AWARD NUMBER: W81XWH-15-1-0437

TITLE: Biomarkers and Brain Mechanisms of Gulf War Illness

PRINCIPAL INVESTIGATOR: Dikoma C. Shungu, Ph.D.

CONTRACTING ORGANIZATION: Weill Cornell Medicine

REPORT DATE: DECEMBER 2022

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
DECEMBER 2022	Final	1SEPT2015 - 31AUG2022

4. TITLE AND SUBTITLE

Biomarkers and Brain Mechanisms of Gulf War Illness

5a. CONTRACT NUMBER
W81XWH-15-1-0437

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dikoma C. Shungu, Ph.D.

E-Mail : dcs7001@med.cornell.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Weill Cornell Medicine
1300 York Ave
NEW YORK NY 10065-4805

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: [**no new findings; original abstract provided**] Gulf War illness (GWI), a chronic and debilitating pain, headaches, impaired memory and thinking, fatigue, respiratory and gastrointestinal symptoms, and skin abnormalities. Exposure and sensitivity to chemical, pharmaceutical and/or environmental toxins in a combat theater of operations is believed to be causative of the illness. The pathobiological mechanisms of GWI are unknown; there are no validated diagnostic tests, nor are there effective treatments or cures. This is a case-control study consisting of 20 Gulf War veterans affected with GWI and 20 matched non-affected Gulf War veterans, who will serve as the normal control group. All subjects will undergo brain positron emission tomography and magnetic resonance imaging scans for assessments of metabolic or neurochemical disturbances that may be associated with GWI. In all consenting participants, a lumbar puncture will be performed to obtain cerebrospinal fluid (CSF), which will be analyzed for abnormalities in biochemical compounds that may be related to GWI. The derived neuroimaging and CSF metabolic or biochemical data will be compared between the groups to determine if there are abnormal changes in GWI veterans compared to controls, which may shed new light onto the pathophysiology of GWI, as well as serve as biomarkers of the disorder.

15. SUBJECT TERMS
Gulf War illness, neuroinflammation, oxidative stress, mitochondrial dysfunction, magnetic resonance imaging (MRI), Positron Emission Tomography (PET)

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
U	U	U	U	**UU**	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION:

The overall objective of this study was to evaluate the suitability of a number of endogenous chemical compounds or metabolites to serve as sensitive central (brain imaging) and peripheral or circulating (cerebrospinal fluid, plasma, urine) biomarkers of pathologic alterations in Gulf War illness (GWI) for use to facilitate early diagnosis, to assess disease progression and/or to monitor therapeutic response in future clinical trials of promising interventions. As originally proposed, this was to be a case-control study designed to enroll 20 Gulf War veterans affected with GWI and 20 matched non-affected Gulf War veterans, who would serve as the normal comparison group. The enrolled participants were to undergo magnetic resonance spectroscopy (MRS) and brain positron emission tomography (PET) scans to assess neurometabolic or neurochemical abnormalities that may be associated with or are specific to GWI. In all consenting participants, a lumbar puncture was proposed to obtain CSF samples, which were to be analyzed for metabolic abnormalities that may be associated with or are specific to GWI. In addition, blood and urine samples were obtained from all participants for assessment of peripheral abnormalities that may be associated with or are specific to GWI. The derived neuroimaging and body fluid biochemical data were to be compared between the groups to determine whether there are abnormal changes in GWI veterans compared to non-affected Gulf War veterans that may shed new light onto the pathophysiology and serve as biomarkers of the disorder.

1. KEYWORDS:

Gulf War illness (GWI); neuroinflammation; oxidative stress; glutathione (GSH); mitochondrial dysfunction; lactate; magnetic resonance imaging (MRI); magnetic resonance spectroscopy (MRS); positron emission tomography (PET).

2. ACCOMPLISHMENTS:

What were the major goals of the project?

Table 1 lists the major goals of the project as proposed in the originally approved Statement of Work (SOW), bur modified as follows to indicate changes that did not alter the major goals or direction of the project, but were significant in that they necessitated adjustments to the project that resulted in modifications of some items listed in the approved SOW:

A. Milestones/Target Dates for Major Activities or Phases of the Project:
Due to great difficulties in recruiting Gulf War veterans to participate in the project, we were not able to meet the originally proposed enrollments target of 20 Gulf War Veterans with GWI and 20 healthy Gulf War veterans during the initial funding period of the project. As a result, we requested three consecutive no-cost extension periods, which were approved by USAMRAA, to enable us to try to meet or approach the proposed enrollment targets,. The end of the third and final approved no-cost extension period was 08/31/2021, which also marked the end of the **active phase** of the project. Therefore, the Timeline of the milestones/target dates for the important activities of the project listed in the following Table is no longer applicable, and all the dates should be replaced with 08/31/2021, the actual end date of the active phase of the project.

B. Performance Sites:

1. New Jersey War Related Illness & Injury Study Center [NJ WRIISC]: after the project was approved for funding, but prior to the start of research activities, Dr. Drew Helmer, at the time Project Lead at NJ WRIISC, who was to assume the primary responsibility for recruiting Gulf War veterans for this project, informed us that at the request of NJ WRIISC, his site would withdraw from participation. As a result, the project was adjusted to make the Mount Sinai School of Medicine (MSSM) site, under the leadership of Dr. Benjamin Natelson, the primary site for the recruitment of Gulf War veterans, with and without GWI.

