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A pilot reverse virtual screening study suggests toxic exposures caused long-term epigenetic changes in Gulf War Illness

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ABSTRACT

Gulf War Illness (GWI) is a chronic illness that affects upward of 32% of deployed Veterans to the 1991 Gulf War (GW). The symptoms are medically unexplained, ranging across cognitive deficits, fatigue, gastrointestinal problems, and musculoskeletal pain. Research indicates that chemical warfare agents play a key role in the onset and progression of GWI. The Khamisiyah ammunition storage that housed chemical warfare agents such as sarin, an acetylcholinesterase (AChE) inhibitor, was demolished during the GW, releasing toxicants into the atmosphere affecting deployed troops. Exposure to other chemical agents such as pyridostigmine bromide, N,N-diethyl-m-toluamide, permethrin and chlorpyrifos, were also prevalent during the war. These additional chemical agents have also been shown to inhibit AChE. AChE inhibition induces an acetylcholine build-up, disrupting signals between nerves and muscles, which in high doses leads to asphyxiation. Little is known about low dose exposure. As bioactive compounds tend to interact with multiple proteins with various physiological effect, we aimed to identify other potential shared targets to understand the extent in which these chemicals could lead to GWI. We followed a reverse screening approach where each chemical is computationally docked to a library of protein targets. The programs PharmMapper and TargetNet were used for this purpose, and further analyses were conducted to mark significant changes in participants with GWI. Previously published work on DNA methylation status in GWI was reanalyzed focusing specifically on the predicted shared targets indicating significant changes in DNA methylation of the associated genes. Our findings thus suggest that exposure to GWI-related agents may converge on similar targets with roles in inflammation, neurotransmitter and lipid metabolism, and detoxification which may have impacts on neurodegenerative-like disease and oxidative stress in Veterans with GWI.

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1. Introduction

Gulf War Illness (GWI) is a chronic multi-symptom disorder that affects roughly 25 percent to 32 percent of the returning Veterans deployed to the 1990–91 Persian Gulf War (GW) [1]. The U.S. is reported to have spent approximately $51.2 billion on GW Veterans to cover pension, disability benefits, and compensation [2], with GWI exacerbating this cost. GWI is diagnosed by the persistence and the rate at which at least three symptoms, including fatigue, musculoskeletal pain, cognitive dysfunction, respiratory, gastrointestinal, and dermatologic problems, are displayed across the GW Veteran population.[1,3]. The etiology of GWI remains unknown but exploring potential causes may reveal novel disease mechanisms, avenues for diagnostics of the illness and/or potential treatment avenues. Here, we examine the potential effect of five GWI related toxicants on shared targets of multiple toxic exposures to acetylcholinesterase (AChE) inhibitors.

During the GW, the Khamisiyah ammunition storage was destroyed, exposing U.S. troops to the chemical warfare agent sarin [4,5]. Many Veterans were exposed to the resulting chemical

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plume in addition to experiencing other possible exposures to chemical agents, such as pyridostigmine bromide (PB) a prophylactic against the lethal effects of nerve agent poisoning, the organophosphate and carbamate pesticides chlorpyrifos and permethrin, and the insect repellants N, N-diethyl-m-toluamide (DEET). Combined with the physiological and psychological stressors associated with war [6], this combination of toxic exposures may in part have given rise to GWI. Thus, while the cause and underlying pathophysiology remains yet to be fully elucidated, examining the effects of these exposures in the context of GWI may shed useful information to aid this Veteran population.

The nerve warfare agent sarin and the safeguard against it, PB, have been suggested as the main chemical exposures to play a role in GWI pathology [4,5]. Sarin is an organophosphorus compound known to target and irreversibly inhibit AChE [7], whereas PB also inhibits AchE but in a reversible manner temporarily blocking sarin binding. This inhibtion results in the continued accumulation of acetylcholine (ACh) in the peripheral synapses [6] leading to the overstimulation of the muscarinic and nicotinic ACh receptors resulting in nicotinic and muscarinic toxicity [7]. This is known as the cholinergic crisis and may cause blurry vision, paralysis, nausea, and SLUDGEM (salivation, lacrimation, urination, defecation, gastrointenstinal upset, emesis, muscle twitching/ miosis) [7]. The symptoms vary with the level of exposure to sarin where significant doses can lead to death by respiratory failure [6]. Sarin also has an effect on neurotransmitters and ion channels, but these mechanisms are less clear [6]. While sarin is known to primarily target AChE, bioactive compounds tend to interact with multiple targets[8], leading to off-target interactions and other adverse reactions. This suggest that there are likely other systems beyond the cholinergic system that are affected by sarin exposure. This idea is consistent with previous work in an animal model of GWI which found that a combination of stress and GW-relevant AChE inhibitors reduced the level of AChE inhibition when compared to the AChE inhibitor alone while still producing GWI-related neuroinflammation, indicating that GWI may result from non-cholinergic actions of AChE inhibitors [9].

