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14. ABSTRACT

Gulf War Illness (GWI) consists of a set of debilitating symptoms that have been associated with deployment to the Persian Gulf theatre of war. There is general agreement that physical exposures play an important role in the etiology of this disease (or diseases), yet studies to identify a single major causative exposure have been largely unproductive. Dr. Grant has developed a blood-based assay to detect and quantify genotoxic carcinogenic exposures, through loss of a molecular epitope on the surface of red blood cells. This glycophorin A (*GPA*) mutation assay has been successfully applied in a wide array of occupational, environmental, medical and accidental exposures to radiation and chemicals. The *GPA* assay provides a cumulative dosimeter of mixed exposures impacting on bone marrow stem cells. Our hypothesis is that such exposures are associated with the incidence of clinical symptoms of GWI. To investigate this possibility, we will analyze two sets of military veterans with the GPA assay, one that was deployed to the Gulf and one which was not. If Gulf War deployment involved exposures with the capacity to damage DNA, such as radiation from depleted uranium ammunition, or polyaromatic hydrocarbons from oil fires, we should detect long term effects in the deployed cohort. Since the *GPA* assay also integrates host effects, we will also measure DNA repair capacity in these subjects. This project therefore has the potential to monitor present day Gulf War veterans to identify those at greatest risk of developing GWI, but also to identify those at risk for this disease prior to deployment.

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Grant, S.G., Ibrahim, O.M., Jin, X.-L., Klimas, N.G., Sullivan, K., and Latimer, J.J. (2021) Elevated somatic mutation and evidence of genomic instability in veterans with Gulf war illness. *Life Sciences* **281**: 119746.

Introduction

Veterans who served in the Gulf war report debilitating health symptoms 2-3x more frequently than military personnel who were not deployed to the Gulf. These symptoms are multi-system and non-specific, involving fatigue, headache, memory issues, sleep disorders and musculoskeletal pain. Gulf war illness (GWI) is a life-altering disease that has been attributed to exposures to radiation and/or chemicals encountered in the field. Since some, but not all personnel manifest this disease, there may be an additional genetic component that increases vulnerability to such exposures, producing life-ling effects. We hypothesize that our approach to environmentally-induced carcinogenesis, measuring the total effect of all genotoxic exposures, as modified by the genetic susceptibility of each exposed individual, can be translated successfully to a study of GWI. From our previous studies, it is clear that genotoxic exposures can induce both short-term and long-term effects, with the long-term effects associated with mutagenesis of the stem cell compartment. Stem cell mutagenicity, as demonstrated by persistent elevations in blood-based somatic mutation frequencies, would be expected to result in pleiotropic premature aging effects that could manifest as non-specific GWI. We therefore proposed to directly measure somatic mutation frequencies in symptomatic and asymptomatic Gulf war veterans and controls to determine whether elevated somatic mutation is indicative of disease or disease severity. We also directly measure DNA repair capacity, specifically of the nucleotide excision repair (NER) pathway, to determine whether natural variation in this protective metabolic function is an important genetical predisposing element in determining who will manifest the clinically relevant symptoms of GWI. Identifying the basis of genetic predisposition would also allow for the sequestering of "high-risk" personnel from exposures more likely to produce clinical disease in future deployments.

Keywords

Genotoxic exposure, genetic predisposition, somatic mutation, DNA repair, stem cells

Accomplishments

What were the major goals of the project?

I. Determine whether symptomatic Gulf war veterans have persistently elevated levels of bone marrow somatic mutation.

II. Determine whether there is an association between elevated somatic mutations frequencies and the number or severity of symptoms in GWI.

III. Determine whether symptomatic Gulf war veterans with elevated somatic mutation frequencies are functionally deficient for DNA nucleotide excision repair.

What was accomplished under these goals?

Goal I:

Adaptation of the *GPA* somatic mutation assay to new flow cytometry platform

We have successfully re-established the *GPA* somatic mutation assay by adapting it to the commercially available BD Accuri C6 flow cytometer. We have developed a new protocol that yields somatic mutation frequency results indistinguishable from those of the previous "DR6" version of the assay on historical controls. The traditional *GPA* assay was optimized for application to individuals who were heterozygous for the MN blood group, which is determined by the glycophorin A (*GPA*) gene. In such individuals, who make up about half of most populations, we quantified variants occurring by loss of expression of the M allele (MN → N/Ø), as well as variants occurring by loss of the M allele in concert with duplication of the N allele (MN → NN). Although this assay is being applied to the present project, it was concurrently applied in a study of autistic children; the present grant will be cited for its contribution to the development of this analytical method (manuscript in preparation).

Ms. XiaoLu Jin has been the primary flow cytometry specialist working on the *GPA* assay, with support and consultation from Ms. Megan Foley.

Blood sampling of GWI patients and controls

We have received a total of 56 experimental blood samples; which included 52 symptomatic and diagnosed patients with GWI and 4 controls, including one asymptomatic Gulf War veteran. 26 of the patient samples and all 4 controls are heterozygous for the MN blood group, and therefore optimal for analysis with the GPA somatic mutation assay. Due to a generalized lack of control samples, we have returned to our sets of historical controls and identified a total of 170 age-appropriate (older than 50 years at time of sampling) individuals as comparators for our affected GWI veterans. In the last year, we collaborated with Dr. Gesulla Cavanaugh to secure additional controls that would also be appropriate for her prospective study of exposure in the local homeless population, which was funded as a pilot study by the Health Professions Division of NSU ("Measuring the extent of premature aging, health literacy and cognitive impairment in the chronically homeless"). Three samples from this study were determined to be appropriate for our GWI population.

