A Psychobiological Approach to Gulf War Illness: Acute Exercise & DNA Methylation

By

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ABSTRACT

Background: Gulf War Illness (GWI) is a form of chronic multi-symptom illness characterized by medically unexplained and heterogenous symptoms (*e.g.,* fatigue, pain, cognitive decline) that affect ~30% of personnel deployed during the 1990-1991 Persian Gulf War. Controlled high-intensity or maximal aerobic exercise (*i.e.,* an exercise challenge) have been used to modulate physiological stress responses in GWI. Investigations of DNA methylation in GWI are seldom, and exercise-induced changes in DNA methylation may provide insight into the molecular interactions underlying GWI pathophysiology.

Purpose: The primary purpose of the present dissertation is to compare differences in DNA methylation following an acute aerobic exercise challenge in Gulf War Veterans with and without GWI.

Methods: Differences in DNA methylation measured via microarray were tested in Gulf War Veterans with GWI (N = 27) compared to control Gulf War Veterans (N = 25) pre-, 30-minutes post-, and 24 hours post-exercise. Associations between DNA methylation, ventilatory equivalents during exercise, and pre-post symptom responses were also tested in Gulf War Veterans with and without GWI.

Results: Gulf War Veterans with GWI had differentially methylated positions (DMPs) in genes with established metabolic and immune functions, and DMP-associated genes with select inflammatory functions (*e.g.,* response to IFN-β) were observed only after exercise. Select

DMPs were significantly associated with fatigue and mood disturbance 30-minutes post-exercise, and pre-exercise a DMP within the oxoglutarate dehydrogenase [*OGDH*] demonstrated modest classification accuracy (Area Under the Curve [AUC] = 0.796) between GWI cases compared to controls.

Conclusions: DMP-associated genes that participate in inflammation and metabolism are consistent with previous GWI investigations, including those that have studied DNA methylation. DNA methylation changes following an exercise challenge support involvement from the immune and metabolic systems in GWI pathophysiology and signify potential heightened risk of chronic comorbidities (*e.g.,* cardiovascular or metabolic disease).

CHAPTER 1: INTRODUCTION

Exposure to environmental toxins during deployment risks future adverse health consequences in active-duty military personnel and Veterans. Recent examples include agent orange in connection with the Vietnam War, nerve agents in the 1990-1991 Persian Gulf War (GW), and burn pits in the post-9/11 Middle East conflicts. Specific to the GW, garrison environmental hazards with known adverse physiological effects are linked to Operations Desert Shield and Storm, including sarin gas, pesticides, depleted uranium, smoke from oil-well fires, and prophylactic use of pyridostigmine bromide (PB).[1] These in-theater exposures are hypothesized contributors to Gulf War Illness (GWI) – a chronic multi-symptom illness characterized by diverse and medically unexplained symptoms (*e.g.,* fatigue, pain, cognitive decline)[2] that afflict 25-32% of the 767,000 soldiers deployed to the Persian Gulf.[1,3]

A unique feature of chronic multi-symptom illnesses—including GWI—is a phenomenon known as post-exertional malaise (PEM). PEM lacks a universally accepted clinical definition but is commonly conceptualized as an acute exacerbation of symptoms following physical or mental exertion.[4,5] Prevalence estimates vary but indicate that up to 96% of Gulf War Veterans (GWVs) with GWI experience PEM.[6] In addition to symptom exacerbation associated with PEM, physiological perturbations not apparent at rest have been reported after physical exertion in GWVs with GWI; thereby suggesting that exercise may be a useful model for exploring pathophysiology.[7–9] Exposure to high-intensity or maximal aerobic exercise in controlled settings (also known as an exercise challenge), has been used to explore GWI physiology of metabolic, autonomic,[10] central nervous,[8,11] respiratory,[12] and immune systems.[9] Nevertheless,

the mechanisms of GWI and PEM remain unknown. There is a clear need for novel methods that may better represent the long-term consequences of hazardous exposures associated with GW deployment.

Investigations of candidate genes associated with neuroendocrine[13–15] (*e.g., PON1, BuChE, AChE, ACE*, etc.) and immune[16–18] (*e.g., TLR4, IL-6R, HLA,* etc.) function indicate allelic variation may mitigate individual susceptibility to environmental hazards and participate in GWI pathophysiology. DNA sequence variation alone is unable to completely explain GWI, which is partially attributed to its limitations accounting for the burden of environmental stress (*e.g.,* potential chemical exposure) during deployment. Epigenetic modifications representative of interactions between exogenous factors and DNA sequence may offer insight about molecular activity underlying GWI pathophysiology.[19] Prioritizing interventions that act on these pathways may advance future GWI treatments.

Epigenetics broadly refers to chemical modifications to DNA that alter gene activity without changing nucleotide sequence.[20] DNA methylation is an epigenetic modification that results in the covalent addition of a methyl group at position 5 of the DNA nucleotide cytosine to generate 5-methylcytosine (5mC). DNA methylation participates in coordination of gene expression in the human genome.[21,22] Variations in DNA methylation have been observed within hours of acute physical stress from exercise,[23] surgery,[24] or infection.[25] Altered DNA methylation levels persist for diverse intervals across hours, months, or a lifespan.[26] Exercise-induced changes in DNA methylation that are also associated with GWI symptom severity (*i.e.,* PEM) would provide stronger evidence for that gene or pathway's pathophysiological significance.

Few studies have investigated DNA methylation or other associated epigenetic mechanisms (*e.g.,* histone modifications) in GWI. An earlier pilot study by Trivedi et al., observed differential methylation of genes related to neuronal cell differentiation, metabolism, and immune function in GWVs with GWI (N = 10) compared to control GWVs (N = 10).[27] Disproportionate levels of gene promoter hypermethylation observed in GWI suggest gene "silencing", which correspond to our previous findings of repressed gene expression.[28] Secondary reverse-screening analyses tested in this cohort by Jean-Pierre and colleagues found associations between GWI epigenetic profiles (*i.e.,* DNA methylation) and common pathways targeted by hazardous chemicals present during the GW (*e.g.,* sarin, PB, DEET, chlorpyrifos, and permethrin).[29] To our knowledge, relationships between DNA methylation and post-exercise symptom exacerbation (*i.e.,* PEM) have not been tested in GWI. *The primary purpose of the present dissertation is to compare differences in DNA methylation following an acute aerobic exercise challenge in GWVs with and without GWI.*

SPECIFIC AIMS:

The present research project includes four specific aims:

Specific Aim 1): To compare baseline levels of blood-based DNA methylation between GWVs with GWI and control GWVs.

Specific Aim 1 Hypothesis: Differentially methylated positions (DMPs) measured at baseline *in peripheral blood* via *Illumina Infinium MethylationEPIC V2* microarray will be identified between GW*Vs with GWI and control GWVs.*

Specific Aim 2): To compare changes in blood-based DNA methylation from pre- to 30 minutes and 24 hours post-exercise in GWVs with GWI and control GWVs.

Specific Aim 2 Hypothesis: DMPs measured via Illumina Infinium Methylation EPIC V2 in peripheral blood will be identified in both between and within group comparisons 30 minutes and 24 hours post-exercise. Based on previous literature, changes in GWVs with GWI will favor hypermethylation compared to control GWVs.

Specific Aim 3): To test differences in cardiopulmonary responses measured during submaximal exercise between GWVs with GWI and control GWVs.

Specific Aim 3 Hypothesis: Cardiopulmonary responses to exercise will indicate less efficient ventilation (i.e., higher ventilatory equivalents) in GWVs with GWI compared to control GWVs.

Sub-Aim 3a): To test associations between ventilatory equivalents measured during submaximal exercise with blood-based DNA methylation levels 1) pre-exercise, 2) post-exercise, and 3) 24 hours post-exercise in GWVs with GWI and control GWVs.

Specific Aim 3a Hypothesis: Ventilatory equivalents will be significantly associated with blood-based DMPs 1) 30 minutes post-exercise and 2) 24 hours post-exercise. DMPs associated with ventilatory equivalents and GWI will be predominantly in genes known to participate in metabolic function.

Specific Aim 4): To test if symptom severity increases from pre- to post-exercise (i.e., 30 minutes 24 hours post-exercise) in GWVs with GWI compared to control GWVs.

Specific Aim 4 Hypothesis: GWVs with GWI who endorse PEM will have an increase in symptom severity (i.e., PEM) after exercise.

Sub-Aim 4a): To test whether changes in blood-based DNA methylation levels from pre- to 30 minutes post- and 24 hours post-exercise are associated with symptom worsening in GWVs with GWI.

Specific Aim 4 Hypothesis: Differences in DNA methylation from pre- to post-exercise will be associated with increased symptom severity in GWVs with GWI who endorse experiencing PEM.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1. Introduction – Gulf War Illness (GWI)

Iraqi President Saddam Hussein launched an invasion of the neighboring nation of Kuwait on August 2, 1990, prompting a multinational military response to liberate Kuwait. By August 7th, 1990, the U.S. mobilized the first of 767,000 American troops to the Persian Gulf to combat the Iraqi invasion - named Operation Desert Shield. Dispatched U.S. naval vessels followed by coalition airstrikes began on January 16th, 1991, marking what is known as Operation Desert Storm. U.S. forces deployed during Desert Storm totaled less than 100 hours of ground combat before prompting a surrender by the Iraqi army. President George H.W. Bush declared a cease-fire on February 28th, 1991, officially concluding the conflict.[30] Despite successful liberation of Kuwait by U.S. and allied forces, soldiers stationed in-country encountered numerous airborne hazards and chemical exposures during the Gulf War. Many of these exposures—including oil well fires, organophosphate nerve gases, and pesticides—are associated with adverse health outcomes.

Several federal post-deployment initiatives (*e.g.,* Department of Defense Comprehensive Clinical Evaluation Program) were tasked with clinically evaluating Veterans for various primary and secondary conflict-related diagnoses.[31] Ultimately, existing medical explanations were insufficient for the broad heterogeneity of these clinically reported symptoms and conditions. Patterns observed within these Veterans supported the existence of a new potential illness associated with deployment to the Persian Gulf.[32] The direct impact of post-war conditions on civilian health in the Persian Gulf is not well established, largely because political instability

fractured infrastructure (*e.g.,* medical services, public health surveillance, etc.) involved in monitoring these trends. Beyond potential exposure to chemical weapons, multiple reports described high atmospheric concentrations of toxic gas and particulate matter within the Persian Gulf (*i.e.,* Kuwait and southern Iraq) in 1991.[33,34] Negative consequences were reported in agricultural soil integrity and livestock, indicating that adverse health outcomes for civilians within the region were plausible.[35,36]

2.1.1. Proposed Gulf War Illness Etiology

Decades of research has had limited success phenotyping symptomatic Gulf War Veterans (GWVs),[37–39] and the evidence instead proposes that Gulf War illnesses (GWI) represent a cluster of heterogenous symptoms associated with deployment to the Persian Gulf region.[40] Research and Veteran communities alike have debated the extent of toxic exposures (*e.g.,* demolition of chemical munitions) as antecedents of GWI.[40,41] Importantly, efforts to identify GWI etiology are inherently limited because exposure to biochemicals (*e.g.,* sarin/cyclosarin, DEET, pesticides), prophylactic medicine (*e.g.,* pyridostigmine bromide [PB] pills), and constituents of the weapons (*e.g.,* depleted uranium) were not measured in-theater; nor were symptoms and other health conditions documented prior to deployment. Nevertheless, the lack of knowledge surrounding toxic exposures in the Persian Gulf and other U.S. conflicts (*e.g.,* Agent Orange in connection with Vietnam, burn pits in the post-9/11 Middle East) have rightfully raised alarms over our readiness to tackle future health risks facing Veterans. In August of 2022, the 117th U.S. Congress enacted the Sergeant First Class (SFC) Heath Robinson Honoring our Promise to Address Comprehensive Toxics (PACT) Act,[42] dedicating approximately $280 billion toward toxic exposure research and

healthcare over the next decade. The PACT Act is an unprecedented step toward recognizing the consequences of deployment and developing solutions for military-connected environmental exposures.

GW exposure investigations have largely relied on self-reported exposure data or surrogate models (*e.g.,* animal models). These hypothesized exposures include psychological stress, oil well fires, depleted uranium exposure, prophylactic PB pill use, organophosphate pesticides, nerve agents, vaccines, infectious disease, or a combination of these factors.[3] A small epidemiological investigation in deployed GWVs (N = 304) found higher GWI incidence for those who reportedly entered Iraq or Kuwait (OR = 5.76; 95% CI = 2.26, 14.7), wore pesticide-treated uniforms (OR = 3.72; 95% CI = 1.91, 7.21), used PB pills (OR = 3.21; 95% CI = 1.97, 5.24), or conducted operations within one mile of exploding SCUD missiles (OR = 2.10; 95% CI = 1.30, 3.39).[43] Relationships between self-reported exposures and individual GWI symptoms have also been tested. Pain severity in GWVs treated by the War-Related Injury and Illness Study Center (N = 608) was significantly associated with both pesticide application (OR = 4.13; 95% CI = 1.78, 9.57) and PB pill use (OR = 2.28; 95% CI = 1.02, 5.09).[44] These correlational relationships should be interpreted cautiously, and the lack of pre-deployment data and reliance on self-report prohibits causal inference.

Over 30 years have passed since the Gulf War, and research efforts have largely shifted from etiology to pathophysiology and treatment. As of 2025, only nine clinical trials have advanced to a phase III trial or beyond. Candidate interventions have included diet, cognitive behavioral therapy, exercise therapy, coenzyme Q10, antibiotics, and mifepristone.[45]

Pathophysiological investigations of GWI have prioritized systems and pathways with known sensitivities to many of the proposed etiologies. One of the greatest barriers undermining GWI pathophysiology research is the absence of a rigorously defined GWI phenotype and thorough symptom characterization. Thus, the next section will provide a brief overview of epidemiological efforts to characterize GWI symptoms and the resulting case definitions. A summary of each GWI case definition is provided in Table 1.

2.1.2. Prevalence, Epidemiology, and Gulf War Illness Case Definitions

Veterans returning from the Persian Gulf reported heterogenous symptom profiles (*e.g.,* pain, fatigue, cognitive issues), and these symptoms presented without clear alternate medical explanation.[46] Similar symptoms were also documented in Veterans from other allied nations (*e.g.,* Canada[47] and the United Kingdom[48]) deployed to the Persian Gulf. Early epidemiological efforts to characterize symptoms in U.S. GWVs (N = 11,441) found that the ten most common symptoms included back pain, runny nose, joint pain, headache, anxiousness, difficulty getting to sleep, feeling tired, skin rashes, fatigue, heartburn, and indigestion.[46]

The cumulative evidence supported a potential "Gulf War Syndrome" based on symptom consistency across multiple deployed GWV cohorts[46,49,50] and the lack of explanation from attributable lifestyle (*e.g.,* alcohol consumption, smoking, etc.) or sociodemographic factors (*e.g.,* age, military rank, branch of service, etc.). Early work by Stretch et al., (1995) defined the "Gulf War Syndrome" as a deployed GWV who experienced six or more symptoms (*i.e.,* five or more physical symptoms and one or more psychological symptoms). Operationalizing the "Gulf War

Syndrome" definition with an algorithmic classification model revealed reliability concerns (*e.g.,* ~31% false-positive rate) and prompted efforts to refine the working case definition.

The prevalence of neurotoxic chemical agents potentially encountered in the Persian Gulf was measured in subsequent cross-sectional surveys, and one study of a Naval Battalion (N = 249) investigated neurological symptoms in connection with self-reported exposure to cholinesterase inhibitors (*e.g.,* acetylcholinesterase inhibitors & butyrylcholinesterase inhibitors).[51] A two-stage factor analysis of common symptoms derived three primary syndromes, classified by impaired cognition (Syndrome 1), confusion-ataxia (Syndrome 2), and arthro-myo-neuropathy (Syndrome 3). Prevalence rate risk ratios (RR) demonstrated intersections between each syndrome and self-reported exposures, such that RR was incrementally greater in Syndrome 2 and 3 based on the magnitude of adverse PB pill use. Haley & Kurt acknowledged limitations due to 1) a small study sample and 2) restricted external validity (*e.g.,* only GWVs from a Naval Battalion), but they highlighted the heterogeneity of symptomatic GWVs. Reducing heterogeneity in future case definition efforts warrant consideration of symptom prevalence reported by GWVs, timeline of symptom presentation post-deployment, and symptom chronicity.

Fukuda and colleagues (1998) introduced the term chronic multi-symptom illness (CMI) in recognition of the broad deployment-related health consequences experienced by GWVs.[52] A cross-sectional analysis of physical and mental health screenings, blood, urine, and stool collection, and serologic testing was used to compared deployed GWVs (N = 1,163) and non-deployed Veterans (N = 2,056). Findings from these symptom and lab-based measures resulted

in two important case definition additions: 1) a clinical case definition in which presenting chronic symptoms (*e.g.,* experienced for a minimum of 6 months) must be reported by at least 25% of GWVs at a prevalence 2.5x more frequently than non-deployed personnel; and 2) a statistical definition using elements identified by a principal components analysis and cross-validated via factor analysis. Inter-definition comparisons confirmed substantial agreement (*kappa* = 0.79), and both techniques were applied to discriminate symptomatic GWVs. Deployment to the Persian Gulf Was significantly associated with increased risk of mild-moderate (OR = 4.08, 95% CI = 3.39-4.93) or severe (OR = 16.18, 95% CI = 8.99-29.14) CMI compared to non-deployed Veterans. Notably, the investigators reported 47% of their sample as symptomatic cases, with 41.8% of cases reporting symptoms related to fatigue, mood-cognition, and musculoskeletal pain. Additional advantages of this approach include capturing 1) symptom breadth and 2) illness severity. As a result, Fukuda and colleagues operationalized the symptom category approach, requiring one or more chronic symptoms in at least two of three categories (*i.e.,* fatigue, mood-cognition, and musculoskeletal). The case definition drafted by Fukuda and colleagues was ultimately adopted by the Center for Disease Control (CDC) as its standard for defining Gulf War CMI.

In a later epidemiological study by Steele et al. (2000), data from a cohort of Kansas GWVs were used to develop a set of research criteria (*i.e.,* colloquially named the Kansas criteria) for what is now known as GWI.[2] Participants included Veterans who were deployed to the Persian Gulf between August 1990 and July 1991 (n = 1,548) and a group of Veterans deployed elsewhere (n = 482). Determination of Kansas criteria "caseness" was based on post-deployment symptom occurrence within six general domains: pain, fatigue, cognitive/mood/neurological, skin,

gastrointestinal, and respiratory related issues. Several exclusionary factors that may otherwise explain these symptoms (*e.g.,* cancer, heart disease, psychiatric diagnoses, etc.) were also defined. To meet Kansas criteria for GWI, Veterans must endorse moderate to severe symptoms in three of six major domains. Presenting symptoms must have also become problematic following deployment to the Persian Gulf War and persisted for at least a year prior to study participation. This evaluation found that deployed GWVs were more likely to meet Kansas criteria (OR = 4.68, 95% CI = 3.25, 6.75), CDC criteria for CMI (OR = 3.26, 95% CI = 2.48, 4.28), and chronic fatigue syndrome criteria (OR = 8.21, 95% CI = 2.58, 26.10) than their counterparts deployed elsewhere. Both CDC (*i.e.,* Fukuda) and Kansas case definitions are supported by the National Academy of Medicine as the best available options for identifying GWI in research settings, and subsequent studies using these criteria estimate a GWI prevalence rate of 25%-32% (175,000-250,000 total Veterans).[3,40]

Table 1. Summary of Gulf War Illness (GWI) Case Definitions & Criteria			
Definition:	**Study:**	**Sample Size:**	**Case Definition:**
Stretch	Stretch et al. (1995)[50]	N = 1,524	At least 5+ physical health symptoms & 1+ psychological health symptom
Haley	Haley et al., (1997)[53]	N = 249	1 of 3 syndromes: 1) impaired cognition, 2) confusion-ataxia, 3) arthro-myo-neuropathy
Center for Disease Control (CDC)	Fukuda et al., (1998)[52]	N = 1,163	1 or more chronic symptoms (6+ months) from 2 of 3 categories: fatigue, mood-cognition, musculoskeletal
Kansas Case Definition	Steele et al., (2000)[2]	N = 1,548	Moderate to Severe (2 on 0-3 scale) in at least 3 of 6 domains: Fatigue, Pain, Neurological/Cognitive/Mood, GI, Respiratory, Skin

2.1.3. Gulf War Illness Pathophysiology

Another major barrier towards developing effective treatments has been the dearth of clarity surrounding GWI pathophysiology.[45] Because GWI etiology remains largely unknown, researchers have relied heavily on previous CMI literature and deductive reasoning (*e.g.*, pain often coincides with inflammation in other illnesses) to select prospective physiological systems for investigation. Disrupted function reported in the central nervous system, autonomic nervous system, neuroendocrine axes, immune system, and gut microbiome may be detectable in GWI at the molecular level through epigenomics (*e.g.,* DNA methylation). This dissertation will test

differences in blood-based DNA methylation between GWVs with and without GWI (Specific Aim 1 & 2), and the biological relevance of DNA methylation in affected GWI pathways requires a basic understanding of potential pathophysiology. Evidence for each system's potential role in GWI symptomology is summarized below.

2.1.3.1. Central Nervous System

Neurocognitive deficits (*e.g.,* impaired cognition, disrupted mood) and potential neurotoxic exposure (*e.g*., sarin/cyclo-sarin gas, DEET/pesticides, PB pills) reported by returning symptomatic GWVs support potential pathophysiological contributions from central nervous system (CNS) in GWI.[54] Self-reported toxic exposure (*e.g*., hearing chemical alarms or geographically located in hazardous areas) has been associated with lower cortical gray matter volume, white matter volume, and poorer performance on sustained attention tasks in deployed GWVs.[55–57] Specific GWI symptoms have also been associated with structural differences in subcortical regions. Cross-sectional comparisons between GWVs with GWI and control GWVs demonstrated lower volumes in select subcortical regions (*e.g*., cerebellum, brainstem, basal ganglia, and thalamus) that were associated with greater fatigue, pain, depressive symptoms, and respiratory difficulty.[58,59] Relative consistency is observed across these investigations of neural structure in GWI, although it is plausible that bidirectional influence from chronic symptoms contributes to structural differences within the CNS. Functional neural responses have also been tested in GWVs and exemplify how these structural variations may manifest as chronic symptoms.

Neural activation[60] and multimodal imaging[61] have demonstrated diagnostic potential for GWI. Activation patterns detected via magnetoencephalography in the bilateral frontal lobe and cerebellum have accurately classified GWI cases compared to controls (*e.g.,* correctly classified 94.2% of the 86 participants).[60] Single-subject machine learning methods applied to multimodal imaging methods (*e.g.,* magnetic resonance imaging [MRI], diffusion tensor imaging [DTI], and neurite density imaging [NDI]) has also demonstrated high accuracy (*i.e.,* 90%) GWI classification rates.[61] Questions about the underlying CNS pathophysiology and association to GWI symptoms, however, remain largely unanswered. Because common GWI comorbidities like anxiety[62] and PTSD[63] may also influence imaging-based techniques, experimental designs adapted methods to assess relationships between neural activation and specific symptoms. GWVs with GWI exhibit differences in functional brain activation during experimental paradigms designed to test neurocognitive function,[8,53,60] fatigue,[64] and pain.[65] One early clinical study reported that responses to neurophysiological testing (*e.g.,* radiological and audio-vestibular tests) broadly reflected impaired cognition, confusion-ataxia, and arthro-myo-neuropathy in GWVs with GWI.[53] Activity abnormalities detected in select CNS regions with established structural variation (*e.g.,* brain stem) and their associated systemic consequences (*e.g.,* neuropathy) may indicate deficits across broader neural networks.

A subsequent study applying an exercise-challenge paradigm examined network activation via functional magnetic resonance imaging (fMRI) during working memory tasks pre- and post-exercise in GWVs with GWI (n = 28) and healthy controls (n = 10).[8] Prior to exercise, both control and GWI participants displayed regional activation in frontal parietal network (FPN) and striatum, but only GWVs with GWI participants exhibited bilateral cerebellar vermis

activation. Post-exercise, GWVs with GWI and control participants continued to recruit FPN regions, but GWVs with GWI also had heightened activation in the left-medial frontal gyrus, bilateral anterior insula, and cerebellum. These findings support that GWVs with GWI have unique neural patterns of regional activation (*e.g.,* FPN, cerebellar vermis, and striatum) after acute exercise, and these activation differences may contribute to impaired working memory or attention symptoms in GWI.

FPN top-down modulation may explain augmented somatosensory response activation during experimental pain paradigms of innocuous and noxious stimuli.[65] Compared to controls, GWVs with GWI exhibited hyperactivation in several somatosensory regions (*e.g.,* primary somatosensory cortex [S1], secondary somatosensory cortex [S2], insula, supplementary motor area [SMA], and inferior parietal lobule [IPL]) in response to noxious to heat stimuli.[65] Compensatory neural adaptations may develop from pain chronicity and ultimately explain regional activation differences between acute pain and those observed with chronic pain (*e.g.,* GWVs with primary chronic musculoskeletal pain).[66] Similarly, chronicity has differentiated neural activation responses when comparing state (*i.e.,* acute) to trait (*i.e.,* chronic) fatigue symptoms. Repeated fatiguing tasks administered during fMRI demonstrated state fatigue was associated with greater activation in regions like the basal ganglia and superior parietal lobule in GWVs with GWI.[64] Psychometric-measured trait fatigue was associated with orbital frontal, precentral, superior temporal, and thalamic activation. Chronically elevated neural activation during routine cognitive tasks in GWVs with GWI may partially explain cognitive fatigue symptoms, though the precise pathophysiological mechanism of action remains unclear.

Molecular mechanisms underlying structural and functional neurobiology within the CNS have also been tested between GWVs with GWI and control GWVs. Metabolic disruptions within the CNS may contribute to structural differences observed between GWVs with and without GWI. Metabolically, one investigation using long echo time proton magnetic resonance (MR) spectroscopy reported that GWVs with GWI had lower N-acetyl aspartate-to-creatine (NAA/Cr) ratios in the basal ganglia and brainstem than healthy control GWVs.[67] These findings indicate lower functional neuronal mass; however, longitudinal studies are required to confirm whether this metabolic deficit coincides with CNS atrophy over time. Although resting state comparisons of amino acid concentrations and microRNA content within cerebral spinal fluid (CSF) were similar between GWVs with and without GWI, acute exercise has been shown to increase glutamate[68] and miR-22-3p[11] (*i.e.,* a microRNA associated oxidative stress responses[69]) in GWVs with GWI. These findings support that CNS sensitivity to accrued oxidative stress may facilitate structural variation within GWI.

2.1.3.2. Peripheral Nervous System (Autonomic Function)

Select symptoms contained within the Kansas Case Definition[2] and other epidemiological studies[53] (*e.g.,* gastrointestinal issues or night sweats) indicate potential autonomic nervous system (ANS) contributions to GWI.[70] Self-reported ANS dysfunction (*e.g.,* Composite Autonomic Symptom Score [COMPASS-31] questionnaire[71]) in GWVs with GWI include orthostatic (*e.g.,* feeling dizzy when standing after sitting), vasomotor (*e.g.,* hot flashes or night sweats), and gastrointestinal symptoms (*e.g.,* nausea).[72,73] GWVs with GWI with greater ANS symptom burden also report lower physical functioning,[72] and this relationship was not

mediated by mental health conditions (*e.g.,* Post-Traumatic Stress Disorder or depression) commonly comorbid in GWI.[73] Clinical ANS assessments have also been performed in GWI and support findings observed via self-report. ANS challenges designed to elicit wide-ranging physiological responses (*e.g.,* orthostatic tolerance,[8,74] impact of inspired CO_2 concentrations on capnography,[74] cold pressor challenge,[75] the Quantitative Sudomotor Axon Reflex Test [QSART][76,77]) have been tested in GWI.

Moderate-to-large decreases in cerebral blood flow velocity (CBFV; Hedge's *g* = -0.62), mean arterial pressure (MAP; Hedge's *g* = -1.01), and dynamic cerebral autoregulation (Hedge's *g* = -1.0) were detected in GWVs with GWI exposed to orthostatic stress (*e.g.,* sit-to-stand challenge).[78] Capnography assessments also confirmed these responses were not attributed to group end tidal CO_2 differences (*i.e.,* a known modulator of cerebral autoregulation). These findings are consistent with earlier reports of reduced cardiac output and increased vasodilation observed during mental and cardiac stress tests of GWVs.[75] Additional investigations found significantly lower QSART scores in GWVs with GWI compared to control GWVs, and these findings may signify damage to distal small cholinergic sudomotor fibers within the ANS contributes to GWI. Notwithstanding the limitations of cross-sectional research, these findings provide support for autonomic dysregulation in GWI and may help explain why symptoms such as impaired cognition, vertigo, and hot flashes are reported by these Veterans. Adrenergic agonists (*e.g.,* phenylephrine) have demonstrated therapeutic effects in other CMI populations,[79] while a 10-week behavioral intervention pilot study (*e.g.,* yoga & cognitive behavioral therapy) found functional ANS improvements (*e.g.,* increase high frequency

heartrate variability) in GWVs with GWI.[80] If replicated in larger samples sizes, behavioral interventions that enhance ANS activity may warrant consideration for treating GWI symptoms.

Physical stress (*e.g.,* acute exercise) has been shown to alter gene expression of known adrenergic ANS modulators, including catechol-o-methyltransferase (*COMT*) in GWI[28] and adrenoceptor alpha 2A (*ADRA2A*) in related conditions like ME/CFS.[81] Subtle baseline group differences compared to stressed adrenergic responses and potential transcription factor influence highlighted by Light et al., (2012)[81] have implications for modeling GWI. For example, in-vivo GWI models have demonstrated that blocking b-adrenergic activity (*e.g.,* beta-blocker propranolol) tangentially reduces inflammation[82] and neuroendocrine (*e.g.,* cortisol) activity.[83] Glucocorticoid receptor-a acts as a transcriptional activator for several genes linked to inflammation,[84] and epigenetic-coordinated activity (*e.g.,* methylation, acetylation, etc.) within these genes is a likely contributor toward the perpetuated stress response.

2.1.3.3. Neuroendocrine System

Neuroendocrine disturbance has been reported in Veterans with GWI, however the relationships with symptoms such as fatigue, problems with cognitive function (e.g., memory and attention), and gastrointestinal issues are unclear.[41] Neuroendocrine disturbance is thought to occur through exposure to PB pills which have known effects on the acetylcholine pathway (ACh) and were routinely administered to GWVs.[41,85] Others have proposed that individual allelic variation in nuclear genes coding for acetylcholinesterase enzymes may have been at higher risk for GWI in response to chemical exposure.[15]

PB, DEET insect repellent, permethrin insecticides, and sarin/cyclosarin are all neurochemical insults potentially encountered during the Persian Gulf War,[1,41] and their impact on the neuroendocrine system may contribute to GWI symptomology.[85] Adverse consequences of ACh accumulation, such as myopathy and swollen mitochondria, are well known.[86] The Department of Defense (DoD) reportedly fielded 5,328,710 PB doses during the Gulf War, and Veterans were instructed to take 30mg doses every 8 hours for protection. Prophylactic PB pill use was intended to protect soldiers from organophosphate-based sarin and cyclo-sarin nerve gases.[41] PB acts as a reversible acetylcholinesterase (AChE) inhibitor in the neuronal synapse, and was administered to GWVs to physiologically outcompete harmful and nearly irreversible organophosphate nerve gases from binding to AChE at toxic levels.[41] Although ACh chemical disruption is a biologically plausible explanation for some GWI symptoms, the mechanisms by which these toxic agents lead to chronic and multi-symptom disease remain poorly understood. Several specific studies warrant detailed discussion about how mechanistic neuroendocrine disruption (*e.g.,* ACh) may participate in GWI pathophysiology and symptomology.

Khan et al. assessed peripheral cholinergic function via Doppler imaging of skin blood flow following methacholine and ACh iontophoresis in 52 people with ME/CFS, 24 Veterans with GWI, 25 agricultural workers with chronic fatigue-like symptoms who reported previous organophosphate exposure (OPE), and 40 healthy volunteers.[6] Veterans with GWI did not have significantly different ACh or methacholine responses compared to the healthy volunteers, suggesting a normal breakdown of ACh by enzymes.

Genetic differences in peripheral cholinergic function, such as cholinesterase coding genes, may also mitigate individual GWI risk.[15] Preliminary genotyping of cholinergic enzyme

activity in GWI, chiefly AChE and Butyrylcholinesterase (BuChE), has been used to identify potential risk factors.[15] BuChE function is unclear, but it is hypothesized to have a protective role against organophosphates, DEET, and other AChE inhibitors.[87] However, early work such as that by Lockridge & Masson (2002) found no unadjusted associations between BuChE genotype and GWI classification.[87] Conversely, later research by Steele et al. (2015) comparing GWVs with GWI (n = 144) and controls (n = 160) found that certain BuChE genotypes may have differing protective capabilities from chemical exposure. Specifically, less common BuChE genotypes in Veterans deployed to the Persian Gulf had a higher risk of being associated with GWI.[15] Further, 69% of Veterans who took PB pills and had more common BuChE genotypes (U/U and U/K) met Kansas criteria for GWI (OR = 2.68; 95% CI = 1.62, 4.44), compared to 92% of Veterans who also took PB pills but had less common genotypes (K/K, U/KA, U/A, A/F, AK/F) (OR = 40.00; 95% CI = 3.58, 447.0).

Associations between GWI and allelic variability in paraoxonase 1 (*PON1*), a gene encoding an enzyme known to hydrolyze organophosphates, have also been tested.[88] A subsample from the U.S. Military Health Survey was used to test relationships between self-reported exposures during the Persian Gulf War and *PON1* polymorphisms in 508 GWI cases and 508 nonpaired controls. A *PON1* polymorphism in codon 192 is known to dictate production of either the 192 glutamine (Q) or 192 arginine (R) isoenzyme, with evidence suggesting RR homozygotes have higher susceptibility to toxic organophosphate nerve agents.[89,90] Haley et al. previously reported that possessing the R allele type (RR homozygous or QR heterozygous) was associated with increased risk of GWI post-deployment, though acknowledging their conclusions were limited by small sample size (GWI = 25; Control = 20)[91].

GWVs who reported hearing chemical alarms during deployment and carried the R allele type had significantly higher risk for GWI (QR heterozygous: prevalence odds ratio = 1.49, p = 0.03; RR homozygous: prevalence odds ratio = 3.49, $p < 0.001$) compared to QQ homozygous allele carriers. Notably, this study reported several instances of GWVs with GWI who 1) did not report hearing chemical alarms (N = 111) and 2) were homozygous QQ allele carriers (N = 43). Accordingly, while QR or RR allele carriers may have an elevated risk for GWI post-deployment, the *PON1* genotype does not account for all GWI cases.

2.1.3.4. Immune System & Inflammation

Inflammation is implicated in various chronic illnesses that affect both Veteran and civilian populations, and it is commonly associated with elevated fatigue,[92] pain,[93,94] disruption of cognition/mood.[95,96] Similarly, greater inflammation in GWVs with GWI has also been associated with chronic symptoms (*e.g.,* fatigue & pain)[97], and the evidence broadly supports involvement from both peripheral and central inflammation (*i.e.,* neuroinflammation). Mixed findings have largely precluded reliance on single immune markers, but consistent immunological differences across the literature substantiate a direct or indirect pathophysiological role in GWI.

GWI studies of peripheral immune activity have tested both innate and adaptive immune responses. Investigations of innate immunity activity have found elevated white blood cell (WBC) counts (*e.g.,* monocytes, neutrophils, and select lymphocytes),[98] greater pro-inflammatory cytokine levels (*e.g.,* IL-6 & TNF-α),[99–101] and greater C-Reactive Protein concentrations[101] in GWVs with GWI. Reduced basal natural killer (NK) cell proportions[99] and post-exercise toll-like receptor 4 (*TLR4*) gene expression[102] have also observed in GWVs with

GWI and partially contradict upregulated innate immunity as a primary pathophysiological source. Innate immune activation in GWVs with GWI may instead reflect compensatory sensitization in response to common household stressors (*e.g.,* bacteria, viruses, etc.), which mirrors a phenomenon observed during allergen-induced *TLR4* activation.[103]

Adaptive immune responses have also been investigated in GWI and may contribute to GWI pathophysiology through maladaptive immunological memory[104] following toxic exposure.[105] Evidence includes elevated T-lymphocyte counts (*e.g.,* CD4+ T-lymphocytes),[99,105,106] higher concentrations of cytokines associated with adaptive immunity (*e.g.,* IL-2, IL-4, IL-10, IL-17F, IFN-γ),[9,99,100,105] and reduced human leukocyte antigen (HLA) allele profiles.[16] Higher overall lymphocyte counts have also been significantly associated with specific symptoms of general health, physical function, and cognitive performance in GWVs with GWI.[107] Th1 response polarity is consistent across the GWI literature (*e.g.,* elevated CD4+ T-lymphocytes, IL-2, IL-10, IFN-γ)[99,105,106] and commonly indicates host defense against viral or bacterial pathogens.[104] Activated Th1 responses may serve as a precursor to fluctuating innate immune activity[108] also observed in GWI.

Several future studies intend to expand immune response characterization[109] and test the clinical efficacy of immune-modulating therapeutics (NCT04254627).[110] Incorporating epigenetics (*e.g.,* DNA methylation) with these efforts may improve diagnosis, prognosis, and treatment optimization in GWVs with GWI. Notably, the oncology field has leveraged DNA methylation as an indicator of disease prognosis[111] and symptom response (*e.g.,* fatigue) during patient treatment.[112] Similar approaches in GWVs with GWI may facilitate better individualized treatment strategies (*i.e.,* precision medicine) with candidate therapies and uncover

relationships between underlying pathophysiology and specific symptoms. This dissertation project will use array-based DNA methylation analyses that encompass thousands of genes across several biological pathways. Interactions between peripheral inflammation and neuroinflammation[113] or mitochondrial oxidative damage[114,115] are plausible in GWI, and blood-based DNA methylation may contextualize multi-system interactions. Because the present project will use whole blood bulk tissue and reference-based cell proportion corrections, future studies utilizing single-cell epigenetic approaches will be critical for advancing a mechanistic understanding of GWI.

2.1.3.5. Gut Microbiome

Unique patterns of gut microbiota in GWVs with GWI may contribute to GI symptoms and indirectly influence other forms of pathophysiology, such as *TLR4*-mediated inflammation. Chemically induced dysbiosis tested in-vivo (i.e., murine models) resulted in greater systemic *TLR4* activity; however, TLR4 knockout models had significantly lower inflammation (*e.g.,* IL-1β) in the frontal cortex and intestinal tract.[116] GWI murine models have also demonstrated that comorbidities (*e.g.,* obesity) or lifestyle behaviors (*e.g.,* poor nutrition) may exacerbate these microbiome differences by upregulating IL-1β, IL-6, brain-derived neurotropic factor (BDNF), and microglial activity.[117] Mechanisms underlying dysbiosis and inflammation remain unclear, but gut dysbiosis may indirectly contribute to cognitive difficulty, fatigue, and pain through these pathways.

A study examining stool samples of three different subgroups of GWVs—healthy GWVs (n = 7), GWVs with GWI but no GI symptoms (n = 5), and GWVs with GWI who had GI symptoms

(n=14)—found that GWVs with GWI who experience GI symptoms had more diverse bacteria populations in the gut microbiome in addition to higher levels of pain and fatigue.[118] Specifically, GWVs with GWI and GI symptoms had larger proportions of proteobacteria, which have been previously associated with both irritable bowel disease and Crohn's Disease.[119] A subsequent validation study (GWVs with GWI = 63; control GWVs = 26) confirmed self-reported fatigue severity was associated with higher microbiota subpopulations (*e.g., Blautia*, *Streptococcus*, *Klebsiella*, etc.).[120] Diagnostic application of these subpopulations were limited and achieved only modest classification accuracy (AUC ROC = 74.8%). Several botanical supplements known to reduce inflammation (*e.g.,* curcumin,[121] Resveratrol,[122] and stinging nettle[123]) have significantly reduced GWI symptoms in early phase clinical studies, and it is plausible that compounds like curcumin provide benefit by acting through the GI tract.[124] Ongoing efforts, including a recently initiated clinical trial designed to enhance microbiota through resistant starches in GWVs with GWI (NCT05820893),[125] will help address whether microbiota modulation improves GWI. Dietary interventions are known to impact blood-based DNA methylation in healthy populations,[126] and future GWI dietary studies should consider blood or stool derived DNA methylation as a biological informant of microbiota-mediated inflammation.