The high probability that GWI veterans were exposed to other organophosphorous toxicants (chlorpyrifos, permethrin, and DEET) is also of particular concern as it has been shown that nearly-three million acute pesticide poisoning occur annually resulting in more than two hundred thousand deaths [10,11]. Like sarin, the underlying mechanism of this acute toxicity is mainly due to inhibition of AChE [12,13] leading to the build-up of ACh in the body. Meanwhile, previous work suggests that the neuroinflammation associated with such exposures stems from off-target interactions [14,15]. Still, even at relatively low levels, these compounds are hazardous to human health. Low dose exposure, from drinking, inhalation, or skin exposure [16], causes minimal inhibition of AChE and no obvious cholinergic symptoms, but has been linked to memory loss, sleep disorders, depression, learning impairment, and decreased motor skills [17] all of which have been reported as symptoms of GWI.

Exogenous chemical compounds bind to at least six molecular targets on average [18] due to their small size [19] and reactivity. Usually, these targets are shared among similar gene families, and have similar structural properties [20]. Due to this indiscriminate behavior, exposure to toxic chemicals has the potential to pose serious consequences even at low dose, and result in an array of symptom subtypes [21]. This is all the case for multiple toxicants that act in combination on shared protein targets. Still, the synergistic effects of multiple exposures are poorly understood. In addition, exposure to such chemicals can alter epigenetic marks in both direct and indirect manners [22,23] with these same or similar epigenetic alterations being found in patients with illnesses of concern [24]. As such it is reasonable to expect that shared targets of multiple toxic exposures possess changes in DNA methylation profiles that may contribute to illness.

Here we build on our ongoing research directed at mapping complex inflammatory mechanisms in GWI and model how this complex mixture of toxic exposures affects the combined human neuro-endocrine-immune system to provide a model framework of GWI etiology for use in biomarker and treatment course prediction, as well as the development of future animal models based on combination exposures more indicative of the GWI profile in both the presence and absence of stress. Specifically, we predict other potential common targets of GWI related chemical toxicants to identify pathways that may be epigenetically modified in GWI. To do this we use a combination of reverse docking virtual screening softwares to acquire a library of potential protein targets of GWI related chemical toxicants and identify the binding interactions that are most likely to occur. Our predictions are then validated by secondary analysis of previously published comparison of DNA methylation profiles of GWI subjects to those of healthy Veteran controls (HVC) [25]. This approach is driven by the computational predictions of the reverse docking programs. The DNA methylation reanalysis focuses only on the predicted shared targets and does not look at global DNA methylation changes in GWI as was done previously in Trivedi et al. [25]. This is therefore a more focused look at specific methylation changes which are predicted to be affected by common GWI related toxicants.

2. Materials and methods

2.1. Ligands

The compound structure files were obtained from the freely accessible PubChem database [26–28] in SDF format (DEET (CID 4284), PB (CID 7550), chlorpyrifos (CID 2730), permethrin (CID 40326), and sarin (CID 7871)).

2.2. Reverse screening

Several reverse screening methods exist to predict putative protein targets of chemical compounds [29]. PharmMapper [30–32] and TargetNet [33] were chosen as they were currently accessible, returned a complete list of predicted probabilities of interaction between chemical and target for all targets searched, and positively predicted interaction between the chemical agents investigated and the known primary target of AChE. PharmMapper assesses model pharmacophores stored in large, reputable databases such as TargetBank, DrugBank, BindingDB, and PDTD to find the best fit molecular interactions for molecules submitted by users [30–32]. The structure of each GWI related toxicant was uploaded to PharmMapper in SDF format. The PharmMapper parameters were set to all targets (v2010,7302), and the number of maximum generated conformations and reserved matched targets were each set to 1000. All other parameters were set to default values. The full list of targets was preserved for further analysis. PharmMapper was accessed at www.lilab-ecust.cn/pharmmapper/ in November 2021. TargetNet uses QSAR models to predict and net the human protein targets of the query molecule to create drug-target interaction profiles [33]. Structures of the GWI related toxicant molecules with hydrogen atoms were converted using open-Babel and submitted in SMILES (.smi) format [34]. The parameters were set to include models with AUC scores greater than or equal to 0.7, using a fingerprint type of ECFP4. TargetNet was accessed at http://targetnet.scbdd.com in November 2021.