Performance of the GPA somatic mutation and UDS DNA repair assays on GWI patients and controls

We have now performed flow cytometric analysis on all of our initial samples and will present somatic mutation data on all samples heterozygous for the MN blood group – 26 affected patient samples, 1 unaffected Gulf War veteran as a control, 3 concurrent age-matched laboratory controls and 170 age-matched historical controls from previous studies (Bigbee et al. 1990; Grant et al., 1992; 1994; 2006; Jensen et al., 1990). Our original hypothesis was

consistent with two outcomes: that affected veterans with GWI would have significantly higher *GPA* somatic mutation frequencies indicative of overall greater bone marrow stem cell mutagenesis than controls; or that the GWI population would have a greater incidence of high mutation frequency "outliers" – this second possibility was the basis of our original proposal to follow up such outliers with analysis of DNA repair capacity, however, it is confounded by the fact that outliers naturally increase in frequency with age (Bigbee et al. 1998).

As shown in Table 1, the GWI population is significantly higher in the frequency of allele loss and total *GPA* mutations. The higher frequency of allele loss and duplication mutations does not quite reach statistical significance. This suggests that the cumulative genotoxic exposures of the affected population are greater and/or more persistent that those of similarly aged people who did not serve in the Gulf War. Allele loss mutations are associated with point (base) mutagens as well as clastogens (chromosome breaking agents) (Grant and Bigbee, 1993). No definitive conclusion can be reached on agents associated with allele loss and duplication, such as recombinogens. Aneugens, which interfere with chromosomal segregation at mitosis, can cause both types of variants. These data have now been published (Grant et al., 2021).

Table 1. *GPA* mutation frequencies measured in veterans affected with GWI and age-matched controls.

	Allele loss frequency[1]	Loss and duplication frequency[1]	Total mutation frequency[1]
Affected GWI N = 26	22.5 ± 4.0	24.4 ± 4.2	47.0 ± 7.2
Age-matched controls N = 174	11.6 ± 1.5	15.9 ± 2.0	27.6 ± 2.4
P[2]	0.005	0.056	0.003

[1] X 10^{-5}

[2] T test assuming equal variances

Of the three new samples obtained as controls, two were heterozygous for the MN blood group. These samples had normal allele loss mutation frequencies of 2.2 and 2.3 X 10^{-6}, normal allele loss and duplication mutation frequencies of 0.7 and 1.5 X 10^{-6} and total *GPA* mutation frequencies of 2.5 and 2.8 X 10^{-6}. These controls will be added to the published data when we update the *GPA* results in a paper focused on RNAseq data presently under analysis.

Goal II:

The data in **Table 1** suggest that affected veterans did indeed sustain greater genotoxic exposure than normal, were more genetically susceptible to genotoxic exposures than normal, or both. The statistical significance in the allele loss and total mutation categories could be driven by a shift in the whole population, indicated by an increase in the main peak of the population, or by a smaller population of high-frequency "outliers." Such outliers occur in all populations, including newborns, and their incidence rises exponentially after age 40 to peak around age 65, in a striking parallel to the age-incidence of adult-onset cancers (Bigbee et al. 1998; Grant 2012, 2017). In particular, such outliers were increased in the long-term survivors of the atomic bombing of Hiroshima (Langlois et al. 1993).

Table 2. Number of high *GPA* mutation frequency "outliers" identified in veterans affected with GWI and age-matched controls.

Allele loss	Mutation frequency $< 30^{1}$	Mutation frequency $\geq 30^{1}$	P^{2}	OR (CI)[3]
Affected GWI	19	7		
Age-matched controls	165	9	0.0014	6.74 (3.61 – 12.62)
Allele loss and duplication	Mutation frequency $< 30^{1}$	Mutation frequency $\geq 30^{1}$	P^{2}	OR (CI)[3]
Affected GWI	19	7		
Age-matched controls	155	19	0.053	3.01 (0.99 – 3.80)
Total mutation	Mutation frequency $< 50^{1}$	Mutation frequency $\geq 50^{1}$	P^{2}	OR (CI)[3]
Affected GWI	17	9		
Age-matched controls	154	10	0.0047	4.08 (2.07 – 8.02)

[1] X 10^{-5}

[2] Fisher's exact test

[3] Odds Ratio (Confidence Interval)

As can be seen in **Table 2**, the frequency of *GPA* somatic mutation outliers, defined as having frequencies equal to or greater than 30 X 10^{-5} for either allele loss or loss and duplication, or equal to or greater than 50 X 10^{-5} for total mutation, is significantly increased in the affected veteran population for the allele loss and total mutation endpoints, despite the fact that this is already the age with the highest natural frequency of such individuals. Once again, loss and duplication just barely fails to reach significance. Converted to predictive values (odds ratios), such outliers have 4-fold and 7-fold greater likelihoods of identifying a person with GWI. Even the not quite significant increase in loss and duplication outliers would still provide a 3-fold greater risk. We suggest that these outliers have suffered a loss of genomic instability in the stem cell compartment of their bone marrow, which confers long-term risk of cancer and other diseases.

Generalized induction of persistently higher somatic mutation frequencies and genomically unstable outliers in the GWI population are not mutually exclusive phenomena, however. As demonstrated in **Table 3**, when the outliers are removed from the analysis, the GWI population is significantly higher in mutation frequency for all three endpoints, indicative of greater exposure to and/or susceptibility to mutagens, clastogens, aneugens and recombinogens in the course of their lifetimes. These results were also included in the Grant et al. 2021 publication.

Table 3. GPA mutation frequencies measured in veterans affected with GWI and age-matched controls after removal of high-frequency "outliers."

	Allele loss frequency	Loss and duplication frequency	Total mutation frequency
Affected GWI	12.6 ± 1.6 N = 19	15.3 ± 1.6 N = 19	28.4 ± 3.2 N = 17
Age-matched controls	8.2 ± 0.4 N = 165	9.4 ± 0.4 N = 155	18.8 ± 0.8 N = 154

P	< 0.001	< 0.001	< 0.001

[1]X 10^{-5}

[2]T test assuming equal variances

Our additional controls samples are not outliers, so have little impact on these calculations. Nevertheless, they will be included when the data is updated for a summary publication.