2.2. Post-Exertional Malaise

While the pathophysiological advances in GWI are promising, many questions about the underlying mechanisms remain, warranting integrative study approaches that assess a psychobiological model of disease. Several groups have leveraged a unique phenomenon of

CMI, including GWI, known as post-exertional malaise (PEM). PEM lacks a universally accepted clinical definition, but it is commonly conceptualized as an acute exacerbation or worsening of symptoms following physical and/or mental effort.[127] Additionally, PEM has considerable heterogeneity across its initiating triggers, length, severity, and symptom specificity.[128,129] PEM prevalence varies across CMIs, but epidemiological investigations estimate that PEM occurs in up to 90% of ME/CFS cases.[130] Key components of PEM include exacerbated fatigue, pain, and cognitive difficulties;[127] however, these results often vary based on measurement standards. Recently developed and validated psychometric instruments for assessing PEM, such as the DePaul Symptom Questionnaire (DSQ) PEM subscale,[131] will likely improve future PEM characterization efforts.

Controlled stressors (*e.g.,* exercise challenges & cognitive tests) designed to manipulate symptoms have become critical tools used to assess PEM.[7–9,64] Pre-post exercise studies of CMI have applied psychometric instruments (*e.g.,* validated questionnaires and adapted visual analog scales [VAS]),[7,81,132] wearable devices,[133] and qualitative interviews[134] to characterize symptom exacerbation (*i.e.,* symptom worsening). Many challenges associated with defining and measuring PEM are unresolved,[129] and a comprehensive review of the current measurement standards are beyond the scope of the present dissertation.

Previous psychobiological investigations of PEM in participants with ME/CFS have demonstrated that an acute bout of exercise negatively affects performance and brain responses to complex cognitive tasks,[135] alters transcriptional activity (*i.e.,* mRNA levels) across genes involved in numerous biological processes,[132,136,137] and adversely affects autonomic function.[6,138] These findings support the use of exercise challenge paradigms to simultaneously

manipulate symptoms and biology underlying PEM. Concordant symptomatic and biological responses observed post-exercise may best depict underlying CMI pathophysiology. DNA methylation—among other epigenetic mechanisms—is a primary regulator of molecular processes, and studies that assess changes in the DNA methylation landscape following acute stress (*e.g.,* vigorous exercise) will be critical in understanding how PEM contributes to GWI pathophysiology. Heterogenous phenotypes like PEM require rigorous characterization, but few GWI studies report PEM time course or severity. To illustrate PEM response variability in GWI, several individual studies are described in the next section.

2.2.1. Post-Exertional Malaise in GWI

PEM is included in the Kansas case-definition criteria but not required to meet criteria for GWI.[2] Prevalence estimates suggest that up to 96% (n=23/24) of GWVs with GWI report experiencing PEM.[6] Similar to the broader CMI field, epidemiological studies of PEM in GWI are reliant on self-reported experience. Few PEM studies in GWI have prioritized individual variation in symptom severity or symptom time course.

An investigation by Lindheimer et al. (2020) tested whether self-reported PEM endorsement differentiated post-exercise symptom responses by comparing GWVs with GWI (n = 39) and control GWVs (n = 28) who completed a vigorous exercise challenge.[139] GWVs completed 30-minutes of submaximal cycling exercise test at 70% of their predicted heart rate reserve (HRR) and symptoms were measured immediately before, after, and 24 hours after exercise. PEM was defined as a greater increase in symptom severity from baseline to post-exercise in GWVs with GWI relative to control GWVs, and PEM endorsement consistent of a

binary response (*i.e.,* "yes" or "no") when GWVs were asked if "feeling unwell after physical exercise or exertion" was a typical experience. Direct comparisons between GWVs with GWI and control GWVs did not reveal differences in symptoms from pre-to post-exercise. Subgroup analyses of GWVs with GWI who endorsed experiencing PEM revealed significantly larger symptom exacerbations both post-exercise (*e.g.,* shortness of breath) and 24 hours post-exercise (*e.g.,* fatigue, neuropathic pain, nausea, difficulty concentrating) compared to control GWVs. Notably, a lack of recovery at 24 hours post-exercise may differentiate GWVs with GWI who endorse PEM from those who do not. Evidence indicates that PEM responses peak between 24-72 hours after exercise in other forms of CMI,[140–142] and subsequent GWI studies have investigated individual variability within PEM time course.

Boruch et al., 2021 expanded on this work and reported significant differences in symptom exacerbation from pre- to post-exercise between GWVs with GWI (N = 43) and control GWVs (N = 31).[143] Symptoms were measured via VAS operationalized from the 29-item Kansas Symptom Questionnaire over 7-days pre-exercise and 7-days post-exercise. The symptom with the greatest change from baseline (*i.e.,* average across 7-days pre-exercise) to post-exercise was defined as each participant's "peak PEM response". GWVs with GWI reported significantly larger peak PEM responses than control GWVs (Hedge's $g = 0.70$, $p < 0.01$; Figure 1A), and the majority of PEM responses occurred between 24-72 hours post-exercise (*i.e.,* post-days 1-3; Figure 1B).

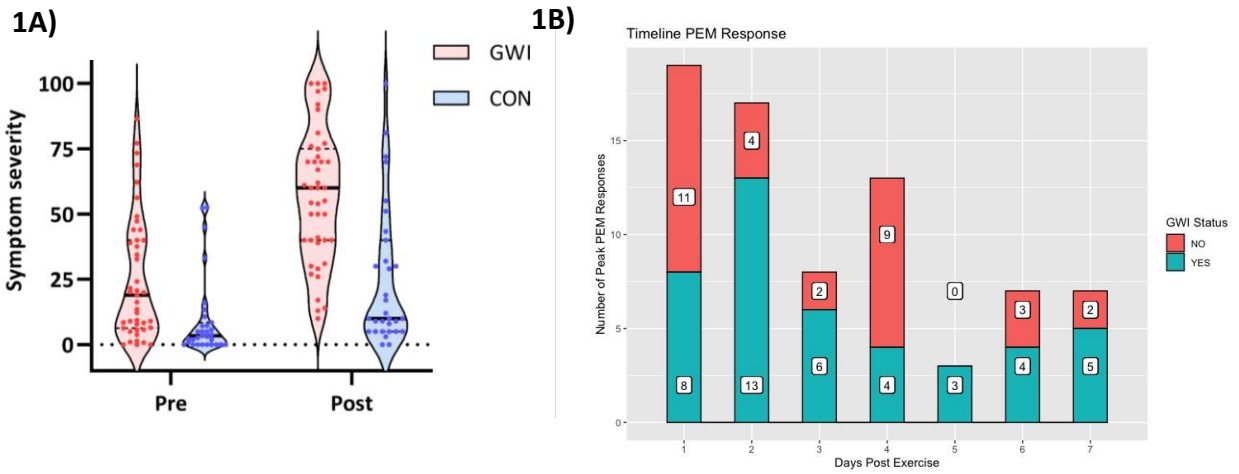


Figure 1A-1B. Peak PEM response severity and time course. **1A)** Symptom severity for GWI and CON groups pre-exercise and post-exercise. The GWI group experienced larger symptom exacerbation from pre-exercise (median = 19, IQR = 6.25, 40) to post-exercise (median = 60, IQR= 40, 75) compared to the CON group (pre-exercise: median=3.33, IQR= 0, 8.5); post-exercise: median=10, IQR= 5, 40), indicating a post-exertional malaise response in the GWI group. **1B)** Each column corresponds to the frequency of participants whose peak symptom response to exercise occurred that day. About 60% (44/74) of peak symptom responses were observed within 72 hours post-exercise.

Note: Adapted from Boruch et al., 2021 (*Life Sciences*) Figure 3 & Figure 4.

The peak PEM response was designated as the dependent variable in a regression model testing potential predictors of PEM. Findings from prior work[12,144,145] were consulted to select four independent variables – (i) cumulative work during exercise, (ii) ventilatory equivalent for carbon dioxide ($\dot{V}E/\dot{V}CO_2$), (iii) peak exercise leg muscle pain, and (iv) the Veteran's Rand 36 Item Health Survey (VR-36) physical component score as model predictors. GWVs with GWI had significantly worse physical health and less efficient ventilatory responses compared to control GWVs, but these metrics did not significantly predict PEM responses (Pooled R^2 = 0.15, Adjusted R^2 = 0.03, p = 0.34). A separate study from the same cohort tested whether changes in gene expression mediated peak PEM responses observed in GWVs with GWI.[102] Using a panel of

a-priori selected genes (N = 13) based on stimulated in-vitro responses[146] and changes after exercise in ME/CFS participants,[81,132,147] differential gene expression in GWVs with GWI (N = 37) compared to control GWVs (N = 25) was tested from pre- to post- and 24 hours post-exercise. Lower relative expression levels in beta-actin (*ACTB*), catechol-o-methyltransferase (*COMT*), and toll-like receptor 4 (*TLR4*) were detected post- and 24 hours post-exercise in GWVs with GWI compared to control GWVs (Figure 2).

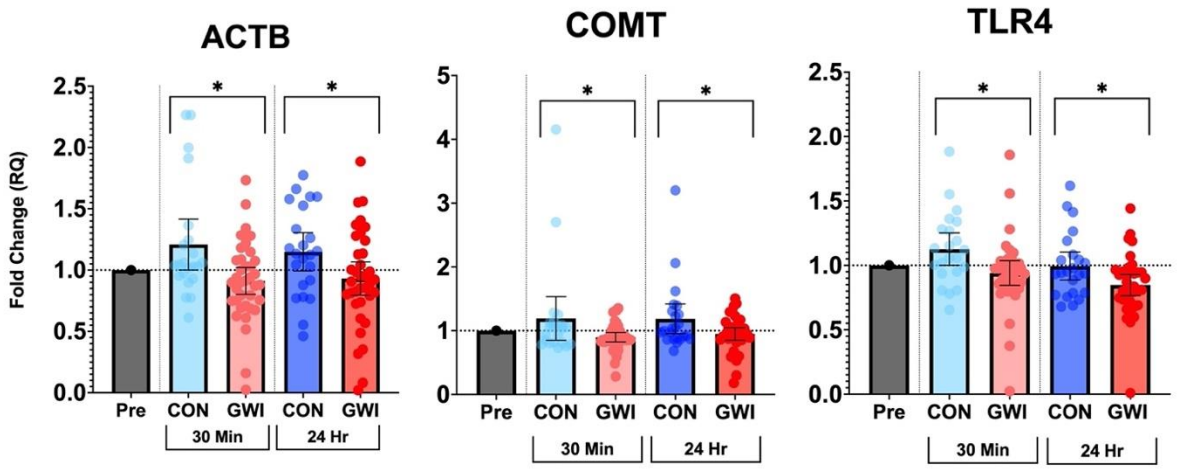


Figure 2. Differentially expressed genes in response to exercise between GWVs with GWI compared to CON GWVs. Pre-exercise gene expression was relativized to a value of 1.0 (gray bar). A significant main effect of group was observed for ACTB (Main Effect: partial η2 = 0.09, 95% CI = 0.01, 1.00; 30 min post: p = 0.02; 24 h post: p = 0.04), COMT (Main Effect: partial η2 = 0.10, 95% CI = 0.01, 1.00; 30 min post: p = 0.02; 24 h post: p = 0.04), TLR4 (Main Effect: partial η2 = 0.10, 95% CI = 0.01, 1.00; 30 min post: p = 0.01; 24 h post: p = 0.03) at both 30 min and 24 h post-exercise as detected by RM-ANOVA.

Note: Adapted from Boruch et al., 2023 Figure 2 (*Brain, Behavior, Immunity – Health*).

These differences in gene expression, however, did not mediate peak PEM responses 24 hours post-exercise. Lack of support for the primary hypothesis may be partially attributed to limitations in the peak PEM response measurement (*e.g.,* symptom heterogeneity, time course variation, ceiling effects) and the narrow scope of genes tested for differential expression.

Future approaches that investigate comprehensive transcriptomics are necessary to understand how changes in gene expression pertain to PEM.

The exercise challenge paradigm has also been used to test individual exertional thresholds (*i.e.,* intensity level) that elicit PEM responses, and these findings suggest that post-exercise symptom exacerbation only occurs for some GWVs with GWI.[148] In a randomized controlled cross-over study, GWVs with GWI (N = 40) completed three different intensities of aerobic exercise (*i.e.,* light, moderate, vigorous) and a seated rest control condition across four total study visits. Symptom measures (*e.g.,* psychometric questionnaires, pain sensitivity, cognitive performance) and plasma inflammatory markers (*e.g.,* IL-6, IL-8, IL-10, TNF-α, C-Reactive Protein) were collected before and after each condition, and symptom questionnaires and physical activity actigraphy were measured outside the lab 7 days post-exercise.

Although group-level changes in symptom responses were not significantly different across exercise compared to seated rest, symptom-specific exacerbation (*e.g.,* fatigue) occurred for select individuals. As an example, fatigue symptoms increased in 46% of GWVs with GWI after vigorous exercise compared to 15% after seated rest. The small increases in fatigue symptoms after rest (6/40 participants) may be partially linked to mental exertion from procedures associated with study participation. PEM may present differently in GWI compared to other forms of CMI, and potential differences may reflect heterogeneity in symptom severity and triggering events.

Significant Condition-by-Time interactions were identified for IL-6 after the moderate and vigorous intensity conditions, and these responses parallel trends observed in acute exercise of healthy individuals.[149] Outside of PEM, acute aerobic exercise lasting up to 60

minutes has a well-documented intensity-dependent relationship with both plasma-based cytokines (*e.g.,* IL-1, IL-6, IL-8, TNF-α) and several lymphocyte subpopulations (*e.g.,* CD4 T-cells, CD8 T-cells, natural killer cells, and neutrophils).[149–151] Elevated cytokines (*e.g.,* IL-6)[151] and lymphocytes (e.g., natural killer cells)[152] may persist up to 2 hours post-exercise in healthy individuals; however, response variation depends on the exercise stimulus and input from other physiological sources (*e.g.,* neuroendocrine system).[149] GWVs with GWI who completed acute maximal aerobic exercise tests had altered inflammatory responses, and peak exercise immune activity (*e.g.,* IL-10 concentrations & CD2 lymphocyte counts) were significantly associated with baseline illness severity.[9] Little is known about relationships between epigenetic modifications and PEM in any form of CMI, but preliminary evidence in ME/CFS (n = 3) indicates PEM may coincide with differential DNA methylation in genes associated with immune function.[153] As such, epigenetic profiles in whole blood are well-suited to better understand molecular contributions of inflammation to GWI pathophysiology (Section 2.1.3.4.) and PEM.

2.3. Introduction of Epigenetics

Developments in the field of epigenetics have expanded on the original "central dogma" of genetics (*i.e.,* DNA, RNA, protein) by providing evidence that external factors (*e.g.,* environment, behavior, etc.) interact with substrates to influence biology. In essence, epigenetics has been described as the mediator between genetics and the environment. Epigenetics broadly refers to chemical modifications to DNA that influence gene transcription without altering DNA sequence. The field of epigenetics encompasses many different mechanisms and modifications, but the present dissertation focuses specifically on a singular

modification (*i.e.,* DNA methylation). As such, DNA methylation quantification methods and application to human health are described in detail. The next section provides brief overviews of different epigenetic mechanisms.

2.3.1. Overview of Epigenetic Mechanisms

There are several important considerations when deciding which epigenetic mechanism is of most interest to study. These include, but are not limited to, the primary research question, phenotype, and type of biological tissue. Beyond DNA methylation, other epigenetic mechanisms include DNA hydroxymethylation, histone modifications (*e.g.,* histone methylation and histone acetylation), and chromatin remodeling. Because of their effects on gene transcription, associated mechanisms like non-coding RNA (ncRNA) are also commonly discussed within epigenetics.

2.3.1.1. DNA hydroxymethylation

DNA hydroxymethylation (*i.e.,* 5-hydroxymethylcytosine [5hmC]), occurs through oxidation of 5-methylcytosine via the Ten-Eleven Translocation (TET) methyl-cytosine dioxygenase family, resulting in the replacement of hydrogen at cytosine position 5 with a hydroxymethyl group.[154] Functionally, the highest levels of 5hmC occur in the central nervous system[154] and are associated with increased gene expression, which may facilitate neuronal functions like memory formation.[155]

2.3.1.2. Histone Modifications

The histone protein family includes four subtypes (*e.g.,* H2A, H2B, H3, and H4), and collected pairs of each histone subtype bind to form octamer structures within the cell nucleus.[156] An octamer entwined by approximately 150 DNA base pairs, a complex often referred to as a nucleosome, are conjoined by linker DNA to form the basic units of a chromosome.[156,157] Epigenetic modifications are known to change nucleosome conformation and influence DNA accessibility to transcription factors.[157] Closed heterochromatin conformation restricts DNA access to transcription factors, while an open euchromatin state facilitates transcription (Figure 3). Histone modifications manipulate DNA accessibility through mechanisms like methylation or acetylation of the amino acid-rich histone tail. Mechanisms like histone-3 lysine-4 acetylation (H3K4ac) or mono-methylation (H3K4me1) induce a euchromatin state for active gene transcription, while trimethylation of histone-3 lysine-9 (H3K9me3) or -27 (H3K27me3) facilitate heterochromatin states associated with reduced gene transcription.[158]

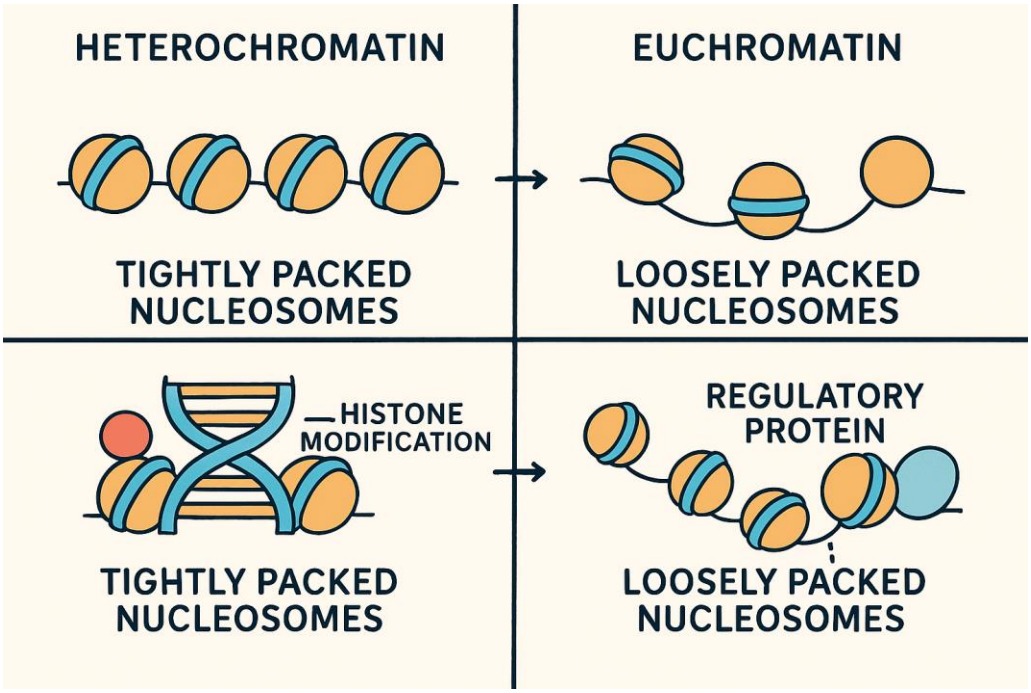


Figure 3. Overview of nucleosome conformation. Closed heterochromatin conformation state (top left) restricts gene transcription by limiting DNA access to transcription factor binding. Histone modifications like acetylation (bottom left) can induce an open euchromatin state (top right) that increase DNA accessibility to transcription factors or other regulatory proteins (bottom right).

2.3.1.3. Mechanisms Related to Epigenetics: non-coding RNA (ncRNA)

Non-coding RNA (ncRNA) is commonly discussed with other epigenetic mechanisms because of its role in modulating gene expression. ncRNAs have several subtypes with diverse functions, including ribosomal RNA (rRNA), small interfering RNA (siRNA), piwiRNA (piRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), long non-coding RNA (lncRNA), and microRNA (miRNA).[156,159] Efforts to characterize ncRNAs and their associated biological processes are ongoing, but both lncRNA and miRNA are known regulators of gene expression.[156] lncRNA span ~17,000 base pairs in length and have several known regulatory

functions in gene transcription. These include increasing transcription factor binding in the gene promoter (*i.e.,* increasing transcription),[156] blocking RNA polymerase binding (*i.e.,* decreasing transcription),[156,159] and X-chromosome silencing.[159] miRNA span ~22 base pairs in length and are often associated with repressed gene transcription (*i.e.,* post-transcriptional gene silencing) through aggregation into RNA-induced silencing complexes.[159,160] The first RNA interference-based therapies have emerged within the last decade,[161] and future treatments that utilize ncRNA's regulatory role may be useful for treating complex disease.

2.3.2. DNA Methylation

DNA methylation, the covalent addition of a carbon-methyl group to position 5 of the DNA nucleotide cytosine (5mC), is widely regarded as one of the most well-studied epigenetic modifications (Figure 4). DNA methylation and gene transcription have generally been described as inversely related; higher levels of DNA methylation in gene promoters (*i.e.,* hypermethylation) generally correlate with down-regulated gene transcription, while lower promoter DNA methylation (*i.e.,* hypomethylation) correlates with up-regulated gene expression. Promoter hypermethylation is thought to directly prevent transcription factor (TF) binding activity or indirectly recruit other binding factors (*e.g.,* methyl binding proteins) that also systematically prevent TF binding. In contrast, hypomethylation may facilitate TF access to the promoter region, allowing for gene transcription. Gene promoters notoriously contain a high frequency of repetitive CpG sites, commonly referred to as CpG Islands, and aggregated methylation within these regions regulates transcription activity.[162,163] Although DNA

methylation occurs in other genomic regions (*e.g.,* exons, introns, intergenic regions), its exact function at these locations is not well-established.

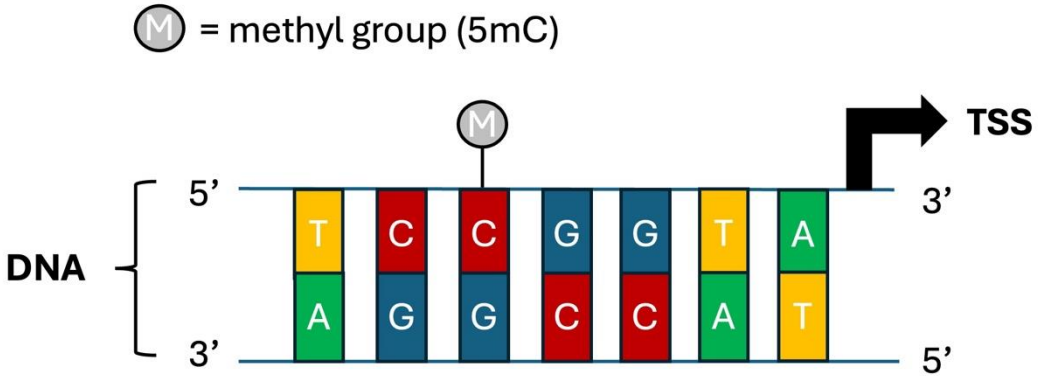


Figure 4. Overview of DNA methylation in gene promoter. Covalent additions of carbon-methyl groups to position 5 of the DNA nucleotide cytosine (5mC) within the gene promoter are often inversely related to gene transcription activity. For example, higher levels of methylation (*i.e.,* hypermethylation) coincide with decreased gene transcription and expression. 5mC blocking transcription factor binding to DNA may partially explain this inverse relationship.

Proteins known as DNA methyltransferases (DNMTs) are the regulators of methylating CpG sites and require methyl donation from substrates like S-adenosyl methionine to covalently attach the methyl group to cytosine position 5. Four primary subtypes have been characterized in humans: DNMT1, DNMT3A, DNMT3B, and DNMT3L.[164] DNMT1 uses an ATP-dependent process to maintain DNA methylation during DNA replication and acts exclusively on hemi-methylated DNA (*i.e.,* DNA with single strand methylation).[165] DNMT3A and DNMT3B both participate in de novo methylation,[166] which add methyl groups to previously unmethylated CpG sites. DNMT3A and 3B activity are principal factors in embryonic stem cell differentiation during development and may facilitate tissue-specific DNA methylation patterns observed across organismal lifespan.[167] Mounting evidence also suggests that DNMT3A may possess

higher CpG specificity based on structural variability observed in DNMT3B (*e.g.,* a catalytic loop in DNMT3B that contains a hydrogen bond).[168] As such, this structural variability and associated heterotetrametric formation between DNMT3A or 3B and DNMT3L potentially contribute to organismal development or disease by facilitating DNMT attachment to DNA.[169]

Knowledge surrounding epigenetics—including DNA methylation—is evolving rapidly and indicates that interactions between epigenetic mechanisms are complex.[170] Other dynamic processes influenced by DNA methylation include crosstalk between genomic regions (*e.g.,* promoter-enhancer interactions) chromatin accessibility (*e.g.,* histone modifications). When taken together, these processes may better explain underlying epigenetic impact on biology.

2.3.3. Methods for Quantifying DNA Methylation

Methodologies for detecting and quantifying DNA methylation have evolved considerably across the last two decades, and notably, selecting the most appropriate DNA methylation methodology is highly dependent on the research question under investigation, cell and/or tissue-type of interest, and desired granularity of genome resolution (*e.g.*, global DNA methylation vs. singular CpG sites, sequencing coverage, sequencing depth, sequencing uniformity).

Further, the evolving methodologies of DNA methylation analyses necessitate bioinformatics approaches capable of tackling high-throughput data. Reviews published by Teschendorff & Relton (2018),[171] Singer (2019),[172] and Li & Tollefsbol (2021)[173] each provide elegant overviews comparing various DNA methylation analysis platforms and associated statistical considerations. While a detailed assessment of each DNA methylation analysis

methodology is beyond the scope of this review, a summary of the primary umbrella categories (*e.g.,* restriction endonuclease-based analysis, affinity enrichment-based analysis, and bisulfite/enzymatic conversion-based analyses) are described below.

2.3.3.1. Restriction endonuclease-based analyses

Restriction endonuclease-based strategies for DNA methylation analysis rely on methylation-sensitive enzymes (*i.e.,* restriction endonucleases) that target specific restriction sites within a DNA locus yet lack the capability of digesting methylated cytosines.[173,174] 5mC blocks restriction endonucleases from binding with recognition sequences, which ultimately indicates methylation status (*i.e.,* methylated or unmethylated). As such, this methodology especially applicable for assessing DNA methylation in targeted regions of interest. Original restriction endonuclease-based methodologies rely on southern blotting or PCR fragment amplification, both of which provide low sequence resolution and require high quality and quantity of input DNA.[173] Combined methods using restriction endonuclease approaches and next generation sequencing (NGS) have demonstrated proficiency for identifying allele-specific DNA methylation across the genome, though this approach remains limited by coverage capability (*i.e.,* availability of genomic enzyme recognition sites) and false-positive associated with incomplete endonuclease digestion.[173,174]

2.3.3.2. Affinity enrichment-based analysis

Affinity enrichment-based methodologies for DNA methylation analysis include methylated DNA immunoprecipitation (MeDIP) and methyl-binding domain sequencing (MBD-

seq).[172] These approaches entail use of monoclonal antibodies with designed affinity for 5mC. Before applying the antibody, these approaches typically require DNA fragmentation into 100-500bp segments, DNA purification, DNA end-repairment, and sequencing adapter ligation. Notably, MeDIP has a stronger affinity for single-stranded DNA, and MBD-seq approaches are applied double-stranded DNA assessment.[173] Greater genomic resolution using affinity enrichment-based approaches is attainable with adaptations to NGS, providing resolutions of ~100bp. These methods, however, are unable to provide single CpG resolution and are subject to bias from both copy number variation and CpG density within surveyed genomic regions.[172] While considerably cost-effective, affinity enrichment-based approaches commonly require larger quantities of input DNA than other discussed methods (*e.g.,* bisulfite conversion).

2.3.3.3. Bisulfite & enzymatic conversion-based analyses

Bisulfite conversion-based analyses of DNA methylation encompass a wide variety of platforms, including bisulfite-equipped pyrosequencing, reduced representation-bisulfite sequencing, microarray-based bead chip analyses, and whole genome bisulfite sequencing (WGBS). During bisulfite conversion, denatured DNA is pre-treated with sodium bisulfite, converting unmethylated cytosines into uracil, while methylated cytosines remain unaffected.[172] After DNA amplification, microarray-based workflows (*e.g., Illumina MethylationEPIC*) require DNA fragmentation and hybridization to bead chips containing CpG-specific specific probes. Fluorescent activity for each probe ultimately delineates the methylation status for the profiled CpG sites. Microarray-based approaches are efficient and well-validated,[175] but their composition of only predefined CpG sites is a noteworthy source of

inherent bias.[173] De novo CpG detection and full genomic coverage (~28,000,000 CpG sites) require sequencing based approaches.

Additional distinctions between microarray and sequencing-based approaches (*e.g.,* WGBS) include typical library preparation and the replacement of converted uracil with thymine prior to sequencing. Procedural alignment (*e.g.,* Bismark Procedure) after sequencing then uses a reference genome to delineate a genomic thymine from a bisulfite converted cytosine across the genome.[21,174] Although bisulfite conversion-based approaches are labor intensive and expensive, they can be performed with low DNA input (*e.g.*, ~500ng) and provide single nucleotide genomic resolution.

In light of DNA fragmentation and damage that is often incurred by bisulfite conversion, enzymatic approaches have also been used to analyze DNA methylation.[176,177] While similar to bisulfite conversion, enzymatic conversion instead relies on carboxymethyltransferase, which converts unmethylated CpG sites to 5-carboxymethyl cytosine (5cxmC). Rather than through bisulfite conversion, 5mC sites are converted to thymine via apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A (A3A) deaminase. PCR and sequencing ultimately detect thymine in lieu of 5mC and 5cxmC rather than unmethylated cytosine following alignment.[176] Direct comparisons of bisulfite conversion and enzymatic conversion are needed to ascertain both advantages and disadvantages of each approach.

2.3.4. Biological Effect Size: DNA Methylation and Phenotypes

In addition to selecting the approach analysis methodology, quantifying the biological meaningfulness of DNA methylation (*i.e.,* effect size, number of differentially methylated CpG

sites, location of CpG sites in gene body, etc.) needed to influence phenotype has been debated. Cross-sectional case-control comparisons have identified differential DNA methylation levels across several disease populations that include mild cognitive impairment/Alzheimer's Disease,[178,179] Post-Traumatic Stress Disorder,[180] cancer,[181] and cardiovascular disease.[182] Large differences in DNA methylation levels (*i.e.,* > 30%) have been reliably detected in certain illnesses like cancer[183] and in individuals who smoke.[184] Segregation between a phenotype and its associated reference comparison (*e.g.,* patient with cancer vs. matched cancer-free control) maximize the likelihood to discern large differences in DNA methylation when comparing cases and controls.

Other phenotypes—such as chronic multi-symptom illnesses—are distributed across a continuous spectrum of severity and are defined by broad clinical parameters that co-occur at generally lower levels in otherwise healthy controls. Investigations measuring DNA methylation within populations like myalgic encephalomyelitis/chronic fatigue syndrome have reported thousands of differentially methylated CpG sites and genes, corresponding to multiple physiological pathways (*e.g.,* immune/inflammatory processes, metabolism, and neurological/neuroendocrine pathways).[185] Notably, differential DNA methylation across CpG sites in participants with ME/CFS reflect small-to-modest effect sizes (*e.g.,* mean methylation differences of ~15% or less) and support that small methylation differences across myriad genes may facilitate ME/CFS disease pathology.[186–188] Efforts by de Vega et al. (2017) have also tested relationships between continuous symptom measures (*e.g.,* RAND-36 Health Survey) and DNA methylation levels in ME/CFS.[187] Significant associations between health-related quality of life and DNA methylation levels were found for genes involved in metabolism (*e.g.,* ATPase H+

transporting V0 subunit e2 [*ATP6V0E2*]) and immune signaling (*e.g.,* interleukin 6 receptor [IL6R]). Modeling complex illnesses with continuous, rather than binary, classifications is a critical next step in deciphering relationships between DNA methylation and disease severity. Leenan et al. (2016) emphasize that subtle widespread alterations in DNA methylation may facilitate biologically significant impacts on phenotype,[189] and this perspective is critical for future studies in parallel fields (*e.g.,* environmental exposure).

Testing whether DNA methylation profiles underlie disease pathogenesis in complex illnesses requires large sample sizes of well-characterized phenotypes under carefully designed studies (*e.g.,* longitudinal or randomized controlled trials). A narrative review by Dr. Michael K. Skinner (2024) outlines ways in which DNA methylation has been correlated to different traits (*e.g.,* epigenome-wide association studies) and may expand insight about population-level origins of human disease beyond previous methods (*e.g.,* GWAS).[190] Skinner notes the important functions DNA methylation plays in human development (*e.g.,* X-chromosome silencing and genetic imprinting) and emphasizes its sensitivity to both behavior and environment in early life. Skinner suggests that early life behaviors like diet and exercise exhibit long-lasting physiological impacts that influence predisposition for diseases (*e.g.,* obesity, metabolic disorders/Type II Diabetes, etc.) later in life partially through DNA methylation. Clinically, Skinner describes how DNA methylation patterns may aid identifying which patients at high risk for breast-cancer would benefit from prophylactic Tamoxifen treatment to prevent disease development. This is one example in which Skinner suggests DNA methylation may shift our current standard of clinical practice from reactionary to a more preventative-based approach. Precision approaches that inform about disease risk, treatment timing, and individual

responsiveness to pharmacological intervention are all examples of potential future clinical applications of DNA methylation. Cumulatively, Skinner emphasizes that large longitudinal/prospective studies capable of capturing DNA methylation (and other epigenetic markers) relative to behavior, environment, and development of disease are needed in order advance our capabilities to the preventative level. Advances in both development and accessibility of largescale/high throughput sequencing technology and integration of bioinformatics tools will be critical for solidifying our understanding of DNA methylation as it pertains to human traits.

Other notable considerations elegantly discussed by Leenen et al. (2016) and Skinner (2024) include 1) sampled tissue type (*e.g.,* tumor tissue vs. systemic whole blood), 2) method of quantification/analysis (*e.g.,* difference in mean methylation vs. variable methylation), 3) resolution (*e.g.,* differentially methylated regions vs. single CpG position), and 4) interactions with heritable genomic sequence (*e.g.,* underlying single nucleotide polymorphisms or methylation quantitative trait loci [meQTLs]).[189,190]

2.3.5. Exercise & DNA Methylation

Exercise and physical activity are pillars of holistic human health, providing myriad benefits for physical function, mental wellbeing, and disease prevention. While the benefits of exercise and physical activity accumulate across one's lifespan, they are also ubiquitous and non-exclusive. Exercise and physical activity procure benefits in both acute and chronic regiments, encompassing many modalities and mechanisms. Although the term "stress" often receives a negative connotation in the context of human health, it would be remissible to argue that exercise and physical activity do not constitute forms of stress. Stress incurred through

physical activity and exercise facilitate adaptation and highlight the intersection between behavioral action (*i.e.,* participating in physical activity or exercise) and subsequent biological reaction.

Repeated exposure to strenuous physical activity (*i.e.*, moderate-to-vigorous physical activity) axiomatically improves overall fitness and strength, which inherently increases biological resilience when encountering extraneous stress in the environment.[191] An accumulating body of evidence supports epigenetics as an intermediary between exercise and its associated health benefits. Continued exploration of epigenetic manifestations from exercise and physical activity will advance our understanding of their biological significance in maintaining health and preventing disease. To date, several studies have investigated acute exercise,[192,193] chronic exercise training,[194–197] and physical activity behavior[198,199] in relation to DNA methylation. Because the literature describing relationships between DNA methylation exercise is relatively limited, several specific studies are discussed in the next sections.

2.3.5.1. Acute Exercise & DNA Methylation

One early investigation tested the effects of an acute exercise stressor on human skeletal muscle cell DNA methylation in a small sample (N = 14) of healthy adults.[200] This two-phase investigation profiled DNA methylation in skeletal muscle cells collected at rest and 20 minutes after a maximal exercise test (Phase 1), followed by pre-post skeletal muscle biopsies in sedentary males (N = 8) who completed both low and high intensity isocaloric exercise challenges (Phase 2). Phase 1 pre-post comparisons of acute exercise detected global hypomethylation and hypomethylation in promoters of genes that participate in energy metabolism (*e.g.,* PPARG coactivator 1 alpha [*PGC-1α*], peroxisome proliferator activated

receptor gamma [*PPARd*], mitochondrial transcription factor A [*TFAM*], pyruvate dehydrogenase kinase 4 [*PDK4*]). Significant hypomethylation up to 3 hours post-exercise was reproduced for *PPARd* and *TFAM* during Phase 2 comparisons, in addition to significant and inversely related levels of gene expression (*i.e.,* increased expression) at the corresponding timepoints. The high-intensity exercise condition elicited larger magnitudes of hypomethylation and increased expression, which support a dose-response relationship. The authors conclude that the dynamic regulation of DNA methylation over gene expression may partially explain physiological adaptations to acute exercise.

Differential methylation in energy metabolism pathways has also been identified during acute resistance exercise challenges. Using a within subject's pre-post crossover design, DNA methylation within skeletal muscle (*i.e.,* vastus lateralis) was analyzed in college-aged male participants (N = 11) across three timepoints (*i.e.,* baseline, 3 hours post, and 6 hours post) in response to two resistance exercise challenges.[193] Participants completed four sets of back squats and leg extensions to volitional exhaustion under high load (HL; 80% of 1 repetition maximum) and low load (LL; 30% of 1 repetition maximum) intensities. Comparisons of LL and HL conditions revealed differential methylation within gene promoters (3h post-exercise: 793 DMPs; 6h post-exercise: 858) and predominantly consisted of hypomethylation (3h post-exercise: 97.4% hypomethylated; 6h post-exercise: 88% hypomethylated). Enriched pathways that overlapped across differentially methylated and differentially expressed genes include mitogen-activated protein kinase (*MAPK*) signaling, which functions as a known regulator of cellular growth and glucose utilization.[201] Cumulatively, DNA methylation levels within skeletal

muscle are responsive to acute aerobic and resistance exercise modalities, and these findings support that DNA methylation participates in muscular adaptations to exercise.

Other investigations have assessed the effects of acute exercise on DNA methylation using whole blood, including one that tested relationships between blood-based DNA methylation and interleukin 6 (IL-6) cytokine levels.[202] Previous acute exercise protocols have reliably demonstrated systemic inflammatory responses indicative of stress (*e.g.,* significantly elevated IL-6 post-exercise);[149,203] participants (N = 8 healthy males) completed a 120 minute submaximal exercise test (60% VO_2max), followed by a 5 kilometer time trial. Enzyme-linked immunosorbent assays (ELISA) were used to assay plasma IL-6 concentrations, and DNA methylation levels were analyzed by bisulfite microarray of ~27,000 CpG sites in whole blood collected across three time points (e.g., pre-exercise, post-exercise, and 24 hours post-exercise). As suggested by previous evidence, significant increases in IL-6 were observed post-exercise and decreased to near baseline levels by 24 hours post-exercise. While differential methylation was not detected post-exercise or 24 hours post-exercise, significant correlations were identified between IL-6 and 11 CpG sites (positive correlation = 3 CpG sites, negative correlation = 8 CpG sites; R^2 range = -0.78 – 0.77). Seven of the 11 CpG sites significantly correlated with IL-6 were annotated to genes with empirically supported roles in immune function. Additional investigation, the authors note, is required to better understand the temporal sequence of DNA methylation and systemic inflammation after repeated exercise exposure.

