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

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13. SUPPLEMENTARY NOTES
None

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) An ELISA Assay of Autoantibodies Against Neural Proteins has been developed, 3) Comparative study of Biomarkers for Gulf War Illness (GWI), Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS) has been demonstrated. Only a few samples of cerebrospinal fluid became available for our study. We plan to collect more samples during the no-cost extension year that we requested and was approved. During this year, we plan to prepare several manuscripts and submit them for publication 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 29 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 results at scientific meetings and in peer-reviewed publications.

15. SUBJECT TERMS
Gulf War Illness, autoantibody, GFAP, Tubulin, tau

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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 and ELISA 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. We plan to obtain more samples during the no-cost extension year that was approved for this year. During this year, we plan to prepare several manuscripts and submit them for publication in peer-refereed journals (See details in the report below). 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 29 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, ELISA, 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* ***Actual 2a****: Adapting western blot for plasma samples* ***Actual 2a****: Beginning analysis of all plasma samples from 100 GWI and 50 controls.*		6-8 10-11 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. ***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)*	4-9	 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: CSF samples still being collected at site 1 so not shared yet.		
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 294 samples (168 GWV; 50 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	
Milestone(s) Achieved: Autoantibodies for CNS proteins of myelinogenesis, astrogliogenesis and neurogenesis data analyzed from three biorepository sites.	9-24	294 autoantibodies run for 9 CNS markers. They have now been merged with demographic datasets for further analysis (see below).
Task 4: Perform CSF Assays	Months	
4a: Perform ELISA assays of 50 CSF samples for neurofascin 155 biomarker.	22-24	Not completed yet because CSF still being collected at site 1 and from other studies.

4b: Merge CSF outcome data with clinical neuroimaging, TBI and exposure data.	24-27	
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 still ongoing with clinical datasets and being prepared for publication. 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. Third year plans have been discussed.	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.** Two yearly progress reports submitted on October 2017 and November 2018.	12-24	12, 24
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, 1 paper published to date, two more in preparation. Possible biomarker selection for GWI and recommendations for treatment development.	18-24	18-24

Milestone Achieved: o *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.*		
Milestone Achieved: *Development of ELISA assay for all neural proteins that we assay as biomarkers for nervous system injury*		
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	Analysis are ongoing and preliminary results are presented below.
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	Analyses are ongoing and preliminary results are presented below.
6c: Discuss results of data analyses and prepare abstracts for DOD and other scientific meetings.	25-32	

6d: Preparation of manuscripts 1. ELISA Assay of Autoantibodies Against Neural Proteins (SOT 2019) • *This manuscript discusses the various steps used and their rational in the analysis of autoantibodies to neural proteins.* 2. Comparative study of Biomarkers for Gulf War Illness (GWI), Chronic Fatigue Syndrome (CFS) and Irritable Ball Syndrome (IBS). • *This manuscript emphasizes the various profiles of autoantibodies of cytoskeletal proteins and their use in the diagnosis of veterans with WGI, CFS and IBS.* 3. Alterations in neuronal white matter proteins: MBP, MAG, Neuronal in GWI • *This manuscript discusses the involvement of white matter in the mechanisms of GWI, CPF, and IBS.* 4 Changes in Cytoskeletal Proteins: Neurofilaments, Tubulin, Microtubule Associated Proteins, and Calcium Calmodulin Kinase II in the GWI. • *This manuscript discusses the involvement of* Cytoskeletal Proteins *in the mechanisms of GWI, CFS and IBS.*		
5. Involvement of Glial Proteins, GFAP and S100B in GWI-(SOT-2018) • *This manuscript discusses the involvement of Glial* Proteins *in the mechanisms of GWI, CFS, and IBS.*	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 of Gulf War. 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 Gulf War with Irritable Bowel Syndrome (IBS) exhibited neural autoantibodies profile that is distinct from both GWI and CFS. 4. Developing of an ELISA assay for autoantibodies and validating it using Western blot method. 5. Analysis of all study results, presentation of results at scientific meetings, submitted publication and final report in progress. 6. 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 1990/1991 Gulf War were sent to Dr. Kimberly Sullivan at Boston University for statistical analysis.	36-48	Performing final data analyses and obtaining CSF samples for analysis is ongoing this year.
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 diagnose of GWI and other related condition of the veterans of the 1990/1991 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 between the sexes 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 5. Veterans with Post traumatic Stress Disorder (PTSD)	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 been discussed.		

