

# Sex-Specific Differences In Plasma Lipid Profiles Are Associated With Gulf War Illness

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## Research Article

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# Abstract

**Background:** Nearly 250,000 veterans from the 1990-1991 Gulf War have Gulf War Illness (GWI), a condition with heterogeneous pathobiology that remains difficult to diagnose. As such, availability of blood biomarkers that reflect the underlying biology of GWI will help clinicians provide appropriate care to ill veterans. In this study, we measured blood lipids to examine the influence of sex on the association between blood lipids and GWI diagnosis.

**Methods:** Plasma lipid extracts from GWI (n=100) and control (n = 45) participants were subjected to reversed-phase nano-flow liquid chromatography-mass spectrometry analysis.

**Results:** An influence of sex and GWI case status on plasma neutral lipid and phospholipid species was observed. Among male participants, triglycerides, diglycerides, and phosphatidylcholines were increased while cholesterol esters were decreased in GWI compared to controls. In female participants, ceramides were increased in GWI cases compared to controls. Among male participants, unsaturated triglycerides, phosphatidylcholine and diglycerides were increased while unsaturated cholesterol esters were lower in GWI cases compared to controls. The ratio of arachidonic acid- to docosahexaenoic acid-containing triglyceride species was increased in female and male GWI cases as compared to their sex-matched controls.

**Conclusion:** Differential modulation of neutral lipids and ratios of arachidonic acid to docosahexaenoic acid in male veterans with GWI suggest metabolic dysfunction and inflammation. Increases in ceramides among female veterans with GWI also suggest activation of inflammatory pathways. Future research should characterize how these lipids and their associated pathways relate to GWI pathology to identify biomarkers of the disorder.

## Introduction

Approximately 30% of the 700 000 veterans of the 1991 Gulf War (GW) suffer from Gulf War Illness (GWI), a chronic multi-symptom condition characterized by cognitive impairment, fatigue, and pain(1). Veterans' exposure to chemical warfare agents, pesticides, and pyridostigmine bromide are among the major contributors to GWI etiology (1–4). Currently, there are no biomarkers for indicating the underlying pathology of this condition. Advances in GWI research suggest lipid dysfunction, bioenergetics deficits, and inflammation as key contributors to its ongoing pathology(5, 6). Blood biomarkers of these biological processes would be a cost-effective and minimally invasive avenue to assist clinicians with accurate detection of GWI.

Evidence suggests that GW pesticides affect cellular lipid metabolism and lipid homeostasis(2, 7–11). Altered lipid homeostasis can result in an abnormal accumulation of lipids that promote cellular inflammation (12, 13). Abnormal plasma and brain lipid profiles after GW pesticide exposure in rodents and in veterans with GWI have been shown to accompany inflammation, neurobehavioral and

bioenergetic deficits(14–18). As such, altered plasma lipid profiles may provide a biomarker signature for the bioenergetic deficits and inflammation associated with GWI.

It is expected that since the pesticides to which GW veterans were exposed tend to disturb lipid homeostasis, owing to their high affinity for lipids, neutral lipids classes, such as triglycerides (TG), diglycerides (DG), and cholesterol esters (CE), and phospholipid (PL) classes would be affected in GWI (2, 9–11, 19, 20). The degree of unsaturation of the fatty acids within the PL classes is known to alter metabolic and inflammatory systems, with many saturated fatty acids (SFA)-containing PL positively associated with chronic metabolic conditions whereas polyunsaturated fatty acid (PUFA)-containing PL are associated with inflammation (21–24). Among these, PUFA containing the omega-3 fatty acid, docosahexaenoic acid (DHA), generate anti-inflammatory or inflammation-resolving lipid metabolites and PUFA containing the omega-6 fatty acid, arachidonic acid (AA), can produce metabolites that promote pro-inflammatory responses(23–25).

Changes in lipid profiles were also expected in relation to the sex of GW veterans, as many classes of plasma lipids are significantly associated with sex(26). Women are up to four times more likely than men to be diagnosed with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), a chronic multi-symptom disease clinically similar to GWI that has been shown to have sex-linked differences in blood lipid profiles(27, 28). Female GW veterans have a higher prevalence of GWI diagnosis and severe GWI-related symptoms than male GW veterans (29). These findings suggest potential sex-linked differences in pathophysiological responses to GW-era chemical exposures, necessitating sex-stratified analyses.

We hypothesized that blood lipid levels would be affected both by GWI status and the sex of GW veterans. We evaluated the levels of several major blood lipid classes and their molecular species in three cross-sectional cohorts of participants classified as GWI or control. This study will further our understanding of plasma lipids as potential biological markers to track the ongoing inflammation and metabolic dysfunction of GWI, which may help to provide better care and management of this illness.