2.3.5.2. Chronic Exercise Training & DNA Methylation

Chronic exercise participation encompasses widespread health benefits, and chronic exercise adaptations include muscular hypertrophy, higher cardiometabolic efficiency, and improvements in mental health.[204,205] Similar to acute exercise, several investigations have tested relationships between DNA methylation and physiological adaptations to both chronic aerobic and resistance exercise modalities. One such investigation by Alibegovic et al. (2010) proposed that aerobic exercise training may reverse hypermethylation within the *PGC-1α* promoter after prolonged sedentary behavior.[194] Three promoter CpG sites were profiled with pyrosequencing in skeletal muscle biopsies from participants (N = 20 healthy males) at baseline, after 10 days of bedrest, and after four weeks of aerobic exercise training (*i.e.,* cycling 30 min/day at 70% of VO_{2max} for 6 days per week). Ten days of bedrest elicited significant hypermethylation in one of three sites compared to baseline. Methylation levels after aerobic training did not reach statistical significance; however, hypomethylation trended toward baseline levels and was significantly correlated with *PGC-1α* expression.

Hypomethylation in genes with metabolic functions following chronic aerobic exercise training was also reported by Nitert et al. (2012).[195] DNA methylation from skeletal muscle (*i.e.,* vastus lateralis) was measured via MeDIP-Chip sequencing in healthy male participants (N = 28) who completed a six-month supervised endurance training program. Following the intervention, 115 of the 134 identified differentially methylated loci were hypomethylated and encompassed several genes with known metabolic functions: mitogen-activated protein kinase 1 (*MAPK1*), myocyte enhancer factor 2A (*MEF2A*), and NADH:ubiquinone oxidoreductase

subunit C2 (*NDUFC2*). Higher expression levels were also detected in *MEF2A* and *NDUFC2*, indicating that chronic aerobic exercise may contribute to upregulated myocyte metabolism.

Chronic aerobic exercise has also been associated with differential DNA methylation in whole blood samples, and these methylation changes may offer clinical insight for mechanistic intervention in cardiovascular disease (CVD)[197] and mild cognitive impairment (MCI).[206] Ferrari et al. (2019) reported that a 12-week aerobic exercise intervention in hypertensive patients (N = 44) and (N = 24) controls resulted in hypermethylation of genes like nitric oxide synthase 2 (*NOS2*), tumor necrosis factor (*TNF*), and endothelin 1 (*EDN1*). Notably, all three genes were positively associated with VO_{2max} post-intervention, while *NOS2* and *EDN1* were inversely associated with systolic and diastolic blood pressure (*i.e.,* higher methylation levels are associated with lower blood pressure post-intervention). Associations between blood-based DNA methylation and clinical indices of hypertension (*e.g.,* blood pressure, VO_{2max}) in response to chronic aerobic exercise demonstrate its potential utility as a prognostic tool during lifestyle or pharmacological interventions.

Work performed by Ngwa et al. (2022) suggests that the same principle may apply to illnesses like MCI, in which chronic aerobic exercise resulted in differential methylation of pathways associated with amyloid precursor protein trafficking.[206] Significant improvements in VO_{2max} and hypomethylation across several genes (*e.g.,* VPS52 subunit of GARP complex [*VPS52*], scavenger receptor class B member 1 [*SCARB1*], attractin [*ARTN*], nuclear receptor subfamily 1 group H member 2 [*NR1H2*], and protein phosphatase 2 regulatory subunit B'delta [*PPP2R5D*]) were identified in participants with MCI who completed a six-month aerobic exercise intervention (N = 11) compared to a stretching regiment (N = 8). Higher

cardiorespiratory fitness is correlated with higher cognitive function[207,208] and brain health (*e.g.,* larger gray matter brain volume[209,210] and fewer white matter hyperintensities[209]), and exercise-induced changes in methylation levels of genes associated with cognitive decline (*e.g.,* VPS52) reported by Ngwa et al. (2022) support future interventional studies for MCI prevention and disease management.

Changes in DNA methylation have also been observed after chronic resistance exercise and reflect potential molecular adaptations to training. Results published by Lindholm & Marabita et al. (2014) reported that eccentric resistance exercise training altered DNA methylation and that increases in citrate synthase activity were significantly correlated with methylation levels at 600 CpG sites.[211] Using a within-subjects design, healthy participants (N = 23; males = 12, females = 11) trained one randomly selected leg (*i.e.,* non-selected leg used as intraindividual control) via supervised one-legged knee extensions for three months. Differential DNA methylation (*e.g.,* 4,919 DMPs), differential gene expression (*e.g.,* 4,076 genes), and enrichment of known transcriptional motifs (*e.g.,* myogenic regulatory factors [MRFs], myocyte enhancer factor 2 [MEF2], erythroblast transformation specific [ETS] family binding domains) were identified in skeletal muscle (*i.e.,* vastus lateralis) analyzed before and after the training intervention. Differentially methylated genes were enriched for several relevant biological processes (*e.g.,* regulation of glycolysis and regulation of cellular carbohydrate catabolic processes) and cellular components (*e.g.,* contractile fiber, myofibril, sarcomere). While changes in methylation appear to be involved in regulating physiological adaptations to resistance training, other factors (*e.g.,* age, sex, underlying genomic sequence, training stimulus) are also important considerations that account for individual variability.

Another investigation by Seaborne et al. (2018) reported that changes in DNA methylation coincide with changes in muscle hypertrophy after analyzing human skeletal muscle during periods of resistance training (loading) and detraining (unloading).[196] In brief, participants (N = 8) underwent a skeletal muscle biopsy at baseline, after acute resistance exercise, after 7-weeks of resistance exercise training, after 7-weeks of detraining (no exercise), and after 7-weeks of retraining (reintroducing resistance training). Interestingly, participants exhibited changes in DNA methylation that correlated with changes in muscle hypertrophy (both gains/losses). Additionally, enriched gene pathways (*e.g.,* PI3K/AKT signaling) were reflective of upregulated metabolism. While limited by a small study sample, this study provides evidence that experimental manipulation of a trait (*e.g.,* muscle hypertrophy) is reflected by parallel changes in DNA methylation. Beyond exercise training, DNA methylation also reflects potential biological implications of lifestyle and habitual behaviors (*e.g.,* physical activity) that reduce risk of developing chronic illness.

2.3.5.3. Physical Activity Behavior and DNA Methylation

Recommended regular physical activity (PA) behavior based on guidelines from the *American College of Sports Medicine* (ACSM) constitutes 1) 150 min/week of moderate-to-vigorous physical activity (MVPA; *e.g.,* 30 minutes of daily moderate intensity activity five days per week or 20 minutes of vigorous aerobic activity three days per week) and 2) performing muscular strengthening or endurance activities at least twice per week for adults ages 18-65.[212] PA is a well-established modifiable risk factor for seven of the top 10 leading causes of mortality in the U.S. as defined by the Centers for Disease Control and Prevention;[213] however,

epidemiological estimates suggest only ~23% of the U.S. population adheres to both the ACSM aerobic and muscle strengthening guidelines.[214] Understanding the biological mechanisms involved in chronic illness risk reduction through regularly performed PA is of great importance to the field and may lead to optimized exercise interventions for at-risk populations.

Reductions in global methylation levels are known to occur with normative aging,[215] but early investigations have found that global DNA methylation levels (*e.g.,* DNA methylation in long interspersed nuclear element-1 [*LINE-1*] retrotransposons) are higher in more physically active individuals[216] and positively correlated with the amount of time spent performing recreational physical activity.[217] Further, epidemiological efforts leveraging data from population-based cohort studies (*i.e.,* Framingham Heart Study) have reported significant associations between lower biological age (*e.g.,* measured via epigenetic clocks) and greater levels of PA.[199] Although only 32.6% of the total participant sample (N = 2,435) met ACSM PA guidelines, participants who performed greater levels of actigraphy-measured MVPA and minimized sedentary time had lower biological age (*e.g.,* extrinsic epigenetic age acceleration [EEAA] and GrimAge). In brief, epigenetic clocks provide an algorithmic estimation of biological age through CpG methylation levels known to fluctuate across lifespan.[218,219] Significant associations between PA and biological age suggest that regular PA behavior may attenuate processes (*e.g.,* oxidative stress) known to contribute to DNA damage and cellular apoptosis.[220] These findings are consistent with results from cohort studies conducted abroad (*e.g.,* Rhineland Study cohort in Bonn, Germany)[221] and suggest that the impact on DNA methylation from regular PA behavior may be one way in which PA mitigates chronic illness risk or other health-related consequences.

Other investigations have also considered how environmental factors (*e.g.,* geographic location and PA feasibility/accessibility) influence PA behavior and DNA methylation. Duncan *et al*. (2022) reported differential DNA methylation in discordant monozygotic twins (N = 140 participants; 70 twin pairs) after grouping based on MVPA behavior and on neighborhood walkability.[198] PA discordance between twins was defined as one twin performing >150 min/week of MVPA while the other performed <150 min/week of MVPA; neighborhood discordance was defined as one twin living in a car dependent or somewhat walkable neighborhood, while the other twin resides in a very walkable or "walker's paradise" neighborhood. BMI discordance was defined as 5kg/m^2 between twin pairs. DNA methylation from buccal cells was analyzed using MeDIP, and differentially methylated regions (1000 bp windows) were stratified by males and females to account for potential sex differences.

Differentially methylated regions were enriched for loci that participate in metabolism, cellular signaling, and cellular transport in both males and females across all discordance parameters (*i.e.,* MVPA, walkability, and BMI). Physiologically, genes associated with obesity risk (*e.g.,* PPARG coactivator 1 alpha [*PPARGC1A*] and peroxisome proliferator activated receptor delta [*PPARD*]) contained differentially methylated regions in both males and females. These findings highlight the potential impacts of habitual PA behavior and environment in chronic illness prevention, independent of genetic predispositions. Increased PA behavior has also demonstrated an additive effect when combined with other modifiable risk factors (*e.g.,* balanced diet) during lifestyle interventions.

Participants (N = 68) enrolled in a 12-week randomized-control trial designed to improve both diet and MVPA levels were randomly assigned to one of three treatment arms: 1)

simultaneously increasing MVPA and fruit/vegetable intake (N = 25), 2) increasing fruit/vegetable intake while sequentially increasing MVPA (N = 31), and 3) a control stress-management and sleep improvement regiment (N = 12).[222] Blood-based DNA methylation (*Illumina Infinium MethylationEPIC* V1 microarray) measured at baseline, three months, and 9 months of the intervention was used to test relationships between lifestyle improvements and regional DNA methylation differences. Compared to the control condition, both treatment groups exhibited significant increases in fruit/vegetable intake (+6.5 servings/day) and MVPA behavior (+24.7 min/day). Differentially methylated regions were identified after three months (154) and nine months (298) of the intervention in pooled analyses of PA treatment arms (*i.e.,* simultaneous and sequential) compared to the control condition. Chronic illness has been associated with several pathways representative of genes containing differentially methylated regions in the present study, including associated cell-cell adhesion, GDP-L-fucose biosynthesis, methyl-malonyl, and PI3K/AKT signaling. These changes in DNA methylation biological contextualize the impact of longitudinal multimodal lifestyle improvements and may serve as future candidate biomarkers for determining lifestyle intervention efficacy.

Biologically, DNA methylation presents as a strong prognostic tool with high potential clinical impact because of its responsivity to external factors (*e.g.,* behaviors, environment, etc.) and governance over downstream molecular processes (*i.e.,* gene transcription). Structured exercise (*i.e.,* acute and chronic) and PA are examples of external factors that have been reliably demonstrated to change DNA methylation and stimulate pathways often associated with chronic illness. Measuring DNA methylation in case-control studies following an exercise challenge may uncover mechanisms that underlie physiological and behavioral changes of

disease phenotypes. This approach may be of relevance to GWI and other CMIs, given the plausible interaction of multiple physiological systems in symptom and disease maintenance.

2.4. DNA Methylation in Gulf War Illness

Previously described investigations of GWI (Section 2.1.3.3. & Section 2.1.3.4.) that applied DNA sequencing and functional assays suggest that single nucleotide polymorphisms and differentially expressed genes of neuroendocrine[13–15] (*e.g., PON1, BuChE, AChE, ACE*, etc.) and immune[16–18] (*e.g., TLR4, IL-6R, HLA,* etc.) systems facilitate GWI progression. Although these findings denote associations between potential environmental stress (*e.g.,* chemical exposure) and genetic susceptibility to GWI, they do not fully elucidate the interaction between the genome and environment. Accordingly, the Department of Defense and Department of Veterans Affairs alike have prioritized researching methods designed to understand the biological consequences of environmental hazards. Resulting initiatives, like the Defense Advanced Research Projects Agency (DARPA) Epigenetic Characterization and Observation (ECHO) program, have emphasized the need to study epigenetic markers in combat-related exposure screening.[223] Epigenetic methodologies may reveal the consequences of environmental or toxicant exposure in GWI pathophysiology.[19] In-vivo animal modeling comprises the majority of studies surrounding epigenetics in GWI, but pilot studies of GWI that measure DNA methylation in human subjects are promising and warrant additional investigation.

While a detailed review of in-vivo GWI studies applying animal models is beyond the scope of the present review, a summary of key findings may better contextualize use of DNA

methylation or other epigenetic methodologies in human studies. Several investigational paradigms using in-vivo animal models (*e.g.,* murine or rats) of GWI measure epigenetic changes following acute exposure to known environmental hazards encountered in-theater (*e.g.,* PB, DEET, permethrin, diisopropyl fluorophosphate, corticosterone, etc.).[224–227] Pierce et al. (2016) reported increased global 5mC content and select micro-RNAs (*e.g.,* miR-124-3p & miR-29b-3p) within the hippocampus but decreased global 5hmC in cortices of rats one year after toxic exposure.[224] Later work by Ribeiro et al. (2021) reported decreased hippocampal levels of brain-derived neurotrophic factor (*Bdnf*) gene promoter H3K9ac occupancy and decreased *Bdnf* protein content of rats exposed to Gulf War toxicants, which supports an epigenetic contribution toward neurological dysfunction in GWI.[227]

High-throughput multi-omic approaches (*e.g.,* transcriptomics, histone modifications, and DNA methylation) in GWI murine models generally report changes to genes that participate in immune support and neuronal function, providing potential biological support for neurocognitive symptoms reported by GWVs with GWI.[225] Epigenetic differences may also be critical for capturing individual variability amid heterogeneity among the proposed GWI etiologies and symptom profiles. Candidate genes identified by Mozhui et al. (2023) that were differentially methylated and expressed in mice exposed to organophosphates and circulating glucocorticoids include tubulin tyrosine ligase like 7 (*Ttll7*), aldo-keto reductase family 1, member C14 (*Akr1c14*), solute carrier family 44 member 4 (*Slc44a4*), and RUN and SH3 domain containing 2 (*Rusc2*).[226] Mozhui et al. proposes that these genes may contribute to individual GWI symptoms, which include impaired cognition (*e.g., Ttll7*), gastrointestinal distress (*e.g., Akr1c14*), and disrupted parasympathetic function (*e.g., Slc44a4* & *Rusc2*). Interactions

between individual genomic differences and the extent of environmental stress (*e.g.,* type of toxin, level of potency, length of exposure, etc.) may also be factors involved in the profile and severity of GWI symptoms. Accordingly, the evidence surrounding in-vivo animal models of GWI supports epigenetic investigations (*e.g.,* DNA methylation) as a viable method for studying illness heterogeneity post-deployment in human participants.

Few human subjects' studies of epigenetics and GWI exist, but preliminary evidence has demonstrated cross-sectional differences in methylation levels in GWVs with GWI (N = 10) compared to controls (N = 10).[27] Biological pathways reported by Trivedi et al. (2019) were consistent with findings from previous animal studies and observed differential methylation in genes associated with neuronal function (*e.g.,* glutamate removal from folates), immune function (*e.g.,* IL-10 signaling & NF-kB signaling), and metabolism (*e.g.,* PPARa/RXRa Activation & PPAR signaling).[27] These findings include disproportionately higher levels of gene promoter hypermethylation in GWVs with GWI, which correspond to our previous findings of repressed gene expression.[28] The initial cross-sectional comparisons of blood-based DNA methylation analyzed by microarray (*i.e., Illumina Infinium MethylationEPIC* V1 microarray) were compared for validity with separate GWI (N = 10) and control (N = 10) blood samples analyzed via pyrosequencing. Methylation levels in CpG sites of two prioritized genes (*e.g.,* zinc finger BTB domain containing 18 [*ZBTB18*] & tumor protein p63 regulated 1 [*TPRG1*]) based on their 1) biological relevance to GWI (*e.g., ZBTB18* participates in neuronal function; *TPRG1* regulates amino acid metabolism) and 2) high promoter CpG density were similar between microarray and pyrosequencing methods, which supports the validity of these findings. These findings support additional investigation into the epigenetic contributions to GWI, and beyond larger

sample sizes, additional approaches may consider testing relationships between DNA methylation and specific GWI symptoms or environmental hazards.

A re-analysis of the DNA methylation findings originally reported by Trivedi et al. (2019)[27] used a reverse screening approach to test relationships between differentially methylated genes and biological pathways often targeted by acetylcholinesterase (AChE) inhibitors (*e.g.,* organophosphates, PB, DEET, chlorpyrifos, and permethrin).[29] While high dose exposure to AChE agents is potentially lethal, Jean-Pierre et al. suggest that physiological damage incurred by prolonged low-dose AChE exposure may be reflected by DNA methylation. Top differentially methylated genes in pathways susceptible to AChE toxicity (*e.g.,* inflammation, lipid metabolism, detoxification) include acetylcholinesterase (*ACHE*), aldo-keto reductase (*AKR*), carboxylesterase 1 (*CES1*), prostaglandin-endoperoxide synthase 1 (*PTGS1*), and monoamine oxidase B (*MAOB*). Pathways enriched with differentially methylated genes had plausible biological roles in GWI symptoms, and Jean-Pierre et al. proposed that these findings provide further support in-theater low-dose AChE exposure as a GWI risk factor.

Little is known about how DNA methylation contributes to PEM in GWI or other forms of CMI. DNA methylation changes under controlled physiological stress (*i.e.,* exercise challenge) may illustrate how pathway activation or suppression contribute to specific GWI symptoms. Compared to cross-sectional comparisons, physiological manipulation through exercise challenge may reveal differential methylation not observed at rest, and if associated with symptom worsening, can elucidate potential targets for intervention. Although the present project is limited to studying GWI, results from this work may benefit other Veteran

populations exposed to hazards in-theater (*e.g.,* burn pit exposure during post-9/11 Operations Iraqi Freedom/Enduring Freedom).

CHAPTER 3: METHODOLODY

The purpose of this dissertation is to investigate the psychobiological contributions of exercise and epigenetics in GWI. Study data and blood samples were sourced from an ongoing pre-post multi-site investigation, examining brain, autonomic, and immune function in GWV with GWI (Department of Veterans Affairs Merit Review Award #I01CX001329; PIs: Cook & Falvo) where participants complete three study visits over a 10-day span. DNA methylation analyses were performed on a subset of participant blood samples and funded by an Airborne Hazards and Burn Pits Center of Excellence Pilot Project Award (#FY2024-003; PI: Boruch). Participant inclusion in DNA methylation analyses was based on 1) sample availability across all three study visits and 2) completion of the visit 2 exercise challenge. All study procedures were approved by the institutional review boards (IRB) and the Research and Development Committees of the University of Wisconsin – Madison (Protocol #2015-1226), VA Madison and the Department of Veterans Affairs, New Jersey Health Care System (#01332). All participants provided informed consent according to the Declaration of Helsinki prior to testing.

3.1. Participant Recruitment & Enrollment:

Veterans who were deployed to the 1990-1991 Persian Gulf Operations Desert Storm/Desert Shield were recruited within the Veteran Integrated Service Networks ‘2’ (New Jersey) and ‘12’ (Wisconsin). Our primary participant identification method of potentially eligible Veterans relied on the VA Informatics and Computing Infrastructure Corporate Data Warehouse to identify names and mailing addresses of Veterans in the VISN 2 and 12 areas who were 1) between the ages of 45–65 and 2) had been deployed to the Persian Gulf War.

Additional recruitment methods included the Defense Manpower Data Center, outpatient referral clinics, and distribution of study fliers to the VA Office of Public Health Gulf War Newsletter service and local Veteran Service Organizations, hospitals, and clinics.

GWVs with GWI were required to meet the Kansas Case Definition criteria,[2] which requires endorsement of moderately severe symptoms (*i.e.,* 2 on a scale of 0-3) in at least three of the following domains: fatigue, pain, neurological/cognitive/mood, skin, gastrointestinal, and respiratory – with symptoms lasting at least 6 months and first presenting during or after the Gulf War. Because two of the most frequently reported GWI symptoms are fatigue and pain, we also required that enrolled Veteran participants reported either fatigue or pain as one of their three major symptoms. A broader aim of the overhead protocol (Department of Veterans Affairs Merit Review Award #I01CX001329; PIs: Cook & Falvo) was to test whether a similar symptom exacerbation response occurred in GWVs with GWI compared to those previously described in the ME/CFS literature. PEM endorsement by GWVs with GWI was not required for study inclusion because repeated symptom measures in response to an acute exercise challenge are not well-characterized. GWVs who did not endorse having an illness associated with Gulf War deployment, related symptoms (*e.g.,* chronic pain, fatigue, and cognitive impairment) and were otherwise healthy were offered enrollment as control GWVs.

3.1.1. Participant Screening:

Participant inclusion/exclusion criteria are summarized in Table 2. Screening for inclusion/exclusion criteria included an initial 20–25 min phone call followed by a 1-h in-person interview during Visit 1. Using the Mini-International Neuropsychiatric Interview (MINI),

potential participants were excluded if they met criteria for bipolar I disorder (current or received previous diagnosis), psychotic disorders, mood disorder with psychotic features, active illicit substance use, or substance dependence in partial remission for less than 1 year.[228] The MINI is a validated instrument used to conduct a neuropsychiatric interview to assess Axis I psychiatric disorders in the DSM-IV and ICD-10, and it has been used consistently in other studies at the William S. Middleton VA. The MINI has sufficient inter-rater (Kappa: 0.88-1.0) and test-retest (Kappa: 0.76-0.93) reliability, and it has demonstrated high levels of agreement with other clinically validated psychological assessments like the Composite International Diagnostic Interview.[229]

3.1.2. Participant Exclusion Criteria:

Exclusionary medications included active use of beta blockers, calcium channel blockers, anti-convulsant medications, unstable use of psychotropic medications (< 3 months), and/or use of multiple sedatives. Per Kansas Case definition criteria, participants were also excluded for chronic conditions that include: Type 1 diabetes, heart disease (other than high blood pressure), cancers (other than non-melanoma skin cancer), rheumatoid arthritis, stroke, lupus, Multiple Sclerosis, Parkinson's Disease, Lou Gehrig's Disease/ALS, seizure disorders, Alzheimer's Disease, liver disease, kidney disease, schizophrenia, bipolar disorder, manic depression, chronic infectious diseases >6 months, and hospitalizations in the last five years for PTSD/clinical depression/substance dependence. Additionally, participants were also excluded for absolute contraindications to exercise testing according to American College of Cardiology/American Heart Association guidelines.[230] Self-reported medical history and active

medication use were cross-referenced with available VA medical chart information (*i.e.,* Department of Veterans Affairs Computerized Patient Record System [CPRS]).

Table 2. Study Participant Inclusion/Exclusion Criteria.

Inclusion:	**Exclusion:**
Deployed to Persian Gulf region during the 1990-1991 Gulf War	Non-deployed Veteran during 1990-1991 Gulf War era
Ages 45-65 years old	Younger than 45; older than 65
Meet Kansas Case Definition Criteria[2]	Current illegal drug/illicit substance use
Presenting symptoms unexplained by known medical cause	Comorbid medical conditions that include: - Psychiatric conditions that may interfere with ability to report symptoms (e.g., psychotic disorders or bipolar disorders) - Conditions listed in the Kansas Case Definition Criteria - Symptoms explained by other conditions NOT in the Kansas Case Definition Criteria (*e.g.,* untreated sleep apnea/insomnia, Gout, Paget disease, hypothyroidism, neuropathy, pulmonary diseases that require treatment - Currently pregnant (female only)
Stable medication use (< 3 months) of: - Psychotropic medications (e.g., antidepressants, antipsychotics, mood stabilizers when NOT used to treat exclusionary psychiatric condition - Opioid Analgesics - >1 prescribed sedative hypnotic - Anti-convulsant medications when NOT used to treat exclusionary medical conditions	Current illegal drug/illicit substance use or active use of prescription drugs with: - Chronotropic effects (*e.g.,* beta blockers, calcium channel blockers, etc.) - Potential to impact symptom reporting (*e.g.,* >1 sedative, non-stable (>3 months) anti-convulsant use)

3.2. Study Design & Procedures:

The overarching study protocol included three total study visits (Figure 5). Visit 1 consisted of screening and consenting, demographic, symptom and physical/mental health questionnaires, and a blood draw. Approximately seven days later, participants returned for visit 2 and completed the exercise challenge. The exercise challenge consisted of a submaximal cardiopulmonary exercise test at 70% of individual heart rate reserve (*i.e.,* vigorous intensity aerobic exercise). A psychometric battery (*e.g.,* fatigue, pain, mood, GWI symptoms, etc.) and blood draws occurred immediately pre-exercise and 30 minutes post-exercise. Participants returned approximately 24-hours later for visit 3 to repeat the psychometric battery and provide a blood sample. Additional procedures conducted both before and after exercise included physical activity (PA) actigraphy, measures of autonomic function, and magnetic resonance neuroimaging. We have previously reported that GWVs with GWI report significant symptom exacerbation following this exercise challenge.[7,143,231] PEM responses have been characterized in N = 110 GWVs who completed the broader overhead protocol (Department of Veterans Affairs Merit Review Award #I01CX001329; PIs: Cook & Falvo). The present study prioritized a participant subset (GWVs with GWI: N = 27; healthy control GWVs: N = 25) for DNA methylation analyses based on 1) availability of blood samples across all three timepoints (*i.e.,* pre-exercise, 30 minutes post-exercise, and 24 hours post-exercise) and 2) completion of the exercise challenge.

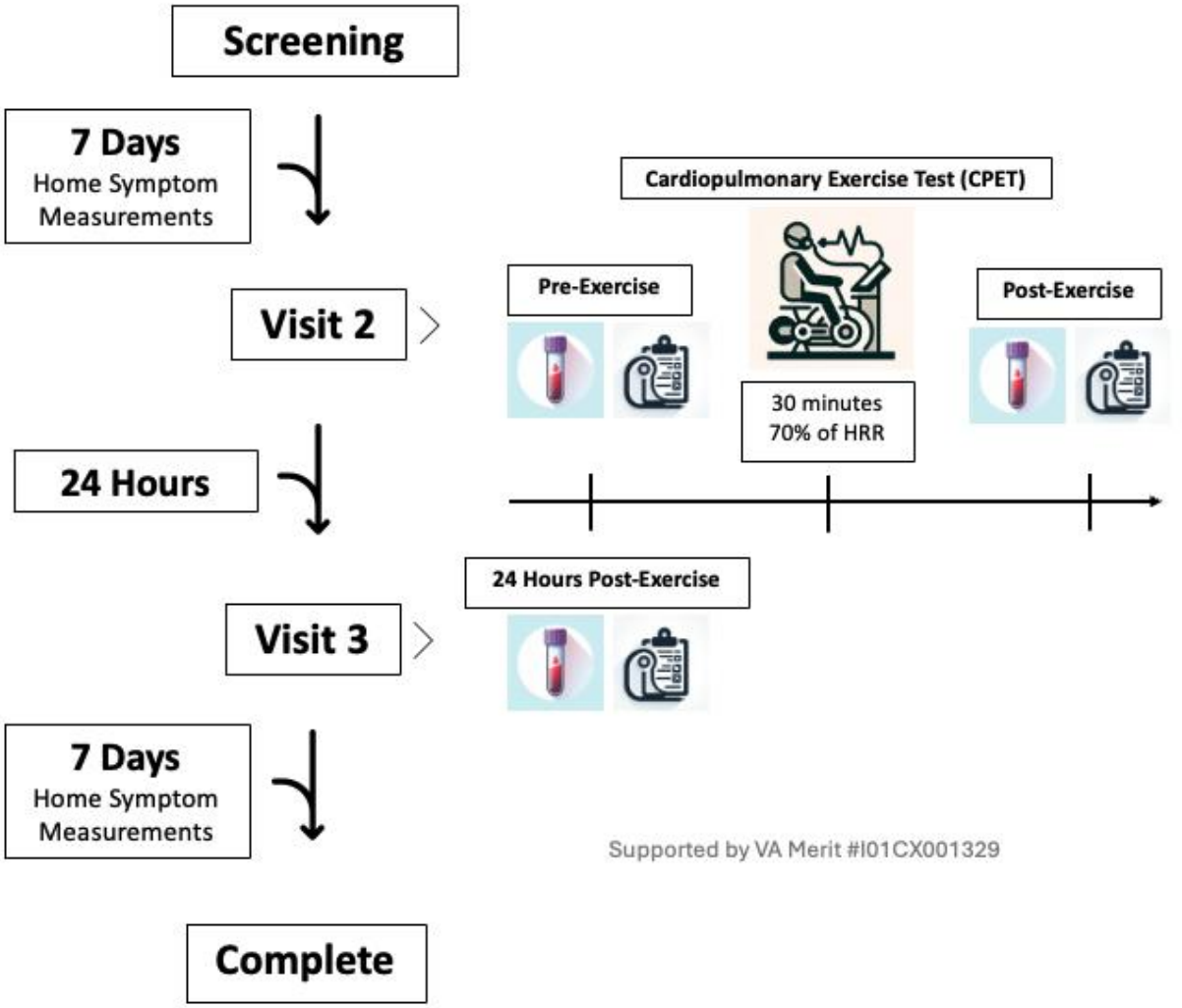


Figure 5. Study design and procedures. Whole blood samples and psychometric responses were collected pre-exercise (Visit 2), post-exercise (Visit 2), and 24 hours post-exercise (Visit 3). Whole blood collected during the screening visit was considered as a potential baseline measure substitute for any failed blood draws during Visit 2 pre-exercise. The 30-minute submaximal exercise challenge was performed at an intensity of 70% of individual heart rate reserve (HRR). Additional data collected as part of the broader study protocol included questionnaires completed at home (7 days pre- and post-exercise), physical activity actigraphy (7 days pre- and post-exercise), autonomic function/cerebral blood flow (*i.e.,* autoregulatory index), and neuroimaging via magnetic resonance imaging scans.

3.3. Exercise Challenge (Visit 2):

3.3.1. Equipment Calibration & Participant Compliance Check:

Room environment (*e.g.,* temperature, humidity, barometric pressure) were recorded and used to calibrate the flowmeter prior to each exercise test. Flowmeter calibration occurs by making multiple comparisons to room air flushed by a 3-liter piston syringe. Oxygen and carbon dioxide sensors were calibrated by the presentation of known gas concentrations.

Participants were also asked to adhere to a 12-hour fast and abstain from caffeine, nicotine/tobacco products, non-prescribed non-steroidal anti-inflammatory drugs, and any routine physical activity/exercise unrelated to employment prior to the exercise challenge. Criteria compliance was confirmed with each participant prior to obtaining height and weight measures at the beginning of Visit 2. A brief overview of Visit 2 procedures, including the exercise test, was provided as an opportunity to confirm participant understanding and comfortability with the protocol.

3.3.2. Submaximal Exercise Challenge Protocol

PEM triggers are found to vary based on both illness duration and severity,[140] but steady-state submaximal exercise challenges have consistently elicited symptom exacerbation in other CMI populations[7,81] and may better reflect physical stressors encountered during daily life. Although other exercise protocols (*e.g.,* maximal cardiopulmonary exercise test) provide greater clinical interpretation, previous evidence indicates that matching CMI and control participants for physical fitness accounts for observed differences in ventilation, exercising heartrate, and O_2 pulse.[232] Significant differences in ventilatory efficiency indices (*e.g.,* $\dot{V}E/\dot{V}CO_2$

& $\dot{V}E/\dot{V}O_2$) between participants with and without CMI occur independent of physical fitness[232] and retain potential clinical utility even when derived from submaximal exercise.[233]

Participants performed the submaximal aerobic exercise challenge at 70% (±5%) of age-predicted heart rate reserve (HRR) on an electronically braked cycle ergometer (Lode Corival, Lode B.V., Groningen, The Netherlands or Ergolselect 200, Ergoline GmbH, Bitz, Germany). Supine resting heart rate (HR) was monitored for 5 minutes, and age-predicted maximum HR was calculated using the following formula: 209–0.70 X Age.[234] Target HR was calculated via American College of Sports Medicine recommendations: Target HR = (HRmax HRrest) x %intensity + HRrest.[235] Following a 2-minute period of resting data collection, exercise began at 50 W and intensity was gradually increased until participants reached their target HR (~5 min). Next, participants completed 30 min of steady-state exercise at the target intensity followed by a 3-min active recovery period at 0W. Participants were instructed to maintain a cycling cadence between 50-70 rpm during both the warm-up and steady-state exercise periods. Workload adjustments ranging from 1-10 W/minute were performed throughout exercise to ensure participants maintained the 70% ± 5% HRR prescribed intensity. Rating of perceived exertion (RPE),[236] leg muscle pain,[237] and overall fatigue[238] were measured every five minutes during exercise and every minute during recovery. These scales and standardized instructions are included in Appendix B.

Electrocardiography (ECG) and a HR rate monitor (POET II; Criticare Systems, Waukesha, WI; T12x; Cosmed, Rome, Italy; Polar, Lake Success, NY) were used to monitor heart rhythm and rate during both exercise and recovery. Blood pressure was monitored every 5 minutes during steady state exercise via manual auscultation. Oxygen consumption ($\dot{V}O_2$), carbon

dioxide production ($\dot{V}CO_2$), ventilation ($\dot{V}_E$), HR, and work rate (WR) measures were obtained breath-by-breath during exercise using a metabolic cart (Parvo Medics TrueOne; Parvo Medics, Sandy, Utah; Quark CPET; Cosmed, Rome, Italy) and a bidirectional non-rebreathing valve attached to an oronasal mask (Hans-Rudolph, Kansas City, MO).

3.3.3. Processing Cardiopulmonary Exercise Testing Data:

Raw data were exported from metabolic measurement devices (.xlsx format) and imported into custom MATLAB (Mathworks, R2020b) scripts for postprocessing. Breath-by-breath data were first interpolated (1 sec intervals) and then primary variables ($\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}_E$, HR) were plotted for visual inspection and removal of errant points. Data were then smoothed (10-sec) and re-plotted to confirm baseline, exercise, and recovery periods. Three steady-state time-points (17-20, 23-25, and 27-30 min) were automatically detected and second-by-second data were averaged over the three time-points (Figure 6).

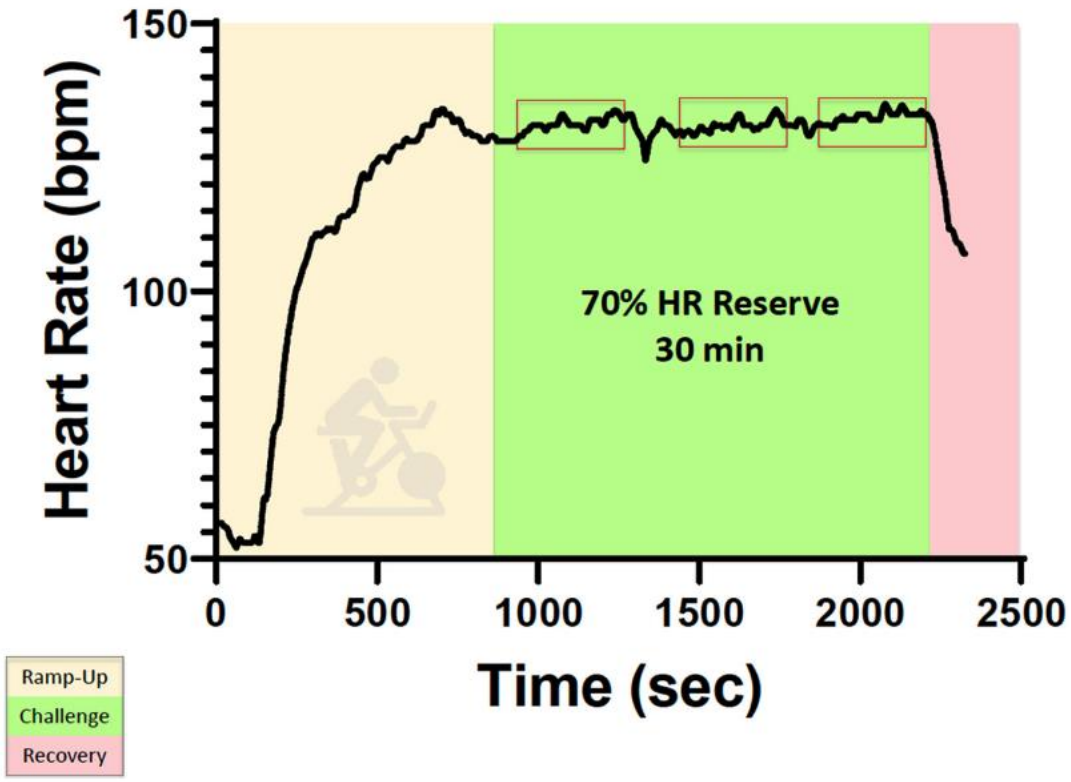


Figure 6. Illustration of Exercise Challenge Protocol. The exercise test began with a 2-minute period of resting data collection, followed by a 5-minute gradual ascension into the prescribed 70% HRR zone, starting at 50 W. Participants completed 30 minutes of steady-state exercise, ending with a 3-minute active recovery period at 0 W. Cardiopulmonary exercise test variables were collected from three different 3-minute steady state periods during the exercise test.

Note: Figure created by Michael J. Falvo, PhD; adapted from Figure 2 in Boruch et al., 2021 (*Life Sciences*)

Peak RPE, fatigue, and leg muscle pain were defined as the highest value recorded during the exercise test. Cumulative work was calculated as the product of cycling power and duration (kJ = W * s). Processed data were then exported for statistical analysis. A satisfactory exercise test was determined by a participant's ability to perform within ± 5% of their 70% HRR.

3.4. Participant Characteristics, Symptoms, and Symptom Time Course:

3.4.1. Baseline Psychometric Measures of Symptoms:

Participant symptoms and overall health were characterized after providing informed consent to study enrollment during Visit 1. Participants completed a variety of psychometric questionnaires to characterize demographics, deployment-related exposures, and mental/physical health symptoms. Copies of each psychometric instrument are located in Appendix A. Self-reported exposures were assessed via the *Gulf War Exposure Questionnaire*.[239] Symptoms assessed via psychometric questionnaires include those of mental/physical function (Veterans Rand 36-item Medical Health Survey [*VR-36*][240]), fatigue (Multidimensional Fatigue Inventory [MFI][241]), perceived deficits (Perceived Deficits Questionnaire [PDQ][242]), mood (Profile of Mood States [POMS][243]), pain (Short Form McGill Pain Questionnaire 2 [SF-MPQ-2][244]), and sleep quality (Pittsburgh Sleep Quality Index [PSQI][245]).

Table 3. Psychometric Instruments for Characterizing GW Exposures & Symptoms

Questionnaire:	Description:	Subscale Used:	Score Range & Interpretation:	Timepoints:	Citation:
Gulf War Exposure Questionnaire	Yes/No to various known exposures present in the Gulf War If YES – asked to rate how many days of exposure were incurred	N/A	0 = No exposure 1 = 1 – 6 Days 2 = 7 – 30 Days 3 = 31+ Days	Visit 2	Hauschild & Sharkey, 2015[239]
Veterans Rand 36-item Medical Health Survey (VR-36)	Health-related quality of life across 36 items that comprise eight subscales, Physical Composite Score (PCS), and Mental Composite Score (MCS)	PCS & MCS	Range: 0 – 100 Higher scores indicate better physical/mental health	Visit 1	Kazis, 2000[240]
Multidimensional Fatigue Inventory (MFI)	Evaluates general fatigue, physical fatigue, mental fatigue, reduced motivation, and reduced activity	Total Score	Range: 20 – 100 Higher scores indicate greater fatigue	Visit 1	Smets et al., 1995[241]
Perceived Deficits Questionnaire (PDQ)	20-items used to measure self-reported cognitive dysfunction	Total Score	Range: 0 – 80 Higher scores indicate greater cognitive impairment		Sullivan et al., 1990[246]

Profile of Mood States (POMS)	Assesses total mood disturbance and subcomponents of tension, depression, anger, vigor, and fatigue	Total Mood Disturbance (TMD)	Range: -30 – 200 Lower scores indicate greater mood disturbance	Visit 2 (Pre & Post-Exercise); Visit 3	McNair et al., 1971[243]
Short Form – McGill Pain Questionnaire (SF-MPQ)	Evaluates sensory, affective, and intensity aspects of pain	Total Pain Score	Range: 0 – 10 Higher scores indicate greater pain symptoms	Visit 2 (Pre & Post-Exercise); Visit 3	Melzack, 1987[247]
Pittsburgh Sleep Quality Index (PSQI)	Assess sleep quality and sleep disturbance over previous month	Total Score	Range: 0 – 21 Higher scores indicate worse sleep quality	Visit 1	Buysse et al., 1989[245]

3.4.2. Symptom Time Course:

To assess acute symptom changes in response to exercise, participants completed psychometric questionnaires pre-exercise, 30 minutes post-exercise, and 24 hours post-exercise (Figure 5). Symptoms recorded at the three timepoints included fatigue (POMS – Fatigue Subscale), mood (POMS – Total Mood Disturbance), and pain (SFMPQ Total Score).