*** 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 nearly 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).
  - *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.
  - *Other achievements.* Currently, we have adapted our autoantibodies assays against neural proteins to ELISA assays. This will give another dimension in assaying large number of samples to these biomarkers. Furthermore, the development of ELISA assay for autoantibodies will allow faster performance of assays and provide more quantitative results that are easily reproducible.
  - 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 patients 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 groups if this finding is replicated in the larger study sample.

- **Development of ELISA Method to detect neural autoantibodies**
  Multiplex Enzyme linked Immunosorbent assay (ELISA) was performed by coating wells of Nunc Immuo-plates, 96-well, microtiter plates (ThermoScientific, Rochester, NY) with 100-500 ng/well of purified, human recombinant proteins expressing specific neural proteins viz., MAPT, S100B, NSE, GFAP, Tubulin, MAG, NEF, CaMkIIα, CaMkIIδ and MAP-2. The optimal dilution of the recombinant protein, sera (serum obtained from GWV) and secondary detection antibody was determined by a checkerboard titration that gave the highest signal to noise ratio to determine the optimum concentration of protein, sera and secondary antibody. In addition, a single lot of antibody directed against these proteins were used to generate quality control standards that gave high and low optical density (high OD = 2.0 to 2.5; low OD = 0.5 to 1.0; and negative OD < 0.2). Briefly, the assay was performed by diluting the recombinant proteins in 15 mM sodium carbonate-35 mM sodium bicarbonate- antigen coating buffer (ACB) pH 9.6. The plates were incubated overnight at 4°C, then washed 3 times (3x) with PBS plus 0.05 % $tween_{20}$ (PBST). Each well was then blocked with 200 µl of sample buffer (SB) (PBST plus 5 % BSA) and allowed to incubate at room temperature for 3 hrs. Test and control sera were diluted at 1:200 in SB, thorough mixed and 100µl was added to each well, left to incubate/bind at 4°C overnight on a plate shaker. The unbound sera in the wells were washed 3x and secondary antibody (HRP conjugated anti-human IgG) was added at a concentration of 200ng/ml (Jackson laboratory, MA) diluted in SB and incubated at 20-22°C for 1 hour. The plate was then washed 5x and developed with 3,3′,5,5′- tetramethylbenzidine, peroxidase substrate (TMB – Invitrogen and incubated in dark until the positive control attained a standard OD. The reaction was stopped using 2N H_2SO_4. Colorimetric development was quantified spectrophotometrically at 450 nm with a Clariostar (BMG plate reader, Germany) using BMG software powered by Matlab programming. The raw data were normalized by subtracting the blank values. Sample to Positive (S/P) ratios were calculated using the following formula: S/P = optical density (OD) of sample - OD of buffer/OD of positive control - OD of buffer. Total IgG levels were determined by quantitating IgG. We also used transferrin as a serum control. All the individual values were normalized to total IgG and transferrin. Final statistical analysis was performed using Matlab, SAS and by R.

The results are from 3 GWI veterans (p1-p3) and a healthy GW veteran (c1-c3)

- **Western blot assay of Autoantibodies against Neural Proteins**

a. Materials and Methods
   Materials
   The sources of standard proteins were the same as previously published (Abou-Donia et al., 2013, 2017).

b. Case and control Samples

Serum samples from 20 GWI cases with GWI and 10 controls were tested in this experiment.

c. Western Blot Assay

To screen for the presence of autoantibodies against a battery of proteins in plasma samples, we applied a Western blot approach as previously reported (Abou-Donia et al., 2013, 2017). Each serum sample was analyzed in triplicate. Each protein was loaded as 10-100 ng/lane except for IgG that was loaded as100 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 plasma 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 membranes were developed by enhanced chemiluminescence using the manufacturer's (Amersham Pharmacia Biotech Cat. No. 34096) 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.

Results

a. Our preliminary results analyzing the current plasma samples are consistent and confirm the results of our preliminary studies using serum samples.
b. ELISA assay using the same samples are being analyzed and are showing promising results for future use.

