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Peripheral Biomarkers of Inflammation Following Blast Exposure in a Clinical Population

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PERIPHERAL BIOMARKERS OF INFLAMMATION FOLLOWING BLAST EXPOSURE IN A CLINICAL POPULATION

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ABSTRACT

Concussions resulting from blast exposures represent a significant source of injury among military service members and the civilian population. Overall, traumatic brain injuries (TBIs) are a significant cause of hospitalization, disability, long-term care, and mortality across all age groups in the United States. Blast induced traumatic brain injury (biTBI) is an increasingly recognized subtype of brain injury, especially among military personnel. Blast exposure may influence a number of neurological processes, such as the inflammatory response, representing a unique biological profile. Outcomes from a TBI vary, even in similar injuries, and biomarkers including proteins and gene expression are increasingly studied to determine potential underlying mechanisms of injury and recovery processes. Biomarkers may yield insight into differential biological pathways in the various severities and subtypes of brain injury. This novel study proposes the examination of clinical and demographic characteristics and the identification of possible biological mechanisms through gene expression and protein analysis following brain injury. This study will be the first to examine gene expression related to inflammatory activation using sequencing and other unique methods to gain insight into immune pathways following blast exposure in clinical populations during the acute and subacute stages of injury. A deeper understanding of the role of inflammatory activation profiles will help direct future research in blast exposure and improve outcomes for individuals affected by this injury.

Keywords: concussion, cytokines, inflammation, biomarkers, RNA sequencing

DEDICATION

This work is dedicated to my kind, loving, and patient husband, Tyler Edwards.

You are the best part of my life.

I would not have dreamed of pursing a PhD without you.

Thank you for believing in me.

This PhD work would not have been possible without the love and support of my dear family and friends. To my Mom and Dad, who have always encouraged me to pursue my dreams. To my brother, Seth, and his wife Cody, whose lives are an inspiration to me. To my grandfather, Kenneth, who is prouder of me than I will ever deserve. To my beloved grandparents in heaven, Louise, Esther, and Chester, who have left me a legacy of loving memories. To countless family members and friends, who have given unending words of encouragement. The kindness and support I have received from each of you means the world to me. I am truly blessed to know and be loved by all of you.

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CHAPTER ONE

INTRODUCTION

Dissertation Manuscript Outline

This dissertation manuscript is prepared in accordance with the guidelines set forth by the Clemson University Graduate School and the School of Nursing Healthcare Genetics Program's Article-Style Format for Dissertation. The manuscript is comprised of five chapters. Chapter One outlines the problem and significance of the research as well as provides an overview of the research methodologies as they relate to each article. Chapter Two provides context for the research in an in-depth literature review of cytokine and gene expression studies in human TBI populations. Chapter Three explores the bench research findings for gene expression inflammatory pathways altered following blast exposure. Chapter Four examines laboratory findings of changes in inflammatory cytokines following concussion and blast exposure. Finally, Chapter Five provides an overview of the research findings and implications for healthcare genetics, considers strengths and limitations of the work, and offers future directions for research. Chapters Two and Three are the articles submitted for consideration of publication, and chapter Four is to be submitted for publication.

Statement of the Problem

Protein and gene expression biomarkers are well-acknowledged in the literature for potential clinical utility among traumatic brain injury (TBI) patient populations (Di Battista et al., 2015). However, the biological role of these biomarkers in mild TBI

pathologies has remained elusive, specifically in concussions occurring with blast exposures in military personnel. This information is needed to improve the health of military personnel who experience concussion, as there are few ways to determine the impact on health. Further research would also help to inform decisions regarding return to duty or training in order to prevent potential negative consequences of additional exposures on neuronal health (DePalma, 2015; Ruff, Riechers, Wang, Piero, & Ruff, 2012). Table 1-1 defines key terms used throughout this manuscript.

Table 1-1.

Key terms in this manuscript**.**

Term	**Definition**
Blast	A shock (i.e. *overpressure*) wave formed by an explosion to cause a solid or liquid quickly converted to a gas form resulting in a release of energy. The shock wave travels at supersonic speeds of 3,000-8,000 m/sec (Ritenour & Baskin, 2008; Wightman & Gladish, 2001)
Blast injury	Within the central nervous system, injury from a shock wave can cause damage including the neurons, blood brain barrier, and cerebrovascular system (Ritenour & Baskin, 2008). Also referred to as blast induced TBI, or biTBI, in the literature.
Blunt force head injury / Closed head injury	A blow to the head results in brain injury. The skull remains intact.
Concussion	Defined by one of the following: 1) an alteration in mental state, loss of memory; 2) loss of consciousness for less than 30 minutes; or, 3) another focal neurological deficit. Also called a mild traumatic brain injury (mTBI) in the literature (Menon, Schwab, Wright, & Maas, 2010).
Cytokine *Pro-inflammatory* *Anti-inflammatory*	Small proteins released by leukocytes (white blood cells) and *glial cells* (i.e. *microglia*) that function in mediating inflammatory response (Woodcock & Morganti-Kossmann, 2013). Cytokines that activate the immune response. Cytokines that induce activity mitigating the immune response, such as clearing debris.
Dendritic spine	Part of a neuron that receives, stores, and sends neurotransmitters.

Gene expression	The information from a gene is transcribed into a functional product. The primary control for this process occurs when the messenger RNA (mRNA) is transcribed, which was studied in Chapter 3.
*Gene network *Hub*	Connected molecular pathway that regulates gene expression. A connection, such as a gene, that has multiple interactions within the network. For example, the gene *AKT1* in Chapter 3.
Glial cells	The most numerous cells in the central nervous system; function in maintenance and support for neural cells. *Microglia* are one type of glial cell.
Interleukin-6 (IL-6)	Traditionally classified as a pro-inflammatory cytokine, though may also have anti-inflammatory properties.
Interleukin-10 (IL-10)	Traditionally defined as an anti-inflammatory cytokine.
Microglia	The primary immune cell type in central nervous system and the first to respond to injury or pathogens. Direct the inflammatory response through release of *cytokines* and other inflammatory-related products (Hendriksen, van Bergeijk, Oosting, & Redegeld, 2017).
Moderate blast exposure	The force of the blast experienced by military personnel described in Chapter 3 (≥5psi). This definition has been specified by the military collaborators involved in this work.
Neddylation	A type of ubiquitination; also regulates dendritic spine development (Vogl et al., 2015).
Nuclear factor kappa light-chain enhancer of activated B cells (NF-κB)	Among its many functions, is a master regulator of cytokines, such as IL-6 and TNFα.
Operation Iraqi Freedom (OIF) and	OEF, also known as the Global War on

Operation Enduring Freedom (OEF)	Terrorism, began in 2001 with targeting al Qaeda and the Taliban in Afghanistan. OIF began in 2003 with the United States invasion of Iraq. Samples for Chapter 4 of this study are from Afghanistan.
Penetrating head injury	The skull is perforated, such as by a high speed projectile, object, or bone fragment.
Traumatic brain injury (TBI)	A biomechanical force to the head, with or without direct impact, resulting in pathological changes in the brain.
Tumor necrosis factor α (TNFα)	Traditionally classified as a pro-inflammatory cytokine
Ubiquitination	The process of removing of oxidized and misfolded proteins following injury, which can protect neurons from reactive oxidative species (ROS)

Note. See also Chapter 3 for a legend to interpret gene networks.

Significance of Concussion and Blast Exposure in the Military

In Chapters Three and Four, the results of studies in military personnel experiencing concussion and blast exposure are discussed. Briefly, concussion, also known as mild TBI (mTBI) in the literature, is considered one of the most prevalent injuries among military personnel serving in recent combat and training environments (Hayward, 2008; Mac Donald et al., 2014). Approximately 80% of concussions occurring among military personnel are caused by blast exposures (Defense and Veterans Brain Injury Center, 2017; Rigg & Mooney, 2011). However, due to co-occurrence of multiple injuries common at the time of a blast exposure, blast injury to the brain is often difficult to study alone (Champion, Holcomb, & Young, 2009). Evidence of long-term neurological effects shown in the literature highlight the need for deeper understanding of the pathophysiology of underlying chronic symptoms and the need to produce data to

inform the development of novel treatments for mTBIs (Carr et al., 2015; Echemendia & Julian, 2001; Giza & Hovda, 2001; Reid et al., 2014; Schatz & Moser, 2011). Although medical care of TBI patients has advanced, at this time there are no FDA-approved pharmaceuticals specifically addressing TBI pathology (Hinson, Rowell, & Schreiber, 2015; Maas, Stocchetti, & Bullock, 2008). Biomarkers, including gene expression and proteins, introduced in Chapter One and detailed in the Chapter Two literature review, may ultimately identify therapeutic targets to improve the care of patients and foster recovery from TBIs (Di Battista et al., 2015; Hinson et al., 2015). Thus, this line of research is vital, as biomarkers will ultimately improve diagnosis, prognosis, and care for patients with concussion.

Significance of the Inflammatory Response in Concussion and Blast Exposure

Recent studies report that serum biomarkers may objectively detect blast exposures, as compared to trauma controls, even in the absence of physical symptoms (Papa et al., 2016; Papa et al., 2012). Taken together with literature indicating the harmful effects of neurological insults over time, further evaluation of potential biomarkers to inform diagnosis and prognosis of concussion and blast exposure is needed (Echemendia & Julian, 2001; Giza & Hovda, 2001; Schatz & Moser, 2011).

Introduction to gene expression.

The Chapter Two literature review highlights the importance of gene expression to TBI research, in addition to cytokine activity. Importantly, considered a master regulator of cytokines, nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) is a transcriptional activator of target genes involved in numerous biological

functions including the development of immune cells such as leukocytes and regulation of the expression of cytokines and chemokines (Barichello, Generoso, Simoes, Elias, & Quevedo, 2013; Kawai & Akira, 2007). Though the NF-κB pathway regulates cytokines and has been implicated in clinical TBI gene expression studies (see review in Chapter Two), the dynamics of the NF-κB pathway together with inflammatory cytokine alterations within the context of clinical blast exposure has not yet been fully explored. Chapter Three describes the details of the gene expression study for this dissertation.

Introduction to cytokines.

In addition to gene expression, the Chapter Two literature review underscores the importance of inflammatory cytokines for understanding neurological recovery processes in persons with TBI. Clinical studies show that inflammatory biomarkers, including immune cell counts and cytokine concentrations, are associated with sustaining a TBI when measured during the acute period, in coordinating recovery during the acute and sub-acute periods, and have been proposed as a possible therapeutic target after TBI (McKee & Lukens, 2016; Schwarzmaier & Plesnila, 2014). Cytokines are especially interesting to the study of concussion as they may serve as practical clinical measures at the bedside as well as reveal underlying inflammatory processes (Hinson et al., 2015; Woodcock & Morganti-Kossmann, 2013). Chapter Four describes the results of the cytokine study for this dissertation.

Summary

In Chapter One, a brief overview was included to underline the significance of this research. Considering the limited amount of current research in inflammatory and

immune pathways related specifically to clinical blast exposure and concussion, literature related to inflammatory markers clinical TBI populations will be reviewed in greater detail in Chapter Two. Chapter Three describes results of the gene expression study for this dissertation, while Chapter Four describes the results of the cytokine study. Research questions as they relate to the studies in Chapters Three and Four are introduced below for guidance through the dissertation manuscript.

Research Questions

The research questions are addressed together in the introduction to better explain the collective goals for this dissertation. Specific Aims are addressed separately in Chapters 3 and 4 as noted below in order to allow details for each study.

Background: Concussions, including those caused by blast exposures, are associated with poor outcomes among the military population. Blast exposures result in altered neurological processes such as inflammatory pathways. Alterations in the NF-κB network, a known regulator of cytokines produced during inflammation, have been identified in clinical TBI. However, the NF-κB gene pathway has not been fully explored following injury specific to blast exposures.

Purpose: The purpose of this study is to examine gene expression related to inflammatory activation using sequencing and protein analyses to gain insight into inflammatory pathways following blast exposure in military personnel.

Aim One (Chapter 3): In a military training environment, determine gene activity changes related to NF-κB through RNA sequencing following blast exposure.

Hypothesis 1a: Blast exposure will result in alterations in NF-κB inflammatory-related gene expression pathways during the sub-acute period.

Aim Two (Chapter 4): In a combat environment, determine changes in NF-kb activity detected through cytokine activity immediately following concussions (blast exposures and blunt force injuries).

Hypothesis 2a: Compared to healthy controls, there will be increased levels of inflammatory cytokines including IL-6, IL-10, and TNFα in the concussed group within 8 hours following injury.

Hypothesis 2b: Compared to healthy controls, levels of inflammatory cytokines including IL-6, IL-10, and TNFα will return to baseline levels within 24 hours of injury in the concussed group.

Hypothesis 2c: Mean change over time for each cytokine (IL-6, IL-10, and TNFα) will be significantly different in the concussed group as compared to the healthy control group.

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A REVIEW OF GENE EXPRESSION AND INFLAMMATORY RESPONSE IN CLINICAL POPULATIONS OF TRAUMATIC BRAIN INJURY

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ABSTRACT

Traumatic brain injuries (TBIs) are a significant cause of hospitalization, disability, long-term medical care cost, and mortality across all age groups in the United States and across the world. Outcomes from a TBI vary, even in patients with similar severity and type of injuries, yet, identifying those patients at highest risk for non-optimal recovery remains difficult. Biomarkers including proteins and gene expression are an increasingly studied area, as they provide a platform to identify underlying mechanisms of injury, patients at risk for poor recovery and recovery processes to inform therapeutics. Initiation of the inflammatory system is fundamental to recovery from TBIs; however, if it is over-activated or prolonged it may compromise recovery and lead to more chronic symptoms. The purpose of this literature review is to examine recent clinical studies of gene expression in traumatic brain injury and related proteomic pathways, with a focus on characterizing the role of inflammation in recovery from TBIs, as well as how it may shape more chronic symptoms. This review identified 5 papers that report altered inflammatory gene regulation and 17 papers that report altered cytokines as related to recovery from TBIs. This paper will link these gene-expression studies to inflammatory activation studies and provide an indication of how these acute changes in gene-activity may shape immune response to TBIs and recovery. A deeper understanding of the role of immune activity following a TBI will ultimately direct future research for the improvement of outcomes for individuals affected by this injury.

Keywords: traumatic brain injury, cytokines, biomarkers, gene expression

CHAPTER TWO

A REVIEW OF GENE EXPRESSION AND INFLAMMATORY RESPONSE IN CLINICAL POPULATIONS OF TRAUMATIC BRAIN INJURY

Introduction

The dissertation research topic is inflammation, as characterized by gene expression and cytokine changes, in military personnel with acute blast exposures and concussions. In undertaking a literature review of this topic, very little information is available to date in clinical populations. Considering the recent reviews of blast exposure in preclinical models, and the limited amount of research in inflammatory pathways related specifically to clinical blast exposure or concussion, a review of gene expression and cytokine changes as it relates to the broader population of clinical TBI was undertaken (Xiong, Mahmood, & Chopp, 2013). This decision to expand to the broader TBI category was made with the knowledge that not all of this information may translate to the specific population represented in this dissertation research. However, the goal was that the knowledge gained from review of studies of human TBI populations would be used establish the current state of the science in order to inform the design and methodologies of the dissertation research. Additionally, rather than relying on one methodology, the integration of -omics data, such as gene expression *and* protein analysis, in the design of research studies has been postulated to strengthen the understanding of the complex relationships between genotype and phenotype (Ritchie, Holzinger, Li, Pendergrass, & Kim, 2015). Any knowledge thus gained from the subsequent research studies, detailed in Chapters 3 and 4, would then add to the growing

field of gene express and cytokine research in persons with blast exposure and concussion.

Significance of concussion and blast exposure in the military.

Concussion, also known as mild TBI (mTBI) in the literature, is considered the signature injury among military personnel serving in recent combat and training environments (Hayward, 2008; Mac Donald et al., 2014). The vast majority (80%) of concussions occurring among military personnel are caused by blast exposures (Defense and Veterans Brain Injury Center, 2017; Rigg & Mooney, 2011). Over 360,000 individuals serving in Operation Iraqi Freedom and Operation Enduring Freedom since the year 2000 have experienced at least one blast injury, with most exposures due to improvised explosive devices (IEDs) (Champion, Holcomb, & Young, 2009; Defense and Veterans Brain Injury Center, 2017; Elder, Stone, & Ahlers, 2014; Hayward, 2008).

Significance of traumatic brain injury.

Traumatic brain injury (TBI) is a significant healthcare issue, effecting 2.5 million Americans each year and leading to 30% of all injury-related deaths (Taylor, 2017). Sources of injury may include falls, motor vehicle accidents, assaults, and blunt trauma. TBIs occur across all populations, regardless of age, ethnicity, socioeconomic status, or sex. TBIs can result in long-lasting disabilities for the injured person, effecting quality of life for both the person and his or her family members (Taylor, 2017). TBI care is estimated to cost up to $76.5 billion each year in the US from medical care costs and loss of work (Coronado et al., 2011; Ma, Chan, & Carruthers, 2014). Significant savings of $2.2 million ($p<0.05$) in projected life care cost for individuals who undergo

rehabilitation therapies in the post-acute TBI stage have been reported (Griesbach, Kreber, Harrington, & Ashley, 2015). A review of the societal economic burden of TBI concluded that successful rehabilitation treatments could result in substantial annual savings for society—up to $302 million (Humphreys, Wood, Phillips, & Macey, 2013). Decreasing the substantial economic burden to society and individuals is an important motivator to improve the clinical care of TBI patients, thereby reducing the symptoms and deficits that can result. Though medical care of TBI patients has advanced, at this time there are no FDA-approved pharmaceuticals or non-pharmacological interventions to reduce the risk of developing symptoms acutely, or to treat symptoms and deficits if they become chronic (Maas, Stocchetti, & Bullock, 2008).

Definition of TBI.

A TBI is defined as a biomechanical force to the head, with or without direct impact, resulting in pathological changes in the brain, and include both blunt force and blast related injuries (McCrory et al., 2013; Menon, Schwab, Wright, & Maas, 2010). TBIs are categorized into mild, moderate, and severe, most often using the Glasgow Coma Scale (GCS), a tool developed by Teasdale and Jennett (1974) classifying subjects based on initial clinical exam. The mTBIs account for approximately 80% of traumatic brain injuries (Ruff, Iverson, Barth, Bush, & Broshek, 2009). As defined by the American Congress of Rehabilitation Medicine, a mTBI is characterized by one of the following: loss of memory, loss of consciousness (<30 minutes), alteration in mental state, or any focal neurological deficit. Exclusion criteria for this category of TBI include: GCS of <13 after 30 minutes, loss of consciousness for >30 minutes, and posttraumatic amnesia

lasting >24 hours (Menon, Schwab, Wright, Maas, et al., 2010). Traditionally mTBIs have been believed to do little or no long-term harm, even though approximately 10% of patients experience ongoing complications (Carroll et al., 2004; Cassidy et al., 2014). Accumulating recent research demonstrates long-term detrimental effects of mTBIs, with the greatest risk in those individuals who sustain multiple events of TBIs (Echemendia & Julian, 2001; Giza & Hovda, 2001; Schatz & Moser, 2011). These long-term effects highlight the need for additional study in the pathophysiology and treatments for mTBIs. Biomarkers obtained in peripheral blood and cerebral spinal fluid (CSF), including gene expression and proteins described in the literature review below, are a current advancement that may aid in the care of individuals with TBIs in order to help improve outcomes. Summarized in this paper are the potential clinical utility of biomarkers for the improved diagnosis, prognosis, and individual treatment plans for patients increasingly supported by the literature, as well as future directions (Di Battista et al., 2015).

TBI from blast exposure.

Brain injury from primary blast injury occurs due to a shock (i.e. blast or overpressure) wave formed by an explosion (Ritenour & Baskin, 2008; Wightman & Gladish, 2001). Resulting tissue damage depends upon factors such as the magnitude of the peak pressure and the duration of the force, as well as enclosures which cause the shock waves to bounce thereby intensifying the risk of injury (Rezaei, Salimi Jazi, & Karami, 2014; Wightman & Gladish, 2001). The energy from a blast enters the body as stress waves and shear waves. Stress waves are longitudinal waves affecting the spaces between tissues and gases, resulting in tissue and microvascular damage; while shear

waves are transverse waves causing disruption in attachments between tissues (Champion et al., 2009; Ritenour & Baskin, 2008; Yeh & Schecter, 2012). Thus, tissues likely to be damaged are those in contact with gaseous regions, such as the middle ear, lungs, and bowel, as well as the central nervous system (Bochicchio et al., 2008; Kirkman & Watts, 2011; Mac Donald et al., 2011; Ropper, 2011; Wightman & Gladish, 2001). Brain injury results when shearing forces from the explosion result in diffuse or axonal injury to the brain, and may also induce cerebrovascular damage and blood brain barrier disruption (Cernak, Wang, Jiang, Bian, & Savic, 2001; Ritenour & Baskin, 2008; Yeoh, Bell, & Monson, 2013). Whether the clinical presentation and pathophysiology of concussion caused by blast is distinct from a penetrating or closed head TBI remains to be determined (Courtney & Courtney, 2015; Mac Donald et al., 2014). However, due to co-occurrence of multiple injuries common at the time of a blast exposure, blast injury to the brain is often difficult to study alone (Champion et al., 2009).

The need for research in mTBI and blast exposure.

One in ten patients with mTBI will continue to experience long-term complications (Carroll et al., 2004; Cassidy et al., 2014). Specifically, evidence of chronic neurological effects has been shown in repeated concussions and post-concussive syndrome as well as chronic low-level blast exposure (Carr et al., 2015; Echemendia & Julian, 2001; Giza & Hovda, 2001; Reid et al., 2014; Schatz & Moser, 2011). These long-term effects underscore the need for deeper understanding of the pathophysiology of underlying chronic symptoms and the need to produce data to inform the development of novel treatments for mTBIs. Although medical care of TBI patients has advanced, at this

time there are no FDA-approved pharmaceuticals specifically addressing TBI pathology (Hinson, Rowell, & Schreiber, 2015; Maas et al., 2008). Recent evidence suggests that serum biomarkers may objectively detect blast exposures as compared with trauma controls (Papa et al., 2016; Papa et al., 2012). Gene expression and protein biomarker research is essential, as biomarkers may ultimately identify therapeutic targets to improve care and foster recovery for persons with TBIs (Di Battista et al., 2015; Hinson et al., 2015; Prieto, Ye, & Veenstra, 2008).

Statement of the Problem.

Although the potential clinical utility of biomarkers such as proteins and gene expression in patient care is well recognized, the role in various TBI pathologies has yet to be fully realized. Inflammation is a key pathway required for recovery from TBIs, but much remains unknown about the characteristics of activation and regulation that likely contribute to acute and long-term recovery. The purpose of this literature review is to examine the current state of clinical TBI research in gene expression and inflammatory biomarkers.

Literature Review

Method.

Considering the limited current research in cytokines and gene expression pathways related specifically to clinical blast exposure or concussion, literature related to inflammatory markers in acute TBI clinical populations was reviewed. The electronic database PubMed was systematically searched from November 1, 2016 to January 30, 2017. The searches were updated September 2017. Search terms for gene expression

studies included: brain injury and gene expression. Search terms for inflammatory protein marker studies included: brain injury, cytokines, IL-6, IL-10, and TNFα. Published, full-text, original research articles in English appearing in peer-reviewed journals over the past 10 years were included. The search was limited to human populations. Articles meeting these criteria were screened for eligibility based on original studies in human populations. Articles for the gene expression review included adults who sustained a mild or moderate TBI. Articles for the inflammatory protein markers review included adolescents and adults who sustained a mild, moderate, or severe TBI, due to the limited number of mild and moderate TBI articles. Articles meeting the eligibility criteria were screened for inclusion. The following criteria caused articles to be excluded: studies of animals and cell lines, pediatric populations, diagnoses other than TBI, research older than 10 years, reviews, case studies, and those that did not include gene expression or inflammatory protein markers.

Results for gene expression. After applying the inclusion and exclusion criteria to 6,596 titles and abstracts, 952 full-text articles were assessed for eligibility, with only 5 of these articles evaluating gene expression in adults with mild to moderate traumatic brain injury.

Results for inflammatory protein markers. Following application of the inclusion and exclusion criteria to 6,452 titles and abstracts, 1,265 full-text articles were eligible for screening, with 17 articles that evaluated inflammatory protein markers in the adult population with mild, moderate, or severe brain injury.

Gene Expression Studies

Background.

Several clinical studies show that gene expression varies after TBI. Gene expression is the process by which a sequence of nucleic acids in a gene (i.e. genotype) are transcribed into ribonucleic acid (RNA) and translated into protein, which ultimately gives rise to the phenotype, or expressed traits, of an organism (Raser & O'Shea, 2005). Thus, methods of measuring gene expression can be accomplished at the RNA level by examining the activity of genes. Often, gene expression methods in the literature are referring to measurement of the messenger RNA (mRNA), the RNA molecules which are translated into proteins (Wickramasinghe & Laskey, 2015). mRNA is measured through a variety of technologies, notably DNA microarray, Northern Blot, real time PCR, and, most recently, high-throughput RNA sequencing methods (RNA-seq) (Bolón-Canedo, Sánchez-Maroño, Alonso-Betanzos, Benítez, & Herrera, 2014; Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008; Wang, Gerstein, & Snyder, 2009).

Clinical Studies.

The literature review returned five clinical studies of gene expression in TBI. Results from the studies are summarized in Table 2-1 and Table 2-2.

Table 2-1.

Summary of gene expression literature review results

Reference	Platform	Specimen Source	Population	Blood Draw Timepoints	Total Number of Differentially Expressed Genes
Cho et al. (2016)	Affymetrix qPCR	Peripheral whole blood	mTBI (total n=66) Young (19-35 years old, n=33) Old (60-89 years old, n=33)	*Acute* <48 hours after injury *Subacute* 1 week after injury	Young: 42 Old: 5 Young: 28 Old: 1
Gill et al. (2016)	Affymetrix	Peripheral blood mononuclear cells	Athletes Sports-related concussion (n=15) Non-concussed controls (n=16)	*Baseline* (before injury) *Acute* <6 hours after injury *Subacute* 1 week after injury	 71 65
Merchant-Borna et al. (2016)	Affymetrix	Peripheral blood mononuclear cells	Athletes Sports-related concussion (n=15) Non-concussed controls (n=16)	*Baseline* (before injury) *Acute* <6 hours after injury *Subacute* 1 week after injury	 71 65
Livingston et al. (2016)	Affymetrix	Peripheral whole blood	mTBI (total n=40) TMI+ (n=17) TMI- (n=23)	*Acute* <48 hours after injury	76
Heinzelmann et al. (2014)	Affymetrix	Peripheral whole blood	Military, mild to moderate TBI blast-TBI (n=19) Controls without TBI (n=17)	*Chronic* symptoms </=18 months after injury	29

Table 2-2

Summary of differentially expressed gene pathways from literature

Reference	Gene Pathways	Representative Genes	Gene name	Fold change	P value
Cho et al. (2016)	Cell signaling, development, growth, and proliferation	*BACH2*	Basic leucine zipper transcription factor 2	1.616	*
	Intracellular regulation of calcium	*S100P*	S100 calcium binding protein P	1.954	*
		S100A8	S100 calcium binding protein A8	1.515	*
		LRRN3	Leucine-rich repeat neuronal 3	2.849	*
		LEF1	Lymphoid enhancer binding factor 1	1.539	*
		NOG	Noggin	1.852	*
Gill et al. (2016)	Nuclear factor kappa light-chain enhancer of activated B cells (NF-ĸB)	*IL8*	Interleukin 8	acute -6.94 subacute -13.80	 1.18E-04 2.08E-07
		CXCL2	Chemokine (C-X-C motif) ligand 2	acute -4.47 subacute -7.11	 1.38E-04 3.77E-07
		NR4A2	Nuclear receptor subfamily 4, group A, member 2	acute -7.12 subacute -6.63	 5.87E-07 5.39E-08
Merchant-Borna et al. (2016)	Inflammatory Response, Infectious Disease, Renal and Urological Disease Glucocorticoid Receptor Signaling Neurological Disease, Cell Death and Survival, Cell Cycle	Hubs: *IL-6* *IL-12* *TRL4* *NF-κB*	 interleukin 6 interleukin 12 toll-like receptor 4 Nuclear factor kappa light-chain enhancer of activated B cells	**	**
Livingston et al. (2016)	Inflammatory pathways related to cellular development Organismal Injury and Abnormalities	*LOC100134822* *FcαR (aka CD89)*	Uncharacterized LOC100134822 Fc fragment of IgA, receptor for	1.62 1.58	5.05E-05 4.81E-08

	Cellular Organization Nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) pathway	*MCTP2* *GPR27*	Multiple C2 domains, transmembrane 2 G-protein-coupled receptor 27	1.54 1.52	1.85E-05 5.88E-05
Heinzelmann et al. (2014)	Ubiquitin pathway	*TNS1* *MARCH8* *TRIM58*	Tensin-1 Membrane-associated ring finger (C3HC4) 8, E3 ubiquitin protein ligase Tripartite motif containing 58	-2.3682 -1.6123 -1.9188	0.00062 0.00065 0.00012

Note. For the Cho et al. (2016) study, values are reported at the 48-hour time period; fold changes were also significant for all reported genes at one week. Positive numbers indicate upregulation; negative numbers indicate downregulation. *p < 0.05; **denotes differently expressed gene network.