Goal III:

Performance of the unscheduled DNA synthesis (UDS) assay of DNA nucleotide excision repair (NER) on GWI patients and controls

Our original hypothesis was that veterans with GWI, especially those with elevated "outlier" levels of bone marrow mutational burden, would be associated with reduced levels of DNA repair. For the NER pathway, we found the opposite, that the GWI population exhibited an increased level of DNA repair capacity over controls, as determined by a functional, live cell assay performed in blood lymphocytes, the "unscheduled DNA synthesis assay" (DNA replication is "scheduled" DNA synthesis). Since induction of this pathway of DNA repair is used as a method of detecting mutagens, we hypothesized that the affected veterans were exhibiting a persistent inductive or "hormetic" effect caused by their cumulative genotoxic exposure in the Gulf theater of war. This would differentiate them from deployed personnel who a) did not get enough exposure to induce their DNA repair systems, or b) experienced a transient induction of DNA repair that returned to normal after the inductive conditions ended. This interpretation is very much in line with work from Dr. Klimas' group indicating that veterans with GWI have a generally altered homeostasis (Craddock et al., 2014). A preliminary report of these data was published in the journal *Military Medicine* (Latimer et al., 2020), and slightly updated data maintaining the same result was presented last year the *DOD/VA Gulf War Illness State of the Science Conference* and is given in **Figure 1**.

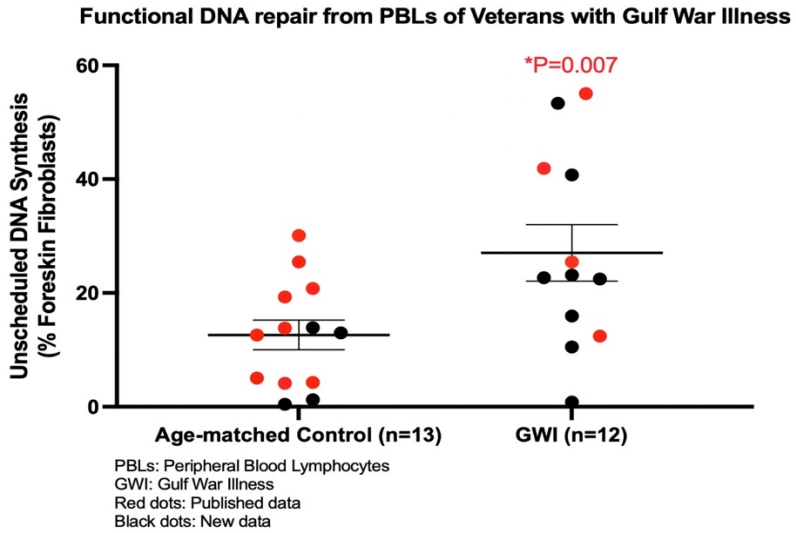

Figure 1. NER capacity as measured by the functional UDS assay in veterans affected with GWI and age-matched controls.

We have now completed the processing of UDS assay data. A total of 25 samples, 12 affected individuals and 13 controls, were quantifiable. These total data confirm the effect that we reported in our preliminary conference proceeding paper (Latimer et al., 2020) with 9 controls and 4 affected samples; that the symptomatic GWI individuals exhibit significant elevated levels of NER despite being decades removed from their wartime exposure. We have suggested that this might represent a persistent hormetic effect, or that this induced state is maintained in the subpopulation by ongoing "normal" exposures.

Goal IIIa:

Performance of RNAseq on DNA nucleotide excision repair (NER) genes in GWI patients and controls

In previous studies on breast cancer, leukemia and now autism (Latimer et al., 2010; Ibrahim et al., 2018), we have found that there is a high degree of correlation between the expression of the 20 core NER genes and functional DNA repair capacity as determined using the UDS assay. We have therefore extracted total mRNA from all samples submitted for *GPA* analysis and have begun RNAseq analysis in our Genomics Core (located on the same floor as our laboratory). In a preliminary analysis of our current study, we found that expression of NER genes was significantly upregulated in veterans with GWI (**Fig**. **2**).

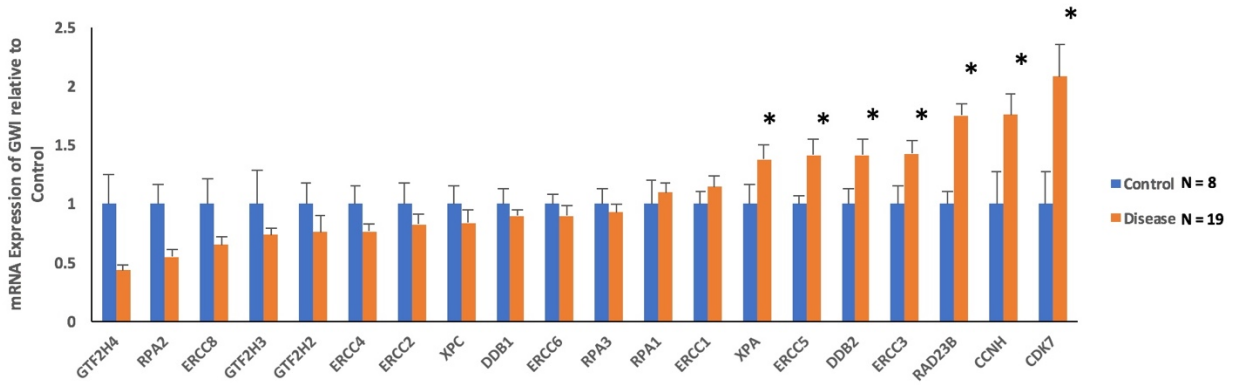

Figure 2. RNA sequencing analysis of affected GWI veterans (red bars) vs. age matched controls (blue bars) for each of the 20 canonical genes in the NER pathway. All gene levels are normalized to the mean of the controls. Overall, 9 of the 20 canonical genes were relatively overexpressed in the affected GWI veterans. The elevation in expression observed of seven of the genes are individually statistically significant: *XPA* ($P = 0.047$), *ERCC5* ($P = 0.037$), *DDB2* ($P = 0.043$), *ERCC3* ($P = 0.017$), *RAD32B* ($P < 0.001$), *CCNH* ($P = 0.017$), *CDK7* ($P = 0.016$).