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 that we generated in our study to diagnose of GWI and other related conditions of the veterans of the 1990/1991 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 across sexes
3. Analysis by Race for GWI Cases Only
4. Differences between GWI Veterans and All Controls Across Race

The following analyses were carried out:

1. GWI Autoantibody Data: Analysis by sex for GWI Cases Only

Sample Source:

Task A. Gulf War Subjects and Controls

A total of 322 subjects and controls were investigated for autoantibodies of brain injury in order to develop a diagnostic system for the GWI and related diseases, i.e., CFS and IBS. GWI

Subjects and controls that were studied, were obtained from Boston GWIC Nova Southeastern University and BIDMC listed in Table 1. The controls included GWI controls, Irritable Bowel Syndrome (IBS), and Chronic Fatigue Syndrome (CFS).

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) Range (Years)	49 (8) 24-80	51 (7) 38-72	39 (14) 22-73	47 (10) 20-63
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 %)

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

Task B
GWI Autoantibody Data: Differences between GWI veterans and all controls across sexes Proteins

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.

Task C.
GWI 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 ±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 Subjects Exposed	GWI Subjects Not Exposed	Comparing those exposed vs. not using ANCOVA adjusting for age, gender, and race (GWI cases only)
N		83	91	
A. Neuronal Proteins				

1. Neurofilament Triplet Proteins (NFP)	Mean ±SD Range	3.67 ± 2.16 1.09 -13.82	3.19 ±2.94 0.30 – 15.15	0.5665
2. Tubulin	Mean ±SD Range	4.45 ± 2.90 0.97 -22.80	3.84 ±3.57 0.32 – 19.81	0.5652
3. Tau	Mean ±SD Range	2.90 ± 1.63 1.07-8.87	9.22 ±5.37 0.33 – 10.55	0.6342
4. MAP-2	Mean ±SD Range	10.16 ± 4.60 1.66 -23.00	1.88 ±2.02 0.88 – 27.42	0.6119
5. Calcium/Calmodulin Kinase 2 (CaMK2)	Mean ±SD Range	1.92 ± 0.88 0.66-3.95	2.14 ±1.30 0.10 – 82.56	0.07512
6. Alpha Synuclein (SNCA)	Mean ±SD Range	2.26 ± 1.16 0.53 -7.74	1.88 ±2.02 0.16 – 9.74	0.204
B. Glial Proteins: Oligodendrocytes:				
1. Myelin Basic Protein (MBP)	Mean ±SD Range	3.86 ± 1.87 1,05 – 13.76	4,75 ±2.02 0.16 – 9.74	0.6119
2. Myelin Associated Glycoprotein (MAG)	Mean ±SD Range	4.18 ± 2.09 1,42 -15.15	5.64 ±2.02 0.24 – 29,95	0. 0006
Glial Proteins: Astrocytes:				
3. Glial Fibrillary Associated Protein (GFAP)	Mean ±SD Range	4.63 ± 2.25 2.04 -13.14	3.95 ±2.95 0.39– 12.48	0.1119
4. Fold Change Glial S100B (S100B)	Mean ±SD Range	1.50 ± 0.40 0.58 -2.41	0.87 ±0.37 0.16– 1.96	< .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."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

- 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.
  5. The Annual SOT, March 2017, Baltimore, MD: A poster was presented (see Appendix).
  4. The Annual SOT, March 2018 in San Antonio, Texas: Two posters were presented (see Appendix)
  5. The annual SOT meeting, March 2019 in Baltimore, MD a poster was presented.

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

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) 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.

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. We plan to present our results that were generated from this study during the annual society of toxicology meeting that will be held in Anaheim, California from March 15-19, 2020.
2. Currently we are preparing three manuscripts to report the results of our studies following finishing statistical analysis of the data.

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."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

- 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 ultimately find, this will provide the opportunity to develop drugs that treat neuronal injury in those specific pathways
    - (neuronal, glial etc); such treatment could be directly applicable to Gulf War veterans in the short-term.
    - Our first publication provides the CNS autoantibodies that we will attempt to validate in our larger study sample and target for treatment development planning.

What was the impact on other disciplines?

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. 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 will report further in a publication.
    - 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 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 brain 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.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/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. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

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 is still obtaining them but there should not be much longer delay before they are ready for sharing.