## **Materials And Methods**

### **Cohort Characteristics:**

All plasma utilized within this study were from the following from the following case-control veteran cohorts: (1) the Roskamp Neurology Clinic (RNC) cohort (n=36), (2) the Boston Gulf War Illness Consortium (GWIC), and (3) the Fort Devens cohort (n = 58) (3, 30, 31). Additional healthy control samples were provided by Dr. Klimas to supplement the control population of the study. GW veteran controls and the healthy civilian controls were grouped together for analysis in the GWIC cohort (n=51). In all cohorts, either the Center for Disease Control (CDC) chronic multi-symptom illness definition or the Kansas GWI criteria were used to diagnose GWI(32, 33). These criteria required that GW veterans must show symptoms in at least 3 of 6 symptom domains (fatigue/sleep problems, somatic pain, neurological/cognitive/mood symptoms, gastrointestinal symptoms, respiratory symptoms, and skin

abnormalities) for GWI diagnosis. Exclusionary criteria have been described previously, primarily that participants were excluded if they reported prior diagnosis with a confounding medical condition(17, 30, 31). Controls were either veterans deployed to the 1991 GW or healthy civilian controls, that did not meet the Kansas GWI criteria or any of the exclusionary criteria. All protocols were conducted in compliance with the relevant guidelines and regulations and were approved by institutional review boards (IRB) at each institution. Informed consent was obtained from all participants.

Data on the sex of the participants was gathered via self-report, with the options being either male or female. As per the National Institute of Health, sex refers to biological differences between males and females(34). As per the World Health Organization, gender refers to the characteristics of men and women that are socially constructed(35). Therefore, this paper evaluating biological lipid levels uses exclusively male/female terminology for participants rather than men/women terminology, as data on the gender of the participants was not collected.

Body mass index data was not available in all cohorts. Participant recruitment, blood collection, and blood storage procedures have been described previously(17, 18, 31). All samples were collected using the same written standard operating procedures for performing phlebotomy, plasma separation and aliquoting. All participants had fasted prior to blood collection. There was no requirement of time of collection.

Due to low numbers in ethnic groups other than Caucasian, we combined participants who were African American, Aleutian Eskimo, American Indian, Asian, Pacific Islander, multiracial or other races into one category, called Non-Caucasian. Only one African American veteran with GWI was present in the RNC cohort. Of the eleven African American participants in GWIC, three were control participants and eight were veterans diagnosed with GWI. Of the six African American participants in the Fort Devens cohort, four were control participants and two were veterans diagnosed with GWI.

## **Lipidomic Analysis and Quantification:**

Plasma samples were randomized prior to use via a group-block design, to ensure that the experimenters were blinded to participants' group membership and other characteristics. The extraction protocol and liquid chromatography-mass spectrometry (LC-MS) run settings have been previously described by our laboratory (18, 36). Briefly, lipid extracts from 10  $\mu\text{L}$  of plasma spiked with class-specific internal standards were resuspended in 200  $\mu\text{L}$  of 70:30 LC-MS mobile phase A:B (36, 37). Mobile phase A is composed of 42% water, 31% acetonitrile, 27% isopropanol, 10 mM ammonium formate, and 0.1% formic acid. Mobile phase B is composed of 90% isopropanol, 10% acetonitrile, 10 mM ammonium formate, and 0.1% formic acid. For LC-MS analysis, an Easy-nanoLC 1000 chromatography pumping system was used with a nanoflex electrospray ionization (ESI) source on a Thermo LTQ/orbitrap mass spectrometer.

Resuspended lipid extracts (4  $\mu\text{L}$ ) were injected onto an Acclaim PepMap<sup>TM</sup> 100 (75  $\mu\text{m}$   $\times$  2 cm, nanoViper, C18, 3  $\mu\text{m}$ , 100  $\text{\AA}$ ) trapping column attached to an Acclaim PepMap<sup>TM</sup> RSLC (75  $\mu\text{m}$   $\times$  15 cm, nanoViper, C18, 2  $\mu\text{m}$ , 100  $\text{\AA}$ ) analytical column for reversed-phase chromatographic separation of lipid

species. The chromatographic gradient started at 30% B, then increased linearly from 1 to 40 minutes from 50 to 98% B, plateauing at 98% B from 40 to 50 minutes, and then decreasing to 30% B for 50 to 65 minutes. All samples were run in triplicate, accompanied by a blank and a quality control sample in each run. Full-scan mass spectrometry (MS) data was acquired in positive ion mode, over a mass range of 300-2,000 at a resolution of 30,000. A maximum inject time of 200 ms was used with 6 microscans/acquired scan. Thermo Xcalibur Qual Browser was used to examine the individual chromatographic data of samples for lipid retention times. Lipid peak areas were integrated using Tracefinder™ software, using target compound lists of analytes of interest, identifying them based on m/z and retention time. Mass accuracy windows of 5 mmu were used. We detected several major classes of lipids including the phospholipid classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), the neutral lipid classes TG, DG, and CE and the sphingolipid classes sphingomyelin (SM), ceramides (Cer) and hexosylceramides (HexCer), which were quantified as previously described(28, 36). The quality control (QC) samples were then used to normalize between batches of data ([sample] x [batch QC/normalizing QC]). Once the coefficients of variance (CV) were calculated per class for the whole cohort, lipid species with an average CV > 25 were excluded from further analysis. Lipid species with less than 90% coverage were also excluded from our analyses.