2. Mount Sinai School of Medicine: In December 2020, the collaborating research team at Mount Sinai School of Medicine, led by Dr. Benjamin Natelson, withdraw from participation in the third USAMRAA-approved no-cost extension period due to insufficient funds to continue supporting that site's investigators' efforts. Because the MSSM team had assumed the responsibility for recruiting participants into the study, the team of the Project PI, Dr. Shungu, at Weill Cornell Medicine (WCM) took over the responsibility to recruit participants for the study – a task that was facilitated by the fact that only 10 healthy Gulf War veterans, requiring no specialized expertise to screen for eligibility remained to enroll.

C. Modified Target Enrollments:

Due to continued challenge to recruit Gulf War veterans into the study, we proposed in June 2020 and obtained USAMRAA approval to revise the total enrollment target of the study from 40 subjects to 30 subjects, consisting of 15 veterans with GWI and 15 healthy Gulf War veterans. To assist with recruitment, we engaged s commercial company, WeHealth, which made a big difference.

Table 1: List the Major Goals of the Project, Modified from the Original SOW as Described

RESEARCH-SPECIFIC TASKS:		
For All Specific Aims: Recruitment & Regulatory Approvals	**Timeline**	**Site(s)**
Major Task 1: GWI and Non-GWI Subject Recruitment	Months	ALL
Subtask 1: Establish formal contact between Mount Sinai School of Medicine (MSSM) and the New Jersey War Related Illness & Injury Study Center [NJ WRIISC] to discuss strategy for recruiting GWI and non-GWI veterans for the study.	1	Dr. Natelson & Dr. Helmer
Subtask 2: Develop complementary or cooperative IRB protocols, including study advertisement material that would enable seamless recruitment/characterization of subjects at NJ WRIISC/MSBI and referral to Weill Cornell Medicine [WCM].	1-3	Drs. Natelson, Helmer & Shungu
Subtask 3: Submit IRB protocols at each participating Institution. **Second-tier DoD human subjects regulatory review and approval conducted by the Office of Research Protections, Human Research Protections Office (HRPO)**.	**3-6**	Drs. Natelson, Helmer & Shungu;

		HRPO
Milestone(s) Achieved: All IRB protocols approved; recruitment starts in earnest by month 6 and will continue to end of project	6-30	Drs. Natelson, Helmer
Specific Aim 1: Neuroimaging Biomarkers Studies		
Major Task 2: Conduct *in vivo* brain ^{11}C-(R)-PK11195 PET to assess **neuroinflammation**		WCM
Subtask 1: Order supply for producing the radioligand and review chemistry and PET scanning protocol.	1-6	Dr. Babich
Subtask 2: Conduct PET scans in 10 GWI and 10 non-GWI veterans	6-30	Dr. Babich
Milestone(s) Achieved: Availability of radioligand on demand to end of study; clear ability to obtain good PET scans, reproducibly, in each subject using the PK11195 PET technique	30	
Major Task 3: To conduct ^{1}H and ^{31}P MRS studies for assessment of **oxidative stress** and **mitochondrial dysfunction** *in vivo*. Assess cerebral blood flow using ASL-MRI.		WCM
Subtask 1: Protocols for achievement of this Major Task are already fully developed and being used in an ongoing study in chronic fatigue syndrome that is identical to the one we are proposing in GWI. The protocol will be reviewed with the MR neuroimaging team to ensure its flawless implementation.	1-6	Dr. Shungu
Subtask 2: Conduct ^{1}H and ^{31}P MRS and scan in 20 GWI and 20 non-GWI veterans to assess oxidative stress and mitochondrial dysfunction; also measure CBF in all 40 subjects using ASL-MRI.	6-30	Dr. Shungu
Milestone(s) Achieved: Clear ability to obtain high-quality ^{1}H and ^{31}P MR spectra, as well as ASL-MRI CBF maps in each enrolled subject.	30	
Specific Aim 2: CSF Biomarkers		
Major Task 4: Collect CSF samples from all consenting subjects for validation of neuroimaging biomarkers.		WCM
Subtask 1: Collect and cryo-freeze CSF samples using lumbar puncture	6-30	Dr. Mangat
*Milestone(s) Achieved: Clear ability to collect and freeze CSF samples for later analyses to determine markers of oxidative stress and neuroinflammation (cytokines, **including IL-17**).*	30	

For Specific Aims 1 & 2: Data Analysis and Hypothesis Testing		
Major Task 5: **Data Analysis, Reduction, Statistical Analyses**.		WCM
Subtask 1: Analyze/process and reduce the data from all the active tasks, combine with the clinical data in a **master** database and perform statistical analyses and hypothesis testing.	30-36	All investigators with Dr. Shungu supervising
Milestone(s) Achieved: Determination of whether: (a) ***neuroinflammation****,* ***oxidative stress*** *and* ***mitochondrial dysfunction*** *play a role in GWI pathobiology; and (b) the outcome measures either individually or in concert can serve as* ***biomarkers*** *for GWI and point to potential* ***brain mechanisms*** *for the illness.* *Submission of at least 3 manuscripts for publication.*	36	

What was accomplished under these goals?