Once again, an effect shown in preliminary form in our published paper (Latimer et al., 2020) with data from 4 controls and 6 GWI subjects has been confirmed upon analysis of the larger population. Indeed, while fewer overall genes in the NER pathway are overexpressed in the final data, more are significantly overexpressed (four of the the five genes reported in the previous compilation plus three more) and to a greater degree. Completion of this work was aided by an NSU President's Faculty Research and Development Grant awarded to Dr. Latimer ("Molecular analyses of the five pathways of DNA repair in Gulf war illness"). We also intend to extend the analysis to the other four pathways of human DNA repair.

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

This project has been performed by technicians in the laboratory, beginning with Ms. Stephanie Sveiven, who is now in graduate school, then Ms. Megan Foley, whom we hired after her completion of a Masters degree in the Oceanographic Institute of NSU, and now Ms. Jin. The flow cytometric assay was updated largely by a doctoral student, Omar Ibrahim, who has graduated with his Ph.D. and now works locally in industry. We currently have 3 other Ph.D. students in the group and we have interns, D.O., M.P.H., Pharm.D. and undergrad students as well as high school students from the University-associated school, spending various amounts of time in the lab. Two Ph.D. students, Abdullah Alhamed, who has completed his degree, and Ali Almutairy, who is still in the lab, were co-authors on our preliminary report on this project (Latimer et al., 2020). Dr. Ibrahim was an author on our recent report on the GPA results in these subjects (Grant et al., 2021). A Masters student in the lab, Yousef Alharbi, has overseen the completion of the UDS work on the project, with contributions from former Ph.D. student, Jowaher Alanazi, and Pharm.D. student, Pegah Manesh. Mr. Almutairy is similarly in charge of completing the RNAseq analysis as part of his Ph.D. thesis, with contributions from Colton Simmons, another Ph.D. student in the group.

This project involves the application of core capabilities from the laboratories of Drs. Grant and Latimer to a new health issue, Gulf War illness. The work takes place within the context of our larger group, however, and other lab members, particularly students, have the opportunity to

observe the application of these techniques to a mixed historical exposure, rather than just acute exposures, like chemotherapy.

We are applying the same core techniques from our ongoing studies on breast cancer and leukemia to this study on GWI, and this has allowed for the participation of graduate students and interns in the preparation of samples and analysis of RNAseq data. Since the UDS assay is labor-intensive and requires hand quantification of silver grains under the microscope, processing of these data is often a group effort.

Drs. Grant and Latimer and their students are regular participants in the Seminar series run by Dr. Klimas' Institute for Neuro-Immune Medicine. Dr. Grant is chair of the subcommittee for faculty research in the Dr. Kiran C. Patel College of Osteopathic Medicine and oversees monthly research Grand Rounds where this work has been presented, as well as a "Research Spotlight" during monthly College faculty meetings. Progress of the project is discussed at weekly group lab meetings and a "Work-in-Progress" meeting for faculty in the Center for Collaborative Research.

How were the results disseminated to communities of interest?

Our ongoing progress is discussed at weekly meetings of the Grant/Latimer lab and monthly meetings that include Dr. Klimas and participating members of her group. Dr. Latimer is the Director of the AutoNation Breast Cancer Institute on the NSU campus, and although this work does not involve cancer, its association with genotoxic exposures is discussed in context with environmental oncology. Similarly, Dr. Klimas is Direction of the Institute for Neuro-Immune Medicine at NSU, and this work is regularly reported and discussed in context with other projects on Chronic Fatigue Syndrome and Gulf War Illness.

We take every opportunity to discuss our work with the greater scientific community in Nova Southeastern University, soliciting feedback and potential collaboration. In the last year, Dr. Klimas hosted a Conference entitled *Sustained Homeostatic Imbalance due to Environmental Exposure Linked to Deployment (SHIELD)* for Gulf War veterans and researchers on the campus of Nova Southeastern University and featured the findings of the present project in an overall plenary presentation. Many members if Dr. Kimas' group attended the *DOD/VA Gulf War Illness State of the Science Conference* which occurred virtually August 18-20. Dr. Grant presented a poster, which in this context consisted of a 10 minute recorded talk with slides.

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

We will concentrate on obtaining new age-matched control samples through the completion of the extended funding period, since when we broke the deidentification codes for our existing samples we found that there were very few controls. We do not believe that this will fundamentally change our results including clarifying the close to significant results we have on the loss and duplication GPA endpoint. The UDS and RNAseq data are highly statistically

significant even with very preliminary data, and we will work to finish the processing and analysis of all samples we have in hand.

Impact

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

We are currently preparing a manuscript involving data derived from the new version of the *GPA* assay. Previous versions of the *GPA* assay have contributed to almost 400 peer-reviewed articles examining accidental, environmental, occupational and medical exposure to genotoxic agents, including radiation and chemicals. We have also used the assay to characterize and even diagnose DNA repair deficiency syndromes that are associated with cancer predisposition and/or premature aging. A direct result of providing facile application of a new GPA assay would be proactive monitoring of the populations listed above, rather than passive retrospective analysis. In all of these venues, but significantly including military personnel on active duty, baseline and ongoing monitoring with assays including but not limited to the GPA assay would allow for individualized management of extent of exposure. It should be noted that the GPA assay was originally developed by the DOE for such an application. This was the topic of an editorial published by Dr. Grant "Translating mutagenesis into carcinogenesis" (Grant, 2012). He further elaborated on the subject of monitoring the process of carcinogenesis as opposed to the accumulation of mutations in specific genes in a recent review paper entitled "The arrow of carcinogenesis" (Grant, 2017).

If we consider the *GPA* data above as most representative of their populations after the removal of outliers, the results are consistent with our initial hypothesis, that soldiers exhibiting GWI are those who either sustained higher or more persistent genetic damage or are particularly genetically (or epigenetically) susceptible to their exposures. Although this is not a particularly novel concept, the identification of DNA repair capacity as a mediator of this effect would allow for initial screening and periodic biomonitoring of individuals likely to undergo such exposure.

What was the impact on other disciplines?