2.3. Consensus vote score

To identify the targets most likely affected by the toxic agents we opted for a simple consensus voting scheme. Here, each non-zero probability of binding between target and each toxicant for either Pharmmapper or TargetNet was counted as a positive vote of association. Summing each positive vote across all toxicants and programs for each target yielded the targets score. Those with the highest vote score were taken to be the most likely target of multiple toxic exposures. The top 20 targets were identified and chosen for review of their methylation sites.

2.4. Cohort recruitment

Full details about cohort recruitment have been published previously [25]. In brief, the study protocol was approved by the Institutional Review Boards of the Miami Veteran Affairs Human Research Protections Program and Nova Southeastern University (Protocol #4987.79). All subjects were recruited from the Miami Veterans Administration Medical Center and gave written informed consent. Subjects taking medications specifically targeting the steroid or immune systems were excluded from the study. GWI cases comprised 20 male Veterans who were deployed to the Persian Gulf Theater between August 8, 1990 and July 31, 1991 and met Centers for Disease Control and Prevention (CDC) and Kansas criteria for GWI [1]. HVC were 20 nondeployed male Veterans matched 1 to 1 to GWI cases on age, ethnicity, and body mass index (BMI).

2.5. Isolation of peripheral blood mononuclear cells, DNA extractions, and genomic DNA methylation profiling

PBMCs were isolated from blood samples as described previously by our group [25]. In brief, genomic DNA was extracted from PBMCs using the Qiagen DNeasy Blood & Tissue Kit (Qiagen cat. no. 69504), according to the manufacturer's instructions. DNA quality and concentration were assessed by Agilent TapeStation 4200 (Agilent Technologies). All DNA samples had DNA Integrity Number above 8 as previously published [25]. A total of 500 ng of genomic DNA was submitted to the Center of Genome Technology of the John P. Hussmann Institute for Human Genomics in the Miller School of Medicine, University of Miami. The EZ DNA Methylation Kit (Zymo Research) was used to bisulfite convert genomic DNA. Following the processing according to Illumina's specifications, DNA was hybridized with the Illumina MethylationEPIC microarrays [35].

2.6. Statistical Analysis.

Normalized CpG site signal intensity data from a previous study [25] was used for all statistical analysis and reanalyzed as follows. Only normalized CpG site data for the targets identified by the reverse docking consensus voting were selected and used for reanalysis. Statistical comparisons were made using heteroscedastic two-tailed t-tests between the GWI and HVC for the identified targets. Multiple comparisons were corrected for using the method of Storey with all p-values at < 0.05 taken as differentially methylated with a false discovery rate of ≤ 10 % [36].

2.7. Functional annotation of affected genes

Targets shown to have differential methylation patterns in GWI versus HVC was annotated using the ConsensusPathDB [37–40] to provide biological pathway information for the gene set. Over-representation analysis incorporating the Kyoto Encyclopedia of Genes and Genomes (KEGG) (73.0) [41] and Reactome (51) [42] pathway sets was used to interpret the function of the epigenetically modified gene set. Here the significance of the observed overlap between the gene module and the members of known pathways, compared to random expectations, was calculated based on the hypergeometric distribution. A minimum overlap of two genes between the gene module and the pathway set at a p-value cutoff 0.01 was required. Specifically, the p-value is calculated as the probability of randomly finding k or more successes from the population in N total draws. Thus, small p-values indicate a greater overlap than expected by chance. Pathway sets containing most of the altered genes, the highest number of genes in the pathway/function overall, and the lowest p-value were taken as the functional annotation of the altered gene set. Pathway annotation was performed only to provide biological pathway information for the altered gene set.

3. Results

Reverse Virtual Screening: The GWI-related chemicals Sarin, PB, chlorpyrifos, permethrin, and DEET were screened against protein targets using the online webservers TargetNet [32] and Pharmapper [30]. TargetNet predicted a list of 623 targets for all chemicals except pyridostigmine bromide (PB), which had 535 predicted targets. PharmMapper predicted a list of 998 targets for each compound except for PB which produced no output for any target. All the target candidates from both programs were compiled as a full list (see Supplementary Data: Tables S1). Targets with a probability of zero were excluded. The full list comprised of 1616 targets.