3.5. Biological Specimens & DNA Methylation Analysis:

3.5.1. Biological Specimens:

Whole blood samples collected from 2016-2022 were obtained via antecubital venipuncture into EDTA tubes and centrifuged at 3300 RPMs for 12 minutes, starting within 7

minutes of collection. After centrifugation, the white blood cell layer was isolated from the whole blood and aliquoted into 10mL conical tubes containing 5mL of RNeasy Lysis Buffer (RLT: QIAGEN, Valencia, CA) supplemented with 50*u*L of β-Mercaptoethanol (B-ME: Sigma-Aldrich, St. Louis, MO) and then flash frozen in liquid nitrogen and stored in a -80°C freezer. We previously published our findings describing the expression of a select gene panel (N = 17) within this sample.[28]

3.5.2. Statistical Power: DNA Methylation

A post-hoc statistical power analysis (R package *EpipwR*[248]) showed that N = 50 participants (GWVs with GWI = 25, control GWVs = 25) provided sufficient statistical power ($1 - \beta = 0.93$) to detect small average DNA methylation differences ($\mu_\delta = 10\%$) at an FDR < 0.05. Under the assumption that 100 of ~935,000 total CpG sites included in the *Illumina MethylationEPIC V2* microarray would return a non-zero association with the GWI phenotype, statistical power was also calculated for average DNA methylation differences of 5% and 15%. Additionally, to understand the potential impact of missing data or anticipated future subset analyses (*e.g.,* GWVs with GWI who endorse PEM), statistical power under the defined parameters was calculated for total sample sizes of 30 and 40 total participants. Power analysis parameters are described in Table 4 and graphically depicted in Figure 7.

Although underpowered ($1 - \beta = 0.62$) to consistently detect small average DNA methylation differences $\leq 5\%$, the repeated measures design and inclusion of relevant covariates in differential DNA methylation analyses provides additional confidence for this

investigations capability to capture small differences. Repeated measures used in the present study provide additional confidence in the reliability of small observed differences.

Table 4. Summary of Statistical Power Analysis for DNA Methylation

Total Sample Size (N)	μ_δ (Avg. DNA Methylation Difference)	Avg. Power (1 - B)	SD Power
30	0.05	0.38	0.05
40		0.53	0.04
50		0.62	0.05
30	0.10	0.83	0.03
40		0.87	0.04
50		0.93	0.03
30	0.15	0.94	0.02
40		0.97	0.01
50		0.98	0.02

Note. Statistical power analysis parameters calculated for DNA methylation in a case-control study with total sample sizes (N) of 30, 40, and 50 participants. Using a false discovery rate (FDR) correction < 0.05, estimated average DNA methylation differences (μ_δ) were tested at three thresholds: 0.05, 0.10, and 0.15. Total sample size of N = 50 was adequately powered to detected average DNA methylation differences 0.10 (10%) between groups.

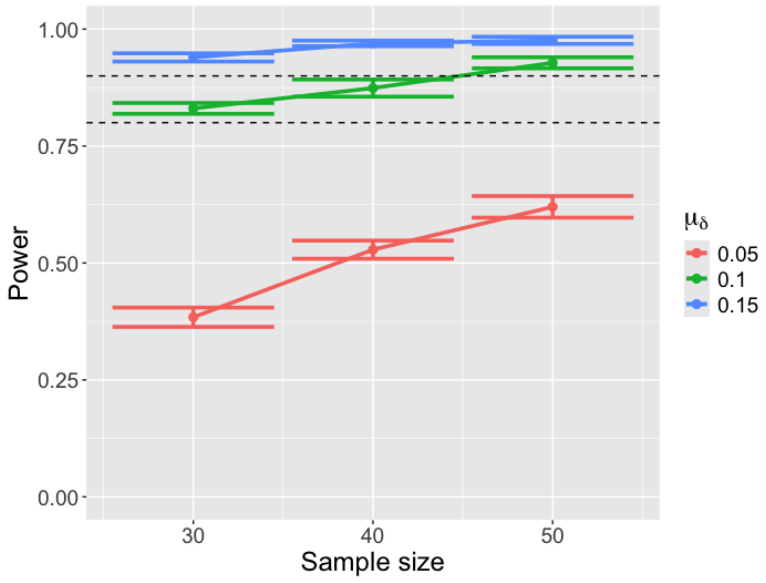


Figure 7. Statistical power comparisons for detecting differences in DNA methylation. Average statistical power (y-axis) with 95% confidence intervals need to detect differences in DNA methylation is plotted relative to total samples sizes (x-axis) of 30, 40, and 50 participants. Each line is color-coded by estimated average DNA methylation differences (μ_δ) of 5% (red), 10% (green), and 15% (blue). The present study is adequately powered ($1 - \beta = 0.93$) to detect 10% on average DNA methylation differences.

3.5.3. DNA Methylation Analysis:

For analyses, we established an intra-agency agreement (IAA) with the Pharmacogenomic Analysis Lab (PAL) of the Central Arkansas Veterans Health System (CAVHS) in Little Rock, AR. In brief, PAL is a VA core lab and Cooperative Studies Program member specializing in a variety of genomic services—including DNA methylation detection—to improve understanding of pathogenesis and treatment of various Veteran health conditions. Blood samples from GWVs with GWI (*N* = 27) and control GWVs (*N* = 25) collected at three timepoints (*i.e.,* pre-exercise, 30-min post-exercise, and 24 hours post-exercise) were forwarded to PAL for DNA methylation analysis via microarray (*Illumina Infinium MethylationEPIC* V2). As mentioned, participant samples were prioritized for DNA methylation analyses based on 1) sample availability across all three study visits and 2) participant completion of the exercise challenge. Compliance with the targeted heartrate range (70% of individual heart rate reserve) was high for both GWVs with GWI (Mean ± SD: GWI = 96.84% ± 4.88%) and healthy control GWVs (Mean ± SD: Control = 98.02% ± 5.40%). DNA methylation assays were completed using the *Illumina Infinium MethylationEPIC* V2 microarray platform comprising ~935,000 probes to detect differential DNA methylation levels in each participant at all three timepoints.

Prior to analysis via microarray, both genomic DNA quantity (Figure 8A) and integrity (Figure 8B) were verified. Genomic DNA extraction yields were sufficient in both quantity (Mean = 51.49mg per sample; Range = 5.88 – 147.36mg; minimum required = 250 nanograms) and quality ($260/280_{avg}$ = 1.91; >1.8 accepted as an indicator of pure genomic DNA).

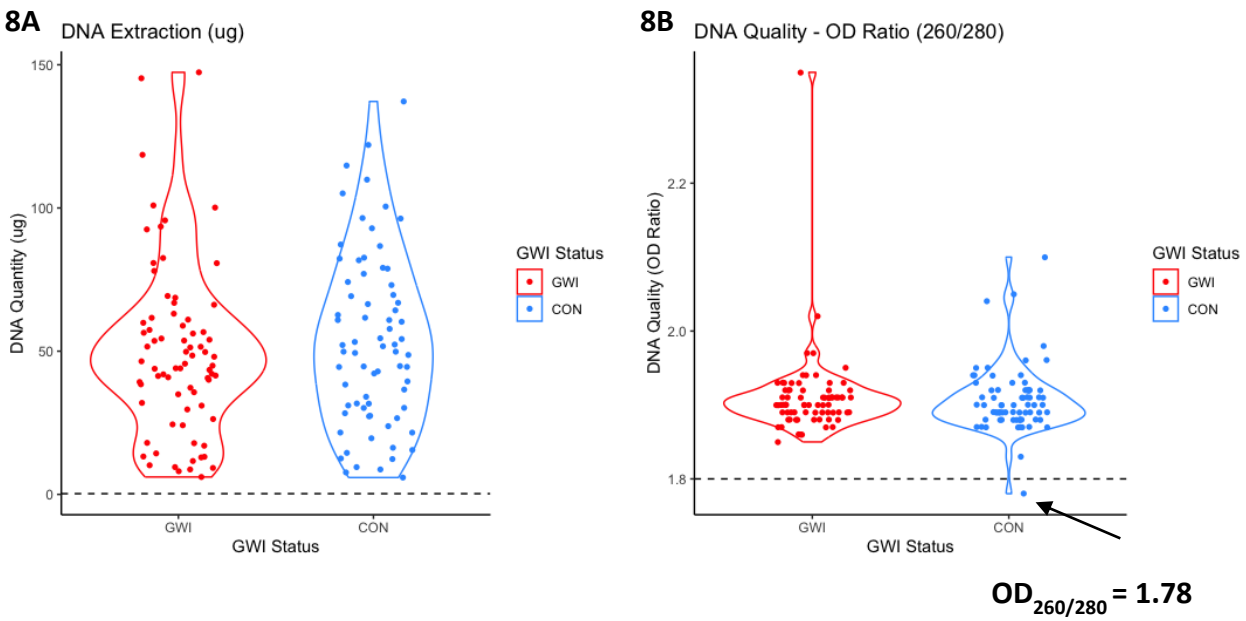


Figure 8A-8B. Extracted genomic DNA yields and integrity. **8A)** DNA yields across all participant samples are presented as violin plots for GWVs with GWI (red) and control GWVs (blue). Sufficient DNA quantities (Mean = 51.49µg per sample; Range = 5.88 – 147.36µg) for each sample were obtained for DNA methylation microarray analyses. **8B)** DNA integrity was confirmed using $OD_{260/280}$ ratios, and ratio values across all participant samples are presented as violin plots for GWVs with GWI (red) and control GWVs (blue). Values > 1.80 indicating high quality (i.*e.,* pure) DNA only one sample provided by a control GWV fell below this threshold (1.78). Proximity to the cutoff allowed for inclusion of this sample in DNA methylation microarray analyses.

250 nanograms of DNA was exposed to sodium bisulfite to convert unmethylated cytosines to uracil, which are read as thymine following polymerase chain reaction (PCR) amplification. PCR amplification of *DAPK1* in all samples confirmed successful bisulfite-mediated conversions required for microarray hybridization (Supplemental Figure S1). Following hybridization to microarrays, Illumina's platform returned excellent scores for both sample independent and dependent quality control metrics. To ensure assay reproducibility and verify sample identity, samples were analyzed in duplicate to compare 65 single nucleotide

polymorphisms within the *Illumina Infinium MethylationEPIC* V2 microarray platform. High intrasample correlation (r^2 = 0.9987) confirmed assay reproducibility among DNA methylation results.

Data generated from DNA methylation microarrays (~26.2 GB) were transferred to the Madison VA Medical Center via password-protected and encrypted media. All data were uploaded to local servers. Under an approved data use agreement (DUA) with Dr. Reid Alisch's (Project Co-Investigator) laboratory in the University of Wisconsin Department of Neurosurgery, all DNA methylation data were uploaded to his laboratory server for analysis.

3.6. Statistical Analysis:

3.6.1. Participant Characteristics, Baseline Symptoms, & Cardiopulmonary Exercise Responses:

Group differences between participant demographics and deployment-related exposure information were tested using the Wilcoxon Sum Rank Test and Fischer's Exact Test. Group differences in baseline symptom levels and cardiopulmonary exercise responses during the exercise challenge were tested using independent samples t-tests. The magnitude of group differences was quantified using Cohen's *d* effect sizes.

3.6.2. Symptom Time Course: PEM

PEM currently lacks validated criteria or a response threshold that designates whether an individual experiences PEM following a controlled stressor. Previously described methods have relied on self-reported PEM endorsement[145] or used only the symptom with the largest change from pre- to post-exercise.[249] Adequate PEM assessment warrants consideration of

several heterogenous features (*e.g.,* time course, symptom profile, symptom severity), and a novel method developed by our group that address several of these features is described in a manuscript currently being prepared for peer-review. Comparisons of existing PEM quantification strategies are beyond the scope of the present dissertation, and this study defined PEM as self-reported endorsement at baseline (*i.e.,* "feeling unwell after physical exercise or exertion").

Validated psychometric questionnaires that assess core GWI symptoms of fatigue (*i.e.,* POMS Fatigue Subscale), pain (*i.e.,* SF-MPQ-2 Total Score), and mood disturbance (*i.e.,* POMS TMD) were the primary symptom outcomes of interest. Changes in symptom severity between groups (*i.e.,* GWVs with GWI who endorse PEM [+PEM], who do not endorse PEM [-PEM], and control GWVs) over time (*i.e.,* pre-, post-, 24 hours post-exercise) were tested for each core symptom using linear mixed effects regression models [R packages *lme4*[250] & *lmerTest*[251]]. Linear mixed effects models were fit with random intercepts to account for individual participant differences.

3.6.3. DNA Methylation: Data Processing & Statistical Analysis

Imported raw intensity files (R package *minfi*)[252] annotated to the hg38 reference genome (R package *IlluminaHumanMethylationEPICv2anno.20a1.hg38*)[253] were used to assess sample quality based on each tested probe's calculated detection p-value. Probes were background and control-corrected, followed by subset-quantile within array normalization (SWAN) to correct for probe-type bias.[252,254] Probes excluded from analyses were determined by 1) exceeding a detection p-value >0.05 in at least one sample; 2) containing a single

nucleotide polymorphism (SNP); 3) reporting DNA methylation at a SNP; 4) originating from a sex chromosome; 5) measuring DNA methylation at a cytosine flanked by a non-guanine nucleotide; 6) functioning as a cross-reactive probe.

DNA methylation levels (*i.e.,* beta-values) were calculated (R packages *minfi*[252] & *SeSAMe*[255]) as the ratio of methylated to total signal (*i.e*., beta-value = methylated signal/(methylated signal + unmethylated signal +100)), where beta values range from 0 (fully unmethylated) to 1 (fully methylated). Beta values were further be converted to M-values (*i.e.,* logit-transformed beta-values) for differential analysis. Linear regression for each tested CpG using a multivariate model was employed using (R package *limma*[256]), and all discrete (*e.g.,* GWVs with GWI vs. healthy control GWVs) and continuous variables (*e.g.,* cardiopulmonary exercise & symptom responses) were treated as the independent variable, while DNA methylation level was the dependent variable. A model adjusted for age, BMI, sex, BeadChip, and white blood cell counts (*i.e.,* reference-based cell proportion estimates for granulocyte, monocyte, natural killer, B-cell, CD8T, and CD4T lymphocyte; R package *FlowSorted.Blood.EPIC*) was used for regression.[252,257–260] Acute exercise has been shown to stimulate immunological activity[149] (discussed in Section 2.2.1), and linear mixed effects regression models [R packages *lme4*[250] & *lmerTest*[251]] were used to test differences in reference-based cell proportion estimates between groups over time. Repeated measures analyses also included correlation estimates between samples derived from the same individual across multiple timepoints (*i.e.,* pre-, post-, and 24 hours post-exercise). Multiple comparisons correction using a false discovery rate (FDR) threshold of <0.05 was used to identify differentially methylated positions (DMPs).

3.6.4. DMP-associated Genes & Post-hoc Analyses:

Post-hoc analyses included annotation of genomic structures containing a DMP across all known isoforms using *annotatr* (version 1.32.0).[261] Gene promoters were defined as regions < 1Kb upstream of the transcription start site (TSS). Genes containing one or more DMPs (FDR < 0.05) were defined as DMP-associated genes (DMGs). Pending the number of returned DMGs, pathway analyses (*e.g.,* gene ontology [GO][262,263] and Kyoto Encyclopedia of Genes and Genomes [KEGG][264]) were considered to contextualize associations of DMGs in GWI with known physiological processes and diseases.

Specific Aim 1: Analysis

Specific Aim 1): To compare baseline levels of blood-based DNA methylation between GWVs with GWI and control GWVs.

Specific Aim 1 Hypothesis: Differentially methylated positions (DMPs) measured at baseline *in peripheral blood* via *Illumina Infinium MethylationEPIC V2* microarray will be identified between GW*Vs with GWI and control GWVs.*

Specific Aim 1 Primary Approach: A regression-based approach using the linear-models for microarray (R-package *limma*[256]) was used to test for differences in DNA methylation between GWVs with GWI and control GWVs at baseline. A linear model comprised of the primary independent variable (*i.e.,* GWI status) and covariates was fit to DNA methylation levels (*i.e.,* M-value) for each CpG site to determine which sites were differentially methylated (FDR < 0.05).

Specific Aim 1 Secondary Approach: Sex-specific differences in autosomal DNA methylation have been previously described across multiple tissue types and highlight the importance of controlling for biological sex in differential DNA methylation analyses.[265–267] Because of the small and unequal distribution of female participants within each group (*i.e.,* GWVs with GWI: N_{female} = 4; control GWVs: N_{female} = 2), a secondary subgroup analysis of male-only participants was performed to account for the potential impact of biological sex.

Specific Aim 1: Variable Descriptions	
Variable:	**Name (Description):**
Dependent Variable (Y)	CpG Site DNA Methylation (M-Value)
Independent Variable (X)	Group Status (GWI vs. Control)
Covariates	Age (Years), BMI (kg/m^2), Sex (male vs. female), white blood cell estimates (6 types), BeadChip
Secondary Analysis: Replicate the primary analysis using male-only participants (GWVs with GWI: N = 23 vs. control GWVs: N = 23).	

Specific Aim 2: Analysis

Specific Aim 2): To compare changes in blood-based DNA methylation from pre- to 30 minutes and 24 hours post-exercise in GWVs with GWI and control GWVs.

Specific Aim 2 Hypothesis: *DMPs measured via Illumina Infinium Methylation EPIC V2 in peripheral blood will be identified in both between and within group comparisons 30 minutes and 24 hours post-exercise. Based on previous literature, changes in GWVs with GWI will favor hypermethylation compared to control GWVs.*

Specific Aim 2 Primary Approach: A regression-based approach using the linear-models for microarray (R-package *limma*[256]) was used to test for differences in DNA methylation between and within groups across each timepoint. A linear model comprised of the primary independent variables that include group (*i.e.,* GWI status), time (*i.e.,* pre-, post-, 24 hours post-exercise), group-by-time, and covariates was fit to DNA methylation levels (*i.e.,* M-value) for each CpG site to determine which sites had differential DNA methylation changes across time and by group (FDR < 0.05).

Specific Aim 2 Secondary Approach: As described in the Specific Aim 1 Secondary Approach, the small and unequal distribution of female participants within each group (*i.e.,* GWVs with GWI: N_{female} = 4; control GWVs: N_{female} = 2) warranted a secondary subgroup analysis using male-only participants to account for the potential impact of biological sex.

Specific Aim 2: Variable Descriptions	
Variable:	**Name (Description):**
Dependent Variable (Y)	CpG Site DNA Methylation (M-Value)

Independent Variable (X)	Group Status (GWI vs. Control) Time (Pre-, Post-, 24hr Post-Exercise Group x Time (2 groups x 3 Timepoints)
Covariates	Age (Years), BMI (kg/m^2), Sex (male vs. female), white blood cell estimates (6 types), BeadChip

Secondary Analysis: Replicate the primary analysis using male-only participants (GWVs with GWI: N = 23 vs. control GWVs: N = 23).

Specific Aim 3: Analysis

Specific Aim 3): To test differences in cardiopulmonary responses measured during submaximal exercise between GWVs with GWI and control GWVs.

Specific Aim 3 Hypothesis: *Cardiopulmonary responses to exercise will indicate less efficient ventilation (i.e., higher ventilatory equivalents) in GWVs with GWI compared to control GWVs.*

Specific Aim 3 Primary Approach: Cardiopulmonary responses to exercise are not well-characterized in GWVs with GWI, but as discussed in Section 3.3.2, published findings from our group support distinct ventilatory patterns (*i.e.,* higher tidal volume and lower respiratory frequency) during a maximal exercise in GWVs with GWI compared to control GWVs.[268] Our published worked performed using submaximal exercise challenge model also described 1) significantly less efficient ventilatory responses during steady state exercise and 2) small-to-

moderate correlations between ventilatory equivalents and peak PEM symptom responses in GWVs with GWI.[249] Although the present study's submaximal steady state exercise challenge protocol has limited clinical utility, previous evidence demonstrates that ventilatory equivalents of carbon dioxide parallel end-tidal PCO_2 during both steady state and non-steady state aerobic exercise.[269] These findings indicate that ventilatory equivalents of carbon dioxide are informative of underlying metabolic factors during both steady state and non-steady state exercise protocols.

One-way analysis of covariance (ANCOVA) was used to test group differences in ventilatory equivalent responses during the exercise challenge [R package *rstatix*[270]]. Participant age, biological sex, and body mass index were also included as model covariates. Cumulative work (kilojoules [kJ]) produced during exercise was also included as a model covariate to help control for confounding effects of individual cardiorespiratory fitness/cumulative work output differences. Linear mixed effects models were fit with random intercepts to account for individual participant differences.

Sub-Aim 3a): To test associations between ventilatory equivalents measured during submaximal exercise with blood-based DNA methylation levels 1) pre-exercise, 2) post-exercise, and 3) 24 hours post-exercise in GWVs with GWI and control GWVs.

Sub-Aim 3a Hypothesis: *Ventilatory equivalents will be significantly associated with blood-based DMPs 1) 30 post-exercise and 2) 24 hours post-exercise. DMPs associated with ventilatory equivalents and GWI will be predominantly in genes known to participate in metabolic function.*

Sub-Aim 3a Approach: Associations between DNA methylation and pulmonary function have been identified in both healthy[271] and clinical populations (*i.e.,* chronic obstructive pulmonary disease [COPD])[272], and investigating relationships between DNA methylation and potential pathophysiological pulmonary responses in GWVs with GWI is warranted. Associations between ventilatory equivalents for carbon dioxide and DNA methylation levels were tested by fitting a separate linear model for each timepoint (*i.e.,* pre-, post-, 24 hours post-exercise). Each model utilized the linear-models for microarray (R-package *limma*[256]) method to test for differences in DNA methylation relative to 1) $\dot{V}E/\dot{V}CO_2$ and 2) $\dot{V}E/\dot{V}CO_2$ stratified by group (*i.e.,* GWV with GWI vs. control GWV). Separate linear models for each timepoint included primary independent variables of ventilatory equivalent for carbon dioxide, group (*i.e.,* GWI status), ventilatory equivalent-by-group interaction, and covariates were fit to DNA methylation levels (*i.e.,* M-value) for each CpG site to determine which sites were differentially methylated (FDR < 0.05).

Sub-Aim 3: Variable Descriptions	
Variable:	**Name (Description):**
Dependent Variable (Y)	CpG Site DNA Methylation (M-Value) • Model 1: Pre-Exercise • Model 2: Post-Exercise • Model 3: 24 hours Post-Exercise
Independent Variable (X)	Ventilatory Equivalent ($\dot{V}E/\dot{V}CO_2$)

	Group Status (GWI vs. Control) $\dot{V}E/\dot{V}CO_2$ x Group
Covariates	Age (Years), BMI (kg/m^2), Sex (male vs. female), Cumulative Work (kilojoules [kJ]) white blood cell estimates (6 types), BeadChip

Specific Aim 4: Analysis

Specific Aim 4): To test if symptom severity increases from pre- to post-exercise (*i.e.,* 30 minutes 24 hours post-exercise) in GWVs with GWI compared to control GWVs.

Specific Aim 4 Hypothesis: *GWVs with GWI who endorse PEM will have an increase in symptom severity (i.e., PEM) after exercise.*

Specific Aim 4 Approach: A detailed overview of analysis steps for symptom time course is described in Section 3.6.2. Symptom Time Course: PEM. Heterogeneity across PEM time course is well-accepted,[5,128] but several previous investigations in people with ME/CFS[81,132,137] and GWVs with GWI[9,102] have demonstrated both symptom and biological responses occur within 24 hours of an exercise challenge. For consistency, timepoints with simultaneous blood samples and psychometric measures of fatigue (*i.e.,* POMS Fatigue Subscale), pain (*i.e.,* SF-MPQ-2 Total Score), and mood disturbance (*i.e.,* POMS TMD) were the focus of the present dissertation. As described, participant PEM classification was based on endorsement of previous PEM episodes at screening.

Sub-Aim 4a): To test whether changes in blood-based DNA methylation levels from pre- to 30 minutes post- and 24 hours post-exercise are associated with symptom worsening in GWVs with GWI.

Sub-Aim 4 Hypothesis: *Differences in DNA methylation from pre- to post-exercise will be associated with increased symptom severity in GWVs with GWI who endorse experiencing PEM.*

Sub-Aim 4a Approach: A penalized least absolute shrinkage and selection operator (LASSO) regression model (R package *glmnet*[273]) was used to select DMPs identified in Primary Aims 1 & 2 that had the strongest associations with each primary symptom outcome (*i.e.,* fatigue, pain, and mood disturbance) in GWVs with GWI. Briefly, feature selection through LASSO regression is performed by applying an *L*1penalty that reduces regression coefficients of weaker predictors toward zero.[274,275] A penalty parameter (λ) for each time point was selected using a 20-fold cross-validation to optimize model fit. This approach has been utilized in genomics to better identify specific loci with the strongest association to a given trait,[276] and additional rationales for LASSO regression included 1) limited GWI sample size (GWI: N = 27) that required cognizance of model overfitting and 2) potential collinearity among selected CpG sites. Predictors (*i.e.,* DMPs) identified through LASSO regression were integrated to separate linear mixed effects models for each primary symptom outcomes (*i.e.,* fatigue, pain, and mood disturbance) to test relationships between symptoms and DNA methylation in GWVs with GWI.

Sub-Aim 4a: Variable Descriptions	
Variable:	**Name (Description):**
Dependent Variable (Y)	Fatigue Symptoms (POMS Fatigue Subscale) Pain Symptoms (SF-MPQ-2 Total Score) Mood Disturbance (POMS TMD)
Independent Variable (X)	CpG Site DNA Methylation Time (Pre-, Post-, 24hr Post-Exercise) DNA Methylation x Time Interaction

CHAPTER 4: Results

4.1. Participant Demographics & Combat Exposures:

GWVs with GWI and control GWVs were similar in age, body mass index (BMI), and proportions of sex, self-reported race/ancestry, and ethnicity. A significantly larger proportion of GWVs with GWI met the American College of Rheumatology Widespread Pain Index (WPI) criteria for fibromyalgia (GWI = 14/27, 52%) compared to healthy control GWVs (CON = 0/25, 0%; $p < 0.001$). Nearly half of all GWVs with GWI (N = 12/27; 44%) endorsed experiencing PEM during screening. Descriptive statistics for participant demographics are provided in Table 5.

Data describing branch of service and self-reported exposures encountered during deployment to the Persian Gulf are summarized for GWVs with GWI and healthy control GWVs in Table 6. Self-reported exposures were similar between groups, though significantly larger

proportions of GWVs with GWI reported hearing chemical alarms (GWI: 25/27, CON: 16/25; $p < 0.05$), seeing Iraqis or Civilians wounded/killed (GWI: 21/27, CON: 12/25; $p < 0.05$), and saw their living areas treated with pesticides (GWI: 15/27, CON: 7/25; $p < 0.05$).

Table 5. Participant Demographics

Participant Demographics	GWI N = 27[1]	CON N = 25[1]	p-value[2]
Age	52.37 (4.12)	53.52 (5.75)	0.7
BMI	30.37 (6.04)	28.45 (4.32)	0.4
Sex			0.7
F	4 / 27 (15)	2 / 25 (8.0)	
M	23 / 27 (85)	23 / 25 (92)	
Race			>0.9
American Indian or Alaskan Native	1 / 27 (3.7)	0 / 25 (0)	
Black or African American	1 / 27 (3.7)	1 / 25 (4.0)	
White	24 / 27 (89)	23 / 25 (92)	
Unknown	1 / 27 (3.7)	1 / 25 (4.0)	
Ethnicity			>0.9
Hispanic or Latino	1 / 27 (3.7)	1 / 25 (4.0)	
Non-Hispanic or Latino	24 / 27 (89)	23 / 25 (92)	
Unknown	2 / 27 (7.4)	1 / 25 (4.0)	
Fibromyalgia (WPI)	14 / 27 (52)	0 / 25 (0)	<0.001
PEM Endorsement (Yes/No)	12 / 27 (44)	0 / 25 (0)	<0.001

[1] Mean (SD); n / N (%)

[2] Wilcoxon rank sum test; Fisher's exact test; Pearson's Chi-squared test

Note. Abbreviations: GWI = GWVs with GWI; CON = healthy control GWVs; F = Female; M = Male; WPI = Widespread Pain Index.

Table 6. Branch of Service & Self-Reported Gulf War Exposures

GW Exposures	GWI N = 27[1]	CON N = 25[1]	p-value[2]
Branch_Summary			0.8
Airforce	2 / 27 (7.4)	1 / 25 (4.0)	
Army	18 / 27 (67)	14 / 25 (56)	
Coast Guard	0 / 27 (0)	1 / 25 (4.0)	
Marines	3 / 27 (11)	5 / 25 (20)	
Navy	4 / 27 (15)	4 / 25 (16)	
Other	0 / 27 (0)	0 / 25 (0)	
Came into Contact with American Vehicles Hit by Friendly Fire	10 / 27 (37)	5 / 25 (20)	0.2
Came into Contact with Dead Animals	8 / 27 (30)	5 / 25 (20)	0.4
Came Into Contact with POWs	17 / 27 (63)	13 / 25 (52)	0.4
Directly Involved in Air Combat	0 / 27 (0)	1 / 25 (4.0)	0.5
Directly Involved in Ground Combat	14 / 27 (52)	11 / 25 (44)	0.6
Had SCUD Missile Explode Within 1 Mile	16 / 27 (59)	13 / 25 (52)	0.6
Heard Chemical Alarms	25 / 27 (93)	16 / 25 (64)	0.012
In Contact with Fresh CARC Paint	11 / 26 (42)	7 / 25 (28)	0.3
Unknown	1	0	
Less than 4 Hours of Sleep in 24 Hour Period	24 / 27 (89)	21 / 25 (84)	0.7
Received One or More Shots in Arm in Theatre	20 / 27 (74)	15 / 25 (60)	0.3
Received One or More Shots in Buttocks in Theatre	16 / 27 (59)	9 / 25 (36)	0.093
Saw American or Allied Troups Wounded or Killed	15 / 27 (56)	9 / 25 (36)	0.2
Saw Dead Animals	23 / 27 (85)	18 / 25 (72)	0.2
Saw Iraqis or Civillians Wounded or Killed	21 / 27 (78)	12 / 25 (48)	0.026
Saw Living Area Fogged or Sprayed with Pesticides	15 / 26 (58)	7 / 25 (28)	0.032
Unknown	1	0	
Saw Smoke from Oil Well Fires	24 / 27 (89)	19 / 25 (76)	0.3
Took Pyridostigmine Pills	20 / 27 (74)	20 / 25 (80)	0.6
Used Pesticide Cream or Spray on Skin	19 / 27 (70)	12 / 25 (48)	0.10
Wore a Flea Collar	6 / 27 (22)	3 / 25 (12)	0.5
Wore Uniform Treated with Pesticides	13 / 27 (48)	8 / 25 (32)	0.2

[1] n / N (%)

[2] Fisher's exact test; Pearson's Chi-squared test

Note. Self-reported exposures encountered during the Persian Gulf War in GWVs with GWI and control GWVs. Abbreviations: GW Exposures = Gulf War Exposures; GWI = GWVs with GWI; CON = healthy control GWVs.

4.2. Participant Symptom Characteristics & Cardiopulmonary Responses to Exercise:

GWVs with GWI reported worse physical/mental health, fatigue, pain, sleep quality, greater perceived deficits, and worse overall sleep quality compared to control GWVs ($p < 0.001$). The magnitude of effect between groups was considered large (Cohen's *d* Range: 1.17 - 2.27). Summary statistics and results from statistical tests of group differences are described in Table 7.

Six participants (GWVs with GWI: N = 4; control GWVs: N = 2) were excluded from analyses due to missing exercise data. Reasons for missing data included: 1) technical issue with HR monitor or oronasal facial mask during exercise (N = 4); 2) hypertensive response to exercise (*i.e.,* systolic blood pressure of 250+ mmHg), warranting termination of test (N = 1); and 3) an illness concern unrelated to the exercise challenge (N = 1). Complete cardiopulmonary responses measured during the exercise challenge were similar between GWVs with GWI (N = 23) and control GWVs (N = 23) and are described in Table 8.

Table 7. Baseline Symptoms **Screening Instrument:**	**GWI:** **Mean (SD)**	**CON:** **Mean (SD)**	***t-statistic:***	***p-value:***	***Cohen's d:***
Kansas Screening Questionnaire - Total Score (0 - 87)	29.07 (12.41)	3.82 (4.17)	9.99	< 0.001	2.73
Fatigue domain (0 – 12)	6.33 (2.18)	1.20 (1.73)	9.44	< 0.001	2.61
Pain domain (0 – 9)	3.76 (2.42)	0.50 (0.61)	6.78	< 0.001	1.85
Neurological/Cognitive/Mood domain (0 – 42)	13.78 (6.88)	1.44 (2.35)	8.78	< 0.001	2.40
Gastrointestinal domain (0 – 9)	2.78 (2.72)	0.28 (0.54)	4.67	< 0.001	1.27
Respiratory domain (0 – 9)	1.78 (1.80)	0.08 (0.28)	4.83	< 0.001	1.32
Skin domain (0 – 6)	0.65 (0.78)	0.32 (0.63)	1.67	0.10	0.46
Symptom Measure:					
Veterans Rand 36 Item Health Survey (VR-36)					
VR-36 Physical Component score (0 – 100)	60.45 (18.22)	90.36 (6.99)	-7.92	< 0.001	-2.17
VR-36 Mental Component score (0 – 100)	53.87 (20.23)	89.31 (8.84)	-8.29	< 0.001	-2.27
Multidimensional Fatigue Inventory (MFI) total score (20 – 100)	67.67 (15.27)	34.00 (11.77)	8.94	< 0.001	2.47
Perceived Deficits Questionnaires (PDQ) total score (0 – 80)	41.48 (14.34)	14.84 (11.50)	7.42	< 0.001	2.05
Profile of Mood States (POMS) total score (-30 – 200)	29.93 (19.51)	-0.84 (10.36)	7.17	< 0.001	1.97
McGill Pain Questionnaire (MPQ) Total score (0 – 10)	2.32 (1.75)	0.28 (0.29)	5.94	< 0.001	1.62
Pittsburgh Sleep Quality Index (PSQI) (0 – 21)	11.59 (4.39)	6.64 (4.07)	4.22	< 0.001	1.17

Note. Questionnaire score ranges are provided in column 1 for severity interpretation. Abbreviations: GWI = GWVs with GWI; CON = healthy control GWVs; SD = Standard Deviation.

Table 8. Participant Cardiopulmonary Responses During Exercise Challenge	**GWI (N = 23) Mean (SD)**	**CON (N = 23) Mean (SD)**	***t-statistic:***	***p_{BH}:***	***Cohen's d:***
$\dot{V}O_2$ (mL•kg•min^{-1})	13.7 (3.3)	14.6 (4.1)	-0.80	0.60	-0.23
$\dot{V}CO_2$ (mL)	1,388 (385)	1,516.25 (381)	-1.14	0.41	-0.34
$\dot{V}E$ (L•min^{-1})	43.94 (15.4)	43.80 (11.7)	-0.04	0.97	-0.01
V_T	1.87 (0.5)	2.08 (0.5)	1.43	0.28	0.42
f_R	27.07 (5.0)	26.13 (5.6)	-0.61	0.69	-0.18
HR (beats per minute)	137 (7.2)	137 (7.4)	-0.20	0.90	-0.06
Average Power (Watts)	80.8 (24.4)	92.73 (29.0)	1.51	0.28	0.44
Cumulative Work (kJ)	251.1 (23.6)	253.1 (18.2)	0.33	0.87	0.10

Note. Group differences of cardiopulmonary exercise responses between Gulf War Veterans with Gulf War Illness and control Gulf War Veterans were compared using independent samples t-tests using and significance thresholds of $p_{BH} < 0.05$ (Benjamini-Hochberg corrected). The magnitude of each group difference was reported using a Cohen's *d* effect size. $\dot{V}O_2$ = oxygen consumption; $\dot{V}CO_2$ = carbon dioxide consumption; $\dot{V}E$ = ventilation; V_T = Tidal Volume; f_R = Respiratory Frequency; HR = Heart Rate (rounded to nearest whole number); kJ = kilojoules

4.3. DNA Methylation: Data Preprocessing, Normalization, and Cell Proportion Estimates

4.3.1. DNA Methylation Data Processing & Normalization:

Preprocessing and normalization steps are depicted in Figure 9. Raw IDAT files integrated into the analysis pipeline contained 936,990 probes across 152 total samples. Following normalization (SWAN Method), samples displayed adequate binomial distributions. Probes were assessed and excluded based on detection quality (detection *p*-value < 0.01), location on a sex chromosome, correspondence to a single-nucleotide polymorphism at the CpG site, or labeled to be cross-reactive. Beta and M-values were computed from probes included in the final analysis (N = 874,899) for probe-wise differential DNA methylation analyses.

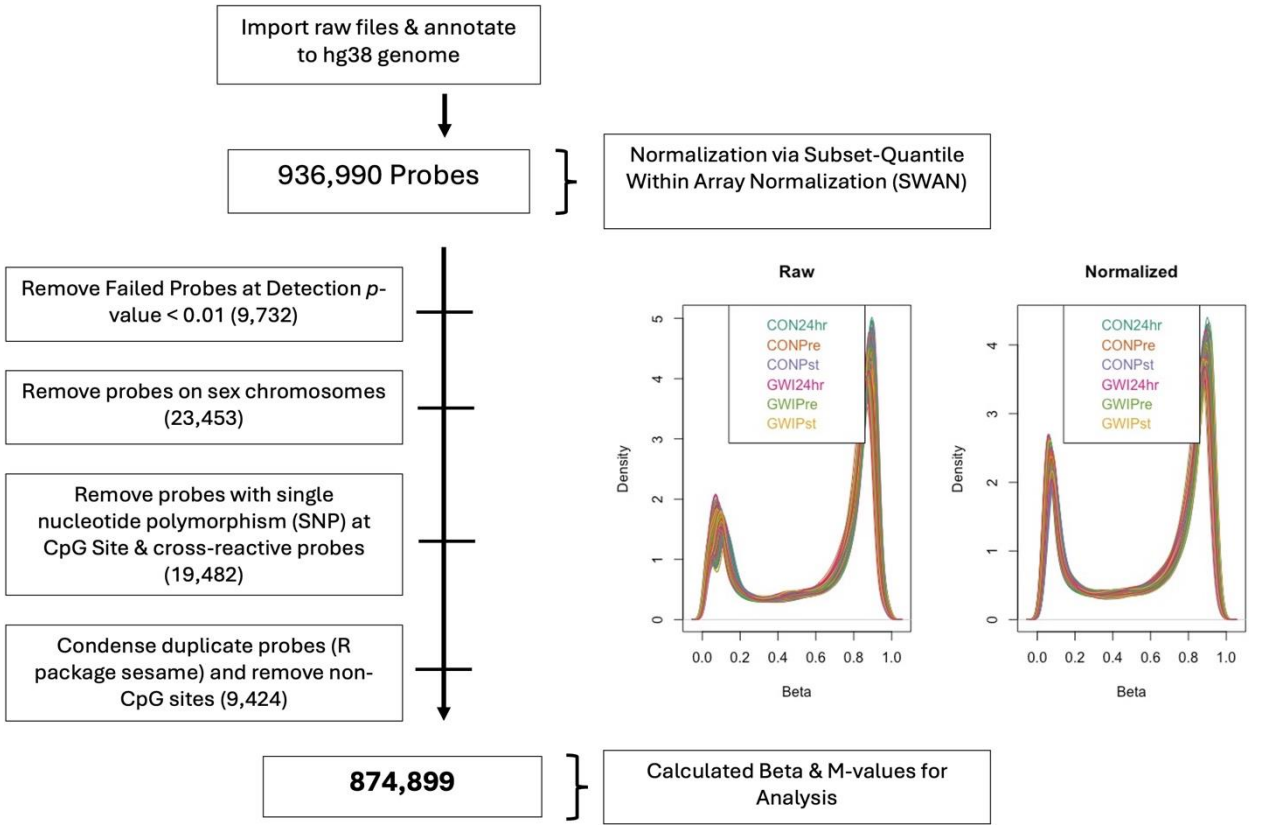


Figure 9. DNA Sample Preprocessing & Normalization. Both beta-values and M-values for 874,899 CpG sites were extracted for differential DNA methylation analyses following normalization (*i.e.,* SWAN procedure), probe exclusion (*i.e.,* failed detection, location on sex chromosome, location on SNP, cross-reactive), and condensing (*i.e.,* averaging) duplicate probes.