The *GPA* assay has, in the past, been used to detect and quantify both known and unknown genotoxic exposures in studies of environmental, occupational, medical and accidental exposures. It has also been used to characterize, and even diagnose, hereditary diseases associated with predisposition to cancer and/or premature aging. It has been used to longitudinally biomonitor cancer patients undergoing genotoxic radio- and/or chemotherapy and victims of radiation accidents and A-bomb survivors. Perhaps because the published protocol required unobtainable reagents and devices, studies using this assay in the U.S. have been declining, although studies in Japan appear to have been maintained and the assay seems to have been embraced in China. Our redevelopment of the assay, upon publication, should reinvigorate its use in these fields, and allow for its application to novel situations, such as the present application to GWI. In particular, the present project is a paradigm for future

application of the assay: applying to individuals who have been exposed to a "complex mix" of agents, and factoring in individual host sensitivities in the form of DNA repair capacity.

What was the impact on technology transfer?

The *GPA* assay was developed as a method of biologically monitoring workers at risk of genotoxic exposure, and has been validated for radiation workers. Since leaving LLNL, we, and others, have also demonstrated its utility in a number of other occupational settings. The assay has been presented at the R&D facility for Quest diagnostics and a patent has recently been awarded to Drs. Grant and Latimer (U.S. Patent **10**,**684**,**293** "Associating somatic gene mutations in glycophorin A with complex multifactorial diseases") specifically for application to diseases where neither the exposure profile nor genetic susceptibility appear to explain incidence. We can only hope that a successful application to a high-profile problem, such as GWI, will spur interest in the wider application of this and other blood-based methods of detecting and quantifying genotoxic damage.

What was the impact on society beyond science and technology?

We have previously presented evidence that a one-time *GPA* analysis can be highly predictive of cancer risk (especially if the individual manifests a high "outlier" phenotype, such as the one we are screening for in GWI). This had been done using histograms and t-tests, but, in his recent review mentioned above, Dr. Grant provided numbers showing that high mutation frequency "outliers" occur 4.24 X more frequently in populations of newly diagnosed cancer patients than in controls (Grant, 2017). This further indicates that the *GPA* somatic mutation assay (and the lymphocyte HPRT somatic mutation assay) is a cumulative dosimeter of genotoxic exposure and damage, integrating host sensitivity factors that might be more significant than the majority of cancer risk factors presently acknowledged. Broader application of this and similar analyses would allow for the identification of individuals whose cumulative exposure has pushed them into a category of high risk, detect when they have had a previously unsuspected genotoxic exposure, or quantify the effect of a known exposure, allowing them to avoid further injury.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

We made changes in the study design based on the preliminary functional and molecular data on DNA repair. This involved applying UDS and RNAseq to all samples rather than a subset identified as unusual through screening *GPA* analysis. In our summary report, we will look at possible interactions between these three markers.

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

Our original study design involved screening a large population with the *GPA* assay and performing nested studies on high mutation frequency "outliers" with the UDS assay. Since we did not receive enough blood samples to reach our original sampling goals, we altered our design to be "deeper" rather than "broader." We therefore are generating data on somatic mutation (GPA assay), DNA repair (UDS assay) and gene expression (RNAseq) on all samples and will analyze them individually and in association.

Changes that had a significant impact on expenditures

We had anticipated performing a smaller nested study of UDS analysis based on a more complete study of *GPA* results. So far, we have managed to perform UDS and RNAseq analysis on all samples to complement the *GPA* results.

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

None.

PRODUCTS:

- **Presentations**

Klimas, N.G., Nathanson, L., Abdullah, L., Ait-Ghezala, G., Grant, S.G., Latimer, J.J., Salguero-Tosta, L.M., Craddock, T., Broderick, G., Morris, M., Chaterjee, S., Ashford, J.W., Adamson, M.M., Chao, L.L., Cook, D., Shungu, D.C., Nile, B.L., Kelly, K.A., Lasley, S.M., O'Callagham, J.P., Balbin, E., and Sullivan, K. (2020) State of the science Gulf War Illness: Moving knowledge to treatment. Presented at the conference on *Sustained Homeostatic Imbalance due to Environmental Exposure Linked to Deployment (SHIELD)*, Nova Southeastern University, Fort Lauderdale, Florida, February 28.

Grant, S.G., Ibrahim, O.M., Jin, X.-L., Foley, M., Abdullah, L., Almutairy, A., Alanazi, J., Klimas, N.G., Sullivan, K., and Latimer, J.J. (2020) Somatic mutation and DNA repair in veterans with Gulf War illness. Presented at the *DOD/VA Gulf War Illness State of the Science Conference*, August 18-20.

- **Papers**

Latimer, J.J., Alhamed, A., Sveiven, S., Almutairy, A., Klimas, N.G., Abreu, M., Sullivan, K., and Grant, S.G. (2020) Preliminary evidence for a hormetic effect on DNA nucleotide excision repair in veterans with Gulf War Illness. *Military Medicine* **185**: e47-e52.

Grant, S.G., Ibrahim, O.M., Jin, X.-L., Klimas, N.G., Sullivan, K., and Latimer, J.J. (2021) Elevated somatic mutation and evidence of genomic instability in veterans with Gulf war illness. *Life Sciences* **281**: 119746.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Stephen Grant, Ph.D.*
Project Role:	*Principle Investigator*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Grant oversees the entire project. He works with Drs. Klimas and Fletcher to obtain blood samples and with Dr. Latimer to coordinate the performance of the UDS assay and extraction of mRNA. This year, he has analyzed GPA assay data provided by Ms. Jin.*
Name:	*Jean J. Latimer, Ph.D.*
Project Role:	*Co-Investigator*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Latimer provides input and expertise for the UDS assay, and supervises the extraction of mRNA. She is primarily responsible for the analysis of RNAseq data for quantification of expression of the genes of the nucleotide excision repair pathway.*

Name:	*Nancy Klimas, M.D.*
Project Role:	*Co-Investigator*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Klimas provides the samples to our lab from her clinic at the Miami Veterans Affairs facility.*

Name:	*Patrick Hardigan, Ph.D.*
Project Role:	*Statistician*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Hardigan has consulted with our lab on data analysis in order to determine the best approaches and comparisons.*

Name:	*Megan Foley*
Project Role:	*Research Assistant I*
Nearest person month worked:	*6*

Contribution to Project:	*Ms. Foley prepares the samples for RNA, spheres for GPA, cultures for the UDS assay. She runs the UDS assay and analyzes the UDS data (which involves coordinating the efforts of students and interns). She is learning how to analyze RNAseq data.*

Name:	*XiaoLu Jin*
Project Role:	*Research Assistant I*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Jin prepares samples and performs the flow cytometric GPA assay. She is learning how to analyze RNAseq data.*

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

No senior/key personnel reported changes in their support.