A vote score was obtained for each target by assigning 1 point for each program and each chemical that yielded a non-zero probability of interaction. As five compounds were screened through two programs, the highest vote score possible for a target is 10. However, as PB did not receive any results from PharmMapper for any target, the effective highest attainable vote score was 9. The names and gene symbols of the top 20 targets with the highest count scores are shown in Table 1. Table 2 gives the webserver application predicted probabilities by compound and program for the top 20 targets, and the consensus vote score for each target as described in section 2.3. Supplementary Data: Tables S1 gives the complete list of the information used to generate Table 2.

Table 1
Gene symbols for the top 20 hits from PharmMapper and TargetNet.

Gene Symbol	Gene Name
ACHE	Acetylcholinesterase
AKR*	Aldose reductase
CES1	Liver carboxylesterase 1
PTGS1	Prostaglandin G/H synthase 1
MAOB	Amine oxidase [flavin-containing] B
AR	Androgen receptor
ALOX15	Arachidonate 15-lipoxygenase
BCL2A1	Bcl-2-related protein A1
RORA	Nuclear receptor ROR-alpha
MMP2	72 kDa type IV collagenase
MIF	Macrophage migration inhibitory factor
NOS2	Nitric oxide synthase, inducible
PLA2*	Phospholipase A2
RAC1	Ras-related C3 botulinum toxicant substrate 1
HTR1E	5-hydroxytryptamine receptor 1E
ALPL	Alkaline phosphatase, tissue-nonspecific isozyme
MAOA	Amine oxidase [flavin-containing] A
ALOX5	Arachidonate 5-lipoxygenase
CA14	Carbonic anhydrase 14
CA2	Carbonic anhydrase 2

*Protein families with multiple members not distinguished by screening software.

Table 2
Predicted probabilities by compound and program for the top 20 targets by vote score.

Target	Chlorpyrifos		DEET		Permethrin		Pyridostigmine		Sarin		Vote Score
	PM	TN	PM	TN	PM	TN	PM	TN	PM	TN	
ACHE	0.614	0.428	0.568	0.126	0.674	0.101		1	0.581	0.766	9
AKR	0.423	0.001		0.996	0.562	0.553		0.514	0.202	0.746	8
CES1	0.428	0.024	0.387	1	0.523	0.005		0.546		1	8
PTGS1	0.380	0.733	0.369	0.407	0.505	0.004		0.972		1	8
MAOB	0.597	0.024	0.575	0.001		0.024		0.977		0.983	7
AR	0.450	0.13	0.412		0.560			0.016	0.958	0.02	7
ALOX15	0.374	0.001	0.346	0.997		0.052		0.011		1	7
BCL2A1	0.426	0.316	0.385	0.993		0.016		1		0.997	7
RORA	0.416	1	0.329	0.983	0.492			0.955		0.403	7
MMP2	0.497		0.488		0.524	0.522		0.001		0.008	6
MIF		0.993		0.972		0.989		0.998	0.663	0.987	6
NOS2		0.483	0.406	0.903	0.540			0.228		1	6
PLA2	0.429	0.046		0.865	0.500			0.154		0.029	6
RAC1		0.48	0.834	0.999	0.413			0.816		0.181	6
HTR1E		0.984		0.999		0.665		0.983		0.998	5
ALPL		0.998		0.999		0.002		0.901		1	5
MAOA		0.925		0.962		0.653		1		0.99	5
ALOX5		0.14		0.995		1		0.099		0.919	5
CA14		0.045		0.025	0.478			0.003		1	5
CA2	0.437		0.368		0.492			0.003		0.139	5

We performed a secondary analysis of previously published differences in DNA methylation profiles from peripheral blood mononuclear cells (PBMCs) that compared between subjects with GWI and sedentary HVC for the top 20 targets identified in Table 2. Heteroscedastic two-tailed t-tests corrected for false discovery were performed for methylation sites of the top 20 targets. Table 3 presents the CpG sites found to be differentially methylated between GWI and HVC for the genes identified by computational screening and listed in Table 2. As the targets AKR and PLA2G identified via Pharmmapper and TargetNet denote families of proteins rather than individual proteins the DNA methylation profiles of all family members were investigated separately. Supplementary Data: Table S2 gives the complete list of the information used to generate Table 3. Among the listed CpG sites, the promoter associated methylation sites are also featured with asterisks. Among them, nine CpG sites associated with their gene promoters had an increase in methylation. Promoter methylation commonly leads to gene silencing, and methylation of CpG islands can also disrupt transcription factor binding and silence gene expression [24]. This suggests the genes AKR, MIF, and PLA2 with the methylated CpG promoters are likely dysregulated in expression for GWI participants.

