCOVA analysis of autoantibody differences between men with GWI and all controls and women with GWI and all controls.

Autoantibody Measure	Men			Women		
	GWI Cases (N = 137) Mean + SD	All Controls (N = 64) Mean + SD	p-Value	GWI Cases (N = 34) Mean + SD	All Controls (N = 81) Mean + SD	p-Value
A. Neuronal Proteins						
Neurofilament triplet proteins (NFP)	3.70 + 2.75	1.80 + 1.96	<0.0001	2.40 + 1.69	1.08 + 0.38	<0.0001
Tubulin	4.51 + 3.52	2.54 + 2.40	0.0001	2.79 + 1.46	1.90 + 1.33	0.0041
Tau proteins (Tau)	2.99 + 1.86	1.50 + 1.25	<0.0001	2.50 + 1.53	1.15 + 0.73	<0.0001
Microtubule-associated protein-2 (MAP)	10.11 + 4.96	5.01 + 5.54	<0.0001	8.19 + 5.18	4.53 + 3.78	0.0001
Calmodulin-dependent protein kinase II (CaMKII)	2.09 + 1.08	1.19 + 0.98	<0.0001	1.85 + 1.27	0.97 + 0.43	<0.0001
Alpha-synuclein (α -syn)	2.58 + 1.62	1.43 + 1.25	<0.0001	2.28 + 1.68	0.98 + 0.44	<0.0001
B. Glial Proteins Oligodendrocytes						
Myelin basic protein (MBP)	4.34 + 2.24	2.15 + 2.37	<0.0001	3.82 + 2.82	1.36 + 0.55	<0.0001
Myelin-associated glycoprotein (MAG)	5.26 + 3.44	2.19 + 1.90	< 0.0001	3.57 + 2.70	2.19 + 1.31	0.0004
Astrocytes						
Glial fibrillary acidic protein (GFAP)	4.30 + 2.37	2.51 + 2.40	<0.0001	4.01 + 2.67	3.11 + 2.41	0.1379
Glial S100B protein (S100B)	1.21 + 0.47	1.19 + 0.45	0.6833	1.04 + 0.58	1.15 + 0.39	0.0952
CNS Autoantibody Neurodegeneration Index (CA_NDI)	41.1 + 15.7	21.5 + 16.8	<0.0001	32.4 + 16.9	18.4 + 7.24	<0.0001

ANCOVA analyses were adjusted for age and race. Values reflect fold change relative to control.

3.2.4. CAI Values by Gender in Men and Women GWI Cases versus Men and Women Controls

When all autoantibodies were combined into the CAI score, men with GWI had the highest values of total combined autoantibodies, as indicated by a CAI score of 41.1, and women with GWI showed a CAI score of 32.4. These values were statistically significantly different by ANCOVA, adjusting for age and race ($p = 0.007$). The CAI score of the GW control men had a mean of 21.4, which was statistically lower than the GWI men ($p < 0.0001$) (Figure 3). The CAI score of the GW control women had a mean score of 12.0, which was statistically lower than the GWI women ($p < 0.021$). The combined male control group had a mean CAI of 21.5, which was statistically significantly lower than the GWI male

group ($p < 0.0001$). In addition, the combined women control group had a mean CAI of 18.7, which was also significantly lower than the GWI women group ($p < 0.0001$). In this study, our results of both men and women with GWI showed that their CAI values were higher than 90% of controls.

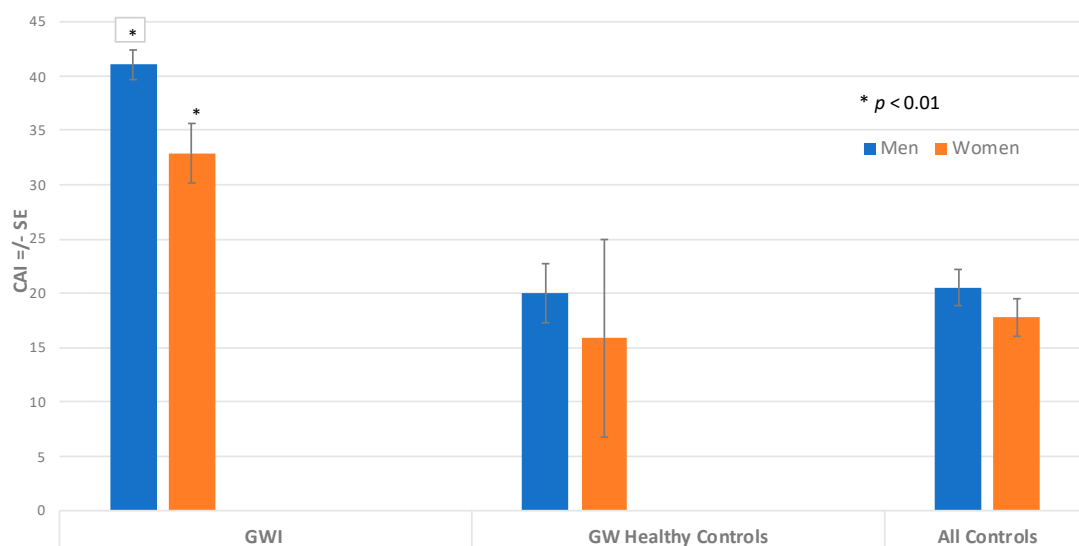


Figure 3. CNS Autoantibody Index (CAI) for Men and Women. Comparing CAI for men and women using ANCOVA, adjusting for age and race. The height of the bar denotes adjusted mean CAI $\pm$ standard error. CAI for GWI is significantly different than CAI for GWI women (* $p < 0.01$).

4. Discussion

This study, using our previously validated plasma CNS autoantibody biomarkers, shows that there was a difference between men and women veterans with GWI, with male veterans having significantly higher levels for 2 out of the 10 autoantibodies (tubulin, MAG) that are related to axonal and oligodendrocyte functions (Figure 2). When combining all of the autoantibodies into the CNS autoantibody neurodegenerative index (CAI), a significant difference between men with GWI and women with GWI was observed, such that men with GWI had more overall autoantibody protein burden and a significantly higher CAI value. Our second set of analyses compared male veterans with GWI to male GW healthy controls and then repeated the analyses for women veterans with GWI compared with healthy GW women. Our next analyses compared male veterans with GWI to all male controls from our prior study (healthy GW veterans, non-veterans with IBS or ME/CFS) [19]. We then performed the same analyses comparing women veterans with GWI to the combined all women control group (non-veterans with IBS and ME/CFS). The results showed that men with GWI had significantly higher levels of autoantibodies for 9 out of the 10 autoantibodies when compared with male healthy GW veterans or with the combined male control group. Women with GWI showed significantly higher values for 2 out of the 10 autoantibodies when compared with women healthy GW veterans and with 8 out of 10 autoantibodies when compared with their respective combined women control group (nonveterans with IBS and ME/CFS). The CAI analyses comparing GWI men with their male control group and GWI women with their respective control group also showed significantly higher values for the GWI men and women, with double the values compared with respective controls. These results add to our prior findings that suggested using a cutoff of 30 or more on the CAI score would distinguish between GWI and other chronic disorders. These results indicate that this cutoff corresponds with both men and women veterans with GWI.

Although men and women veterans with GWI had higher autoantibody levels than their respective control groups, they only differed from each other on two CNS autoantibody proteins. However, the total combined CAI score was a significantly higher value for

the men with GWI. The two significantly different proteins included tubulin and MAG, which are related to axonal and oligodendrocyte functioning, which have been associated with history of mild traumatic brain injuries (mTBI) and to chemical weapons exposures. These results correspond with our prior studies showing increased rates of mTBI and chemical weapons exposures during the war, particularly in male veterans with GWI [30,36,37]. In fact, this multiple-hit hypothesis suggested those with both mTBI and chemical weapons exposure would report more chronic health symptoms that may be related to these axonal and myelin-related pathobiological markers. Correspondingly, Carney et al. compared combat experiences, occupational, and other service-related exposures and health care use of GW male and female veterans and reported similar military experiences but with male veterans more often participating in combat related activities. However, no significant gender differences were found in exposure to solvents/petrochemicals, infectious diseases, neurotoxins, heat stress, trauma, or radiation [38]. In addition, the women veterans with GWI differed from the GW women healthy controls on two markers, including a glial marker (GFAP) and a neuronal cytoskeletal marker (Tubulin). These results suggest that women with GWI appear to be showing more neuronal cytoskeletal and neuroinflammatory changes when compared to healthy GW controls or women with IBS or ME/CFS. The male veterans with GWI differed from the GW healthy males with higher values on all autoantibodies except for the acute glial BBB marker S100B and the CAI value, which was twice that of the controls. When the women with GWI were compared with the combined control group, they showed higher levels of autoantibodies on all markers except the glial markers GFAP and S100B. Again, their CAI value was nearly twice that of the combined controls. Similar to the prior analysis, the male veterans with GWI did not differ from their respective controls for the glial activation and acute BBB marker S100B. This suggests that male GWI veterans may be showing more chronic glial activation, neuronal damage, and neuroinflammation than their male control healthy and symptomatic counterparts with IBS and ME/CFS because S100B is a marker of current BBB disruption and GFAP is a marker of current neuroinflammation [39,40]. This is because GFAP is secreted by activated astrocytes, which leads to neuroinflammation [41–43].