What other organizations were involved as partners?

Dr. Klimas' group is recruiting patients and controls through two additional partnering institutions:

- **Organization Name:** Miami VAMC
- **Location of Organization:** Miami, FL
- **Partner's contribution to the projec**t: collaboration

- **Organization Name:** Boston University (Dr. Kim Sullivan)
- **Location of Organization:** Boston, MA
- **Partner's contribution to the projec**t: collaboration

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Elevated somatic mutation and evidence of genomic instability in veterans with Gulf War illness☆

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ABSTRACT

Aims: Gulf War illness (GWI) is thought to be associated with exposures experienced by soldiers deployed in the 1991 Gulf War. A major question is how these exposures continue to influence the health of these individuals three decades later. One potentially permanent effect of such exposures is the induction of genetic mutations. We investigated whether veterans with GWI exhibited persistently elevated levels of somatic mutation.

Materials and methods: We applied the blood-based glycophorin A (*GPA*) somatic mutation assay to a cohort of veterans diagnosed with GWI and a set of both concurrent and historic age-matched controls. This assay quantifies red blood cells with a phenotype consistent with loss of one allele at the genetic determinant for the MN blood group, the *GPA* gene.

Key findings: As a population, those affected with GWI exhibited an uninduced mutation frequency at the *GPA* locus that was effectively twice that observed in controls, a result that was statistically significant. This result was influenced by an increase in the incidence of individuals with aberrantly high mutation frequencies, seemingly higher than would be expected by dose extrapolation and consistent with the induction of localized genomic instability in the hematopoietic bone marrow stem cells. When these "outliers" were removed from consideration, the remaining affected population retained a significantly higher mean allele loss mutation frequency, suggesting that both dose-dependent bone marrow genotoxicity and induction of genomic instability are contributing to the elevation in mutation frequency in these affected veterans.

Significance: This study provides evidence that manifestation of GWI is associated with increased cumulative exposure to agents capable of inducing persistent mutations in bone marrow stem cells. Whether these mutations are involved in the clinical aspects of the condition or are simply biomarkers of overall exposure has yet to be determined. The increased incidence of genomic instability suggests that this persistent mutation can have important delayed effects on cellular integrity.

1. Introduction

Gulf War illness (GWI) is a life-altering condition, affecting up to one-third of Gulf War veterans, characterized by long-term and persistent symptoms that include fatigue, headaches, memory problems, musculoskeletal pain, and respiratory, gastrointestinal and dermatologic complaints [1–3]. Personnel deployed to the Gulf War experienced a plethora of potentially debilitating exposures, including to heat, sun, sand, dust and particulates, vaccinations, oil well fires, chemical and biological weapons, depleted uranium, noise, chemical agent resistant coating (CARC) paint, pyridostigmine bromide, pesticides, toxic embedded fragments, infectious diseases (although these

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were limited by pesticide use), and other occupational hazards, not including biological weapons [4,5]. Many studies have attempted to find associations between individual exposures and occurrence or aspects of GWI [5,6], such as exposure to depleted uranium and somatic mutation at the X-linked *HPRT* gene [7]. In contrast, our approach, informed by our experience with multistep carcinogenesis [8], hypothesizes that GWI is a product of the cumulative exposure of these individuals, especially to exposures known to have persistent lifetime effects.

One potentially persistent effect of environmental exposures that can be cumulatively measured is genotoxicity, the induction of permanent changes in the DNA sequence of affected individuals. There are a number of methods for detecting and quantifying cumulative lifetime mutagenesis [9–14], but one of the most widely applied technologies is the blood-based glycophorin A (*GPA*) assay [15]. The *GPA* assay flow cytometrically analyzes red blood cells labeled with allele-specific monoclonal antibodies, counting cells with aberrant phenotypes consistent with loss of one allele at this highly polymorphic blood group locus. It has been applied to many environmental, occupational, accidental and medical exposures to radiation and chemicals; significantly to survivors of the atomic bombing of Hiroshima and Nagasaki, where it demonstrated dose-dependent increases over 40 years after the events [16,17].

We therefore applied the *GPA* somatic mutation assay to veterans affected with GWI, to determine whether they exhibited evidence of significantly elevated genotoxic exposure that may be indicative of and/or related to their clinical symptoms.

2. Human samples and mutational analysis

We obtained fresh blood samples from 52 veterans diagnosed with GWI by the Kansas criteria [18] from ongoing studies at Boston University and the Miami VA [19,20]. After typing, 26 of these samples were heterozygous MN at the *GPA* locus, ideal for identification of variant cells that have lost expression of one allele. Three concurrent normal controls were also obtained, as well as one asymptomatic veteran of the Gulf War; all of these were typed before draw to ensure they were MN heterozygotes. Blood samples were processed and analyzed for *GPA* mutation frequencies by established methods [21], except as adapted to the use of a commercial BD Acurri C6 flow cytometer (BD, Franklin Lakes, NJ). The *GPA* assay can quantify variants arising by both allele loss and allele loss and duplication mechanisms; this report concerns only the allele loss variants.

Due to a deficiency of control samples, we returned to our sets of historical controls [22–26] and identified a total of 170 age-appropriate (older than 50 years at time of sampling) individuals as comparators for our affected GWI veterans.