One study aimed to compare gene expression in older (60-89 years old) and younger (19-35 years old) cohorts of mTBI patients within 24 hours of injury (Cho et al., 2016). Notably, being "older," has consistently been linked to a greater risk of poor recovery in clinical TBI studies (Hukkelhoven et al., 2003; McIntyre, Mehta, Janzen, Aubut, & Teasell, 2013). Cho et al. (2016) found that, compared to younger patients, the older patients experienced overall worse recovery from injury as determined by magnetic resonance imaging (MRI) one-week following the TBI. The MRI findings were linked to differential gene activation, including several genes involved in the inflammatory response [measured using GeneChip 3' IVT Plus Expression kit (Affymetrix, Santa Clara, CA, USA)]. First, *LRRN3* and *LEF1*, genes implicated in regulation of inflammation, were highly upregulated in the younger cohort as compared to the older cohort. Lesser upregulation of these inflammatory genes suggests a decreased ability of the older population to modulate inflammatory responses. Second, there was a decreased

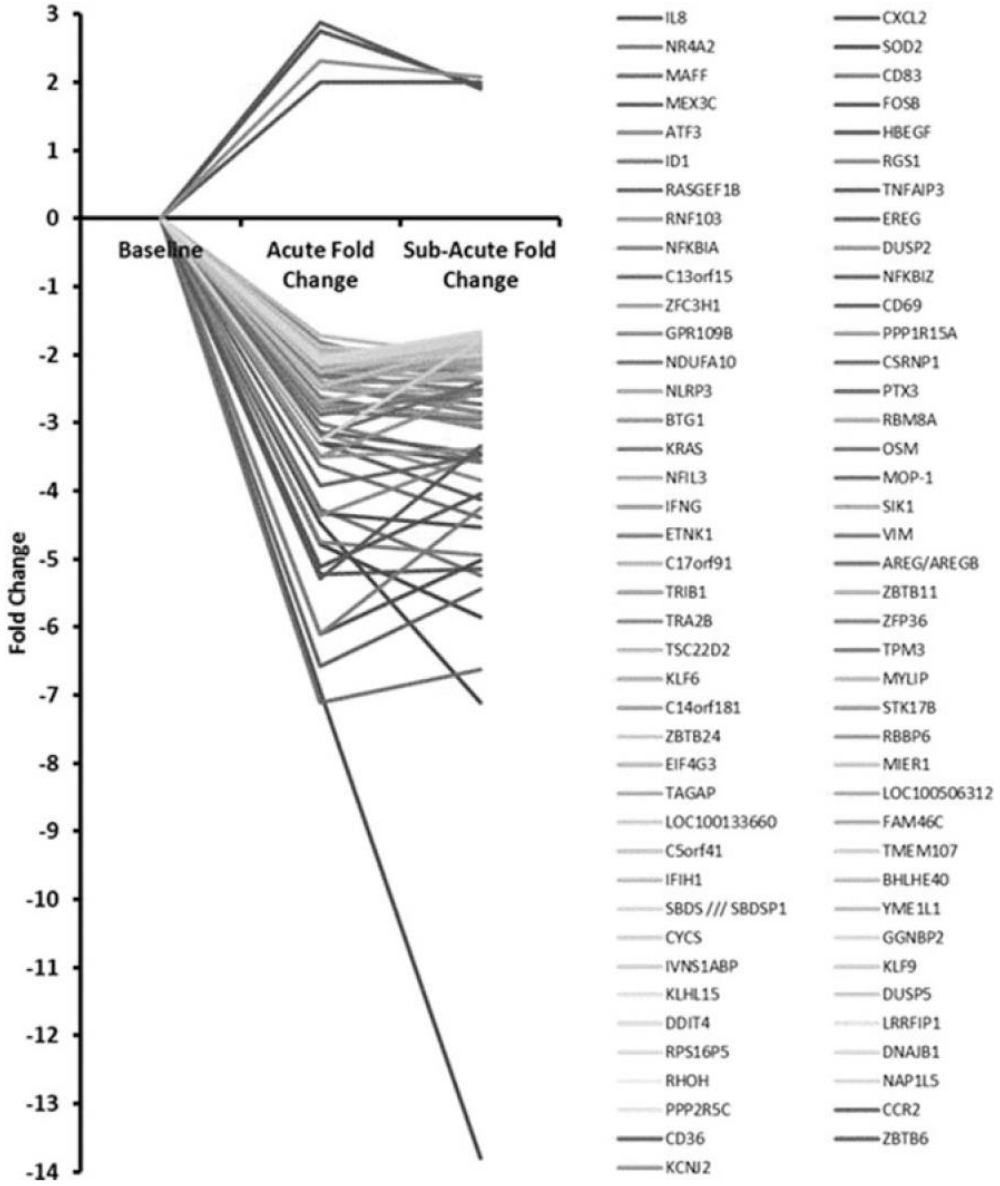
expression of *BACH2* gene that transcribes basic leucine zipper transcription factor 2 (BACH2) in older as compared to younger adults at 48 hours following injury. BACH2 is expressed in B cells, and it modulates the proinflammatory response in preclinical models (Muto et al., 2004; Roychoudhuri et al., 2013; Vahedi et al., 2015). Decreased *BACH2* gene expression may therefore lead to suppression of the neuroprotective humoral immune response in TBI. Third, in older individuals, neuronal recovery may be impaired through upregulation of the genes S100 calcium binding protein P (*S100P*) and S100 calcium binding protein A8 (*S100A8*). Both genes are part of the *S100* gene family involved in the regulation of intracellular calcium levels (Zimmer, Eubanks, Ramakrishnan, & Criscitiello, 2013), and previously associated with neuronal recovery following injury (Di Battista et al., 2015). *S100P* also activates signaling pathways such as NF-κB. Considered a master regulator of cytokines, NF-κB is a transcriptional activator of target genes involved in numerous biological functions including the development of immune cells such as leukocytes and regulation of the expression of cytokines and chemokines (Barichello, Generoso, Simoes, Elias, & Quevedo, 2013; Kawai & Akira, 2007). For example, one preclinical TBI study found that regulatory T cells decreased the expression of proinflammatory cytokines through suppression of the NF-κB pathway (Yu, Cao, Ran, & Sun, 2016). These gene expression results from Cho et al. (2016) suggest that regulation of immune and inflammatory responses as well as neuronal repair following TBI may vary across age groups, with maladaptive responses in older adults associated with worse outcomes. Further comparison of gene expression profiles, including inflammatory-related pathways such as NF-κB, across young and old

age groups may yield insight into the biological mechanisms which lead to better versus worse outcomes following TBI. Comparing these types of gene expression studies to neuroimaging findings may also yield further insight into neuronal changes occurring following injury.

A second study of acutely concussed collegiate athletes also implicated changes in inflammatory gene expression following injury (Gill et al., 2016). Biomarkers in whole blood collected following concussion was compared to baseline levels collected pre-season. Following head injury, 28 differentially expressed genes [Affymetrix HG U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA)] were associated with the inflammatory response, including the NF-κB pathway, as seen in Figure 2-1 (Gill et al., 2016). In the third gene expression study, further gene network analysis of this athlete population revealed interleukin 6 (*IL-6*), interleukin 12 (*IL-12*), and toll-like receptor 4 (*TRL4*) as hubs (see Table 1-1 for definitions) at 6 hours post-injury, in addition to *NF-κB* at both 6 hours and 7 days post-injury (Merchant-Borna et al., 2016) (see Figure 2-2). Together, these four hubs modulate both the innate immune response and the transition to the adaptive immune response, suggesting alterations in immune system functioning may influence neuronal recovery during the acute period (Merchant-Borna et al., 2016).

Figure 2-1.

Differential Gene Expression Following Concussion in Athletes



Note. Used with permission by:

Gill, J., Merchant-Borna, K., Lee, H., Livingston, W. S., Olivera, A., Cashion, A., . . . Bazarian, J. J. (2016). Sports-Related Concussion Results in Differential Expression of Nuclear Factor-kappaB Pathway Genes in Peripheral Blood During the Acute and Subacute Periods. *J Head Trauma Rehabil, 31*(4), 269-276. doi:10.1097/htr.0000000000000191

Figure 2-2.

Altered Gene Expression in the NF-κB Pathway Following Concussion in Athletes

Gene symbol	Gene title	Acute fold change	*P*	Subacute fold change	*P*
IL8	Interleukin 8	−6.94	1.18E-04	−13.80	2.08E-07
CXCL2*	chemokine (C-X-C motif) ligand 2	−4.47	1.38E-04	−7.11	3.77E-07
NR4A2*	nuclear receptor subfamily 4, group A, member 2	−7.12	5.87E-07	−6.63	5.39E-08
SOD2*	superoxide dismutase-2, mitochondrial	−4.78	1.26E-04	−5.85	3.65E-06
MAFF	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog F	−6.57	2.98E-06	−5.45	1.06E-06
CD83*	CD83 molecule	−4.27	8.12E-07	−5.23	5.87E-09
MEX3C	mex-3 RNA binding family member C	−5.22	4.16E-05	−5.14	5.18E-06
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	−6.11	1.15E-05	−5.02	3.74E-06
ATF3*	activating transcription factor 3	−4.76	5.56E-06	−4.95	3.51E-07
HBEGF*	heparin-binding EGF-like growth factor	−4.33	1.11E-05	−4.53	6.94E-07
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	−3.63	1.31E-04	−4.40	2.93E-06
RGS1*	regulator of G-protein signaling 1	−6.10	6.32E-06	−4.26	6.99E-06
RASGEF1B	RasGEF domain family, member 1B	−3.29	3E-07	−4.12	9.09E-06
TNFAIP3*	tumor necrosis factor, alpha-induced protein 3	−5.12	4.05E-07	−4.04	2.84E-07
RNF103	ring finger protein 103	−3.16	3.01E-04	−3.84	6.51E-06
EREG*	Epiregulin	−3.29	5.52E-04	−3.58	3.40E-05
NFKBIA*	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	−3.01	1.61E-06	−3.56	1.02E-08
DUSP2*	dual specificity phosphatase 2	−4.36	7.26E-06	−3.49	5.83E-06
C13orf15	regulator of cell cycle RGCC	−3.93	3.29E-07	−3.48	3.91E-07
NFKBIZ*	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	−3.14	9.21E-06	−3.44	3.10E-07
ZFC3H1	zinc finger, C3H1-type containing	−3.49	3.91E-06	−3.38	3.89E-07
CD69*	CD69 molecule	−5.28	1.40E-06	−3.34	8.57E-06

[a]An asterisk following the name of a differentially expressed gene identifies inflammatory-related genes.

Note. Used with permission by:

Merchant-Borna, K., Lee, H., Wang, D., Bogner, V., van Griensven, M., Gill, J., & Bazarian, J. J. (2016). Genome-Wide Changes in Peripheral Gene Expression following Sports-Related Concussion. *J Neurotrauma, 33*(17), 1576-1585. doi:10.1089/neu.2015.4191

Altered gene expression has also been observed in a fourth study of a subpopulation of mTBI patients with traumatic meningeal injury (TMI). Livingston et al. (2016) found 76 differentially expressed genes [Affymetrix HG-U133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA)] in patients positive for TMI (TMI+) (n=17) as compared to mild TBI patients with no neuroimaging findings (n=23). The altered genes

were found to be involved in three main gene networks. Four genes with the greatest fold changes were *LOC100134822*, *FcαR*, *MCTP2*, and *GPR27*, with *FcαR* implicated in inflammatory processes (Ben Mkaddem, Rossato, Heming, & Monteiro, 2013). In addition, three of these genes (*FcαR*, *MCTP2*, and *GPR27*) mapped to inflammatory processes in cellular development using Ingenuity Pathway Analysis (IPA). The most significantly altered gene pathway in TMI+ patients was the nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) pathway. While its role in TMI is yet to be studied, both findings suggest a potential biological pathway specific to patients with a meningeal injury; further studies are needed. To date, this is the only study examining gene expression in patients with a meningeal injury (Livingston et al., 2016).

Finally, in addition to these four acute/subacute studies, a chronic TBI study found 29 differentially expressed genes [Affymetrix GeneChip Human Gene U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA)] in a cohort of military personnel with medical history of blast-TBI (n=19) as compared to control military personnel with no TBI (n=17) (Heinzelmann et al., 2014). Genes within the ubiquitin pathway (*TNS1, C3HC4, MARCH8*, and *TRIM58*), which functions in the removal of oxidized and damaged proteins following neuronal injury, were notably down-regulated in the blast-TBI population, suggesting a role for this pathway in chronic symptoms following blast-TBI (Heinzelmann et al., 2014). Although this study differs from previously discussed studies regarding population type (military vs. civilian), outcomes over time (chronic vs. acute/subacute outcomes), and injury type (blast vs. closed head), the differential gene

expression results following neuronal insult contributes to the accumulating evidence for the roles of multiple biological pathways in TBI recoveries.

Summary.

Results from these five studies show that gene expression changes are observed in individuals following mild TBIs and concussions. Specifically, inflammatory gene-activity is related to response to these brain injuries. It is interesting to note that alteration of the NF-κB pathway is implicated in all four of the acute/subacute studies. Notably, the NF- κB pathway has been previously associated with the regulation of proinflammatory cytokines in meningitis (Barichello et al., 2013) as well as blood-brain barrier permeability (Merrill & Murphy, 1997). Based on the current state of research found here, and previous neurological-related work, further examination of the role of this NF-κB pathway in acute/subacute mTBI recoveries is warranted. These studies used variations of the Affymetrix microarray platform to examine gene expression differences. This platform has limitations, including batch effect, a recognized systematic error of microarray technology which occurs when many samples are processed in separate "batches" (Chen et al., 2011). No clinical TBI gene expression studies have yet utilized a more global RNA sequencing methodology. Therefore, additional studies are needed that use other methods for analyses, such as RNA-seq, and also include cohorts of patients with mild TBI and blast exposure.

Cytokines in Traumatic Brain Injury

The inflammatory response after a brain injury results in biological changes that are interrelated, including those of proteins and gene activity (Jassam, Izzy, Whalen,

McGavern, & El Khoury, 2017). Therefore, gene activity and proteins have complementary activities that coordinate the response to brain injury. Current studies examining gene-activity across the genome have consistently implicated inflammatory pathways, including NF-κB, a major regulator of cytokines (Kawai & Akira, 2007). For this reason, this section of the review is focused on inflammatory proteins, to more comprehensively understand the biological underpinnings that shape onset of symptoms following and recovery after TBI.

Summary of cytokine review results.

Results of the cytokine literature review are summarized in Table 2-3. To give a brief overview of the results, 17 studies of cytokines in adult clinical TBI were found. Notably, there were only two studies in mild TBI, with the remainder of the studies in moderate to severe TBI. There was one blast study and two military studies. Most studies (15) focused on measurement of cytokines in the acute time period, although some of these studies (5) considered chronic outcomes at 6-12 months. Two studies measured cytokines in the chronic period. Increased levels of IL-6 were found in 14 studies, increased IL-10 in 10 studies, and increased TNFα in 8 studies. Details of these studies are listed in Table 2-3. Considering the complexity of the biological response following TBI, the discussion will focus on the understanding of these cytokine findings within the wider context of TBI inflammatory processes, as well as broader, potential clinical applications. Thus, the review is organized to address important considerations for moving the research forward, including: acute and chronic studies, bio-specimen source, biological pathways, interventions, as well as age and TBI severity.

Introduction to Inflammatory Cytokines and Concussion

Inflammation is instrumental in the TBI recovery process. Clinical TBI studies associate inflammatory biomarkers, such as cytokine concentrations and immune cell counts, with TBI during the acute period, as well as during the acute and sub-acute recovery periods (McKee & Lukens, 2016). In further support of the critical role of inflammation in TBI recovery, modulation of the inflammatory response has been proposed as a possible therapeutic target after TBI (Schwarzmaier & Plesnila, 2014). Cytokines are especially interesting to the study of concussion as they may serve as practical clinical measures at the bedside as well as reveal underlying inflammatory processes. The role of cytokines in the inflammatory response following brain injury is well-recognized (Hinson et al., 2015; Woodcock & Morganti-Kossmann, 2013). Briefly, cytokines, small proteins released by leukocytes and glial cells that function in mediating inflammatory response, are a well-documented research area in preclinical and limited clinical TBI studies (Lenzlinger, Morganti-Kossmann, Laurer, & McIntosh, 2001; Woodcock & Morganti-Kossmann, 2013).

Inflammatory response to TBI.

What are Cytokines? Cytokines are a variety of proteins (including interleukins, interferons, and growth factors) secreted by immune cells that are involved in signaling between cells during the immune response to injuries, such as a TBI. Cytokines are generally categorized as having pro-inflammatory (such as IL-1,-12; TNFα, INF-γ) or anti-inflammatory effects (IL-10; TGF-β) (Hernandez-Ontiveros et al., 2013; McKee & Lukens, 2016), with some having both pro-and anti-inflammatory effects that assist in

communication between inflammatory and anti-inflammatory activities (IL-6) (Brandt & Pedersen, 2010). Both pro- and anti-inflammatory cytokines are produced by microglia and other glia, such as astrocytes, following insult to the brain, and the two types of cytokines work in concert to determine the fate of affected neurons. Anti-inflammatory cytokines shift the balance toward neuroregenerative and neuroprotective biological pathways and pro-inflammatory cytokines shift the balance toward apoptosis and cell death. Together these cytokines work to maintain the balance of inflammation (Hernandez-Ontiveros et al., 2013).

What is the inflammatory response in TBI? Inflammation plays a central role in the recovery of patients from a TBI; observable through the activities of immune cells and cytokines. A TBI initiates a cascade of inflammatory events that are closely regulated (McKee & Lukens, 2016; Plesnila, 2016). Specifically, TBIs cause the release of substances, damage-associated molecular patterns (DAMPs), also known as alarmins, from injured cells that then trigger a subsequent immune response (Bianchi, 2007; Tang, Kang, Coyne, Zeh, & Lotze, 2012). The DAMPs signal pattern recognition receptors on microglia within the central nervous system (CNS) to produce pro- and anti-inflammatory cytokines and chemokines; resulting cytokines and chemokines then activate and recruit immune cells to the injured tissues (Kigerl, de Rivero Vaccari, Dietrich, Popovich, & Keane, 2014). Within 24 hours, peripheral immune cells, such as neutrophils, are recruited first across the blood-brain barrier (Plesnila, 2016) to the site of injury (Clark, Schiding, Kaczorowski, Marion, & Kochanek, 1994; McKee & Lukens, 2016; Peruzzotti-Jametti et al., 2014). Within the CNS, astrocytes and microglia,

phagocytic immune cells of the brain (Hernandez-Ontiveros et al., 2013), become activated 3-5 days post-injury while the number of neutrophils diminishes. T cells, B cells, and monocytes, normally found in peripheral circulation, are also found at the injury site at 3-5 days (McKee & Lukens, 2016). Of note, CNS produced cytokines and activated microglia have been found to remain elevated for months to years following injury, indicating an unusually lengthened immune response to TBI in human patients (Gentleman et al., 2004; Johnson et al., 2013; Ramlackhansingh et al., 2011). This is associated with long-term symptoms (Bombardier et al., 2010; Bryant, 2008) and cognitive deficits that patients may experience following TBI (Smith, Johnson, & Stewart, 2013). Current research suggests that injury to the brain results in an inflammatory response, which is beneficial when regulated appropriately. If inflammation is prolonged in time, or it is either excessive or insufficient in the degree of activation, it can indicate poor clinical neurological outcome (McKee & Lukens, 2016; Santarsieri, Kumar, Kochanek, Berga, & Wagner, 2015).

A note on "immune privilege." In further support of the importance of cytokines and inflammation to brain injury recovery, the long-held theory of central nervous system (CNS) immune privilege has been challenged. Current evidence shows that peripheral immune cells cross the blood brain barrier (BBB), and immune cells within the brain reach the periphery (Carson, Doose, Melchior, Schmid, & Ploix, 2006; Louveau, Harris, & Kipnis, 2015). Microglia activated following brain insult release a cascade of pro- and anti-inflammatory cytokines, regulating the innate immune response (Hendriksen, van Bergeijk, Oosting, & Redegeld, 2017; Hernandez-Ontiveros et al., 2013). Potential

clinical utility of these markers depends on factors such as the specificity to type and severity of injury as well as ability to be correlated with other protein markers of injury (Woodcock et al., 2013).

Measurement of cytokines in clinical studies. In clinical TBI studies, inflammatory and immune responses are monitored through serum, cerebrospinal fluid (CSF) and blood levels, and include: immune cell counts (neutrophils, B cells, and T cells), concentrations of cytokines: [interleukin (IL) -1, -6, -8, -10, -18, tumor necrosis factor α (TNFα), granulocyte colony-stimulating factor (G-CSF)], inflammasomes, type 1 interferon (INF), and transforming growth factor β (TGFβ) (McKee & Lukens, 2016). Technologies that have been used to measure inflammatory biomarkers in TBI patients including, but not limited to: flow cytometry (aka cytometry bead-based array) such as the BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokine Kit (BD Biosciences, San Diego, CA) (Ferreira et al., 2014; Schneider Soares et al., 2012); multiplex bead array assays (Wisniewski et al., 2007) including the Luminex™ bead array assay (Millipore, Billerica, Massachusetts) (Kumar, Rubin, Berger, Kochanek, & Wagner, 2016; Santarsieri et al., 2015); the enzyme-linked immunosorbent assay (ELISA) (Wisniewski et al., 2007), and Simoa, an ultrasensitive paramagnetic bead-based ELISA (Quanterix Corporation, Cambridge, MA) (Devoto et al., 2016). Important components of laboratory measures include reliability (the reproducibility of the results) and validity (measurement of the intended value; includes sensitivity and specificity) (Kane & Radosevich, 2010).

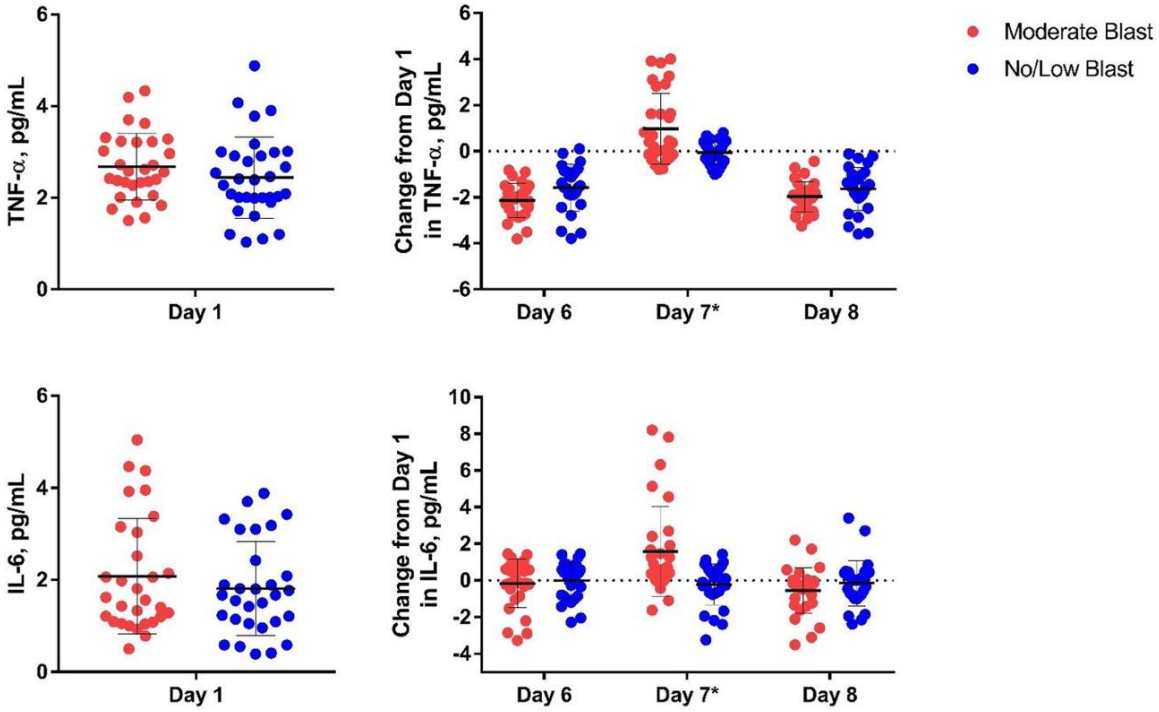
Studies of Inflammatory Markers and TBI Outcomes.

Cytokines are elevated following blast exposure.

A study in a military blast population demonstrates significantly increased concentrations of pro-inflammatory (TNFα, IL-6), and anti-inflammatory (IL-10) cytokines, with level alterations dependent upon degree of blast exposure, immediately following that exposure in a military training environment (Figure 2-3) (Gill et al., 2017), though this finding is yet to be confirmed in additional clinical populations.

Figure 2-3.

Comparison of TNFα and IL-6 in moderate versus no/low blast exposures



Note. Permission obtained from Gill, J., Motamedi, V., Osier, N., Dell, K., Arcurio, L., Carr, W., . . . Yarnell, A. (2017). Moderate blast exposure results in increased IL-6 and TNFalpha in peripheral blood. *Brain Behav Immun*. doi:10.1016/j.bbi.2017.02.015

Considering the limited clinical research in blast exposure, studies in the wider TBI population are considered.

During the acute phase, serum and plasma cytokines are increased and can relate to TBI outcomes.

Cytokines are associated with TBI outcomes when measured in the acute period. For example, in a study of severe TBI male patients (n=24), significantly higher plasma levels of IL-10, -8 and -6 measured both at hospital admission and 24 hours post-injury were found in non-survivors as compared to those patients who survived (Ferreira et al., 2014). Results from a similar study in patients with a range of TBI severities, including mild (n=18), moderate (n=16), and severe (n=93), revealed that increased serum IL-10 levels at 10 and 30 hours post-injury were significant predictors of mortality in the severe TBI cohort (Schneider Soares et al., 2012). Additional evaluations of severe TBI patients show that elevated plasma IL-10 and TNFα are associated with poor 6 month outcomes (Di Battista et al., 2016); increased serum IL-6 is associated with poor neurological outcomes (Lustenberger et al., 2016); and increased serum IL-6, IL-10, and TNFα is associated with unfavorable 6-month outcomes (Santarsieri et al., 2015).

Although published previous to the eligibility dates for inclusion in this review, it is important to note that these studies have built on 15+ years of previous research associating elevated cytokines with poor outcomes. For example, similar to Ferreira et al. (2014), a prior study of moderate and severe TBI patients demonstrated that elevated plasma IL-6 concentrations (>100 pg/mL) on day 1 following a TBI were associated with death within one week of the injury (Woiciechowsky et al., 2002). Likewise, plasma elevations of IL-6 and IL-12, and a decrease of malone dialdehyde (MDA) (indicator of oxidative stress) were reported within 24 hours following injury in patients who did not survive following a severe isolated TBI (n=15) as compared to survivors (n=7) (Arand, Melzner, Kinzl, Bruckner, & Gebhard, 2001). These seven studies suggest that elevations

of both pro-inflammatory and anti-inflammatory cytokines relate to greater mortality and poor neurological outcomes. Collectively, these studies suggest that elevations of peripheral IL-10 and/or IL-6 levels may be useful when evaluating severe TBI prognosis, and specifically in relation to mortality. IL-10 is considered an anti-inflammatory cytokine, to function as a negative regulator of pro-inflammatory cytokine production, while IL-6 is considered to have both pro-inflammatory and anti-inflammatory activities to signal immune cells, including microglia, to the injury site (McKee & Lukens, 2016). To clarify these complex relationships, the need for additional research in mild to moderate TBI populations is evident.

Sample Source and heterogeneity are relevant concerns in TBI cytokine research. Given that cytokine levels have been associated with TBI outcomes in clinical studies, the implication of clinical utility of these biomarkers has stimulated many questions including which bio-specimen source is optimal for the measurement of cytokines. Although many studies evaluate serum or plasma concentrations of cytokines, circulating concentrations of cytokines may differ from cerebrospinal fluid (CSF) or brain tissue concentrations as a direct result of the blood brain barrier (BBB) and the blood meningeal barrier (BMB) though these differences remain poorly understood (Jensen, Massie, & De Keyser, 2013).

Previous work in severe TBI patients that examined cytokines in serum and CSF, an increase in levels of anti-inflammatory markers (IL-1ra, s-TNF-r-1, and IL-10) were found in the serum. This finding was not consistent in the CSF of patients with extracranial injuries compared to patients with only isolated head injury and no additional

injuries. Additionally, in CSF alone, concentrations of the pro-inflammatory marker (IL-1β) and anti-inflammatory markers (IL-1ra, s-TNF-r-1, and IL-10) were higher in all patients compared to controls regardless of the presence of additional injuries. Included in these marker findings were patients with an increased ICP as well as patients with an unfavorable outcome at 6 months (Shiozaki et al., 2005) which suggests that extracranial injury may be responsible for observed elevated serum cytokine levels.

Thus, more recent studies in TBI patients have considered comparison of CSF and plasma or serum sources, with elevated levels observed in CSF as compared to plasma or serum. Elevated levels of CSF IL-6, and serum IL-10 and TNFα were associated with poor outcome at 6 and 12 months (Kumar et al., 2015). IL-6, IL-10, and TNFα CSF concentrations were elevated in TBI patients compared to controls during first 6 days after injury (Juengst, Kumar, Failla, Goyal, & Wagner, 2015). All biomarkers measured, including increased CSF IL-6, IL-10, and TNFα associated with poor 6-month outcomes, with IL-6 remaining elevated at day 5 (Nwachuku et al., 2016). Overall, these studies seem to give evidence of higher levels of cytokines present in the CSF as compared to serum or plasma.

Considering well-known heterogeneity among TBI studies, such as age as observed in some of the studies in this review (Note the delineation of ages in Table 2-3), it may be interesting to note differences in pediatric vs adult studies. For example, in contrast to the Shiozaki et al. (2005) study, a population of pediatric patients with isolated severe TBI (n=14) showed that an increase in plasma or CSF concentrations of IL-1β and IL-6 occurring between the 2- and 24-hr post injury was associated with greater injury

severity (GCS<5) and worse outcome at 6 months (GOS≤3) (Chiaretti et al., 2005). While Shiozaki et al. (2005) did not measure IL-6, the IL-1β concentration was increased in CSF, but not serum, from adult patient cohorts with and without additional injuries and worse outcomes. IL-1β was also increased in the plasma of the pediatric population, even though the pediatric patients were isolated head injuries, while the results in the adult population imply elevated serum cytokines are likely due to extracranial injuries (Chiaretti et al., 2005; Shiozaki et al., 2005). These studies confirm the need for ongoing/continuing research to further understand the role of the BBB in isolated vs. extracranial head injuries among heterogeneous populations, such adult vs. pediatric, and clarification of the optimal source for measuring inflammatory biomarkers.

Three other studies measured cytokine levels in the brain tissue of severe patients. Using intracranial microdialysis to measure IL-6, significantly higher levels ($p=0.04$) were observed in the brain parenchyma of severe TBI survivors as compared to non-survivors (Winter, Pringle, Clough, & Church, 2004). These results suggest a neuroprotective role for IL-6 within the injured brain. In a more recent study using cerebral microdialysis, post-TBI cerebral production of cytokines was also supported (Helmy, Carpenter, Menon, Pickard, & Hutchinson, 2011). Similar to Winter et al. (2004), more work in 2011 showed a trend of increased IL-6 (did not reach significance) in first 24 hours after injury, followed by gradual decline, which also did not associate with poor outcomes (Perez-Barcena et al., 2011). Contrasting results by Shiozaki et al. (2005) suggest that extracranial injury contributes to the increased peripheral cytokine levels. More recent evidence suggests that isolated brain injury may, in fact, contribute

to the altered circulating (plasma or serum) cytokine levels that are observed post-TBI. For example, Di Battista et al. (2016) found that poor outcomes and mortality in moderate and severe TBI patients (n=166) were associated with elevated plasma levels of IL-1β, IL-10, and TNFα within 24 hours of admission. These cytokine increases are possibly associated with the activation of the sympathetic nervous system, as evidenced by the positive association of increased levels of epinephrine and norepinephrine with elevated levels of cytokines (Di Battista et al., 2016). Overall, evidence from these recent seven studies, found in Table 2-3, seems to suggest cytokines are altered in serum, CSF, and brain tissue following TBI in the acute period, and that these altered levels may have prognostic value in the acute or chronic time periods following TBI. However, additional studies are needed to confirm associations of specific cytokines with TBI outcomes, and to elucidate the specific biological functions of various cytokines in the inflammatory and recovery processes following TBI. Further research is also needed to differentiate the roles of cytokines in mild and moderate TBI, as most studies have focused on severe TBI cohorts. Future research studies should take into account the following: 1) recent development of higher-sensitivity techniques; 2) potential confounding factors within and between patient cohorts such as the presence of additional injuries, age, gender, how outcomes are measured, and the timing of sample collection [recognized issues in the literature (Loane & Faden, 2010)]; and, 3) the potential interrelationship of the inflammatory response with other biological pathways.