With regard to integrating our findings within the larger scope of GWI research relevant to sex differences, it has been shown that there are differences in immune inflammatory markers and in overall rates of health symptoms between GW veteran men and women [5,8,13,44–46]. The current study adds to the literature in that GW veteran men have higher overall values of autoantibodies (as assessed by the CAI ratio), and that the findings are consistent with men and women potentially experiencing increased autoantibodies to a similar degree, but with a lesser increase in autoantibodies in women associated with greater symptom burden. These results suggest that further study of the sensitivity of the CAI cutoff of 30 for both men and women veterans with GWI is warranted.

Autoantibodies to neural proteins in addition to being blood biomarkers are also indications of neurodegeneration and may explain the mechanisms of brain diseases and aging effects [26,47]. Currently, the only consistent risk factors for GWI are chemical exposures and a history of mTBI [30,36,37,48–51]. These chemicals include: pyridostigmine bromide, pesticides including the insecticides permethrin and lindane as well as the insect repellent, DEET (*N,N*-diethyl-*m*-toluamide), and organophosphate (OP) insecticides and nerve gases, sarin and cyclosarin, which are now known to adversely affect the CNS in significant or combined dosages [3,14,15,48,51–58].

Investigations into the mechanisms by which OP compounds cause neurodegeneration have established that OPs increase the activity and expression of calcium-calmodulin Kinase II (CaMKII) that causes hyperphosphorylation of neural proteins, leading to their aggregation and slowing of axonal transport, resulting in neuronal cell death [51,59–62]. In agreement with this is our prior finding that airline crews who were exposed to OPs developed autoimmune antibodies to neural proteins³⁴. Increased autoantibodies to neuronal proteins such as Tau, NFP, MAG, MBP, and GFAP are consistent with the brain imaging study in another cohort of airline crews which showed decreased brain white

matter microstructure and cerebral perfusion, which may be potential causes of cognitive impairments and mood deficits reported by the aircrews [63]. The significance of this finding is that GFAP is a marker of astrocyte activation and astrocytes can directly interact with and restrict brain vasculature including capillaries that are involved in cerebral perfusion and neuroinflammation.

Health symptom complaints reported by GW veterans are consistent with sequelae following exposure to pesticides such as organophosphates, pyrethroids, and DEET [51,64,65]. Increased autoantibodies against neurofilament, tau, tubulin, and myelin basic proteins, which are biomarkers for myelin and neuronal cytoskeletal disruptions including microtubule instability, axonal degeneration, and altered axonal transport, have been found in many cell and animal studies of toxicant-induced models of GWI [66–74]. Our results are consistent with previous reports showing increased various autoantibodies in a smaller and then larger study of GW veteran blood samples [16,19,75–78]. To our knowledge, this is the first study to assess the sex effects of these autoantibodies in veterans with GWI compared with healthy and symptomatic comparison groups.

In summary, the results of the present investigation on the effect of gender on blood biomarkers showed that the levels of autoantibodies were significantly higher for both men and women GW veterans compared to controls¹⁹. When there was a sex difference, male veterans with GWI exhibited higher levels of autoantibodies as well as the overall CAI score than women veterans with GWI. This is consistent with the situation in the war theatre, that although men and women had similar military experiences, men more often participated in combat and had higher rates of mTBI [30,36–38]. This is not to suggest that women veterans do not have increased levels of other symptoms of this multi-factorial disorder but rather that male veterans appear to be showing more CNS autoantibody differences than women veterans as indicated by higher CAI total values.

Limitations

Like most studies, our study had limitations. We had small group sizes in some analyses that reduced our power to see differences between the groups. This was especially true for the women's comparisons. Because the main focus of this study was to assess GWI vs. symptomatic control groups, we did not have a non-veteran healthy control group for comparison, which may have provided more clarity to the results had that group been included. In this study, we used the Kansas criteria for GWI that should have excluded known cases of disorders including Alzheimer's Disease (AD), Parkinson's Disease (PD), and other chronic illnesses that could have accounted for their chronic symptoms [27]. In this study, we demonstrated associations between autoantibodies, particularly in GWI veterans, as compared to healthy and symptomatic controls stratifying by sex. However, the temporal relationship remains unclear between these conditions and production of autoantibodies. More research is needed to determine if these are blood-based CNS proteins from exposures 30 years ago or if there are ongoing CNS changes that are eliciting these autoantibody proteins. Other larger studies with women veterans would also help further validate the pathobiological impact of GWI on women veterans.

A major strength of our study is that it represents both healthy and symptomatic GW veteran groups as well as symptomatic non-veteran controls with ME/CFS or IBS. This suggests that both men and women veterans with GWI differ not only from their healthy GW veteran controls but also have more CNS differences than other groups of men and women with chronic multi-symptom illnesses. In addition, the CNS autoantibody analyses were performed with the laboratory staff blinded to the case status of all participants.

5. Conclusions

This year marks the 30th anniversary of the 1990/1991 Gulf War. Throughout most of this time, there has been a lack of diagnostic tools to accurately diagnose the disorder, which has hindered both accurate diagnoses and much-needed treatments for veterans. Our laboratory was one of the first to propose and document that GWI is related to

chemical exposures during the war and that those exposures adversely affected the CNS. In addition, we have documented differences in autoantibodies between GWI and GW-healthy and symptomatic controls. This study documents that there is a sex effect for these groups with men and women with GWI showing higher levels of autoantibodies than their respective control groups and with male veterans with GWI showing the greatest burden of autoantibodies. After further validation, we are hopeful that our newly developed CNS Autoantibody Neurodegeneration Index (CAI) summary score cutoff of 30 or more can be utilized to develop objective diagnostic markers of GWI and to compare treatment trial effectiveness for both men and women GW veterans.

Author Contributions: Conceptualization, M.B.A.-D., K.S., J.M., L.A.C., E.K. and N.G.K.; data curation, E.Q. and D.D.N.; formal analysis, E.Q., J.L. and J.M.; funding acquisition, M.B.A.-D. and K.S.; investigation, M.H.K.; methodology, M.B.A.-D., E.S.L., J.M., L.A.C., E.K., M.A., N.G.K., D.D.N. and K.S.; project administration, M.B.A.-D., E.S.L., M.H.K., J.L., L.A.C., E.K., M.A., D.D.N. and K.S.; resources, M.A. and N.G.K.; supervision, M.B.A.-D.; K.S.; writing—original draft, M.B.A.-D., M.H.K., C.G.Z. and K.S.; writing—review and editing, E.S.L., M.H.K., E.Q., J.M., L.A.C., E.K., C.G.Z., M.A., N.G.K., D.D.N. and K.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Boards of Duke University Health System Institutional Review Board for Clinical Investigations on 10/9/2017 and from the Boston University Medical Campus Institutional Review Board on 1/19/2018.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data are available upon request from the corresponding authors.

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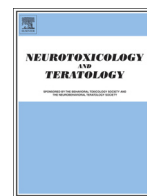
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Screening for novel central nervous system biomarkers in veterans with Gulf War Illness

Mohamed B. Abou-Donia^{a,*}, Lisa A. Conboy^b, Efi Kokkotou^c, Eric Jacobson^{d,i}, Eman M. Elmasry^e, Passent Elkafrawy^f, Megan Neely^g, Cameron R. 'Dale' Bass^h, Kimberly Sullivan^h^a Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, United States^b Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States^c Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States^d Department of Global Health and Social Development, Harvard Medical School, United States^e Department of Microbiology, Zagazig University, Zagazig, Egypt^f Department of Math and Computer Science, Menoufia University, Shebin ElKom, Egypt^g Department of Biostatistics & Bioinformatics, Duke University Medical Center, United States^h Biomedical Engineering Department, Duke University, United Statesⁱ Department of Environmental Health, Boston University School of Public Health, Boston, MA, United States

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ABSTRACT

Gulf War illness (GWI) is primarily diagnosed by symptom report; objective biomarkers are needed that distinguish those with GWI. Prior chemical exposures during deployment have been associated in epidemiologic studies with altered central nervous system functioning in veterans with GWI. Previous studies from our group have demonstrated the presence of autoantibodies to essential neuronal and glial proteins in patients with brain injury and autoantibodies have been identified as candidate objective markers that may distinguish GWI. Here, we screened the serum of 20 veterans with GWI and 10 non-veteran symptomatic (low back pain) controls for the presence of such autoantibodies using Western blot analysis against the following proteins: neurofilament triplet proteins (NFP), tubulin, microtubule associated tau proteins (Tau), microtubule associated protein-2 (MAP-2), myelin basic protein (MBP), myelin associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP), calcium-calmodulin kinase II (CaMKII) and glial S-100B protein. Serum reactivity was measured as arbitrary chemiluminescence units. As a group, veterans with GWI had statistically significantly higher levels of autoantibody reactivity in all proteins examined except S-100B. Fold increase of the cases relative to controls in descending order were: CaMKII 9.27, GFAP 6.60, Tau 4.83, Tubulin 4.41, MAG 3.60, MBP 2.50, NFP 2.45, MAP-2 2.30, S-100B 1.03. These results confirm the continuing presence of neuronal injury/gliosis in these veterans and are in agreement with the recent reports indicating that 25 years after the war, the health of veterans with GWI is not improving and may be getting worse. Such serum autoantibodies may prove useful as biomarkers of GWI, upon validation of the findings using larger cohorts.