3. Results and discussion

A comparison of the mean *GPA* allele loss mutation frequencies observed in our GWI and control populations in given in Fig. 1. The controls for this study, 170 age-matched historical controls, 3 concurrent controls (2 in the correct age range) and 1 asymptomatic Gulf War veteran exhibited an average allele loss mutation frequency of $11.6 \pm 1.5 \times 10^{-6}$ (mean ± standard error). This is higher than has previously been reported for control populations [22–26], but consistent with their age, considering the known increase in *GPA* mutation frequency with age that has been observed in previous studies [27,28]. The corresponding mean allele loss frequency in the affected population was almost 2-fold higher, at $22.5 \pm 4.0 \times 10^{-6}$, which was statistically significantly using a *t*-test assuming equal variances.

In previous studies of chemotherapy-induced mutation, we found two distinct mechanisms of action [22,29]. The first was a transitory induction that completely disappeared approximately 3 red blood cell lifetimes after exposure. We interpret this as occurring through mutagenesis of proliferating and differentiating hematopoietic progenitor

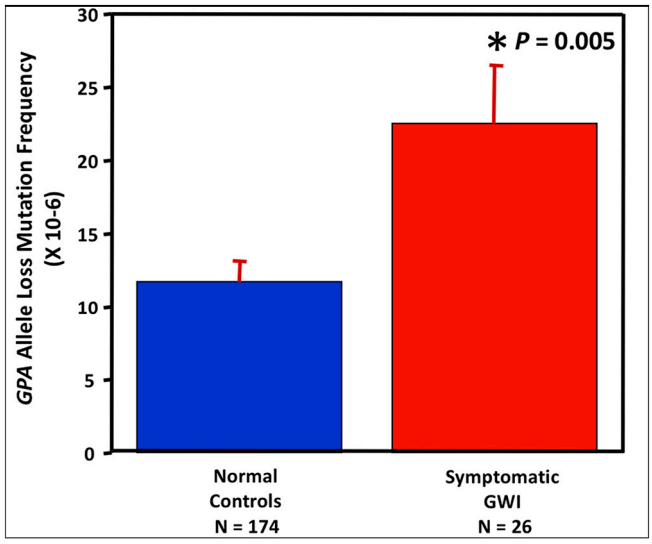

Fig. 1. *GPA* allele loss somatic mutation frequencies in symptomatic GWI veterans and controls.

cells that have committed to terminal differentiation. In some cases, however, mutation frequencies never completely returned to baseline, suggesting that the exposure has affected the relatively quiescent bone marrow stem cells [26,30]. In studies where the exposure occurred long ago, we are relying on the latter effect to provide a lifelong indicator of genotoxicity. In one such study, the evaluation of survivors of the atomic bombing of Japan [16], although a dose-dependent increase in *GPA* allele loss somatic mutation was observed, it was also noted that there was much greater variability in this population than has been seen in other studies. One factor was acknowledged to be the variability in dose due to the limitations of dose reconstruction, and another was thought to involve the stochastic effects of natural reductions in the size of the pool of bone marrow stem cells with age [31]. Both of these factors are relevant to the present study. The increased mutational variability in the population from Hiroshima and Nagasaki largely manifested itself as an increase in the number of individuals with very high mutation frequencies, which we have labeled as "outliers." We have since recognized that such outliers occur in all normal populations and that their prevalence is also highly age-dependent, with an exponential increase starting at about age 45 that peaks around age 65 [32], reminiscent of the age-associated incidence of most solid tissue cancers [33]. We have also found that elevated mutation frequencies consistent with this "outlier" phenotype are characteristic of the DNA repair-deficient, cancer-prone and/or premature aging syndromes Bloom syndrome [34], ataxia telangiectasia [35], Fanconi anemia [36] and Werner syndrome [37]. We therefore suggest that these outliers are indicative of the development of genomic instability among bone marrow stem cells.

The original definition of an outlier was an individual with an allele loss mutation frequency of greater than the mean of the control population + three standard deviations [32], but in order to apply this threshold homogenously, we have standardized it to those with a mutation frequency of greater than 3×10^{-5} [36]. If we apply this threshold to our control and affected populations (Fig. 2), we can see that the proportion of high mutation frequency outliers is much greater in the GWI group than in controls, despite the fact that this age group already has the highest background prevalence of such individuals. Indeed, as shown in Table 1, the increased prevalence of outliers among the affected GWI population is statistically significant, and suggests that outliers are almost 7-fold more likely to appear in the affected population than unaffected.

The next question is, therefore, does this outlier effect completely account for the difference in somatic mutation between the affected and

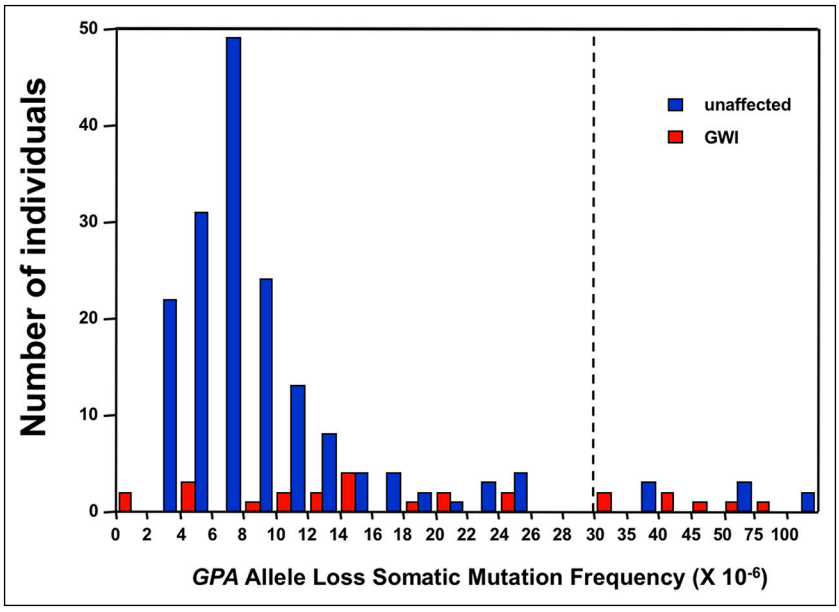

Fig. 2. Distribution of *GPA* allele loss somatic mutation frequencies in symptomatic GWI veterans and controls.