To categorize lipids based on their degree of unsaturation, totals were calculated by adding up values for all lipid species measured belonging to that lipid class. Saturated fatty acids (SFA) were calculated by totaling the lipids with no double bonds; monounsaturated fatty acids (MUFA) were calculated by totaling the lipids with one double bond. Polyunsaturated fatty acids (PUFA) were calculated by totaling all measured lipid species with three or more double bonds. For lipid classes known to contain AA or DHA, their respective categorizations were made by totaling individual species known to contain the relevant type of fatty acid, as based on previous MS/MS confirmations.

## **Statistical Analysis:**

Chi-square and one-way analysis of variance (ANOVA) tests were used, as appropriate, to compare between the three cohorts and to compare GWI and healthy controls for descriptive purposes. Each lipid species was assessed for normality and those found to be skewed were log transformed for parametric analysis. All lipidomics data were analyzed using mixed linear modeling (MLM) as described previously to examine the independent effects of sex and GWI diagnosis as well as to account for random effects associated with repeated measurements in lipidomics and metabolomics datasets (17, 36, 38, 39). The Benjamini-Hochberg (B-H) correction procedure was performed to limit the false discovery rate to 0.05. The webtool MetaboAnalyst 5.0 was used to perform a hierarchical clustering analysis of the individual lipids with a coverage superior to 90%(40). Clusters were determined based on the similarity of lipids as expressed by the Euclidean distance. Lipids that were adjacent based on the Ward algorithm were combined in clusters and an ANOVA was applied to select the top 50 lipids significantly modulated by sex and diagnosis represented in the dendrogram and heatmap.

## **Results**

*Basic Demographics:*

General demographic characteristics of the three cohorts are presented in Table 1. The difference in ages between the RNC participants and the GWIC participants was found to be significantly different ( $p < 0.01$ ). The Fort Devens and GWIC cohorts were found to be significantly different for race ( $p < 0.05$ ). Overall, the GWIC cohort was younger and had a higher proportion of non-Caucasian participants than the other two cohorts (Table 1). The percentage of female participants ranged from 13.7% to 27.8% across the 3 cohorts (Table 1).

**Table 1: Descriptive table of demographic variables of the three cohorts: RNC, GWIC, and Fort Devens.**

The mean age and standard deviation (SD) are reported. Significant differences are indicated by either single asterisks ( $p < 0.05$ ) or double asterisks ( $p < 0.01$ ).

Cohort	RNC	GWIC	Fort Devens
<b>Total No.</b>	36	51	58
<b>GWIC cases, No. (%)</b>	31 (86.1%)	31 (60.8%)	38 (65.5%)
<b>Mean Age in years (SD)</b>	55.4 (1.2)	50.3 (1.1)	55.9 (1.2)
<b>Sex, No. (%)</b>	**		
<b>Female participants</b>	10 (27.8%)	7 (13.7%)	13 (22.4%)
<b>Male participants</b>	26 (72.2%)	44 (86.3%)	45 (77.6%)
<b>Race, No. (%)</b>	*		
<b>White/Caucasian</b>	31 (86.1%)	34 (66.7%)	50 (86.2%)
<b>Non-Caucasian</b>	5 (13.9%)	17 (33.3%)	8 (13.8%)

When all cohorts were combined (Table 2), there were no significant differences for age and race between GWI cases and controls. However, the sex ratio was found to be significantly different between GWI cases and controls, as 16% of GWI cases were female compared to 31% of controls ( $p < 0.05$ ). All subsequent analyses were performed by stratifying GWI cases and controls by sex.

**Table 2: Descriptive table of demographic variables of GWI cases and controls from the RNC, GWIC, and Fort Devens cohorts combined.** Significant differences are indicated by single asterisks ( $p < 0.05$ ).

	<b>GWI Cases</b>	<b>Controls</b>
<b>Total No.</b>	100	45
<b>Mean Age in years (SD)</b>	53.07 (7.09)	55.57 (10.83)
<b>Sex, No. (%)</b>	*	
<b>Female participants</b>	16 (16.0%)	14 (31.1%)
<b>Male participants</b>	84 (84.0%)	31(68.9%)
<b>Race</b>		
<b>White/Caucasian</b>	80 (80.0%)	35 (77.8%)
<b>Non-Caucasian</b>	20 (20.0%)	10 (22.2%)

*Sex-specific differences were observed for the degree of unsaturation and AA and DHA content of several major classes of lipids in veterans with GWI*

Figure 1 shows heatmaps of plasma profiles of eight different lipid classes that are differentially affected by sex and diagnostic differences among male and female participants. The total amount of PC, DG and TG were significantly increased while the total amount of CE was significantly decreased in male GWI compared to male controls (Figure 1A). Among female participants, only total Cer were significantly increased in GWI compared to controls. We also evaluated the degree of fatty acid unsaturation in individual lipid species since neutral lipids are considered reservoirs of PUFA lipids (Figure 1B). Compared to male controls, male veterans with GWI had a significant increase in the unsaturated lipids, consisting of DG-PUFA, TG-MUFA, TG-PUFA and PC-MUFA, while CE-PUFA was significantly decreased. Among female participants, TG-SFA levels were significantly lower in GWI compared to controls. The PUFA-containing lipid species were further grouped by their AA or DHA-content for each lipid class (Figure 1C). Levels of DG-AA, DG-DHA, TG-AA and TG-DHA were significantly increased while CE-AA and CE-DHA were significantly decreased in male GWI cases compared to male controls. The ratios of PE-AA/PE-DHA and TG-AA/TG-DHA were both significantly increased in male GWI compared to male controls. The TG-AA/TG-DHA ratio was also significantly increased in female GWI compared to female controls.