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1. Introduction

Approximately one third of the 697,000 US military personnel who served in the Gulf War (GW) from August 1990 to June 1991, have reported persistent symptoms for many years after the war (RAC, 2008; IOM, 2012, RAC, 2016; White et al., 2016). This complex of symptoms, known as Gulf War Illness (GWI), include memory and attention problems, profound fatigue, chronic muscle and joint pain, severe headaches,

persistent diarrhea, respiratory difficulties and skin rashes. GWI is primarily diagnosed by symptom report and no validated objective diagnostic biomarkers currently exist that fully segregate cases from controls. This study was designed to identify objective central nervous system (CNS) biomarkers of GWI using clues from prior clinical studies with GW veterans and from animal studies that modeled chemical exposures experienced by GW veterans.

Clinical studies have reported impaired cognitive functioning and reduced MRI volume and altered white matter microstructural integrity in organophosphate (OP) pesticide, sarin nerve agent and pyridostigmine bromide (PB) anti-nerve gas pill-exposed GW veteran cohorts (White et al., 2016; Sullivan et al., 2013; Chao et al., 2010;

* Corresponding author at: Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, United States.

E-mail address: donia@duke.edu (M.B. Abou-Donia).

Heaton et al., 2007; Proctor et al., 2006; Sullivan et al., 2003). Animal studies demonstrated that exposure to higher doses of the prophylaxis pill pyridostigmine bromide (PB), the insect repellent, DEET, and the insecticide permethrin and/or chlorpyrifos led to significant brain damage in animal models of GWI (Abou-Donia et al., 1996a,b). Further studies using 60 days of subchronic dermal exposure to DEET and permethrin, alone or in combination, at dose levels approximately equivalent to the exposures that occurred during the Gulf War in a rat-model of GWI, caused the following: (1) a diffuse neuronal cell death in the motor cortex, the different subfields of the hippocampal formation, and the Purkinje cell layer of the cerebellum, accompanied by sensorimotor deficits; (2) significant reduction of MAP-2-positive immunoreactive structures indicating atypical expression of MAP-2 in dendrites of surviving neurons, within the cerebral cortex and the hippocampus that was characterized by a beaded, disrupted, or wavy appearance; (3) a significant upregulation of GFAP-positive expression in structures in the CA3 subfield of the hippocampus, the motor cortex and the dentate gyrus (Abdel-Rahman et al., 2001, 2002a,b, 2004a,b; Abou-Donia et al., 2000, 2001, 2002, 2004; Terry et al., 2003). Similar results were exhibited in animals treated with sarin alone or accompanied by cited-above chemicals, with and without stress (Abdel-Rahman et al., 2004a).

The cytoarchitecture of the CNS is maintained by a complex cellular milieu that involves neuronal and glial cells that must maintain proper communication in order to function properly (Abou-Donia and Lapadula, 1990; McMurray, 2000). CaMKII phosphorylates cytoskeletal proteins, such as MAP-2, tau and tubulin. CaMKII accounts for 12% of all proteins in the brain. CaMKII has the ability to coordinate and transduce upstream Ca and reactive oxygen species (ROS) signals into physiological and pathophysiological downstream responses in the nervous system and cardiovascular biology and disease (Abou-Donia, 1995; Erickson et al., 2011). Tubulin, the major component of microtubules, is responsible for axonal migration and longitudinal growth and is involved in axonal transport. Although tubulin is present in virtually all eukaryotic cells, the most abundant source is the vertebrate brain, where it consists of approximately 10–20% of its total soluble protein (McMurray, 2000). Microtubule-Associated Protein-2 (MAP-2) is found in dendritic compartments of neurons. A loss of MAP-2, is a reliable indication of irreversible neuropathology and is a sensitive marker of seizure-related brain damage (Ballough et al., 1995). Tau Protein, a normal axonal protein, is involved in stabilization and assembly of axonal microtubules. Levels of tau proteins are elevated in the cerebrospinal fluid (CSF) and serum following TBI (Liliang et al., 2011) and has been used for diagnosis of Alzheimer's disease. Myelin basic protein (MBP) is an abundant myelin membrane proteolipid produced by oligodendroglia in the CNS and Schwann cells in PNS and may confirm the clinical assessment of neurodegenerative disorders such as multiple sclerosis and stroke (Jauch et al., 2006). Myelin Associated Glycoprotein (MAG) is selectively localized in periaxonal Schwann cell and oligodendroglial membranes of myelin sheaths, suggesting that it functions in glia–axon interactions in both the PNS and CNS (Schachner and Bartsch, 2000). Glial fibrillary acidic protein (GFAP) is expressed almost exclusively in astrocytes, where it is induced by neural injury and released upon disintegration of the astrocyte cytoskeleton (Rempe and Nedergaard, 2010). GFAP plays an essential role in maintaining shape and motility of astrocytic processes and contribute to white matter architecture, myelination and blood brain barrier (BBB) integrity (O'Callaghan et al., 2015). After traumatic brain injury (TBI), GFAP's serum concentration peaks at 2–6 h and has a half-life of <2 days (Diaz-Arrastia et al., 2014). S-100B exerts both detrimental and neurotrophic effects, depending on its concentration in brain tissues (Adami et al., 2001). After release, S-100B acts as a trophic factor for serotonergic neurons, and plays a role in axonal growth and synaptogenesis during development. Thus, traumatic acute injury results in great destruction of astrocytes leading to massive release (50 to 100 fold) of S-100B into plasma, whereas S-100B levels in psychiatric disorders were

only about 3 times higher in patients compared to controls (Uda et al., 1998; Arolt et al., 2003), correlating well with its neuroprotective action. Specifically, S-100B stabilizes tau and MAP-2. Its half-life in the serum is 2 h (Zurek and Fedora, 2012).

A recent study of airline pilots and other flight crew members chronically exposed to organophosphates through combustion of engine oil and hydraulic fluid that contain organophosphate esters resulted in symptoms similar to those reported by GW veterans (fatigue, headaches, confusion and memory problems). Interestingly, these crew members showed significantly elevated numbers of autoantibodies in their blood serum of CNS damage markers including those associated with axonal transport (microtubule associated protein-2 (MAP-2), tubulin, neurofilament triplet proteins (NFP) and microtubule associated protein-tau (tau protein)) and those exclusively associated with CNS glial activation and neuroinflammation (myelin basic protein (MBP), and glial fibrillary acidic protein (GFAP) (Abou-Donia et al., 2013). A follow-up histopathology autopsy study was performed on a deceased pilot with organophosphate exposure that confirmed CNS damage and demyelination (Abou-Donia et al., 2014). Specifically, the histopathology results showed axonal degeneration and demyelination and the post-mortem and pathological examination of the nervous system confirmed the autoantibody biomarker results.

Recent studies with GW veterans have shown persistent signs and symptoms characteristic of CNS injury including brain imaging and cognitive studies (White et al., 2016; Chao et al., 2010, 2011, 2014, 2016; Heaton et al., 2007; Sullivan et al., 2003). There are, however, no validated objective diagnostic tests to identify acute or chronic sequelae of brain injury in this veteran group. Diagnosis of brain injury using cranial computed tomography (CT) scan and magnetic resonance imaging (MRI) techniques such as diffusion tensor imaging (DTI), have not been able to clinically diagnose veterans with GWI because there have been no proven cutoff values for volumetric or other imaging parameters that have been able to provide the required near 100% accuracy in terms of sensitivity/specificity at the individual level to distinguish cases from controls needed for a diagnostic test. Imaging studies have been able to show differences and altered CNS functioning between veterans with GWI and healthy controls but have not yet been able to identify the groups diagnostically because of the significant overlap between the groups (Chao et al., 2010, 2011, 2014, 2016; Heaton et al., 2007). Hence, it is important to develop clinically available, simple and inexpensive biomarkers for detection of neuronal and glial injury essential in the diagnosis and understanding of the temporal progression of CNS damage in GWI. Recently, serum biomarkers such as cytoskeletal proteins, resulting from axonal degeneration, have been used in diagnosing brain injury (particularly traumatic brain injury). The use of these biomarkers is usually measured in serum shortly after brain injury, because they have short half-lives (Zurek and Fedora, 2011; Diaz-Arrastia et al., 2014).