Cytokines may persist in the chronic period post-TBI. In addition to inflammatory cytokines during the acute period following TBI, three studies described

evidence that inflammatory cytokines, measured in serum, contributed to TBI outcomes in the chronic period, greater than three months following injury. The most recent study was of a military population deployed less than 16 months prior to sample collection, increased plasma levels of IL-6 and TNF-α were found in military personnel experiencing high PTSD as compared to low PTSD in those military personnel with TBI (Devoto et al., 2016). The increased level of IL-6 in the chronic period is interesting considering similar observations in acute period studies. These similar observations suggest dysregulation of the immune system resulting from an inflammatory state left chronically unresolved from the acute response to injury (Gentleman et al., 2004; Johnson et al., 2013). The work by Devoto et al. (2016) demonstrates the association of chronic inflammation, indicated by elevated cytokine levels in post-TBI persons with comorbid conditions, such as PTSD and depression, highlighting the need to detect and alleviate chronic inflammation after TBI. Another study of patients with severe TBI (n=19) with measurements taken at admission, 3 months, and 6 months, found persistently increased plasma levels of TNF-α, IL-6, INF-γ, and IL-1b at 3 and 6 months (Licastro et al., 2016). Elevated cytokine levels were associated with a slower rate of cognitive recovery and poorer cognitive functioning neuropsychological tests at 12 months. Increased levels of TNF-α and INF-γ were also associated with poor functional recovery at 12 months, using measurements from the Functional Independence Measure and Disability Rating Scale (Licastro et al., 2016). Although limited in quantity, chronic phase studies are similar to acute phase studies as they seem to point to an important role of chronic inflammation, as measured by circulating cytokines, in TBI recoveries.

Inflammation is not independent of other biological pathways. Inflammatory markers are part of well-regulated systems that coordinate to promote recovery, but also can become dysregulated, and thus may explain poor recovery even in the chronic period of TBI. In support of this concept, inflammatory markers may influence other types of pathways, such as those in the endocrine system. For example, the hypothalamic-pituitary-adrenal (HPA) axis regulates cortisol secretion and is known to have a central role in the body's response to physical and psychological trauma (Yeager, Pioli, & Guyre, 2011). Cytokines such as IL-1β, IL-6, and TNFα are known to have bidirectional interactions with the HPA axis, which provide regulation of inflammation (Black, 1994). Specifically, immune cells have glucocorticoid receptors that cortisol activates to reduce inflammation (Walker & Spencer, 2018). These cytokines also feedback on the HPA axis, to further regulate cortisol activity, as well as inflammation, such that both systems are regulated sufficiently (Yeager et al., 2011). During states of disease and sickness, inflammatory cytokines increase along with resistance to glucocorticoid. This likely occurs through the interference of inflammatory cytokine pathways with glucocorticoid receptor pathways, contributing to the progression of disease (Pace, Hu, & Miller, 2007; Yeager et al., 2011). In support of this interaction between cytokines and the HPA axis, an acute phase study of severe TBI patients (n=91) correlated a greater inflammatory load score (ILS), calculated by averaging serum concentrations of interleukin (IL)-6, IL-10, soluble Fas (sFas), soluble intracellular adhesion molecule (sICAM)-1, tumor necrosis factor alpha (TNF-α), and CSF cortisol levels for days 0-6 post injury, to poor outcomes at 6 months as measured by the Glasgow Outcome Score-Extended (Santarsieri et al.,

2015). In this study both a high or low mean cortisol level was found to mediate this effect on ILS, implicating the neuroendocrine and immune systems together in TBI outcomes. Thus, both under- and over reactive immune/inflammatory responses may result in poor outcomes, and cortisol levels may be important in understanding inflammation during acute recovery from TBIs (Santarsieri et al., 2015). Another possible influence on the endocrine system post-TBI is brain-derived neurotrophic factor (BDNF), a neurotrophin expressed in the brain that functions in the plasticity and survival of neurons (Hempstead, 2015), which has been found to influence the HPA axis (Colzato, Van der Does, Kouwenhoven, Elzinga, & Hommel, 2011; Gray, Milner, & McEwen, 2013; Shalev et al., 2009). In a follow-up study to Santarsieri et al. (2015), increased serum cortisol and decreased serum BDNF at days 0-3 post injury were linked to poor clinical outcomes (Kumar et al., 2016). Elevated CSF BDNF has been previously associated with greater risk for mortality after a severe TBI (Failla, Conley, & Wagner, 2016). Therefore, regulation of immune function, in part through endocrine function, is important during the acute period, and relates to a greater likelihood of poor recovery.

Interventions for inflammation.

Finally, interventions, including pharmacological and nonpharmacological methods may modulate inflammatory responses, thereby altering outcomes following TBI. For example, a randomized controlled trial of severe TBI patients demonstrated that patients (n=65) receiving pre-hospital resuscitation with hypertonic saline (n=30) experienced significantly reduced serum levels of TNF-α and IL-10 as compared to the group who received normal saline (n=35) (Scarpelini et al., 2010). Hypertonic saline has

the potential to confer beneficial anti-inflammatory and immune modulation effects in addition to the fluid shift from intracellular to intravascular and interstitial spaces (Strandvik, 2009). However, the impact on acute or chronic outcomes was not reported (Scarpelini et al., 2010). Conversely, a separate study found hypertonic saline did not alter 6-month outcomes or survival (GOSE, DRS) in severe TBI patients (n=1087). However, serum inflammatory markers were not measured (Williams et al., 2010). Thus, the influence of hypertonic saline on TBI patient outcomes as well as inflammatory cytokines remains to be answered. Results of a randomized controlled study of a pharmacological agent, ulinastatin, administered every 8 hours revealed TNF-α, IL-2, and IL-6 levels as well as incidence of multiple organ dysfunction syndrome (MODS) and systemic inflammatory response syndrome (SIRS) were decreased in the treatment (n=32)versus control group (n=28), as measured at admission and 10 days post-injury outcomes (Tu, Diao, Yang, Sun, & Zhang, 2012).

Summary and Relevance to Nursing

TBIs are a significant cause of hospitalization, disability, long-term care, and mortality across all age groups in the United States (Taylor, 2017). Factors including genetic predisposition, the timing and relative concentrations of immune and inflammatory markers, and environmental influences, can modulate neurological recovery processes following TBI, and these complicated relationships among the aforementioned factors remain largely uncharacterized (McKee & Lukens, 2016; Santarsieri et al., 2015). Results of this literature review show that pro- and anti-inflammatory cytokines (IL-6, IL-10, and TNF- α) are elevated in the acute period

following injury, may remain chronically unresolved, and are associated with poorer outcomes. Interventions for inflammation are currently in development. Taking note of how these interventions influence the balance of inflammatory cytokines in a variety of tissues will be critical in future studies to optimize patient outcomes. At this time, studies of gene expression following TBI in clinical populations are few, yet promising, warranting further exploration into the biological pathways including, but not limited to inflammation, altered following TBI. These studies will yield insight into pathways that can then be studied at a proteomic biomarker level, such as cytokines, which will allow for the development of diagnostics and therapeutics to directly help the patient. As demonstrated in this review, biomarkers, including but not limited to cytokines, are an increasingly studied area to determine potential underlying mechanisms of injury and recovery processes after TBI. Additionally, biomarkers may yield insight into differential biological pathways in the various severity and subtypes of brain injury (Di Battista et al., 2015). Although the potential clinical utility of biomarkers in patient care is well recognized, the roles in TBI pathologies has yet to be fully realized. A deeper understanding of biological profiles will help direct future research to aid health care providers, nurses, and other medical personnel in improved diagnosis, monitoring and treatment for individuals with TBI.

Table 2-3.

Recent literature examining cytokines in clinical TBI

Reference	Population	Biomarkers	Specimen Source and Collection Times	Significant IL-6, IL-10, and TNFα Results
(Devoto et al., 2016) ***Inflammation Relates to Chronic Behavioral and Neurological Symptoms in Military with Traumatic Brain Injuries.***	**Mild and moderate TBI** N=83 Cases = 63 Controls = 20 All male, military personnel; cases mean age 33.2 yrs; controls mean age 31.6 yrs	**IL-6;** **IL-10;** **TNFα**	Plasma **Chronic**: <16 months following deployment	IL-6 and TNF-α levels higher in TBI vs. control: ↑ IL-6, p = 0.007 ↑ TNFα, p = 0.003 PTSD following TBI associated with higher levels of IL-6 and TNF-α: ↑ IL-6, p = 0.001 ↑ TNFα, p = 0.013
(Di Battista et al., 2016) ***Inflammatory cytokine and chemokine profiles are associated with patient outcome and the hyperadrenergic state following acute brain injury.***	**Moderate and Severe TBI** N = 187 Cases = 166 Controls = 21 74.7% male; age 16-67 yrs.	IL-1β; IL-2; IL-4; IL-5; IL-8 **IL-10**; IL-12p70; IL-13; **TNFα**; IFN-γ; IP-10; MCP-1; MCP-4;	Plasma **Acute**: hospital admission, 6, 12, and 24-hours post-injury	Elevated IL-10 in all injured patients as compared to controls: ↑ IL-10, p < 0.001 Elevated IL-10 and TNF-α associated with poor outcome at 6 months (GOS-E): ↑ IL-10, p < 0.05 ↑ TNFα, p < 0.05

Reference	Population	Biomarkers	Specimen Source and Collection Times	Significant IL-6, IL-10, and TNFα Results
		MDC; MIP-1β; TARC		
(Ferreira et al., 2014) ***Increased levels of interleukin-6, -8 and -10 are associated with fatal outcome following severe traumatic brain injury.***	**Severe TBI** N = 37 Cases = 24 Control = 13 Males, 18-74 yrs.	IL-1b; **IL-6**; IL-8; **IL-10**; IL-12p70; **TNF-α**	Plasma **Acute**: hospital admission (5.6 hour mean time from injury), 24 and 72 hours post-injury	Il-6, IL-10, and TNFα elevated in TBI patients at admission compared to controls: ↑ IL-6, $p < 0.05$ ↑ IL-10, $p < 0.05$ ↑ TNFα, $p < 0.05$ Il-6 and IL-10 elevated in TBI patients with fatal injuries, compared to TBI survivors, at admission and 24 hours: ↑ IL-10, $p < 0.05$ ↑ TNFα, $p < 0.05$

Reference	Population	Biomarkers	Specimen Source and Collection Times	Significant IL-6, IL-10, and TNFα Results
(Gill et al., 2017) ***Moderate blast exposure results in increased IL-6 and TNFalpha in peripheral blood.***	**Blast Exposure** N = 62 Cases = 30 Controls = 32 Military; males, 30.55 yrs. (mean)	**IL-6;** **IL-10;** **TNFα**	Serum **Acute:** day of blast, 24 hours later	Elevated IL-6 and TNFα in cases immediately following blast as compared to controls: ↑ IL-6, $p < 0.01$ ↑ IL-10, $p < 0.01$
(Helmy et al., 2011) ***The cytokine response to human traumatic brain injury: temporal profiles and evidence for cerebral parenchymal production.***	**Severe TBI** N = 12 Males and females, 18-61 yrs.	42 cytokines, including: **IL-6;** **IL-10;** **TNFα**	Plasma; Microdialysate **Acute:** daily for 5 days	Peaks of cytokines (2x higher concentrations than the median) in brain microdialysate were noted on the following days: Day 1: ↑ TNFα Day 2: ↑ IL-6 Day 4-5: ↑ IL-10 Il-6 and IL-10 were 10x higher in brain microdialysate than plasma, $p < 0.001$.
(Hergenroeder et al., 2010) ***Serum IL-6: a candidate biomarker for intracranial pressure elevation following isolated traumatic brain injury.***	**Severe TBI** N = 42 Cases = 28, Controls = 14 35 males and 7 females, 14-56 yrs.	**IL-10**; IL-13; IL-15; IL-16; IL-1α; IL-1β; Il-1ra; IL-2; IL-3; IL-4; Il-5;	Serum **Acute**: first 24 hours after injury, and daily for 5 days	IL-6 levels within 17 hours of injury associated with elevated ICP after TBI: ↑ IL-6, $p = 0.002$

Reference	Population	Biomarkers	Specimen Source and Collection Times	Significant IL-6, IL-10, and TNFα Results
		IL-6; IL-7		
(Juengst et al., 2015) ***Acute inflammatory biomarker profiles predict depression risk following moderate to severe traumatic brain injury.***	**Moderate and Severe TBI** N= 56 CSF Cases = 41 Serum Cases = 50 Controls = 15 Males and females, 18–70 yrs.	IL-1 β; IL-4; IL-5; **IL-6**; IL-7; IL-8; **IL-10**; IL-12; **TNFα**; sVCAM-1; sICAM-1; sFAS.	CSF; Serum **Acute**: CSF was collected twice daily, serum collected once daily; for up to 6 days post injury	IL-6, IL-10, and TNFα CSF concentrations elevated in cases compared to controls during first 6 days after injury: ↑ IL-6, $p < 0.05$ ↑ IL-10, $p < 0.05$ ↑ TNFα, $p < 0.05$
(Kumar et al., 2015) ***Acute CSF interleukin-6 trajectories after TBI: Associations with neuroinflammation, polytrauma, and outcome.***	**Severe TBI** N = 129 Cases = 114 Controls = 15 Males and females, 18-70 yrs.	IL-1β; IL-4; IL-5; **IL-6**; IL-7; IL-8; **IL-10**; IL-12;	CSF; Serum **Acute**: samples collected every 12 hours, up to 5 days post injury	CSF IL-6 levels elevated in cases compared to controls at each day following injury: ↑ IL-6, $p < 0.001$ CSF IL-10, and TNFα elevated in TBI sub-group with high IL-6 levels:

Reference	Population	Biomarkers	Specimen Source and Collection Times	Significant IL-6, IL-10, and TNFα Results
		TNF-α; sVCAM-1; sICAM-1; sFAS		↑ IL-10, $p < 0.05$ ↑ TNFα, $p < 0.05$ Elevated levels of CSF IL-6, and serum IL-10 and TNFα also associated with poor outcome at 6 and 12 months (GOS): ↑ IL-6, $p < 0.001$ ↑ IL-10, $p < 0.05$ ↑ TNFα, $p < 0.05$ Elevated serum IL-6 associated with poor 6-month outcome: ↑ IL-6, $p = 0.015$
(Licastro et al., 2016) ***Peripheral Inflammatory Markers and Antioxidant Response during the Post-Acute and Chronic Phase after Severe Traumatic Brain Injury.***	**Severe TBI** N = 19 Males and females, 19-61 yrs.	**TNFα;** **IL-6;** INFγ; IL-1b	Plasma **Chronic:** First sample collected at 15-66 after TBI, followed by 3 and 6 months later	Increased levels of all cytokines measured associated with poor cognitive outcomes: ↑ IL-6, $p = 0.0170$ ↑ TNFα, $p = 0.0033$

Reference	Population	Biomarkers	Specimen Source and Collection Times	Significant IL-6, IL-10, and TNFα Results
(Lustenberger et al., 2016) ***The effect of brain injury on the inflammatory response following severe trauma.***	**Severe TBI** N = 123 Isolated TBI = 26 TBI with polytrauma = 36 Polytrauma, no TBI = 61 Males and females, 16-66 yrs.	**IL-6**; CRP; leukocytes	Serum **Acute**: upon admission, and days 1-3 post injury	IL-6 levels significantly different between the groups at admission and for 3 days post-injury, with peak at 1 day: ↑ IL-6, $p < 0.05$ Increased IL-6 levels significantly related to multiple organ failure, sepsis and neurological outcomes (GOS) in TBI cohorts: ↑ IL-6, $p < 0.05$
(Nwachuku et al., 2016) ***Time course of cerebrospinal fluid inflammatory biomarkers and relationship to 6-month neurologic outcome in adult severe traumatic brain injury.***	**Severe TBI** N = 32 cases *Biomarkers compared to laboratory standards* Males and females, 17-80 yrs.	IL-1β; **IL-6**; **TNF-α**; IFN-γ; IL-12p70; **L-10;** IL-8	CSF **Acute**: samples collected days 1-5 post-injury	Increased IL-6, IL-10, and TNF-α (and all biomarkers) associated with poor 6-month outcome (GOS) ($p < 0.05$), with IL-6 remaining elevated at day 5. ↑IL-6, $p < 0.05$

Reference	Population	Biomarkers	Specimen Source and Collection Times	Significant IL-6, IL-10, and TNFα Results
(Perez-Barcena et al., 2011) ***Lack of correlation among intracerebral cytokines, intracranial pressure, and brain tissue oxygenation in patients with traumatic brain injury and diffuse lesions.***	**Severe TBI** N = 16 Feasibility study, no controls Males and females, 15-65 yrs.	IL-1; **Il-6**; IL-8; **IL-10** IL12; **TNFα**	Serum; Microdialysate **Acute**: samples collected every 24 hours for 7 days	Increased IL-6 (did not reach significance) in first 24 hours after injury, followed by gradual decline. No association between IL-6 and ICP, brain oxygenation, or edema.
(Santarsieri et al., 2015) ***Variable neuroendocrine–immune dysfunction in individuals with unfavorable outcome after severe traumatic brain injury.***	**Severe TBI** N = 115 Cases = 91 Controls = 24 Males and females, 16-75 yrs.	**IL-6**; **IL-10**; sFas; ICAM-1; **TNF-α**; Cortisol	CSF; Serum **Acute**: CSF collected twice daily, up to 6 post-injury Serum collected once daily, up to 6 days	↑ IL-6, IL-10, and TNF-α higher in cases compared to controls, $p < 0.01$ IL-6, IL-10, and TNF-α significantly associated with unfavorable 6-month outcome (GOS) and CSF cortisol: ↑ IL-6, $p < 0.01$ ↑ IL-10, $p < 0.01$ ↑ TNFα, $p < 0.05$

Reference	Population	Biomarkers	Specimen Source and Collection Times	Significant IL-6, IL-10, and TNFα Results
(Schneider Soares et al., 2012) ***Interleukin-10 Is an Independent Biomarker of Severe Traumatic Brain Injury Prognosis.***	**Mild, Moderate, and Severe TBI** N = 127 Cases = 93 Controls = 34 Males and females, 18-79 yrs.	**IL-10**; **TNF-α**	Serum **Acute**: hospital admission, and two additional samples up to 4 days later	Elevated IL-10, but not TNFα, correlated significantly with GCS severity: ↑ IL-10, $p < 0.0001$ Increased IL-10 levels associated with greater mortality rate in severe TBI: ↑ IL-10, $p = 0.01$
(Stein et al., 2011) ***Relationship of Serum and Cerebrospinal Fluid Biomarkers with Intracranial Hypertension and Cerebral Hypoperfusion After Severe Traumatic Brain Injury.***	**Severe TBI** N = 24 Mostly male (95.8%), 18-83 yrs.	**TNFα;** IL-1β; **IL-6**; IL-8; **IL-10**	CSF; Serum **Acute**: hospital admission and twice daily for 7 days	Increased serum TNFα levels correlate with increased ICP and decreased CPP: ↑ TNFα, $p < 0.001$

Reference	Population	Biomarkers	Specimen Source and Collection Times	Significant IL-6, IL-10, and TNFα Results
(Yan et al., 2014) ***Post-traumatic hypoxia is associated with prolonged cerebral cytokine production, higher serum biomarker levels, and poor outcome in patients with severe traumatic brain injury.***	**Severe TBI** N = 62 Cases = 42 Controls = 20 Males and females, 16-74 yrs.	IL-2; IL-4; **IL-6**; IL-8; **IL-10;** TNF; INFγ; GM-CSF; NSE; S100; MBP	CSF **Acute**: Daily from hospital admission to 5 days post-injury	IL-6 and IL-10 CSF concentrations (and all cytokines) were significantly increased compared to controls at each day, with higher concentrations trending during the first 24-48 hours: ↑ IL-6, ↑ IL-10, $p < 0.05$
(Yousefzadeh-Chabok et al., 2015) ***The Relationship Between Serum Levels of Interleukins 6, 8, 10 and Clinical Outcome in Patients With Severe Traumatic Brain Injury.***	**Severe TBI** N = 44 Mostly (97.7%) male, ≥ 14yrs.	**Il-6**; IL-8; **IL-10**	Serum **Acute**: 6 hours post injury	Increases in Il-6 correlate with unfavorable 6-month outcome (GOS): ↑ IL-6, p=0.03

Note. Glasgow outcome score (GOS); Glasgow outcome score-extended (GOS-E); intracranial pressure (ICP): cerebral profusion pressure (CPP): cerebral spinal fluid (CSF): Interleukin (IL); tumor-necrosis factor α (TNFα); soluble vascular adhesion molecule-1 (sVCAM-1); soluble intracellular adhesion molecule-1 (sICAM-1); and soluble Fas (sFAS); c-reactive protein (CRP)

Declaration of Conflicting Interests:

The Authors declare that there is no conflict of interest.

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A MODERATE BLAST EXPOSURE RESULTS IN DYSREGULATED GENE NETWORK ACTIVITY RELATED TO CELL DEATH, SURVIVAL, STRUCTURE, AND METABOLISM

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ABSTRACT

Blast exposure is common in military personnel during training and combat operations, yet biological mechanisms reacted to cell survival and function that coordinate recovery remain poorly understood. This study explored how moderate blast exposure influences gene expression; specifically, gene-network changes following moderate blast exposure. On day 1 (baseline) of a 10-day military training program, blood samples were drawn, and health and demographic information collected. Helmets worn throughout training measured overpressure in pounds per square inch (psi). On day 7, some participants experienced moderate blast exposure (peak pressure ≥5 psi). On day 10, 3 days post-exposure, blood was collected and compared to baseline with RNA-sequencing to establish gene expression changes. Based on dysregulation data (RNA-sequencing) and top gene-networks [Ingenuity® Pathway Analysis (IPA®)], a subset of genes was validated (NanoString). Five gene-networks were dysregulated; specifically, two highly significant networks: 1. Cell death/survival (score: 42), including 70 genes, with 50 downregulated, and 2. Cell structure, function, and metabolism (score: 41), including 69 genes, with 41 downregulated. Genes related to ubiquitination, including neuronal development/repair: *UPF1* (UPF1, RNA Helicase and ATPase) were upregulated while *UPF3B* (UPF3 Regulator Of Nonsense Transcripts Homolog B) was downregulated. Genes related to inflammation were upregulated, including *ARRB1* (arrestin β1), implicating inflammation in recovery. *AKT1*, a gene coordinating cellular recovery following TBIs, was upregulated. Moderate blast exposure induced significant gene expression changes including gene-networks involved in cell death/survival and

cellular development/function. The present findings may have implications for understanding blast exposure pathology and subsequent recovery efforts.

Key words: Blast; overpressure; gene-expression; RNA-sequencing; NanoString

CHAPTER THREE

A MODERATE BLAST EXPOSURE RESULTS IN DYSREGULATED GENE NETWORK ACTIVITY RELATED TO CELL DEATH, SURVIVAL, STRUCTURE, AND METABOLISM

Introduction

When an individual is in close proximity to a blast, the resulting overpressure (i.e. shock wave) can cause injury to the brain and/or body (Jones, Fear, & Wessely, 2007). The increased use of improvised explosives, sophisticated weaponry, and explosive entry techniques has led to increased risk of blast exposure. Specifically, in military personnel who deployed to recent conflicts of Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF), an estimated 300,000 service members were exposed to at least one blast from adversary attack (Tenielien & Jaycox, 2008). Blast overpressure from firing weapons is increasing commensurate with increases in weaponry power. High intensity blast exposure events can damage connective tissues, including the central nervous system, resulting in cerebrovascular damage and blood brain barrier disruption. Significant overpressure can result in tearing of the long axons of neurons (diffuse axonal injury) leading to the associated deficits and comorbidities of a traumatic brain injury (TBI) (Mac Donald et al., 2016; Yurgil et al., 2014). There is evidence suggesting blast-induced TBI (biTBI) has distinct features from blunt-force or penetrating TBI (Courtney & Courtney, 2015). However, it is difficult to evaluate the consequences of blast in isolation using human subjects, as there is often concomitant blunt force or penetrating TBI when objects are propelled and contact the skull (e.g. shrapnel) or the individual is thrown. These challenges contribute to the relatively poor understanding of the

pathophysiologic responses to blast and lack of therapies to treat blast-exposed individuals. Moreover, the response and subsequent recovery from blast exposure represent an important line of research that remains to be further explored and may elucidate the biological mechanisms associated with blast.

Differential gene expression is reported in a small number of clinical TBI studies (Cho et al., 2016; Gill et al., 2016; Livingston et al., 2016; Merchant-Borna et al., 2016), with few studies relevant to blast TBI (Carr et al., 2015; Gill, Cashion, et al., 2017; Heinzelmann et al., 2014). Gene expression regulation is imperative to appropriate cellular response to external mechanical, environmental, or biological stimuli, and the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) complex is a main transcription factor of these adaptive gene expression changes (Hayden & Ghosh, 2008). More specifically, the NF-κB complex is a transcription factor central to numerous cellular pathways influencing cell survival and proliferation, including inflammatory and immune responses, gene activation, and ubiquitination (Hayden & Ghosh, 2008). Animal models demonstrate that the NF-κB complex regulates the innate immune response through upregulation of proinflammatory cytokines including tumor necrosis factor (TNF) (Bohuslav et al., 1998), interleukin 1 (IL-1) (Lawrence, 2009), and interleukin 6 (IL-6) (Baeuerle & Baltimore, 1996). In addition, mutations and epigenetic changes within the NF-κB pathway have been linked to immune and inflammatory diseases (Courtois & Gilmore, 2006). Cytokines are among a number of factors that may activate NF-κB. NF-κB becomes activated when ubiquitin degrades its inhibitory protein, IκK, freeing NF-κB to enter the nucleus and activate gene transcription (Baeuerle &

Baltimore, 1996; Chen, Bhoj, & Seth, 2006). Study of gene expression changes following blast exposure may elucidate some of these complexities surrounding the roles and relationships of ubiquitin and inflammatory cytokines following blast exposure.

Within clinical studies of TBI, changes in the NF-κB network are reported in a limited number of studies (Cho et al., 2016; Gill et al., 2016; Livingston et al., 2016; Merchant-Borna et al., 2016), but have not yet been examined in biTBI. Preclinical studies of blast exposures have demonstrated altered gene expression, including cognitive impairment (Bailey, Sujith Sajja, Hubbard, & VandeVord, 2015; Tweedie et al., 2016) and immune function (Struebing et al., 2017). Recent work in military training that involves personnel exposure to blast has demonstrated that ubiquitin carboxy-terminal hydrolase-1 (UCH-L1) is weakly correlated with repeated exposure to low-level blast (Carr et al., 2015), consistent with previous work in TBI (Papa et al., 2012) and blast exposure (Heinzelmann et al., 2014; Tate et al., 2013). In particular, Heinzelmann et al. (2014) found protein ubiquitination genes (associated with neuronal recovery, central regulator in IPA) to be downregulated in military personnel with chronic symptoms following blast head injury. UCH-L1 is predominately expressed in the neurons and neuroendocrine cells within the brain (Doran, Jackson, Kynoch, & Thompson, 1983; Leroy, Boyer, & Polymeropoulos, 1998) and is an enzyme responsible for protein degradation, thus providing a role in ubiquitin stability within neurons and maintaining neuronal health (Osaka et al., 2003). In animal models, a mutation in the *UCH-L1* gene causing a truncated protein is associated with neurodegeneration, likely due to the buildup of ubiquitin and subsequent lack of protein clearance (Saigoh et al., 1999). Given

this limited number of clinical studies, this study sought to further examine differential gene expression pathways in a blast exposed population. The purpose of this study was to examine gene networks involving cell death and survival as well as cell structure, function, and metabolism to investigate the role of these networks specific to biTBI.

Materials and Methods

To address the gaps in the knowledge surrounding the consequences of exposure to isolated blast, a unique cohort of military personnel engaged in training on advanced techniques for breaching buildings with controlled explosives was utilized. The breaching activities were conducted under close supervision and with personal protective equipment and established safety procedures, eliminating the chance of concomitant blunt-force or penetrating TBI. Moreover, recruiting from a training environment, as opposed to real-world combat, facilitated accurate measurement of isolated blast exposures using helmets equipped with pressure sensors (see **Blast measurement**). This novel sampling also facilitated a collection of baseline data, including pre-exposure blood draws to support assessment of gene expression changes after blast. During the two-week training program, some participants (n = 29) experienced a moderate blast exposure with peak pressure exceeding 5 pounds per square inch (psi), which exceeded the training range limit of 4 psi and was more than 200% greater than typical exposures measured in such training (e.g., Carr et al., 2015). These 29 cases were studied for gene expression changes related to cell death and survival as well as cell structure, function, and metabolism from training day 1 to training day 10. Unbiased RNA-sequencing (RNA-seq) was used to detect dysregulated genes (Gill, Cashion, et al., 2017). Ingenuity pathway analysis (IPA)

of dysregulated genes was used to identify gene networks, two of which were validated in the present study using NanoString's nCounter® system.

Participants.

All study protocols were reviewed and approved by the Institutional Review Boards (IRBs) at the Naval Medical Research Center and Walter Reed Army Institute of Research (NMRC#2011.0002; WRAIR#1796) as described in a past publication (Carr et al., 2015). Prior to study participation, each participant provided informed consent. The parent study from which the present study is drawn was comprised of (N = 108) male active-duty military service members who were engaged in two-week blast training programs, as either a student or instructor. The goal of the course was to teach advanced techniques for explosive breaching, a tactic used to gain access into secured structures. All participants provided demographic and health history data at baseline, as well as blood samples. For the present study, participants (n = 29) examined were those who experienced a moderate blast exposure (≥ 5psi). These 29 individuals provided blood samples at the end of training (day 10) that were used in the present study to examine gene expression changes from baseline to 3 days post-moderate blast exposure.

Self-reported data provided by participants at baseline included demographic, health, and blast-history information. Demographic data included age, military rank, and educational status. Health information collected included smoking status and history of TBI (see Table 3-1). Previous blast exposure data was also obtained through self-reports on how many blast exposures had been experienced during breaching and artillery fires using the following ordinal scale: 0, 1-9, 10-39, 40-99, 100-199, 200-399, and 400+ blast

exposures. Details regarding the surveys used to collect data have been previously described (Carr et al., 2015).

Blast measurement.

Objective blast data was collected using standard Army combat helmets equipped with bilateral sensors capable of measuring blast parameters greater than a threshold of 0.4 psi on either sensor. Helmets were worn throughout training and the average of the right and left sensor was used as data to approximate levels of explosive blast each participant experienced; the sensitivity of the sensors is based on the technological specifications of the device itself (micro Data Acquisition System, μDAS; Applied Research Associates, Inc., Albuquerque, NM) as well as considerations for signal-to-noise ratios and effect on data interpretation.

Laboratory Methods.

Blood Sampling.

Whole blood samples were collected at baseline and at the end of 2-week training; 3 days after moderate blast. Blood was collected in PAXgene tubes and stored in a -80°C freezer until the time of batch processing.

RNA-seq.

Random fragmentation of complementary deoxyribonucleic acid (cDNA) followed by 5' and 3' adapter ligation was used to create a cDNA library. Average fragment length was 150-170bp. RNA integrity was assessed using Agilent Technologies 2100 Bioanalyzer and the mean value was 8.9 with standard error of 0.05. Samples from 29 participants on day 1 and day 10 were sequenced for mRNA using the Illumina

HiSeq®2500 Next Generation Sequencing system (Illumina Inc., San Diego, CA). Using this system, we performed RNA-seq to read paired-ends; we read 101 bases per each end. Sequencing data used in the study were deposited in the Gene Expression Omnibus (GEO) with GEO ID GSE89866.

Ingenuity Pathway Analysis.

Dysregulated genes were further explored using IPA® software, build version 389077M, content version 27821452, released 2016-06-14, Qiagen, Redwood City, CA). Two pathways of interest were identified (see "Results" for details and Figures 3-1 and 3-2).