ACCOMLISHMENTS & FINAL PROGRESS REPORT

1. Overview and Recapitulation of the Proposed Research Objectives

The overall hypothesis of this GWIRP-NIA project was that neuroinflammation, oxidative stress and mitochondrial dysfunction play pathogenic roles in GWI. To test this overall hypothesis, we proposed the following Specific Aims**:** (a) to use *in vivo* brain ^{11}C-(R)-PK11195 positron emission tomography (PET) to measure the binding potential of the radioligand as a marker of neuroinflammation; (b) to use proton magnetic resonance spectroscopy (^{1}H MRS) to measure *in vivo* brain levels of glutathione (GSH) – the primary and most abundant antioxidant in living tissue – as a marker of oxidative stress; (c) to use ^{1}H MRS to measure *in vivo* brain levels of lactate and N-acetylaspartate (NAA) as markers of mitochondrial dysfunction; (d) to use ^{31}P MRS to measure *in vivo* brain levels of ATP, creatine phosphate (PCr) and inorganic phosphate (Pi) as complementary indices of mitochondrial dysfunction, and phosphomonoesters and phosphodiesters as indices of lipid peroxidation and oxidative stress; and (e) to measure cerebral blood flow with arterial spin-labeling MRI to assess whether hypoperfusion is also implicated. In addition, to validate the preceding neuroimaging measures, markers of inflammation and oxidative stress were also to be targeted in the cerebrospinal fluid (CSF) samples obtained by lumbar puncture in consenting participants. A case-control study design consisting of 20 veterans affected with GWI and of a group of 20 matched non-affected Gulf veterans who served as the normal control group was proposed. At project completion, the enrolled subjects had undergone PET and MR neuroimaging scans, provided body fluid (urine, blood/plasma and, in those who consented to undergo lumbar puncture, CSF) samples, and took a battery of standardized tests of overall health and functional disability. The derived outcome measures were compared between the groups to determine whether there are abnormal changes in the Gulf War veterans with GWI compared to Gulf War veterans without GWI, which would implicate oxidative stress, and/or neuroinflammation and/or mitochondrial dysfunction in the disorder, as postulated.

2. Enrollment

For this study, we had aimed for **target enrollments** of 20 Gulf War veterans affected with GWI and a group of 20 matched non-affected Gulf War veterans to serve as the normal control group. During the third USAMRAA-approved no-cost extension of the project, the total target enrollment was revised to 30 subjects, consisting of 15 Gulf War veterans affected with GWI and 15 healthy Gulf War veterans.

After we fulfilled all regulatory requirements (local IRB and USAMRMC HRPO approvals), participants recruitment began in May 2016 and ended in August 2021. At the completion of the activate phase of the study on 08/31/2021, the **actual/final enrollment** consisted of 15 Gulf War veterans with GWI and 13 healthy Gulf War veterans (**Table 2**), who fulfilled the eligibility criteria for participation, consented to enter the study, and underwent the proposed study assessments.

Table 2: Actual and Target Enrollments (Revised)

Diagnostic Group	Target Enrollments	Actual Enrollments
Veterans with GWI	**15**	**15**
Healthy Veterans	**15**	**13**
Total	**30**	**28**

The demographics and ethnic breakdown of the enrolled participants are provided in the Cumulative Inclusion Enrollment Report (**Table 3**)

3. Individual Study Assessments

Clinical Status Assessment Using Survey Questionnaires

Study participants completed following the survey questionnaires, which consisted of a battery of standardized tests designed to assess overall health and functional disability:

1. The CDC CFS Symptom Inventory: CDC CFS Symptom Inventory is a 19-item self-report that measures the frequency and intensity of symptoms related to chronic fatigue syndrome (CFS), which shares many symptoms with GWI, including sleep disturbance, problems remembering or concentrating, muscle aches and pains, joint pain, sore throat, tender lymph nodes and swollen glands, and headaches.
2. Multidimensional Fatigue Inventory (MFI): MFI is a 20-item self-report designed to measure fatigue along the following dimensions: General Fatigue, Physical Fatigue, Mental Fatigue, Reduced Motivation and Reduced Activity..
3. RAND SF-36: The RAND 36-Item Health Survey taps eight health concepts: physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/fatigue, and general health perceptions.

Table 3 Inclusion Enrollment Report

PART A. TOTAL ENROLLMENT REPORT: Number of Subjects Enrolled to Date (Cumulative) by Ethnicity and Race				
Ethnic Category	**Females**	**Males**	**Sex/Gender Unknown or Not Reported**	**Total**
Hispanic or Latino	0	5	0	5 **
Not Hispanic or Latino	2	21	0	23
Unknown (individuals not reporting ethnicity)	0	0	0	0
Ethnic Category: Total of All Subjects*	2	26	0	28 *
Racial Categories				
American Indian/Alaska Native	0	0	0	0
Asian	0	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0	0
Black or African American	0	7	0	7
White	2	14	0	16
More Than One Race	0	0	0	0
Unknown or Not Reported	0	0	0	0
Racial Categories: Total of All Subjects*	2	21	0	23 *

PART B. HISPANIC ENROLLMENT REPORT: Number of Hispanics or Latinos Enrolled to Date (Cumulative)				
Racial Categories	**Females**	**Males**	**Sex/Gender Unknown or Not Reported**	**Total**
American Indian or Alaska Native	0	0	0	0
Asian	0	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0	0
Black or African American	0	1	0	1
White	0	3	0	3
More Than One Race	0	1	0	1
Unknown or Not Reported	0	0	0	0

3. Individual Study Assessments (continued)

Clinical Status Assessment Using Survey Questionnaires (continued)

4. Inventory of Depressive Symptomatology, Self-Report (IDS-SR): IDS-SR is a validated instrument designed to assess the severity of depressive symptoms.