However, many years have elapsed since the time that GW veterans returned from deployment and became ill therefore, this particular approach cannot apply to GWI. Based on results from both chronic and acute injury, we used our novel autoantibody biomarker panel described above for brain injury to test for the indication of CNS damage in veterans with chronic GWI (Abou-Donia et al., 2013, 2014). One prior study compared autoantibodies of myelin basic protein (MBP) and striated muscle antibodies in GW veterans and reported higher MBP and muscle antibodies in veterans with GWI (Vojdani and Thrasher, 2004). Autoantibodies have previously been recognized as potential objective biomarkers of GWI (Golomb, 2012). Therefore, we hypothesized that chemical exposure to pesticides, anti-nerve gas pills and/or sarin nerve gas during deployment in veterans with GWI caused an excitotoxic cascade (through potential glutamatergic, oxidative stress and proinflammatory cytokine signaling) resulting in neurodegeneration and apoptotic loss of brain cells, leading to blood brain barrier leakage of specific neuronal and glial proteins into circulation, with subsequent formation of autoantibodies (AB) against these

proteins (Abou-Donia et al., 2013; Banks and Lein, 2012; Golomb, 2008; Terry, 2012; Binukumar and Gill, 2010; Soltaninejad and Abdollahi, 2009). In this study, we determined circulating IgG- class autoantibodies in serum from 20 GWI cases and 10 symptomatic (low back pain) controls against the following 9 brain proteins: neurofilament triplet proteins (NFP), tubulin, microtubule associated protein-tau (tau proteins), microtubule associated protein-2 (MAP-2), calcium/calmodulin Kinase II (CaMKII), myelin basic protein (MBP), myelin associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP) and S-100B.

2. Materials and methods

2.1. Materials

The sources of proteins were: NFP (bovine spinal cord), tau protein (human), MAP-2 (bovine serum), tubulin (bovine brain), and MBP (human brain), from Sigma-Aldrich (Saint Louis, Missouri); CaMKII (Human) recombinant Protein and MAG recombinant Protein from Novus Biologicals, Littleton, CO, GFAP (human) from Biotrend Chemikalien GmbH, (Cologne, Germany) and S-100B (human brain) from American Qualex International, Inc. (San Clemente, California). Horseradish peroxidase-conjugated goat anti-human IgG, and enhanced chemiluminescence reagent were obtained from Amersham Pharmacia Biotech (Piscataway, New Jersey). SDS gels, 2–20% gradient (8 × 8), and tris-glycine 15 mM were obtained from Invitrogen (Carlsbad, California). All other materials were purchased from Amersham.

2.2. Ethics statement

Approval for the use of stored blood samples for this study was obtained from the Duke University Medical Center Institutional Review Board.

2.3. Case and control samples

Serum samples from 20 GWI cases with GWI and 10 non-veteran symptomatic controls with lower back pain were tested in this pilot study. GW veteran serum samples were collected from a study of acupuncture treatment in veterans with GWI from 2010 to 2012 (Conboy et al., 2012). Control serum samples were derived from a separate study of non-veteran patients with chronic lower back pain who served as 'symptomatic low back pain' controls from 2011 to 2013 (Jacobson et al., 2015). Veterans with GWI will be referred to as 'cases' and low-back pain symptomatic controls will be referred to as 'controls'.

2.4. Description of the patient cohorts

2.4.1. GWI-case cohort

"The Effectiveness of Acupuncture in the Treatment of Gulf War Illness" PI: Conboy, (8/21/2010–12/26/2012) N = 104; Study Site: New England School of Acupuncture (NESA). Cases were recruited through the Defense Manpower Data Base (DMDC) personnel listings and advertisements. Cases were screened for GWI symptoms and were required to meet the CDC diagnostic criteria for chronic multi-symptom illness (CMI) in order for inclusion in the parent study and in the current study (Conboy et al., 2012; Fukuda et al., 1998). Inclusion in the current study also required that veterans were deployed to the 1990–1991 Gulf War. CMI is characterized by one or more symptoms of at least 6 months duration from at least two of three symptom categories: 1) fatigue; 2) mood-cognition; 3) musculoskeletal pain.

Symptoms were not necessarily required to have started during or after the Gulf War deployment. Exclusionary criteria included that the veteran was 1) currently enrolled in another clinical trial 2) Had another disease that likely could account for the symptoms, as determined by the Medical Monitor 3) Severe psychiatric illness (in the last 2 years psychiatric hospitalization, suicidal attempt, alcohol or substance

abuse, use of antipsychotic medication) 4) Unable to complete the protocol based on the evaluation of the Medical Monitor.

2.4.2. cLBP-cohort

"Structural Integration for chronic low back pain" PI: Jacobson (3/4/2011–6/21/2013) N = 46. Study Site: Spaulding Rehabilitation Hospital (SRH). In this cohort, 46 outpatients from the Boston area with chronic nonspecific low back pain were randomized to parallel 20-week long treatment groups of structural integration (SI) plus outpatient rehabilitation (OR) versus OR alone. The details of the study are described in a recent publication (Jacobson et al., 2015). Inclusion criteria for the parent study included: (i) Men and women aged 18–65, (ii) cLBP of ≥6 months duration, not attributed to infection, neoplasm, severe radiculopathy (as indicated by frequent severe pain radiating down a leg), fracture, or inflammatory rheumatic process, (iii) bothersomeness of back pain self-rated on average over the preceding 6 months ≥3 on an 11-point ordinal scale (0 = none, 10 = worst imaginable), (iv) prior arrangement to enter a course of outpatient physical therapy for low back pain at a Boston area rehabilitation clinic, (v) English language fluency and mental capacity sufficient to provide informed consent and participate in the study. Exclusion Criteria for the study included: (i) Impaired hearing, speech, vision, and mobility sufficient to interfere with participation in the study, (ii) current or anticipated receipt of payments from Worker's Compensation or other insurance for disability attributed to low back pain, (iii) prior treatment with any SI therapy, (iv) plans to initiate additional treatment for back pain during the period of the study other than outpatient rehabilitation care, particularly massage or other manual therapies (e.g., chiropractic or osteopathic manipulation), (v) exclusions for safety: unresolved musculoskeletal pathology of the lower limbs, current pregnancy, any implanted medical device, osteoporosis, any hypercoagulation condition, eczema, skin infection, deep vein thrombosis, burns or other acute trauma including unhealed bone fractures or open wounds, psoriasis, psychiatric illness not well controlled, or current episode of exacerbated major depressive disorder.

2.5. Collection and storage of samples

Samples from the GWI-cohort and the cLBP-cohort were all collected from the Boston area at the same time period at two different sites from 2010 to 2013. All sites followed exactly the same protocol for venipuncture, blood handling, serum separation, aliquoting and storage at –80 °C. The same phlebotomy and sample protocol was distributed in writing to all sites. All samples analyzed were baseline blood samples collected pre-intervention therapy. Samples used for this study have not been previously thawed and are free of hemolysis by visual inspection (Tuck et al., 2009).

2.6. Participant demographics

The participant demographics indicate that a total of 20 veterans with GWI, 18 males and 2 females, compared to 6 females out of 10 cLBP controls participated in the study. The age of the GWI cases ranged from 38 to 61 (mean ± SD 46.0 ± 6.8) compared to 25 to 64 (mean ± SD 50 ± 11.4) years for controls; all study participants were white (Table 1). Seventy percent of veterans with GWI reported taking PB

Table 1
Study participant demographics.^a

Demographics	Cases	Controls
Age (mean ± SD)	46 (6.4)	50 (11.4)
Gender (% female)*	10	60
Race (% Caucasian)	100	100

Age range of Cases = 38–61 years and Controls = 25–64 years in 2010–2013 when the blood was collected.

^a A total of 20 cases and 10 controls participated in the study.

* Cases were significantly different from controls for gender $p < 0.05$ but not for age.

pills during the war ($n = 14$). The groups differed with respect to gender ($X^2 = 8.5$; $p < 0.05$) with significantly more women in the control group but did not differ with respect to age (t -value = -1.3 ; $p > 0.05$).

2.7. Western blot assay

To screen for the presence of autoantibodies against a battery of proteins, we applied a Western blot approach as previously reported (Abou-Donia et al., 2013). Each serum sample was analyzed in triplicate. Each protein was loaded as 10 ng/lane except for IgG that was loaded as 100 ng/lane. Proteins were denatured and electrophoresed in SDS-PAGE (4% to 20% gradient) purchased from Invitrogen (Carlsbad, CA). One gel was used for each serum sample. The proteins were transferred into polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech Piscataway, New Jersey). Nonspecific binding sites were blocked with Tris-buffered Saline-Tween (TBST) (40 mM Tris [pH 7.6], 300 mM NaCl, and 0.1% Tween 20) containing 5% non-fat dry milk for 1 h at 22 °C. Membranes were incubated with serum samples at 1:100 dilutions in TBST with 3% non-fat dry milk overnight at 4 °C. After five washes in TBST, the membranes were incubated in a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Amersham Pharmacia Biotech (Piscataway, New Jersey). The dot blots were probed with anti-human IgG (H + L) HRP conjugate antibody (Cat. No. 31410, Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) for 1 h at RT, incubated with ECL reagent (Cat. No. 34096). The membranes were developed by enhanced chemiluminescence using the manufacturer's (Amersham Pharmacia Biotech) protocol and a Typhoon 8600 variable mode imager. The signal intensity was quantified using Bio-Rad image analysis software (Hercules, California). All tests were performed with the investigators blinded to participant diagnosis.