*Hierarchical clustering analysis identifies sex-specific lipid species signature of GWI*

Hierarchical clustering analyses identified two major clusters (cluster 1 and cluster 2) within the top 50 lipid species significantly modulated by sex and diagnosis (Figure 2). Lipids within both clusters were affected by sex differences, irrespective of GWI diagnosis. Cluster 1 was primarily composed of the neutral lipid class CE and the sphingolipid class SM and showed a phenotype of increased levels in female GWI and decreased levels in male GWI. Cluster 2 was primarily composed of the neutral lipid classes DG and TG which were increased in male participants compared to female participants, irrespective of GWI diagnosis. Lipids associated with each cluster further separated into 3 subgroups. The CEs of group A show controls to be generally similar regardless of sex while the lipids differed in

concentration in the GWI veterans based on sex. Lipids associated with groups B and E were both principally influenced by sex regardless of the diagnostic status. Group B was composed of an ether PC and SM lipids, while Group E was composed of DGs and TGs. Lipids in group C differed between female GWI and female controls. In group D, neutral lipids of the male and female GWI groups were both increased compared to their sex-matched controls. Both group D and E were composed of the neutral lipids, DG and TG, both notably differed in their range of degree of unsaturation, with lipids in group D consisting of more double bonds and those in group E consisting of fewer double bonds. Group F consisted of 2 lipid species that are atypical of any other group, but do not match each other either. PE34:2 is highly increased in the female controls and decreased in all other groups, while TG 46:0 is highly decreased in the female GWI group and increased in all other groups.

Of the top 50 lipids significantly modulated by sex and diagnosis, more lipid species were significantly modulated in male GWI cases (Figure 3A) than in female GWI cases (Figure 3B) as compared to their respective sex-matched controls. Among male participants, TG and DG were significantly increased by 1.18-fold at minimum (TG 58:9,  $\log_2FC=0.24$ ) and all CEs were significantly decreased by 0.88-fold at minimum (CE 20:3,  $\log_2FC=-0.19$ ) in GWI veterans versus controls. A few TGs and SMs were significantly increased by a minimum of 1.18-fold change (TG 56:8,  $\log_2FC=0.24$ ) in female GWI participants compared to female controls, except for TG 46:0, which was significantly decreased by 0.37-fold ( $\log_2FC=-1.45$ ).

## Discussion

In this study, we analyzed plasma lipid profiles of veterans with GWI and control participants. Most of the differences pertained to the neutral lipids (CE, TG and DG), where the degree of unsaturation and ratios of AA-to-DHA were changed in GWI compared to controls. Our study shows that male veterans with GWI had significant increases in total neutral lipid content compared to male controls. Female veterans with GWI had elevated levels of Cer and lower levels of SFA TG species as compared to female controls. These sex-specific plasma lipid compositions in GWI veterans require further attention as they may indicate progressive metabolic and inflammatory processes, particularly in male veterans with GWI.

In the plasma, neutral lipids primarily appear in lipoprotein compartments due to their hydrophobic nature. The neutral lipid class of triglycerides are major dietary fats, but they are also synthesized in the liver (41). Because dietary TGs are digested to free fatty acids and monoglycerides in the intestines, plasma TG levels within lipoprotein particles are reflective of TG synthesis in liver via acylation with DG (42). In plasma, an excess of TG, such as seen in the male veterans with GWI, is thought to reflect overproduction of very low-density lipoprotein (VLDL) corresponding with the production of CE-depleted low-density lipoprotein (LDL) particles (41). This production of CE-depleted LDL may explain the significant decrease in CE in male veterans with GWI. In the current study, female participants, irrespective of their diagnosis, had lower levels of TG and DG compared to their male counterparts. This has also been reported by the Genetics of Lipid-Lowering Drugs and Diet Network (GOLDN) study of plasma lipidome, who attributed this to normal sex-linked lipid differences (26). In terms of the clinical

implications of elevated TG in male veterans with GWI, elevated plasma TG is thought to be a risk factor for cardiovascular disease(43). This may require further monitoring by clinicians as veterans with GWI age. Elevated TG and the generation of CE-depleted LDL particles are also the defining features of dyslipidemia associated with insulin resistance and type 2 diabetes mellitus (41).