NanoString.

A subset of genes examined in RNA-seq data were selected to validate gene expression changes using a direct digital detection system (Nanostring Technologies, Seattle, WA). In selecting genes to validate, the extent of dysregulation, biological plausibility, and the position of the protein within the IPA® pathway diagrams were considered. Two pathways were identified, one focused on cell death and survival and another focused on basic structure, function, and development. A panel was designed for each pathway to include 50 markers of interest, plus a total of 10 reference/housekeeping genes for data normalization (Table 3-2 and Table 3-3). Probes for the 50 genes of interest and the housekeeping genes were designed and manufactured by Nanostring Technologies. NanoString was used to determine the mean copy number of each mRNA probe of interest based on manufacturer's protocol. The standard manufacturer protocol

was followed for sample preparation, hybridization, and detection (see Supplement for more detailed information regarding housekeeping genes and NanoString methods).

Statistical Analysis

Overview.

The Statistical Package for the Social Sciences (SPSS; version 22; IBM Corporation, Armonk, NY) and Nanostring's nSolver^{TM} Analysis Software (version 3; Nanostring Technologies, Seattle, WA) were used for all analyses.

RNA-seq Analysis.

The moderate blast exposed cases (n = 29) met quality control (QC) criteria based on the RNA Integrity Number (RIN) and were subsequently sequenced. In total, between 52.5 million and 75.5 million read counts were completed for each sample; in 94.95% of base calls, an accuracy of at least Q30 was achieved. To establish bioinformatics QC, FastQC (version 0.11.5, Babraham Bioinformatics, Cambridgeshire, UK) was used. Data was aligned to a reference genome (hg19) using an open-source aligner, STAR, (version 2.5) (Dobin et al., 2013). To count the number of reads mapped to genes, HTSeq software was used (version 0.6.1p1) (Anders, Pyl, & Huber, 2015). DESeq2 (version 1.12.3) (Love, Huber, & Anders, 2014) was used to identify differentially expressed genes, with the Wald test used to determine statistical significance, p values adjusted for multiple testing using the Benjamini-Hochberg procedure, and a cutoff value of false discovery rate (FDR) of 0.05.

Nanostring Validation Analysis.

Raw data was analyzed using nSolver™ 3.0 digital analyzer software using standard settings and quality control parameters. It was normalized against housekeeping genes. Fold changes and p-values were calculated using a t-test adjusted for multiple comparisons using the Benjamini-Yekutieli false discovery rate method for samples before and after blast exposure, with statistical significance defined at the level of $p<0.05$.

Results

Demographic results.

Participants in the study were male military service members with a mean age of 31.2 and a mean length of service of 11.2 years (Table 3-1). Almost half of participants (46.3%) had a history of greater than 40 prior blast exposures. No significant differences based on demographic information were noted among the cohort (Gill, Cashion, et al. (2017).

RNA-seq results.

Results of the RNA-seq analysis demonstrated significant gene-activity changes ($p<0.05$) following a moderate blast with multiple networks being dysregulated (Table 3-4). The present study reports on two of the most significant gene-network activity changes (Figures 3-1 and 3-2) determined by Ingenuity® Pathway Analysis (IPA®) software (IPA®, Qiagen, Redwood City, CA). In total, five pathways were identified, including two sets of two pathways that shared overlapping functions and were subsequently merged together to form two pathways of interest in the present study. One merged pathway centered on cell death and survival; this pathway was comprised of

genes implicated in apoptosis, necrosis, autophagy, mitophagy, ferroptosis, survival, regeneration, and recovery, with a score of 42 (Figure 3-1). The second merged pathway focused on development, metabolism and cell structure/function; this pathway consisted of genes involved in cytoskeleton, organelles, cellular metabolism, lipid metabolism, heat shock, cell motion, cell growth, and differentiation, with an IPA score of 41 (Figure 3-2).

NanoString was used to validate the RNA sequencing results. Nanostring analysis showed 32 significantly differentially expressed genes in the Cell Death and Survival network ($p < 0.05$) and 35 significantly differentially expressed genes in the Cell Structure, Function, and Metabolism network ($p < 0.05$), validating differential expression of these two gene networks following blast exposure.

Discussion

In this study, activity changes are reported in two gene networks after moderate blast exposure in military personnel engaged in training. Differentially regulated networks after blast included cell death and survival (see Figure 3-1), which is related to nonsense mediated decay, as well as cellular structure, function, and development (see Figure 3-2). Genes within these networks relate to ubiquitination, apoptosis, as well as activity related to ribosomes, mitochondria, and inflammation. Findings from this study provide novel insight for understanding the biological changes that occur following blast, which for some individuals, may result in biological changes that increase their risk for neurological or behavioral symptoms and deficits. These findings may ultimately contribute to characterizing the cellular mechanisms of blast exposure to improve diagnosis, monitoring, and prognosis of military personnel exposed to blast.

A number of genes related to ubiquitination are increased in activity following blast exposure, including tripartite motif containing 12 (*TRIP12)*, a gene encoding an E3 ubiquitin-protein ligase involved in ubiquitin fusion degradation. Protein ubiquitination initiates the removal of oxidized and misfolded proteins following injury, and its processes can protect neurons from reactive oxidative species (ROS) which accumulate following blast exposure in pre-clinical models (Kochanek et al., 2013). These findings support the previous report of increased UCH-L1, the primary protein for ubiquitination, following repeated low-level blast (Carr et al., 2015). This finding suggests that there may also be overlap with the biological mechanisms related to recovery from TBIs in civilians, as UCHL1 increases are one of the most often reported changes following a TBI (Diaz-Arrastia et al., 2014; Toman, Harrisson, & Belli, 2016). In contrast, as reported in a previous publication, the activity of genes related to ubiquitin were lower in activity in military personnel with TBIs, with many related to blast exposures, and chronic symptoms (Heinzelmann et al., 2014). Therefore, it may be that ubiquitin activity is critical to acute recovery from biTBIs, and that in some individuals, there is a reduction in activity that may place them at higher risk for chronic symptoms. In support of this, pre-clinical studies show that reductions or inactivation of ubiquitin activity results in poor outcomes, including behavioral deficits, possibly indicating long-term neurodegenerative processes (Svetlov et al., 2010).

Additional genes that may relate to neuronal recovery are altered in activity following a moderate blast in this report. Specifically, gene activity changes are observed within the nonsense mediated decay (NMD) pathway, including *UPF1* and *UPF3B*,

which are responsible for neuronal specific cell development and repair through a reciprocal pattern of activity (Kurosaki & Maquat, 2016). Previous studies show an interaction in the activity of these two genes, such that when one gene is less active, the other gene will compensate, preserving the activity of this network; the present findings mirror these previous studies. The present findings show that *UPF1* was increased in activity, whereas *UPF3B* was downregulated. These findings suggest that in response to the blast, injury mechanisms may have been initiated (inflammation, aberrant cellular formation, and cell death), and this initiation may result in an upregulation of *UPF1*, in an effort to preserve the activity of the NMD pathway. Subsequently, the expression of *UPF3B* is suppressed, hindering possible detrimental neurological effects. These findings suggest complex gene-activity changes following blast exposure that may be occurring to promote recovery, implicating the need for additional studies to understand the temporal relationship of these changes and their relation to neuronal recovery.

Another gene downregulated in military personnel following blast within the structure, function, and development pathway was *NAE1* (NEDD8 Activating Enzyme E1 Subunit 1), a protein associated with the neddylation pathway. Vogl, A.M., et al. showed that neddylation was a critical regulator of dendritic spine development, reporting that in *NAE1* knockout mice, there were cognitive deficits as well as synaptic and neurotransmitter impairments (Vogl et al., 2015). The down-regulation observed in the military population could suggest similarly that exposure to blast hinders the neddylation pathway and might suggest a marker of injury resulting directly from blast exposure. Additionally, recent in vitro work suggests Il-1β may inhibit NEDD8 and neddylation in

conjunction with increased ubiquitination; while activation of NEDD8 downregulates the NF-κB pathway (Yan, Guan, Peng, & Zheng, 2017). This is of interest, as genes within the NF-κB network also show activity changes, with most genes becoming more active. The NF-κB network is a dominant activator of the immune system following TBI and this activity is essential as it initiates secondary injury mechanisms required for neuronal recovery. However, if activity of this pathway is too high, or too long-lasting, it can be detrimental to neuronal recovery (Jassam, Izzy, Whalen, McGavern, & El Khoury, 2017). One gene in the pathway, *ARRB1*(arrestin β1), is increased following blast exposure. This gene has been reported to play a role in the beta-adrenergic receptor kinase (BARK) mediated desensitization of beta-adrenergic receptors. In TBI patients, catecholamines surge after injury has been linked to immunosuppression and greater mortality risk that is reversed through β-blocker treatment (Schroeppel et al., 2010). *ZBTB7B* (zinc finger and BTB domain containing 7B) is also upregulated after a blast and is linked to reductions in CD8-cytotoxic activity (Wang et al., 2008), which could be a mechanism to prevent further cellular damage after blast injury.

Another gene related to immune activity with increased activity is *AKT1*, a hub that included approximately 14 connections in the structure, function, and development network. *AKT1* encodes for a serine-threonine protein kinase (AKT1), which is known to regulate a vast number of cellular processes including neuronal survival, glucose uptake, protein and fatty acid synthesis, cell proliferation, and the previously mentioned role in apoptosis (Oeckinghaus & Ghosh, 2009; Vergadi, Ieronymaki, Lyroni, Vaporidi, & Tsatsanis, 2017). Additionally, *AKT1* may function in the inflammatory response as an

upstream activator of the nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) (Lian et al., 2015). Interestingly, in this population, significantly elevated levels of the cytokines tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) have been reported during the acute period following moderate blast (Gill, Motamedi, et al., 2017). This finding is relevant as NF-κB is recognized as a master regulator of cytokines including TNFα and IL-6 (Neumann & Naumann, 2007; Oeckinghaus & Ghosh, 2009). Studies of the NF-κB pathway have implicated the pathway in regulation of proinflammatory cytokines during meningitis (Barichello, Generoso, Simoes, Elias, & Quevedo, 2013) and in blood-brain barrier permeability (Merrill & Murphy, 1997). Additionally, the NF-κB pathway has been found to be dysregulated in clinical studies of acute and subacute TBI (Cho et al., 2016; Gill et al., 2016; Livingston et al., 2016; Merchant-Borna et al., 2016). Upregulation of *AKT1* in this sample suggests activation of the NF-κB pathway; a finding that supports these prior studies, though the specific role of *AKT1* in blast effects on the central nervous system remains to be examined.

Other genes related to NF-κB pathway also show increased activity, including the *Flt3* (dimer) that encodes for a receptor tyrosine kinase. *Flt3* is implicated in multiple signaling pathways including regulation of the proliferation and survival of hematopoietic cells, which ultimately relates to the number of intermediate monocytes (Zawada et al., 2016). This has possible implications, as intermediate monocytes promote production of inflammatory cytokines within the NF-κb network, including TNF-α and Il-1β (Wong et al., 2012), suggesting the possibility of a pro-inflammatory response through increased production of intermediate monocytes.

Genes related to apoptosis are observed to change in activity following blast; findings of interest as preclinical models show blast exposure results in astrocytic and microglial activation, oxidative stress, axonal and vascular damage, and inflammation, which ultimately contribute to programmed cell-death (Agoston & Elsayed, 2012; Agoston, Gyorgy, Eidelman, & Pollard, 2009; Goodrich et al., 2016; Saljo, Mayorga, Bolouri, Svensson, & Hamberger, 2011). Specifically, there is an activation of caspase complexes, a family of cysteine-dependent proteases, which have been previously associated with neuronal and oligo-dendroglial cell death in both pre-clinical and human brain injuries (Schoch, Madathil, & Saatman, 2012). Otherwise referred to as apoptosis executioners, caspase-3 and -7 are both indirectly activated by *MBIP*, a major hub of the cell structure, function, and metabolism network. Increased expression in caspase-3 and -7 complexes have also been previously linked to TBIs in pre-clinical models (Clark et al., 2000; Larner, McKinsey, Hayes, & KK, 2005) and to mortality in patients with severe TBIs (Zhang et al., 2006). Increased activity in other apoptosis genes following blast include *EPB41L3*, or erythrocyte membrane protein band 4.1-like 3, and *EPB41L3*, a tumor suppressor gene strongly expressed in the brain that promotes apoptotic pathways and inhibits cellular proliferation (Li et al., 2011). These findings suggest that moderate blast results in expression of apoptosis inducing genes, and that mitigating these activities may be protective.

Lastly, several mitochondrial genes and genes connected with the mitochondrial gene network are dysregulated, including *COA5, HIBH, RPL6, RPL35*, as well as mitochondrial ribosomal genes *MRPL50, MRPL1, MRPL3*, and *MRPL46*. Although the

function of mitochondria is not yet well understood in blast exposures, it is worth noting that mitochondrial dysfunction has been implicated in preclinical TBI pathology. Previous studies have indicated that following TBI an influx of intracellular calcium leads to disruption of the mitochondrial membrane potential, impairing ATP production and creating ROS, activating cell death pathways and leading to neuronal damage associated with cognitive impairments (Ohta et al., 2013; Walker & Tesco, 2013). The biological mechanisms specific to blast effects on the central nervous system in the context of mitochondrial genes is not yet known.

Conclusion

The findings reported here provide further characterization of gene activity that occurs following moderate blast exposure, including changes in the activity of key pathways for ubiquitination, NF-κB, apoptosis and mitochondrial activity. This study had a unique design, as it allowed for evaluation of changes in gene-activity following a moderate blast exposure, by comparing gene-activity to baseline prior to blast exposure. These findings highlight the need for future studies in larger samples that include the collection of additional acute days of gene expression data, to complement consideration of acute and chronic symptomology and neuronal changes. This study's gene expression findings related to ubiquitination and inflammatory pathways add to previous TBI literature even though there was no acute TBI diagnosis in this cohort. Further study of such blast-associated effects and the role of these networks and associated proteins is warranted.

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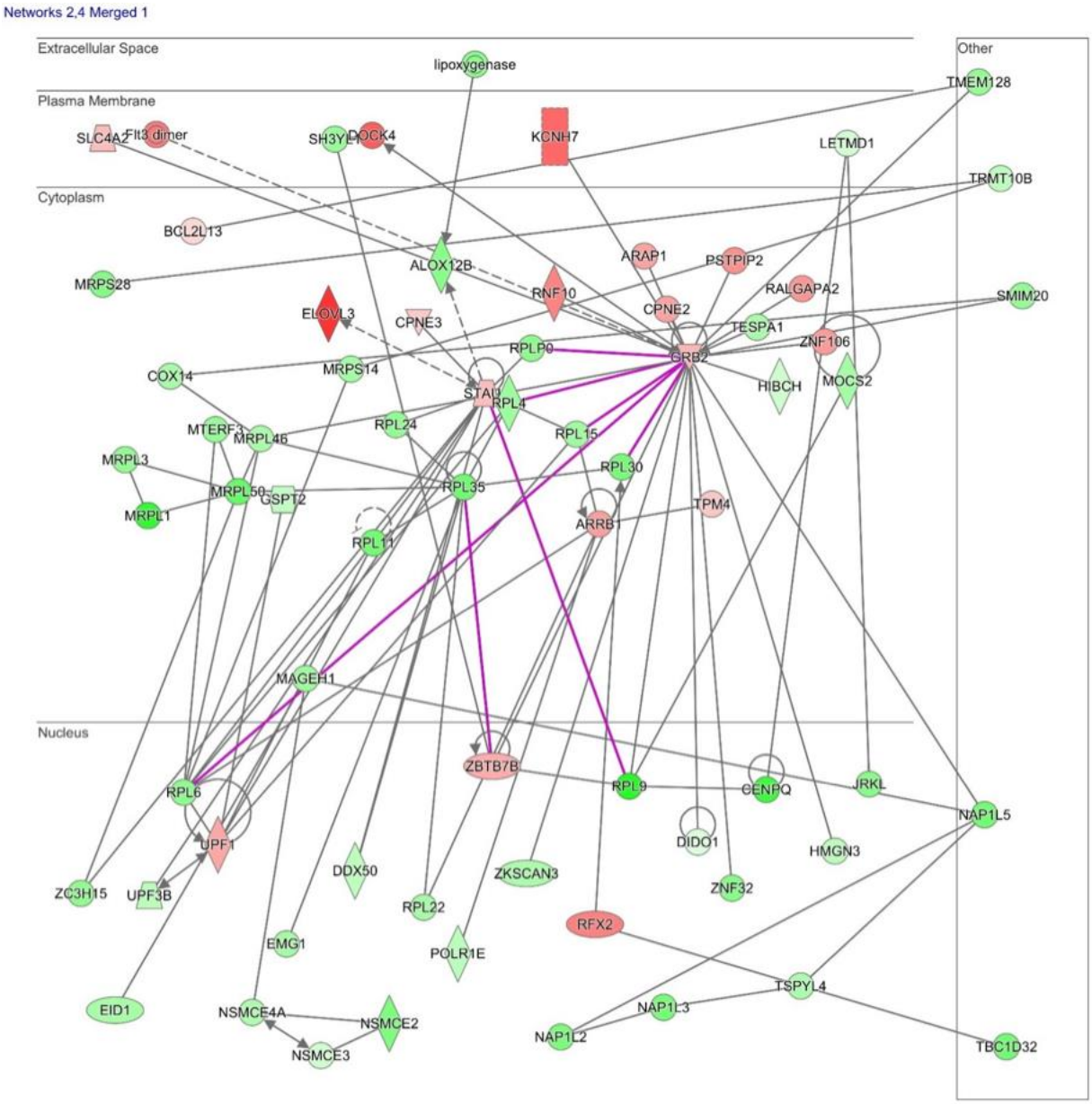
Table 3-1.

Demographic and previous explosive exposure of participants exposed to moderate blast

Variables	**Moderate Blast (N=29)**
Mean Age in Years (SD)	31.2 (4.4)
Mean Years of Service (SD)	11.2 (4.7)
Number of Prior Explosive Breaches	**Artillery Fires, % (n)**
0-9	20.7% (6)
10-39	34.8% (10)
40-99	17.2% (5)
100-199	20.6% (6)
200-399	6.9% (2)

Figure 3-1.

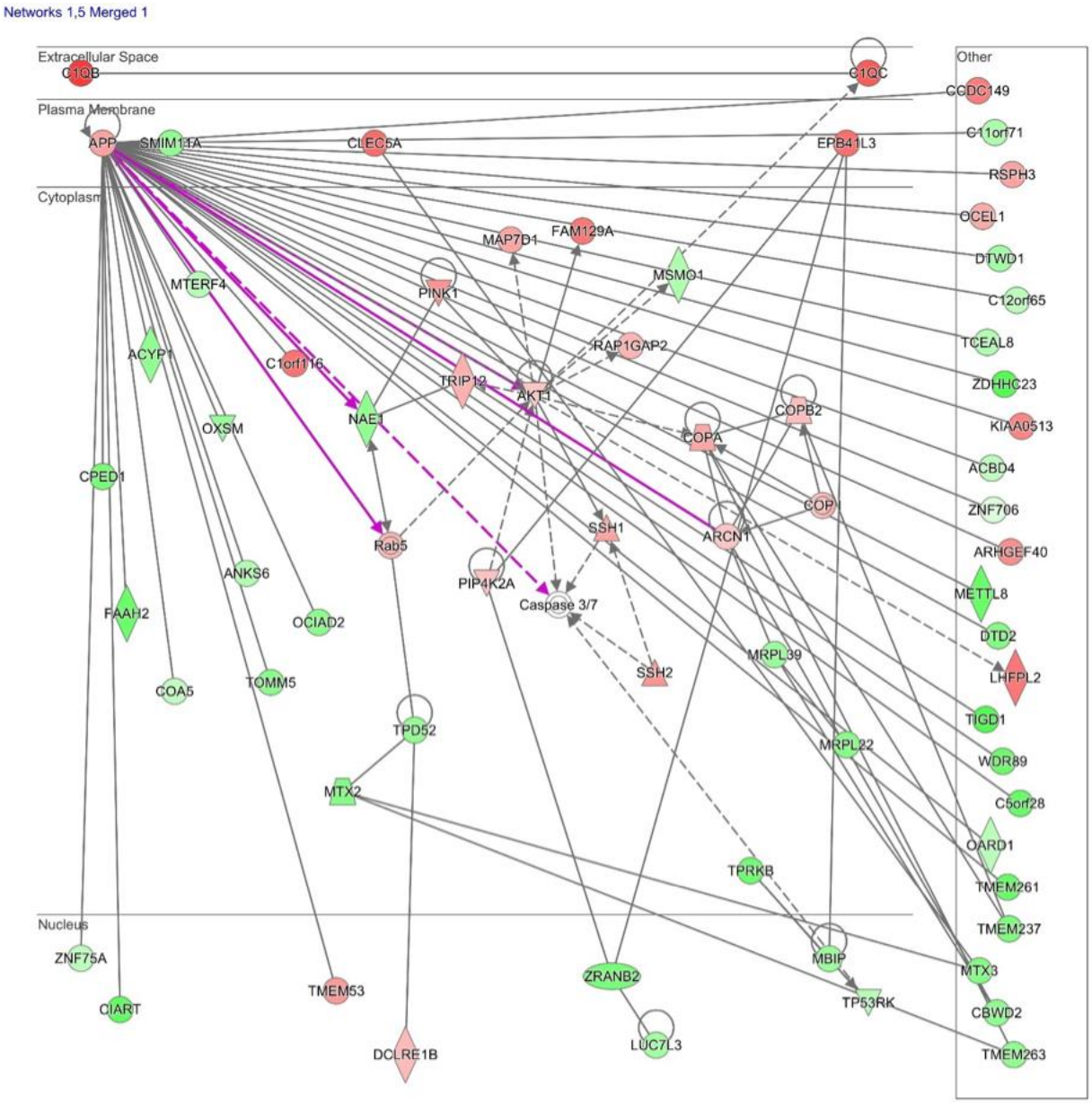
Cell death and survival pathway



Note. Ingenuity® pathway analysis (IPA®) figure shows dysregulated cell death and survival pathway following moderate blast. Genes described in the text included: *UPF1, UPF3B, ARRB1, ZBTB7B, flt3, HIBCH, RPL6, RPL35, MRPL1, MRPL3, MRPL36,* and *MRPL50*. See Legend for IPA networks for symbol meanings.

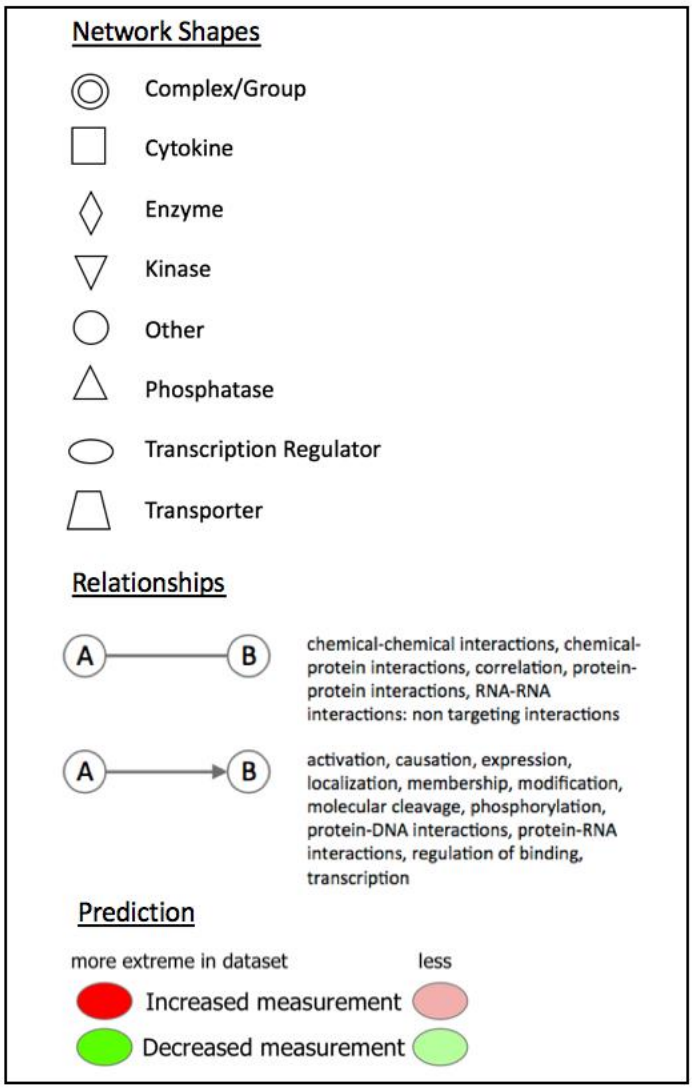
Figure 3-2.

Structure, function, and development pathway



Note. Ingenuity® pathway analysis (IPA®) figure shows dysregulated structure, function, and development pathway following moderate blast. Genes described in the text include: *TRIP12, NAE1, AKT1, MBIP, COA5*, and *EPB41L3*. See Legend for IPA networks for symbol meanings.

Note: Legend for IPA® Networks, Figures 3-1and 3-2.



Note. Legend indicates main features of the IPA® network, including molecular shapes, targeted and non-targeted relationships between molecules, and color showing increased or decreased measurement. Adapted from the Qiagen, Inc. IPA® legend http://ingenuity.force.com/ipa/IPATutorials?id=kA250000000TN2wCAG

Table 3-2.

Genes included in the cell death and survival pathway.

Gene Symbol	Gene Name	Ref Seq Accession	HKG	log2 Fold Change	Adjusted p-value
ABCF1	ATP Binding Cassette Subfamily F Member 1	NM_001090.2	Yes		
ACBD4*	Acyl-CoA Binding Domain Containing 4	NM_024722.2	-	-0.199499195	0.020347576
ALAS1	5'-Aminolevulinate Synthase 1	NM_000688.4	Yes	0.242752898	0.028367932
ALOX12B*	Arachidonate 12-Lipoxygenase, 12R Type	NM_001139.2	-	-0.346622555	0.039772707
ALOXE3*	Arachidonate Lipoxygenase 3	NM_001165960.1	-	-0.228199903	1
ARAP1*	ArfGAP With RhoGAP Domain, Ankyrin Repeat And PH Domain 1	NM_001040118.2	-	0.269598353	0.04521624

ARRB1*	Arrestin Beta 1	NM_004041.3	-	0.278700332	0.006452812
BCL2L13*	BCL2 Like 13	NM_001270733.1	-	0.179780094	0.04946175
BIRC3*	Baculoviral IAP Repeat Containing 3	NM_182962.2	-	-0.507459422	0.00558523
DECR1	2,4-Dienoyl-CoA Reductase 1, Mitochondrial	NM_001359.1	Yes		
DIDO1*	Death inducer-obliterator 1	NM_001193369.1	-	-0.09345587	0.045208841
FLT3*	Fms Related Tyrosine Kinase 3	NM_004119.2	-	0.389648012	0.018925186
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	NM_002046.3	Yes		
GRB2*	Growth Factor Receptor Bound Protein 2	NM_002086.4	-	0.214453639	0.048161569
GUSB	Glucuronidase beta	NM_000181.3	Yes	0.194071193	0.03424108
HIBCH*	3-Hydroxyisobutyryl-CoA	NM_014362.3	-	-0.137136152	0.048761345

	Hydrolase				
HMGN3*	High Mobility Group Nucleosomal Binding Domain 3	NM_004242.3	-	-0.201298014	0.02317844
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	NM_000194.1	Yes		
IPO8	Importin 8	NM_006390.2	Yes		
KCNH7*	Potassium Voltage-Gated Channel Subfamily H Member 7	NM_033272.2	-	0.441079018	0.038059517
MAGEH1*	MAGE Family Member H1	NM_014061.3	-	-0.294631712	0.003622067
-93 miR	MicroRNA 93	NR_029510.1	Yes		
MOCS2*	Molybdenum Cofactor Synthesis 2	NM_004531.4	-	-0.266516936	0.049007307

MRPL1*	Mitochondrial Ribosomal Protein L1	NM_020236.3	-	-0.62382527	0.00331706
MRPL3*	Mitochondrial Ribosomal Protein L3	NM_007208.2	-	-0.330469686	0.030366265
MRPL46*	Mitochondrial Ribosomal Protein L46	NM_022163.3	-	-0.260611172	0.010945064
MRPL50*	Mitochondrial Ribosomal Protein L50	NM_019051.1	-	-0.46102798	0.010773055
MRPS14*	Mitochondrial Ribosomal Protein S14	NM_022100.1	-	-0.277593921	0.003523616
MRPS28*	Mitochondrial Ribosomal Protein S28	NM_014018.2	-	-0.368377224	0.0463031
NAP1L2*	Nucleosome Assembly Protein 1 Like 2	NM_021963.3	-	-0.394155658	0.040740338

NAP1L3*	Nucleosome Assembly Protein 1 Like 3	NM_004538.4	-	-0.403261661	0.04324522
NSMCE3*	NSE3 Homolog, SMC5-SMC6 Complex Component	NM_138704.2	-	-0.138835375	0.069775418
NSMCE4A*	NSE4 Homolog A, SMC5-SMC6 Complex Component	NM_017615.2	-	-0.244479492	0.016903185
PARK2*	Parkin RBR E3 Ubiquitin Protein Ligase	NM_004562.2	-	-0.462238206	0.039312303
PGK1	Phosphoglycerate Kinase 1	NM_000291.2	Yes	0.19447752	0.023843395
PSTPIP2*	Proline-Serine-Threonine Phosphatase Interacting Protein 2	NM_024430.3	-	0.327102801	0.029452324
RFX2*	Regulatory Factor X2	NM_000635.3	-	0.371048376	0.028931549
RNF10*	Ring Finger Protein 10	NM_014868.3	-	0.363231062	0.019697501

RPL11*	Ribosomal Protein L11	NM_000975.2	-	-0.422877581	0.016695613
RPL15*	Ribosomal Protein L15	NM_001253379.1	-	-0.285314324	0.023453576
RPL22*	Ribosomal Protein L22	NM_000983.3	-	-0.264541621	0.021464081
RPL30*	Ribosomal Protein L30	NM_000989.2	-	-0.408465221	0.017083487
RPL35*	Ribosomal Protein L35	NM_007209.3	-	-0.43080228	0.010643658
RPL4*	Ribosomal Protein L4	NM_000968.2	-	-0.289569243	0.020267917
RPL6*	Ribosomal Protein L6	NM_000970.3	-	-0.340254818	0.018412596
RPL9*	Ribosomal Protein L9	NM_000661.4	-	-0.714413772	0.001773623
SH3YL1*	SH3 And SYLF Domain Containing 1	NM_001159597.1	-	-0.311600753	0.038059517
STAU1*	Staufen Double-Stranded RNA Binding Protein 1	NM_017454.2	-	0.19259478	0.016276538
TBP	TATA-Box Binding Protein	NM_001172085.1	Yes		
TESPA1*	Thymocyte Expressed,	NM_001098815.2	-	-0.261036039	0.010073305

	Positive Selection Associated 1				
TPM4*	Tropomyosin 4	NM_003290.2	-	0.161122161	0.016276538
TRMT10B	TRNA Methyltransferase 10B	NM_144964.3	-	-0.196738227	0.026654574
TSPYL4*	TSPY Like 4	NM_021648.4	-	-0.247290787	0.008592219
UPF1*	UPF1, RNA Helicase And ATPase	NM_002911.3	-	0.254526328	0.03643324
UPF3B*	UPF3 Regulator Of Nonsense Transcripts Homolog B (Yeast)	NM_080632.2	-	-0.2020123	0.026669595
ZBTB7B*	Zinc Finger And BTB Domain Containing 7B	NM_015872.2	-	0.243249222	0.025478126
ZC3H15*	Zinc Finger CCCH-Type Containing 15	NM_018471.2	-	-0.335063911	0.026607119
ZKSCAN3*	Zinc Finger With KRAB And	NM_001242895.1	-	-0.253800344	0.031068942

	SCAN Domains 3				
ZNF106*	Zinc Finger Protein 106	NM_022473.1	-	0.294418924	0.015098223
ZNF32*	Zinc Finger Protein 32	NM_006973.2	Yes	-0.355641394	0.012837388

Note. HKG=house-keeping gene. *Validated by NanoString

Table 3-3.