2.8. Specificity of serum autoantibodies

Previously we checked the specificity of the serum autoantibody by performing peptide/antigen competition assay, in which the serum was spiked with the target protein or peptide (Abou-Donia et al., 2013). The serum from random healthy controls was mixed with or without tau, MAP or MBP. The serum/protein mix was centrifuged at 15,000 rpm to pellet any immune complexes. The supernatants were then carefully removed and used in Western blotting.

2.9. Calculations

The mean value of the optical density measurement from the triplicate testing was used for each serum sample tested and normalized by total IgG. Thus, the results are expressed as mean values of triplicate assays of optical density arbitrary units normalized to total serum IgG.

2.10. Power analysis

A total of 20 GWI cases were available for testing in this convenience sample. Effect size calculations were based on two-sample t -test assuming a common standard deviation between groups. The power analysis assumes that cases and controls are not matched. In a t -test of difference between two independent means, selecting power of 80%, 2-sided alpha 0.05, and size of 20 vs 10, the study was powered to detect an effect only if at least 1.12 SD.

2.11. Statistics

Grouped data are reported as mean $\pm$ SD. The values from cases were compared to the control group using t -tests and Pearson correlation analyses (SigmaStat, Systat Software) and p -values were

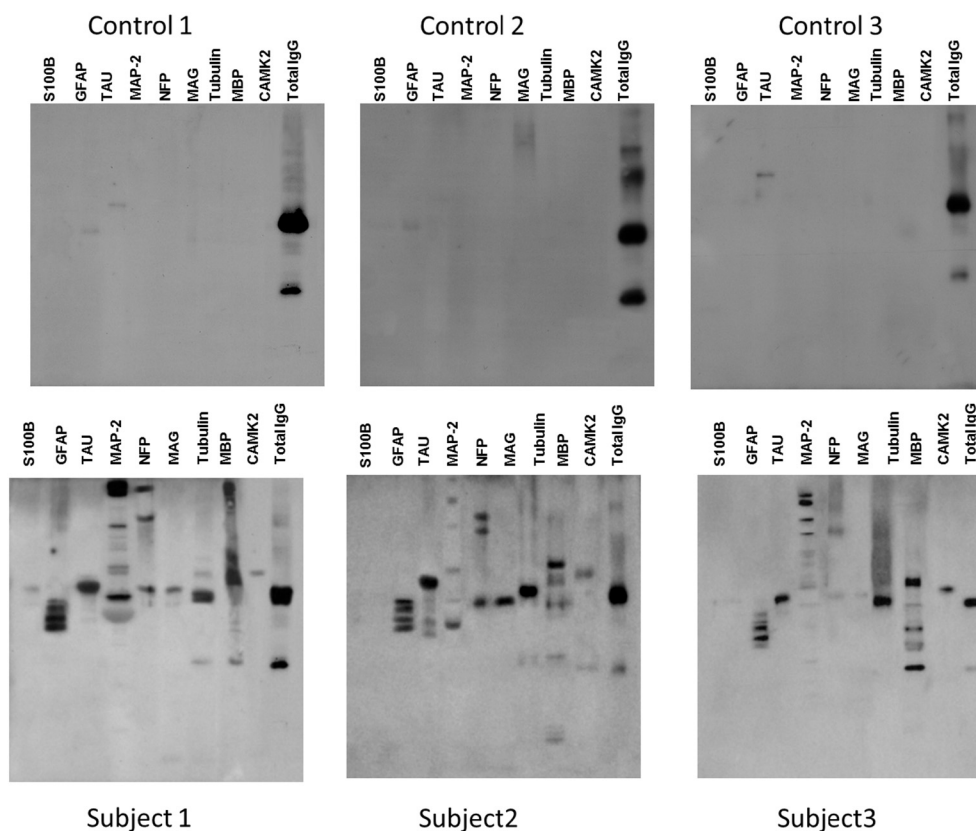


Fig. 1. A representative sample of Western blot gels from three cases showing that the majority of GWI serum reacted intensely to neural proteins, while most control serum showed a weak or no reaction.

Table 2Chemicals, environmental and other exposures of cases during the Gulf War.^a

Chemical exposures	Exposed		Environmental and other exposures	Exposed	
		%			%
Pyridostigmine bromide (PB)	14	70	Khamisiyah notification letter	8	40
Organophosphorus pesticides (OP)	7	35	Contaminated food/water	18	90
Carbamates	7	35	Vaccines	18	90
Pyrethroids	4	20	Malaria	12	60
DEET	11	55	Sand	18	90
Sarin	9	45	Tent heater	11	55
Depleted uranium (DU)	6	30	Jet fuel	14	70
Solvents	10	50	Oil fires	18	90

^a A total of 20 veterans with GWI participated in the study.

calculated. Pairwise correlations among the nine biomarkers were assessed. A 2-sided p value <0.05 was considered significant. Due to the exploratory nature of this pilot study, analyses were not adjusted for multiple comparisons.

3. Results

As previously described, we assessed the specificity of the serum autoantibody by performing peptide/antigen competition assay, in which the serum was spiked with the target protein or peptide. The serum bound to tau eliminated the tau band in the Western blot (see Fig. 1) while the band of MAP-2 or MBP were present and not affected. The serum bound to MAP-2 eliminated the MAP-2 band in the Western blot while the band of tau or MBP was present. The serum bound to MBP eliminated the MBP band in the Western blot while the bands of tau and MAP-2 were present. These results indicate that each autoantibody in the serum was specifically neutralized by its target protein in serum sample and was no longer available to bind to the epitope present in the protein on the Western blot. This confirmed that the assay used in this study, was specific and accurately determined autoantibodies against tested proteins in serum samples.

To detect autoantibodies in serum, we probed Western blots with individual serum samples. A total of 30 human serum samples (20 veterans with GWI and 10 non-veteran symptomatic low-back-pain controls) underwent measurement of the levels of the serum circulating IgG-class autoantibodies against nine neuronal- and glial- specific proteins. Table 2 lists the number of GWI cases who were exposed to chemical and environmental exposures. It shows that 14 cases (70%) used PB as a prophylaxis against possible exposure to nerve agents and nine cases reported being exposed to the nerve agent sarin. In addition, a total of eight cases reported receiving notification from the Department of Defense (DOD) that they were potentially exposed to sarin and other chemicals due to their proximity to the Khamisiyah, Iraq underground weapons depot where a chemical weapons cache was destroyed in March 1991 (US DOD, 2002). Eight cases reported exposure to depleted uranium. All of the cases reported exposure to one or more insecticides or a mixture of pesticides including organophosphates, carbamates,

pyrethroids and organochlorines. Eleven cases used the insect repellent DEET. All cases underwent environmental and other exposures listed in Table 2. Other chemicals that the cases reported exposure to included oil well fires, sand, tent heaters, jet fuel, and solvents. Some veterans reported exposure to malaria and 18 reported being vaccinated. Serum from GWI cases showed significantly increased levels of autoantibodies against all cytoskeletal proteins except those against S-100B compared to non-veteran symptomatic (low back pain) controls (Table 3). Due to the gender differences between the cases and controls, analyses were also run with just the males in the groups. Although there was only a small number of males ($n = 4$) in the control group which could be problematic in this type of analysis, results of this comparison showed a very similar pattern of significant differences in all autoantibodies (GFAP $p < 0.001$; Tau $p < 0.001$; MAP $p < 0.002$; MAG $p < 0.001$; PNF $p < 0.006$; Tubulin $p < 0.003$; MBP $p < 0.01$; S-100B $p = 0.31$). The majority of GWI serum reacted intensely to neural proteins, while most control serum showed a weak or no reaction. Fig. 1a and b present Western blots results from three representative GWI cases and three controls. The levels of serum autoantibodies in GWI cases and controls to neural-specific proteins expressed as mean values $\pm$ SD of triplicate assays of optical density arbitrary units normalized to total serum IgG optical density ranged from 0.30 for S-100B and 4.09 for GFAP for the cases compared to 0.30 and 0.62, respectively for controls are listed in Table 3 and shown in Fig. 2. The percentage of autoantibodies against neural proteins of cases compared to controls (in descending order) were: CaMKII, 927, GFAP 660, Tau 483, Tubulin 441, MAG 360, MBP 250, NFP 245, MAP-2 230, S-100B 103. Fig. 3 presents the mean values $\pm$ SD ($p < 0.001$) of fold increase of autoantibodies against neural proteins for the cases compared with the controls. Serum from controls had no or low levels of circulating autoantibodies to nervous system-specific biomarkers. Autoantibodies against CaMKII were more predominant in the cases' serum than in controls' serum (Fig. 3).