The target genes identified as differentially methylated in GWI (Table 3) were then annotated using the ConsensusPathDB [37] over representation analysis with results shown in Fig. 1 and full data for this image given in Supplementary Data Table S3. To illustrate information about the most enriched pathways and the number of specific genes enriched within each pathway the pathway nodes in Fig. 1 are organized counterclockwise by the number of candidate genes contained, while the size of each pathway node is scaled based on the total number of genes in the pathway set, and the pathway nodes are colored based on the percentage of the total pathway genes that the candidate genes make-up. As can be seen in the figure the pathway-based sets of the genes shown to be differentially methylated in GWI exhibit similarities in function where the overlap in pathways of the differentially methylated genes widely refer to metabolism of lipids and amino acids. Specifically, the metabolism of lipids such as phospholipid, ether lipid, linoleic acid, and amino acids such as tryptophan, glycine, and arginine. In addition, the synthesis and metabolism of bile acids, the metabolism of steroids, and the metabolism of fat-soluble vitamins are noted. Bile acids are steroid acids that promote the absorption of lipids including fat-soluble vitamins and the breakdown of cholesterols through their synthesis [43–45]. Furthermore, the related serotonergic synapse, tryptophan metabolism, and MAOA and MAOB pathways are also noted, as tryptophan is the only precursor for serotonin and serotonin is catabolized by MAO [46].

4. Discussion

Here we use an approach driven by computational analysis to predict potential shared targets of GWI related toxicant exposure to elucidate novel pathways that may be dysregulated due to changes in DNA methylation patterns leading to illness. Chemicals tend to be multi-target compounds [18]. As such, GWI related toxicants such as sarin, chlorpyrifos, DEET, permethrin, and PB are likely to have multiple targets in the body. To investigate this possibility and the role that these chemical agents may play in the pathogenesis of GWI, these five compounds were screened using two reverse screening web servers to obtain potential shared protein target candidates. The DNA methylation profiles of the top 20 targets were chosen for further analysis, 15 of which showed a significant difference in DNA methylation sites between GWI and HVC.

Expectedly, AChE had the highest voting score. The organophosphates sarin and chlorpyrifos as well as the prophylactic pyridostigmine bromide are AChE inhibitors, therefore it is understandable for these compounds to have AChE as a target candidate [47]. DEET has also shown interactions with AChE, but more as a weak and reversible AChE inhibitor that likely has various approaches to its adverse effects in mammals [48]. Though not classified as an AChE inhibitor, permethrin has been reported to have similar effects as organophosphates in neurotoxicity through increasing the levels of acetylcholine and AChE [49].

The identification of MIF, a pro-inflammatory cytokine, as an affected target with DNA methylation changes in its promoter region is of particular interest in the context of GWI. A previous proteome profile on the frontal cortex, corpus striatum, and hippocampus of rats after sarin exposure also exhibited MIF as a sarin target [50], indicating that MIF was one of the upregulated proteins in the hippocampus. Another study indicated that the expression of MIF varies based on the time following sarin exposure [51]. Specifically, Spradling et al. [51] gathered data from hippocampal samples following sarin-induced seizure over a 24-hour time

Table 3
Differential methylation sites between GWI and HVC.

Gene Symbol	Hypermethylated	Hypomethylated
ACHE	cg11227411, cg19008168	
AKR1A1	cg22837372, cg24332710*, cg23317297*, cg13179880*	
AKR1B1	cg15700802	
AKR1B10		cg00185189, cg25171118
AKR1B15	cg17730961, cg16588864, cg08527618, cg27018005	cg18108329
AKR1C1		cg11761372
AKR1C2	cg10158681	
AKR1CL1		cg12357263
AKR1D1	cg02355442	cg01143233
AKR1E2	cg19697109	
AKR7A3	cg20313364, cg01798255	
ALOX15	cg23358572, cg09872233, cg09000800, cg15843823, cg11369867, cg15799267, cg26926665	
ALPL	cg17725163, cg22460923, cg24722348, cg16997101, cg20645065, cg06035250, cg14781605	
AR	cg01329836, cg20878850, cg09418623, cg27271368, cg08063601, cg01086868, cg07780118, cg04886514, cg12589433	cg25686125
CA14	cg12317021, cg08678949, cg05037389, cg20791007	
HTR1E	cg10135483	
MAOA	cg15014034	
MAOB		cg04484695, cg24538132
MIF	cg17492289*	
MMP2		cg13262328, cg05977220
PLA2G12A	cg16243646*, cg23820716*	
PLA2G15	cg26843401, cg22379719, cg07093942*	
PLA2G16	cg18840359, cg13765695*	
PLA2G1B	cg13900420, cg15733611	
PLA2G2F	cg24514073	
PLA2G3	cg00727590	
PLA2G4C	cg11854772	
PLA2G4D		cg00934746
PLA2G5	cg13242924	
PLA2G6	cg01243677, cg11226597*	
PLA2G7	cg05947443, cg21269330, cg07219955, cg26780998, cg03700944	cg15922518
PLA2R1	cg20257553, cg10825876, cg11228250, cg24235037, cg14584448, cg12991125, cg26873705, cg22279027	
PTGS1	cg08972579, cg19417324, cg08836767	cg04679156
RAC1	cg20947553	
RORA	cg20041802, cg22728551, cg01871688, cg27280396, cg17149655, cg26113056	cg04324336