Genes included in the structure, function, and development pathway.

Gene Symbol	Gene Name	Ref Seq Accession	HKG	log2 Fold Change	Adjusted p-value
ABCD4*	ATP Binding Cassette Subfamily D Member 4	NR_003256.2	-	-0.199499195	0.020347576
ABCF1	ATP Binding Cassette Subfamily F Member 1	NM_001090.2	Yes		
ACYP1*	Acylphosphatase 1	NM_001107.3	-	-0.329474401	0.019194616
AKT1*	AKT Serine/Threonine Kinase 1	NM_001014432.1	-	0.17005921	0.038615925
ALAS1	5'-Aminolevulinate Synthase 1	NM_000688.4	Yes	0.242752898	0.028367932
ANKS6*	Ankyrin Repeat and Sterile	NM_173551.3	-	-0.235773531	0.040849382

	Alpha Motif Domain Containing 6				
APP*	Amyloid Precursor Protein	NM_000484.3	-	0.275234781	0.033723847
ARCN1*	Archain 1	NM_001655.4	-	0.171105347	0.046801529
C12orf65*	Chromosome 12 open reading frame 65	NM_152269.4	-	-0.204227132	0.026071674
C1QB*	Complement Component 1, Q Subcomponent, B Chain	NM_000491.3	-	0.584216904	0.011252651
CIART*	Circadian Associated Repressor of Transcription	NM_144697.2	-	-0.475138427	0.038059517
CLEC5A*	C-Type Lectin Domain Family 5 Member A	NM_013252.2	-	0.43694439	0.013654046
COA5*	Cytochrome C Oxidase Assembly Factor 5	NM_001008215.2	-	-0.188436396	0.030279095

COPA*	Coatamer Protein Complex Subunit Alpha	NM_004371.3	-	0.2593244	0.019024708
COPB2*	Coatamer Protein Complex Subunit Beta	NM_004766.2	-	0.192983112	0.012365191
DCLRE1B*	DNA Cross-Link Repair 1B	NM_022836.3	-	0.198400973	0.017155237
DECR1	2,4-Dienoyl-CoA Reductase 1	NM_001359.1	Yes		
EPB41L3*	Erythrocyte Membrane Protein Band 4.1 Like 3	NM_012307.2	-	0.431151189	0.006476354
FAAH2	Fatty Acid Amide Hydrolase 2	NM_174912.3	-	-0.499379188	0.005745525
FAM129A*	Family with sequence similarity 129, member A	NM_052966.2	-	0.40324029	0.024746754
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	NM_002046.3	Yes		
GUSB	Glucuronidase Beta	NM_000181.3	Yes	0.194071193	0.03424108

HPRT1	Hypoxanthine Phosphoribosyltransferase 1	NM_000194.1	Yes		
IPO8	Importin 8	NM_006390.2	Yes		
KIAA0513*	KIAA0513 Ortholog	NM_014732.3	-	0.361322535	0.014586154
LHFPL2*	Lipoma HMGIC Fusion Partner-Like 2	NM_005779.2	-	0.400554788	0.000715584
LUC7L3*	LUC7 Like 3 Pre-MRNA Splicing Factor	NM_006107.2	-	-0.279039102	0.042125529
MAP7D1*	MAP7 Domain Containing 1	NM_018067.3	-	0.26655588	0.039772707
MBIP*	MAP3K12 Binding Inhibitory Protein 1	NM_001144891.1	-	-0.304655557	0.017155256
miR-93		NR_029510.1	Yes		
MRPL22*	Mitochondrial Ribosomal Protein L22	NM_014180.3	-	-0.361248608	0.023357416

MRPL39*	Mitochondrial Ribosomal Protein L39	NM_017446.3	-	-0.30416698	0.015173431
MSMO1*	Methylsterol Monooxygenase 1	NM_001017369.1	-	-0.237679234	0.041114756
MTX2*	Metaxin2	NM_006554.4	-	-0.369413967	0.019530729
MTX3*	Metaxin3	NM_001010891.4	-	-0.370201897	0.032915527
NAE1*	NEDD8 Activating Enzyme E1 Subunit 1	NM_001018159.1	-	-0.324495292	0.007350252
OARD1*	O-Acyl-ADP-Ribose Deacylase 1	NM_145063.2	-	-0.205410287	0.0115853
OCIAD2	Ovarian Carcinoma Immunoreactive Antigen-Like Protein 2	NM_152398.2	-	-0.349639139	0.004637306
OXSM*	3-Oxoacyl- Acyl Carrier	NM_017897.2	-	-0.306169512	0.028418587

	Protein Synthase, Mitochondrial				
PGK1	Phosphoglycerate Kinase 1	NM_000291.2	Yes	0.19447752	0.023843395
PIP4K2A*	Phosphatidylinositol-5-Phosphate 4-Kinase Type 2 Alpha	NM_005028.3	-	0.180982773	0.033210105
RAB5A*	RAS-Associated Protein RAB5A	NM_004162.4	-	0.18204685	0.136291748
RAP1GAP2*	RAP1 GTPase Activating Protein 2	NM_015085.4	-	0.219298444	0.011143681
RSPH3*	Radial Spoke 3 Homolog	NM_031924.4	-	0.272080541	0.032117983
SSH1*	Slingshot Protein Phosphatase 1	NM_018984.3	-	0.269175026	0.010309573
SSH2*	Slingshot Protein	NM_033389.3	-	0.315283525	0.024375198

	Phosphatase 2				
TBP	TATA-Box Binding Protein	NM_001172085.1	Yes		
TCEAL8*	Transcription Elongation Factor A Like 8	NM_153333.2	-	-0.242696303	0.044714506
TIGD1*	Tigger Transposable Element Derived 1	NM_145702.1	-	-0.53120337	0.012433895
TMEM237*	Transmembrane Protein 237	NM_001044385.1	-	-0.394403177	0.032253724
TMEM261*	Transmembrane Protein 261	NM_001318058.1	-	-0.464675435	0.002668268
TMEM263	Transmembrane Protein 263	NM_152261.2	-	-0.34231287	0.048588716
TOMM5*	Translocase Of Outer Mitochondrial Membrane 5	NM_001001790.2	-	-0.354166369	0.045960581
TP53RK*	TP53 Regulating Kinase	NM_033550.3	-	-0.213093565	0.017767807
TPD52*	Tumor Protein D52	NM_005079.2	-	-0.330475184	0.038005975
TPRKB*	TP53RK Binding Protein	NM_016058.2	-	-0.443196546	0.006016182

TRIP12*	Tripartite Motif Containing 12	NM_004238.1	-	0.231188855	0.003223298
ZDHHC23	Zinc Finger CCHC-Type Containing 23	NM_173570.3	-	-0.520810011	0.004066651
ZNF706*	Zinc finger protein 706	NM_001042510.1	-	-0.10910689	0.031066687
ZRANB2*	Zinc Finger RANBP2-Type Containing 2	NM_005455.4	-	-0.40446744	0.019490303

Note. HKG=house-keeping gene. *NanoString validation.

Table 3-4.

IPA® Network Scores

Network	**IPA Network Score**
Metabolic	45
Cell Death and Survival	42
Post-Translational Modification	42
Cancer, Cell Death and Survival	42
Immunological Diseases	37
Merged Networks	**IPA Network Score**
Cell Death and Survival	42
Cell Structure, Function, and Metabolism	41
Note. Network scores are numerical values used to rank fit of molecules to the network. The scores are calculated using an algorithm based on Fisher's Exact Test. Eligible molecules are compared to the Ingenuity Knowledge Base of over 1 million molecules curated from literature findings. Highly interconnected genes imply significant biological function.	

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INTERLEUKIN-6 ASSOCIATED WITH ACUTE CONCUSSION IN MILITARY COMBAT PERSONNEL

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CHAPTER FOUR

INTERLEUKIN-6 ASSOCIATED WITH ACUTE CONCUSSION IN MILITARY COMBAT PERSONNEL

Introduction

Concussion, or mild traumatic brain injury (mTBI), is recognized as one of the most prevalent injuries among military members serving in Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF), yet biomarkers related to these injuries and the related recovery processes remain elusive (DePalma, 2015; Jones, Fear, & Wessely, 2007). The most common cause of concussions sustained by deployed personnel worldwide is blast exposures, especially by improvised explosive devices (IEDs) (Ritenour & Baskin, 2008). A blast exposure can directly result in a concussion, and it may also contribute to blunt force injuries if the soldier comes into contact with objects resulting from the exposure to the blast, i.e. being thrown into objects or being hit by objects from the blast (Champion, Holcomb, & Young, 2009; Ramasamy, Harrisson, Clasper, & Stewart, 2008). Blast exposure effects multiple organs and tissues, including the central nervous system, which is well documented in preclinical models (Mac Donald et al., 2011). It is also increasingly recognized that military personnel can sustain multiple blast exposures as well as concussions during combat deployments, and the consequences of these injuries are just now being determined (Carr et al., 2016). Over time, blast exposures as well as blunt force injuries are associated with neurological symptoms that are garnering concern for the health and well-being of military personnel and veterans (Carr et al., 2015; Mac Donald et al., 2016; Mac Donald et al., 2014). At this time, limited objective measures exist for identification of individuals who may be at high risk

for developing complications and poor outcomes following concussion, including those affected by concussion and blast exposures during deployment. Thus, identifying underlying biological changes that occur following a concussion or mTBI are crucial to identify those military personnel who may

be at the most risk, who require increased monitoring and preventive interventions, and for ongoing monitoring of individuals who may be at high risk for poor outcomes (Prieto, Ye, & Veenstra, 2008).

Peripheral biomarkers show promise in distinguishing patients with traumatic brain injuries (TBIs) who require additional monitoring and interventions, yet most previous studies primarily include severe patients (Papa et al., 2016; Papa et al., 2012). Thus, protein biomarkers may be useful in monitoring those at high risk for poor outcomes, which may be especially beneficial among concussed individuals who may not otherwise follow up on mild subjective symptoms (Menon, Schwab, Wright, & Maas, 2010). Specifically, studies of blood-based inflammatory protein biomarkers may implicate the underlying inflammatory processes following concussions that are important for acute recovery (Ferreira et al., 2014; Woiciechowsky et al., 2002) and may relate to chronic symptoms (Kumar, Boles, & Wagner, 2015; Licastro et al., 2016). Not only may inflammatory biomarkers help monitor outcomes, but they may also help identify inflammatory pathways that may be targeted for therapies (McKee & Lukens, 2016). Of interest are pro- and anti-inflammatory cytokines, as they have been implicated in the underlying balance of inflammatory processes which occur following a TBI (Hinson, Rowell, & Schreiber, 2015; Woodcock & Morganti-Kossmann, 2013). For

example, preclinical brain injury studies of interleukin (IL)-6 indicate some IL-6 activity is beneficial for recruiting immune cells and improving outcomes, especially in the acute phase (Penkowa et al., 2003). However, harmful outcomes may result from either IL-6 deficiency, as demonstrated in IL-6 knockout mice (Penkowa, Giralt, Carrasco, Hadberg, & Hidalgo, 2000), or chronic IL-6 overexpression (McKee & Lukens, 2016; Penkowa et al., 2003). Likewise, the study of IL-10 in preclinical brain injury models has shown poor outcomes in IL-10 knockout mice, with IL-10 administration improving neurological function and decreasing lesion volume (Kline et al., 2002). In human studies, elevated levels of IL-6, IL-10, and TNFα, among others, have been associated with poor outcomes in severe cases of TBI (Arand, Melzner, Kinzl, Bruckner, & Gebhard, 2001; Ferreira et al., 2014; Woiciechowsky et al., 2002). However, fewer studies have evaluated cytokines in concussions. This lab has previously reported that elevated levels of plasma inflammatory cytokines, IL-6 and TNFα, are concurrent with chronic neurological symptomology among military personnel who experienced blunt force and/or blast injury (within 16 months of deployment) (Devoto et al., 2016). This lab also reported an association between moderate blast exposure and acute increases in levels of IL-6 and TNFα within 16 months of deployment in a military training population (Gill et al., 2017). Yet, peripheral levels of IL-6, IL-10, and TNFα have not yet been measured during the first 24 hours following concussion sustained during a military combat deployment.

To better understand the role of inflammatory cytokines in concussions, cytokines levels of (IL-6, IL-10, and TNFα) were measured acutely following a medically

diagnosed concussion, during transport to a medical facility, and then again 24 hours later to characterize the relationship between cytokines and recovery from acute brain injury. This project is needed to expand the understanding of peripheral inflammatory biomarker levels in a cohort of deployed military personnel who sustain concussions. Findings from this line of research will provide the basis to identify the biological underpinnings of inflammatory processes occurring in the acute stage of recovery from concussion sustained in austere environments like military deployment where blast has become a primary cause of injury, which is necessary to improve recovery trajectories.

Methods

Participants.

This study protocol was reviewed and approved by the Research Institutional Review Boards at the US Army Medical Research and Materiel Command (M-10216) and the Walter Reed Army Institute of Research (WRAIR #2028, #2529). Each study participant provided informed consent prior to participation. This unique, observational cohort study consisted of: 1) deployed military personnel who sustained a concussion, provider diagnosed, without other major medical diagnosis and received acute medical care (n=45) and 2) healthy control participants in the same deployment environment who did not sustain concussion or other illness or injuries (n=49). Both groups were deployed to units in the same region of operations in Afghanistan during 2012. Participants had blood draws at two time points: 1) time point 1 was at the time of medical care, less than 8 hours after concussion, or at the time of initial encounter for the healthy control group and 2) time point 2 was at 24 hours following the time of the first blood draw.

Blood sampling.

Whole blood was drawn and processed for serum, using standard protocols, within one hour of the blood draw. Serum was aliquoted and stored at -80 C until batch processing and analyses.

Laboratory methods.

IL-6, IL-10, and TNFα concentrations were measured using Simoa technology. Simoa™ (Quanterix, Lexington, MA), an ultrasensitive single-molecule enzyme-linked immunosorbent assay, as previously described (Mondello et al., 2014). The IL-6, IL-10, and TNFα assays have low limits of detections (0.006pg/mL, 0.0022pg/mL, and 0.011pg/mL, respectively). Samples were run in duplicate, and the personnel running analyses were blinded to group. Average coefficient of variance (CV) were 4.75%, 4.43%, and 4.78% for IL-6, IL-10 and TNFα, respectively. Samples with CVs > 15% were excluded.

Statistical methods.

SPSS version 25 (IBM Corporation, Chicago, IL) was used to conduct statistical analyses, and GraphPad Prism version 7.0d (Graph Pad Software, San Diego, CA) was used to create figures. Baseline demographic characteristics were compared between healthy and concussed groups using Pearson's chi square (race and gender) and ANOVA (age). Distributions did not require adjustment for normality. The differences in concentrations of IL-6, IL-10, and TNFα at two time points (time point 1 = <8 hours after injury with time point 2 = 24 hours following time point 1) were compared between the healthy and concussed groups using Mann Whitney U tests. Mean difference was

calculated by subtracting each participant's cytokine concentration at time point 2 from the concentration level at time point 1, resulting in a variable that reflects the total change in each cytokine between the groups. This calculated change resulted in the creation of a variable that could then be compared between the groups to determine if there were differences in the change in cytokine concentrations between these groups. A Mann Whitney U test was conducted to evaluate if there was a significant change in the cytokines in the concussed group compared to the healthy control group. Since groups were similar in demographic characteristics, we did not include any covariates in these models.

Results

Demographics.

The sample included primarily male (96.8%) participants who were active duty service members (n=94) deployed to Afghanistan. The mean age was 26.41 years (SD=6.364) with a range of 19 to 48 years of age. Here, military personnel who were medically diagnosed with a concussion and received acute care (n=45) were compared to healthy controls with no diagnosis of concussion (n=49) deployed to the same combat station. The two groups did not differ in demographic features including sex, race, or age (see Table 4-1). All participants within both groups had a Glasgow Coma Score (GCS) of 15. The concussed personnel were diagnosed by a healthcare provider <8 hours following injury. Of the concussed personnel, 33 (73.3%) participants were exposed to blast during the injury event, with the others reporting a blunt force injury (see Table 4-2).

Table 4-1.

Demographic Data

	Healthy Controls (N=49)	Concussion (N=45)	Significance
Mean age in years (SD)	26.63 (6.978)	26.36 (5.747)	p=0.841[a] F=0.041[a]
Sex			p=0.066[b]
Male	49 (100%)	42 (93.3%)	
Female	0 (0%)	3 (6.7%)	
Race			p=0.297[b]
White	35 (71.4%)	21 (67.7%)	
Black	5 (10.2%)	0 (0.0%)	
Hispanic	6 (12.2%)	5 (7.5%)	
Pacific Islander	2 (4.1%)	1 (3.2%)	
Asian	1 (2.0%)	1 (3.2%)	
Middle Eastern	0 (0.0%)	1 (3.2%)	
Other	0 (0.0%)	2 (6.5%)	

Note. The percentages in each column refer to the proportion of individuals in each sex and race category. [a]Anova [b]Pearson's chi square *p value significant at the $p<0.05$ level.

Table 4-2.

Clinical Data

Reason for visit	Concussion (N=45)
Blast exposure	n=33 (73.3%)
Blunt force injury, only	n=12 (26.7%)

Note. The percentages in each column refer to the proportion of individuals with each reason for visit.

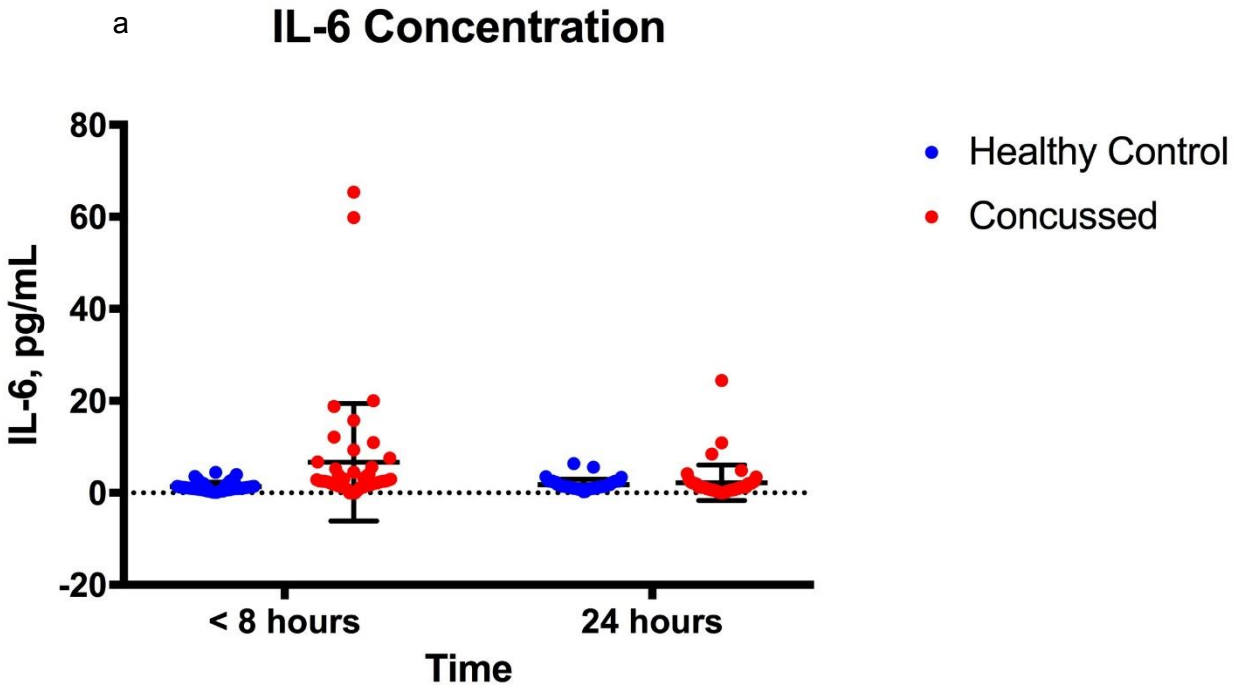
Inflammatory protein changes following mild concussion.

Comparisons at time point 1 and time point 2. Differences in IL-6, IL-10, and TNFα between healthy and concussed groups were evaluated at each time point (time point 1 = <8 hours after injury; time point 2 = 24 hours following time point 1). At time-point 1, IL-6 concentrations were significantly greater in the concussed group (M=3.92, SD=9.30) compared to the healthy control group (M=1.48, SD=0.50; $U = 420.00$, z= -5.12, p<0.001) (see Figure 1a). Compared to healthy controls, the concussed group did not significantly differ at time point 1 in concentrations of IL-10 (p=0.358) or TNFα (p=0.382) (see Figure 4-1,b-c). At time point 2, no significant differences were detected between concussed and healthy controls for IL-6 (p=0.075), IL-10 (p=0.937), or TNFα (p=0.390) concentrations (see Figure 4-1,a-c).

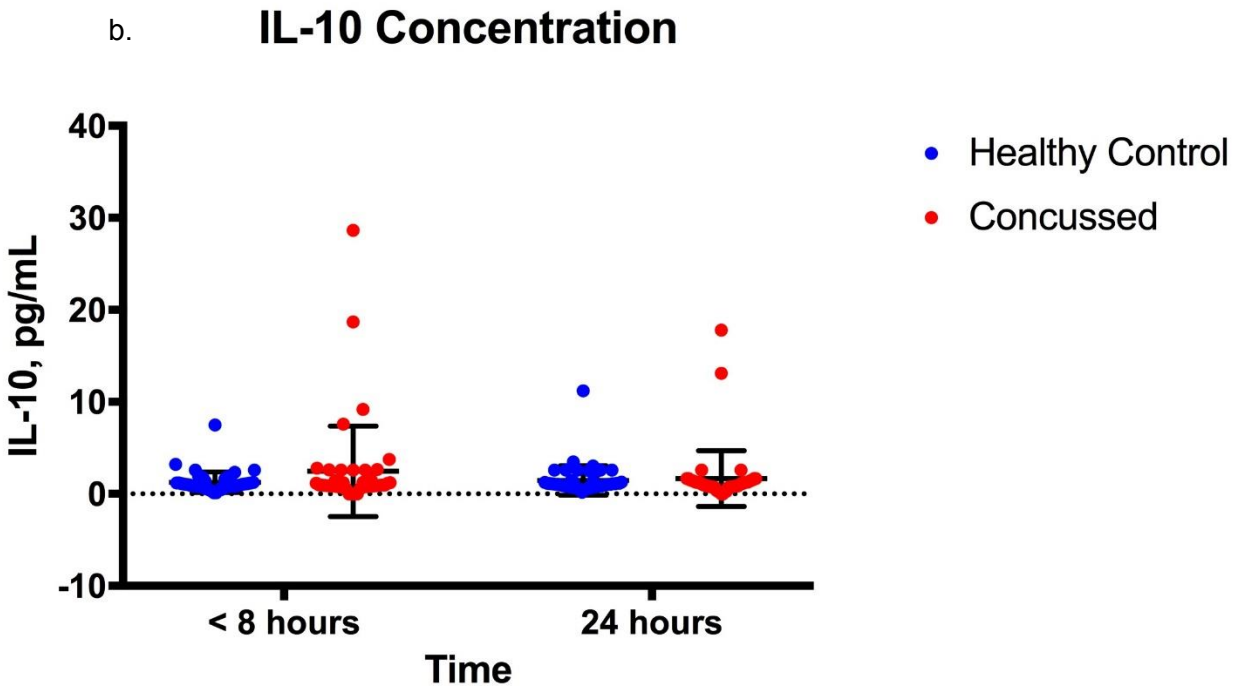
Figure 4-1 a-c

Comparison of Cytokines between Concussed and Healthy Controls at Two Time Points

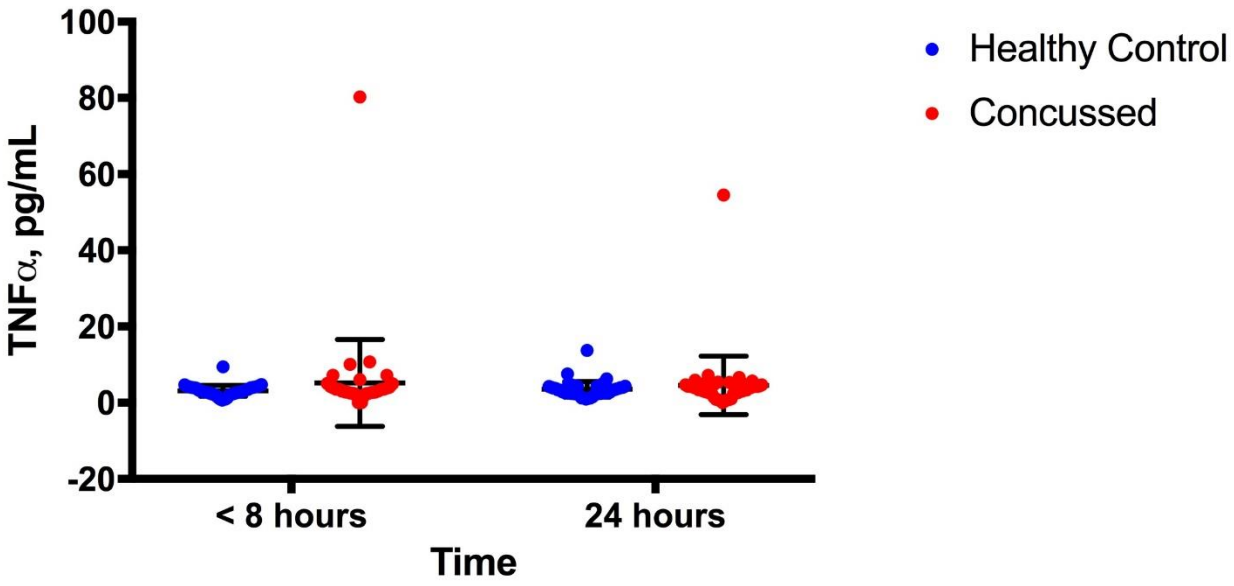
a



b.



c. **TNFα Concentration**



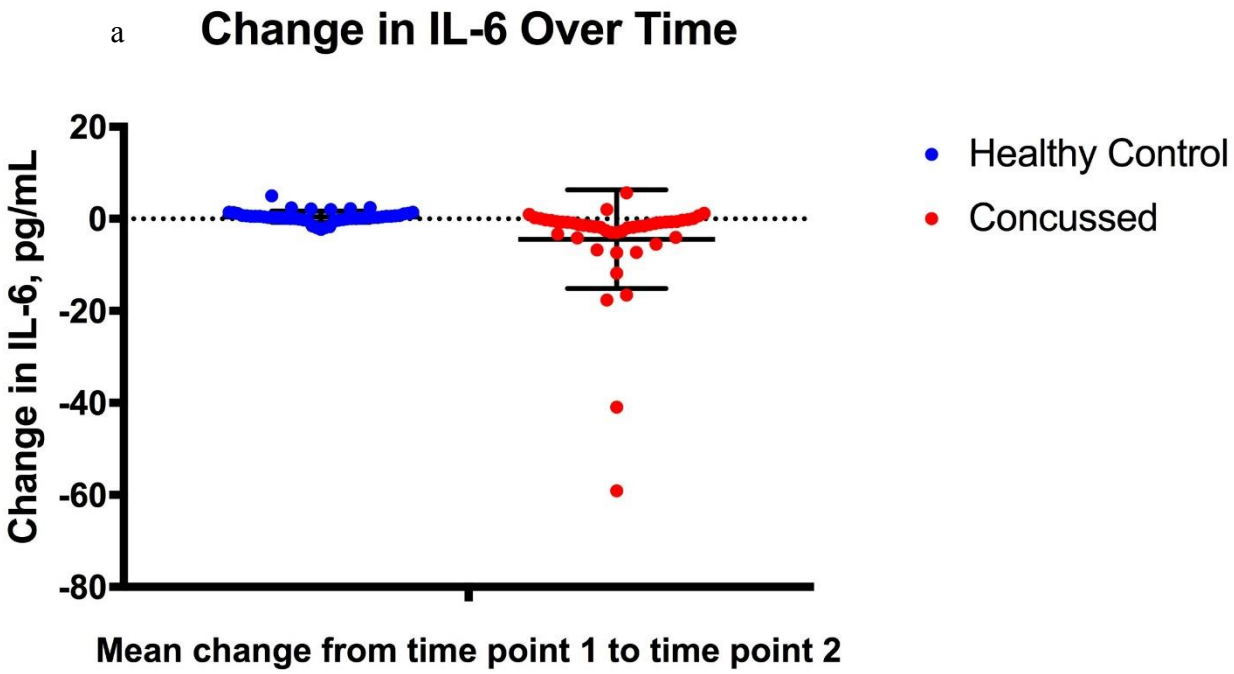
Note. Time point 1 is <8 hours after injury. Time point 2 is 24 hours after time point 1. Mann Whitney U tests were conducted to compare each cytokine's concentration between the healthy and concussed groups at each time point for a. IL-6, b. IL-10, and c. TNFα. IL-6 concentration was significantly higher in the concussed group at time point 1 at $p<0.0001$.

Mean Change Across Time. The mean difference between time point 1 and time point 2 was compared between the concussed and healthy control groups for IL-6, IL-10, and TNFα. A Mann Whitney U test was conducted to determine that the mean difference in IL-6 was significantly different in the concussed group as compared to the healthy control (M= -1.94, SD=7.91; $U = 315.00$, z= -5.96, $p<0.001$) (see Figure 4-2a). However, there was no difference between groups in the change of IL-10 ($p=0.158$) or TNFα ($p=0.777$) (See Figure 2b-c). The percentage change in IL-6 was -67.7% in the concussed group compared to 33.5% in the healthy controls.