Fig. 4 shows that Tubulin and GFAP had the highest values in the GWI cases compared with the controls. Pairwise correlations among the nine autoimmune biomarkers were significant only for the pair Tau and MBP. When comparing the correlation between each pair, only tau and MBP were significantly linearly correlated to each other (Fig. 5). Fig. 5 shows that the control values of those two biomarkers were <1 optical density unit, whereas GWI cases had values strongly linearly correlated with each other such that on average tau was elevated up to 10 times higher than controls in some GWI cases, and MBP was also elevated up to 5 times higher for the same cases vs the controls.

Finally, when each biomarker was compared separately between individual cases and controls for potential fold-increase cut-points to discriminate the groups, results indicated that tubulin values had some of the highest-fold increased values in the individual GWI cases compared with the individual control values although only 60% of the individual cases ($n = 12$) showed that effect (Fig. 6a). However, in 9 (out of the 20) cases tubulin values were elevated by a factor of 3 to 9-fold higher than the controls. In Fig. 6b, GFAP was elevated the most in cases compared to controls. In fact, GFAP was higher in all of the cases compared with all of the controls with 20 out of 20 cases having 2 to 7 fold higher

Table 3Statistical analysis of the levels^a of serum autoantibodies (AA) in controls^b and GWI cases^b to neural-specific proteins.

	NFP	Tau	Tubulin	MBP	MAG	MAP2	GFAP	S-100B	CaMKII
Cases	1.42 $\pm$ 0.24	2.52 $\pm$ 0.31	3.48 $\pm$ 0.78	1.75 $\pm$ 0.30	1.44 $\pm$ 0.28	2.18 $\pm$ 0.29	4.09 $\pm$ 0.33	0.30 $\pm$ 0.03	1.02 $\pm$ 0.20
Mean $\pm$ SD									
Controls	0.58 $\pm$ 0.09	0.60 $\pm$ 0.09	0.79 $\pm$ 0.11	0.70 $\pm$ 0.11	0.40 $\pm$ 0.04	0.086 $\pm$ 0.09	0.62 $\pm$ 0.11	0.29 $\pm$ 0.04	0.11 $\pm$ 0.03
Mean $\pm$ SD									
p values	0.02	0.0001	0.001	0.001	0.007	0.002	0.00001	0.40	0.015

^a The results are expressed as mean values of $\pm$ triplicate assays of optical density arbitrary units normalized to IgG optical density as fold of healthy controls.^b Values from cases were compared to the control group using t -tests; most were highly significant $p < 0.001$ (2-sided), except for S-100B that was not significantly different from controls. Cases were significantly different from controls with respect to gender $p < 0.05$ but not with respect to age.

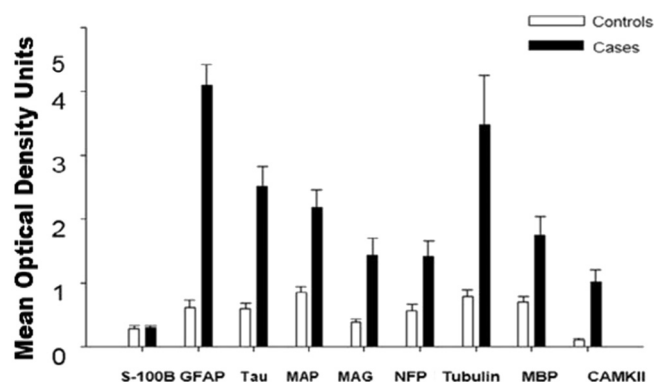


Fig. 2. Mean autoantibodies against neural proteins from cases and controls expressed in mean optical density units.

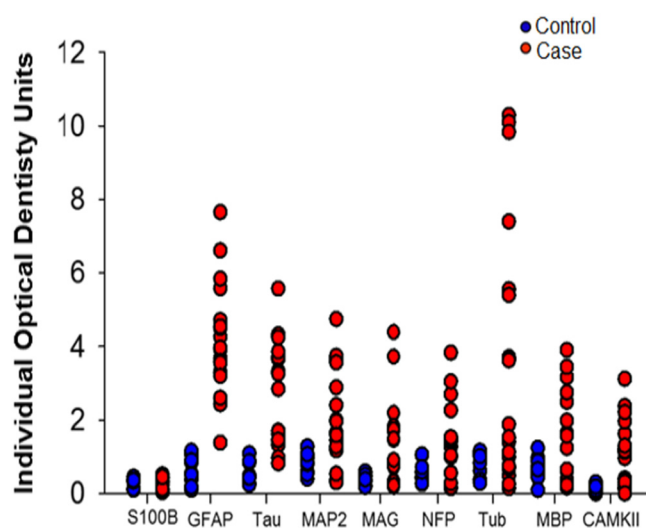


Fig. 4. The levels of autoantibodies of neural proteins of GW cases and of controls expressed as optical density units.

value than the control mean. Thus GFAP values completely distinguished the cases from the controls. GFAP values did not overlap in cases vs controls in this small sample; however, the separation in the ranges was small relative to the substantial standard deviations. In Fig. 6c, tau was higher than controls in 18 cases and 50% of the cases had double the value of tau compared with the controls. In Fig. 6d, MAP was higher than the controls in 15 cases and 75% of the cases had a 0.5 to 11-fold higher value than the controls. In Fig. 6e MAG was higher than controls in 15 cases and 75% of the cases had up to a 10-fold higher value than the controls. In Fig. 6f NFP was higher than controls in only 50% of the cases ($n = 10$) and they showed 0.5 to 11-fold higher values than controls. MBP was higher than controls in 12 cases and 60% of the cases were higher than controls with 2 to 5-fold higher values than controls. CAMKII was higher than controls in 16 cases and 50% of the cases had a 3 to 30-fold higher value than the controls. S100B values were not statistically significant as the values overlapped with cases and controls.

4. Discussion

This pilot study reports significantly elevated levels of autoantibodies against neurotypic- and gliotypic-specific proteins in serum from a sample of 20 veterans with GWI and 10 non-veteran symptomatic (low back pain) controls with musculoskeletal symptoms rather than CNS symptoms. The increased levels in GWI cases compared to controls ranged from 9.27 fold for CaMKII to 6.6 fold for GFAP to 2.45 fold for neurofilaments. Autoantibody levels against S-100B were not different in GWI cases than controls (1.03 fold) consistent with its neural protective role and in agreement with presence of chronic injury and

absence of acute brain injury in veterans with GWI (Zurek and Fedora, 2011; Diaz-Arrastia et al., 2014; Stalnacke et al., 2006, 2004; Coch and Leube, 2016). Previous studies, using animal models of GWI, showed that exposure to the neurotoxins that were present in the GW environment, caused deficits in behavioral outcomes that were accompanied by neuronal and glial degeneration (Abdel-Rahman et al., 2001, 2002a,b, 2004a,b; Abou-Donia et al., 2000, 2001, 2004). Following neurodegeneration, there is accumulation of cellular neurological waste products or debris such as misfolded or hyper-phosphorylated proteins that form toxic stable aggregates (Nedergaard, 2013; Edgar et al., 2004). This extracellular debris send damage signals that cause the CNS immune cells - microglia to become activated and act as profound antigen presenting cells that secrete pro-inflammatory cytokines (IL-1b, TNF-a and IL-6) and mediators (reactive oxygen species, ROS) resulting in the recruitment of T-lymphocytes (Milligan and Watkins, 2009; Banks and Lein, 2012). Multiple exposures to these waste proteins can cause microglia and astrocytes to become primed to react more strongly after each subsequent exposure (Watkins and Maier, 2003). This can result in a persistent neuroimmune response and chronic neuroinflammation contributing to chronic health symptoms, such as those seen in GW veterans (Johnson et al., 2016; Milligan and Watkins, 2009; Maier and Watkins, 1998; Watkins and Maier, 2003). These waste proteins are eventually released into circulation due to defects in the

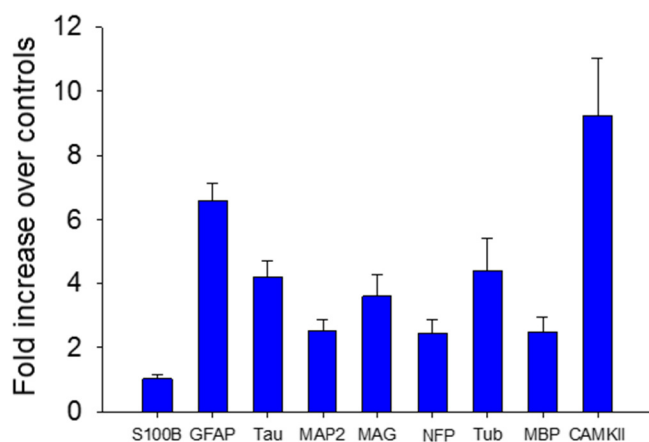


Fig. 3. Folds increase of autoantibodies against neural proteins from cases relative to controls.