Veterans with GWI may be more sedentary and exercise less than healthy controls due to their condition, so this TG increase could reflect heightened body weight in veterans with GWI. A positive association between high TG and elevated body mass index (BMI) has been previously reported (44, 45). As early as 1995, it was observed that veterans with severe GWI had slightly higher BMI than non-cases (33). Unfortunately, not all the GWI cohorts in this study collected BMI data and so we cannot control for BMI as a covariate in this study. A recent health survey of GW veterans from the Fort Devens cohort suggested increases in the prevalence of high blood pressure, diabetes, and cardiovascular disease over the last 30 years (4). The Fort Devens Cohort was also assessed to have significantly higher rates of diabetes in both male and female GW veterans than the general population (46). Collectively, these studies suggest that a regular monitoring of blood lipids is required to better assess their contributions to the risk of developing cardiovascular disease and metabolic disorder among veterans with GWI.

In the current study, SFA-containing TG species were decreased in female veterans with GWI. The role of SFA in promoting cardiovascular disease remains controversial as the source of these SFA-containing lipids and the individual identity of each SFA influences their potential role in promoting cardiovascular disease (21, 47). As the SFA-TG species measured ranged from TG 46:0 to TG 54:0, and therefore consisted of 46 to 54 carbons split amongst 3 fatty acid chains, it is likely that the SFA-TG species measured contained long-chain fatty acids, which are over 12 carbons in length (47). However, their significance in the clinical presentation of female GWI is unclear. Future studies aimed at different aspects of dietary modification in male vs. female GW veterans may be required to better understand the role of SFA-containing TG species in the health of veterans with GWI.

Male GW veterans with GWI had elevated levels of PUFA within the DG and TG classes. Since omega-6 AA or omega-3 DHA comprise a large proportion of PUFA, we focused on lipids that contain AA and DHA as their fatty acid side chains. Our previous studies have shown that AA and DHA content within certain PL classes were associated with GWI (17). We now show that ratios of AA-to-DHA were increased in TG among male and female GW veterans with GWI. The ratio of AA-to-DHA was also increased in PE among male GWI veterans. As AA and DHA compose 50% and 40% of brain PUFAs respectively, sufficient AA is necessary for the growth, repair, and maintenance of neurons and DHA is used in neurotransmission (48). However, AA metabolites, such as prostaglandins, are known to activate pro-inflammatory pathways whereas DHA metabolites, such as resolvins, contribute to inflammation resolution pathways (24, 49). In addition, studies have shown that patients with a low DHA/AA ratio, (i.e. a higher AA-to-DHA ratio) had a higher risk of acute coronary syndrome than those with a high DHA/AA ratio, and this was significant for men in particular (50). These changes in AA and DHA composition could reflect a generalized pro-inflammatory state in veterans with GWI, which would be consistent with reports of markers of chronic-low grade inflammation being associated with GWI diagnosis (51, 52).

Another indication of pro-inflammatory states in GWI would be the increase in Cer in female GWI veterans, as ceramides are known to stimulate the production of pro-inflammatory cytokines, such as IL-6 and TNF- $\alpha$ , in macrophages and are correlated with IL-6 levels in serum (53, 54). Production of long-chain ceramides is known to induce apoptosis (55). The increase in the long chain ceramides measured in this study (d32:0-44:2) may therefore indicate increased cell death in female GWI veterans. Boon and colleagues also found that ceramide elevation is another marker of the dyslipidemia of type 2 diabetes mellitus and promotes insulin resistance (53).

This study provides key information about blood biomarkers associated with adverse metabolic and inflammation-related lipid profiles. However, there are certain limitations which restrict the generalizability of our findings to the current cohort only. First, due to a lack of availability of BMI data across cohorts, we were unable to adjust for BMI differences as a potential confounder between male and female veterans. However, as our findings are largely consistent with previous studies showing sex-specific differences in lipid profiles, it is therefore unlikely that BMI differences significantly confound this relationship and may be inherent to lipid dysfunction. Another limitation of our study includes unavailability of dietary data and medication data, particularly on the use of statins and omega-3/fish oil supplements. Since statins lower TG levels in blood and our findings suggest increases of TG in male veterans with GWI, we anticipate that it is unlikely that statin use affected our study results. Similarly, we have previously shown that AA-to-DHA ratios are decreased after DHA supplementation (39). Since we observed an increase in AA-to-DHA ratios in male GWI veterans, it is similarly unlikely that omega-3 or fish oil supplement was a confounder in our study. As CE levels were lower in male GWI veterans, measuring free cholesterol would be useful in future studies to determine if either the level of free cholesterol or the rate of cholesterol esterification had decreased. Unfortunately, we could not reliably detect the labelled free cholesterol in our assay but will correct this in future studies. While we have previously examined free fatty acids in a smaller cohort of GW veterans, which suggested that free forms of PUFA were indeed affected in GWI (18), a large study evaluating free fatty acids and their bioactive lipid metabolites will help better elucidate the role of these fatty acids in ongoing inflammatory processes associated with GWI.