4.3.2. Reference-Based Cell Proportion Estimates:

Reference-based estimated cell proportions for T-cells (*i.e.,* CD4+, CD8+), Natural Killer (NK) cells, B-cells, Monocytes, and Neutrophils at each timepoint (Figure 10) were derived from Beta-values, and these estimates were included as covariates in differential DNA methylation analyses models. Mixed effects regression models confirmed that estimated proportions for each cell type did not differ significantly between groups over time.

Figure 10. Reference-based Cell Proportions

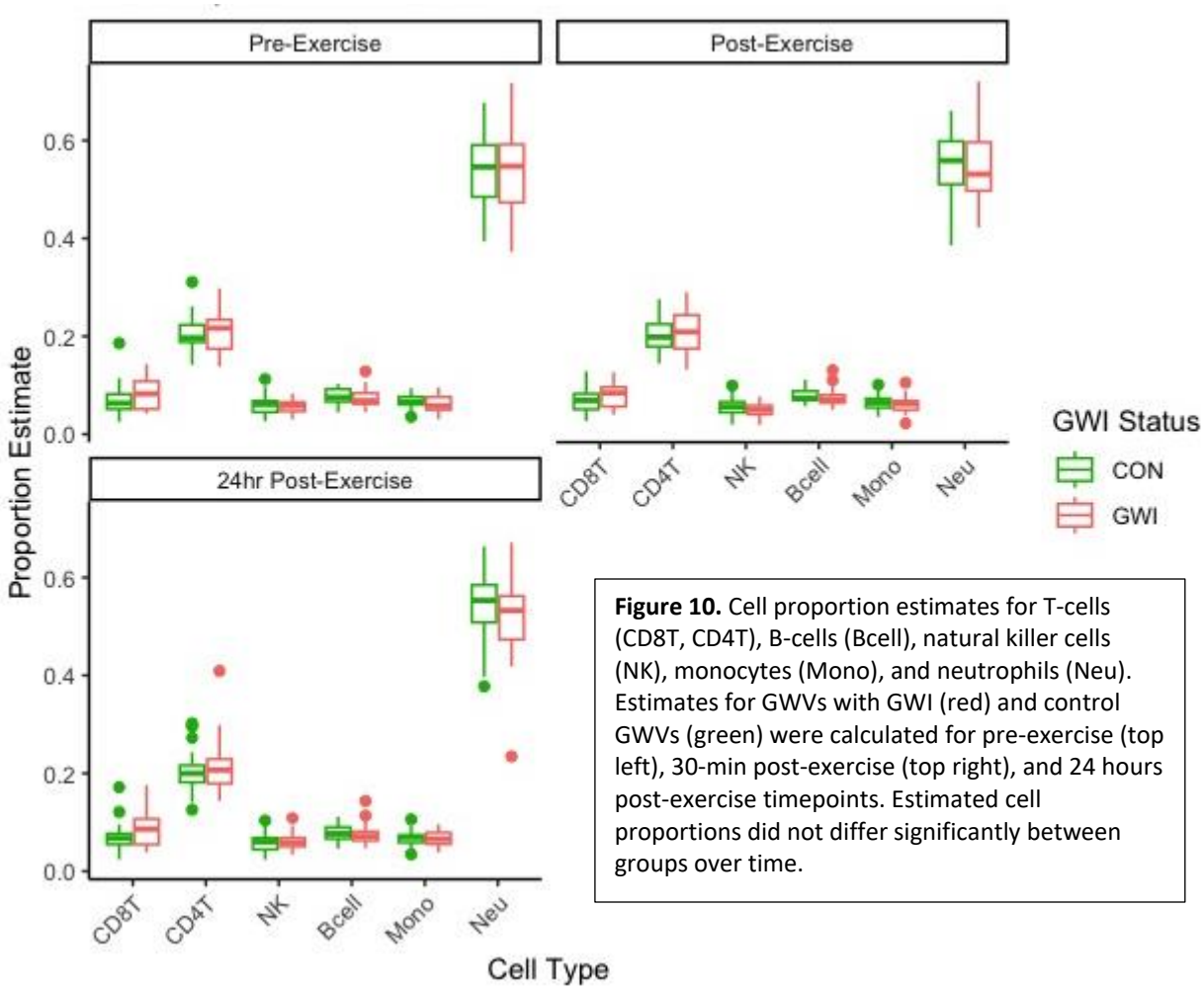


Figure 10. Cell proportion estimates for T-cells (CD8T, CD4T), B-cells (Bcell), natural killer cells (NK), monocytes (Mono), and neutrophils (Neu). Estimates for GWVs with GWI (red) and control GWVs (green) were calculated for pre-exercise (top left), 30-min post-exercise (top right), and 24 hours post-exercise timepoints. Estimated cell proportions did not differ significantly between groups over time.

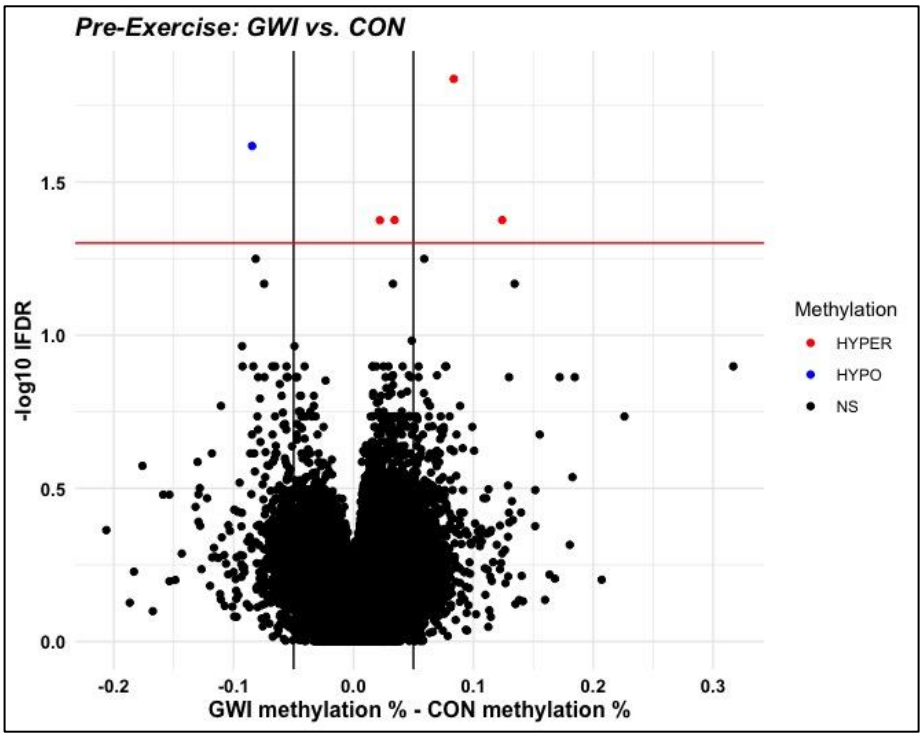
4.4. Results: Specific Aim 1 & 2 Summary

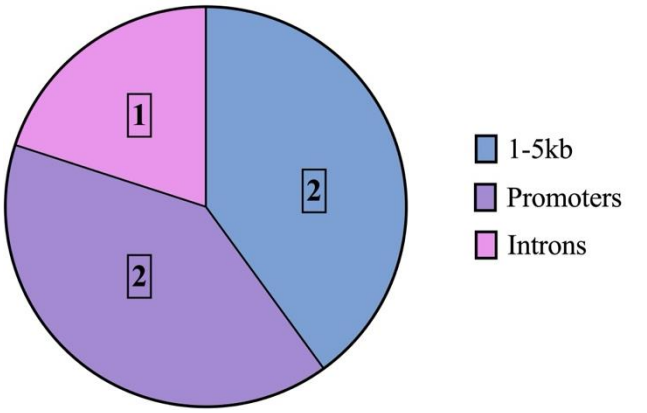
DMPs identified between GWVs with GWI and control GWVs at baseline (Specific Aim 1) and in response to the exercise challenge (Specific Aim 2) are summarized in Supplemental File 1A. Descriptive information for DMPs identified in each comparison (*e.g.,* genomic location, mean DNA methylation difference, annotation to known protein-genes, etc.) is provided in Supplemental File 1B. GO and KEGG pathway analyses performed on DMP-associated genes in a given comparison are described in Supplemental File 1C.

4.5. Results: Specific Aim 1

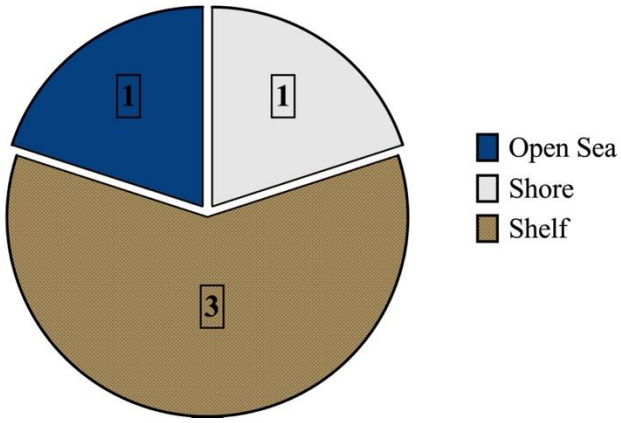
Five DMPs were observed in GWVs with GWI compared to control GWVs prior to exercise (Supplemental File 1A), and each DMP annotated to a protein-coding gene (Table 9). Four DMPs were hypermethylated (*i.e.,* greater DNA methylation levels in GWVs with GWI compared to control GWVs), while one DMP was hypomethylated (*i.e.,* lower DNA methylation levels in GWVs with GWI compared to control GWVs). Relative effect sizes (*i.e.*, % DNA methylation difference between GWI and CON) for DMPs and non-statistically significant CpG sites are plotted in Figure 11A. Four of the five total DMPs identified in GWVs with GWI pre-exercise were in 1) gene promoter (1500 base pairs upstream or 200 base pairs downstream of transcription start site [TSS]) or 2) 1-5 kilobases (kb) upstream of the transcription start site (Figure 11B). Relative to genomic regions with high concentrations of CpG sites (*i.e.,* CpG Islands), DMPs identified pre-exercise were in CpG shores (0 – 2 kb from Island edge), CpG shelves (2 – 4kb from Island edge), and in the CpG Open Sea (>4kb from Island edge) (Figure 11C).

11A.





11B. DMP Location (Gene Body)



11C. DMP Location (CpG Island)

Figure 11A-11C. Summary of differentially methylated positions (DMPs) pre-exercise between GWI and CON. **11A)** Volcano plot of 874,899 CpG sites displays mean DNA methylation difference (x-axis) and statistical significance between (y-axis; -log10(FDR)) at pre-exercise between GWVs with GWI and control GWVs. Vertical black lines indicate a ± 5% mean DNA methylation difference, and the horizontal red line displays the -log10 of an FDR < 0.05. **11B)** Gene body distribution of DMPs identified in pre-exercise comparisons. **11C)** Relation of DMPs identified pre-exercise to CpG Island.

Table 9. DMP-Associated Genes (Pre-Exercise)		
Gene Symbol:	**Description:**	**Associated DMP Methylation:**
OGDH	Oxoglutarate Dehydrogenase	Hypermethylated
RANBP17	Ras-related Nuclear Protein Binding Protein 17	Hypomethylated
PDGFRA	Platelet-Derived Growth Factor Receptor Alpha	Hypermethylated
RGS10	Regulator of G Protein Signaling 10	Hypermethylated
SLC12A8	Solute Carrier Family 12 Member 8	Hypermethylated

Subgroup analyses of male-only participants (GWVs with GWI: N = 23; control GWVs: N = 23) returned three DMPs (Supplemental File 1B), and two of these DMPs were in protein-coding genes present in analysis of the total sample (*i.e., RANBP17 & SLC12A8*). One DMP located in an intergenic region was the only unique DMP returned by male-only subgroups analyses.

4.6. Results: Specific Aim 2

4.6.1. Main Effect: Group

Group Comparisons: 30-min Post-Exercise

Nine DMPs were observed in GWVs with GWI compared to control GWVs 30-min post-exercise. Six of these DMPs were hypermethylated and three DMPs were hypomethylated in GWVs with GWI compared to control GWVs. Relative effect sizes (*i.e.*, % DNA methylation difference between GWI and CON) for DMPs and non-statistically significant CpG sites are plotted in Figure 12A. Four of the nine DMPs identified 30-min post-exercise in GWVs with GWI were located in regulatory regions associated with gene activity (*i.e.,* promoters, 1-5kb

upstream of TSS; Figure 12B), while six of the nine DMPs were located within 4kb of CpG Islands (Figure 12C).

Seven of the nine DMPs identified in 30-min post-exercise comparisons are within a protein coding gene (Table 10A), while two other DMPs were within a 1) pseudogene (*LOC650226*) and 2) intergenic region. Three DMPs previously identified in pre-exercise comparisons were also present in 30-min post-exercise comparisons and mirrored identical methylation level directionality in their respective genes (*i.e., OGDH*, *PDGFRA*, *RANBP17*). Genes containing DMPs that were unique to 30-min post-exercise group comparisons were primarily associated with metabolic functions and include Cbl proto-oncogene (*CBL*), glutamine-fructose-6-phosphate transaminase 2 (*GFPT2*), and insulin-like growth factor binding protein 3 (*IGFBP3*).

Two DMPs 30 minutes post-exercise were returned by subgroup analyses of male-only participants (Supplemental File 1B). One DMP located in the protein-coding gene *RANBP17* was also present in analyses of the total sample, and the sole unique DMP was annotated to an intergenic region.

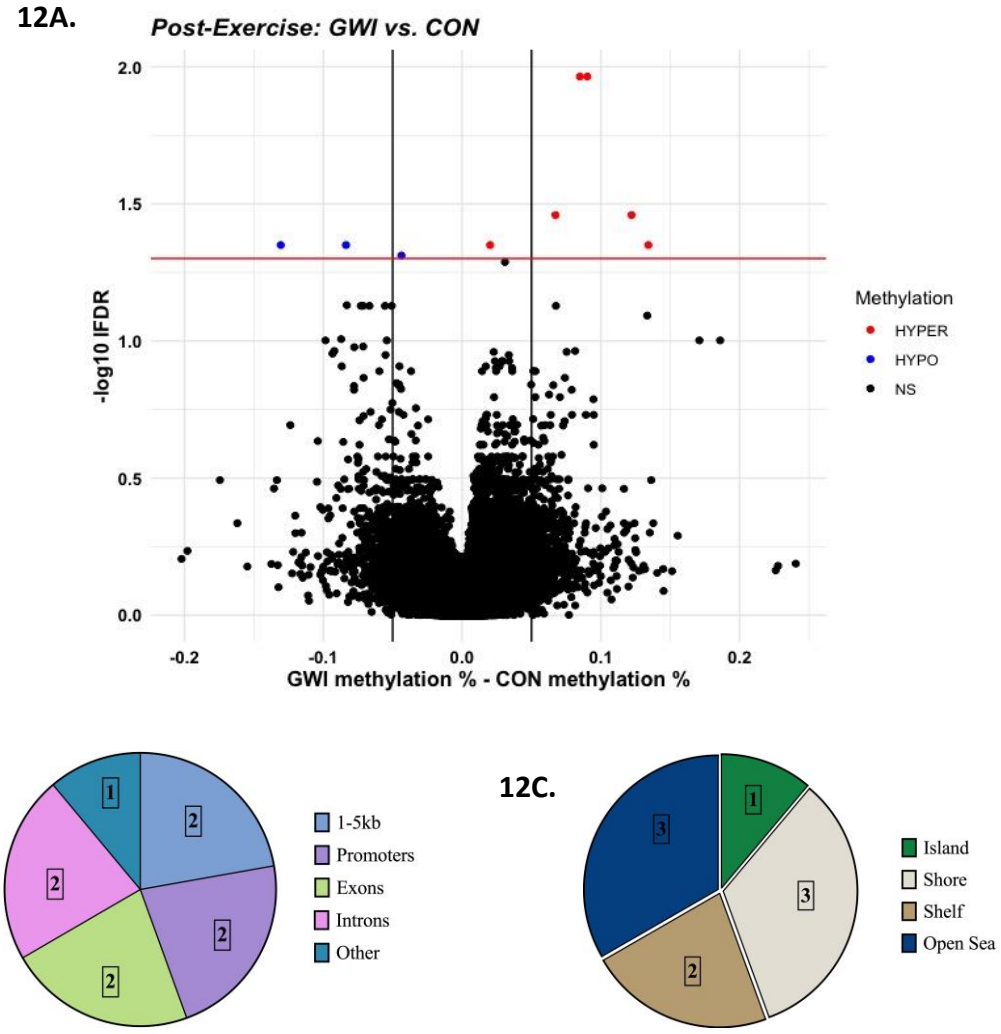


Figure 12A-12C. Differentially methylated positions (DMPs) 30-min post-exercise between GWI and CON. **A)** Volcano plot of 874,899 CpG sites displays mean DNA methylation difference (x-axis) and statistical significance between (y-axis; -log10(FDR)) at 30-min post-exercise between GWVs with GWI and control GWVs. Vertical black lines indicate a ± 5% mean DNA methylation difference, and the horizontal red line displays the -log10 of an FDR < 0.05.**B)** Gene body distribution of DMPs identified in 30-min post-exercise comparisons. **C)** Relation of DMPs identified 30-min post-exercise to CpG Island.

Table 10A. DMP-Associated Genes (30-min Post-Exercise)		
Gene Symbol:	**Description:**	**Associated DMP Methylation:**
SETBP1	SET Binding Protein 1	Hypermethylated
OGDH	Oxoglutarate Dehydrogenase	Hypermethylated
PDGFRA	Platelet-Derived Growth Factor Receptor Alpha	Hypermethylated
RANBP17	Ras-related Nuclear Protein Binding Protein 17	Hypomethylated
LOC650226	Pseudogene - Ankyrin Repeat Domain Containing 26	Hypomethylated
CBL	Cbl Proto-Oncogene	Hypermethylated
GFPT2	Glutamine-Fructose-6-Phosphate Transaminase 2	Hypermethylated
IGFBP3	Insulin-like Growth Factor Binding Protein 3	Hypomethylated

Group Comparisons: 24hr Post-Exercise

Two hypermethylated DMPs were observed in GWVs with GWI compared control GWVs at 24hr post-exercise. Relative effect sizes (*i.e.*, % DNA methylation difference between GWI and CON) for DMPs and non-statistically significant CpG sites are plotted in Figure 13. Each DMP annotated to a protein-coding genes: 1) 1-5kb upstream of the TSS in *OGDH* (DMP previously observed at pre- and 30-min post-exercise group comparisons) and 2) in the promoter of Guanine Nucleotide-Binding Protein, Alpha Stimulating [*GNAS*] (Table 10B).

Male-only participant subgroup analyses returned one DMP 24hr post-exercise (Supplemental File 1B) located in the *RANBP17* protein coding gene. No unique DMPs were identified 24hr post-exercise in male-only subgroup analyses.

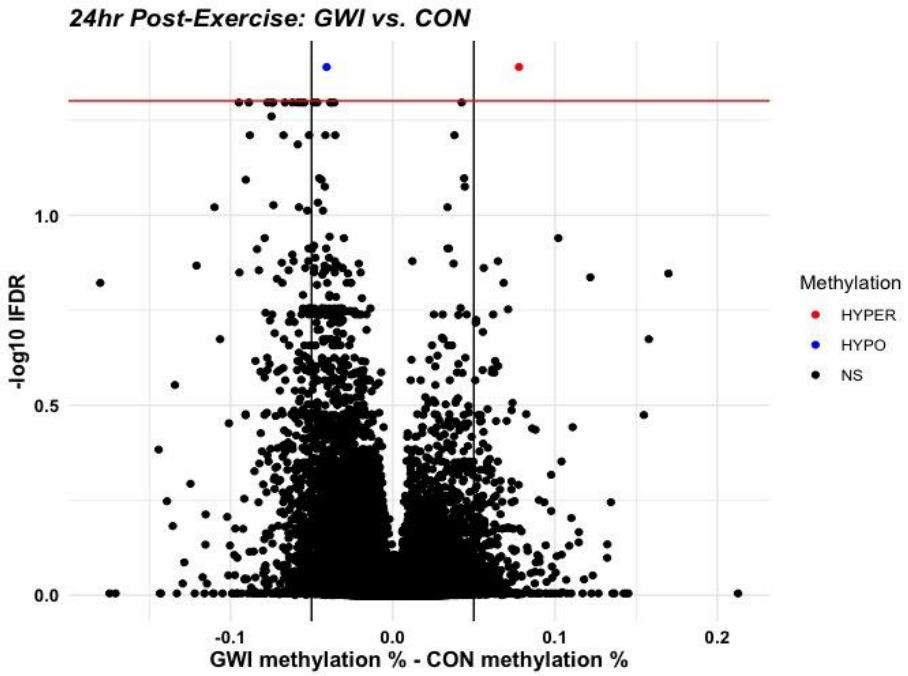


Figure 13. Volcano plot of 874,899 CpG sites displays mean DNA methylation difference (x-axis) and statistical significance between (y-axis; -log10(FDR)) at 24hr post-exercise between GWVs with GWI and control GWVs. Vertical black lines indicate a ± 5% mean DNA methylation difference, and the horizontal red line displays the -log10 of an FDR < 0.05.

Table 10B. DMP-Associated Genes (24hr Post-Exercise)

Gene Symbol:	**Description:**	**DMP Methylation Gene Body | Island Location:**
OGDH	Oxoglutarate Dehydrogenase	Hypermethylated 1-5bk | Shelf
GNAS	Guanine Nucleotide-Binding Protein, Alpha Stimulating	Hypomethylated Promoter | Shore

4.6.2. Main Effect: Time

Four DMPs (GWI = 1; CON = 3) were identified in pre- to 30-min post-exercise comparisons (Figure 14A-14B), and three DMPs (GWI = 1; CON = 2) were identified in 30-min post- to 24hr post-exercise comparisons (Figure 14C-14D). DMPs identified in pre- to 30-min post-exercise comparisons were annotated to three protein-coding genes (Table 11). The lone DMP found in GWVs with GWI annotated to G protein-coupled coupled receptor 65 (*GPR65*), and the two control GWV-associated DMPs annotated to protein coding genes were in collagen type XXIII alpha 1 chain (*COL23A1*) and plastin 1 (*PLS1*). Three DMPs (GWI = 1; CON = 2) identified in 30-min post- to 24hr post-exercise comparisons were in three unique from those identified in pre- to 30-min post-exercise comparisons (Table 11). The GWI-associated DMP was in the disco-interacting protein 2 homolog C (*DIP2C*) gene and control GWV-associated DMPs were in the serine and arginine rich splicing factor 8 (*SRSF8*) and insulin-like growth factor 1 receptor (*IGF1R*) genes, respectively.

No significant DNA methylation changes in male-only participants were identified within groups from pre- to 30-min post-exercise. DNA methylation did not change significantly from 30-min post- to 24hr post-exercise in male-only control GWVs. Five GWI-associated DMPs, however, were detected in comparisons of 30-min post- to 24hr post-exercise (Supplemental File 1A & 1B) and included four unique DMP-associated protein coding genes: proline rich coiled-coil 2A (*PRRC2A*), cholinergic receptor nicotinic alpha 5 subunit (*CHRNA5*), RNA binding motif protein 20 (*RBM20*), and decaprenyl diphosphate synthase subunit 2 (*PDSS2*).

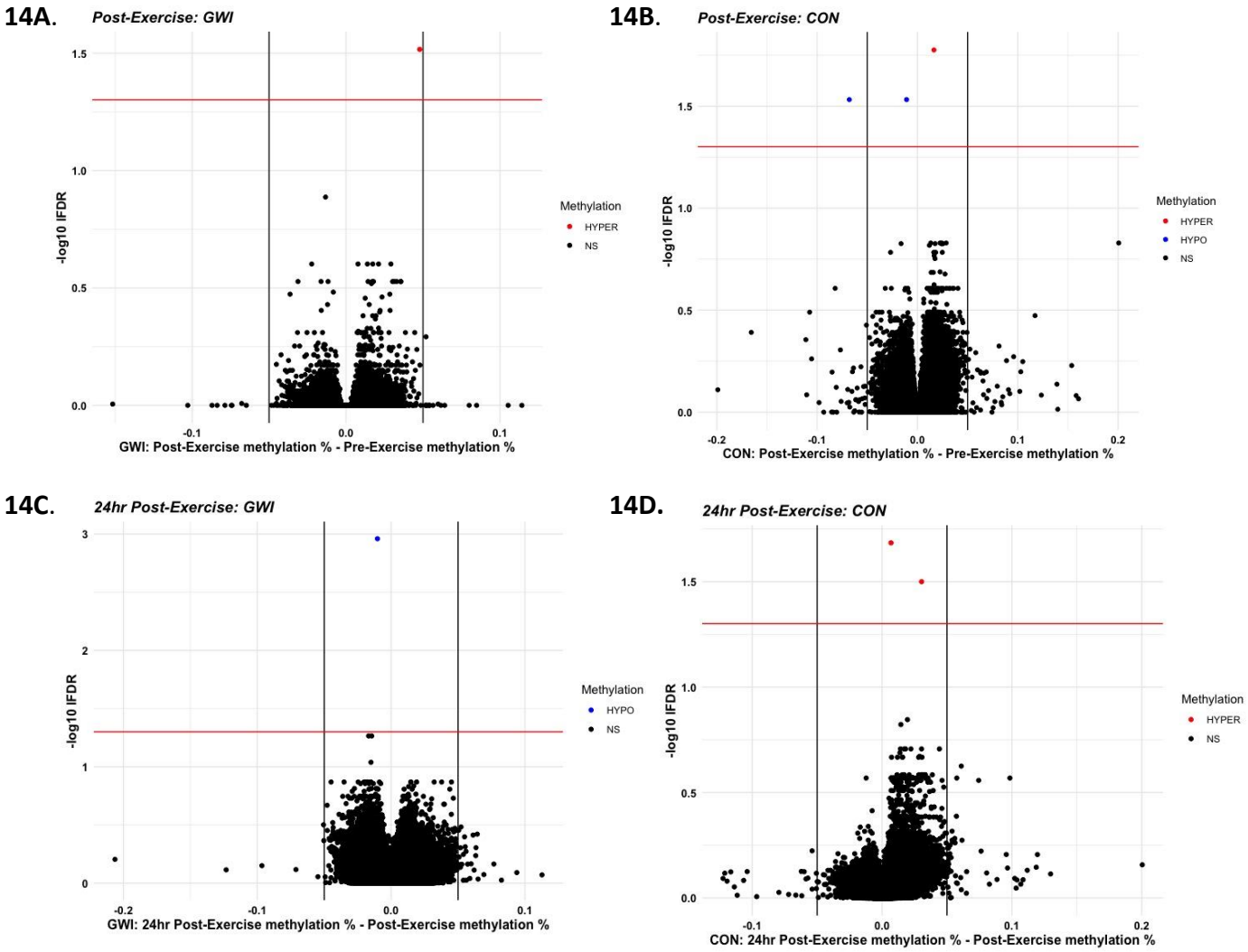


Figure 14A-D. Volcano plot of 874,899 CpG sites displays mean DNA methylation difference (x-axis) and statistical significance between (y-axis; -log10(FDR)) from pre- to 30-min post-exercise. Vertical black lines indicate a ± 5% mean DNA methylation difference, and the horizontal red line displays the -log10 of an FDR < 0.05. **A)** DMPs (N = 1) from pre- to 30-min post-exercise in GWVs with GWI. **B)** DMPs (N = 2) from pre- to 30-min post-exercise in control GWVs. **C)** DMPs (N = 1) from 30-min post- to 24hr post-exercise in GWVs with GWI. **D)** DMPs (N = 2) from 30-min post- to 24hr post-exercise in control GWVs.

Table 11. DMPs Significant Over Time Within GWI and CON Groups			
Pre- to 30-min Post-Exercise:	**Gene Symbol:**	**Description:**	**DMP Methylation Gene Body | Island Location:**
GWI	*GPR65*	G Protein-Coupled Receptor 65	Hypermethylated Exon 2 | Open Sea
CON	*COL23A1*	Collagen Type XXIII Alpha 1 Chain	Hypermethylated Intron | Open Sea
	PLS1	Plastin 1	Hypomethylated Promoter | Shore
30-min to 24hr Post-Exercise:			
GWI	*DIP2C*	Disco-Interacting Protein 2 Homolog C	Hypomethylated Intron | Island
CON	*SRSF8*	Serine and Arginine Rich Splicing Factor 8	Hypermethylated Exon 1 | Shore
	IGF1R	Insulin-like Growth Factor 1 Receptor	Hypermethylated Intron | Open Sea

4.6.3. Interaction: Group-by-Time

No significant DMPs were identified between GWVs with GWI and control GWVs from pre- to 30-min post-exercise comparisons; however, 14 hypomethylated DMPs in group-by-time comparisons between GWVs with GWI and control GWVs from 30-min post- to 24hr post-exercise (Figure 15A). DMPs (13 / 14 DMPs; ~93%) were primarily located in CpG Open Seas (Figure 15B), but these DMPs were distributed across several genomic structures that include 1-5kb upstream of the TSS (2 / 14 DMPs), gene promoters (1500bp upstream or 200bp downstream of TSS; 4 / 14 DMPs), exons (2 / 14 DMPs), and introns (6 / 14 DMPs). Significant DMPs identified between GWVs with GWI and control GWVs from 30-min post- to 24hr post-exercise corresponded to 13 genes (Supplemental File 1B), and only one of 13 DMP-associated genes was shared across main effect comparisons of group and time (*e.g.,* Disco-Interacting Protein 2 Homolog C [*DIP2C*]; significant Time main effect comparisons of GWVs with GWI from pre- to 30-min post-exercise).

Pathway enrichment was tested using Gene Ontology (GO)[262,263] and KEGG Pathway Analyses.[264] GO Analyses returned five significant terms (FDR corrected *p*-value < 0.05; Figure 16A-16B). Functions and DMP-associated genes included cadherin binding (*e.g.,* ABL interactor 1 [*ABL1*], cadherin 18 [*CDH18*], staphylococcal nuclease and Tudor domain containing 1 [*SND1*]) and interferon-beta responses terms (*e.g.,* ubiquitin conjugating enzyme E2 G2 [*UBE2G2*] & calpain 2 [*CAPN2*]).

Terms returned from KEGG Pathway Analyses were non-significant at adjusted significance thresholds (FDR-adjusted *p*-value = NS). Given the exploratory nature of this

analysis, KEGG terms significant at unadjusted thresholds (raw *p*-values < 0.05) included folding/sorting/degradation (DMP-associated genes: *UBE2G2, CAPN2*), neurodegenerative disease (DMP-associated genes: *UBE2G2, CAPN2*), and bacterial infectious disease (DMP-associated genes: Rho GTPase Activating Protein 10 [*ARHGAP10*]). Complete results for both GO Analyses and KEGG Pathway Analyses are provided in Supplemental File 1C.

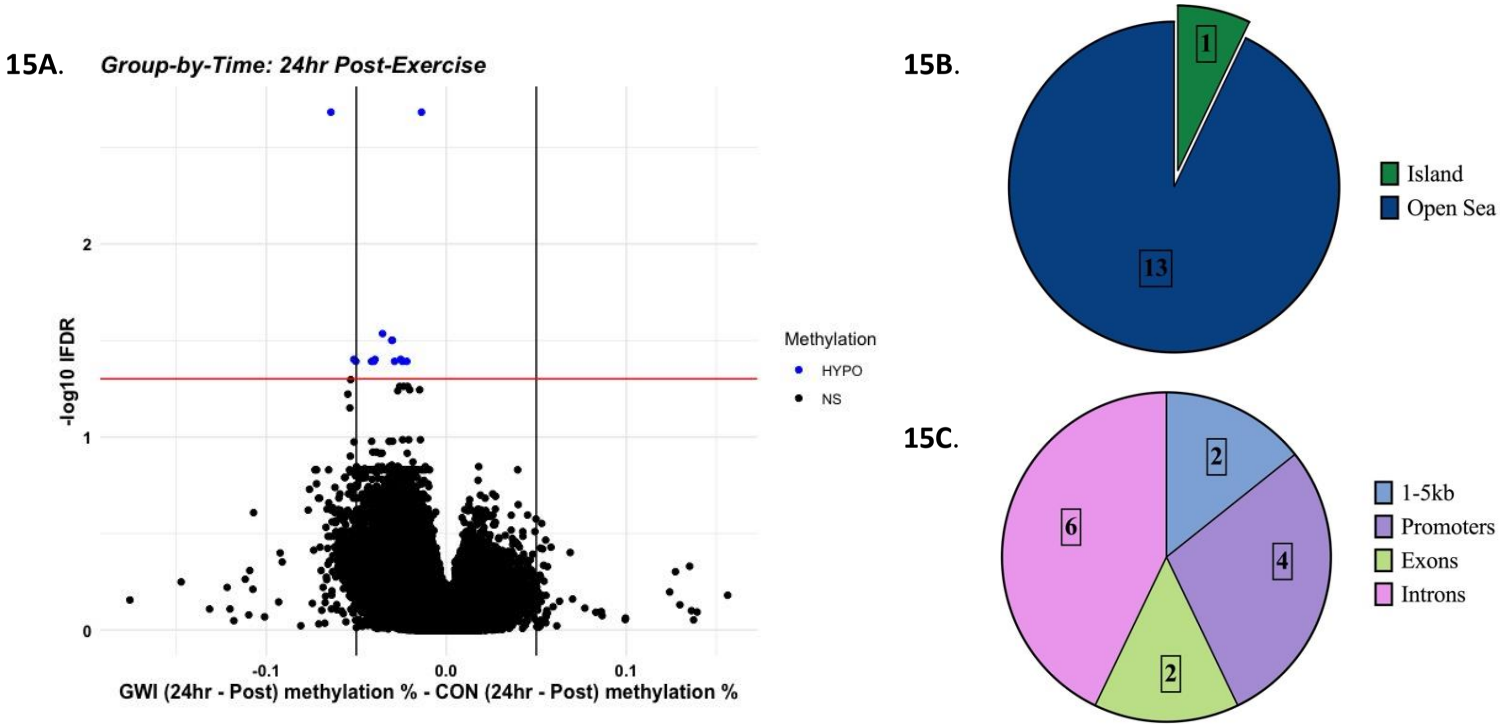
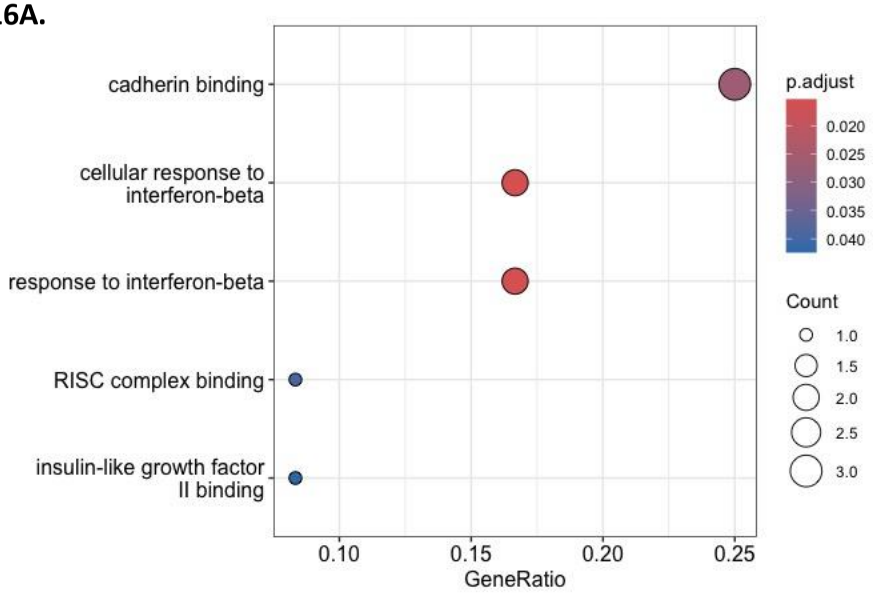


Figure 15A-C. Distribution of significant and non-significant CpGs in group-by-time comparisons. **A)** Volcano plot of 874,899 CpG sites displays mean DNA methylation difference (x-axis) and statistical significance between (y-axis; -log10(FDR)) from 30-min post- to 24hr post-exercise between GWVs with GWI and control GWVs. Vertical black lines indicate a ± 5% mean DNA methylation difference, and the horizontal red line displays the -log10 of an FDR < 0.05. **B)** Relation of DMPs identified 24hr post-exercise to CpG Island. **C)** Gene body distribution of DMPs identified in group-by-time comparisons of post- to 24hr post-exercise.

16A.



16B.

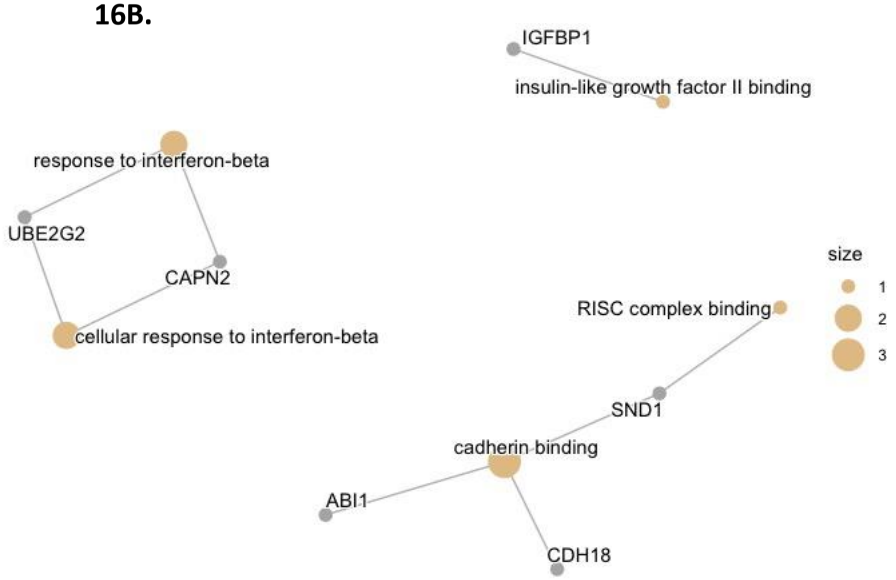


Figure 16A-B. Gene Ontology (GO) analyses of DMP-associated genes between GWVs with GWI and control GWVs from 30-min post- to 24hr post-exercise returned 5 significant terms. **A)** Dot plot describing gene ratios (x-axis) for all significant GO terms (y-axis). Dot size corresponds to the number of genes within the GO term, and term significance (False Discovery Rate [FDR] adjusted *p*-value < 0.05) is depicted by the color gradient. **B)** Network plot of DMP-associated genes within each significant GO term. Significantly enriched nodes (tan) vary in size based on the number of DMP-associated genes (grey) identified within each term and are connected directly by corresponding lines. Genes connected to multiple lines are implicated in multiple node functions. Node significance was determined based on FDR-adjusted p-values ($p < 0.05$).

Significant group-by-time changes in male-only GWVs with GWI were detected in nine DMPs from 30-min post- to 24hr post-exercise (Supplemental File 1A), and three of the nine DMPs identified were unique from analyses performed in the complete sample (Supplemental File 1B). The three unique DMPs were located within two protein-coding genes (*i.e., CHRNA5* and *PRRC2A)* and a long intergenic non-protein coding RNA gene (*i.e.,* LINC00456).