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

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

- **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**

  Abou-Donia MB, Conboy LA, Kokkotou E, Jacobson E, Elmasry EM, Elkafrawy P, Neely M, Bass CR, Sullivan K. 2017. Screening for novel central nervous system biomarkers in veterans with Gulf War Illness .Neurotoxicol Teratol.61:36-46. See attachment 1.

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.

- **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. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

7. 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."

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*

Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award).*

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

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

Blood Serum and CSF Biorepository Sites

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

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

Study Sites Responsibilities

Site 1: Dr. Sullivan and her BUSPH team will be responsible for providing the serum blood and cerebrospinal fluid samples from GWIC study participants who have agreed to share their specimens with the GWIC biorepository to be used in future studies including the proposed study. Specifically, she will oversee the recruitment and blood draws/lumbar punctures of study participants from the GWIC study and the processing of serum and CSF samples that will be shared for the proposed study. Dr. Sullivan will also assist with the experimental design, data analysis, interpretation and presentation of study results in collaboration with Dr. Abou Donia and the other study investigators. **Tasks 1-6**

Site 2: Dr. Abou-Donia will be responsible for receiving the serum and plasma samples from all sites and performing autoantibody analyses using western blot/ELISA analyses for 450 serum samples (250 GWI, 200 controls). He will also assist with the experimental design, interpretation of data, report and manuscript writing and presentation of results at scientific meetings. **Tasks 2, 3, 5, 6**

Site 3: Dr. Fields will be responsible for receiving the serum and CSF samples from all study sites and performing ELISA assays for 450 serum/plasma samples and 50 CSF samples. He will assist with the experimental design, interpretation of data, report and manuscript writing and presentation of results at scientific meetings. **Tasks 2-6**

Sites 4 and 5: Drs. Klimas, Conboy and Kokkoutu will provide serum samples from their respective biorepositories for study analyses, will assist with interpretation of data, report and manuscript writing.

2. Special Reporting Requirements: None

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

No Change has taken place.

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

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
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)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

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

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

Blood Serum and CSF Biorepository Sites

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Bldg. 35, Room 2A211, MSC 3713
35 Lincoln Drive
Bethesda, MD 20892

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

Study Sites Responsibilities

Site 1: Dr. Sullivan and her BUSPH team will be responsible for providing the serum blood and cerebrospinal fluid samples from GWIC study participants who have agreed to share their specimens with the GWIC biorepository to be used in future studies including the proposed study. Specifically, she will oversee the recruitment and blood draws/lumbar punctures of study participants from the GWIC study and the processing of serum and CSF samples that will be shared for the proposed study. Dr. Sullivan will also assist with the experimental design, data analysis, interpretation and presentation of study results in collaboration with Dr. Abou Donia and the other study investigators. **Tasks 1-6**

Site 2: Dr. Abou-Donia will be responsible for receiving the serum and plasma samples from all sites and performing autoantibody analyses using western blot/ELISA analyses for 450 serum samples (250 GWI, 200 controls). He will also assist with the experimental design, interpretation of data, report and manuscript writing and presentation of results at scientific meetings. **Tasks 2, 3, 5, 6**

Site 3: Dr. Fields will be responsible for receiving the serum and CSF samples from all study sites and performing ELISA assays for 450 serum/plasma samples and 50 CSF samples. He will assist with the experimental design, interpretation of data, report and manuscript writing and presentation of results at scientific meetings. **Tasks 2-6**

Sites 4 and 5: Drs. Klimas, Conboy and Kokkoutu will provide serum samples from their respective biorepositories for study analyses, will assist with interpretation of data, report and manuscript writing.

8. 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.

9. 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: The following paper has been published, Abou-Donia et al., 2017, entitled: ". Abou-Donia MB, Conboy LA, Kokkotou E, Jacobson E, Elmasry EM, Elkafrawy P, Neely M, Bass CR', Sullivan K. (2017). Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. Neurotoxicol Teratol.;61:36-46"

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Appendix 2:

The following presentation will be presented at the Annual Meeting of the Society of Toxicology at Anaheim, California on March 15– 19, 2020.

Gulf War Illness A Potential Autoimmune Disease: Neurodegeneration-Induced Autoantibodies against Neural Proteins.