Neuroimaging Assessments

I. Magnetic Resonance Spectroscopy (MRS): ^{1}H MRS and ^{31}P MRS imaging scans to derive our primary indices of CNS oxidative stress and mitochondrial dysfunction were successfully conducted in all enrolled subjects according to procedures described in the funded proposal. The following outcome measures were derived:
   - ^{1}H MRS: (a) *in vivo* brain levels of glutathione (GSH) as a marker of oxidative stress and (b) *in vivo* levels of ventricular lactate and cortical N-acetylaspartate (NAA) as markers of mitochondrial dysfunction.
   - ^{31}P MRS imaging: (a) *in vivo* brain levels of ATP, creatine phosphate (PCr) and inorganic phosphate (Pi) as indices of mitochondrial metabolism dysfunction; and (b) phosphomonoesters and phosphodiesters as indices of lipid metabolism.

II. ^{11}C-(R)-PK11195 Positron Emission Tomography (PET): *in vivo* evidence of neuroinflammation was assessed through measurement of the binding potential of the PET ^{11}C-(R)-PK11195 radioligand using positron emission tomography (PET) as described in funded application.

Body Fluid Samples Collection:
The following body fluid samples were collected as indicated and then stored at -80 ºC:

1. CSF: to derive central markers of oxidative stress (consenting subjects only): due to the to the discomfort associated with lumbar puncture, which was required to collect CSF samples, the wide majority of participants opted out of undergoing the procedure. **Therefore, collection of CSF samples was deemed as not viable and discontinued**.
2. Blood/plasma: to derive peripheral markers of oxidative stress (all subjects)
3. Urine: to derive peripheral markers of oxidative stress (all subjects)

Table 4 provides a summary of the number of subjects who completed each of the major study assessments.

Table 4: Number of Subjects per Study Assessment

Group	No. of Clinical Assessments	No. of MR Scans	No. of PET Scans	No. of plasma samples	No. of urine samples	No. of CSF samples
Veterans with GWI	15	14	15	15	15	discontinued
Healthy Veterans	13	13	7	13	13	discontinued
Total	28	27	22	28	28	discontinued

4. Data Analyses and "Reduction"

The data obtained from the individual study assessments (clinical questionnaires, neuroimaging, body fluids) as described in the preceding section were appropriately "reduced", and then the resulting numerical values were entered into a master database, which was used to conduct statistical analyses for hypothesis testing.

Clinical Assessment Using Survey Questionnaires:

- The CDC CFS Symptom Inventory: The inventory questionnaires were scored according to published guidelines to produce scores ranging from 0 to 304, with higher scores indicating greater severity.
- Multidimensional Fatigue Inventory (MFI): Each item of the MFI is rated from 0 to 5 yielding possible scores ranging of 0 to 100, with higher scores indicating greater severity.
- RAND SF-36: The scores for each of the 8 concepts (physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/fatigue, and general health perceptions) that constitute the RAND SF-36 health survey are directly transformed into a 0-100 scale on the assumption that each question of the survey carries equal weight. The lower the score the more disability, with a score of zero indicating maximum disability and a score of 100 indicating no disability.
- Inventory of Depressive Symptomatology , Self-Report (IDS-SR): The IDS-SR survey is scored by summing responses to 28 of 30 items to obtain a score ranging from 0 to 84, with higher scores indicated greater severity of depression.

Neuroimaging Data

- MRS Data Analyses: The ^{1}H MRS and ^{31}P data were processed and then quantified as fully described previously[1], to yield the level of each metabolite as the area under its peak normalized to the water signal in each volume of interest for ^{1}H MRS data or to the total phosphate signal in a spectrum for ^{31}P MRS. Both measures are directly proportional to the concentration of the metabolite in the sampled tissue.
- ^{11}C-(R)-PK11195 (PET Data Analyses: Parametric PET images of regional ^{11}C-(R)-PK11195 non-displaceable binding potential (BP_{ND}) values for group comparisons were generated using published methods[2] in which the cerebellar cortex serves as the reference region. BP_{ND} values were compared between the groups, with significantly higher values of BP_{ND} in patients than in controls indicating the presence of neuroinflammation.

Circulating Markers of Oxidative Stress in Plasma and Urine Samples:
Although not explicitly stated in the application, we did obtain participants' blood to derive plasma samples, as well as urine samples, which we intended to determine peripheral or circulating markers oxidative stress to complement our central measures of same from MRS levels of brain glutathione. Specifically, we were interested in determining in plasma and urine samples the levels of 8-iso-prostaglandin-$F_{2\alpha}$-isoprostane and its isomers (collectively, **isoprostanes**), which are byproducts of free radical-mediated peroxidation of cell membrane-associated arachidonic acid and are considered the "gold standard" among biomarkers of oxidative stress.[3,4]

For the most reliable determination of the isoprostanes in our body fluid samples, used the commercial services of the Eicosanoid Core Laboratory of Vanderbilt University Medical Center (Nashville, TN), which had discovered the compounds[3]. The laboratory uses the gas chromatographic/negative ion chemical ionization mass spectrometric (GC/NICI-MS) method, which they had developed, and is considered the "gold standard" for determination of isoprostanes. The following isoprostanes were determined:

- 8-iso-prostaglandin-$F_{2\alpha}$ (8-iPF2α, for short)
- 5-iso-prostaglandin-$F_{2\alpha}$ (5-iPF2α-VI, for short)
- 8,12-iso-Isoprostane-$F_{2\alpha}$-VI (8,12-iPF2α-VI, for short)
- 2,3-dinor-5,6-dihydro-15-F2t-isoprostane (2,3-dinor-iPF2α-III, for short)

5. Statistical Analyses

Prior to the analyses, the normality of all data was assessed using the Shapiro–Wilks test. Type I error rate (i.e., chance findings) was protected against by limiting the statistical tests only to our specified hypotheses. Differences in the means of the outcome measures between the two groups were assessed using one-way analysis of variance (ANOVA). For all normally distributed secondary outcome measures, two-group comparisons were performed using general linear models (ANOVA), followed by post-hoc comparisons with Tukey's honestly significant difference test. Additional factors and covariates, if any, were chosen based on their previously reported influence on outcome measures. In the case of inhomogeneity of variance, Welch tests were performed for global group comparisons and Games–Howell tests for post-hoc pairwise comparisons. Group comparisons for non-normally distributed data used the Kruskal–Wallis nonparametric test, with Mann–Whitney U-tests for post-hoc analyses. Exploratory associations between continuous variables were conducted using Pearson's product–moment correlation (r) or, for non-normally distributed data, Spearman r. All analyses were two-tailed, with the level of significance set at $\alpha < 0.05$, unless otherwise noted. All statistical analyses were conducted using SPSS version 28.0 (SPSS Inc., Chicago, IL, USA).

6. Results and Discussion

A. Sample Demographics and Clinical Characteristics

Table 5 provides selected demographic l characteristics of Gulf War veterans with GWI (**GWI Vets**) and Gulf War veterans without GWI (**Healthy Vets**), who participated in the study.

Table 5: Demographic characteristics of Study Participants

Variables	GWI Vets	Healthy Vets	*p*-value
Age (mean ± SD)	53.7 ± 4.6	50.1 ± 4.5	0.08
BMI (mean ± SD)	29.7 ± 2.7	30.6 ± 3.8	0.44
Males	13	13	
Females	2	0	
Total (N)	15	13	

Two group were comparable for age, although the GWI Vets were slightly older at the trend level ($p = 0.08$); relatively well-matched for sex, with 13 of 15 GWI Vets being males (2 females) and all 13 Healthy Vets being males; and well-matched for body mass index (BMI, $p = 0.44$).

The clinical characteristics of the participants' physical, mental and general health status, as well as social functioning, are provided in **Table 6**.

Table 6: Clinical Characteristics of the Participants

Clinical Characteristic	GWI Vets	Healthy Verts	*p*-value
CDC (SD) – total score (SD)	73.9 (42.0)	23.4 (37.1)	0.003*
RAND SF-36			
Limitations due to physical health (SD)	33.3 (40.8)	78.9 (40.0)	0.005*
Limitations due to emotional problems (SD)	35.6 (40.8)	79.5 (40.0)	0.008*
Fatigue (SD)	28.0 (25.6)	63.6 (22.3)	0.001*
Emotional well-being (SD)	66.9 (24.0)	78.8 (17.2)	0.152
Social Functioning (SD)	50.0 (30.3)	80.8 (29.1)	0.011*
Physical functioning	58.3 (24.6)	83.1(29.8)	0.024*
Pain (SD)	44.7 (28.5)	71.3 (30.5)	0.025*
General health (SD)	46.7 (25.5)	76.9 (17.3)	0.001*
Multidimensional Symptom Inventory (MFI)			
Reduced activity (SD)	11.6 (5.6)	7.2 (3.9)	0.027*
Mental fatigue (SD)	13.7 (5.2)	7.4 (3.7)	0.001*
Physical fatigue (SD)	14.1 (5.0)	6.8 (4.0)	< 0.001*
General fatigue (SD)	15.4 (4.5)	8.8 (4.6)	0.001*
Reduced motivation (SD)	10.9 (5.2)	6.5 (2.3)	0.009*
Inventory of Depressive Symptoms, Self-report (SD)	28.5 (13.2)	9.7 (11.0)	<0.001*

An asterisk (*) next to a *p*-value denotes a statistically significant comparison.

Consistent with the well-established overlap of GWI symptoms with those of chronic fatigue syndrome (CFS), GWI Vets exhibited relatively strong CFS-associated symptoms, as assessed with the CDC CFS Symptom Inventory, compared to Healthy Vets. In general, health quality as assessed with our battery of questionnaires across multiple domains (**Table 6**) was markedly reduced in the GWI Vets group relative to the Healthy Vets group. GWI Vets reported lower physical and social functioning, greater limitations due to physical health and emotional problems, greater physical, mental and general fatigue, as well as worse pain and general health scores than Healthy Vets (**Table 6**). GWI Vets were also more depressed. They did not differ from Healthy Vets only on emotional well-being scores. The findings are consistent with GWI being a highly debilitating

illness and a significant healthcare concern in need of scientific breakthroughs that can advance our understanding of the disorder's pathophysiological underpinnings, which may point to effective treatments.

B. Testing of Primary Hypotheses

The overall hypothesis of this GWIRP-NIA project was that oxidative stress, neuroinflammation, mitochondrial dysfunction are implicated in GWI pathophysiology. To test this hypothesis, we derived several outcome measures, which will be presented and discussed in this section.