4.7. Results: Specific Aim 3

Group differences for ventilatory equivalents during the exercise challenge are described in Table 12. GWVs with GWI exhibited higher ventilatory equivalents (*i.e.,* less efficient ventilation) compared to control GWVs, but group differences were only statistically significant in comparisons for ventilatory equivalents of oxygen ($p < 0.05$). Mean differences between both ventilatory measures were of moderate magnitude ($\dot{V}E/\dot{V}CO_2$: $\eta^2 = 0.07$ & $\dot{V}E/\dot{V}O_2$: $\eta^2 = 0.07$). Among the tested covariates (*e.g.,* age, BMI, sex, and cumulative work), only BMI explained a significant proportion of variance ($p < 0.05$) between the group differences.

Table 12.	**GWI (N = 23)** Mean (SD)	**CON (N = 23)** Mean (SD)	***F*-statistic:**	***p***	η^2
$\dot{V}E/\dot{V}CO_2$	31.32 (4.18)	29.08 (4.00)	3.81	0.06	0.07
$\dot{V}E/\dot{V}O_2$	28.96 (4.65)	26.58 (4.11)	4.10	< 0.05	0.07

Note. One-way analysis of covariance (ANCOVA) for ventilatory equivalents of carbon dioxide ($\dot{V}E/\dot{V}CO_2$) and oxygen ($\dot{V}E/\dot{V}O_2$). GWVs with GWI had significantly higher mean ventilatory equivalents of oxygen compared to control GWVs. Moderate mean differences (partial η^2 effect size) were observed for both ventilatory equivalent measures between GWVs with GWI and control GWVs.

Ventilatory equivalents of carbon dioxide were selected to be tested in association with DNA methylation because of their clinical utility in both maximal and steady-state exercise protocols. Ventilatory equivalents of carbon dioxide greater than 30 during cardiopulmonary exercise tests are associated with adverse health consequences (*e.g.,* hospitalization for major cardiac event),[277] and the number of participants above this threshold in each group were also quantified (Table 13; Figure 17).

Table 13. Participants with Elevated Ventilatory Equivalents for Carbon Dioxide ($\dot{V}E/\dot{V}CO_2$)

Average $\dot{V}E/\dot{V}CO_2$:	**GWI (N = 23)**	**CON (N = 23)**
> 30	13 (56.5%)	9 (39.1%)
> 34	7 (30.4%)	5 (21.7%)
> 40	1 (4.3%)	0 (0%)

Note. Numbers and percentage of total participants within each group for ventilatory equivalents of carbon dioxide thresholds >30, >34, and >40.

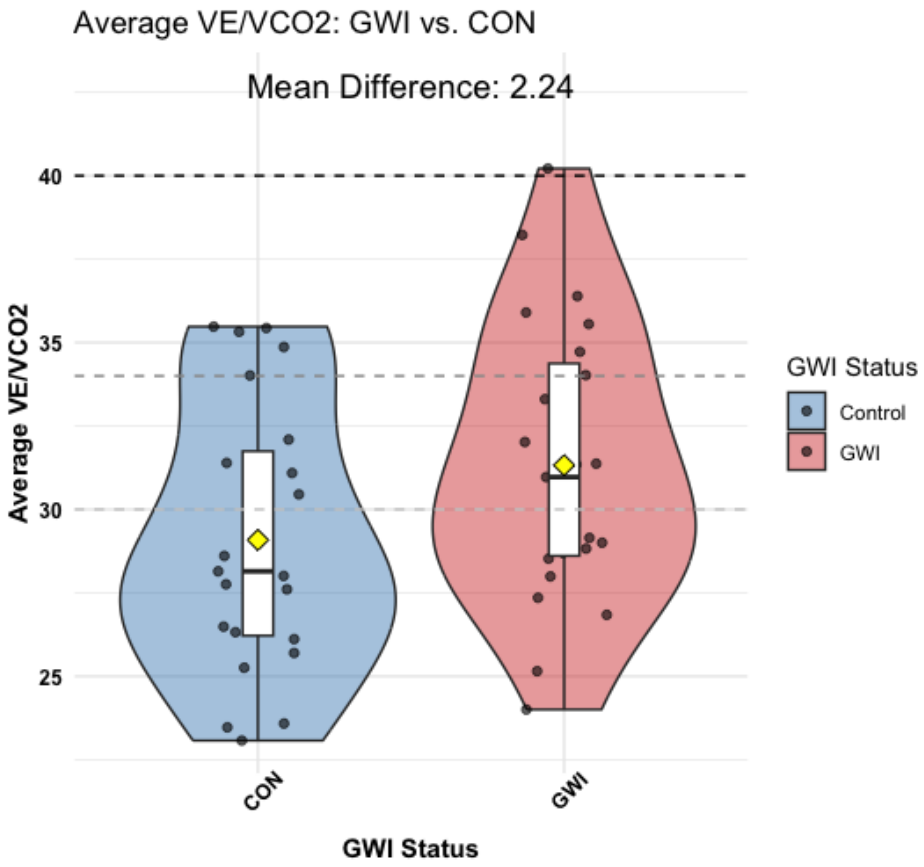


Figure 17. Violin plots of average ventilatory equivalents of carbon dioxide produced during the submaximal exercise challenge for GWVs with GWI (N = 23; red) and control GWVs (N = 23; blue). GWVs with GWI had less efficient ventilation than control GWVs (mean difference = 2.24). Yellow diamonds indicate mean ventilatory equivalents of carbon dioxide for each group. Medians and interquartile ranges are depicted by box plots within each violin plot. Dashed lines indicate ventilatory equivalents of carbon dioxide values > 30 (light gray), > 34 (gray), and > 40 (black).

4.7.1. Sub-Aim 3a:

DNA methylation levels measured pre-, 30-min post-, and 24hr post-exercise were not significantly associated with 1) ventilatory equivalents of carbon dioxide or 2) ventilatory equivalents-by-GWI interactions (*i.e.,* $\dot{V}E/\dot{V}CO_2$ x GWI Status). A relaxed statistical threshold of $p < 0.001$ and B-statistic > 1.5 (*i.e.,* log-odds of 82% probability that the CpG is differentially

methylated for the interaction term) to explore potential relationships between DNA methylation and ventilatory equivalents-by-GWI interactions. Total DMPs identified at each timepoint using $p < 0.001$ and B-statistic > 1.5 are described in Table 14.

TABLE 14. Differentially Methylated Positions Associated with Ventilatory Equivalents of Carbon Dioxide-by-GWI Status

	Pre-Exercise:	**30-min Post-Exercise:**	**24hr Post-Exercise:**
FDR < 0.05	0	0	0
P < 0.001 & B > 1.5	80	21	49

NOTE: Number of DMPs at time timepoint using the original threshold (FDR < 0.05) and exploratory threshold (P-Value < 0.001 & B-Statistic > 1.5). Abbreviations: FDR = false discovery rate; *P* = raw p-value; B = B-statistic (log-odds probability that the CpG is differentially methylated for the tested term).

DMPs observed under this exploratory significance threshold for ventilatory equivalents-by-GWI interactions were distinct at each timepoint (Figure 18), and a long intergenic non-protein coding RNA (*LINC03108*) was the only shared DMP-associated gene in comparisons of 30-min to 24hr post-exercise.

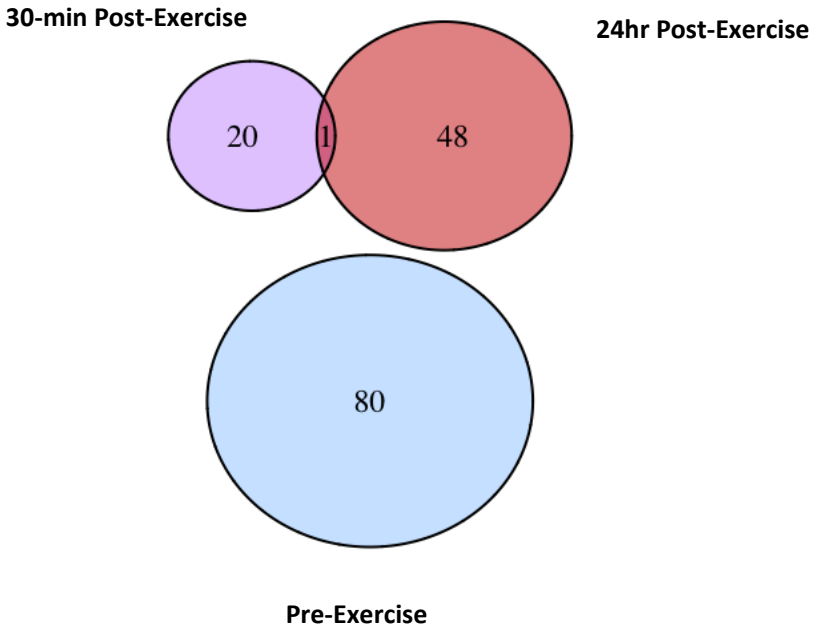


Figure 18. Venn Diagrams of DMP-associated genes ($p < 0.001$ & B-statistic > 1.5) observed pre- (blue), 30-min post- (purple), and 24hr post-exercise (red) for differential DNA methylation analyses of ventilatory equivalents of carbon dioxide in GWVs with GWI. One DMP-associated gene (LINC03108) was shared between 30-min post- and 24hr post-exercise comparisons.

Enriched GO (Supplemental File 1E) and KEGG (Supplemental File 1F) pathways were identified for DMP-associated genes pre- and 30-min post-exercise linked to the primary interaction term. Ventilatory equivalents-by-GWI interactions were associated with pre-exercise DNA methylation levels in genes that participate in vacuolar membrane, lysosomal membrane, and lytic vacuole membrane cellular components (Figure 19A; Supplemental File 1E). Specific examples are genes that encode for the low-density lipoprotein receptor related protein 1 (*LRP1*) and phospholipase D1 (*PLD1*). KEGG pathway analyses did not return significantly enriched terms for pre-exercise DMP-associated genes found with ventilatory equivalents-by-GWI interactions.

Ventilatory equivalents-by-GWI interactions were associated with 30-min post-exercise DMPs in epidermal growth factor receptor (*EGFR*), glutamate metabotropic receptor 4 (*GRM4*), and phosphate cytidylyltransferase 1A choline (*PCYT1A*). These gene are known to participate in calcium/chloride channel activity (GO; Figure 19B), integrin binding (GO, Figure 19B), GnRH signaling pathways (KEGG; Figure 20A-20B), choline metabolism in cancer (KEGG; Figure 20A-20B), parathyroid hormone synthesis (KEGG; Figure 20A-20B), and phospholipase D signaling pathways (KEGG; Figure 20A-20B).

No enriched GO or KEGG pathway terms were returned for DMP-associated genes 24hr post-exercise tested with the primary interaction term, but individual genes of interest included interferon regulatory factor 4 (*IRF4*) and 2 binding protein 2 (*IRF2BP2*).

19A. Gene Ontology (GO) of Pre-Exercise DMP-Associated Genes

19B. Gene Ontology (GO) of 30-min Post-Exercise DMP-Associated Genes

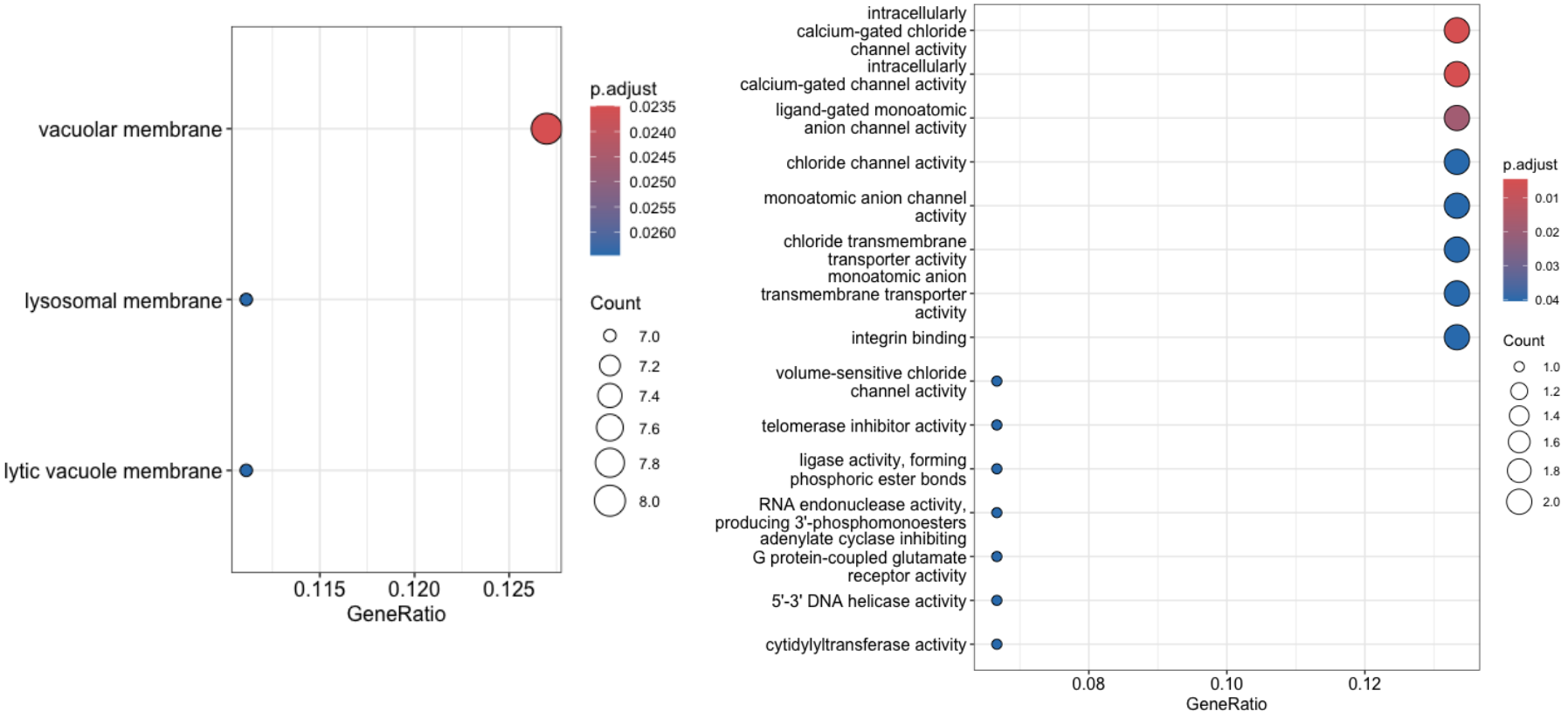
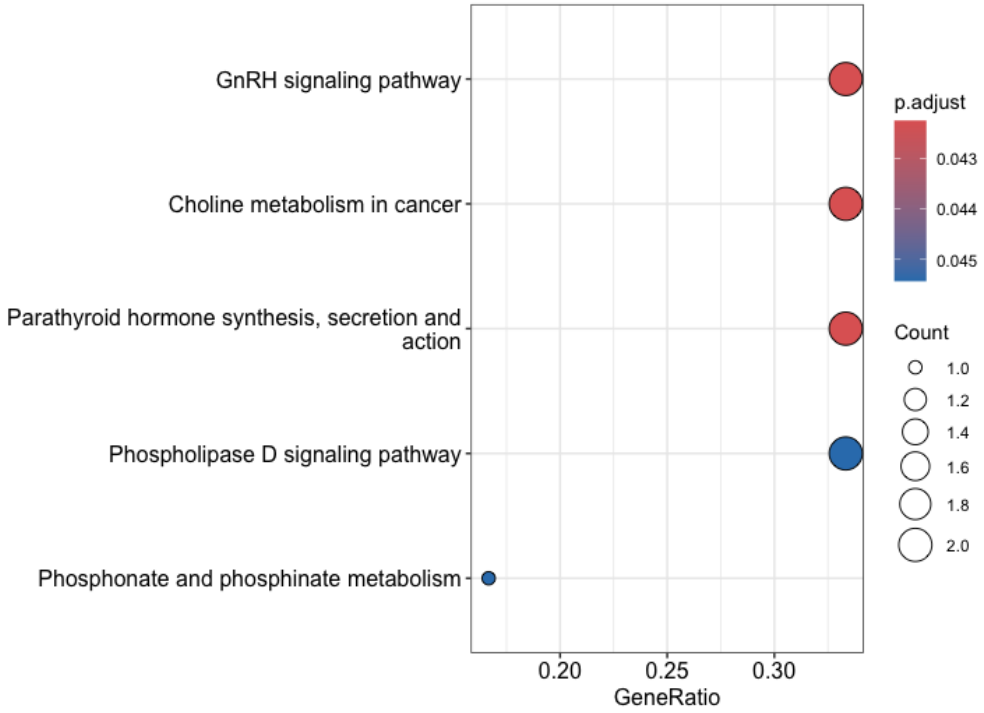


Figure 19A-19B. Gene Ontology (GO) dot plots of enriched pathways using pre- (**19A**) and 30-min post-exercise (**19B**) DMP-associated genes returned for interactions between ventilatory equivalents of carbon dioxide and GWI status. Dots represent enriched GO terms (y-axis) ordered based on gene ratio (x-axis) size. Dot color corresponds to FDR-adjusted p-value (p.adjust) and dot size represents the number of DMP-associated genes present within each respective GO term.

20A.



20B.

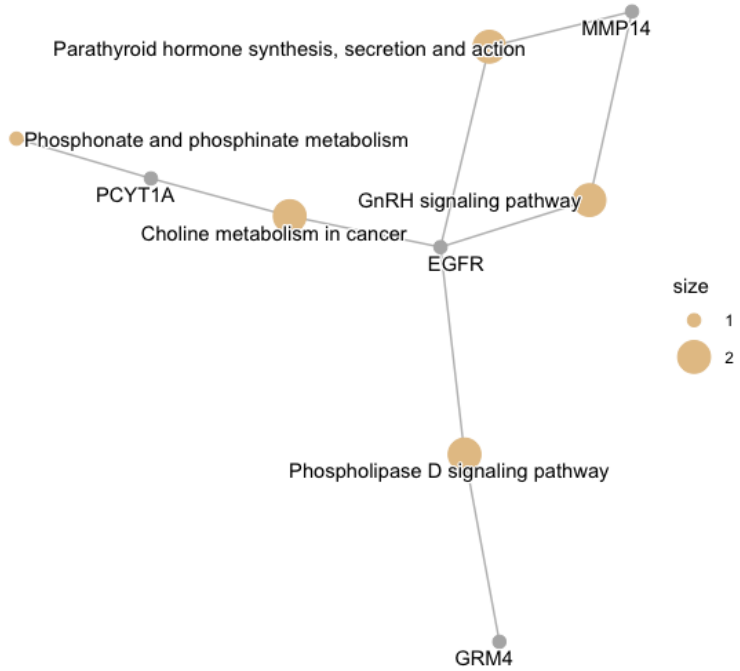


Figure 20A-20B. Kyoto Encyclopedia of Genes & Genomes (KEGG) dot and network plots of enriched pathways using 30-min post-exercise DMP-associated genes returned for interactions between ventilatory equivalents of carbon dioxide and GWI status. Dots represent enriched GO terms (y-axis) ordered based on gene ratio (x-axis) size. Dot color corresponds to FDR-adjusted p-value (p.adjust) and dot size represents the number of DMP-associated genes present within each respective GO term.

4.8. Results: Specific Aim 4

Individual participant responses before and after (*i.e.,* 30 minutes and 24 hours post-exercise) the exercise challenge are graphically depicted with violin plots for the *POMS Fatigue Subscale* (Figure 21A), *POMS TMD* (Figure 21B), and *SFMPQ Total Score* (Figure 21C). Complete linear mixed effects regression model results are described in Supplemental Table S1A (*POMS Fatigue Subscale*), Supplemental Table S1B (*POMS TMD*), and Supplemental Table S1C (*SFMPQ Total Score*).

4.8.1. GWVs with GWI: No PEM Endorsement (PEM-)

Significant main effects of group were observed in GWVs with GWI who did not endorse PEM for the *POMS Fatigue Subscale* (β = 5.89, Standard Error (*SE*) = 1.40, $p < 0.01$), *POMS TMD* (β = 19.88, *SE* = 4.52, $p < 0.01$), and *SFMPQ Total Score* (β = 0.89, *SE* = 0.34, $p < 0.01$) compared to control GWVs. Neither the main effects of Time nor the Group-by-Time interaction were statistically significant across any of the three psychometric symptom measures.

4.8.2. GWVs with GWI: PEM Endorsement (PEM+)

Significant main effects of group were observed in GWVs with GWI endorsed PEM for the *POMS Fatigue Subscale* (β = 7.11, Standard Error (*SE*) = 1.50, $p < 0.01$), *POMS TMD* (β = 26.93, *SE* = 4.86, $p < 0.01$), and *SFMPQ Total Score* (β = 1.00, *SE* = 0.37, $p < 0.01$) compared to control GWVs. Significant group-by-time effects contradicted the primary hypothesis and indicated that GWVs with GWI who endorsed PEM reported significant decreases in *POMS*

Fatigue Subscale (β = -2.66, *SE* = 1.33, $p < 0.05$) and *POMS TMD* (β = -7.84, *SE* = 3.34, $p < 0.05$) scores from pre- to 30-minutes post-exercise compared to control GWVs.

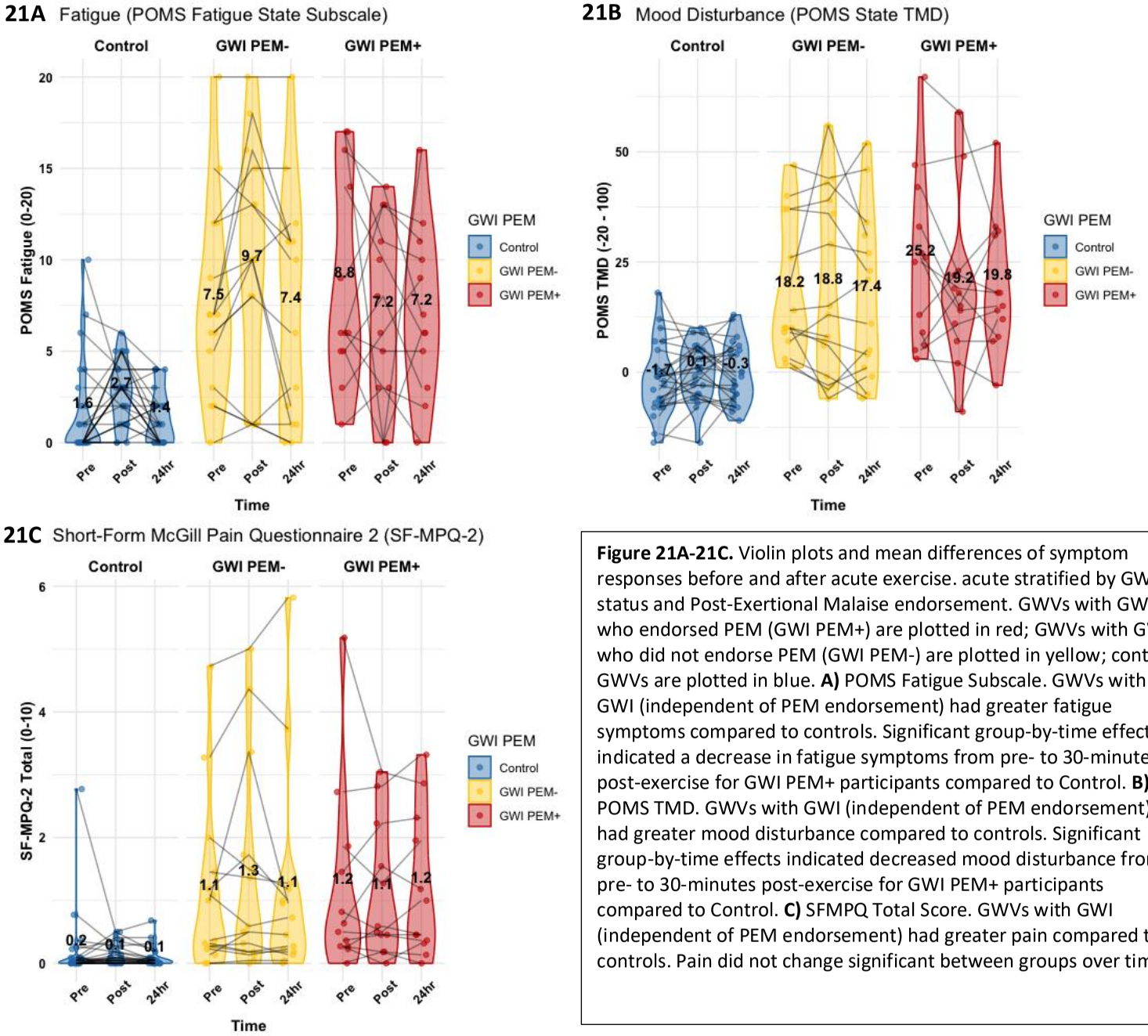


Figure 21A-21C. Violin plots and mean differences of symptom responses before and after acute exercise. acute stratified by GWI status and Post-Exertional Malaise endorsement. GWVs with GWI who endorsed PEM (GWI PEM+) are plotted in red; GWVs with GWI who did not endorse PEM (GWI PEM-) are plotted in yellow; control GWVs are plotted in blue. **A)** POMS Fatigue Subscale. GWVs with GWI (independent of PEM endorsement) had greater fatigue symptoms compared to controls. Significant group-by-time effects indicated a decrease in fatigue symptoms from pre- to 30-minutes post-exercise for GWI PEM+ participants compared to Control. **B)** POMS TMD. GWVs with GWI (independent of PEM endorsement) had greater mood disturbance compared to controls. Significant group-by-time effects indicated decreased mood disturbance from pre- to 30-minutes post-exercise for GWI PEM+ participants compared to Control. **C)** SFMPQ Total Score. GWVs with GWI (independent of PEM endorsement) had greater pain compared to controls. Pain did not change significant between groups over time.

4.8.3. Sub-Aim 4a: Relationships Between Symptoms & DNA Methylation in Response to Exercise

To best understand the relationship between blood-based DNA methylation levels and GWI symptoms, LASSO regression and subsequent mixed effects models were limited to only the GWVs with GWI. Using the 32 GWI-associated DMPs identified in response to exercise (Specific Aim 1 & Specific Aim 2), significant predictors were returned for both fatigue (30 min Post-Exercise: 12 DMPs) and mood disturbance (30 min Post-Exercise: 15 DMPs & 24hr Post-Exercise: 3 DMPs). No significant associations between DMPs and pain symptoms in GWVs with GWI were observed. Prior to analysis, values were scaled by subtracting the variable mean from each response and dividing by the standard deviation. LASSO regression penalty parameters for each symptom over time were selected using 20-fold cross-validation (Table 15).

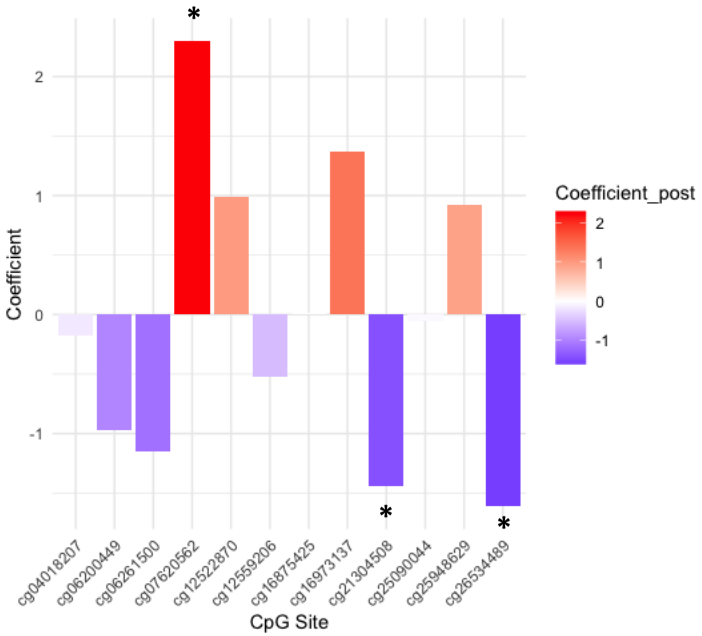
Table 15. *LASSO Regression Penalty Parameter (λ)*

	Pre-Exercise:	**30min Post-Exercise:**	**24hr Post-Exercise:**
POMS Fatigue	2.44	0.42	2.54
POMS TMD	8.63	0.95	4.45

To maintain a predictor-observation ratio of 1:10, the top three DMPs with the largest coefficients were selected as candidate predictors for mixed effects models of fatigue and mood disturbance symptoms. The top three DMPs demonstrated the strongest relationships 30 minutes post-exercise and were identical for fatigue (Figure 22A) and mood disturbance

symptoms (Figure 22B). All returned CpG coefficients for fatigue and mood disturbance symptoms are described in Supplemental File 1D.

22A) Fatigue LASSO Coefficients for Selected CpGs



22B) TMD LASSO Coefficients for Selected CpGs

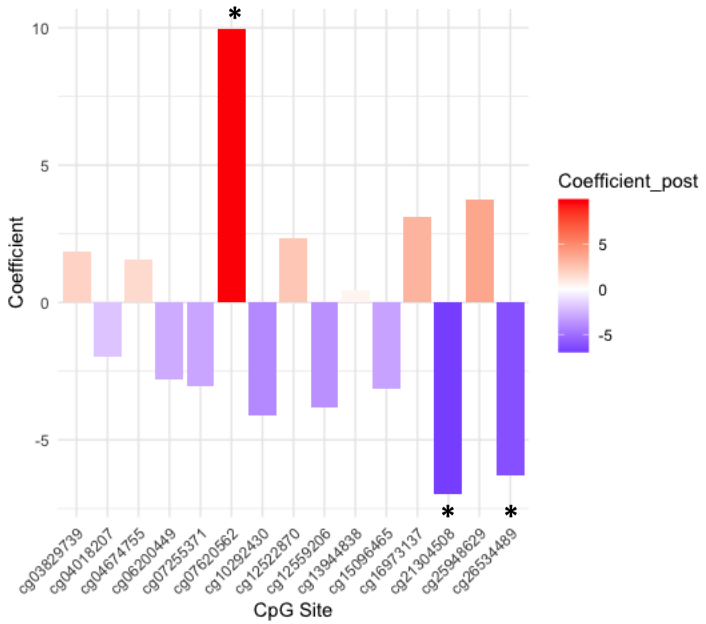


Figure 22A-22B. LASSO regression coefficients of differentially methylated CpG sites (*i.e.,* DMPs) selected as predictors for fatigue and mood disturbance symptoms. Positive LASSO coefficients were interpreted as associations between higher levels of DNA methylation and higher (*i.e.,* more severe) symptom scores. Negative LASSO coefficients were interpreted as associations between lower levels of DNA methylation and higher (*i.e.,* more severe) symptom scores. Identical CpG sites (cg07620562, cg21304508, cg26534489) were returned for both **22A)** fatigue symptoms and **22B)** mood disturbance symptoms. * Signifies each of the three selected CpG sites.

The top three DMPs selected as predictors for mixed effects regression models of fatigue and mood disturbance were each located in a protein-coding gene: solute carrier family 12 member 8 (*SLC12A8;* cg07620562), muscleblind-like splicing regulator 2 (*MBNL2*; cg21304508), and guanine nucleotide-binding protein alpha stimulating (*GNAS*; cg26534489). Compared to pre-exercise, higher levels of DNA methylation in cg07620562 (*SLC12A8*) were significantly associated with more severe fatigue symptoms (β = 0.28, Standard Error (SE) = 0.14, $p < 0.05$) 30 minutes post-exercise (Figure 23A). More severe mood disturbance was significantly associated with lower levels of DNA methylation in *MBNL2* (cg21304508; β = -0.28, Standard Error (SE) = 0.14, $p < 0.05$) and *GNAS* (cg26534489; β = -0.41, Standard Error (SE) = 0. 13, $p < 0.01$) at 30 minutes post-exercise (Figure 23B).

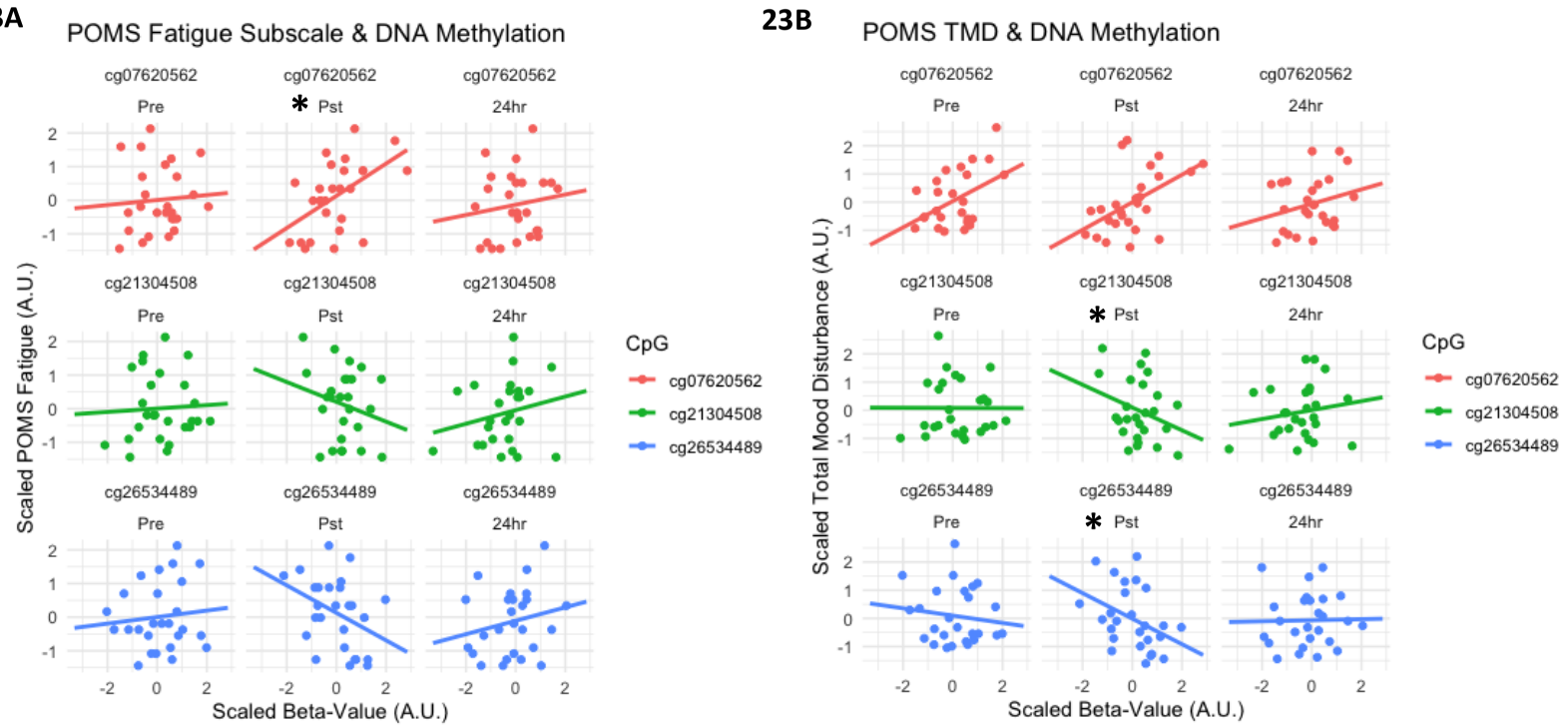


Figure 23A-23B. Correlations between DNA methylation in three CpG sites selected by LASSO regression (cg07620562, cg21304508, cg26534489) and symptoms before and after (*i.e.,* 30 minutes post- and 24hr post-exercise) an acute exercise challenge. **23A)** Significant associations between fatigue symptoms and DNA methylation levels in cg07620562 were identified 30 minutes post-exercise. **23B)** Significant associations between mood disturbance and DNA methylation levels in cg21304508 and cg26534489 were identified 30 minutes post-exercise. * = significant relationship between symptom DNA methylation level at specified CpG site.

4.8.4. Sub-Aim 4a: Exploratory Group Classification

Blood-based DNA methylation levels within the *OGDH* of GWVs with GWI were stable over time (intra-class correlation [ICC] = 0.97, $p < 0.001$, 95% confidence interval [CI] = 0.94, 0.99), despite completing a vigorous aerobic exercise challenge. *OGDH* stability and replicated differences between GWVs with GWI and control GWVs supported its potential use for group discrimination, and classification accuracy of pre-exercise *OGDH* DNA methylation levels was tested using an exploratory binomial logistic regression model. Cases and controls were converted to a binary outcome (GWI = 1, Control = 0). Data were partitioned by a 70:30 train-test split, and the binomial logistic regression model was fit to the training set with a cut threshold of 0.50 (Figure 24A). Cases and controls were correctly distinguished at an accuracy rate of ~81% (Accuracy = 0.81, Sensitivity = 0.84, Specificity = 0.77), and overall model performance evaluated using an area under the receiver operating curve (ROC) was fair-to-good (AUC = 0.796; Figure 24B).

Figure 24A) Logistic Regression Classification

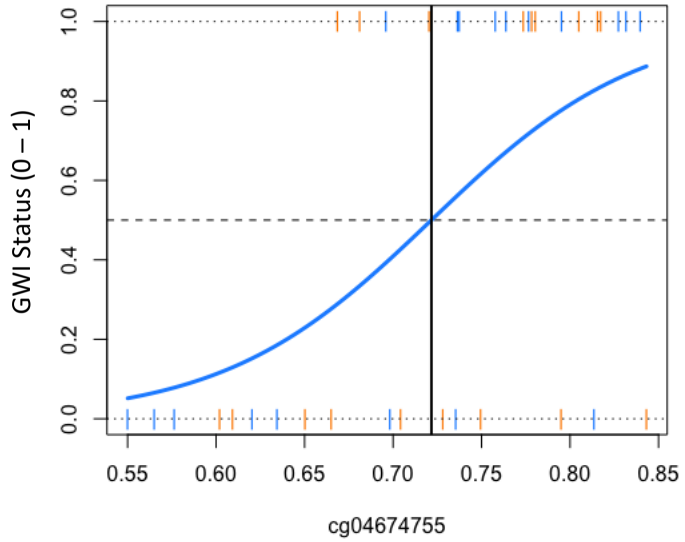


Figure 24B) Receiver Operating Curve (ROC)

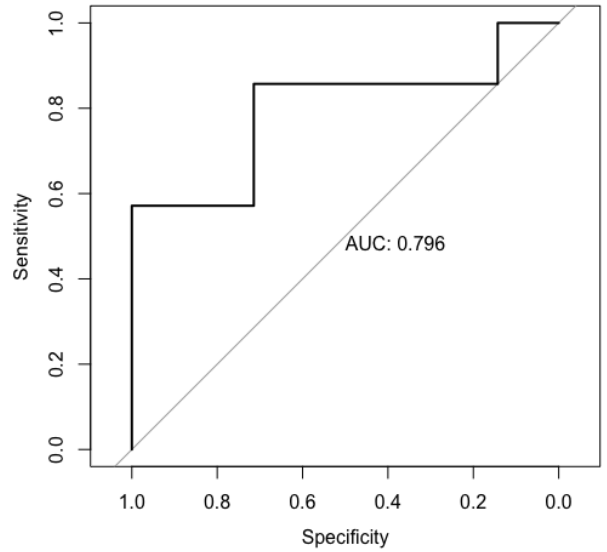


Figure 24A-24B. GWI classification accuracy of DNA methylation levels at a singular CpG site within the 2-oxoglutarate dehydrogenase (*OGDH*) gene. **24A)** Logistic regression classification accuracy of GWI cases. DNA methylation levels (x-axis) of a DMP (cg04674755) located in the *OGDH* gene were fit to a binary operator (y-axis) for GWI (1) or Control (0) using a logistic regression model. The blue curved line represents predictive probabilities for case status based on DNA methylation levels in the DMP in the *OGDH* gene. The vertical black line represents a decision boundary for a predictive probability of 0.5. **24B)** Receiver operating characteristic (ROC) curve was used to evaluate model sensitivity (y-axis) and specificity (x-axis). Higher values indicate better classification capacity, and an AUC = 0.796 for the present model is considered good (0.80 < AUC < 0.90) to fair (< 0.70 AUC < 0.80).

CHAPTER 5: DISCUSSION

This dissertation's primary purpose was to compare differences in DNA methylation following an acute aerobic exercise challenge in GWVs with GWI (N = 27) and control GWVs (N = 25). DNA methylation levels differed between GWVs with GWI and control GWVs at baseline (Specific Aim 1) and in response to an acute aerobic exercise challenge (Specific Aim 2). Male-only subgroup analyses were performed to better account for biological sex differences, and subtle DNA methylation level changes associated with GWI were detected within (*i.e.,* Time Effect) and between groups (*i.e.,* Group-by-Time Effect) from 30-min post- to 24hr post-exercise.