*Promoter associated CpG sites.

course and exhibited MIF expression levels to be upregulated at the beginning of the time course and downregulated for the remaining time [51]. In addition, the researchers showed that the change in expression of MIF has been correlated to the neuropathological changes induced by nerve agents such as sarin. MIF has.

been shown to not only be time dependent, but also concentration and temperature dependent with exposure to organophosphates, such as diisopropyl fluorophosphate (DFP) [52]. MIF has also been shown to correlate to neurological disorders such as amyotrophic lateral sclerosis (ALS) [53]. ALS is a neurodegenerative disease that leads to the loss of upper and lower motor neurons [53]. The protein that has been linked to this disease is the misfolded superoxide dismutase 1 (SOD1) motor expressing proteins [53]. MIF appeared to be the chaperone to the mutant SOD1 by binding directly to it and preventing the development of these misfolded SOD1 proteins [53,54]. So, the lack of MIF expression resulted in the accumulation of misfolded SOD1 and extremely low MIF protein levels in the motor neurons [53–55] GW Veterans were found to be at a higher risk of developing ALS within the ten years post-war [56]. Also, a spatial analysis demonstrated that the risks of developing ALS were higher for units in or near Khamisiyah, Iraq, the area in which troops may have been exposed to chemical warfare agents from the destroyed munition dumps [57]. The correlation between the exposure to organophosphates and higher instances of ALS amongst farmers, soccer players, and GW Veterans also displayed the possible role of sarin in the development of ALS [58]. It has also been inferred that MIF may contribute to the prevention of ALS [53]. As organophosphates are likely to influence MIF activity and MIF is noted to lower the development of ALS, the interplay among organophosphate, MIF, and ALS need to be further studied.

Amine oxidase (flavin containing) B (MAOB), a metabolic enzyme primarily known to catabolize neurotransmitters such as dopamine and serotonin [59], also shows a relation to ALS and other neurodegenerative disorders which may have bearing on the higher prevalence of these disorder in those suffering from GWI [1]. MAOB has been shown to be irreversibly inhibited by organophosphate pesticides [60]. Our observed hypomethylation of the MAOB gene may be a direct response to this inhibition indicative of increased expression to compensate for pesticide exposure. As many drugs have been developed to inhibit MAOB as a neuroprotective treatment to neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and ALS [61], increased expression may lead to the opposite effect and increase the likelihood of these illnesses. In support of this finding recent mouse models of GWI have shown that a combination of PB, DEET, permethrin and the sarin analog DFP cause serotonergic dyshomeostasis, monoamine disbalance, and neuroinflammation in multiple brain regions, as well as acute peripheral serotonin imbalance [62].

Veterans with GWI have also shown abnormalities in various biochemical pathways, 78 % of which were constituted to lipids [63]. The metabolic impact of lipids such as ceramides, sphingomyelins, and phospholipids were increased with GWI [63]. Carbonic anhydrase is one enzyme found to be involved in mechanisms such as metabolism, respiratory gas exchange, and cellular defenses against oxidative stress [64]. Interactions between carbonic anhydrase and pesticides like organophosphates have been observed and shown to inhibit carbonic anhydrase activity [64]. In humans, carbonic anhydrases have been associated with the activation of cellular defense systems using antioxidants such as glutathione and vitamins in response to oxidative stress damage [65]. Oxidative stress is known to be induced by pesticides in wildlife and their induction along with AChE inhibition have been observed in organophosphorus pesticide manufacturing workers as well [64,66].