M B. Abou-Donia[1], M.V. Brahmajothi[1], L A. Conboy[2], E. Kokkotou[2] Eric Jacobson[3], Nancy Klimas[3] and Kimberly Sullivan [4]. [1] Duke University Medical Center, Durham, NC, [2]Harvard Medical School, Boston, MA, [3]Nova Southeastern University, [4]Boston University School of Public Health, Boston, MA

In the present study, we used western blot assay to screen plasma for the presence of autoantibodies (AA) against the following neural 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), calcium-calmodulin kinase II (CaM-KII) and glial S100B protein. Plasma reactivity was measured as arbitrary chemiluminescence units. The study included 180 GWI and 52 non-veteran asymptomatic served as controls. None of the patients showed any significant change in the level of AA against S100B. GWI patients showed increased AA in descending order: MAP-2 > MBP > NFP > Tubulin > Tau > MAG > SNCL > GFAP > CaMKII. We have demonstrated that GW veterans with GWI had consistent pattern of increased autoantibodies against neural proteins. Although these autoantibodies may be markers of disease and may also be a contributor to pathogenesis of GWI i.e, they may be the cause for the symptom. If the autoantibody is the cause of the disease and not merely an epiphenomenon, then the targeted antigen should be present in a functional pathway that is relevant to the disease. Such autoantibodies can cross the damaged BBB into brain resulting brain alterations characteristic of GWI. Our conclusion is sonsistent with recent studies (Israeli, Lubus:21, 190-194, 2010; Apostolos et al., 2017). We hypothesize that neurodegeneration, resulting from combined chemicals of veterans of the 1990/1991 Gulf War, is the cause of GWI. Following brain injury neural proteins cross the breached BBB into circulation, triggering the formation of IgG autoantibodies. Subsequently, these autoantibodies enter the brain causing GWI. We propose these circulating autoantibodies as a potential target for treatment of GWI or could lessen the severity, and as biomarkers for screening, diagnosis and treatment of GWI.

(Supported in part by DOD Contract No. W81XWH-15-1-0641).

Appendix 3:The following presentation was presented at the Annual Meeting of the Society of Toxicology at Baltimore, Maryland on March 10– 15 2019.

Increased Autoantibodies to Glial fibrillary Acidic Protein (GFAP) in Plasma of Veterans with Gulf War Illness (GWI).

Abou-Donia MB[1], Barmouth MV[1], and Sullivan K[2].

[1] Duke University Medical Center, Durham, NC, [2]Boston School of Public Health, Boston, MA,

Following the 1991-1992 Gulf War, approximately one third of the U.S. troops out of 700,000, complained of chronic symptoms, which is now considered as Gulf War Illness (GWI). This illness is characterized by multisymptomatic disorder that consists of joint pain, gastrointestinal problems, memory and difficulty concentrating. In this study, we identified and determined the levels of autoantibodies against two main glial proteins. One being glial fibrillary acidic protein (GFAP), which is an astrocytic protein that mainly contributes to white matter architecture, myelination, maintains mechanical strength of astrocytes and provides integrity to the blood brain barrier. The other one is S100B protein, which is expressed primarily in astrocytes, known to interact with and stabilize proteins associated with astrogenesis, thus promoting the maturation of the brain. Many studies have reported increase in S100B during the acute phase of the brain damage. Initially has been hypothesized that the serum concentration of S100B may also be a predictive value of brain injury however, because of its short half-life of two hours in the serum, it's use in characterizing is limited to a short-term estimation and is usually detected at the onset or at the acute phase of the injury. We determined the level of autoantibodies specific to GFAP and S100B using a western blot assay in the plasma of veterans with GWI (n=68) compared to the non-veteran asymptomatic controls (n=26). We observed increased level of autoantibodies specific to GFAP protein in the plasma of veterans with GWI compared to controls. The increase was nearly nine-fold compared to healthy controls for GFAP and the significance was estimated by the Fischer's exact test to be $p<0.0001$, but the autoantibodies specific to S100B showed insignificant increase compared to the healthy controls consistent with the chronic condition of the GWI. Because of the long-lasting nature of IgG, detection of anti-GFAP autoantibodies provides a blood marker for brain injury characteristic of GWI. autoantibodies Conclusively,

autoantibodies to GFAP may signify a chronic CNS damage. Because of the greater anti-GFAP immu(Supported in part by DOD Contract No. W81XWH-15-1-0641).