GWVs with GWI had less efficient ventilation during the acute aerobic exercise challenge (Specific Aim 3), and although not statistically significant, these differences were of moderate magnitude. DNA methylation levels across each timepoint were not significantly associated with ventilatory equivalents of carbon dioxide in GWVs with GWI—the primary interaction term—at an FDR < 0.05 significance threshold selected a-priori. DMP-associated genes identified under an exploratory significance threshold have functional roles in vacuolar/lysosomal cellular processes (*i.e.,* pre-exercise), ion channel activity (*i.e.,* 30-min post-exercise), and metabolic signaling (*i.e.,* 30-min post-exercise) pathways. GWVs with GWI had greater overall fatigue, pain, and mood disturbance than control GWVs (Specific Aim 4), but contrary to the primary hypothesis, GWVs with GWI who also endorsed previously experiencing PEM reported less-severe fatigue and mood disturbance 30-min post-exercise. Despite these reductions in fatigue mood disturbance 30-min post-exercise, symptom levels between GWVs with GWI who did and did not endorse PEM were similar across time. DNA methylation levels

within three select CpG-sites known to respond to physiological stress were significantly associated with fatigue and mood disturbance reported 30-min post-exercise by GWVs with GWI. Classification accuracy was tested for a DMP within the *OGDH* gene that had stable DNA methylation levels over time, and its fair-to-good performance supports potential future application as a GWI-associated biomarker.

5.1. Discussion: Differences in DNA Methylation Between GWVs with GWI and Control GWVs at Baseline (Specific Aim 1)

Differences in blood-based DNA methylation between GWVs with GWI and control GWVs were identified at baseline and support the primary hypothesis. Several of the five DMP-associated genes identified in pre-exercise comparisons of GWVs with GWI and control GWVs (*i.e.,* 2-oxoglutarate dehydrogenase [*OGDH*], RAN binding protein 17 [*RANBP17*], platelet-derived growth factor receptor alpha [*PDGFRA*], regulator of G-protein signaling 10 [*RGS10*], solute carrier family 12 member 8 [*SLC12A8*]) are known to participate in GWI-relevant biological processes. For example, *OGDH* encodes a protein subunit localized to the mitochondrial matrix and acts as a catalyst for the conversion of alpha-ketoglutarate to succinyl-CoA and carbon dioxide.[278] Exercise-training studies demonstrate that increased *OGDH* activity coincides with improved fitness,[279] while preliminary clinical evidence suggests that *OGDH* deficiency leads to metabolic acidosis[280] and hyper-lactic acidemia.[281] Phenotypic consequences of *OGDH* deficiency include neurological symptoms and hypotonia.[282] Decreased *OGDH* gene activity (*i.e.,* via hypermethylation leading to reduced transcription/expression) is

consistent with documented GWI characteristics that include altered metabolic activity[283] and impaired neurological symptoms/functions.[284]

RGS10 encodes a protein that participates in G-coupled protein signal transduction[285] and may function as a suppressor of microglial-mediated neuroinflammation.[286] *RGS10* inhibition of cyclooxygenase-2 is known to suppresses systemic inflammatory signaling (*e.g.,* TNF-α) associated with microglial activation.[287] Evidence also supports neuroinflammation as a pathophysiological source in GWVs with GWI,[113] and promoter hypermethylation that represses *RGS10* expression may mechanistically contribute to microglial-mediated neuroinflammation.

RANBP17 is a nuclear transport receptor,[288] and disruptions to its expression are associated with the movement disorder DYT1 dystonia.[289] In-vitro RANBP17 overexpression in motor neurons from patients with DYT1 improved nuclear transport and DYT1 neuronal function.[289] Neurological and cognitive symptoms are a core features of GWI,[2,284] and *RANBP17* promoter hypomethylation in peripheral blood may reflect systemic compensatory responses to increased central *RANBP17* expression.

GWVs with GWI in the present study reported several neurological symptoms (e.g., Kansas Screening Questionnaire), worse overall mental function (*e.g.,* VR-36 MCS), perceived cognitive deficits (*e.g.,* PDQ), and greater mood disturbance (*e.g.,* POMS TMD). Direct measures of neuroinflammation (*e.g.,* positron emission tomography imaging) were beyond the scope of the present dissertation, but peripheral immune cells with differential DNA methylation levels in genes that encode known drivers of neuroinflammation and disrupted neurological function are consistent with baseline symptoms in GWVs with GWI. A multi-omics approach testing

whether differential DNA methylation affects expression of these genes is currently underway, and future investigations assessing both peripheral and neuroinflammation may be warranted.

5.2. Specific Aim 2: Differences in DNA Methylation Between GWVs with GWI and Control GWVs in Response to an Exercise Challenge

Differences in blood-based DNA methylation between GWVs with GWI and control GWVs were observed both 30 minutes and 24 hours after completing the aerobic exercise challenge. Changes in DNA methylation between groups over time (*i.e.,* group-by-time interaction effect) were the main effect of interest and support the Specific Aim 2 primary hypothesis of finding differential DNA methylation responses to exercise.

5.2.1. Post-Exercise Between & Within Group Differences:

Compared to pre-exercise, three unique DMP-associated genes known to participate in metabolism were identified 30-minutes post-exercise between GWVs with GWI and control GWVs: *CBL*, *IGFBP3,* and *GFPT2*. *CBL* is involved in proteasome degradation through E3 ubiquitin ligase,[290] and it participates in insulin sensitivity through GLUT4 receptor translocation.[291] Increased *CBL* expression was associated with both increased fitness and glucose utilization in participants with gestational diabetes mellitus who completed a 6-month aerobic exercise intervention.[292] *IGFBP3* encodes a versatile protein that contains both an IGF and thyroglobulin type-I protein binding domain,[293] and IGFBP3-IGF1 interactions have been shown to mediate intracellular cascades that lead to apoptosis.[294] Higher circulating IGFBP-3 protein levels are associated with worse health outcomes in diabetic patients,[295] and in-vivo

Alzheimer's Disease models have demonstrated that interactions between circulating IGFBP3 and the mitochondrial peptide Humanin contribute to systemic insulin resistance.[296] *GFPT2* encodes a hexosamine biosynthetic pathway rate-limiting enzyme that convert glutamine and fructose-6-phosphate into glucosamine-6-phosphate during glycolysis,[297] and *GFPT2* sequence variation in has been associated with Type 2 Diabetes Mellitus susceptibility.[298] Cumulatively, these metabolic DMP-associated genes (*e.g., CBL, IGFBP3, GFPT2*) were observed only after stimulation via the exercise challenge, and they provide evidence for potential metabolic disruption in GWVs with GWI.

GWVs with GWI have higher comorbidity rates of chronic metabolic syndromes and their precursors (*e.g.,* pre-diabetes or Type II diabetes) compared have to control GWVs,[299] but parameters used to determine criteria for these metabolic syndromes (*e.g.,* oral glucose tolerance tests[300]) were beyond the scope of the present study. Conceptually, vigorous exercise may have provided necessary metabolic stimulation to observe these differences that would have been otherwise undetected at rest. Acute aerobic exercise has been shown to elicit differential DNA methylation in genes associated with metabolic function in healthy participants,[200] but the parallel control group in the present study allows the conclusion that observed metabolic responses in GWVs with GWI were distinct from otherwise healthy control GWVs. Previous chemical exposure has been associated with impaired mitochondrial integrity in GWVs with GWI,[115,301] and DMP-associated genes identified in the present study (*e.g., CBL, IGFBP3, GFPT2*) are plausible indicators of stress sensitivity in response to exercise. Integrating these findings with other peripheral measures of metabolic function (*i.e.,* mitochondrial

function, targeted metabolomics, etc.) are necessary to better understand their relevance to GWI pathophysiology.

Of the two DMPs between GWVs with GWI and control GWVs that persisted at 24 hours post-exercise, only one DMP was novel and annotated to the protein coding gene *GNAS*. *GNAS* encodes one of three protein subunits (*i.e.,* alpha subunit) involved in adenylate cyclase, an enzyme with multifaceted functions that include hormone production and regulation of glucose/triglycerides.[302] Allelic variation within exon 5 of *GNAS* has been associated with β-blockade activity, and an investigation of men with hypertension reported that carriers of the *GNAS* T allele had exacerbated antihypertensive responses to light-intensity acute exercise.[303] Genetic sequencing to obtain allele type was beyond the scope of the present study, but differential DNA methylation within the GNAS gene 24 hours after the vigorous exercise challenge supports possible cardiovascular distress in GWVs with GWI. Beyond clinical parameters for cardiovascular and pulmonary disease, physical fitness and hypertension are important considerations for future investigations.

5.2.2. Changes in DNA Methylation Between Groups Over Time (*i.e.,* Group-by-Time)

Significant changes in DNA methylation were only detected from 30 minutes post- to 24hr post-exercise between GWVs with GWI compared to control GWVs. Fourteen DMPs were located within 13 unique protein-coding genes with differential effect sizes ranging from 1% - 6%. Evidence supports that small differences in DNA methylation (< 10%) can have significant biological implications,[189,304] and relative effect sizes observed in the present study are comparable to findings in other forms of chronic multi-symptom illness (*e.g.,* ME/CFS)[186] and

complex psychopathology.[189,305] Several DMP-associated genes observed in the group-by-time effect participate in common pathways with potential relevance to GWI pathophysiology. These include: 1) response to interferon-β (*e.g., CAPN2*, *UBE2G2*) and 2) cadherin binding (*e.g., ABL1, CDH18, SND1*).

Interferon-beta (IFN-β), an inflammatory cytokine that induces intracellular pathogenic responses (*e.g.,* Type I immune responses),[306] is a precursor to several additional cytokines elevated in GWVs with GWI (*e.g.,* IL-1β, IL-6,TNF-α).[99,101,307] Our recent work in GWVs with GWI has also demonstrated that acute moderate-to-vigorous exercise challenges upregulate immunoregulatory factors (*e.g.,* IL-6) involved in Type I immune responses.[148] *CAPN2* and *UBE2G2* are two notable DMP-associated genes in GWVs with GWI related to several IFN-β initiated processes that include innate immune cell response coordination (*i.e., UBE2G2*),[308] T-lymphocyte migration/adhesion (*i.e., CAPN2*),[309,310] and calcium-dependent apoptosis during systemic hyperthermia (*i.e., CAPN2*).[311–313] Cadherin binding (*i.e.,* cell-cell adhesion) is another critical component of Type I immune responses activated by IFN-β.[314] Increased vascular permeability during Type I inflammatory responses is accompanied by lymphocyte migration into peripheral tissues,[306,315] and mechanistically, proteins involved in cadherin binding (*i.e.,* ABI1[316] & CDH18[317]) promote cell-cell adhesion to facilitate lymphocyte infiltration. It remains unclear if altered immune activation by these DMP-associated genes directly or indirectly contribute to disease pathology, but these elevated inflammatory responses observed up to 24hr post-exercise in GWVs with GWI may provoke symptoms through higher susceptibility to adverse physical stress or signaling from the periphery that stimulates neural inflammation.[9,318,319]

5.3. Specific Aim 3: Ventilatory equivalents measured during submaximal exercise in GWVs with and without GWI

Although not statistically significant, group differences in ventilatory equivalents of carbon dioxide and oxygen are evidence of less efficient ventilation in GWVs with GWI and partially support the primary hypothesis. As described previously (Section 4.2.), missing cardiopulmonary responses during the exercise challenge reduced group sample sizes to N = 23 for both GWVs with GWI and control GWVs, but we have previously reported reduced ventilatory efficiency in this GWI cohort.[145,249] These modest reductions in sample size may partially explain the lack of statistical significance, and higher ventilatory equivalent values (*i.e.,* less efficient ventilation) quantified by moderate effect sizes (partial η^2) in the present study are consistent with previous responses observed in GWVs with GWI.[145,148,249]

5.3.1. Sub-Aim 3a: Associations between ventilatory equivalents measured during submaximal exercise with blood-based DNA methylation levels

Ventilatory equivalents of carbon dioxide measured during maximal exercise testing protocols have prognostic utility in chronic conditions like congestive heart failure[320] and COPD,[321] and values > 30 may confer greater cardiopulmonary disease risk.[322,323] Submaximal steady state exercise protocols limit the clinical utility of most cardiopulmonary responses to exercise; however, consistency between ventilatory equivalents of carbon dioxide and end-tidal PCO_2 during both steady state and non-steady state aerobic exercise[269] support its use as a viable measure of ventilatory efficiency and exercise tolerance in the present study.

Exacerbated respiratory symptoms (*e.g.,* shortness of breath) after aerobic exercise have been reported by GWVs with GWI,[145] and post-exercise symptom exacerbation of both respiratory and non-respiratory symptoms (*i.e.,* PEM) have small-to-moderate correlations with ventilatory equivalents in GWVs with GWI.[249] However, previous research has not found exercise parameters, including ventilatory equivalents, to be predictive of symptoms responses to acute exercise challenge.[249] To date, cardiopulmonary responses to exercise have not been predictive of PEM.

Acute and chronic aerobic exercise are known to elicit differential DNA methylation levels (Section 2.5.3.), but beyond work by Ferrari et al. (2019),[197] few studies have directly tested relationship between DNA methylation and cardiopulmonary responses to exercise. Ferrari et al. (2019)—described in Section 2.5.3.2.—reported that differential blood-based DNA methylation levels (*e.g., EDN1, NOS2, TNF*) were significantly associated with VO_{2peak} after 12-weeks of aerobic exercise training in patients with CVD. Ventilatory responses during acute cardiopulmonary exercise may be useful in understanding GWI pathophysiology beyond PEM,[268] and genes containing differential DNA methylation that are associated with these ventilatory responses should be prioritized in future mechanistic assessments of this relationship.

Blood-based DNA methylation levels measured pre- (Model 1), 30-minutes post- (Model 2), and 24hr post-exercise (Model 3) were not significantly associated with ventilatory equivalent-by-GWI interactions under the a-priori significance threshold (FDR < 0.05). Meta-analyses of several diverse cohort studies have identified significant relationships between blood-based DNA methylation and pulmonary function (e.g., FEV_1),[324] but relationships

between blood-based DNA methylation and ventilatory equivalents are not well characterized. Mean ventilatory equivalent group differences were of moderate magnitude, but relatively few participants from each group had ventilatory equivalents > 34 (GWVs with GWI: 7/23 (30.4%); control GWVs: 5/23 (21.7%). Greater representation of clinically significant ventilatory efficiency responses may be required to discern meaningful relationships with blood-based DNA methylation, and this may be a future research avenue in investigations of pulmonary function. Alternatively, it is also plausible that other measures of pulmonary function (*e.g.,* FEV_1,)[325] or cardiorespiratory fitness (*e.g.,* VO_{2max})[197] are better equipped for utilization with blood-based DNA methylation.

An exploratory significance threshold ($p < 0.001$ & B-statistic > 1.5) was used to investigate candidate DMPs associated with ventilatory equivalent-by-GWI interactions and warrant cautious interpretation. Pre-exercise ventilatory equivalent-by-GWI interactions returned DMP-associated genes were enriched for vacuolar/lysosomal cellular components pathways. These pathways and their individual constituents (*e.g., LRP1* & *PLD1*) potentially signify lipid-mediated metabolic stress. Disrupted vacuolar and lysosomal storage and degradation of macromolecules (*e.g.,* lipids) are known to initiate immunostimulatory reactive oxygen species production[326,327] and cellular apoptosis.[328] For example, *LRP1* encodes an endocytic receptor involved in lipid and apoptotic cell clearance,[329] and *LRP1* DNA methylation has been positively associated with high-density lipoprotein levels in patients with metabolic syndromes.[330] *LRP1* polymorphisms have also been associated with Alzheimer's Disease risk,[331] and proposed mechanisms for increased risk suggest impaired *LRP1* activity contributes to lipid-mediated neuroinflammation.[332,333] Likewise, *PLD1* encodes a targeted phospholipase enzyme

that is responsive to metabolic stress[334] (*e.g.,* nutrient deprivation) and mitochondrial-produced reactive oxygen species.[335]

Similarly, lipid-mediated metabolic stress and inflammation were also indicated 30-min post-exercise based on enriched KEGG terms like phospholipase D signaling and underlying DMP-associated genes that include *GRM4* and *EGFR*. *GRM4*, a group III metabotropic glutamate receptor,[336] has complex functions when expressed on non-neuronal cell-types that include LPS-induced microglial activation.[337] Phospholipase D activity exerts downstream regulator effects on EGFR,[338] a cell-surface glycoprotein that binds to epidermal growth factors.[339] *EGFR* mutations are well-documented in non-small cell lung cancers,[340,341] and more recent evidence supports that EGFR signaling is associated pulmonary mucus production and fibrosis in patients severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) following COVID-19 infection.[342] Interactions between EGFR signaling and type I immune responses (*e.g.,* IL-6 & TNFα) are potential contributors to respiratory pathology in these conditions.[342–345] Chronic consequences following severe COVID-19 infections (*i.e.,* Long COVID) are distinct from GWI, but based on reported similarities in symptoms and pathophysiology between these conditions,[346] EGFR-mediated inflammation may also be considered in future GWI studies. In addition to DMP-associated genes in case-control comparisons (Specific Aim 2), two DMP-associated genes returned in 24hr post-exercise ventilatory equivalent-by-GWI interactions that participate in Type I interferon responses (*i.e.,* interferon regulatory factor 4 [*IRF4*][347] & interferon regulatory factor 2 binding protein 2 [*IRF2BP2*][348]) provide evidence of acute inflammation related to EGFR signaling following the exercise challenge. Less efficient ventilation observed in GWVs with GWI

may be partially mediated through EGFR activity and its downstream consequences (*e.g.,* inflammation and metabolic distress), but this claim warrants further investigation.

Independent of the measurement timepoint, commonalities among DMP-associated genes identified in ventilatory equivalent-by-GWI interactions include functions related to lipid metabolism and inflammation. Notably, dysregulation within each of these systems is well-supported in GWI.[9,101,349] Whether dysregulation within these pathways function as primary or secondary pathologies remains unknown, but increased risk of chronic comorbid cardiovascular (*e.g.,* heart attack), pulmonary (*e.g.,* chronic bronchitis), and metabolic (*e.g.,* diabetes) conditions are also concerns in GWI disease management.[350] For example, chronic inflammation and lipid toxicity are hallmark characteristics of CVD,[351,352] and as noted earlier, patients with CVD exhibit less efficient ventilation.[277,353] Further validation to affirm the accuracy and strength of relationships between DNA methylation and cardiopulmonary responses, however, is a necessary first step.

5.4. Specific Aim 4: Symptom Changes in Response to an Acute Aerobic Exercise Challenge

No significant increases in symptom severity from pre- to post-exercise (*i.e.,* 30 minutes 24 hours post-exercise) were observed in GWVs with GWI compared to control GWVs. Contrary to the primary hypothesis, GWVs with GWI who endorsed PEM at screening reported significant decreases in fatigue and mood disturbance symptoms from pre- to 30-min post-exercise compared to control GWVs. The lack of symptom worsening in GWVs with GWI has several plausible explanations.

How to best defined PEM is an ongoing and vigorous debate, and the primary challenges preventing a standardized definition include heterogeneity among individual PEM response initiation, length, severity, and symptom specificity.[128,129] PEM onset may occur within minutes[7,139] or be delayed by several days[249,354] and persist for days following an initiating event.[133,143] Symptoms in the present study were quantified within 24hr after the acute exercise challenge; it is possible that PEM occurred beyond this time. Evidence of delayed PEM responses in GWVs with GWI have been described in our previous work and show that the greatest symptom changes are often observed between 48-72hr post-exercise.[249]

Group-level fatigue, pain, and mood disturbance symptoms in GWVs with GWI who do and do not endorse PEM were comparable to our group's previous work within this GWV cohort.[145] Decreased mood disturbance following exercise has been previously reported in GWI,[145] and these findings contradict post-exercise responses of participants with ME/CFS.[7,355] As such, mood disturbance may be less germane to PEM responses in GWVs with GWI. Additionally, similarities between fatigue and mood disturbance observed in the present study (*i.e.,* decreases at 30-min post-exercise in GWVs with GWI who endorse PEM) can likely be attributed to these measures being derived from the same psychometric questionnaire (*i.e.,* POMS short form). GWVs with GWI have reported significant pain symptom exacerbations in our group's previous efforts to characterize PEM responses.[143,145] Pain symptoms measured in the present study were significantly greater in GWVs with GWI but relatively stable at the group-level in response to exercise. Investigating pain-specific subdomains (*e.g.,* muscle pain, joint pain, neuropathic pain, etc.) may clarify whether pain-related PEM responses are more pertinent to GWVs with GWI.

Beyond steps to account for time course and individual response variation, it is also plausible that PEM is not a core component for all GWVs with GWI. Acute symptom exacerbations are evident in other CMI populations,[356,357] but findings from our group's recent randomized controlled crossover experiment investigating three different acute aerobic exercise intensities found that the negative consequences of exercise were comparable to seated-rest.[148] This study acknowledged that some participants may exhibit post-exercise symptom exacerbation, but the low frequency of observed PEM responses in this context may explain their absence in the present study. It is also possible that PEM occurred outside of the 24-hour window of measurement used in the present study. Ongoing work by our group to build a more comprehensive construct of GWI symptom burden that considers existing symptoms, symptom time course, and potential exacerbation due to PEM is a necessary step toward improving PEM measurement and eventually illness management strategies and treatment approaches. Examples may include optimizing exercise prescription precision to mitigate PEM risk, while also reducing the risk of chronic illness commonly associated with sedentary behavior.[148,358]

5.4.1. Sub-Aim 4a: Symptom Changes in Response to Exercise and Association with DNA methylation levels GWVs with GWI

Penalized LASSO regression was used to test which DMPs had the strongest associations with symptoms in GWVs with GWI, and as discussed, this feature selection method has been used broadly in both genomic[359] and epigenomic[276,360] studies of complex phenotypic traits. The present study observed significant associations between several DMPs with both fatigue and

mood-disturbance symptoms in GWVs with GWI. The top three DMPs and associated genes (*i.e.,* cg07620562 [*SLC12A8*], cg21304508 [*MBNL2*], cg26534489 [*GNAS*]) with the largest LASSO coefficients were identical for fatigue and mood-disturbance symptoms, although this can likely be attributed to their codependence within the POMS-BF (*i.e.,* higher fatigue increases overall TMD score). Notably, altered activity within these three DMP-associated genes are related to multiple aspects of physiological stress that include ROS neutralization (*i.e., SLC12A8*[361,362]), response to hypoxic microenvironment (*i.e., MBNL2*[363,364]), and pseudo-hormonal resistance (*i.e., GNAS*[302,365]). Genes that participate in metabolic functions aligns with previous reports that mitochondrial impairment may act as a primary driver of GWI symptom severity.[114] Specific mechanistic contributions to fatigue and mood disturbance by each DMP-associated gene are unclear but may represent heighted cumulative physiological stress following acute exercise.

5.4.2. Exploratory Analysis: DNA Methylation Classification Accuracy in GWVs with GWI

Feature selection based on biological relevance of the gene or pathway containing a DMP and the outcome (*e.g.,* phenotype) is a limitation of LASSO regression and comparable approaches. Notably, DNA methylation levels within oxoglutarate dehydrogenase (*OGDH;* cg04674755) were consistent within GWVs with GWI, and differential DNA methylation between groups was observed across all three timepoints (*i.e.,* pre-, 30 minutes post-, 24hr post-exercise). *OGDH* encodes an enzyme critical to the citric acid cycle of aerobic metabolism,[366] and previous studies have demonstrated enzymatic OGDH activity is responsive to aerobic exercise.[279] Interestingly, DNA methylation levels within this DMP were stable both before and after the exercise challenge in GWVs with GWI. Stability within accessible bulk

tissue (*i.e.,* whole blood) support its potential as a GWI biomarker, and ROC-based classification accuracy between cases and controls was fair-to-good (AUC = 0.796). This single CpG site discriminated GWVs with GWI and control GWVs at rates comparable to investigations leveraging multiple blood-based measures,[16,98,367] but these findings require replication to confirm validity in a larger cohort. Future efforts will include testing whether classification accuracy improves with the addition of other CpG sites or physiological measures (*e.g.,* plasma CRP).

5.5. Strengths, Limitations, & Future Directions

Strengths of the present study include well-characterized phenotypes (*i.e.,* GWI) using multiple psychometric instruments, adequate statistical power needed to reliably detect case-control DNA methylation differences, repeated measures of DNA methylation in tissue (*i.e.,* whole blood) potentially implicated in GWI pathogenesis, and the use of an exercise challenge intended to stimulate systemic physiology. Primary limitations within the present study that warrant consideration in future pathophysiological investigations of GWI include use of reference-based blood cell proportion estimates and a PEM measurement window limited to 24hr post-exercise.

Reference-based blood cell proportion corrections are efficacious for controlling bulk-tissue heterogeneity in healthy and pathological conditions (*e.g.,* Rheumatoid Arthritis) that may otherwise bias DNA methylation findings.[258,259] Previous GWI investigations have reported variability across several immune cell subpopulations that may have pathophysiological significance,[9,99] and individual complete blood cell counts (CBCs) would provide additional

confidence in these blood-based DNA methylation results. Cell lysis during biospecimen processing precluded retrospective individual CBCs, but efforts to investigate cell responsivity to immunological stimulation (*i.e.,* TruCulture®[368]) in a separate GWI subsample are ongoing. Advancements in high throughput sequencing technology capable of using low DNA inputs from flow-cytometry or magnetic-assisted blood cell sorting procedures may also be applicable in future studies of DNA methylation in GWI.

PEM in GWVs with GWI (Specific Aim 4) was determined based on endorsement of a single Kansas Questionnaire item at screening and quantified by psychometric symptom responses (*i.e.,* fatigue, pain, mood disturbance) measured pre, 30 min post-, and 24hr post-exercise. As part of a screening tool, this singular Kansas Questionnaire item was not independently validated or intended for PEM classification. More recently developed questionnaires (*e.g.,* DSQ PEM Subscale[131,369]) have been validated to determine whether participant's with ME/CFS have previously experienced PEM, and comparing response consistency between the DSQ PEM Subscale and PEM endorsement on the Kansas Questionnaire would improve PEM characterization in GWVs with GWI. To the best of our knowledge, associations between repeated symptom measures and DNA methylation levels have not been tested GWVs with GWI. Testing associations between concurrent symptom responses and DNA methylation measurements was a necessary first step, as PEM responses are most consistently reported within 24-72hr after a triggering event.[4,143,354] PEM responses beyond 24hr post-exercise were not measured in the present study, and subsequent investigations will characterize symptom responses up to 72hr post-exercise within this GWV cohort to test whether these responses are associated with DNA methylation.

Microarray-based methods for quantifying DNA methylation levels in the present study were deliberate based on accessibility (*e.g.,* microarray analysis platform, computing resources, etc.) and comparability to previous GWI literature.[27] Although DNA methylation microarrays are well-validated,[175,370–372] they encompass <4% of the nearly 28,000,000 CpG sites located throughout the genome.[266] Whole genome methylation sequencing studies are currently scant in the literature, but evidence from these methods in clinical populations like Schizophrenia[373] demonstrates their potential for deciphering how the DNA methylation landscape contributes to human health and disease.

CHAPTER 6: CONCLUSIONS

6.1. Conclusions:

Differences in DNA methylation identified before and after an acute exercise challenge in GWVs with GWI signify potential pathophysiological pathways. DMP-associated genes that participate in inflammation and metabolism are consistent with previous GWI investigations, including those that have studied DNA methylation. Associations between DNA methylation, ventilatory equivalents during the exercise challenge, and symptoms were representative of stress responses that may reflect both the burden of GWI and have risk implications for other forms of chronic illness (*e.g.,* cardiovascular or metabolic disease). DNA methylation may also have future diagnostic applications for GWI but require replication. Planned next steps include testing whether blood-based levels of DNA methylation correspond to transcriptional activity in GWI, and if successful, these genes and their associated pathways may be candidate pathways considered for intervention.

Supplemental Figures:

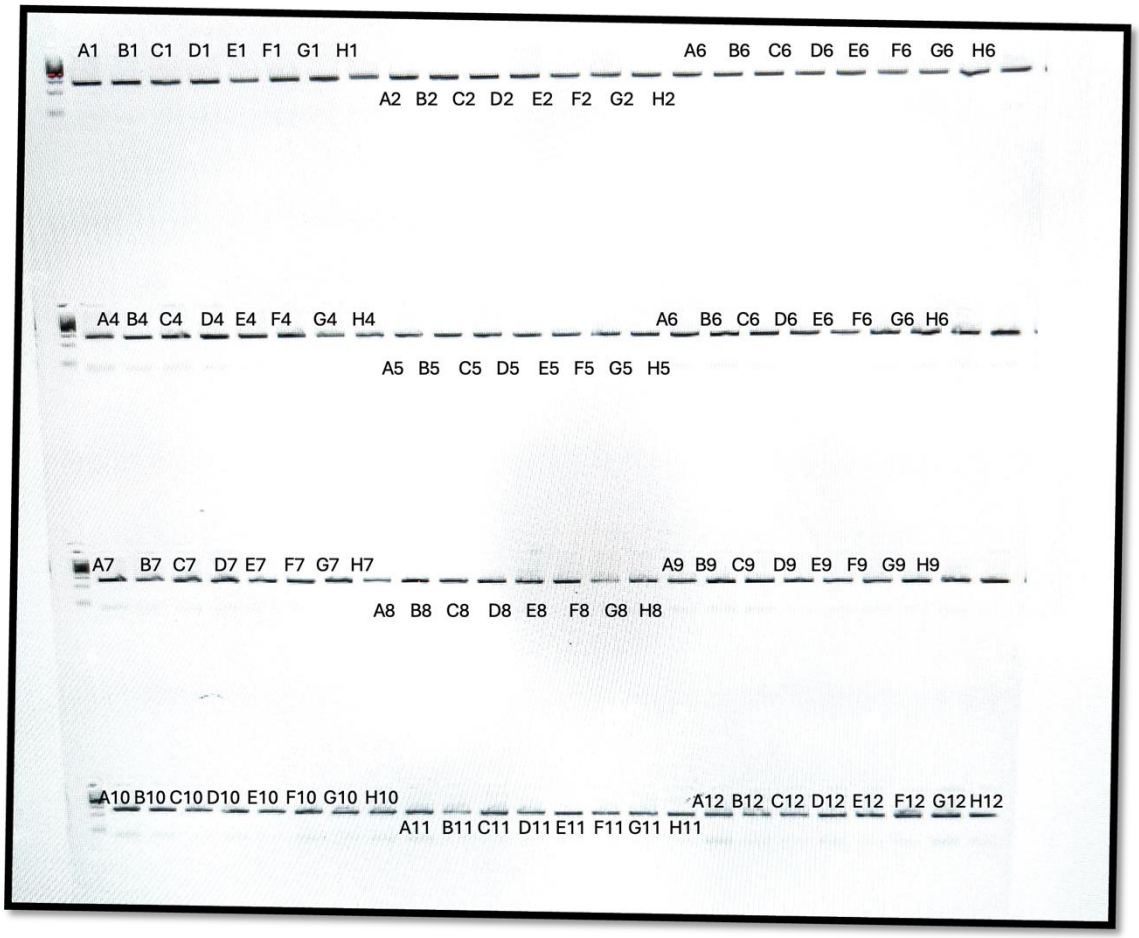


Plate 1: DAPK1 PCR 2% Agarose Gel

Supplemental Figure S1. DAPK1 PCR to confirm sodium bisulfite conversion. Sodium bisulfite conversion of DNA prior to microarray analyses were performed using the EZ-96 DNA Methylation-Lightning Kit (cat. No. D5033). DAPK1 PCR performed on 2% agarose gel is used to confirm successful bisulfite conversions within DNA. Using Plate 1 as an example, strong solid bands indicate sufficient bisulfite conversions.

Supplemental Tables:

Supplemental Table S1A: Profile of Mood States (POMS) Fatigue Subscale

Effect:	***β***	***SE***	***df***	***t***	***p***
(Intercept)	1.64	0.86	1.92	84.09	0.06
GWI PEM-	5.89	1.40	4.22	84.09	< 0.01
GWI PEM+	7.11	1.50	4.73	84.09	< 0.01
Time 2 (30-Minutes Post-Exercise)	1.08	0.76	1.43	96.23	0.16
Time 3 (24 hours Post-Exercise)	-0.33	0.77	-0.44	96.67	0.66
GWI PEM- x Time 2	1.05	1.24	0.85	96.23	0.40
GWI PEM+ x Time 2	-2.66	1.33	-2.01	96.23	< 0.05
GWI PEM- x Time 3	0.07	1.26	0.06	96.85	0.96
GWI PEM+ x Time 3	-1.17	1.33	-0.87	96.38	0.38

Note. POMS Fatigue group differences over time (*i.e.,* group-by-time) were tested using linear mixed effects regression models. Abbreviations: GWI PEM- = GWVs with GWI who do not endorse PEM; GWI PEM+ = GWVs with GWI who endorse PEM; Time 2 = 30-minutes Post-Exercise; Time 3 = 24 hours Post-Exercise; β = slope coefficient; *SE = standard error; df = degrees of freedom; t = t-value; p = p-value*

Supplemental Table S1B: Profile of Mood States (POMS) Total Mood Disturbance

Effect:	***β***	***SE***	***df***	***t***	***p***
(Intercept)	-1.68	2.77	-0.61	67.65	0.55
GWI PEM-	19.88	4.52	4.39	67.65	< 0.01
GWI PEM+	26.93	4.86	5.54	67.65	< 0.01
Time 2 (30-Minutes Post-Exercise)	1.76	1.90	0.92	96.06	0.36
Time 3 (24 hours Post-Exercise)	0.94	1.93	0.49	96.31	0.63
GWI PEM- x Time 2	-1.16	3.11	-0.37	96.06	0.71
GWI PEM+ x Time 2	-7.84	3.34	-2.35	96.06	< 0.05
GWI PEM- x Time 3	-2.37	3.18	-0.75	96.42	0.46
GWI PEM+ x Time 3	-6.44	3.36	-1.92	96.14	0.06

Note. POMS Total Mood Disturbance group differences over time (*i.e.,* group-by-time) were tested using linear mixed effects regression models. Abbreviations: GWI PEM- = GWVs with GWI who do not endorse PEM; GWI PEM+ = GWVs with GWI who endorse PEM; Time 2 = 30-minutes Post-Exercise; Time 3 = 24 hours Post-Exercise; β = slope coefficient; *SE = standard error; df = degrees of freedom; t = t-value; p = p-value*

Supplemental Table S1C: Short Form McGill Pain Questionnaire (SF-MPQ) Total Score

Effect:	***β***	***SE***	***df***	***t***	***p***
(Intercept)	0.20	0.21	0.95	58.74	0.35
GWI PEM-	0.89	0.34	2.62	58.74	< 0.05
GWI PEM+	1.00	0.37	2.72	58.74	< 0.01
Time 2 (30-Minutes Post-Exercise)	-0.07	0.11	-0.61	96.06	0.54
Time 3 (24 hours Post-Exercise)	-0.11	0.11	-0.96	96.20	0.34
GWI PEM- x Time 2	0.30	0.18	1.68	96.06	0.10
GWI PEM+ x Time 2	-0.02	0.19	-0.11	96.06	0.91
GWI PEM- x Time 3	0.11	0.18	0.61	96.26	0.55
GWI PEM+ x Time 3	0.11	0.19	0.57	96.11	0.57

Note. SF-MPQ Total Score group differences over time (*i.e.,* group-by-time) were tested using linear mixed effects regression models. Abbreviations: GWI PEM- = GWVs with GWI who do not endorse PEM; GWI PEM+ = GWVs with GWI who endorse PEM; Time 2 = 30-minutes Post-Exercise; Time 3 = 24 hours Post-Exercise; β = slope coefficient; *SE = standard error; df = degrees of freedom; t = t-value; p = p-value*

Appendix A: Psychometric Instruments & Questionnaires

Kansas Case Definition for Gulf War Illness & Scoring

Gulf War Illness Questionnaire

Study ID: _____

Please answer these questions as completely as possible.

If you are **not sure** of the answer to any question, please give your best estimate.

If you have **no idea** of the correct answer, indicate by writing "DON'T KNOW" in the blank.

If you **prefer not to answer** any question, indicate by writing "NO ANSWER" in the blank.

The Persian Gulf Region

Persian Gulf War Time Line

Questions About Your Military Service Between August, 1990 and July, 1991

Please refer to the map and timeline on the previous page to help you answer the following questions.

	NO	YES
1. Did you deploy to the Persian Gulf region any time between August, 1990 and July, 1991?	❍	❍
2. In what month and year did you first arrive in the region?	_____(month)	_____(year)
3. In what month and year did you last leave the region?	_____(month)	_____(year)

Please indicate if you were ever located in the following countries or areas of the Persian Gulf Theater between August of 1990 and July of 1991, and the total length of time you spent in each area. Use the accompanying map, and the letter indicated for each country or region.

(NOTE: Answer NO for areas that you flew over, but did not touch down.)

4. While in the Persian Gulf region, were you ever located in ..... ? (Mark NO or YES for each)	**NO, I was never there**	**YES, I was there for :** 1 - 6 days	7 - 30 days	31 days or more
a. Saudi Arabia: Eastern area (area **A** on map)	❍	❍	❍	❍
b. Bahrain (area **B** on map)	❍	❍	❍	❍
c. Kuwait (area **C** on map)	❍	❍	❍	❍
d. Iraq (area **D** on map)	❍	❍	❍	❍
e. Saudi Arabia: Northern area (area **E** on map)	❍	❍	❍	❍
f. Saudi Arabia: Central area (area **F** on map)	❍	❍	❍	❍
g. Saudi Arabia: Western area (area **G** on map)	❍	❍	❍	❍
h. At sea: in the Persian Gulf (area **H** on map)	❍	❍	❍	❍
i. At sea: other (specify_____)	❍	❍	❍	❍
j. Other location (specify_____)	❍	❍	❍	❍

5. While you were in the Persian Gulf region, did you experience any of the following?

[Please mark NO or YES for each]	NO	YES	IF YES, About how many days? 1 - 6 days	7 - 30 days	31 days or more
Saw smoke from oil well fires	○	○ →	☐	☐	☐
Heard chemical alarms sounded	○	○ →	☐	☐	☐
Had SCUD missile explode within one mile of you	○	○ →	☐	☐	☐
Was directly involved in ground combat	○	○ →	☐	☐	☐
Was directly involved in air combat	○	○ →	☐	☐	☐
Saw American or Allied troops who had been badly wounded or killed	○	○ →	☐	☐	☐
Saw Iraqis or civilians who had been badly wounded or killed	○	○ →	☐	☐	☐
Came into contact with prisoners of war	○	○ →	☐	☐	☐
Saw dead animals	○	○ →	☐	☐	☐
Came into direct contact with dead animals	○	○ →	☐	☐	☐
Saw destroyed enemy vehicles	○	○ →	☐	☐	☐
Came into direct contact with destroyed enemy vehicles	○	○ →	☐	☐	☐
Came into direct contact with American vehicles hit by friendly fire	○	○ →	☐	☐	☐
Used pesticide cream or spray on your skin	○	○ →	☐	☐	☐
Wore a uniform treated with pesticides	○	○ →	☐	☐	☐
Wore a flea collar	○	○ →	☐	☐	☐
Saw the area in which you lived fogged or sprayed with pesticides	○	○ →	☐	☐	☐
Received one or more shots in the arm while in theater	○	○ →	☐	☐	☐
Received one or more shots in the buttocks while in theater	○	○ →	☐	☐	☐
Took pyridostigmine pills (also called NAPP pills) *(Little white pills in a foil package, used to protect against nerve gas.)*	○	○ →	☐	☐	☐
Used or came into contact with freshly applied CARC paint *(Chemical Agent Resistant Coating)*	○	○ →	☐	☐	☐
Had less than four hours of sleep in a 24-hour period	○	○ →	☐	☐	☐

6. In August, 1990, what was your branch of service?	Army ❍	Navy ❍	Air Force ❍	Marines ❍
7. In August, 1990, were you in the enlisted ranks, or an officer?			Enlisted ❍	Officer ❍
8. In August, 1990, were you in the Regular military, the Reserves, or the National Guard?		Regular ❍	Reserves ❍	National Guard ❍

9. What type of unit did you serve with while in the Persian Gulf area? _____

10. What was your primary military occupation while in the Gulf area? _____

Questions About Your Health

Please indicate below if you have had a persistent problem with each of the following symptoms over the past six months. If you have had the problem, please rate it as mild, moderate, or severe and indicate if it first became a problem before you deployed to the Gulf, or during/after your deployment.