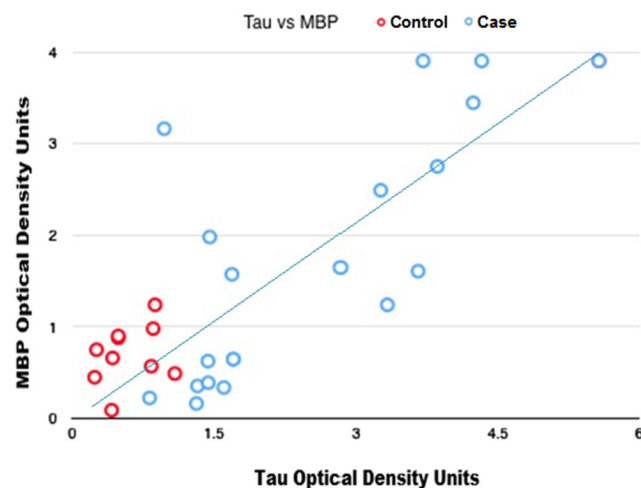


Fig. 5. Paired correlations of Tau and MBP optical density levels in cases relative to controls.

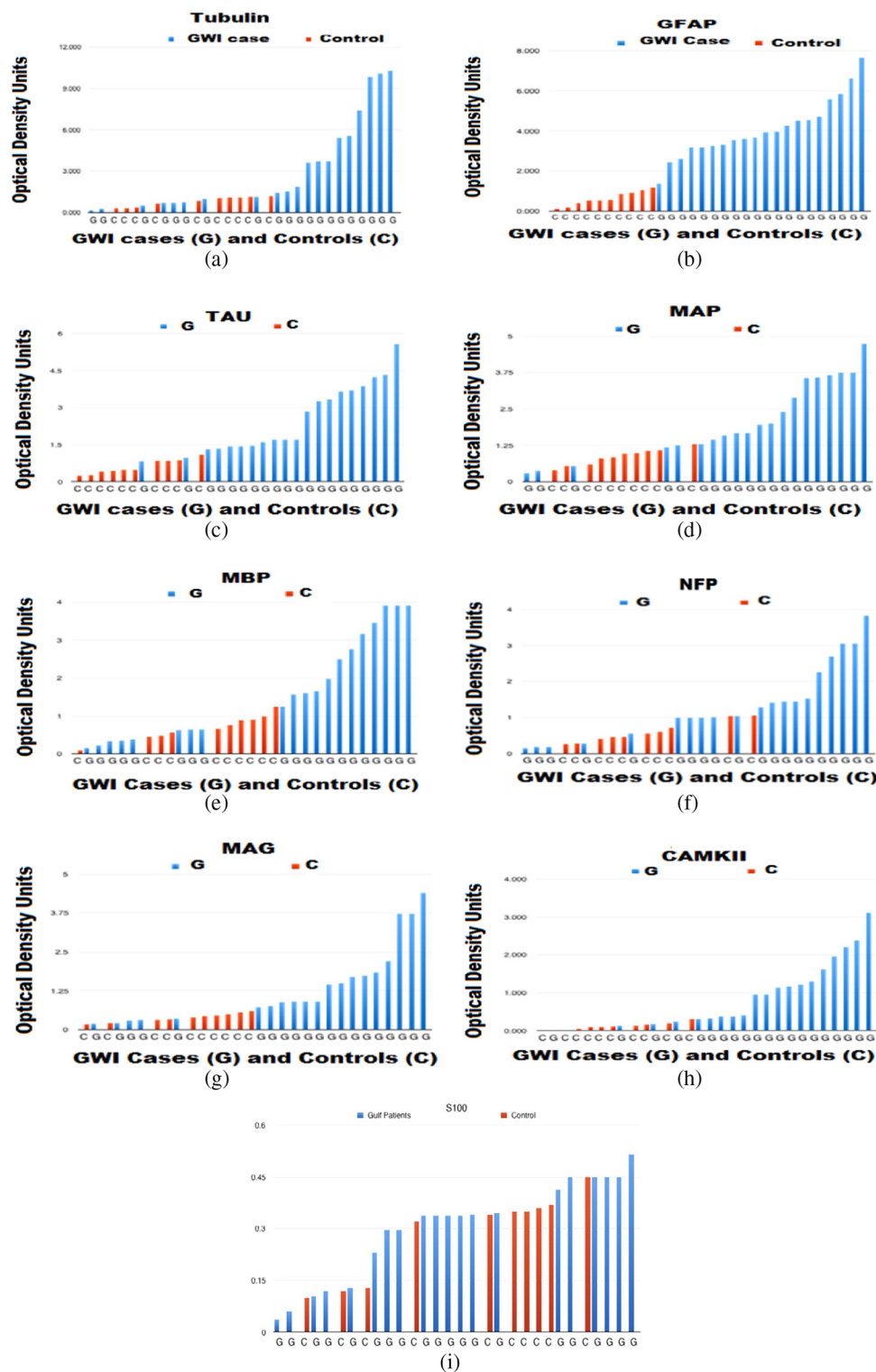


Fig. 6. a) Tubulin levels were higher than all controls in 12/20 cases. b) GFAP levels were higher than all controls in 20/20 cases. c) Tau levels were higher than all controls in 17/20 cases. d) MAP levels were higher than all controls in 15/20 cases. e) MBP levels were higher than all controls in 12/20 cases. f) NFP levels were higher than all controls in 10/20 cases. g) MAG levels were higher than all controls in 15/20 cases. h) CAMKII levels were higher than all controls in 16/20 cases. i) S100B levels overlap with cases and controls.

brain-blood barrier induced by astrocyte alterations. Waste proteins in the brain ultimately reach the liver through a mechanism known as the “glymphatic system” where they are degraded (Nedergaard, 2013). However, the released proteins that could serve as markers of injury are present in the short-term and cannot be used as biomarkers in the case of chronic GWI (Zurek and Fedora, 2011; Diaz-Arrastia et al.,

2014). Thus detection of autoantibodies can serve as surrogate markers for these circulating waste proteins as described in this study.

The highest increase in autoantibodies was against CaMKII which was 9.27 times higher than that of controls followed by GFAP which was 6 times higher than controls. This result is consistent with the veterans' exposure during their deployment to the Gulf War to

organophosphorus compounds such as pesticides, and the nerve agent sarin that have been shown to increase the activity and mRNA expression of CaMKII (Patton et al., 1983, 1985, 1986; Gupta et al., 1998; Barbier et al., 2009) as well as enhanced CaMKII-induced phosphorylation of NFP, tubulin (Serrano et al., 1986) and tau activity leading to the aggregation, deregulation and accumulation of NFP (Abou-Donia et al., 1993; Norgren et al., 2003) and tubulin in the axon (Abou-Donia, 1993; Jensen et al., 1992; Gupta et al., 2000; Grigoryan and Lockridge, 2009). Aggregated neurofilaments result in slowing of axonal transport as has been illustrated in GW-relevant animal and cell neurotoxicant models (Gupta et al., 1997; Reagan et al., 1994; Terry et al., 2012; Gao et al., 2016; Edgar et al., 2004). GW-relevant exposure models have also been associated with astrocyte activation (Zakirova et al., 2015; Ojo et al., 2014).

Neuronal proteins studied in this pilot analysis represented various anatomical regions of the neuron with distinct functions which can be instructive with regard to the pathobiology of GWI (Lapadula and Abou-Donia, 1992). All of the proteins used are involved in axonal structure and function and are released as products of neural degeneration of various regions of the neuron. MAP-2 is present in the dendrites; CaMKII, tau, tubulin, and neurofilament proteins are located in the axon; myelin basic protein (MBP) and myelin associated glycoprotein (MAG) are an integral part of myelin (McMurray, 2000). Furthermore, the central nervous system-specific glial protein, GFAP and S-100B are secreted by astrocytes after neuronal injury (McMurray, 2000). Following axonal and myelin degeneration, neuronal and glial proteins are released and once in circulation, activated lymphocytes, B and T cells lead to the formation of autoantibodies against these proteins (Schwartz and Shechter, 2010a,b).