Central and Peripheral Markers of Oxidative Stress in GWI

a. In Vivo ^{1}H MRS Measures of Cortical Glutathione as a Marker of Central Oxidative Stress

MRS-derived occipital cortex glutathione (GSH) normalized to the water signal (W) in the same volume of interest (i.e., GSH/W) was lower at trend level (p = 0.16) in GWI Vets ([1.29 C 0.18]x10^{-3}) than in healthy Vets ([1.40 ± 0.19]x10^{-3}) (**Figure 1**). The statistical power for the of these GSH data was somewhat affected because data points for two Healthy Vets had to be excluded from the analysis due to poor spectral quality. The results presented in the text and in **Figure 1** are, therefore, for **15 GWI Vets** and **11 of 13 Healthy Vets**. It is likely that an outright statistically significant deficit of cortical GSH in GWI Vets would be found in a larger sample to definitively document central oxidative in GWI.

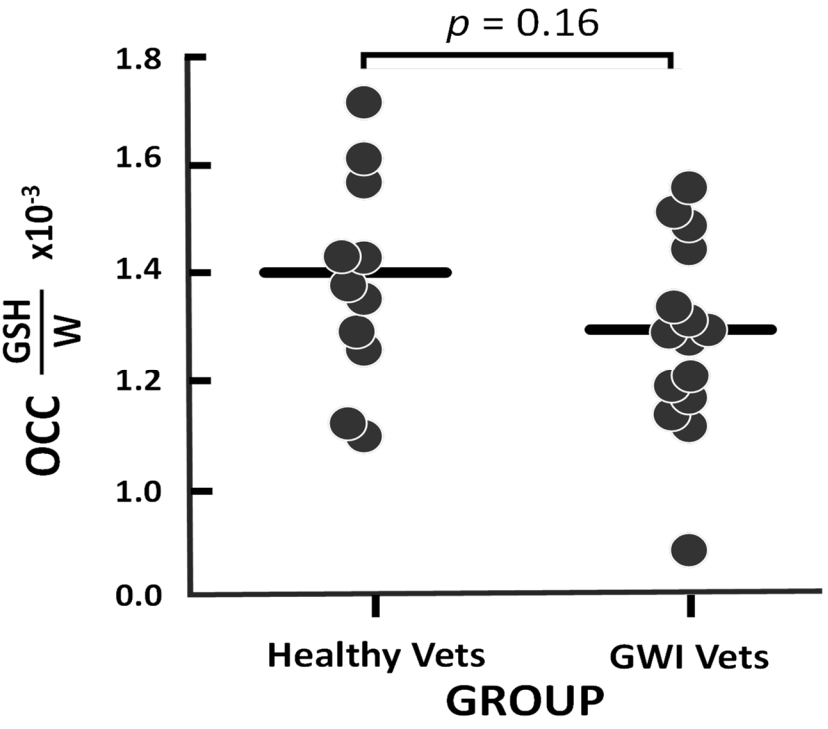

Figure 1. Scatter plot comparing occipital cortex glutathione (GSH) levels in GWI Vets with those in Healthy Vets.

b. Peripheral pr Circulating Markers of Oxidative Stress

Table 7 provides the levels of the following isoprostanes -- sensitive markers of oxidative stress -- expressed relative to the concentration of creatinine (Cr) in the body fluid samples.

- 8-iso-prostaglandin-$F_{2\alpha}$ (8-iPF2α, for short)
- 5-iso-prostaglandin-$F_{2\alpha}$ (5-iPF2α-VI, for short)
- 8,12-iso-Isoprostane-$F_{2\alpha}$-VI (8,12-iPF2α-VI, for short)
- 2,3-dinor-5,6-dihydro-15-F2t-isoprostane (2,3-dinor-iPF2α-III, for short)

Only the results for the markers in urine samples are presented because the analysis of data derived from plasma samples were negative (i.e., not statistically significant different between the groups for all compounds).

Table 7: Means ± SD of Markers of Oxidative Stress in Urine Samples

Compound	GWI Vets (ng/mg Cr)	Healthy Vets (ng/mg Cr)	*p*-value
8-iPF2α	0.67 ± 0.24	0.52 ± 0.19	0.099
5-iPF2α-VI	1.39 ± 0.63	1.02 ± 0.31	0.072
8,12-iPF2α-VI	20.29 ± 12.07	13.38 ± 4.35	0.065
2,3-dinor-iPF2α-III	9.37 ± 3.94	6.72 ± 1.19	0.028

The presented data show trend-level elevations of three of the markers (8-iPF2α, $p = 0.099$; 5-iPF2α-VI, $p = 0.072$; 8,12-iPF2α-VI, $p = 0.065$), and a statistically significant elevation of 8,12-iPF2α-VI ($p = 0.028$) in GWI Vets relative to Healthy Vets. That 8,12-iPF2α-V (see **Figure 2**), also known as 2,3-dinor-5,6-dihydro-15-F2t-isoprostane, in the urine samples of GWI Vets was statistically higher than in Healthy Vets is noteworthy because levels of this metabolite in urine samples have been reported to be a more sensitive biomarker of oxidative stress than the more widely targeted and measured 8-iPF2α[5]. In addition to being consistent with this suggestion, these results strongly implicate peripheral oxidative stress or redox dysregulation in GWI.