## Conclusion

This study identifies distinct sex-specific plasma lipid profiles associated with GWI diagnosis, with significantly elevated TG and DG and lowered CE as a distinctive pattern in male GW veterans with GWI. Triglyceride AA-to-DHA ratios were also increased in both male and female GW veterans with GWI. Since these lipid changes are associated with metabolic syndrome and inflammation, our findings suggest veterans with GWI have maladaptive biological processes that involve lipid transport and metabolism. While mechanistic work is needed to better elucidate lipid dysfunction in GWI, these blood lipid signatures could aid clinicians in tracking ongoing inflammation and metabolic dysfunction associated with GWI. As such, further research is needed to characterize how these lipids and their associated pathways relate to GWI pathology to identify potential biomarkers for tracking general health related outcomes in GWI.

# Abbreviations

GW: Gulf War Illness

GW: Gulf War

TG: Triglycerides

DG: Diglycerides

CE: Cholesterol esters

PL: Phospholipids

SFA: Saturated Fatty Acids

PUFA: Polyunsaturated fatty acids

DHA: Docosahexaenoic acid

AA: Arachidonic acid

ME/CFS: myalgic encephalomyelitis/chronic fatigue syndrome

RNC: Roskamp Neurology Clinic

GWIC: Gulf War Illness Consortium

CDC: Center for Disease Control

LC-MS: Liquid chromatography-mass spectrometry

ESI: electrospray ionization

MS: Mass spectrometry

PC: Phosphatidylcholine

PE: Phosphatidylethanolamine

SM: Sphingomyelin

Cer: Ceramides

HexCer: Hexosylceramides

QC: Quality control

CV: Coefficients of Variance

MUFA: monounsaturated fatty acids

ANOVA: Analysis of variance

MLM: mixed linear modeling

B-H: Benjamini-Hochberg

SD: Standard deviation

VLDL: very low-density lipoproteins

LDL: low-density lipoproteins

GOLDN: Genetics of Lipid-Lowering Drugs and Diet Network

BMI: Body mass Index

## **Declarations**

### *Ethics approval and consent to participate*

An informed consent was collected for each participant and was reviewed the IRB of each respective site

### *Consent for publication*

Not applicable

### *Availability of data and materials*

Not applicable

### *Competing interests*

Not applicable

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### *Authors' contributions*

Sarah Oberlin: Conceptualization, Investigation, Formal Analysis, Writing – Original Draft, Writing – Review and Editing, Visualization, Data Curation  
Aurore Nkiliza: Formal Analysis, Writing – Original Draft, Writing – Review and Editing, Visualization

Megan Parks: Formal Analysis, Writing – Original Draft, Writing – Review and Editing, Visualization

James E. Evans: Methodology, Formal Analysis, Data Curation, Writing – Review and Editing,

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Fiona Crawford: Conceptualization, Methodology, Funding acquisition, Investigation, Resources, Supervision, Project administration, Writing – Review and Editing,

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*Data Availability Statement:* The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

*Disclaimer:* The contents of this poster do not represent the views of the Department of Veterans Affairs or the United States Government.