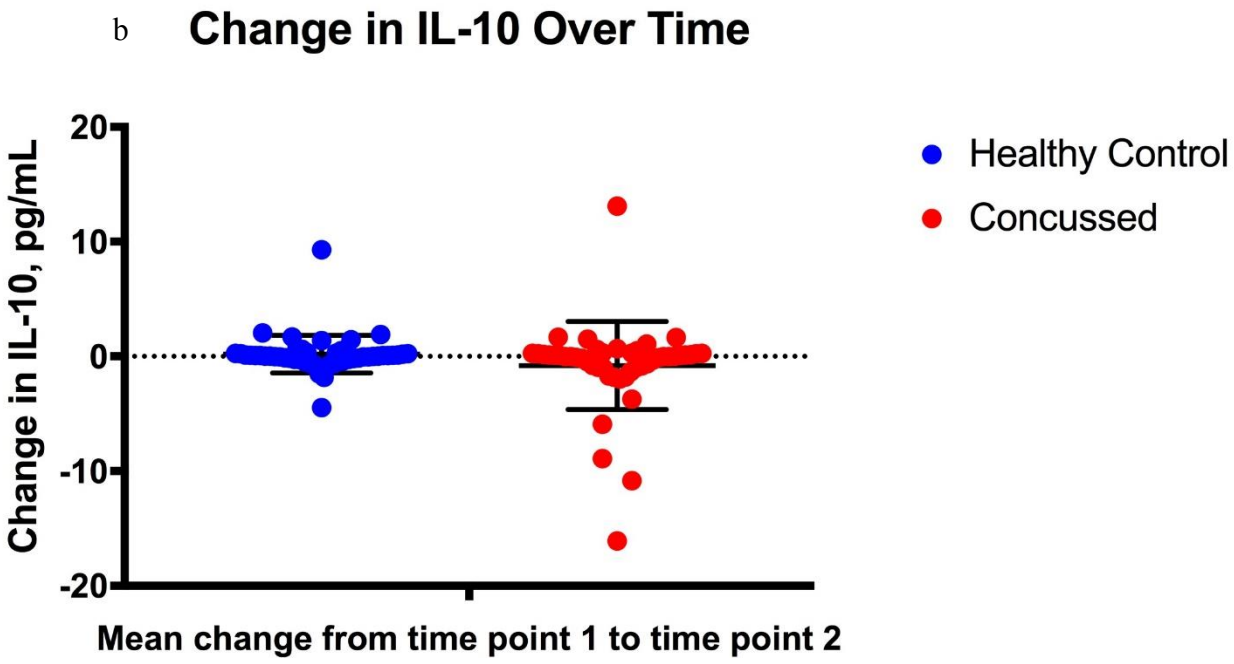
Figure 4-2 a-c

Mean Difference in Each Cytokine Over Time from Time Point 1 to Time Point 2

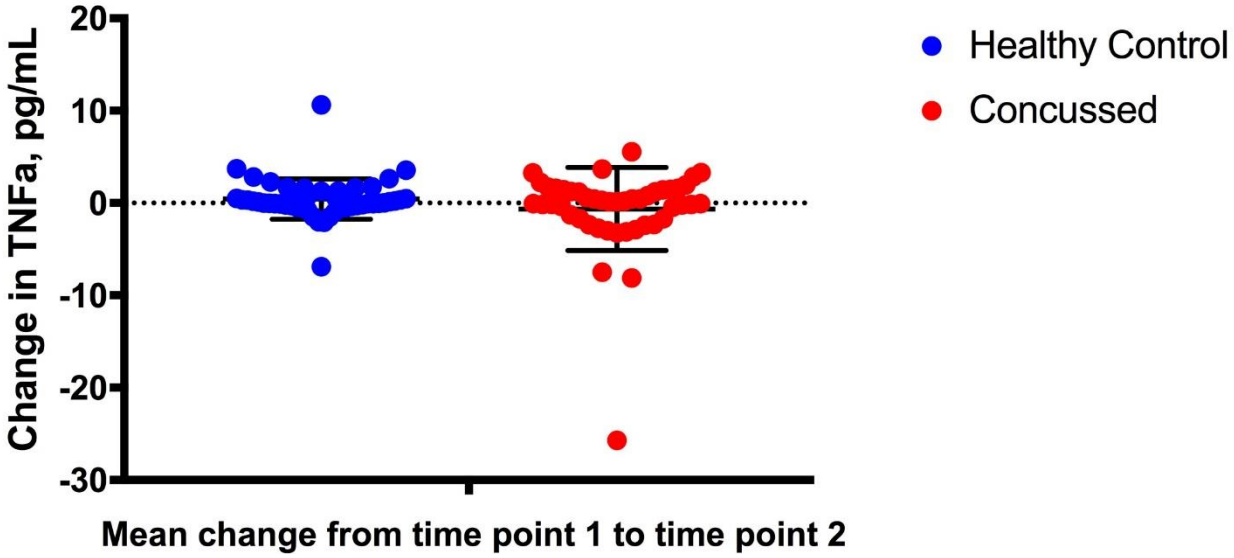
a



b



c. **Change in TNFa Over Time**



Note. Time point 1 is <8 hours after injury. Time point 2 is 24 hours after time point 1. Mean difference = (each participant's cytokine concentration at time point 2) -(each participant's concentration level at time point 1). Mann Whitney U tests were conducted to determine if there were differences in the mean change variable between these groups. a. IL-6, b. IL-10, and c. TNFα. IL-6 was significantly different in the concussed group as compared to the healthy control at $p<0.0001$.

Discussion

The findings of higher IL-6 within 8 hours of a medically diagnosed concussion sustained during combat deployment is consistent with previous studies that report acutely elevated levels of IL-6 in severe TBI patients (Arand et al., 2001; Ferreira et al., 2014; Woiciechowsky et al., 2002). In fact, this study is the only one known that reports acute biomarker findings in a deployed cohort of military personnel with concussion. There are a variety of factors that make a deployed population unique, and for this study, paramount is the high rate of blast exposure. This finding is in line with this lab's previous report that linked IL-6 elevations to a moderate blast exposure sustained during

training by an undiagnosed population which did not include blunt force. That elevation was then followed by a decrease in IL-6 in sampling on subsequent days to below baseline levels (Gill et al., 2017). Therefore, the present findings indicate that concussions sustained during deployment, highly comorbid with blast, result in elevations of IL-6, following by a decrease in concentrations within 24 hours. This finding suggests that IL-6 is coordinating recovery from concussions, as well as blast exposures, and that understanding these complex relationships may be important to improving care provided to military personnel with complex, and often overlapping injuries sustained in combat stations.

IL-6 is involved in the modulation of pro- and anti-inflammatory activity following a TBI, with evidence pointing to the importance of the balance of IL-6 levels in the promotion of recovery following TBIs and concussions (McKee & Lukens, 2016). Cytokines, such as IL-6, orchestrate the acute inflammatory response to brain injury (Helmy, Carpenter, Menon, Pickard, & Hutchinson, 2011; Hinson et al., 2015). In support of this, preclinical models that knock out IL-6 activity result in poor behavioral performance following a TBI (Ley, Clond, Singer, Shouhed, & Salim, 2011) as well as increased apoptosis and delayed neuronal regeneration (Penkowa et al., 2000). Detrimental outcomes also occur with elevated IL-6 activity following a TBI, including delays in motor coordination and neuronal tissue repair in preclinical models (Penkowa et al., 2003; Yang, Gangidine, Pritts, Goodman, & Lentsch, 2013). There is also evidence of increased IL-6 concentration in human post-mortem brain tissue obtained following a severe TBI that resulted in mortality, compared to patients who died from non-central

nervous system causes (Frugier, Morganti-Kossmann, O'Reilly, & McLean, 2010). There may be long-term health consequences that result if IL-6 remains imbalanced, as findings of elevated IL-6 may be indicative of chronic neurological symptoms or deficits (Devoto et al., 2016).Therefore, findings from the current study showing that an IL-6 elevation occurs within hours of a concussion, and are then similar to healthy controls at 24 hours later, suggests that IL-6 is playing a role in recovery from these mild injuries. Considering these early findings in concussion, additional studies with longer follow up are warranted to understand the role of IL-6 in recovery and links to long-term consequences.

Conversely, here is reported that IL-10 and TNFα were not significantly different between the concussed and healthy military cohorts. This differs from a previous report of elevated levels of TNFα in military personnel following blast exposure, along with elevated IL-6 levels (Gill et al., 2017). One explanation may be differences between the samples in the two studies. Gill et al. (2017) studied military personnel in a well-controlled training environment, with no reported incidences of blunt injuries and no medical diagnosis, while the present cohort included military personnel diagnosed with a concussion. These reported differences in injury event characteristics may account for a lack of TNFα differences between the concussed and healthy military personnel. Likewise, IL-10 was not significantly different between the groups in the present study, a finding that replicated Gill et al. (2017). As would be expected from non-significant findings of IL-10 and TNFα cohort differences at time point 1, mean change over time was also not significant between the healthy and concussed cohorts. The absence of an

upregulation of IL-10 concurrently with the IL-6 elevation is suggestive of possible immune dysregulation. Both pro- and anti-inflammatory cytokines are produced by microglia following insult to the brain, and the two types work in concert to determine the fate of affected neurons, with anti-inflammatory cytokines shifting the balance toward neuroregenerative and neuroprotective biological pathways, and pro-inflammatory cytokines shifting the balance toward apoptosis and cell death (Hernandez-Ontiveros et al., 2013). Specifically, elevations concurrently in IL-6 and IL-10 have been observed in studies of severe TBI (Ferreira et al., 2014) and, increases in serum IL-10 seem positively correlated with more severe TBI (Di Battista et al., 2016; Schneider Soares et al., 2012). Thus, in the current study, the lack of an increase of IL-10 is not surprising based on the previous literature, and the mild injury in these cases suggests a state of immune dysregulation that may have consequences that require larger sampling and more in-depth clinical measures. IL-10 is traditionally classified as exerting anti-inflammatory effects, while IL-6 is traditionally defined as pro-inflammatory characteristics (Brandt & Pedersen, 2010; Hernandez-Ontiveros et al., 2013; McKee & Lukens, 2016), with increasing evidence for IL-6 anti-inflammatory characteristics (Brandt & Pedersen, 2010). IL-10 may confer neuroprotective effects in animal models (Barrett et al., 2017; Chen et al., 2014; Zou et al., 2017). Thus a lack of increase in IL-10 suggests that there may be immune dysregulation that may relate to clinically relevant implications that should be determined in future studies.

There are a number of factors in the current study that limit interpretations of these findings, including a relatively small sample size, yet this is the first study to report

acute biomarkers obtained from active duty military personnel who sustained concussions during a military deployment. Additional limitations in the scope of this study include a lack of neuroimaging, such as computerized tomography (CT) scans, as well evaluation of long-term outcomes. The nature of the combat environment may limit specificity in the current study, as blast exposure and blunt injuries often occur concurrently in the same injury event. Differences between injury types may account for discrepancies with the literature, though it is outside the scope of this study to delineate effects of blast from blunt force injury causes.

In conclusion, reported here is a significant elevation of IL-6 levels in concussed military personnel less than 8 hours following injury. This is the first reported observation of peripheral levels IL-6, -10, and TNFα in a combat environment to determine biomarkers of concussions sustained during combat station deployments, in a cohort that had high rates of comorbid blast exposure. The present finding of IL-6 elevation warrants further exploration of inflammatory cytokines in combat injuries involving concussion and blast, especially in future studies designed to account for the aforementioned limitations. Future studies may examine acute and chronic neurological symptomology associated with inflammatory cytokine levels, distinguish individuals at high risk for developing neurological complications, and identify underlying biological pathways to mitigate inflammation and improve outcomes. The present findings of elevated IL-6 may be further explored in larger cohorts, as well as to determine inflammatory pathways that may be targeted for therapies.

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CHAPTER FIVE

DISCUSSION

Concussions are a significant health concern worldwide, especially among military personnel (Defense and Veterans Brain Injury Center, 2017; Taylor, 2017). Given the limited FDA-approved interventions, substantial societal economic burden, and risk of long-term neuronal health consequences, it is essential that new therapeutic approaches are explored and developed (Carroll et al., 2004; Cassidy et al., 2014; Ma, Chan, & Carruthers, 2014; Maas, Stocchetti, & Bullock, 2008). This research aimed to build on the biological foundation necessary for future developments in treatment, monitoring, and prognosis by investigating underlying inflammatory processes, through gene expression and protein analysis, immediately following concussions with blast exposures. As a result, this research has contributed to emerging knowledge of inflammatory response in military personnel experiencing acute concussion.

Summary of Key Outcomes

The purpose of the dissertation research was to examine alterations in inflammatory processes following traumatic brain injury. During the course of this research, gaps were identified in current literature specific to gene expression and inflammatory cytokines following concussion in military personnel. Additionally, we were presented with the unique opportunity to analyze samples collected both in a military training environment, as well as overseas during combat in Afghanistan. Each factor is discussed below, as well as the response in the research program, and a brief overview of key outcomes resulting from each study.

1. **Gap Identified**: While a literature review of multiple clinical studies examined the significance of the inflammatory response in persons with TBI, there are few research publications which describe inflammatory-related gene expression and proteins in clinical populations with mild TBI or concussion.

   Response: The research results in Chapter 2 addressed Gap 1 by examining the current state of the literature over the last 10 years in two ways: 1) gene expression studies in mild TBI and, 2) inflammatory protein markers in mild TBI.

   Key Outcomes: In Chapter 2, a review of the current literature demonstrated that inflammatory cytokines, including IL-6, IL-10, and TNFα, may be elevated in the acute time period following mild TBI or concussion and that chronically elevated levels are associated with poor outcomes. In addition, gene expression studies showed that alterations do occur following mild brain injury, including inflammatory pathways. One of these inflammatory pathways is NF-κB, which is considered a master regulator of inflammatory cytokines. However, studies in inflammatory cytokines, and especially gene expression, are limited in number at this time, with findings requiring validation in additional studies. Further, most studies are limited to the civilian population, and they do not consistently delineate between different subtypes of injury including blast exposure. Thus, examination of cytokines and gene expression in military personnel with concussion from blast exposure is needed to continue to build on this knowledge.

2. **Gap Identified**: Although concussion is recognized as the signature injury in military personnel serving in recent conflicts, gene expression data related to recovery processes remain poorly studied, especially in the most common cause of injury—blast exposure.

   Response: Chapter 3 explored gene expression alterations following moderate blast exposure in a military training population.

   Key Outcomes: Chapter 3 describes the contribution to existing literature which demonstrates that gene expression is altered following brain injury. This dissertation study showed two differentially regulated gene networks following moderate blast exposure: 1) cell death and survival, and 2) cellular structure, function, and development. These gene networks included alterations in key biological pathways related to ubiquitination, neuronal recovery, and immune and inflammatory pathways. Specifically, gene expression changes were observed that activate immune and inflammatory pathways involving the NF-κB pathway and the *AKT1* gene. These findings build on previous work in the same population of moderate blast exposure showing increased concentrations of inflammatory cytokines IL-6 and TNFα (Gill et al., 2017).

3. **Gap Identified**: Inflammatory cytokines (IL-6, IL-10, and TNFα) in the acute stage following concussion have not been examined in deployed military personnel who experienced concussion and blast exposures.

Response: In Chapter 4, measured IL-6, IL-10, and TNFα concentrations were described at two acute time points following provider-diagnosed concussion in military combat personnel.

Key Outcomes: Results identified in Chapter 4 contributed to existing work demonstrating acute elevations of IL-6 following concussion. Specifically, the findings showed significantly increased concentrations in IL-6 less than 8 hours following concussion, which were highly comorbid with blast (>70% of concussed individuals reporting blast). This increased IL-6 concentration was followed by a decrease in IL-6 concentration within 24 hours. IL-6, in balance with other cytokines, is known to modulate the inflammatory process following brain injury; this is also supported in preclinical models (Ley, Clond, Singer, Shouhed, & Salim, 2011; McKee & Lukens, 2016; Penkowa, Giralt, Carrasco, Hadberg, & Hidalgo, 2000; Penkowa et al., 2003). Thus, findings from this study suggest that IL-6 is coordinating recovery from concussions, including those caused by blast exposures. Further research could contribute to the understanding of the cytokine balance, important to improving care of the complex, and often interrelating, concussion and blast injuries sustained by military personnel in combat stations. Importantly, this study is unique in that it is the only one at this time to measure cytokines in deployed military personnel with concussion.

Significance of Key Outcomes

The research outcomes within this dissertation provide several key contributions to knowledge about inflammatory responses following concussion, including:

1. The first review of gene expression and inflammatory cytokines in mild traumatic brain injury clinical populations;
2. Contribution to the growing body of research demonstrating altered gene expression networks following blast exposure in human populations;
3. Evidence of altered inflammatory gene pathways, including the regulator of cytokines NF-κB, following blast exposure in a military training population;
4. The only study to date that measures inflammatory cytokines in military personnel deployed to combat stations who experienced concussion highly comorbid with blast; and,
5. Evidence of increased IL-6 concentrations in the acute period following concussion comorbid with blast exposure.

Strengths and Limitations of the Research

The strengths and limitations of each chapter has been discussed previously but the cumulated strengths and limitations of the overall research program are discussed here.

The research program has multiple strengths. First, the research papers have addressed complex questions using different study designs and research methodologies. During the course of this study, this lab was presented with the unique opportunity to analyze samples collected both from military training personnel as well as combat

personnel overseas in Afghanistan. Thus, questions regarding activation of the inflammatory response following blast exposure and concussion were able to be addressed in two cohorts of military personnel: 1) A well-controlled training environment with only blast exposures; and, 2) A real-world combat setting with concussions and blast exposures representing the experience of deployed military personnel. Second, the controlled training environment represents a unique opportunity to explore the impact of blast alone, as there were no other known blunt force injuries observed. In the training environment, samples obtained in PAXgene DNA tubes were collected pre and post moderate blast exposure, which allowed for the analysis of gene expression changes before and after the moderate blast exposure. Previous reports in the same population that indicate an increase in inflammatory cytokines (IL-6 and TNFα), together with the present study findings of activation of inflammatory pathway genes, strengthened the evidence for altered inflammatory systems following blast exposure in human populations. Third, the combat setting, to date, is the only deployed military population in which blood has been collected for the purpose of measuring biomarkers following concussion. Importantly, inflammatory cytokines (IL-6, IL-10, and TNFα) were able to be examined in a real-world combat environment, with concussions often occurring simultaneously with other sources of injury and involving blast exposures. Data from this dissertation study aligned with previous reports of acute increases in IL-6, suggesting that further exploration of IL-6 is relevant to future research in concussions and blast exposure.

There are several limitations in this program of research. In addition to the strengths described above, the use of two cohorts presented challenges. First, there are differences in the descriptions of the injuries between the two cohorts. While the combat population had provider-diagnosed concussions, largely comorbid with blast exposure, there was a lack of concussion diagnosis in the military training population exposed to moderate blast. Despite these differences, it is important to note that both cohorts experienced blast exposures. Second, the nature of the combat environment creates unavoidable differences from a controlled training environment. For example, the force of the blast exposure was not able to be measured in the combat environment as it is in a training setting. Additionally, blunt force injury is difficult to delineate from blast exposures in a combat setting. Though the nature of this sample presented some limitations, the majority of combat personnel experienced blast exposure as the cause of concussion so met eligibility for inclusion in this research study. Rarely does the opportunity arise to study the effects of blast in human populations. Thus, despite the differences between the cohorts, these researchers believe that both the training and combat populations have made significant contributions to understanding inflammatory processes following blast, and that knowledge gained from each study will help guide future studies moving forward.

Implications for Healthcare Genetics and Future Directions for Study

This research program has presented a number of novel findings in the context of understanding activation of the inflammatory response following concussion and blast exposure in the military. Namely, knowledge gained from these studies to be considered

in moving the research forward include activation of inflammatory gene networks and changes in IL-6 over time. However, there is a pressing need to investigate the relationship between inflammatory processes to recovery from concussion and blast exposures. Specifically, the research methodology of examining gene expression and protein products has application to the field of Healthcare Genetics in its potential for translation from bench to bedside care.

Research agenda.

The literature review in Chapter 2 looked at clinical traumatic brain injury studies of gene expression and related proteomic pathways. Those results identified a continued need to conduct additional research studies in both gene expression and cytokine activity following concussions in human populations. This dissertation research contributed to that need through two studies in the military population described in Chapter 3 and 4. However, as identified in Chapter 2, additional studies, both in civilian and military populations, with standardized identification of various brain injury subtypes and severities, are recommended. Additionally, there is a need to map outcomes with gene expression and cytokines over time, a need which is reflected in Chapters 3 and 4 of this research program.

Results in Chapter 3, which identified differentially regulated gene networks following blast exposure in a military training environment, suggested a need for further evaluation of gene expression in larger cohorts, with additional acute days of blood sample collection. Collection of data over time would allow for a more in-depth analysis of gene expression changes over time. With collection of the participants' symptoms

using objective and measureable tools, the gene changes could be mapped to poor outcomes. Finally, exploration in gene network changes would give indications of the underlying biological processes. Similar to cytokines and the changes related to the NF-κB network in the Chapter 3, protein products of those gene changes could be measured—a technique which would have clinical utility for healthcare personnel caring for patients at the bedside. Specifically, in application to the field of Healthcare Genetics, a future study could be designed to capture gene expression data and cytokines at the same time points within the same cohort.

Per results in Chapter 4, it is recommended that inflammatory cytokines in military personnel with concussion and blast exposures be further explored in larger cohorts. Future studies should be designed to account for the limitations mentioned above, including delineating blast from other subtypes of concussion. Acute and chronic neurological symptoms may be collected and associated with inflammatory cytokine levels over time. This information may help to characterize individuals at risk for developing neurological complications, as well as further elucidate the underlying inflammatory pathways that may be targeted for therapies in order to improve outcomes. Finally, as referred to in Chapter 3 above, the measure of protein biomarkers may have potential clinical utility for identification and/or monitoring of inflammatory processes over time. The elevated IL-6 concentration in this study is interesting given the similar increase in IL-6 seen in a previous report by Gill et al. (2017), as well as the increased expression of inflammatory-related genes in Chapter 3 of this work. Thus, there may be reason to further explore the question of cytokines informing concussions.

Conclusion

Concussion and blast exposure in the military remains an important concern for military personnel, as well as civilians, around the world. The acute period following injury is crucial for appropriate activation of the inflammatory response, with prolonged imbalances in the inflammatory response likely leading to poor outcomes. This research program resulted in 3 papers, each of which focused on the essential need to further elucidate inflammatory gene expression and cytokine responses to acute concussion. With research from Chapters 2 and 3, Chapters 3 and 4 respectively, indicating significantly altered gene expression networks and increased IL-6 during the acute time period, this research contributes to the existing literature and provides direction for continued exploration. The research findings and potential future directions will have application to the field of Healthcare Genetics for researchers and health care professionals seeking to develop, and eventually implement, therapeutics to improve patient outcomes following concussion.

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APPENDICES

Appendix A

Abbreviations

BBB: blood brain barrier

CNS: central nervous system

GCS: Glasgow Coma Scale

GOS: Glasgow Outcome Scale

ICP: intracranial pressure

IL-6: interleukin 6

IL-10: interleukin 10

IPA: ingenuity pathway analysis

ISF: interstitial fluid

TBI: traumatic brain injury

mRNA: messenger ribonucleic acid

mTBI: mild traumatic brain injury

NF-κB: nuclear factor kappa light-chain enhancer of activated B cells

OEF: Operation Enduring Freedom

OIF: Operation Iraqi Freedom

ROS: reactive oxygen species

TNFα: tumor necrosis factor α

Tregs: Regulatory T cells

Appendix B

IRB

DEPARTMENT OF THE ARMY
WALTER REED ARMY INSTITUTE OF RESEARCH
503 ROBERT GRANT AVENUE
SILVER SPRING, MD 20910-7500

REPLY TO ATTENTION OF

MCMR-UWZ-C 13 February 2018

MEMORANDUM FOR Walter Carr, MAJ, MS, Chief, Military Psychiatry, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research (WRAIR), 503 Robert Grant Ave., Silver Spring, MD 20910-7500

SUBJECT: Project Qualifies as Research Not Involving Human Subjects, **WRAIR #2529.**

1. A determination was made that the project **WRAIR #2529** entitled, "Analysis for a Comparative Evaluation of Blood Biomarkers and Automated QEEC from Concussed and Non-Concussed Cohorts in a Combat Zone (version 1.2, dated 09 February 2018), does not require review by the WRAIR Institutional Review Board (IRB) in accordance with WRAIR Policy Letter #12-09, as the project involves analysis of pre-existing de-identified data and specimens where the investigator and study team do not have access to any identifiable information; therefore, this research activity does not meet the definition of research involving human subjects and 32 CFR 219 does not apply.

2. The primary objectives of this project are to complete final analysis and documentation of assay results for specimens collected under U.S. Army Medical Research and Materiel Command (USAMRMC) protocol M-10216 (WRAIR #2028), entitled "A Comparative Evaluation of Blood Biomarkers and Automated QEEC from Concussed and Non-Concussed Cohorts in a Combat Zone ". Per the existing Department of Defense Institutional Agreement for IRB Review (IAIR) between WRAIR and USAMRMC, WRAIR relied on the Headquarters USAMRMC Institutional Review Board (IRB) for the ethical review of that protocol. The Headquarters, USAMRMC IRB closed protocol M-10216 (WRAIR #2028) on 18 September 2017. The study data and specimens collected under protocol M-10216 (WRAIR #2028) have been de-identified by removing and destroying the link between subject identity and subject data and serum samples. De-identified specimens collected under protocol M-10216 (WRAIR #2028) were provided for biomarker analyses to the National Institute of Health/National Institute of Nursing Research (NIH/NINR) Laboratory for Tissue Injury, as described in the protocol. The NIH Office of Human Subjects Research (OHSR) determined the work conducted at the NINH NINR to be not human subjects research in a memorandum dated 17 February 2015. The work to be completed in this project will consist of quantitative comparison of NINR assay results and complementary de-identified data previously collected.

3. This project is funded through institutional resources at NINR and WRAIR; there is no transfer of funds between institutions and no costs to itemize.

4. This project was found to be scientifically feasible and valid, militarily relevant, and appropriately resourced by Jeffrey L. Thomas, COL, MS, Director, Center for Military Psychiatry and Neuroscience, on 31 January 2018.

5. No additional information is needed at this time. However, should the study team gain access to any personal identifiers or codes linking the participants with their specimens, the submitted project would need an independent determination by either the WRAIR Institutional Review

Board Chair or the Director, Human Subjects Protection Branch (HSPB), as to whether or not the investigator is engaged in human subjects research, and whether or not the WRAIR IRB review and approval are required. The HSPB reserves the right to review the project records to re-assess the determination of research not involving human subjects. The WRAIR HSPB also reserves the right to review the project records and re-assess the NHSR determination as part of post approval compliance monitoring. The PI is responsible for maintaining records that confirm that the executed activities match the project that was evaluated and found to be research not involving human subjects.

6. The point of contact for this action is Anna Sanner, M.D., M.P.H, at 301-319-9866 and Anna.V.Sanner.ctr@mail.mil.

TUZSON.TIBERIU.1265005116
Digitally signed by TUZSON.TIBERIU.1265005116
Date: 2018.02.13 09:18:02 -05'00'

TIBOR TUZSON, MD
Exemption Determination Official
Human Subjects Protection Branch
Walter Reed Army Institute of Research

OHSR RESPONSE TO REQUEST FOR REVIEW OF RESEARCH ACTIVITY
INVOLVING HUMAN SUBJECTS

FAX:

Exempt: #: 12767

To: Gill, Jessica
NINR
60/254

From: Office of Human Subjects Research (OHSR)

Nature of Research Activity:
The overall objective of this project is to examine concentrations of proteins including tau and GFAP following acute traumatic brain injuries (TBIs) in military personnel who were deployed to Afghanistan. Subjects had 2 blood samples, with the first occurring within 12 hours of the TBI, and the second 24 hours following the TBI. These samples were collected during deployment to Afghanistan under a protocol with the primary investigator of Dr. Walter Carr "A Comparative Evaluation of Blood Biomarkers and Automated QEEG from

Original Request Received in OHSR on: 1/26/2015

Responsible NIH Research Investigator(s): Jessica Gill, PhD, CRNP NINR

OHSR review of your request dated Mon, Jan 26, 2015 has determined that:

☒ Federal regulations for the protection of human subjects do not apply to above named activity. The OHSR determination of Not Human Subjects Research is based on the interpretation of 45 CFR 46 under "Research Involving Coded Private Information or Biological Specimens" (OHRP, Revised October 16, 2008) and Guidance on Engagement of Institutions in Human Subjects Research (October 16, 2008). NOTIFY OHSR VIA AN E-MAIL AMENDMENT OF ANY CHANGES THAT MAY ALTER THIS RESEARCH ACTIVITY.

☐ The activity is designated **EXEMPT**, and has been entered in the OHSR database. PLEASE NOTIFY OHSR OF ANY **SIGNIFICANT** CHANGES THAT MAY ALTER THE EXEMPT STATUS OF THIS RESEARCH ACTIVITY.

☐ **NOT EXEMPT**. OHSR recommends IRB review. Please forward your request to the Chair of your IRB, who may ask you to provide additional information in order to determine whether expedited or full review is appropriate.

☐ Confidentiality Agreement

☐ Reliance

☐ Amendment

☐ Other

Office Person JE Admin Assist. CB

Note:
2/17/2015: Walter Carr, Walter Reed Army Institute of Research

Julie M. Eiserman | Policy Analyst, OHSRP | 2/17/2015
Signature | Title | Date

Domestic/International:
Domestic

Human Subjects Data: Yes
Biologic Material: Yes

OHSR Use Only
☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6

Eiserman, Julie (NIH/OD) [C]

From: Gill, Jessica (NIH/NINR) [E]
Sent: Wednesday, February 18, 2015 7:41 AM
To: Eiserman, Julie (NIH/OD) [C]
Subject: RE: Follow Up re: Request for Determination for OHSRP #12767

Julie- No, he has de-identified the samples so that there are not longer any identifiers for the subjects.
Thanks
-Jessica

From: Eiserman, Julie (NIH/OD) [C]
Sent: Wednesday, February 18, 2015 7:40 AM
To: Gill, Jessica (NIH/NINR) [E]
Subject: RE: Follow Up re: Request for Determination for OHSRP #12767

Your collaborator won't have access to the code key as the PI of the other study?

Sent with Good (www.good.com)

From: Gill, Jessica (NIH/NINR) [E]
Sent: Wednesday, February 18, 2015 7:20:05 AM
To: Eiserman, Julie (NIH/OD) [C]
Subject: RE: Follow Up re: Request for Determination for OHSRP #12767

Julie- Yes, they will be coded, so the correct answer is b. I apologize for this error, please let me know how I may be of help in correcting it.
Thank you
-Jessica

From: Eiserman, Julie (NIH/OD) [C]
Sent: Tuesday, February 17, 2015 10:05 PM
To: Gill, Jessica (NIH/NINR) [E]; Olivera, Anlys (NIH/NINR) [F]; Livingston, Whitney (NIH/NINR) [F]; Martin, Christiana (NIH/NINR) [F]
Subject: Follow Up re: Request for Determination for OHSRP #12767

Hello,

I am reviewing your request for determination and I just want to confirm something about this request related to your answer (below).
9. Select the best description that applies to the specimens or data:

(a) _X_ Specimens, data or information will not contain any identifiable information, **and cannot be linked to individual subjects by you or your collaborators**.
(b) ___ Specimens, data or information will be coded, however that **code cannot be used by either the provider or the receiver** to identify specific individuals.
(c) ___ Specimens, data or information will be **coded so that the provider of the samples/data can link them to specific individuals** but the receiver will not be able

to do so.

I just want to confirm that the specimens and data will be coming to you completely anonymous rather than coded since your collaborator is the PI of the project and would likely have access to identifiers. In addition, because you will be receiving data and specimen, you would likely need to receive everything coded rather than anonymous so you can link the specimens and data to each other. If I am misunderstanding something, please let me know.