Appendix 4:

Abstract: Annual Society of Toxicology Meeting, San Antonio, TX, March 2018

Biomarkers for Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS) compared to Gulf War Illness in Veterans of Gulf War (GWI).

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Gulf war illness is primarily diagnosed by symptom report and no clear objective diagnostic biomarkers currently exist. 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. In the present study we screened the sera of patients with GWI for the presence of such autoantibodies. A total of 20 patients with GWI and 10 non-veteran symptomatic controls were investigated. The presence of autoantibodies was evaluated by 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 kinaseII (CaM-KII) and glial S100B 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 S100B. The percentage of autoantibodies against neural proteins of the subjects compared to controls in descending order are: CaMKII 927, GFAP 660, Tau 483, Tubulin 441, MAG 360, MBP 250, NFP 245, MAP-2 230, S-100B 1.03. This pilot study is the first to demonstrate the presence of serum autoantibodies to central nervous system-specific proteins in veterans with GWI. These results confirm at least a prior history of neuronal injury/gliosis in these veterans and add to 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 circulating autoantibodies may be used as biomarkers for the diagnosis of GWI, upon validation of the findings of the present study using larger cohorts *(*Supported in part by DOD Contract No. W81XWH-15-1-0641).

Key Words: Biomarkers, Chronic Fatigue Syndrome (CFS), Irritable Bowel Syndrome (IBS), Gulf War Illness

Appendix 5:

Abstract: Annual Society of Toxicology Meeting, San Antonio, TX, March 2018

Quantitative measurement of neuronal autoantibodies by ELISA in the sera of patients with Gulf War Illness (GWI).

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For Gulf War Veterans (GWV), the effects of war continued long after they returned home. In addition to the psychological ramifications, veterans and civilian workers showed exacerbated effects of medically unexplained chronic multi-system disorders. The causality of illness may be due to the compounds they were exposed to, which inhibit acetylcholine (AChE) or modulate the pharmacokinetics of substances that control the metabolic activation or breakdown of AChE inhibitors. Such compounds include chlorpyrifos, sarin, cyclosarin, sulfur mustard, pyridostigmine bromide, DEET, opiates as therapeutics and the enzymes responsible for drug metabolism such as cytochrome P450 reductases, liver microsomal oxidases, etc. Inaccessibility of the nervous system has impeded the evaluation of cellular and molecular changes that result in neurodegeneration. Discharges of neural proteins during this process can induce autoimmune response that can be measured in the serum. When we screened for novel nervous system biomarkers in the sera of GWVs from our pilot study, we found 2 to 9-fold increase of autoantibodies to the neuronal specific proteins. We have now developed ELISA to determine and quantitate serum autoantibodies against microtubule associated proteins (MAP2), microtubule associated protein tau (Tau), tubulin and glial fibrillary acidic protein (GFAP). This method quantitatively distinguishes IgG levels of the autoantibody titers at 0.1 microgram level of the specific neuronal proteins. Determination of specificity was achieved by absorption studies to estimate the threshold level (Supported in part by DOD Contract No. W81XWH-15-1-0641).

Appendix 6:

Abstract: Annual Society of Toxicology Meeting, Baltimore, MD, March, 2017

Neural Autoantibodies in Veterans with Gulf War Illness. M.B. Abou-Donia[1], K. Sullivan[2.] L. Conboy [3], E. Kokkotou.[4]

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A number of studies have linked exposures of chemical and biological toxins to increased risk of auto-immunity and elevated levels of antibodies to neural antigens. Currently, autoimmune diseases affect 5-7% of the world's population that are usually accompanied by circulating autoantibodies. Neural autoantibodies are also present in approximately 2-3% worldwide, but dare not involved in brain pathology. This current study measured levels of 8 types of neural autoantibodies in a group of 20 Gulf War patients with Gulf War

Illness (GWI). The patients were exposed to a variety of toxicants including insecticides, insect repellant, pyridostigmine bromide, a prophylaxis for nerve agents and the nerve agent sarin. The results indicate that neural markers were significantly elevated in peripheral blood samples of GWI cases. Of interest, the highest fold increase (8 times that of controls) was found in GFAP, a known marker of astrocyte activation. Other markers of axonal transport damage were also significantly increased in GWI cases including MAP, Tau, tubulin and NFP. Myelin basic protein, a marker of oligodendrocytes, was also increased. These results strongly corroborate with the GWI hypotheses that neuroinflammation in GWI potentially results in white matter and axonal transport damage. Autoantibodies directed against self-antigens can also cause local activation of complement. *B cells play a key role in autoimmunity, B cell lineage may contribute to the development either as antigen presenting cells or cytokine secreting cells or autoantibody producing cells. Immunotherapies that deplete B cells may be an effective strategy to combat autoantibodies.* (Supported in part by DOD Contract No. W81XWH-15-1-0641).