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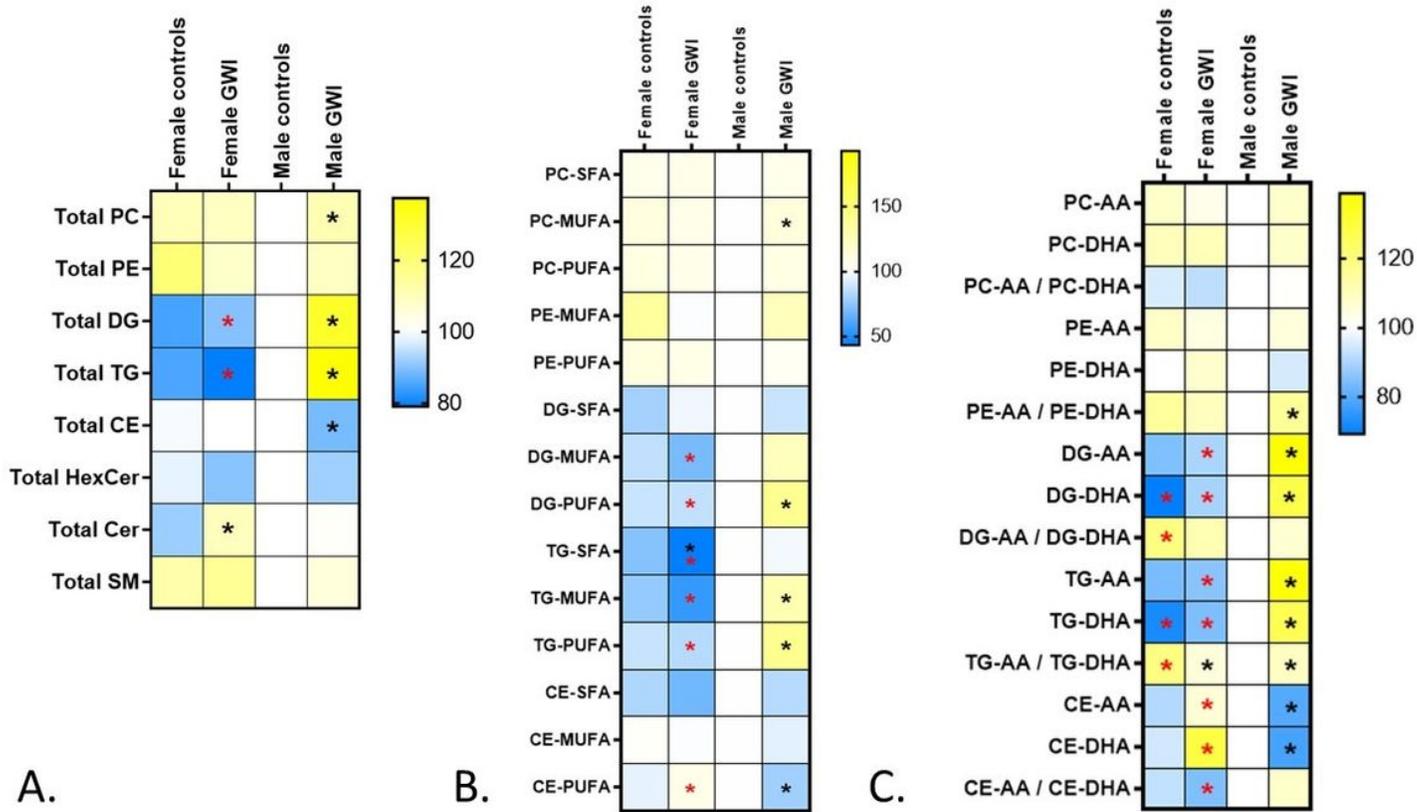
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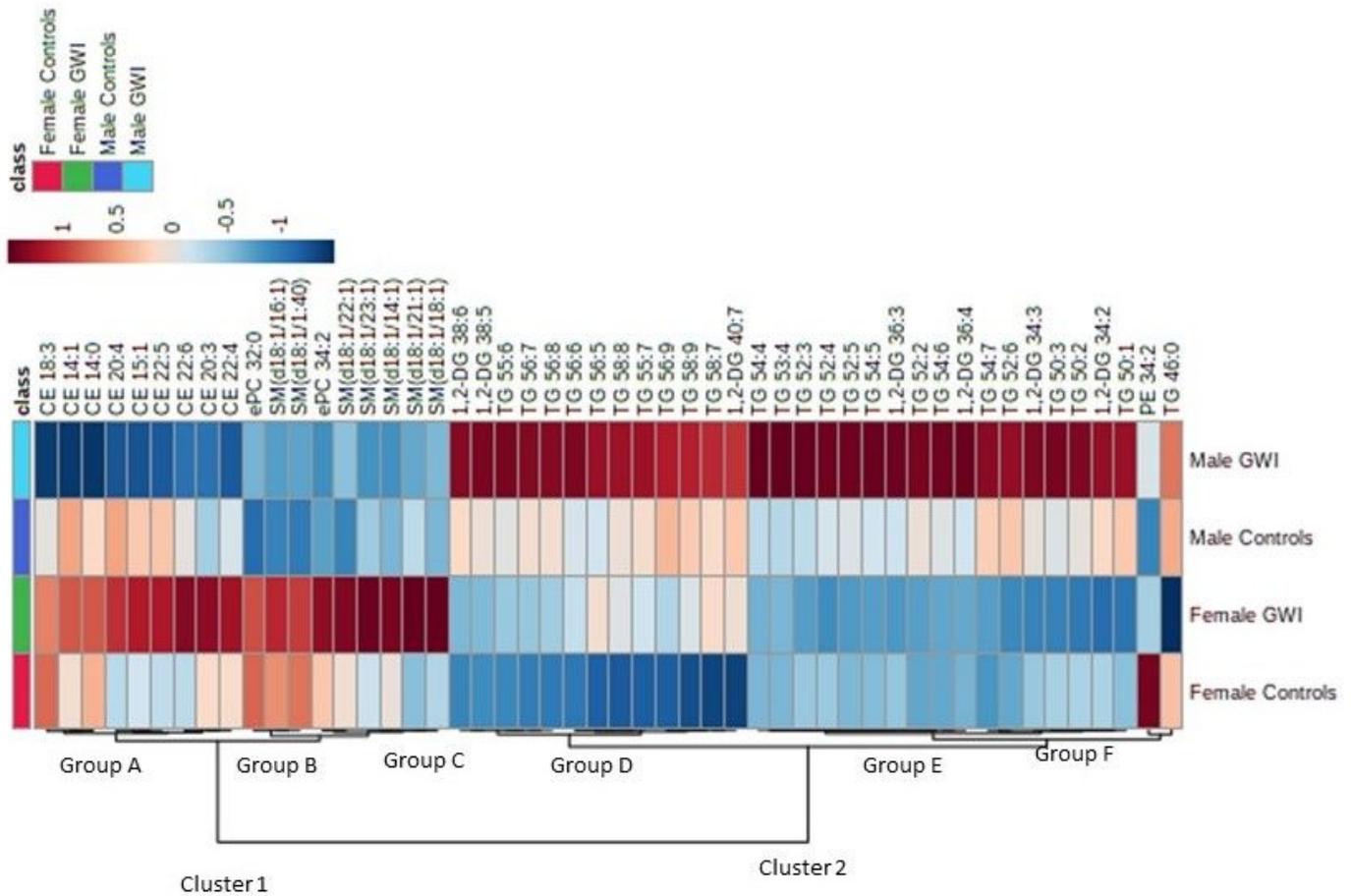
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## Figures