The AKR superfamily is also involved in metabolism, specifically the biosynthesis processing, and the detoxification of substrates such as glucose, steroids, and environmental pollutants [67]. Changes in AKR expression has also been observed following exposure to the organophosphate pesticide, malathion [68]. The study used normal human mammary epithelial cells and identified the changes with DNA microarrays and real time polymerase chain reaction, displaying the increased expression of three genes where two of which were AKR, specifically AKR1C1 and AKR1C2 [68]. Thus, the pesticide malathion may result in changes to AKR, altering AKR's effectiveness in breaking down and detoxifying both endogenous and exogenous substrates [68].

Finally, the PLA2 family of enzymes plays a crucial role in the digestion and metabolism of phospholipids [69]. It can also act as a precursor to prostaglandins [70]. PTGS1 is an enzyme that cat-

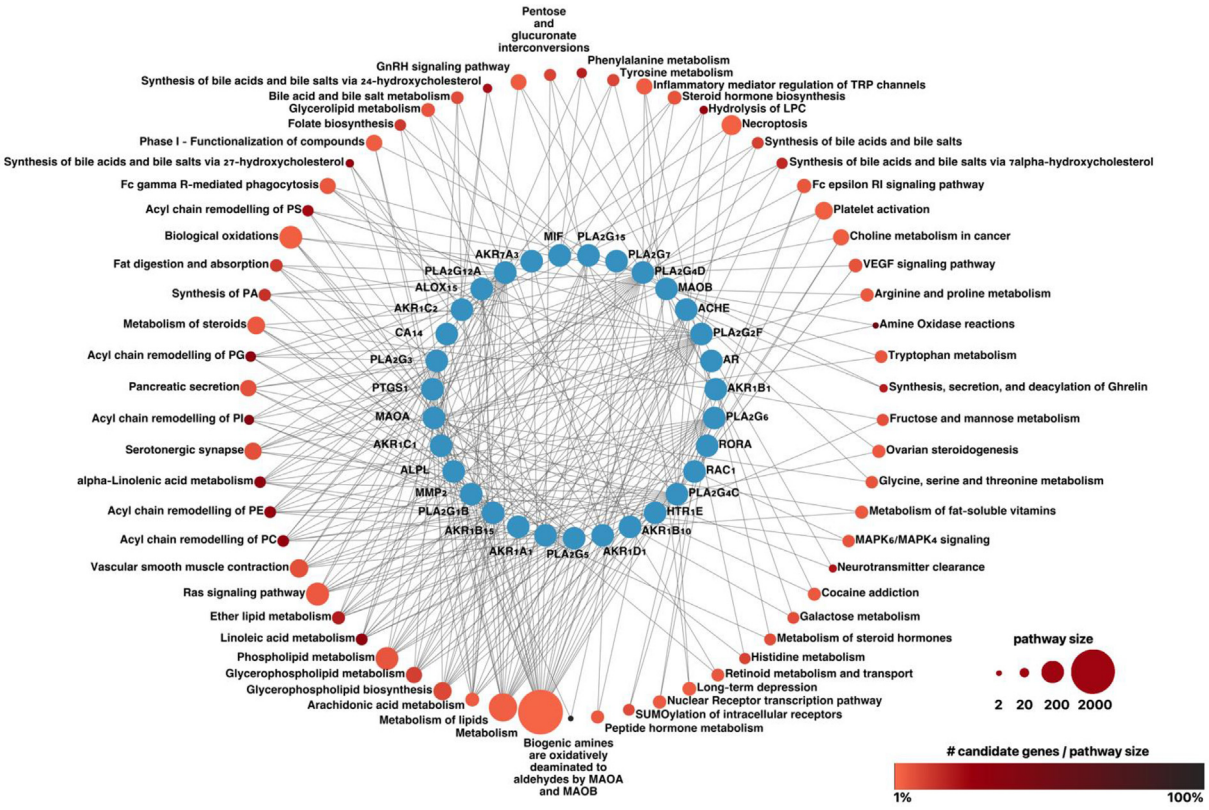

Fig. 1. Overlap of enriched pathway-based sets containing genes shown to be differentially methylated in GWI. Full data for this image is given in Supplementary Data Table S3.

alyzes the conversion of arachidonate to prostaglandin [71]. A study has been done with glyphosate-based herbicides resulting in changes altering the gene expressions relating to lipid metabolism and inflammation [72]. It was noted that there was a significant increase in the gene expression levels of PTGS1 [72]. ALOX15 is an enzyme known to metabolize polyunsaturated fatty acids to generate the formation of lipid mediators [73,74]. ALOX15 and its resulting products have also been associated with the regulation of inflammation [75]. It has been noted as a suppressor of inflammation where the lipid mediators formed can be used to terminate cancer, and its downregulation is observed in colorectal cancer [73].