Increased autoantibodies against nervous system-specific proteins leads to structural consequences in various regions as follows: increased autoantibodies against neurofilaments proteins, tau, CaMKII and tubulin are indicative of axonal degeneration; increased autoantibodies against MAG and/or MBP suggest demyelination, increased autoantibodies against MAP-2 suggest dendritic degeneration, increased autoantibodies against GFAP suggest astrogliosis, and the low or no-increased levels of autoantibodies against S-100B is consistent with chemical-induced brain injury (Zurek and Fedora, 2011; Diaz-Arrastia et al., 2014; Stalnacke et al., 2006, 2004). The linear correlation pattern of tau and MBP in this study suggests an important potential effect of axonal degeneration followed by demyelination that would correspond with prior neuroimaging studies in neurotoxicant exposed GW veterans (Heaton et al., 2007; Chao et al., 2010). Furthermore, these structural changes of the nervous system lead to functional alterations. Hence axonal degeneration in the cerebral cortex leads to: motor and sensory abnormalities, ataxia, deficit in posture, locomotion, and skilled fine motor movements (fingers, speech, facial expression) and weakness; degeneration of the limbic system including the hippocampus leads to: learning and memory deficits, and neurobehavioral (mood, emotion and judgment) abnormalities; increased autoantibodies against MAP-2 suggests damage to the dendrite-rich Purkinje cells in the cerebellum resulting in: gait and coordination abnormalities, staggering gate and ataxia (McMurray, 2000; Abou-Donia, 2015). Increased autoantibodies against GFAP indicate astrogliosis and potential neuroinflammation and/or glial scarring. GFAP contributes to white matter architecture, myelination and blood brain barrier (BBB) integrity (O'Callaghan and Sriram, 2005; Amourette et al., 2009; Lamproglou et al., 2009). Consequently, blood levels of GFAP in healthy individuals are very low. GFAP levels were higher in GWI cases and completely discriminated between the cases and controls in this study. This is particularly relevant because disorders with higher levels of GFAP include memory disorders such as Alzheimer's and vascular dementia that have significant axonal neurodegeneration and neuroinflammation (Mecocci et al., 1995). Increased autoantibodies against S-100B suggest traumatic brain damage and can help to differentiate between acute and chronic brain injury (Stroick et al., 2006; Stalnacke et al., 2006, 2004; Zurek and Fedora,

2011; Diaz-Arrastia et al., 2014; Coch and Leube, 2016). Their lack of increase in this study suggests against acute traumatic brain injury in veterans with GWI.

Important mechanistic clues from animal and cell studies of these GW-relevant neurotoxicants have shown deficits in axonal transport, as well as aberrations in neurofilaments and microtubules, which are the structural railways for axonal transport (Gupta and Abou-Donia, 1995a, b; Gearhart et al., 2007; Grigoryan and Lockridge, 2009; Prendergast et al., 2007; Jiang et al., 2010). Mitochondria are also delivered by axonal transport to provide the energy required to power the biochemical reactions necessary for the functioning of the axon and have shown altered functioning in GW-relevant neurotoxicant models (Middlemore-Risher et al., 2011). GW-relevant chronic low-level organophosphate exposure has also been associated with mitochondrial compromise from oxidative stress induction and with neuroinflammation resulting in cell damage or cell death resulting in debris of waste proteins in the extracellular spaces (Laetz et al., 2009; Kaur et al., 2007; Banks and Lein, 2012). In fact, one hypothesis of GWI suggests that mitochondrial damage and oxidative stress in the brain and the periphery have caused the chronic symptoms of GWI; notably, increased autoantibodies were expressly cited among objective markers and mediators in this model (Golomb et al., 2014; Golomb, 2012; Koslik et al., 2014).

Another hypothesis of GWI suggests that the neurotoxicants acted synergistically to create a self-perpetuating neuroinflammatory state, which in turn has an ongoing negative impact on brain cells including neurons (microtubules, motor proteins, mitochondria) and glia (microglia, astrocytes, oligodendrocytes) and blood-brain barrier function (O'Callaghan and Sriram, 2005). Clinical studies have also found consistent results with GW veteran cohorts who showed impaired cognitive functioning and reduced volume and altered white matter microstructural integrity on MRI in OP pesticide, sarin nerve agent and PB pill exposed cohorts (White et al., 2016; Sullivan et al., 2013; Chao et al., 2010; Heaton et al., 2007; Proctor et al., 2006; Sullivan et al., 2003). These prior results suggest clear CNS alterations in neurotoxicant-exposed GW veterans which correlated with behavioral outcomes that are related to neurodegeneration and perhaps with both a chronic neuroinflammatory and Mitochondrial/OS hypothesis.

The only other study that we are aware of that compared CNS autoantibodies in GW veterans compared MBP and striated and smooth muscle antibodies and reported higher MBP and muscle antibodies in veterans with GWI when compared with controls (Vojdani and Thrasher, 2004). The current study validates the prior MBP findings and expands on those findings with a larger panel of 8 additional CNS autoantibody markers. Collectively, these findings suggest that alterations in white matter as evidenced by circulating autoantibodies to MBP appear to be associated with GWI. This finding corresponds with both leading hypotheses for GWI given that white matter alterations can be associated with oxidative stress and neuroinflammation as a result of glial activation and signaling of both proinflammatory cytokines and oxidative stress (Milligan and Watkins, 2009). The additional finding of this study that higher Tau autoantibody levels were significantly linearly correlated with higher MBP autoantibody levels in GWI cases suggests that axonal degeneration may be occurring before demyelination in veterans with GWI and warrants further more conclusive study to distinguish it from the more myelin-specific toxic leukoencephalopathies (Schmahmann et al., 2008; Filley, 2013). These findings also correspond with MRI findings of differences on both white and gray matter brain volumes in neurotoxicant-exposed GW veterans (Heaton et al., 2007; Chao et al., 2010, 2011, 2014, 2016). These findings also clearly suggest that glia and astrocytes in particular should be further studied in GWI given significantly higher levels of GFAP in the GWI cases that correspond with prior animal models of GWI (Abdel-Rahman et al., 2001, 2002a, 2002b, 2004a, 2004b; Abou-Donia et al., 2000, 2001, 2002, 2004; Zakirova et al., 2015; Ojo et al., 2014) and with recent studies illustrating the ability of astrocytes to donate mitochondria to damaged neurons (Hayakawa et al., 2016).

4.1. Limitations and future directions

This study, like all studies has important limitations. Although the present pilot study can serve as a proof-of-concept it has a small sample size and non-matched subject groups for age, gender and for CNS symptoms. This is particularly important as it has also been shown that CNS autoantibodies have been reported to be age-related in animal models (Lal and Forster, 1988). In addition, the convenience comparison group utilized in this study had musculoskeletal symptoms and not CNS symptoms therefore, it remains to be shown that these CNS autoantibody markers can clearly distinguish between GWI cases and additional groups with CNS specific symptoms. However, the strong results including 9-fold higher levels of CAMKII, 6-fold higher levels of GFAP and 4-fold higher levels of tau and tubulin that were presented in this study warrant further research for a blood-based objective marker of GWI in larger, well-characterized veteran cohorts. These results suggest a possible new avenue for further development of an objective biomarker of GWI. The identification of this small panel of neural-specific autoantibody biomarkers in GWI shows promise for further validation in larger study samples that are more carefully matched for subject demographics (particularly age), different types of control groups (i.e. healthy and CNS symptomatic groups) and that classify cases by both the CDC and the more specific Kansas GWI criteria which also specifies the time period of deployment which may be relevant to particular OP and other deployment-related exposures (Steele, 2000; Fukuda et al., 1998). Future directions will be to compare these CNS autoantibody markers with specific behavioral outcomes including cognitive performance and brain imaging of gray and white matter volume and microstructural integrity to further validate these suspected brain-immune-behavioral outcomes.

5. Conclusions

In conclusion, in this pilot study GWI was significantly associated with 2–9 fold increased serum autoantibodies against 8 neuronal and glial-specific proteins (CaMKII, GFAP, Tau, Tubulin, MAG, MBP, NFP, MAP-2) and not with a marker of more acute damage (S-100B). The autoantibodies that were found here to be elevated in GWI, targeted proteins/ antigens that play critical roles in the structure and function of the neuron including axonal transport and myelination. Many of them are explicit markers for neurodegenerative disorders, consistent with axonal and myelin degeneration of myelinated neurons and with astrogliosis, cell signaling and neuroinflammation. These same proteins have been shown to be affected in other clinical groups and animal models with similar organophosphate and carbamate exposures (Abou-Donia et al., 2013, 2014). These results validate prior reports of increased MBP autoantibodies in GWI cases and suggest that oligodendrocyte signaling, glia and white matter alterations should continue to be further studied in GWI and validated with health symptom and behavioral outcomes (Vojdani and Thrasher, 2004). The results also indicate that veterans with GWI may be continuing to show brain neuronal degeneration and glial activation that would be consistent with recent reports of chronically persistent and in some cases worsening health of these veterans (Smith et al., 2013; Ozakinci et al., 2006; Li et al., 2011; Kang et al., 2009; Dursa et al., 2016; White et al., 2016). These results suggest a possible avenue for further development of a panel of objective biomarkers of GWI upon further validation in larger study samples that are more carefully matched for subject demographics.

Conflict of interest statement

The authors report no relationships that could be construed as a conflict of interest.

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