Appendix 7

A. Annual Society of Toxicology Meeting, New Orleans, LA, March, 2016

A Pilot Study of Novel Brain Neurodegenerative Biomarkers in Veterans with Gulf War Illness.

MB. Abou-Donia[1], K Sullivan [2], L A. Conboy[3], and E Kokkotou[4]

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Upon their return from the 1990-1991 Gulf War (GW), hundreds of thousands of American military personnel complained of symptoms with unknown etiology, known as the Gulf War Illness (GWI). The hallmark of GWI is neural degeneration that is consistent with symptoms related to nervous system injury and confirmed by experimental studies. A major problem in identifying veterans with GWI is the difficulty in its diagnosis. This report presents the results of a pilot, investigative and descriptive study of assays performed to detect circulating autoantibodies to a panel of nine proteins associated with the nervous system in sera of a group of 20 veterans of the Gulf War who reported having symptoms War Illness (GWI) and 10 symptomatic controls who did not have GWI. Various types of proteins present in axons, dendrites and myelin sheath that are affected by neuronal degeneration were used. In sera samples from the GWI subjects and symptomatic non-veteran controls using Western blotting, immunoglobin IgGs were measured against: 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 -2 (CAM-2) and glial S100B protein. Also, α-synuclein, a marker for Parkinson' disease was included. The results show significantly elevated levels of circulating Ig-G-class autoantibodies in the veterans with GWI, compared to controls. This preliminary study demonstrates a relationship between clinical condition, and the level of serum autoantibodies to nervous system-specific proteins. These results showing the development of neuronal injury and gliosis in the subjects are consistent with recent reports indicating 20 years after the Gulf War, the health of the- veterans who developed GWI is getting worse. It is

concluded that that these serum circulating autoantibodies may be used as biomarkers for confirming GWI upon further validation. (Supported in part by DOD Contracts No. W81XWH-15-1-0641 and W81XWH-15-1-0640).

Abstract 8

International Neuropsychological Society (INS) annual mid-year meeting, July 2016.

London Abstract: A Pilot Study of Novel Brain Neurodegenerative Biomarkers in Veterans with Gulf War Illness: M. B. Abou-Donia[1], K. Sullivan [2], L. A. Conboy[3], E. Kokkotou[4,] and E. M, El-Masry[5] Duke University Medical Center, Durham, NC, [2] Boston University School of Public Health, Boston, MA, [3]Harvard Medical School, Boston, MA, [4] Harvard Medical School. Boston, MA ; [5] Zagazig University, Zagazig, Egypt

Objective: To determine circulating autoantibodies in ten proteins associated with nervous system in sera of a group of 20 veterans of the Gulf War who reported having symptoms of and 10 symptomatic non-veteran controls with lower back pain. 2. Participants and Methods: Subjects and controls were obtained from the Beth Israel Deaconess Medical Center and Harvard Medical School bio-repository. Control serum samples came from a separate study of non-veteran patients with chronic lower back pain who served as 'symptomatic' controls from one of the authors (EK). The proteins were separated using Western blot assay. 3. Results: Mean levels of autoantibodies in the subjects compared to controls were in descending order: CaMKII (9.27) > GFAP (6.60) > Tau (4.83) > Tubulin (4.41) > MAG (3.60) > MAP-2 (2.53) > MBP (2.50) > NFP (2.45) > S100B (1.03). 4. Conclusions: This study demonstrates a relationship between clinical condition, and the level of serum autoantibodies to nervous system-specific proteins. It is concluded that that these serum circulating autoantibodies may be used as biomarkers for confirming GWI when validated in larger samples. (Supported in part by DOD Contracts No. W81XWH-15-1-0641 and W81XWH-15-1-0640).