Table 1
Number of high *GPA* mutation frequency "outliers" identified in veterans affected with GWI and age-matched controls.

Allele loss	Mutation frequency <30[a]	Mutation frequency ≥ 30[a]	P[b]	OR (CI)[c]
Affected GWI	19	7		
Age-matched controls	165	9	0.0014	6.74 (3.61–12.62)

[a] $\times 10^{-6}$.
[b] Fisher's exact test.
[c] Odds ratio (confidence interval).

unaffected populations? To explore this question, we compared the two populations after removal of the outliers in each. The results, shown in Fig. 3, demonstrated that there is still a significantly elevated allele loss somatic mutation frequency in the remaining GWI population, $12.6 \pm 1.6 \times 10^{-6}$ versus $8.2 \pm 0.4 \times 10^{-6}$ ($P < 0.001$). Thus, the difference between the two populations seems to involve both greater persistent mutation in the bone marrow stem cells of the affected population, as well as an increased incidence of individuals with unusually high somatic mutation frequencies, perhaps due to focal development of genomic instability.

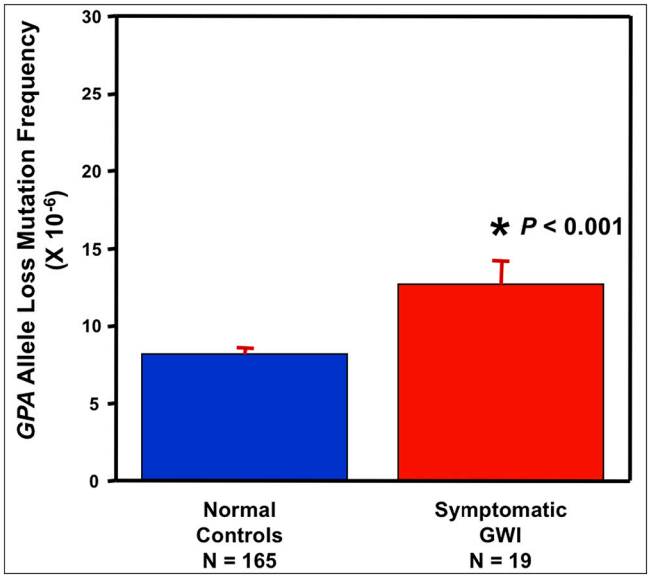

Fig. 3. *GPA* allele loss somatic mutation frequencies in symptomatic GWI veterans and controls after removal of high somatic mutation frequency "outliers".

Many of the agents encountered by military personnel deployed to the Gulf theatre are known to be genotoxic, including smoke from oil fires [38,39], pesticides [40,41], jet fuel [42,43], depleted uranium [7,44–46], and even noise [47]. We have suggested that the allele loss variants we quantified in our GWI population are representative of the long-term, persistent effects of some or all of the exposures they encountered in the Gulf, but another possibility is that they exhibit a hypersensitivity to mutational induction not unlike that seen with other endpoints attributable to chemical exposures [48]. In our previous work on mutational induction with chemotherapeutic agents [30] we found that the ability to induce mutations in differentiating vs. stem cells (or both) was agent-specific, such that we cannot say what subset of exposures we are detecting and quantifying under either scenario. However, it should be enough that there is a clear linkage between high allele loss mutation frequencies at the *GPA* locus and the occurrence of GWI.

High somatic mutation frequencies, especially "outlier" status, has previously been associated in normal populations with cancer incidence [49]. Current consensus on GWI is that there is no evidence of excess cancer incidence in this group [50–52]. In a previous study [53], we examined the DNA nucleotide excision repair capacity of some of our symptomatic Gulf War veterans, and were surprised to find that they actually had greater DNA repair capacity than controls. We attributed this to a persistent induction of DNA repair capacity consistent with both high initial exposure and a generalized alteration in homeostasis [54]. This persistently elevated repair capacity would both explain the lack of cancer in these individuals, reducing the frequency of the new mutations necessary to complete a carcinogenic pathway, and argue against a hypersensitivity model for our somatic mutation results.

This study would be stronger if our results were compared to a contemporaneous set of controls, ideally of veterans deployed to the Gulf who have not suffered with GWI. That would allow us to rule out the possibility that the mutational effects we have observed in veterans with symptomatic GWI are not common to all Gulf War veterans. Similarly, we would have liked to control for demographic factors

beyond age, such as tobacco smoking, which has been shown to induce somatic mutation at the *GPA* locus [55–58] and was found to be an important confounding variable in a previous occupational study [59]. Surveys of Gulf war veterans suggest that about 40–50% are present or former smokers [60,61], and that the proportion is similar among those with GWI [62]. Since our historic control population were all collected between 1990 and 2000, a large proportion, 63%, identified as either present or former smokers, suggesting that controlling for a smoking effect might actually increase the disparity in somatic mutation in the GWI cohort.

4. Conclusion

Gulf War veterans with symptomatic GWI exhibit persistently elevated allele loss somatic mutation frequencies consistent with increased exposure to agents genotoxic to bone marrow stem cells. A concurrent increase in the prevalence of high somatic mutation frequency outliers further suggests a greater incidence of focal genomic instability in this population. Thus, our data suggest that GWI veterans have elevated somatic mutation via two mechanisms: a simple mechanism of increase, mutational induction in long-lived bone marrow stem cells, and a more esoteric mechanism, the induction of genomic instability in some stem cells.

CRediT authorship contribution statement

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Declaration of competing interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Authors thank Nova Southeastern University for supporting this work with a President's Faculty Research and Development Grant, as well as the Department of Defense Gulf War Illness Research Program through grant W81XWH-16-1-0678. S.G.G. and J.J.L. conceived of this project, samples were procured by N.G.K. and K.S., sample preparation and analysis were performed by O.M.I. and X.L.J. and data were analyzed and the manuscript was written by S.G.G. The NSU HPD Research Committee is also gratefully acknowledged for ongoing support of equipment maintenance.

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