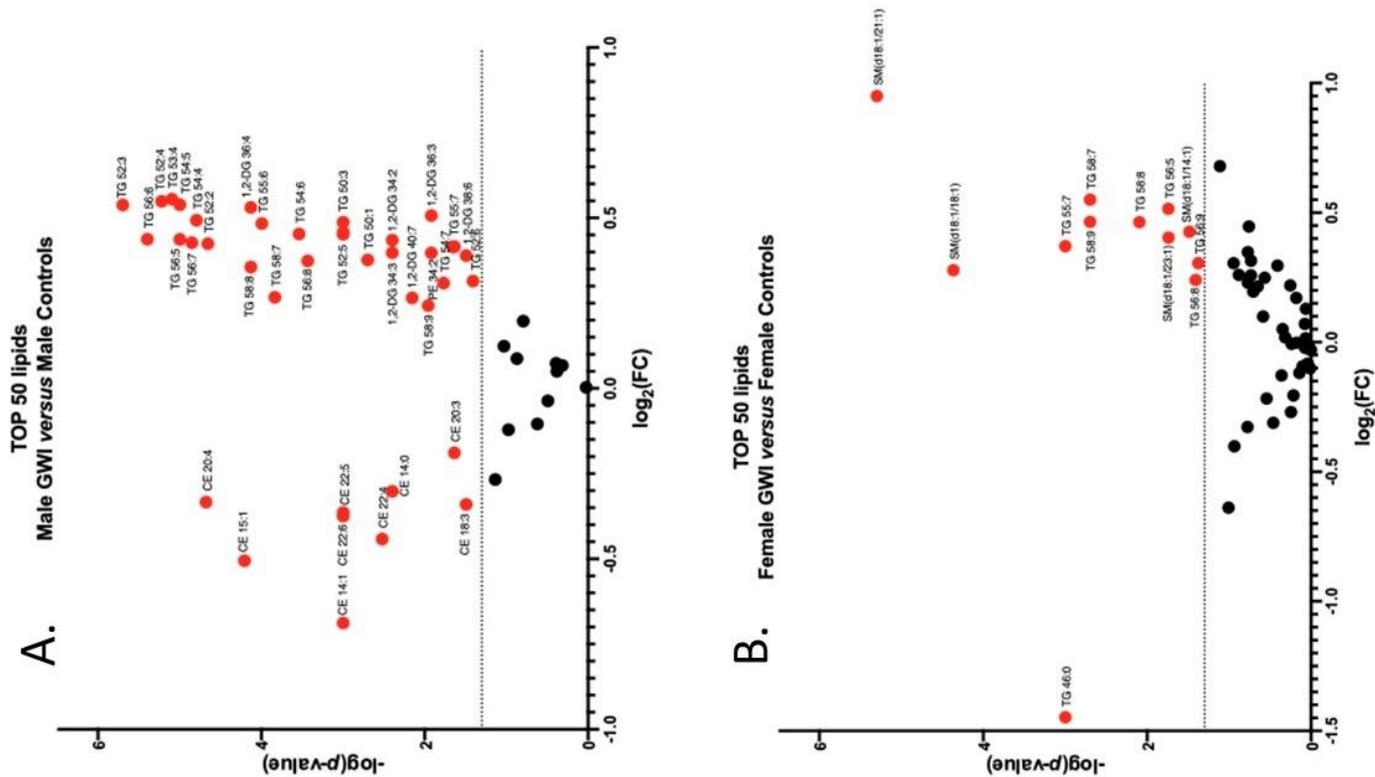
**Figure 1**

Heatmap visualization of phospholipids and neutral lipids in plasma of GWI cases and controls. The heatmaps represent the average concentrations relative to male controls of the (A) total level of each lipid classes, (B) lipids stratified by their degree of unsaturation and (C) lipids containing arachidonic acid (AA) or docosahexaenoic acid (DHA) as well as their AA-containing lipids/DHA-containing lipids ratio in each group. Black asterisks (\*) indicate significant differences between GWI cases and their sex-matched controls ( $p < 0.05$ ). Red asterisks (\*) indicate significant differences between males and females within the same diagnostic group ( $p < 0.05$ ).



**Figure 2**

Hierarchical clustering of the top 50 lipids. The relative concentration value of each lipid from the top 50 lipids significantly modulated by sex and diagnosis identified by MetaboAnalyst has been projected onto the heatmap for each group. The color legend identifies in red the lipids with higher concentration and identifies in blue the lipids with lower concentration.



**Figure 3**

Fold Change of the top 50 lipids by sex. Relative changes of the top 50 lipids significantly modulated by sex and diagnosis in males (A) and females (B) between GWI participants and GW cases are shown by fold change (FC) on the volcano plots. Each dot represents one individual lipid from the list of top 50 lipids identified by MetaboAnalyst. The red dots (•) indicate the significant changes relative with p-value threshold set at 0.05 (horizontal dotted line).