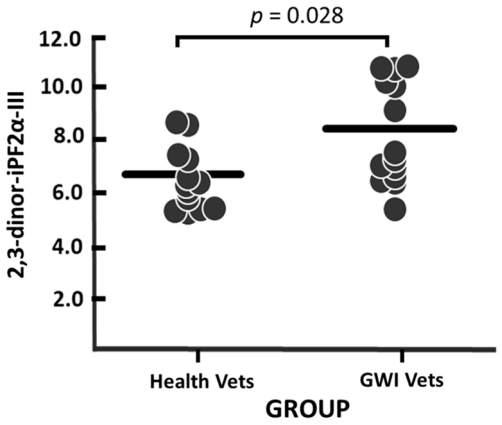

Figure 2: Scatter plot comparing urine levels of 2,3-dinor-iPF2α-III, a markers of oxidative stress, in GWI Vets with those in Healthy Vets.

Together, our findings of a trend-level decrease of cortical GSH – a marker of brain tissue oxidative stress – and trend-level and significant elevations of urine markers of oxidative stress in GWI Vets relative to Healthy Vets are potentially consistent with our hypothesis that oxidative stress is a pathophysiological mechanism in GWI. Confirmation of these findings in larger studies would provide a rationale for investigating therapeutic strategies that would be based on elevating cellular or tissue antioxidant reserves (e.g., through supplementation with the *in situ* GSH synthesis precursor or prodrug, N-acetylcysteine) as a protective intervention against oxidative stress.

<u>^{11}C-(R)-PK11195 PET Assessment of Neuroinflammation in GWI</u>

One of the key objectives of his project was to use ^{11}C-(R)-PK11195 PET to attempt to validate our hypothesis that, along with oxidative stress, neuroinflammation plays a pathophysiological role in GW. However, our analyses comparing parametric PET images of regional ^{11}C-(R)-PK11195 non-displaceable binding potential (BP_{ND}) values between GWI Vets and Healthy Vets yielded no differences in any brain region, suggesting that neuroinflammation may not be implicated. However, it could well be that our failure to find evidence of neuroinflammation in GWI might be due to our use the ^{11}C-(R)-PK11195 radioligand, which is increasingly considered to be a less sensitive marker of neuroinflammation than newer and so-called "second generation" radioligands, such as ^{11}C-DPA-713 or ^{11}C-DPA-713, which are less lipophilic and, thus, have better tissue penetration.

To examine this possibility, we searched the literature for studies that used ^{11}C-(R)-PK11195 or second generation PET radioligands to assess neuroinflammation in GWI and related illnesses, such as CFS and fibromyalgia (FM). In one study study[6], ^{11}C-(R)-PK11195-PET was used to assess neuroinflammation in 9 patients with CFS and 10 healthy controls, and found increases in BP_{ND} values ranging from 45% to 199%, suggesting neuroinflammation in the disorder. By contrast, another study that also used ^{11}C-(R)-PK11195-PET to assess neuroinflammation in 9 patients with CFS and 9 healthy controls, not only failed to replicate the robust increases in ^{11}C-(R)-PK11195 BP_{ND} of up to 199% in the prior study, but it found no increases at all[7]. In another study[8] using the second generation ^{11}C-DPA-713 PET radiotracer, credible evidence of neuroinflammation was found despite a small sample of 4 patients with CFS and 5 healthy controls. Lastly, studies using ^{11}C-PBR28, another second generation PET radioligand, reported evidence of neuroinflammation in fibromyalgia and GWI[9,10]. Studies that used second generation tracer more consistently reported evidence of neuroinflammation in GWI-like disorders.

Therefore, even though relatively small, the results of prior studies seem to be consistent with the view that the ^{11}C-(R)-PK11195 radioligand that we used in this study might be less reliable at detecting neuroinflammation than newer, second generation PET radioligands. Our failure to find evidence of neuroinflammation in GWI in this study is thus far from being dispositive or conclusive. Larger studies using second generation PET radioligands (e.g., ^{11}C-DPA-713, ^{11}C-PBR28) are warranted to determine whether a more sensitive technique would be able to establish conclusively whether neuroinflammation plays pathophysiological or pathogenic role in GWI.

Neuroimaging Assessment of Mitochondrial Dysfunction in GWI

Because oxidative stress and associated accumulation of free radicals can lead to mitochondrial dysfunction[11], we had postulated that along with oxidative stress, mitochondrial dysfunction may play a role in GWI. Therefore, we had proposed (a) to ^{1}H MRS to use measure ventricular lactate, which would be increased as a result of upregulation of glycolytic metabolism to compensate for impaired mitochondrial energy production[12], and (b) N-acetylaspartate (NAA), a putative neuronal integrity marker, which is synthesized in neuronal mitochondria and would be decreased in mitochondrial dysfunction[12]; and (c) to measure using ^{31}P MRS levels of ATP, creatine phosphate (PCr) and inorganic phosphate, which are involved in cell energy metabolism and would be affected by impaired mitochondrial energy production. However, our measurements and analyses of the levels of these markers of mitochondrial dysfunction revealed no abnormalities. Therefore, the results of this study do not support a significant role for mitochondrial dysfunction in GWI.

Exploratory Associations between Neuroimaging, Body Fluid Measures and Clinical Variables

With a sample size of, at most, 15 GWI Vets and 13 Healthy Vets, this study did not have sufficient statistical power to enable a meaningful assessment of within-group associations among our objective outcome measures and clinical variables. Selected correlation analyses were not promising. Therefore, we did not explore such correlations, which will require larger studies to assessed meaningfully.