11. Over the past six months, have you had a persistent problem with ..... ? ***(Mark NO or YES for each)***

			How would you rate this problem (if "Yes")?			When did you first have this problem?				
Symptoms	*No*	*Yes*	*Mild*	*Moderate*	*Severe*	*Before Gulf War (GW)*	*During GW*	*1-5 yrs after GW*	*5-10 yrs after GW*	*10-20 yrs after GW*
Fatigue	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Feeling unwell after physical exercise or exertion	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Problems getting to sleep or staying asleep	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Pain in your joints	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Body pain, where you hurt all over	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Headaches	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Feeling dizzy, lightheaded or faint	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Eyes very sensitive to light	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Blurred or double vision	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Numbness or tingling in your extremities	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Tremors or shaking	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Low tolerance for heat or cold	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Night sweats	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Physical or mental symptoms after breathing in certain smells or chemicals	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Skin rashes	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Other skin problems	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Diarrhea	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Nausea or upset stomach	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Abdominal pain or cramping	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Difficulty breathing or catching your breath	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Frequent coughing when you don't have a cold	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Wheezing in your chest	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Difficulty remembering recent information	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Difficulty concentrating	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Trouble finding words when speaking	O	O→	☐	☐	☐	☐	☐	☐	☐	☐

	No	Yes	Mild	Moderate	Severe	Before Gulf War (GW)	During GW	1-5 yrs after GW	5-10 yrs after GW	10-20 yrs after GW
Feeling down or depressed	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Feeling irritable or have angry outbursts	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Feeling moody	O	O→	☐	☐	☐	☐	☐	☐	☐	☐
Feeling anxious	O	O→	☐	☐	☐	☐	☐	☐	☐	☐

12. In general, how would you describe your health **before you went to the Persian Gulf?** Excellent ❍ Good ❍ Fair ❍ Poor ❍

13. In general, how would you describe your health **now?** Excellent ❍ Good ❍ Fair ❍ Poor ❍

14. Were you a regular smoker **before** you deployed to the Persian Gulf region? NO ❍ YES ❍

15. Were you a regular smoker **while** you were in the Persian Gulf region? NO ❍ YES ❍

16. Are you **currently** a regular smoker? NO ❍ YES ❍

Gulf War Exposure Questionnaire

Gulf War Exposure

While you were in the Persian Gulf region, did you experience any of the following?

[Please mark NO or YES for each]	NO	YES	IF YES, About how many days? 1 - 6 days	7 - 30 days	31 days or more
Saw smoke from oil well fires	❍	❍ →	❒	❒	❒
Heard chemical alarms sounded	❍	❍ →	❒	❒	❒
Had SCUD missile explode within one mile of you	❍	❍ →	❒	❒	❒
Was directly involved in ground combat	❍	❍ →	❒	❒	❒
Was directly involved in air combat	❍	❍ →	❒	❒	❒
Saw American or Allied troops who had been badly wounded or killed	❍	❍ →	❒	❒	❒
Saw Iraqis or civilians who had been badly wounded or killed	❍	❍ →	❒	❒	❒
Came into contact with prisoners of war	❍	❍ →	❒	❒	❒
Saw dead animals	❍	❍ →	❒	❒	❒
Came into direct contact with dead animals	❍	❍ →	❒	❒	❒
Saw destroyed enemy vehicles	❍	❍ →	❒	❒	❒
Came into direct contact with destroyed enemy vehicles	❍	❍ →	❒	❒	❒
Came into direct contact with American vehicles hit by friendly fire	❍	❍ →	❒	❒	❒
Used pesticide cream or spray on your skin	❍	❍ →	❒	❒	❒
Wore a uniform treated with pesticides	❍	❍ →	❒	❒	❒
Wore a flea collar	❍	❍ →	❒	❒	❒
Saw the area in which you lived fogged or sprayed with pesticides	❍	❍ →	❒	❒	❒
Received one or more shots in the arm while in theater	❍	❍ →	❒	❒	❒
Received one or more shots in the buttocks while in theater	❍	❍ →	❒	❒	❒
Took pyridostigmine pills (also called NAPP pills) *(Little white pills in a foil package, used to protect against nerve gas.)*	❍	❍ →	❒	❒	❒
Used or came into contact with freshly applied CARC paint *(Chemical Agent Resistant Coating)*	❍	❍ →	❒	❒	❒
Had less than four hours of sleep in a 24-hour period	❍	❍ →	❒	❒	❒

Determining Gulf War Illness Case Status Using the Kansas GWI Case Definition

The Kansas Gulf War illness (GWI) case definition was developed from a population-based survey of over 2,000 Kansas veterans who served during the time of the 1990-1991 Gulf War. The case definition was identified empirically in 2000 as the pattern of self-reported symptoms that best distinguished veterans who deployed to the Gulf War theater from those who did not.[1]

The Kansas GWI case definition has both exclusionary and inclusionary components. Veterans are excluded from consideration as a GWI case if they have been diagnosed by a physician with (1) chronic conditions (e.g., cancer, heart disease) that are not associated with Gulf War service but can produce diverse symptoms (e.g. fatigue, cognitive problems, pain) similar to those affecting Gulf war veterans, or (2) conditions that might interfere with respondents' ability to report their symptoms (e.g. serious psychiatric conditions).

Inclusionary criteria require that veterans endorse moderately severe and/or multiple symptoms in at least 3 of 6 symptom domains. A template for identifying exclusionary conditions and determining scores for each of the 6 symptom domains is provided below.

1. Identify exclusionary conditions: Veterans who report any of the following conditions, diagnosed by a physician, are excluded as GWI cases.

Diabetes
Heart disease (other than high blood pressure)
Stroke
Lupus
Multiple sclerosis
Rheumatoid Arthritis
Parkinson's Disease
ALS, or Lou Gehrig's Disease
Seizure disorder
Alzheimer's Disease
Cancer (other than skin cancer)
Melanoma
Liver disease
Kidney disease
Schizophrenia
Bipolar disorder or manic depression
Hospitalized for PTSD or posttraumatic stress disorder in the past 5 years
Hospitalized for depression in the past 5 years
Hospitalized for alcohol or drug dependence in the last 5 years
Any chronic infectious disease lasting 6 months or longer
Any other current exclusionary condition lasting 6 months or longer

[1] Steele, L. Prevalence and Patterns of Gulf War Illness in Kansas Veterans: Association of Symptoms with Characteristics of Person, Place, and Time of Military Service. Am. J. Epidemiol 2000;152:992-1002.

2. Score individual symptoms. Calculate symptom score totals for each of six domains:

Veteran-reported symptoms that have persisted over the previous six months:	NO response	YES response: Scores for symptom severity		
	Score	Mild	Moderate	Severe
Fatigue Domain				
Fatigue	0	1	2	3
Feeling unwell after physical exercise or exertion	0	1	2	3
Problems getting to sleep or staying asleep	0	1	2	3
Not feeling rested after you sleep	0	1	2	3
				Total score: Fatigue Domain
Pain Domain				
Pain in your joints	0	1	2	3
Pain in your muscles	0	1	2	3
Body pain, where you hurt all over	0	1	2	3
				Total score: Pain Domain
Neurological/cognitive/mood Domain				
Headaches	0	1	2	3
Feeling dizzy, lightheaded, or faint	0	1	2	3
Eyes very sensitive to light	0	1	2	3
Blurred or double vision	0	1	2	3
Numbness or tingling in your extremities	0	1	2	3
Tremors or shaking	0	1	2	3
Low tolerance for heat or cold	0	1	2	3
Night sweats	0	1	2	3
Having physical or mental symptoms after breathing in certain smells or chemicals	0	1	2	3
Difficulty concentrating	0	1	2	3
Difficulty remembering recent information	0	1	2	3
Trouble finding words when speaking	0	1	2	3
Feeling down or depressed	0	1	2	3
Feeling irritable or having angry outbursts	0	1	2	3
				Total score: Neuro/cogn/mood Domain
Skin Domain				
Skin rashes	0	1	2	3
Other skin problems	0	1	2	3
				Total score: Skin Domain

Veteran-reported symptoms that have persisted over the previous six months:	NO response Score	YES response: Scores for symptom severity Mild	Moderate	Severe
Gastrointestinal Domain				
Diarrhea	0	1	2	3
Nausea or upset stomach	0	1	2	3
Abdominal pain or cramping	0	1	2	3
			Total score: Gastrointestinal Domain	
Respiratory Domain				
Difficulty breathing or catching your breath	0	1	2	3
Frequent coughing when you don't have a cold	0	1	2	3
Wheezing in your chest	0	1	2	3
			Total score: Respiratory Domain	

Number of symptom domains having score of 2 or greater _____

Summary of Kansas GWI case criteria:

1) **No excluding diagnosed conditions, and**

2) **A total score of 2 or greater in at least 3 (of 6) symptom domains.**

American College of Rheumatology (ACR) 2010 Criteria for Fibromyalgia

AMERICAN COLLEGE OF RHEUMATOLOGY (ACR)
PRELIMINARY DIAGNOSTIC CRITERIA FOR FIBROMYALGIA[1]

The information contained on this form was derived from Wolfe F, Clauw DJ, Fitzcharles M-A, et al. The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity. *Arthritis Care Res.* 2010;62(5):600-610.

PART 1: WIDESPREAD PAIN INDEX

HOW TO CALCULATE THE PATIENT'S WIDESPREAD PAIN INDEX (WPI)

1. Using the list of 19 body areas, identify the areas where the patient felt pain over the past week. As a visual aid, front/back body diagrams are included.

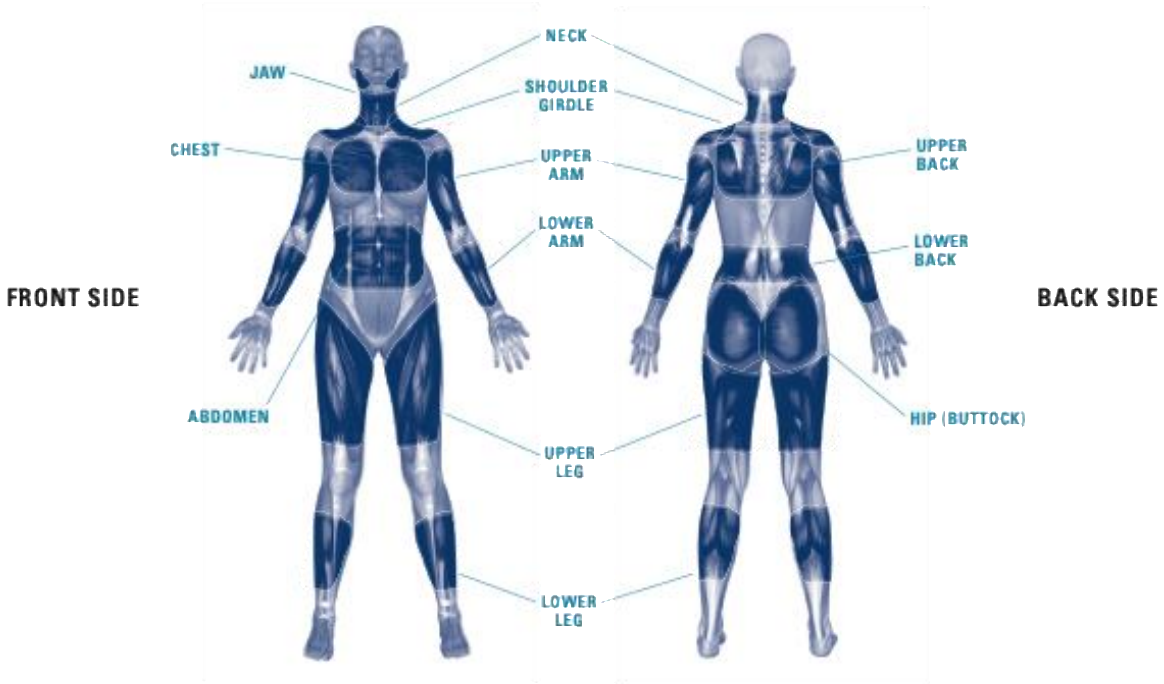
   — Each area identified on the list counts as 1

2. Total the number of body areas (the WPI score can range from 0 to 19).

Write the patient's WPI score here: _____.

Identify the areas where the patient felt pain over the past week

❑ Shoulder girdle, left	❑ Lower arm, right	❑ Lower leg, left	❑ Abdomen
❑ Shoulder girdle, right	❑ Hip (buttock), left	❑ Lower leg, right	❑ Neck
❑ Upper arm, left	❑ Hip (buttock), right	❑ Jaw, left	❑ Upper back
❑ Upper arm, right	❑ Upper leg, left	❑ Jaw, right	❑ Lower back
❑ Lower arm, left	❑ Upper leg, right	❑ Chest	



PART 2A: SYMPTOM SEVERITY SCALE (LEVELS OF SEVERITY)

HOW TO MEASURE THE PATIENT'S LEVEL OF SYMPTOM SEVERITY

1. Using a scale of 0 to 3, indicate the patient's level of symptom severity over the past week in each of the 3 symptom categories. Choose only 1 level of severity for each category.

   — The score is the sum of the numbers that correspond to the severity levels identified in all 3 categories

2. Total the scale numbers for all the 3 categories and write the number here: _____

Fatigue

- ❑ 0 = No problem
- ❑ 1 = Slight or mild problems; generally mild or intermittent
- ❑ 2 = Moderate; considerable problems; often present and/or at a moderate level
- ❑ 3 = Severe; pervasive, continuous, life-disturbing problems

Waking unrefreshed

- ❑ 0 = No problem
- ❑ 1 = Slight or mild problems; generally mild or intermittent
- ❑ 2 = Moderate; considerable problems; often present and/or at a moderate level
- ❑ 3 = Severe; pervasive, continuous, life-disturbing problems

Cognitive symptoms

- ❑ 0 = No problem
- ❑ 1 = Slight or mild problems; generally mild or intermittent
- ❑ 2 = Moderate; considerable problems; often present and/or at a moderate level
- ❑ 3 = Severe; pervasive, continuous, life-disturbing problems

PART 2B: SYMPTOM SEVERITY SCALE (OTHER SOMATIC SYMPTOMS)

HOW TO DETERMINE THE EXTENT OF THE PATIENT'S OTHER SOMATIC SYMPTOMS

Using the symptoms list on the following page, determine the extent of other somatic symptoms the patient may have experienced over the past week.

1. Determine the quantity of somatic symptoms using the list on the following page.
2. Using your best judgment, calculate the score that matches the quantity of those somatic symptoms and write the number here: _____

Add the scores from Parts 2a and 2b (the Symptom Severity score, or SS score, can range from 0 to 12).
Write the patient's SS score here: _____

OTHER SYMPTOMS

❑ Muscle pain	❑ Depression	❑ Itching	❑ Dry eyes
❑ Irritable bowel syndrome	❑ Constipation	❑ Wheezing	❑ Shortness of breath
❑ Fatigue/tiredness	❑ Pain in upper abdomen	❑ Raynaud's	❑ Loss of appetite
❑ Thinking or memory problem	❑ Nausea	❑ Hives/welts	❑ Rash
❑ Muscle weakness	❑ Nervousness	❑ Ringing in ears	❑ Sun sensitivity
❑ Headache	❑ Chest pain	❑ Vomiting	❑ Hearing difficulties
❑ Pain/cramps in abdomen	❑ Blurred vision	❑ Heartburn	❑ Easy bruising
❑ Numbness/tingling	❑ Fever	❑ Oral ulcers	❑ Hair loss
❑ Dizziness	❑ Diarrhea	❑ Loss/change in taste	❑ Frequent urination
❑ Insomnia	❑ Dry mouth	❑ Seizures	❑ Bladder spasms

Based on the quantity of symptoms, the patient's score is:

❑ **0 = No symptoms**
❑ **1 = Few symptoms**
❑ **2 = A moderate number of symptoms**
❑ **3 = A great deal of symptoms**

WHAT THE PATIENT'S SCORE MEANS

The patient's WPI score (Part 1): _____ **The patient's SS score (Parts 2a and 2b):** _____

A PATIENT MEETS THE DIAGNOSTIC CRITERIA FOR FIBROMYALGIA IF THE FOLLOWING 3 CONDITIONS ARE MET:

1a. The WPI score (Part 1) is greater than or equal to 7 and the SS score (Parts 2a and 2b) is greater than or equal to 5.

OR

1b. The WPI score (Part 1) is from 3 to 6 and the SS score (Parts 2a and 2b) is greater than or equal to 9.

2. Symptoms have been present at a similar level for at least 3 months.

3. The patient does not have a disorder that would otherwise explain the pain.

Reference: 1. Wolfe F, Clauw DJ, Fitzcharles M-A, et al. The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity. *Arthritis Care Res.* 2010;62(5):600-610.

Veterans Rand 36-Item Health Survey (VR-36)

WRIISC HEALTH QUESTIONNAIRE V20120717 0 6 0 1

Section 6: General Health

1. In general, would you say your health is? (Fill in one answer.)

O Excellent O Very Good O Good O Fair O Poor

2. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much? (Fill in one answer for each activity.)

ACTIVITY	YES, LIMITED A LOT	YES, LIMITED A LITTLE	NO, NOT LIMITED AT ALL
a. **Vigorous activities** (such as running, lifting heavy objects, participating in strenuous sports?)	O	O	O
b. **Moderate activities** (such as moving a table, pushing a vacuum cleaner, bowling, or playing golf?)	O	O	O
c. Lifting or carrying groceries?	O	O	O
d. Climbing **several** flights of stairs?	O	O	O
e. Climbing **one** flight of stairs?	O	O	O
f. Bending, kneeling, or stooping?	O	O	O
g. Walking **more than a mile**?	O	O	O
h. Walking **several blocks**?	O	O	O
i. Walking **one block**?	O	O	O
j. Bathing or dressing yourself?	O	O	O

WRIISC HEALTH QUESTIONNAIRE V20120717 0602

3. **During the past 4 weeks**, how much of the time have you had any of the following problems with your work or other regular daily activities *as a result of your physical health*? (Fill in one answer for each problem.)

	NO, NONE OF THE TIME	YES, A LITTLE OF THE TIME	YES, SOME OF THE TIME	YES, MOST OF THE TIME	YES, ALL OF THE TIME
a. Cut down the **amount of time** you spent on work or other activities	O	O	O	O	O
b. **Accomplished less** than you would like	O	O	O	O	O
c. Were limited in the **kind** of work or other activities	O	O	O	O	O
d. Had **difficulty** performing the work or other activities (for example, it took extra effort)	O	O	O	O	O

4. **During the past 4 weeks**, how much of the time have you had any of the following problems with your work or other regular daily activities ***as a result of any emotional problems*** (such as feeling depressed or anxious)? (Fill in one answer for each problem.)

	NO, NONE OF THE TIME	YES, A LITTLE OF THE TIME	YES, SOME OF THE TIME	YES, MOST OF THE TIME	YES, ALL OF THE TIME
a. Cut down the **amount of time** you spent on work or other activities	O	O	O	O	O
b. **Accomplished less** than you would like	O	O	O	O	O
c. Didn't do work or other activities as **carefully** as usual	O	O	O	O	O

5. **During the past 4 weeks**, to what extent has your **physical health or emotional problems** interfered with your normal social activities with family, friends, neighbors, or groups? (Fill in one answer.)

O Not at all O Slightly O Moderately O Quite a bit O Extremely

6. How much bodily pain have you had **during the past 4 weeks**? (Fill in one answer.)

O None O Very Mild O Mild O Moderate O Severe O Very Severe

7. **During the past 4 weeks**, how much did *pain* interfere with your normal work including both work outside the home and house work)? (Fill in one answer.)

O Not at all O A little bit O Moderately O Quite a bit O Extremely

WRIISC HEALTH QUESTIONNAIRE V20120717 0603

8. These questions are about how you feel and how things have been with you **during the past 4 weeks**. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time **during the past 4 weeks**... (Fill in one answer for each item.)

ACTIVITY	ALL OF THE TIME	MOST OF THE TIME	A GOOD BIT OF THE TIME	SOME OF THE TIME	A LITTLE OF THE TIME	NONE OF THE TIME
a. Did you feel **full of pep**?	O	O	O	O	O	O
b. Have you been a **very nervous person**?	O	O	O	O	O	O
c. Have you felt so down in the dumps that **nothing could cheer you up**?	O	O	O	O	O	O
d. Have you felt **calm and peaceful**?	O	O	O	O	O	O
e. Did you have a **lot of energy**?	O	O	O	O	O	O
f. Have you felt **downhearted and blue**?	O	O	O	O	O	O
g. Did you feel **worn out**?	O	O	O	O	O	O
h. Have you been a **happy person**?	O	O	O	O	O	O
i. Did you feel **tired**?	O	O	O	O	O	O

9. **During the past 4 weeks**, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting friends, relatives, etc.)? (Fill in one answer.)

O All of the time O Most of the time O Some of the time O A little of the time O None of the time

WRIISC HEALTH QUESTIONNAIRE V20120717 0 6 0 4

10. Please choose the answer that best describe how ***true or false*** **each** of the following statements is for you. (Fill in one answer for each problem.)

	DEFINITELY TRUE	MOSTLY TRUE	NOT SURE	MOSTLY FALSE	DEFINITELY FALSE
a. I seem to get sick a little easier than other people	○	○	○	○	○
b. I am as healthy as anybody I know	○	○	○	○	○
c. I expect my health to get worse	○	○	○	○	○
d. My health is excellent	○	○	○	○	○

11. Now we'd like to ask you some questions about how your health may have changed. (Fill in one answer for each item.)

	MUCH BETTER	SOMEWHAT BETTER	ABOUT THE SAME	SOMEWHAT WORSE	MUCH WORSE
a. **Compared to one year ago**, how would you rate your *health in general* now?	○	○	○	○	○
b. **Compared to one year ago**, how would you rate your *physical health* in general now?	○	○	○	○	○
c. **Compared to one year ago**, how would you rate your *emotional problems* now (such as feeling anxious, depressed, or irritable)?	○	○	○	○	○

Multi-Dimensional Fatigue Inventory

Multi-Dimensional Fatigue Inventory

The next questions are about how you have been feeling lately. Please place one "X" for each statement.

The more you agree with the statement, the more you should place an "X" in the direction of "yes, that is true." The more you disagree with the statement, the more you should place an X in the direction of "no, that is not true."

Take for example the statement: "I FEEL RELAXED."

If you think that this statement is entirely true, that you have been feeling relaxed lately, you would place an "X" in the box labeled "1."

yes, that is true ☒ ☐ ☐ ☐ ☐ **no, that is not true**
1 2 3 4 5

1. I feel fit.	yes, that is true	☐ ☐ ☐ ☐ ☐ no, that is not true 1 2 3 4 5
2. Physically I feel only able to do a little.	yes, that is true	☐ ☐ ☐ ☐ ☐ no, that is not true 1 2 3 4 5
3. I feel very active.	yes, that is true	☐ ☐ ☐ ☐ ☐ no, that is not true 1 2 3 4 5
4. I feel like doing all sorts of nice things.	yes, that is true	☐ ☐ ☐ ☐ ☐ no, that is not true 1 2 3 4 5
5. I feel tired.	yes, that is true	☐ ☐ ☐ ☐ ☐ no, that is not true 1 2 3 4 5
6. I think I do a lot in a day.	yes, that is true	☐ ☐ ☐ ☐ ☐ no, that is not true 1 2 3 4 5

7. When I am doing something, I can keep my thoughts on it.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
8. Physically I can take on a lot.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
9. I dread having to do things.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
10. I think I do very little in a day.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
11. I can concentrate well.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
12. I am rested.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
13. It takes a lot of effort to concentrate on things.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
14. Physically I feel I am in a bad condition.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
15. I have a lot of plans.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
16. I tire easily.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true

17. I get little done.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
18. I don't feel like doing anything.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
19. My thoughts easily wander.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true
20. Physically I feel I am in an excellent condition.	yes, that is true	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	no, that is not true

Perceived Deficits Questionnaire (PDQ)

PDQ-1

ID#: _____ Test#: 1 2 3 4

PERCEIVED DEFICITS QUESTIONNAIRE (PDQ)

INSTRUCTIONS

Everyone at some point experiences problems with memory, attention, or concentration, but these problems may occur more frequently for individuals with neurologic diseases like MS. The following questions describe several situations in which a person may encounter problems with memory, attention or concentration. If you are marking your own answers, please circle the appropriate response (0, 1, 2,...) based on your cognitive function during the past 4 weeks. If you need help in marking your responses, tell the interviewer the number of the best response. Please answer every question. If you are not sure which answer to select, please choose the one answer that comes closest to describing you. The interviewer can explain any words or phrases that you do not understand.

During the past 4 weeks,
how often did you....

		Never	Rarely	Some-times	Often	Almost always
1.	lose your train of thought when speaking?	0	1	2	3	4
2.	have difficulty remembering the names of people, even ones you have met several times?	0	1	2	3	4
3.	forget what you came into the room for?	0	1	2	3	4
4.	have trouble getting things organized?	0	1	2	3	4
5.	have trouble concentrating on what people are saying during a conversation?	0	1	2	3	4
6.	forget if you had already done something?	0	1	2	3	4
7.	miss appointments and meetings you had scheduled?	0	1	2	3	4

PDQ-2

During the past 4 weeks, how often did you....

		Never	Rarely	Some-times	Often	Almost always
8.	have difficulty planning what to do in the day?	0	1	2	3	4
9.	have trouble concentrating on things like watching a television program or reading a book?	0	1	2	3	4
10.	forget what you did the night before?	0	1	2	3	4
11.	forget the date unless you looked it up?	0	1	2	3	4
12.	have trouble getting started, even if you had a lot of things to do?	0	1	2	3	4
13.	find your mind drifting?	0	1	2	3	4
14.	forget what you talked about after a telephone conversation?	0	1	2	3	4
15.	forget to do things like turn off the stove or turn on your alarm clock?	0	1	2	3	4
16.	feel like your mind went totally blank?	0	1	2	3	4
17.	have trouble holding phone numbers in your head, even for a few seconds?	0	1	2	3	4
18.	forget what you did last weekend?	0	1	2	3	4
19.	forget to take your medication?	0	1	2	3	4
20.	have trouble making decisions?	0	1	2	3	4

ID#: _____ Test#: 1 2 3 4

PERCEIVED DEFICITS QUESTIONNAIRE - 5-ITEM VERSION (PDQ-5)

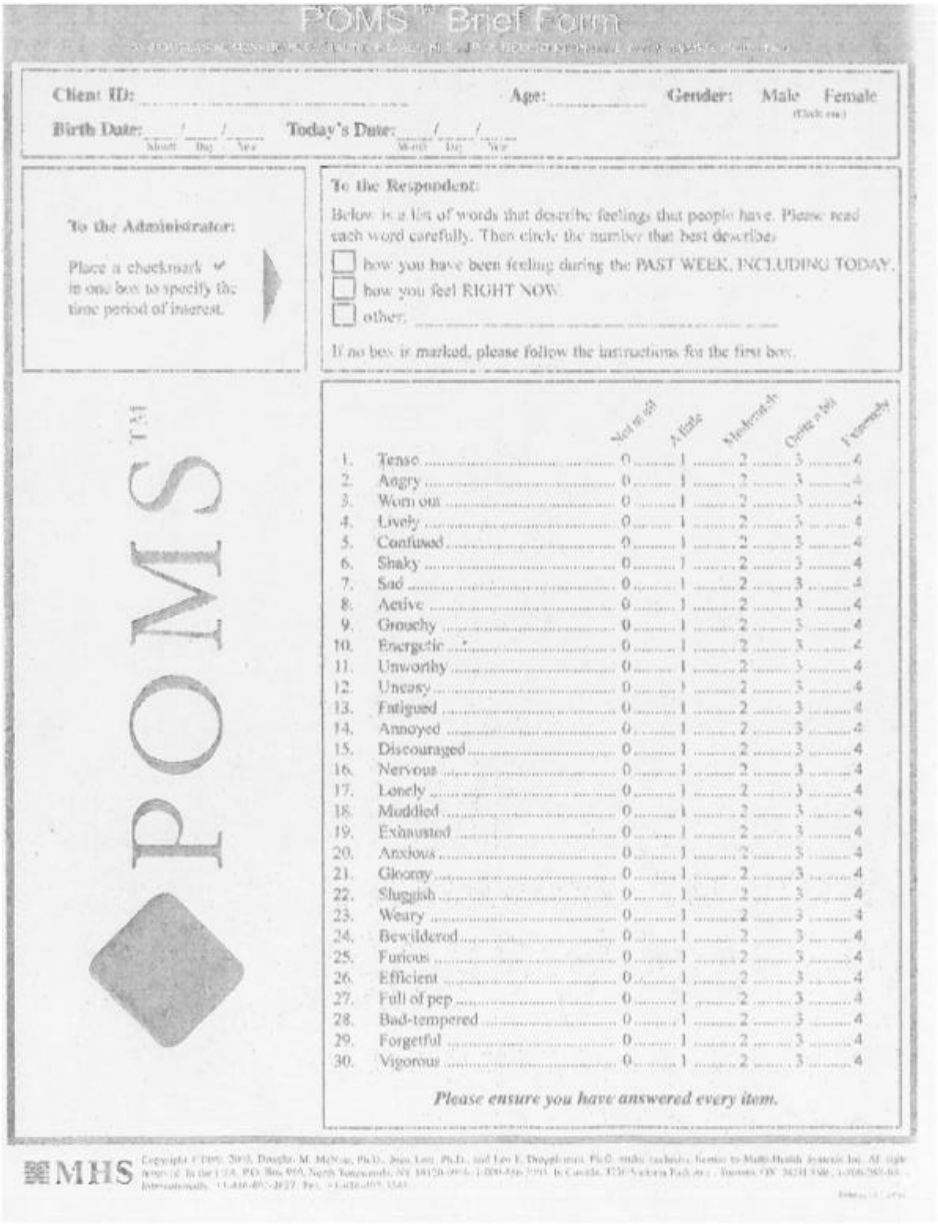
INSTRUCTIONS

Everyone at some point experiences problems with memory, attention, or concentration, but these problems may occur more frequently for individuals with neurologic diseases like MS. The following questions describe several situations in which a person may encounter problems with memory, attention or concentration. If you are marking your own answers, please circle the appropriate response (0, 1, 2,...) based on your cognitive function during the past 4 weeks. If you need help in marking your responses, tell the interviewer the number of the best response. Please answer every question. If you are not sure which answer to select, please choose the one answer that comes closest to describing you. The interviewer can explain any words or phrases that you do not understand.

During the past 4 weeks, how often did you....

		Never	Rarely	Some-times	Often	Almost always
1.	have trouble getting things organized?	0	1	2	3	4
2.	have trouble concentrating on things like watching a television program or reading a book?	0	1	2	3	4
3.	forget the date unless you looked it up?	0	1	2	3	4
4.	forget what you talked about after a telephone conversation?	0	1	2	3	4
5.	feel like your mind went totally blank?	0	1	2	3	4

Profile of Mood States (POMS)



POMS™ Brief Form

Client ID: _____ Age: _____ Gender: Male Female (Circle one)

Birth Date: ___/___/___ (Month / Day / Year) Today's Date: ___/___/___ (Month / Day / Year)

To the Administrator:

Place a checkmark ✔ in one box to specify the time period of interest.

To the Respondent:

Below is a list of words that describe feelings that people have. Please read each word carefully. Then circle the number that best describes:

- ☐ how you have been feeling during the PAST WEEK, INCLUDING TODAY.
- ☐ how you feel RIGHT NOW.
- ☐ other: _____

If no box is marked, please follow the instructions for the first box.

POMS™

		Not at all	A little	Moderately	Quite a bit	Extremely
1.	Tense	0	1	2	3	4
2.	Angry	0	1	2	3	4
3.	Worn out	0	1	2	3	4
4.	Lively	0	1	2	3	4
5.	Confused	0	1	2	3	4
6.	Shaky	0	1	2	3	4
7.	Sad	0	1	2	3	4
8.	Active	0	1	2	3	4
9.	Grouchy	0	1	2	3	4
10.	Energetic	0	1	2	3	4
11.	Unworthy	0	1	2	3	4
12.	Uneasy	0	1	2	3	4
13.	Fatigued	0	1	2	3	4
14.	Annoyed	0	1	2	3	4
15.	Discouraged	0	1	2	3	4
16.	Nervous	0	1	2	3	4
17.	Lonely	0	1	2	3	4
18.	Muddled	0	1	2	3	4
19.	Exhausted	0	1	2	3	4
20.	Anxious	0	1	2	3	4
21.	Gloomy	0	1	2	3	4
22.	Sluggish	0	1	2	3	4
23.	Weary	0	1	2	3	4
24.	Bewildered	0	1	2	3	4
25.	Furious	0	1	2	3	4
26.	Efficient	0	1	2	3	4
27.	Full of pep	0	1	2	3	4
28.	Bad-tempered	0	1	2	3	4
29.	Forgetful	0	1	2	3	4
30.	Vigorous	0	1	2	3	4

Please ensure you have answered every item.

MHS

Pittsburgh Sleep Quality Index (PSQI)

WRIISC HEALTH QUESTIONNAIRE V20120717 1 2 0 1

Section 12: Sleep

The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all questions.

1. During the past month, what time have you usually gone to bed at night?

   BED TIME (24 HOUR FORMAT): ☐☐ : ☐☐ (H H : M M)

2. During the past month, how long (in minutes) has it usually taken you to fall asleep each night?

   NUMBER OF MINUTES: ☐☐☐☐

3. During the past month, what time have you usually gotten up in the morning?

   GETTING UP TIME (24 HOUR FORMAT): ☐☐ : ☐☐ (H H : M M)

4. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spent in bed.)

   HOUR OF SLEEP PER NIGHT: ☐☐

For each of the remaining questions, fill in one best response. Please answer all questions.

5. During the past month, how often have you had trouble sleeping because you...

	NOT DURING THE PAST MONTH	LESS THAN ONCE A WEEK	ONCE OR TWICE A WEEK	THREE OR MORE TIMES A WEEK
Cannot get to sleep within 30 minutes	○	○	○	○
Wake up in the middle of the night or early morning	○	○	○	○
Have to get up to use the bathroom	○	○	○	○
Cannot breathe comfortably	○	○	○	○
Cough or snore loudly	○	○	○	○
Feel too cold	○	○	○	○
Feel too hot	○	○	○	○
Had bad dreams	○	○	○	○
Have pain	○	○	○	○
Other reason(s)...	○	○	○	○

Please describe other reason(s): _____

_____

_____

6. During the past month, how would you rate your sleep quality overall? (Fill in one answer.)

Very good	Fairly Good	Fairly Bad	Very Bad
○	○	○	○

7. During the past month, how often have you taken medicine to help you sleep (prescribed or "over the counter")? (Fill in one answer.)

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
○	○	○	○

8. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity? (Fill in one answer.)

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
○	○	○	○

9. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done? (Fill in one answer.)

No problem at all	Only a very slight problem	Somewhat of a problem	A very big problem
○	○	○	○

10. Do you have a bed partner or roommate? (Fill in one answer.)

No bed partner or roommate	Partner/roommate in other room	Partner in same room, but not same bed	Partner in same bed
○	○	○	○

If you have a roommate or bed partner, ask him/her how often in the past month you have had...
(Fill in one answer for each item.)

	NOT DURING THE PAST MONTH	LESS THAN ONCE A WEEK	ONCE OR TWICE A WEEK	THREE OR MORE TIMES A WEEK
Loud snoring	○	○	○	○
Long pauses between breaths while asleep	○	○	○	○
Legs twitching or jerking while you sleep	○	○	○	○
Episodes of disorientation or confusion during sleep	○	○	○	○
Other restlessness while you sleep...	○	○	○	○

Please describe: _____

_____

_____

Short-Form McGill Pain Questionnaire-2 (SF-MPQ-2)

R.H. Dworkin et al. / PAIN® 144 (2009) 35–42

Short-Form McGill Pain Questionnaire-2 (SF-MPQ-2)

This questionnaire provides you with a list of words that describe some of the different qualities of pain and related symptoms. Please put an X through the numbers that best describe the intensity of each of the pain and related symptoms you felt during the past week. Use 0 if the word does not describe your pain or related symptoms.

1. Throbbing pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
2. Shooting pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
3. Stabbing pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
4. Sharp pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
5. Cramping pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
6. Gnawing pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
7. Hot-burning pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
8. Aching pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
9. Heavy pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
10. Tender	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
11. Splitting pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
12. Tiring-exhausting	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
13. Sickening	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
14. Fearful	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
15. Punishing-cruel	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
16. Electric-shock pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
17. Cold-freezing pain	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
18. Piercing	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
19. Pain caused by light touch	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
20. Itching	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
21. Tingling or 'pins and needles'	none	0	1	2	3	4	5	6	7	8	9	10	worst possible
22. Numbness	none	0	1	2	3	4	5	6	7	8	9	10	worst possible

[1] McDermott Center for Evidence-Based Medicine, Assessment, and Pain Assessment in Clinical Trials (IMMPACT).
Information regarding permission to reproduce the SF-MPQ-2 can be obtained at www.immpact.org

Appendix B: Standardized Questionnaire Instructions

Ratings of Perceived Exertion Instructions

- This first scale is used to rate your perceived exertion, which is how hard and strenuous the exercise feels to you (**show RPE scale**). The perception of exertion depends mainly on the strain and fatigue in your muscles and on your feelings of breathlessness.
- As you can see, this rating scale contains the numbers 6-20, where 6 is no exertion at all (e.g., sitting still and resting) and 20 is your maximal exertion (e.g., as hard as you could exert yourself).
- We'll prompt you to rate your level of exertion by showing you the scale and asking you **"how hard are you currently working"?**

6	No exertion at all
7	
8	Extremely light
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard (heavy)
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion

Borg RPE scale

Leg Muscle Pain Instructions

- We will also ask you about the intensity of pain you feel in your exercising leg muscles (i.e., quadriceps, hamstrings, calves). This is defined as the intensity of hurt that you feel in your leg muscles. When rating these pain sensations, be sure to attend only to the specific sensations in your exercising leg muscles and not report other pains you may be feeling (e.g., knee pain).
- The numbers on the scale represent a range of pain intensity from "very faint pain" (number ½) to "extremely intense pain-almost unbearable" (number 10). When you feel no pain in your legs, you should point to the number 0. If your legs feel extremely strong pain that is almost unbearable, you should point to the number 10.
- We'll prompt you to rate your leg muscle pain by showing you the scale and asking you **"how much pain are you currently feeling in your leg muscles?".** It is very important that your ratings of pain intensity reflect only the degree of hurt you are feeling in your legs at that specific moment in time. Do not use your ratings as an expression of feelings of fatigue or relief that the exercise task is completed.

MUSCLE PAIN INTENSITY SCALE

0 NO PAIN AT ALL

½ VERY FAINT PAIN (just noticeable)

1 WEAK PAIN

2 MILD PAIN

3 MODERATE PAIN

4 SOMEWHAT STRONG PAIN

5 STRONG PAIN

6

7 VERY STRONG PAIN

8

9

10 EXTREMELY INTENSE PAIN (almost unbearable)

λ UNBEARABLE PAIN

Overall Fatigue Instructions

- Finally, your will use this scale to assess your overall feelings of fatigue. **[show fatigue scale]** This scale ranges from 0 = "no fatigue" to 10 = "highest fatigue imaginable" at the other end. In this context, fatigue is defined as the feeling of having a reduced capacity to complete activities. In other words, think about the mental and physical tasks that you normally perform on a daily basis. A higher rating of fatigue would mean that you don't feel as though you can get done what you're normally able to do. Do not use your ratings as an expression of pain or exertion. Keep in mind, this rating is for your overall feelings of fatigue – it is not specific to your exercising muscles.
- We will prompt you for a fatigue rating by showing you the scale and asking **"what is your current level of overall fatigue?"**
- Please keep in mind that even though we are measuring fatigue responses to exercise, we will also use this scale to measure how fatigued you are feeling prior to exercise. Any questions?

OVERALL FATIGUE

0 NO FATIGUE

1

2

3

4

5

6

7

8

9

10 HIGHEST FATIGUE IMAGINABLE

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