Julie M. Eiserman, MA, CCRP [C]
Health Science Policy Analyst
Office of Human Subjects Research Protections
10 Center Drive, Bldg. 10, Suite 2C146
Bethesda, MD 20892-1154
Office Phone: 301-402-3444
Fax: 301-402-3443
OHSRP website: https://federation.nih.gov/ohsr/nih/index.php (NIH login required)
Public site: http://ohsr.od.nih.gov/

REQUEST FORM: OHSRP DETERMINATION FOR RESEARCH-USE OF DE-IDENTIFIED SPECIMENS OR DATA

Date of Request: 1-26-2015

Requestor's name: Jessica Gill e-mail: gillj@mail.nih.gov

Role: X Investigator __Administrative support __Other, explain: _____

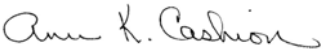
Name of NIH Senior Investigator: Jessica Gill
(*The investigator must be an NIH employee*)

IC: NINR **Laboratory/Branch:** Tissue Injury Branch

Building & Room No.: 60, 254 **Tel. No.:** 451-8452 **FAX No.:** 301-451-1678

Is the NIH Senior Investigator an NIH employee (FTE)? __X__Yes _____No

Senior Investigator Signature: 
(*Signature of Investigator who will conduct research, Dr. Jessica Gill*)

Supervisor Signature:
____  _____
(*Signature of official for IC, e.g., Lab/Branch Chief, Dr. Ann Cashion*)

Name of NIH investigator conducting research if not the NIH Senior Investigator: *(i.e, junior investigator, contractor investigator, fellow, student)*
Anlys Olivera, Ph.D, IRTA Postdoctoral Fellow, Whitney Livingston, post-bac IRTA, and Christiana Martin, post-bac IRTA

Please provide the name and e-mail of any others who should receive a copy of the OHSR determination: Hyung-Suk Kim, kimy@mail.nih.gov

1. **What role will the NIH investigator(s) have in this research project?** *(check all that apply)*
   __x_ Analyze samples/data
   ___ Consultant/advisor to collaborator(s)
   __x_ Author on publication(s)/manuscript(s) pertaining to this research
   ___ Investigator or the NIH holds an IND/IDE for this research
   ___ Other, please describe: _____

2. **Title:** An Examination of Neurological Proteins Related to Traumatic Brain Injuries in Military Personnel Deployed in Afghanistan

3. Describe in lay terms the research activity that will be performed:

The overall objective of this project is to examine concentrations of proteins including tau and GFAP following acute traumatic brain injuries (TBIs) in military personnel who were deployed to Afghanistan. Subjects had 2 blood samples, with the first occurring within 12 hours of the TBI, and the second 24 hours following the TBI. These samples were collected during deployment to Afghanistan under a protocol with the primary investigator of Dr. Walter Carr "A Comparative Evaluation of Blood Biomarkers and Automated QEEG from Concussed and Non-Concussed Cohorts in a Combat Zone, Walter Reed Army Institute of Research Protocol #2028."

4. Proposed start date: 2/20/15 **Proposed completion date:** 2/09/16

5. Specify the nature of the specimens or data: *(select all that apply)*

___ iPSC lines ___ hESC ___ Fetal Tissue
___ WES/WGS ___ GWAS
X Other human specimens *(e.g. tissue, blood, derivatives),* describe: *Blood*
X Data *(e.g. clinical or research information or laboratory results)* describe:
De-identified data, including demographics (age, sex, race), and traumatic brain injury-related information
_ Other, describe:

6. Will specimens or data be? (*select all that apply*)

Collected Yes__ No
Received Yes_X_No__
Sent Yes__No__

7. If receiving or sending, list the collaborating investigator(s):

Name Institution/IC Address/e-mail FWA number*****
Walter Carr Walter Reed Army Institute of Research walter.s.carr.mil@mail.mil, FWA=_00000152

8. Do the specimens, data or information:

Already exist? Yes X__ No__

If "no", explain:_____

9. Select the best description that applies to the specimens or data:

(a) _X_ Specimens, data or information will not contain any identifiable information, **and cannot be linked to individual subjects by you or your collaborators**.
(b) _X_ Specimens, data or information will be coded, however that **code cannot be used by either the provider or the receiver** to identify specific individuals.
(c) ___ Specimens, data or information will be **coded so that the provider of the samples/data can link them to specific individuals** but the receiver will not be able to do so.

10. If c is selected above, please follow the instructions below:
Projects involving coded research specimens obtained from a non-NIH collaborator will require a de-identification agreement. Please e-mail your collaborator(s) the following agreement language modified to reflect the nature of your collaboration. Attach the completed agreement to this submission.

De-identification Agreement:

Provider of coded specimens or data:

I, [Name] of [Institution], holder of the code-key or cipher for the coded [specimens, data (*specify*)], promise not to release the identity of the subjects from whom the coded [specimens, data (*specify*)] originated, until the subjects decease to [Recipient Name] at [Recipient Institution].

Recipient of coded specimens or data:

I, [Name] of [Institution], recipient of the coded [specimens, data (*specify*)], promise not to request the identity of the subjects from whom the coded [specimens, data (*specify*)] originated, until the subjects decease from [Sender Name] at [Sending Institution].

11. If data are being extracted from existing records, who will extract the data? (*if applicable*)
(a) ___ NIH Investigator
(b) _x__ non-NIH Collaborator
(c) ___ NIH Contractor
(d) __Other, specify:

If a or c, will an Honest Broker or data use agreement be used? Yes__ No__

If yes, complete and attach the Honest Broker Assurance or Data Use Agreement to this submission; e-mail ohsr_nih_ddir@od.nih.gov to request the form.

12. Where are the subjects of this research activity located? Subjects were recruited

while deployed as active duty military personnel deployed in Afghanistan.

13. If human subjects are located elsewhere (not at NIH), will you have direct contact or intervention with them? (For example, as subject's physician, obtaining specimens directly from the subject?) Yes__ No X

14. Do the specimens, data or information come from:

___ NIH BTRIS
___ NIH Medical Records
X_ Repository
If an NIH Repository, specify: _____
___ Pathological waste
___ Autopsy material
___ Publicly available source
___ Originate from an IRB-approved protocol?
___ Other_____

15. Will the results of the research be returned to the provider(s) of the specimens or data?

(a) ___ No, results will not be returned to the provider(s)
(b) X_ Yes, aggregated results will be returned to the provider(s)
(c) ___ Yes, results that are linked to identifiable individuals, will be returned to provider(s)
(d) ___Yes, the results of this project will be returned to an active NIH IRB-approved protocol? If yes, protocol ID: _____

If b or c, is the NIH project consistent with the IRB/EC-approved protocol at the collaborating institution? Yes_x_ No__

16. Per NIH guidance, are all conflicts of interest by NIH employees, if any, resolved?

X_ Yes _____No**

**A Federalwide Assurance (FWA) is issued by the U.S. Department of Health and Human Services (DHHS)/ Office of Human Research Protections (OHRP) to institutions which receive Federal funds/support to conduct human subjects research. To search for the FWA# for domestic or international institutions go to http://ohrp.cit.nih.gov/search/fwasearch.aspx?styp=bsc*

***If the answer is "No", note that OHSRP will be unable to make a determination and research may not proceed until all conflicts are resolved. For more information, see the October 2011, A Guide to Preventing Financial and Non-Financial Conflict of Interest in Human Subjects Research at NIH. For assistance review the list of Ethics Coordinators and find the contact for your IC: http://ethics.od.nih.gov/coord.pdf*

OHSR (NIH/DDIR)

From: Gill, Jessica (NIH/NINR) [E]
Sent: Monday, January 26, 2015 4:47 PM
To: OHSR (NIH/DDIR)
Subject: review of possible exempt protocol
Attachments: gill_CARR_OHSRP.doc

Hello- I am attaching an application for the review of a possibly exempt protocol . Please let me know if any questions arise or if other information would be of help. Thank you.
-Jessica

OHSR (NIH/DDIR)

From: OHSR (NIH/DDIR)
Sent: Friday, January 30, 2015 12:34 PM
To: Gill, Jessica (NIH/NINR) [E]
Subject: Req for Determination Rec'd_OHSRP 12767

Good afternoon Dr. Gill,

This email is to verify that OHSR has received your Request for Determination and it is currently being processed as **OHSRP #12767.** Please use this number in any future correspondence regarding this study.

Protocol Title: An Examination of Neurological Proteins Related to Traumatic Brain Injuries in Military Personnel Deployed in Afghanistan

Thank you.
Sincerely,
Chris Brentin
OHSRP - National Institutes of Health
Bldg 10, Suite 2C146
Bethesda, MD 20892
Office Telephone: 301-402-3444
Office Fax: 301-402-3443

The NIH is committed to maintaining the highest standards for the protection of human subjects.


Please consider the environment before printing this e-mail

Original Submission Date: 11/10/2010
Version # 14.0 & Date: 01/23/2017

MEMORANDUM FOR OFFICE OF RESEARCH ADMINISTRATION, NAVAL MEDICAL RESEARCH CENTER

SUBJECT: Application and Request for Approval of Human Subjects Research

STUDY SITE(s): _X_ NMRC, _X_ WRAIR, _X_ NIH/NINDS

Protocol Number: NMRC.2011.0002 (WRAIR #1796; NINDS #12-N-0065)

Protocol Title: Experienced Breacher Injury Study: Evaluation of the Bio-Effects from Chronic Exposure to Low-Level Blast

Principal Investigators

LCDR Peter B Walker PhD MSC USN
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CITI: 29 April 2015

MAJ Angela M. Yarnell, MSC, USA
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TABLE OF CONTENTS

LIST OF ACRONYMS

ANAM	Automated Neuropsychological Assessment Metrics
ANOVA	Analysis of Variance
ARA	Applied Research Associates, Inc.
ASVAB	Armed Services Vocational Aptitude Battery
CDMRP	Congressionally Directed Medical Research Programs
CDP	Computerized Dynamic Posturography
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computerized Tomography
CTSIB	Clinical Test for Sensory Integration of Balance
DANA	Defense Automated Neurobehavioral Assessment
deoxy-Hb	deoxyhemoglobin
DGI	Dynamic Gait Index
DHHS	Department of Health and Human Services
DHI	Dizziness Handicap Inventory
DHSP	Division of Human Subjects Protection
DOD	Department of Defense
DON	Department of the Navy
DSC	Dynamic Susceptibility Contrast
DTI	Diffusion Tensor Imaging
DTP	Dual Tasking Posturography
DVBIC	Defense and Veterans Brain Injury Center
EEG	Electroencephalography
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
fMRI	functional Magnetic Resonance Imaging
FTSST	Five Times Sit to Stand Test
GAD	Generalized Anxiety Disorder
HIPAA	Health Insurance Portability and Accountability Act
HRP	Horse Radish Peroxidase
HRPP	Human Research Protections Program
ImPACT	Immediate Post-concussion Assessment and Cognitive Test
IRB	Institutional Review Board

IV	Intravenous
LOS	Limits of Stability
MRI	Magnetic Resonance Imaging
mTBI	mild Traumatic Brain Injury
NCAA	National Collegiate Athletic Association
NCAT	Neurocognitive Assessment Tool
NIH	National Institutes of Health
NINDS	National Institute of Neurological Disorders and Stroke
NMRC	Naval Medical Research Center
OEF	Operation Enduring Freedom
OIF	Operation Iraqi Freedom
ORA	Office of Research Administration
oxy-Hb	oxyhemoglobin
PTSD	Post Traumatic Stress Disorder
rCBF	regional Cerebral Blood Flow
REM	Rapid Eye Movement
SAE	Serious Adverse Events
SHA	Sinusoidal Harmonic Acceleration
SOT	Sensory Organization Test
SPEM	Smooth Pursuit Eye Movement
SWI	Susceptibility Weighted Imaging
UCMJ	Uniform Code of Military Justice
uDAS	miniature Data Acquisition System
USAMRMC	United States Army Medical Research and Materiel Command
USASOC	United States Army Special Operations Command
USMA	United States Military Academy
USMC	United States Marine Corps
VEMP	Vestibular Evoked Myogenic Potentials
VNG	Videonystagmography
WRAIR	Walter Reed Army Institute of Research

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2. ABSTRACT

2.1 Purpose

The purpose of this study is to evaluate the cognitive and neurophysiological effects of chronic exposure to repeated low-level blast overpressure. The results of previous studies (NMRC.2007.0006; NMRC.2009.0011; NMRC Project #60) show converging evidence for a neurophysiological effect from cumulative exposure to blast that is consistent with anecdotal reports of cognitive impairments by members of the professional community known as "Breachers". These studies were undertaken as a result of a request by the Breacher instructors who had subjective complaints of memory impairment and on occasion, balance and sleep difficulties. However, the number of instructors was small and a larger group evaluation is needed at this time to verify whether breaching activities may result in increased risk for cognitive impairment. The proposed study will expand on these findings by examining a larger cohort of experienced Breachers who may be incurring a cumulative effect of low-level blast exposure over the course of several years in the profession. Analysis of this unique population will yield a greater effect size than previous studies of Breacher instructors with the goal of identifying the mechanisms underlying cognitive deficits specifically related to repeated low-level blast exposure and identify the most efficacious means of detecting mild traumatic brain injury (mTBI) in soldiers.

2.2 Research Design

Volunteers will be recruited from the military and civilian law enforcement Breacher communities for a multi-phase, cross-sectional study of chronic exposure to low-level blast overpressure ("breaching blast"). Experienced Breachers are those with at least 4 years of experience with exposure to low-level blast from breaching either in the field or as instructors for explosive entry training courses. Phase A of the study will include field assessments of Breachers during explosive entry training to measure breaching environments and blast exposure and evaluate the acute effects of low-level blast exposure. Phase B will involve subjects travelling to the National Institute of Neurological Disorders and Stroke (NINDS) of the National Institutes of Health (NIH) in Bethesda, MD for neuropsychological testing, neuroimaging, blood components analysis, vestibular and auditory testing, and a sleep assessment. Subjects will also be invited back to NINDS for a 1-year follow-up assessment to look at the progression of the effects.

2.3 Methodology / Technical Approach

We will evaluate individuals from the military and civilian law enforcement Breaching communities with extensive breaching experience and compare their cognitive performance with that of age, gender, and service length matched individuals with exposure to non-blast related overpressure (e.g. artillery units) and those with no prior exposure to overpressure. For Phase A, we will evaluate between 100 to 150 breachers and between 25 and 50 artillery personnel during breacher and artillery training. In addition, we will evaluate between 25 and 50 unexposed individuals for a total of up to 250 subjects. For Phase B, we will evaluate a minimum of 15 subjects from each of the three groups (breachers, artillery personnel, and unexposed individuals) for a total of at least 45 subjects, with an upper limit of 60 subjects (20 per group). Subjects for Phase B may come from the subject pool for Phase A, however, subjects are not required to participate in Phase A in order to be eligible for Phase B. In addition, subjects from all 3 groups will be asked to bring a companion to NIH for an interview to capture changes in daily functioning that subjects may not be able to self-assess, which could yield an additional 60 subjects. However, subjects are not required to bring a companion to participate in the Phase B; therefore, the actual number of companions that will be evaluated is unknown. Companions can also participate in the study remotely. The sum of the maximum number of possible subjects over all groups in both phases is 370.

During Phase A of the study, staff from the Naval Medical Research Center (NMRC) and the Walter Reed Army Institute of Research (WRAIR) will conduct daily field assessments during explosive entry training to evaluate the acute effects of breaching in an experienced population. These assessments will include neuropsychological tests of cognitive and emotional functioning, symptomology, vestibular

system assessments, eye-tracking, analysis of sleep patterns, and blood components sample analysis for biomarkers of brain injury. In addition, blast measurement experts from Applied Research Associates, Inc. (ARA) will accompany the research team to gather data on blast pressure using pressure sensors on the subject and in the environment to estimate the magnitude and frequency of the overpressure energy transmitted to the head.

In Phase B, subjects will travel to NINDS in Bethesda, MD for a multi-day visit for a series of evaluations to measure cognitive and neurophysiological changes related to exposure. These procedures are described in detail in Appendix A and will include neuropsychological testing, blood components analysis for biomarkers, vestibular and auditory testing, a sleep assessment (polysomnography), and neuroimaging studies using diffusion tensor imaging (DTI), susceptibility weighted imaging (SWI), perfusion imaging, imaging with Gadolinium contrast, and functional magnetic resonance imaging (fMRI). To participate in this study, volunteers will be required to consent to both DOD and NINDS protocols; however, they can opt out of individual procedures for any reason.

All procedures outlined in this protocol are subject to modification or replacement with methods that are similar in time commitment and method of administration to tasks contained in the current version of the protocol. We will not substitute tasks that introduce additional risks beyond that of the approved tasks without explicitly requesting their use via an amendment to this protocol.

3. OBJECTIVES AND SPECIFIC AIMS

The objective of this study is to determine the cognitive and neurophysiological effects of chronic exposure to low-level blast overpressure in the professional community of "Breachers" (explosive entry personnel). The primary goal is to detect differences in cognitive performance and neurological functioning between experienced Breachers and well-matched control groups to substantiate and guide surveillance.

The specific aims of the study are as follows:

Phase A

Specific Aim #1: Replicate and augment NMRC.2007.0006, by examining the acute effects of breaching on cognitive and emotional functioning in individuals with chronic exposure to low-level blast overpressure using blast exposure characterization in conjunction with neuropsychological testing, vestibular system assessments, eye-tracking, and sleep pattern analysis.

Specific Aim #2: Characterize multiple breaching blast environments, as well as a non-blast generated overpressure environment, and measure variations in individual exposure levels due to tactical and environmental factors.

Specific Aim #3: Develop acute time-courses of blood biomarker levels that are associated with brain injury by collecting blood samples from subjects before, during, and after breaching blast exposure.

Phase B

Specific Aim #4: Examine long-term effects of chronic exposure to breaching blast on neurophysiological and cognitive functioning using neuropsychological testing, structural and functional neuroimaging, blood components sample analysis, vestibular and auditory testing, and a sleep assessment.

Specific Aim #5: Determine the most effective techniques for detecting neurophysiological and cognitive changes specific to breaching blast exposure by contrasting the experimental population with a well-matched control group consisting of individuals with extensive exposure to overpressure not related to blast (e.g. artillery units), as well as a control group with no history of overpressure exposure.

Specific Aim #6: Capture changes in daily functioning that the subjects may not be able to self-assess by conducting interviews with a close companion using questionnaires that target the companion's perception of the primary subject's daily function and by comparing responses to questionnaires that both the companion and subject answer.

Specific Aim #7: Examine the progression of long-term neurophysiological and cognitive changes in experienced Breachers by conducting a 1-year follow-up assessment.

4. MEDICAL APPLICATION / MILITARY RELEVANCE

In both training and operations, Warfighters are repeatedly exposed to blast events in the course of carrying out their duties. Very little data exists on the effect of this exposure on the physiological function of the human body, and none of the available data addresses the risk of cognitive impairment as a result of chronic repeated blast exposures. In 2005 and 2006, Breachers from both military and civilian law enforcement units began expressing some sensitivity to the risk of injury as a result of multiple blast exposures. Because Breachers apply explosives as a means of gaining access to barricaded or hardened structures, these specialists can be exposed to as many as a dozen 0.3 to 10 pound charges per day during training exercises and even larger numbers during night time operations. Although the Breachers' concerns are based upon anecdotal data and self-diagnosis, the symptoms they report, including sleep pattern disruption and short term memory loss, are similar to those reported by the Defense and Veterans Brain Injury Center (DVBIC) and others in the military community in regard to veterans returning from the recent and ongoing conflicts in Afghanistan and Iraq.

To address the profound issues related to the diagnosis and treatment of TBI, the United States Congress, through Public Law 110-252, established the Center for Neuroscience and Regenerative Medicine (CNRM) as a collaborative intramural program in May 2008. The CNRM is a contributing program resources for the execution of this study to include use of the CNRM funded MRI scanner, personnel and data sharing; however, no CNRM funds are being utilized in the performance of this study. Imaging data will be processed and stored by the CNRM at the NIH Clinical Center.

The concerns raised by Breachers present a unique opportunity for the blast injury research and medical communities to gather blast injury data on human subjects in a fully characterized blast environment. Analysis of this blast injury data will serve to answer the Breachers' question, "Are we being injured in our breaching maneuvers?" and will provide some characterization of the blast effects. This information can then be applied to improve our understanding of non-penetrating, non-impact neurological injuries occurring in the combat environment and develop appropriate mitigation strategies.

5. BACKGROUND AND SIGNIFICANCE

Significance of breacher research

There is limited published literature on the neurophysiological effects of blast exposure in humans and none of that literature represents repeated exposure to low-level blast. Breachers, more formally known as explosive entry personnel, are a unique population who are by occupational definition exposed to controlled blast. Instructors who train new breachers, by virtue of their job description, are *routinely*

exposed to low-level blast. Although this blast exposure does not result in clinical injury, the cadre of breacher instructors at USMC Weapons Training Battalion reported concerns with potential for injury from this repeated blast exposure. It is on the basis of these anecdotal reports that the original study of bio-effects from repeated exposure to blast was conducted. Those anecdotal reports included memory difficulty, sleep disturbance, and characteristics similar to those reported by the Defense and Veterans Brain Injury Center (DVBIC) for patients with traumatic brain injury returning from OEF/OIF. The primary objective of that study was to collect data during USMC breacher training to support the evaluation of potential for injury, with particular attention to breacher instructors. A multi-disciplinary collaboration was employed to meet this objective, including investigative teams for blast environment characterization, neurocognitive assessment, auditory/vestibular assessment, toxicological evaluation, and neuroimaging evaluation.

Reports from this ongoing study and others conducted by NMRC and WRAIR are currently in preparation for submission as publications. The results of these studies are largely a function of converging evidence, that is, complimentary observations across measures and across modalities. This converging evidence points to a previously undocumented phenomenon in this professional community. It also illustrates that further exploration of this issue is warranted. There are many benefits to studying this further including: risk management, the preservation of health and safety for members of this professional community, and the potential to generalize findings to blast-related post-concussion disorder and mild traumatic brain injury. Obtaining a larger sample of control subjects, which is also a part of this proposed protocol, is necessary to improve the quality of analyses of these data sets and assist in identifying subtle changes in central nervous system function.

Primary injury from blast

Primary injury from exposure to blast is not well understood and remains controversial, especially in respect to injury to the brain (Warden, 2006). Primary injury from blast is only beginning to be documented with neuroimaging techniques (Warden et al., 2009) and animal models are in development (Ahlers et al., 2008). The principal means to characterize this injury for clinical and research purposes is through behavioral evidence. The study proposed here will address primary blast injury as specific to the breacher training environment. Results of this study may be generalizeable to primary blast injury from other settings, an ancillary objective of this research. The importance of this ancillary objective is underlined in the documented blast exposures among U.S. service personnel deployed to operations in Afghanistan and Iraq (OEF/OIF).

A potential injury resulting from repeated exposure to low-level blast in the breacher training environment should be expected to be a relatively small effect. A large effect, a noticeable injury or impact on behavior, that occurred in any repeated fashion would be expected to have been recorded by training command personnel and appropriately prevented through revision in procedures. Regular operations yielding noticeable injuries would not be sustainable and, through logical consideration alone, should not be expected. A small injury or effect, developing slowly over time and exposure and to differing degrees across individuals, might be expected to escape notice. A slow to develop small effect to which some individuals are resilient might be detectable only with targeted objective measurement.

The type of insidious injury potentially at issue here may be present in breacher instructors, as a function of their routine exposure to low-level blast. Instructors for breacher training activities are exposed to repeated controlled low-level blast with each training session, for each group of new breacher trainees. Also, for breacher instructors, such repeated exposure to blast in a training setting can be expected to occur following a successful career of blast exposure as a breacher in operational settings. Those operational exposures would be less controlled than in the training environment. The breacher trainees cycling through this training environment would not have the same history of blast exposure or frequency of exposure. The trainees are much greater in number than the instructors and their absence of any small

injury might further mask the detection of an effect among the instructors from an occupational exposure to blast.

Relevance of sports concussion studies

The sports concussion literature can guide our understanding of blast injury hypothesized in the original study of breacher bio-effects. The research literature on closed head injury includes multiple terms of concussion, post-concussion syndrome, and mild traumatic brain injury (mTBI). These diagnostic labels have significant overlap in meaning, associated symptoms, and assessment methods, so such research is relevant to the present study, even though the injury mechanisms differ. (The breacher training environment presents potential for primary blast injury but not secondary, tertiary, or quaternary injury.) It is useful to point out now that there is also overlap in symptomology between post-concussion syndrome and post traumatic stress disorder (PTSD) but that the subject population of primary focus in this research, breacher instructors, is exposed to blast in the controlled settings of a training environment so the contributions of PTSD to the present study will be minimized.

In a specific study from the sports concussion literature McCrea's (McCrea et al., 2003) NCAA concussion study tracked 1631 collegiate football players from baseline on assessments of memory, cognitive processing, mental flexibility, verbal fluency as well as balance and other symptoms. These data showed not only changes in these assessments as a function of concussion but also showed a time course of recovery, using a daily testing schedule not dissimilar to that proposed in the present study. Also relevant to the proposed effort, two studies of military populations susceptible to sports concussion (Bleiberg et al., 2004; Warden et al., 2001) showed decrement in cognitive function association with concussion. Bleiberg (Bleiberg et al., 2004) administered preseason baseline testing with the Automated Neuropsychological Assessment Metrics (ANAM) to 729 athletes who were members of the United States Military Academy (USMA). ANAM is a computer-based behavioral assessment of neurocognitive performance, reflecting brain function. Following baseline, those who sustained head injury and those who were not injured (control group) were subsequently administered ANAM at regular intervals. In this repeated testing, cognitive impairment was present in the injured group on the day of injury and 1-2 days post-injury (Bleiberg et al., 2004). The injured subjects recovered from their cognitive impairment 3-7 days post-injury. In this study of USMA head injury using ANAM, concussion was demonstrated not only by a decrease in performance on the ANAM, but also by a lack of practice effects.

A meta-analysis of sports concussion literature Broglio (Broglio & Puetz, 2008) showed that the demonstration of effects of concussion on neurocognitive status were moderated by several factors: the inclusion of control groups, time from baseline testing to date of injury, and method of neurocognitive testing administration. A separate meta-analysis of the neuropsychological effects of sports concussion Belanger (Belanger & Vanderploeg, 2005) showed that there are impairments across several different neuropsychological domains, with the largest deficits in the following areas: global functioning, memory acquisition, and delayed memory. Also, concussed athletes were found to fully recover neuropsychologically within 7-10 days following injury. The effect sizes of concussion on neuropsychological performance for single assessments were double that of serial assessments; this finding is likely due to the practice effects from repeated administration of the neuropsychological tests. The studies that included subjects with previous head injuries had larger effect sizes than those that did not include such subjects; it was concluded by the authors that this finding indicates that prior head injury is associated with poorer cognitive performance (Belanger & Vanderploeg, 2005). These meta-analysis findings – ability-specific impairment, recovery from injury, practice effects in serial assessments, testing modality and individual differences in impairment as a function of previous injury – all have direct bearing on the present study.

Justification for proposed experimental procedures

Blast characterization (Phase A)
The purpose of the environment instrumentation is to characterize the blast environment to which the breachers are exposed, thus supporting the first aim of the study: examining the acute effects of breaching. The addition of the environmental characterization data addresses the primary shortfall associated with pure clinical blast injury studies, which is the ambiguity of the blast conditions associated with the observed neurophysiological changes. At this time, we do not know which components of the blast are dominant causal factors in the onset of mild TBI from blast, but based upon the physics of blast and research by the Naval Medical Research Center using a porcine model, blast overpressure is believed to be the most likely component. We will measure individual blast exposure levels while subjects are performing breaching techniques and correlate these levels with symptom reports and neuropsychological data collected before, during, and after breacher training.

Neuropsychological Tests (Phase A & B)
The neuropsychological tests for this protocol include the Automated Neuropsychological Assessment Metrics (ANAM4) TBI Battery and the Immediate Post-concussion Assessment and Cognitive Test (ImPACT 2.0). We selected the ANAM4 TBI Battery as a central tool in this protocol given the 20+ year history of ANAM development in DOD medical research activities, National Rehabilitation Hospital's specific efforts in ANAM4 validation for TBI, and DVBIC's extensive use (N>8,000) of ANAM4 TBI Battery with paratroopers in ongoing evaluations at Ft. Bragg. Key references for the type of cognitive-behavioral symptoms associated with TBI and mild TBI come from research and clinical observation in sports medicine described above. It must be noted that the two studies with military populations and ANAM reported above both suffer from methodological weaknesses and also that there are still unresolved issues in the use of computerized test batteries for clinical assessment of cognitive function. A thorough review of ANAM, its use, and approaches to analyses is available in a special issue of Archives of Clinical Neuropsychology (Kane, 2007). This ANAM-dedicated supplement includes 11 papers that provide a comprehensive review of ANAM, including a review paper focusing on the use of ANAM with concussion (Cernich et al., 2007). From consideration of this literature, key recommendations in the use of ANAM4 are captured in the proposed research.

In addition to the ANAM4 TBI Battery, we are also proposing to include the Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT 2.0) (Lovell, 2006) and the Defense Automated Neurobehavioral Assessment (DANA). ImPACT is a computerized neuropsychological test battery developed in the early 1990's by the University of Pittsburg Medical Center that was specifically designed for the evaluation of sports concussion. This battery has recently been adapted for the military for the assessment of mTBI and is currently in use as part of a baseline neurocognitive testing program by the United States Army Special Operations Command (USASOC). ImPACT has been shown to be sensitive to the acute effects of concussion and has been validated as a reliable measure of neurocognitive performance related to concussion (Iverson et al., 2004; Iverson et al., 2005; Lovell et al., 2006). Furthermore, studies using reliable change indices demonstrated that repeated administrations over a 2-week period revealed no practice effects (Iverson et al., 2002). From consideration of this literature, we are proposing to use ImPACT as part of the neuropsychological tests included in this protocol. DANA is a behavioral assessment tool developed for DOD use in field settings to reflect personnel impairment and level of functioning. DANA's development leverages what the DOD has learned through the employment of ANAM and other neurocognitive assessment tools (NCATs) for the evaluation of head injury. DANA is a flexible platform and can accommodate many uses, including a 40-minute exhaustive assessment and as a 5-minute surveillance assessment. The 40-minute DANA augments what will be learned from the ANAM and ImPACT; however, the ANAM and ImPACT are principal measures in this protocol and the 40-minute DANA is a supporting measure. If there are operational requirements limits in personnel availability in the before and after training paradigm, ANAM and ImPACT would be used preferentially;

the 5-minute DANA is relatively brief and non-intrusive and is expected to be used without operational requirements limits in personnel availability.

In addition to cognitive impairment following mild traumatic brain injury, mood disturbances may occur as well. Moore (Moore et al., 2006) found in their review of the literature on mTBI and anxiety that the prevalence of anxiety among those with mTBI was 23%, higher than an estimated rate for a non-injured population. The authors also found that PTSD, the re-experiencing of traumatic events, ranges in frequency from 20-84% among mTBI patients (Moore et al., 2006). The authors point out that the co-morbidity rate of depression and anxiety ranges 33-65% and that the majority of studies of mTBI focus on depression and anxiety separately. In one of the few studies that focused on both of these disorders within TBI, Jorge (Jorge et al., 2004) found that all subjects who met the criteria for generalized anxiety disorder (GAD), defined as excessive worry over issues in everyday life, also met criteria for depression. From consideration of this literature, a series of questionnaires and cognitive/emotional test batteries will be used to capture mood and other behavioral disturbances.