Primary Study Limitation

This study was limited by the excruciating difficulty of recruiting Gulf War veterans, resulting in inadequate statistical power to meaningfully test a number of our hypotheses.

C. Conclusion

In summary, the results of this study support oxidative stress and redox dysregulation, but not neuroinflammation (though the jury is still out) or mitochondrial dysfunction, as a potential mechanism in the pathophysiology of GWI.

D. Literature Cited

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

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

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

Nothing to Report

4. IMPACT:

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

Several aspects of this study may have an impact on GWI research and our understanding of the condition, as our project represented a departure from nearly all the studies that have been conducted in the disorder to date:

1. Multimodal experimental approach: This study leveraged the investigators' extensive expertise and experience in neuroimaging and in GWI and related disorders (CFS and fibromyalgia) to conduct what may be the first comprehensive and multimodal attempt at developing and validating a number biomarkers of GWI, based on measuring, all at once, multiple objective outcome measures that reflect the metabolic, physiologic and clinical abnormalities postulated to be manifestations of biological dysfunctions that underpin the disorder. Potential benefits of the proposed approach include the opportunity to (a) retain promising outcome measures for further exploration as potential diagnostic, treatment response monitoring or subtyping/classification "biomarkers" for GWI. Importantly, our multimodal experimental approach as used in the study offers the possibility derived a wealth of metabolic, physiologic, biochemical and clinical data in GWI that are necessary to advance the understanding of a disorder about which there is currently a paucity of objective biomedical data acquired simultaneously in the same cohorts.

3. Study was mechanistic: This study was highly innovative in that it was almost entirely model-driven, drawing both in concept and in research strategy from our prior studies in closely related multi-symptom illnesses (CFS, FM), to identify measurements that can be made to test the validity of the model. Thus, an impact of this research for GWI is to point to promising areas of further investigations that can advance our understanding of the pathobiology of the disorder.

4. Study offered the possibility to differentiate GWI from other chronic multi-symptom illnesses: A central question and unmet need in the study of GWI and other closely related multi-symptom illnesses is whether and how these illnesses can be objectively differentiated from one another. While there is extensive symptom overlap among the various multi-symptom illnesses, which now include "Long COVID"[13], there is ample evidence that each is a distinct medical entity, with a highly heterogeneous clinical presentation. Because the model that we proposed to investigate for GWI and the experimental approach that we used to try to validate this model were virtually identical to those that we had used and are currently using in our studies of CFS and FM, we have the opportunity to compare and try to differentiate GWI from CFS and FM based on the objective neuroimaging and clinical data obtained through this research project. In other words, our proposed research looks beyond a single multi-symptom illness. It pits them against each other in manner that may enable their objective differentiation, since we would be comparing identical objective biomarkers rather than subjectively reported of clinical symptoms.

What was the impact on other disciplines? .

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

There were no changes to the approach; only a discontinuation of a study procedure. The collection of CSF samples had to be discontinued because the majority of participants opted out of undergoing lumbar puncture, which is a procedure that is not without discomfort but is required to collect CSF samples.

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

The study was delayed, and progress slowed due to the great challenge of locating and recruiting eligible Gulf War veterans with or without GWI. Studies of this disease would benefit greatly from the establishment of a network for facilitating the recruitment of participants.

Changes that had a significant impact on expenditures

It was necessary to reallocate some funds to engage a professional recruitment service to assist in locating eligible participants. Without this adjustment, it is unlikely that this study would have gotten as far as it did.

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

Not applicable

Significant changes in use of biohazards and/or select agents

Not applicable

6. PRODUCTS:

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

  Journal publications.

  In progress.

  Books or other non-periodical, one-time publications. *Report any book, monograph,).*

  Nothing to report

  Other publications, conference papers and presentations. .

  Nothing to report

- **Website(s) or other Internet site(s)**

  Nothing to Report.

- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

FOR PERIOD 09/01/2015 TO 8/31/2022

Name: Dikoma C. Shungu, Ph.D.
Project Role: PI
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0001-9452-2245
Nearest person month worked: 1 calendar Month
Contribution to Project: Dr. Shungu oversaw all the MR Neuroimaging aspects the proposed research, as well as its day-to-day coordination of the study.

Name: Xiangling Mao, M.S.
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0003-2274-8282
Nearest person month worked: 1 Calendar Month
Contribution to Project: Ms. Mao performed work in the area of regulatory activity, MR Scan and data processing.

FOR PERIOD 09/01/2015 TO 8/31/2018

Name: Yeona Kang, Ph.D.
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1 calendar Month
Contribution to Project: PET data processing

FOR PERIOD 09/01/2015 TO 8/31/2020

Name: Benjamin H. Natelson, M.D.
Project Role: Sub-site PI at BIMC
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1 Calendar Month
Contribution to Project: Dr. Natelson performed work in the area of subject recruitment and characterization.

Name:	Sarah Khan
Project Role:	Study coordinator at BIMC
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 Calendar Month
Contribution to Project:	Ms. Khan performed work in the area of subject recruitment.
Name:	Diana Vu
Project Role:	Study coordinator at BIMC
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 Calendar Month
Contribution to Project:	Ms. Khan performrf work in the area of subject recruitment.
Name:	Michelle Blate
Project Role:	Nurse-practitioner at MSBI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 Calendar Month
Contribution to Project:	Ms. Blate performed work in the area of subject recruitment.

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

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
N/A

QUAD CHARTS:

N/A

9. APPENDICES:

NONE