The above findings suggest that exposure to the five GWI-related toxic compounds studied here can target and effect genes and proteins with roles in inflammation, the metabolism of neurotransmitters and lipids, and detoxification. However, while the findings of this pilot study are promising and encourage further investigations in this area, the limitations of this study must be noted. The first is that GWI is a complex multi-symptom disorder exhibiting a variable constellation of symptoms that include fatigue, musculoskeletal pain, gastrointestinal and cognitive dysfunctions, and therefore it is unlikely that all those with GWI have the exact same underlying biological dysfunctions. While it would be worthwhile to check for any correlation between individual methylation status and GWI phenotype, the current pilot study is underpowered for this type of analysis. To further confound the problem, the onset of GWI occurred over 30 years ago and many of these veterans are now experiencing common co-morbidities associated with aging such as obesity, diabetes, hypertension, hyperlipidemia, and various cancers. The effect that aging has on this unique and vulnerable population remains a matter of significant concern, including its effect on DNA methylation status of the identified proteins. However, despite the role aging plays in GWI DNA methylation changes, the results presented herein are in comparison to an age and BMI matched cohort of healthy sedentary veteran controls which aims to reduce the contribution of these co-morbid factors.

Secondly, and most importantly for understanding the context of this study is the relationship between the changes at an epigenetic level associated with toxicant exposure and potential protein-toxicants interactions. Current research indicates that environmental exposures can be associated with DNA-methylation and expression changes impacting human health [23,76]. As such it may be viewed that a toxicant causes cellular dysfunction by interfering with the transfer of information to and from the genome to the function of an active protein leading to an adaptive response as the cell attempts to compensate for the effects of the toxicant [77 78]. As such, here we used DNA methylation data as a measure of the consequence of exposure associated with adaptive response to GWI related toxicants. However, there are limitations that must be noted with this approach. The first is, regardless of whether DNA methylation changes are associated with the root cause of the toxicity or downstream of the initial event, DNA methylation changes alone do not necessarily equate to expression changes in the associated genes and proteins. Subsequent analysis looking specifically at gene expression and protein expression/function are warranted to confirm the potential effects of the DNA methylation changes in GWI noted herein. Furthermore, even if overall gene and protein expression/function are affected by the epigenetic changes caused by GWI related toxic exposures this alone may not lead to health impairments but may confer positive adaptive responses expected to have a survival advantage [79]. Again, this suggests investigating correlation between individual methylation status and GWI phenotype.

5. Conclusions

Overall, the results of our analysis suggest that sarin, PB, chlorpyrifos, permethrin, and DEET share common targets that have significantly different DNA methylation status in GWI compared to HVC. Our findings suggest that exposure to GWI-related toxic compounds, such as AChE inhibitors, DEET, and permethrin may converge on similar target molecules with roles in inflammation, the metabolism of neurotransmitters and lipids, and detoxification which may have impacts on neurodegenerative-like disease and oxidative stress in Veterans with GWI. While these findings used only a limited set of protein targets, and DNA methylation status was confirmed only in a small sample, the results presented here show congruence with the greater literature on the effects of toxic exposures for the compounds examined and their role in GWI pathogenesis. Moreover, the results support the conclusions of previous work that has suggested that while GWI is likely the result of exposure to agents that impact AChE activity, the long-term symptoms associated with GWI may result from non-cholinergic targets of these chemicals [9]. Future studies using this methodology with expanded protein target datasets, additional GWI related chemical compounds, and larger sample size for DNA methylation analysis is warranted.

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Institutional Review Board Statement

This study was conducted according to the guidelines of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards of the Miami Veteran Affairs Human Research Protections Program and Nova Southeastern University (Protocol #4987.79). All subjects were recruited from the Miami Veterans Administration Medical Center and gave written informed consent.

Informed Consent Statement

All subjects were recruited from the Miami Veterans Administration Medical Center and gave written informed consent.

CRediT authorship contribution statement

Modeline Jean-Pierre: Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Lindsay T. Michalovicz:** Writing – review & editing, Funding acquisition. **Kimberly A. Kelly:** Writing – review & editing, Funding acquisition. **James P. O'Callaghan:** Resources, Writing – review & editing, Funding acquisition. **Lubov Nathanson:** Formal analysis, Writing – review & editing, Funding acquisition. **Nancy Klimas:** Methodology, Resources, Writing – review & editing. **Travis J. A. Craddock:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.11.006.

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