Biomarkers (Phase A & B)

Evidence is accumulating that TBI initiates a physiologic cascade that can be detected in blood components. Initial findings of research with this professional community have shown evidence for a positive relationship between blast exposure, elevated symptomology, performance deficits, and elevation of specific biomarkers in blood serum (including UCH-L1, SBDP150, SBDP120, MAP-2, EMAP-11, GFAP, and VCAM). This research has been conducted by WRAIR in partnership with Banyan Biomarkers (Alachua, FL, USA) and was most recently presented at the Advance Technology Applications for Combat Casualty Care 2010 Conference (St. Pete's Beach, FL). More recent pilot studies with mTBI patients and also with rodent models have indicated mTBI-related changes in other biomarkers (S100 beta, neuron specific enolase, brain derived neurotrophic factor, monocyte chemotactic protein, and peroxiredoxin 6) and in epigenetic and gene expression (using genes identified from separate studies with rodents exposed to repeated blast), and methylation analysis (which allows identification potential epigenetic changes that might be specific to human blast-related TBI). These results suggest that blood components biomarkers could serve as field-able diagnostic tools for mild traumatic brain injury that could augment non-field-able conventional diagnostic tools, such as CT and MRI, which may not be sensitive to mild and diffuse brain injury. Therefore, we will analyze blood components samples for a panel of biomarkers that will provide extensive information on blast-induced brain injury and potential mechanisms of injury.

Neuroimaging (Phase B)

To achieve maximal sensitivity and specificity for the detection of TBI, the current study incorporates multiple magnetic resonance imaging (MRI) neuroimaging endpoints, including diffusion tensor imaging (DTI), perfusion imaging, susceptibility weighted imaging (SWI), imaging with Gadolinium contrast, and functional magnetic resonance imaging (fMRI). These endpoints have been efficacious in demonstrating changes in mild TBI that are otherwise occult using routine anatomical computerized tomography (CT) and MRI approaches (Arfanakis et al., 2002; Inglese et al., 2005; McAllister et al., 1999; McAllister et al., 2001; Sigmund et al., 2007).

DTI is a recently developed MRI-based quantitative technique that can measure macroscopic axonal organization in nervous system tissues. Diffusion is the random microscopic translational motion of molecules (in MRI, usually water) in a fluid system and in the biological tissues. The DTI sequence is particularly effective in the detection of microstructural disruption of white matter (Arfanakis et al., 2002). Choice of this sequence is based upon recent data generated in a porcine model of mild blast-induced TBI clearly demonstrating traumatic axonal injury occurs following experimental low-level blast exposure. This sequence relies upon the normal anisotropic movement of water within brain white matter tracts. While water normally moves longitudinally down the length of white matter tracts, microstructural

disruption of white matter tracts will cause a reduction in this normal anisotropic movement of water. This loss of normal anisotropy may be quantified through the DTI approach. DTI has proven effective in detecting changes across the spectrum of TBI, from moderate to severe, which are occult on standard T1 and T2 MRI sequences.

Perfusion imaging techniques are sensitive to microscopic levels of blood flow (Hoeffner, 2005). Arterial spin labeling as a recently developed perfusion MRI technique measures perfusion without the need for an exogenous tracer by labeling the water in the arterial blood entering the brain, to provide an endogenous tracer of perfusion (Keston et al., 2003). Perfusion imaging can provide insights into the relationship between cognitive function and blood flow in the brain (Hillis, 2007). It has long been recognized that reduction in regional cerebral blood flow (rCBF) is associated with impairment of neural function in that area of brain. The reduced rCBF (hypoperfusion) can be secondary to dysfunction, as exemplified by the temporal and parietal hypoperfusion, for example, seen in studies of patients with Alzheimer's disease (Grossman et al., 2001). Adequate blood flow is necessary for both neural function and neural viability. Tissue receiving blood flow that is between 10 and 30% of the normal blood flow rate is getting just enough to survive, but not enough to function (Astrup et al., 1977). Therefore, imaging of blood flow can reveal areas of dysfunctional tissue that may be responsible for cognitive deficits after blast injury.

The SWI sequence is particularly effective in the detection of microhemorrhage within the brain (Sigmund et al., 2007). Microhemorrhage is a known feature of diffuse brain injury. This sequence capitalizes upon differences in magnetic susceptibility between deoxyhemaglobin and the surrounding neurological tissues. The SWI approach combines magnitude and phase information from a high-resolution, 3D T2 weighted gradient echo sequence to dramatically increased contrast of magnetically susceptible tissues.

Gadolinium-based contrast agents are used during MRI to increase the sensitivity for detecting differences between tissues and are used by radiologists to look at changes in blood vessels in the brain. Using this contrast agent can enhance the image in the area near a leak or proliferation of blood vessels, indicating a disruption of the blood-brain barrier (Giesel et al., 2010). Blast injury has been shown to causes increased permeability of the blood-brain barrier (Hicks et al., 2010). However, the duration of this effect and its association with clinical and other markers of injury are not understood. Therefore, we propose to administer Gadolinium contrast during structural MRI scanning to explore the hypothesis that cumulative exposure to low-level blast causes chronic increased permeability in the blood-brain barrier.

While the previous sequences provide exquisite sensitivity in detecting microstructural changes in brain tissues, fMRI is highly sensitive at detecting changes in neurological activity within the brain. The principle of fMRI is similar to SWI in that it detects susceptibility differences associated with deoxyhemaglobin within the brain. However, in contrast to increases in deoxyhemaglobin at sites of hemorrhage, fMRI detects decreases in deoxyhemaglobin that accompany the increased delivery of oxygenated blood to areas of high neurological activity. fMRI is typically performed during and following the performance of specific tasks. These tasks are designed to test particular neurological function which may relate to motor function, sensation, or cognition. In the current investigation, tasks will be employed which assess working memory, executive functioning, and social functioning given the recommendation of clinicians experienced with current military TBI patient populations and anecdotal evidence of symptoms reported by experienced Breachers. Previous use of fMRI in the study of brain function has shown that fMRI is a technique useful for identifying prefrontal dysfunction related to executive cognitive abilities in TBI patients without structural lesions on MRI (Fontaine et al., 1999; McAllister et al., 1999; McAllister et al., 2001), whereas CT scans and conventional MRI are only weakly related to executive function deficit in TBI patients (Fontaine et al., 1999; Vilkki, 1992).

Sleep (Phase A & B)

Sleep disturbances are observed in 50% of the TBI population (Castriotta et al., 2007), however, the sleep architecture that characterizes specific degrees of TBI (mild, moderate, and severe) has been addressed by few studies. A recent meta-analysis concerning sleep disturbances and TBI suggested that mild TBI correlates more strongly with sleep disruption than severe forms of TBI (Orff et al., 2009), giving credence to anecdotal accounts of sleep disturbances reported by Breacher Instructors and revealing a further need to dissect the sleep architecture of TBI subpopulations to determine a acute and long term treatment strategies. We will study acute effects of blast exposure on sleep-wake patterns and circadian rhythms during breacher training by assessing movement using wrist-worn actigraphy devices. Actigraphy is the use of a portable device that records movement over extended periods of time to give an accurate measure of sleep patterns and circadian rhythms (Morgenthaler et al., 2007) and has been validated against the gold-standard polysomnography for recording sleep/wake under field conditions (Signal et al., 2005). In addition, subjects who participate in Phase B who have a significant sleep disturbance as indicated by actigraphy data collected during Phase A or self-report, will undergo a sleep assessment using a one night polysomnographic recording to rule out the presence of overt sleep disorders (e.g., obstructive sleep apnea, periodic leg movements during sleep, etc.).

Vestibular and Auditory Assessments (Phase A & B)

Breachers wear hearing protection during all breaching maneuvers however, exposure to blast presents an inherent risk to the auditory and vestibular systems. Both military and law enforcement Breachers report incidents of transient post-blast auditory and balance problems (observation and USMC Dynamic Entry School verbal report, June 6, 2007) and recent studies have demonstrated a link between blast exposure and vestibular disorders (Hoffer et al., 2010; Scherer & Schubert, 2009; Sylvia et al., 2001). Furthermore, research shows that athletes demonstrate decreased stability up to three to five days post injury, which may be the result of ineffective use of one or more of their sensory systems (Guskiewicz et al., 1997). There is strong evidence demonstrating the impact of balance deficits on functional performance and increased risk of re-injury (Goldie et al., 1994; Lehmann et al., 1990). Therefore, to evaluate potential effects from this exposure, the auditory and vestibular systems will be assessed in this protocol using a sensory integration of balance test using the Portable BioSway Device, as well as self reports as part of a daily symptom questionnaire (e.g. dizziness, tinnitus, noise sensitivity). In addition, subjects who participate in Phase B will be assessed using computerized dynamic posturography as well as clinical tests of balance function and a self-reported questionnaire to evaluate the impact of symptoms on quality of life. Similar assessments have been shown to be successful in characterizing sequelae with TBI (Basford et al., 2003; Jury & Flynn, 2001; Newton, 1995; Wober et al., 1993) and vestibular disorders (El-Kashlan et al., 1998; Furman, 1995; Yardley et al., 1998). Additional tests will also be employed to assess peripheral vestibular and auditory functioning and to distinguish disorders of the peripheral and central vestibular systems.

Eye-Tracking Test (Phase A)

TBI has been shown to increase performance variability in visuomotor tasks that require sustained and focused attention (Robertson et al., 1997; Stuss et al., 1989). Because predictive visual tracking requires both intact attention and working memory (Barnes, 2008), it has been suggested that visual tracking performance can be used to supplement conventional evaluations of mTBI (Heitger et al., 2009). In addition, increased performance variability during predictive visual tracking has been demonstrated in individuals with mTBI and correlated with white matter track vulnerability (Maruta et al., 2010). Therefore, we will use a portable eye-tracking system that uses a highly predictable circular pursuit paradigm to evaluate anticipatory eye-tracking. This paradigm involves the anticipation of target motion, which requires higher cognitive input than visual-feedback controlled smooth pursuit eye movements. This test will provide additional insight into the link between blast exposure and higher cognitive processes known to be mediated by the prefrontal cortex.

Justification for the use of human subjects
Human subjects are required for this protocol to understand the impact of years of cumulative exposure to low-level blast generated overpressure that service members and law enforcement personnel experience. While animal experimentation with artificially generated overpressure can provide dose-response curves that exceed safety thresholds for humans, it is critical to compliment this research with human subjects that have cumulative exposure over several years.

Potential Benefits
There is no direct benefit to subjects for participating in this study. The documentation of neurocognitive change or other injury in this study that can be reasonably associated with exposure to blast would be an important first step in a means to mitigate risks in future training and in breaching operations. Enhancement of protection from blast exposure would be a benefit for military members and civilian law enforcement personnel assigned to Breacher duty and for all exposed to operational blasts. Payment to subjects is not considered a benefit because it is a fair compensation for time and inconvenience associated with participating in this research.

6. PLAN

6.1 New Investigational Drugs / Investigational Devices Exemption Status

N/A

6.2 Selection of Subjects

6.2.1 Type of the Subject Population

The target population for this study consists of individuals from military and civilian law enforcement Breacher communities with at least 4 years of experience in the breaching profession and extensive exposure to breaching blast. Breachers with less experience will also be included in Phase A of the study. In addition, the study will include a control group consisting of experienced active duty or prior active duty military personnel with extensive exposure to non-blast generated overpressure (e.g. artillery units) and a second control group consisting of experienced active duty or prior active duty military personnel with no prior exposure to overpressure. We will also include companions of the primary subjects.

6.2.2 Inclusion and Exclusion Criteria (see Eligibility Checklist, Appendix B)

a. Inclusion Criteria

Experimental Group: Breachers
To be included in the experienced Breacher Group, individuals must be active duty or prior active duty military personnel or civilian law enforcement personnel, between the ages of 18 and 60, with at least 4 years of experience in the breaching profession and actively involved in breacher training and/or operations (minimum of annual exposure). An alternate criterion to years of breacher experience is exposure to a significant number of breaching blasts, specifically, exposure to 400 breaching blasts or more within a career, will be considered “experienced” by the investigators. Individuals who are eligible to participate in breacher training will be allowed to participate in Phase A regardless of inclusion/exclusion criteria in order to preserve training group integrity, unless they decline to provide informed consent.

Control Group 1: Artillery
To be included in Control Group 1, individuals must be active duty or prior active duty military personnel that are demographically similar to the Breacher Group in terms of age, gender, service length, and operational and/or deployment experience, and have at least 4 years experience with exposure to concussive environments not related to blast (e.g. artillery units) (minimum of annual exposure). An alternate criterion to years of experience is exposure to a significant number of concussive evolutions, specifically, exposure to 400 or more within a career, will be considered "experienced" by the investigators. Individuals who are eligible to participate in artillery training will be allowed to participate in Phase A regardless of inclusion/exclusion criteria in order to preserve training group integrity, unless they decline to provide informed consent.

Control Group 2: Unexposed
To be included in Control Group 2, individuals must be active duty or prior active duty military personnel or law enforcement personnel that are demographically similar to the Breacher Group in terms of age, gender, service length, and operational and/or deployment experience. Operational experience is defined as years of experience actively involved in military or law enforcement operations and/or number of operations with the condition that operations include direct mission engagement roles rather than support roles. Military deployment or law enforcement patrol are examples of direct mission engagement roles and shore logistics or office based call center are examples of support roles.

Companion Group (Phase B)
To be included in the companion group, individuals must be considered a close companion of an experimental or control group subject over the age of 18 with knowledge of the subject's daily functioning (e.g. spouses, family members, domestic partners, close friends, etc.).

b. Exclusion Criteria

In order to preserve training group integrity, all individuals participating in breacher or artillery training will be invited to participate in Phase A of the study. The following exclusion criteria are applicable only to Phase B.

Experimental/Control Groups
- Children will be excluded from this study
- History of moderate or more severe brain injury with loss of consciousness greater than 5 minutes
- Current diagnosis of other CNS disorder (e.g. epilepsy)
- A medical condition that would make participation detrimental to the subject (e.g. severe clinical depression, unstable heart disease)
- MRI contraindications (see MRI Safety Questionnaire, Appendix B; includes pregnancy, screening test will be performed prior to MRI)

Control Group 1
- Previous experience with explosive entry training
- Exposure to blast from Breaching (greater than 40 individual blasts)

Control Group 2
- Previous experience with explosive entry training
- Exposure to blast or overpressure of any kind (greater than 40 individual blasts)

Companion Group (Phase B)

- None

6.2.3 Recruitment

a. Equitable Selection of Subjects

Children will be excluded from this study as a consequence of not being eligible to participate in explosive entry training. Women who meet eligibility criteria will be included as primary subjects and will also be included in the companion group. There is no exclusion of any minority from participation in this protocol.

b. Recruitment Procedures

This collaborative research team is already in contact with individuals who will be eligible to participate in this protocol, by virtue of blast-related engineering programs (ARA), active research protocols, and interaction at annual breacher meetings. Investigators and other personnel named on this protocol will advertise this study by word of mouth and approved advertisements (e.g., information sheet). Individuals who believe they are eligible and are interested in this research would contact the research team and would be invited to participate.

Phase A will differ from Phase B in that environmental characterization will include coordination with a breaching site and chain of command or supervisory support in addition to individual consent. For Phase A, an in-person meeting will be arranged between members of the research team and the representatives from the unit conducting breacher training. In that meeting, copies of this protocol and informed consent forms will be provided and the protocol procedures will be discussed. The discussion will resolve the feasibility of the protocol for that site and logistics required to support the study. The unit representatives will also be briefed on the possibility of individual subjects being invited to travel to NIH to participate in Phase B. Providing that protocol criteria are met and procedures are feasible and accepted, Informed Consent will be reviewed. If consent is granted by unit representatives, scheduling and other arrangements will be made. Informed Consent and eligibility criteria will then be reviewed for each individual participating in the training before any research participation. Any individual not consenting to participate will not be affected by this research, in terms of either the conduct of research procedures or participation in training activities. In order to avoid influence from senior leadership, officers and senior non-commissioned officers from the subjects' units and/or the training group will not be present during the consent process. In addition, officers, non-commissioned officers, and training supervisors who are participating in the study will be consented separately and will not be present during the consenting of subordinates. Recruitment of the control groups will be conducted in a similar fashion by coordinating with units that conduct artillery training as well as a unit at one of the performance sites that can provide personnel who would be eligible to participate as unexposed control subjects. Initial contact with these units will be conducted via informal word of mouth advertizing. Interested parties can follow up with the research team via coordination with unit commanders as described above.

For Phase B, interested persons will be recruited as individuals. Interested individuals will be contacted by the Research Contact and eligibility criteria and Informed Consent will be reviewed. Additional information about the study can be provided to the individual over the phone or via e-mail if requested. One additional criterion for this DOD protocol is MRI compatibility. The items on a standard of care MRI Safety Questionnaire (Appendix B) will be reviewed and the questionnaire will be provided to the individual. Similarly, persons for the Companion group will also be contacted by the Research Contact and invited to participate as individuals. The initial contact to the Companion

will, of course, be made by the subject who has already agreed to participate. Companions who are unable to travel to NIH may participate in the study remotely. "Off-site" companions will be screened over the phone and will exchange study documents with the investigators via mail. For any individual agreeing to participate in Phase B, scheduling, travel arrangements, and question and answer will be completed over the telephone by the Research Contact.

Recruitment may be by advertisement in multiple media formats including Facebook, Twitter, newspapers, newsletters, and radio. Recruitment may also include word of mouth, oral presentations and/or distribution of approved recruiting materials at events, meetings, and briefings wherein the desired recruit population might reasonably be expected to attend. In accordance with DoD Instruction 3216.02, an ombudsman will be present for the recruitment of Service members in a group setting. All advertisements, both general and specific to this study, will have been reviewed and approved by the IRB prior to their use.

Additionally, the approved flyers and written advertisements will be used in color as submitted, or may be printed in black and white. The color of the ads may vary. Color changes will not be used to change the emphasis of an ad. The size of the ads may vary, but all parts of the ads, including fonts and pictures, will be changed proportionately to the rest of that ad. Disproportionate changes in size will not be used to change the emphasis of an ad. The flyer and the IRB approved written ads may be placed in print publications of recruitment venues such as authorized military bases, base newspapers or magazines, as well as on the US military (.mil) domain websites for the military bases, their newspapers, magazines, or Facebook pages. It is recognized that posting recruitment notices must be in accord with the recruitment venue's policies and may require specific approval before proceeding.

c. Compensation

Military service members may not be compensated for their participation in research while "on duty" with the exception of compensation for blood draws. During Phase A of the study subjects will be compensated $25 per blood draw. Military service members must be on official leave status during their participation in Phase B of the study, and they must have their supervisor's and Unit Commander's written approval. For participation in Phase B, compensation for primary research subjects will be provided in accordance with NIH and DOD guidelines, and will include $70 per day of participation. Total possible compensation ($70.00/day up to 5 days, plus an additional $50 for completion of the sleep study) = $400.00. Individuals who participate in the 1-year follow-up visit will be compensated according to the same guidelines described above. This visit is expected to last 3 days for a total possible compensation of $210.Companions of primary subjects will not be compensated for participating in the companion group interviews.

Study related expenses for primary subjects and companions participating in Phase B will be paid for by NIH, including travel to and from NIH, hotel fees, and the NINDS standard per diem reimbursement for 3 meals per day.

6.2.4 Consent Process

Information about this protocol, including purpose, risk, benefit, eligibility criteria, contact points, and volunteers' right to decline participation or withdraw at any time with no consequence, will be provided to prospective subjects either in person or through an initial email to interested subjects. During the consent process, the Consent Form describing in detail the study procedures and risks is given to the subject and written documentation of informed consent and HIPAA authorization are required prior to enrolling in the study. A copy of the informed consent document will be given to the subjects for their records. Separate Consent Forms will be used for participation in each Phase of

the study, as individuals may only be eligible or available to participate in specific portions of the study. The consent procedures will be the same for the experimental and control groups.

For Phase A, arrangements to obtain Informed Consent from individual volunteers will first be made with the Commanding Officer of the training site after obtaining permission for protocol activity. Informed Consent will be obtained at the training site by a member of the research team listed as a "Consenter" (see Section 9. Roles and Responsibilities). Subjects will be assigned a random study number at the time of consent. This procedure prevents coercion as the Consenter is not in the volunteers' chain of command or connected to any medical treatment to which they are entitled. Subjects may be asked to participate in multiple evolutions of Phase A (e.g. if the research team revisits a field site for additional data collection) and can do so under the original consent form as long as it is valid. As there is no training site for the unexposed control group, consent for these subjects will be conducted in a suitable location, such as the unit's headquarters facility.

For Phase B, volunteers will be asked to sign both the DOD consent form for this protocol and the NINDS consent form (NINDS consent forms are included in Appendix A). The consent process for Phase B is described in detail in the NINDS protocol. Interested individuals will be contacted by the Research Contact and questions about research participation, if any, will be addressed and arrangements for travel to NIH will be made. Informed Consent will be obtained when the individual is on site at NIH in Bethesda. "Off-Site" companions will be consented over the phone.

6.3 Study Design and Methodology

6.3.1 Study Design

This is an observational study that will evaluate neurophysiological and cognitive changes related to chronic exposure to low-level blast overpressure by comparing experienced Breachers to a well-matched control group using a battery of neuropsychological assessments, physiological markers, and experimental procedures.

6.3.2 Study Methodology/Procedures

Subject Participation
This protocol consists of 2 phases. Subjects may participate in the entire study as per their availability and eligibility, or may elect to only participate in one portion of the study. Subjects are not required to participate in Phase A in order to enroll in Phase B, and vice versa. Subjects may participate in Phase A first and then choose to enroll in Phase B, or vice versa, depending on their availability and the training schedule of their operational group. Furthermore, subjects may be asked to participate in multiple data collection evolutions (e.g. multiple visits to field sites by the research team for Phase A; 1-year follow-up visit to NIH by the subject for Phase B).

A goal of Phase A is to evaluate up to 150 breachers, 50 artillery personnel, and 50 unexposed controls using neuropsychological measures and blood components analysis in order to develop a time-course of biomarker levels that are associated with brain injury. In order to achieve this goal, and to maximize the efficient use of resources during site visits to training facilities, we will also include individuals who do not meet criteria for experienced operators with extensive exposure. In order to preserve training group integrity, all individuals participating in breacher or artillery training will be invited to participate in Phase A of the study. From this pool of subjects, operators and instructors with at least 4 years of experience and who meet eligibility criteria may be invited to travel to NINDS to participate in Phase B. Recruitment for Phase A will continue after the enrollment goals for Phase B have been met.

Demographics Form and Head Injury Questionnaire
After Informed Consent has been obtained, subjects will be asked to complete a Demographics Form and Head Injury Questionnaire (Appendix B) that will ask them to provide information about their breaching history, other blast exposure, operational and deployment history, history of major medical issues, history of sleep patterns, and history of head injury (dates and duration will be recorded when is present). In addition, the questionnaire will include items related to cognitive and psychological health, including elements of the Post Traumatic Stress Disorder scale (Bombardier et al., 2006) and the Beck Depression Inventory (Beck et al., 1996). Subjects will also be asked to complete the Combat Exposure Checklist, which measures the frequency of stressful events experienced during deployments. If possible, scores from the Armed Services Vocational Aptitude Battery (ASVAB) , standard predeployment baseline assessments, or an equivalent law enforcement aptitude test will be recorded for pre-exposure baseline functioning. These data are collected in support of interpretation of primary research data.

Phase A: Field Assessments
In Phase A, a research team consisting of staff from NMRC, WRAIR, and ARA will travel to various breacher training facilities (for example: Ft. Benning, GA; Marine Corps Base Quantico, VA; Fort Bragg, NC; Montgomery County Police Department, MD) to conduct daily field assessments before, during, and after explosive entry training and concomitant blast exposure. Individual sites will be added to the protocol as each collaboration is formalized. Our research team is currently in the process of establishing a formal partnership with the United States Army Special Operations Command (USASOC), which will provide access to various sites where breacher training is conducted. The field assessments will include symptomology, neuropsychological tests, vestibular system assessments, eye-tracking, sleep pattern analysis, and blood components analysis for biomarkers. These procedures are described in the following subsections of this document. Additionally, during the training period, the research team will instrument the training environment to measure blast exposure. An important principle guiding this research protocol is to make no changes to the standard protocols for explosive entry training and to minimize additional burdens (e.g., 1-hour end-of-the-day test session) on the volunteers participating in this research. Parallel data collection using all of the above mentioned procedures will occur daily for 5 days prior to the start of breacher training, on breaching days, and for up to 7 days after training is complete in order to establish a baseline and observe the time-course of signal changes. A typical breacher training evolution involves a 2-week course with approximately 5 days of exposure to breaching blast (see section 6.3.5 Study Time Line), however, training schedules and amount of exposure varies between training groups. Participation in any of the data collection sessions or individual procedures will be subject to the requirements of the operational community and may be refused without consequence by any individual subject or for all subjects at a particular site by the training group commander.

Subjects from control group 1 will be assessed during artillery training with the same procedures as the Breacher subjects. Control group 2 will be assessed according to the same methods and scheduling, albeit absent any connection to blast or other exposure to overpressure. Arrangements for an appropriate location for data collection for control group 2 will be made with the participating unit. As there will no blast measurements taking place for this group, a classroom would be sufficient for the 1-hour of daily testing.

Phase B: Hospital Assessments
In Phase B, subjects will travel to NINDS in Bethesda, MD to undergo 5 days of neurophysiological and cognitive assessments including neuropsychological tests, blood components analysis for biomarkers, vestibular and auditory testing, a sleep assessment, and neuroimaging studies using DTI, SWI, perfusion imaging, imaging with Gadolinium contrast, and fMRI. The details of these

procedures are described in the NINDS protocol attached as Appendix A. A companion will be invited to accompany each subject to NINDS and asked to complete questionnaires that may capture changes daily functioning that subjects are not able to self-assess. Subjects will be invited back to NINDS 1 year following their initial visit for follow-up testing. As with Phase A, participation in any of the procedures may be refused without consequence.

6.3.3 Collection of the Human Biological Specimens

For Phase A, no greater than 10ml of blood per collection will be acquired once a day from subjects via venipuncture to the volunteer's extremity (e.g., antecubital vein) by a military phlebotomist or other individual certified to draw blood, with the exception of an additional 10 ml drawn on the first and the last days of sample collection (i.e., an additional 20 ml). For a typical 2-week training evolution, with maximum daily participation before, during, and after blast exposure, approximately 19 blood draws would take place, for a total of 210ml of blood. However, the specific number of blood draws will vary between training groups depending on the length of the training course, subject availability, and feasibility as determined by the researchers and training directors. These samples will be sent to the following laboratories where they will be assayed to look for internal indicators for changes after neurological insult at a molecular and cellular level: Banyan Biomarkers in Alachua, Florida; James J. Peters VA Medical Center/Mount Sinai School of Medicine in Bronx, NY; National Institute of Nursing Research, National Institutes of Health, Bethesda, MD. The samples will also be used to quantify biomarkers in blood from subjects to see how they correlate to measures of injury severity, progression, and outcome. The samples will be stored by study identification code, but the key that links the specimen by code to the individual's information will also be stored (separately) at NMRC/WRAIR so data will be identifiable for the duration of their storage. For Phase B, a single 20ml sample will be collected using the same procedures and will be sent to the collaborating laboratories described above to be assayed in the same way as described for Phase A. All samples will be destroyed once assayed.

See Appendix B for details of the Banyan Biomarkers standard operation procedures for serum collection and storage. Once analyzed, the blood samples will be destroyed. Note that collection of cerebrospinal fluid (CSF) is described in this appendix but will not be executed in this protocol. Collection and storage of other blood components (peripheral blood mononucleated cells) will be by parallel methods but with difference in collection container (e.g., green top vacutainer v red top or tiger top vacutainer).

6.3.4 Data Collection

Phase A: Field Assessments

Physical Characteristics of Exposure

This protocol for the environmental characterization will use two pressure sensors per individual. The pressure transducers sensing the exposure will be mounted to the left and right exterior surface of the helmet. Since the entire system is located on the exterior of the helmet, the drilling of holes that could potentially compromise the ballistic performance of the shell will not be necessary. Also, because the entire system is located on the exterior of the helmet, there is no risk of the system causing discomfort to the wearer. The output from the transducer will be recorded and digitized by a miniature data acquisition system (uDAS) mounted to the rear surface of the helmet. The sampling rate of the uDAS system is 1 million samples per second. Each unit is self triggered so a trigger cable, which is a tripping hazard, is not required. The uDAS system and sample output from the unit are shown in the Figure below. Each pressure gage weighs approximately 0.0025 ounces (0.08 grams) and has

diameter of 6.6 millimeters and a thickness of 0.84 millimeters. The entire uDAS system, including the power supply and automatic trigger, weighs 0.40 kilograms (15 oz).

The proposed sensor system was designed for, approved in the associated protocol for, and used in the Congressionally Directed Medical Research Programs (CDMRP) sponsored program, Brain Injury Biomarkers and Behavioral Characterization on mTBI in Soldiers Following Repeated, Low-Level Blast Exposure (WRAIR #1635).

Data down-loads from each helmet system will occur at the end of every test day when the batteries in the units are recharged. The charging and download will occur through a common USB download port which mates with a docking station that has enough ports to automatically charge and download data from all of the helmets at one location. The docking station will have an automated link to a secure server at ARA's office in Denver, Colorado. This system allows the coded laboratory-quality pressure data to be recorded on breachers and transmitted without having any of the research team permanently located at the test site for the duration of the study. Data in this study will be stored in Denver, CO by the individual's study identification code and processed by trained personnel.

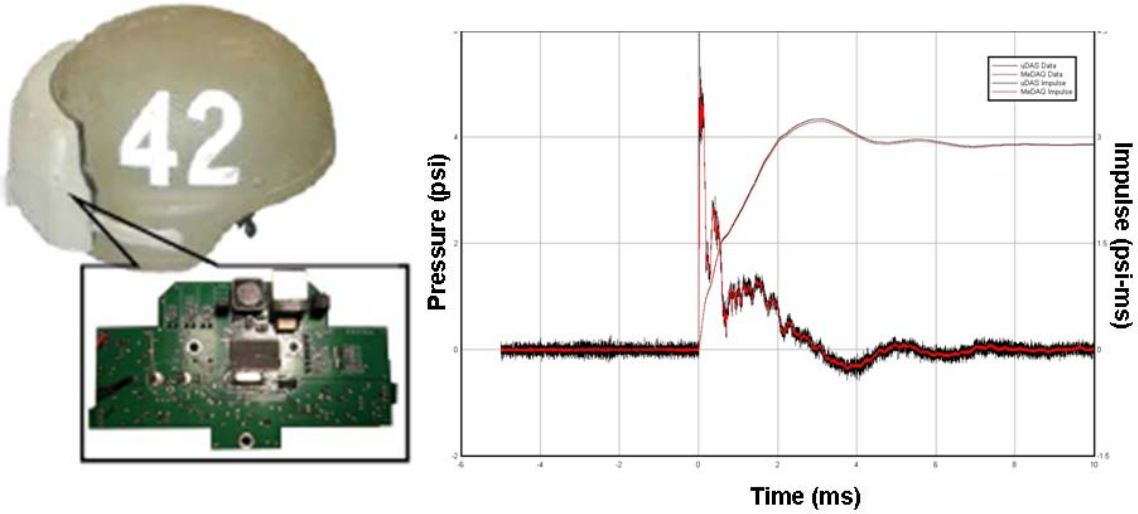


Figure 1: uDAS System and sample pressure output

The data from the systems will be used to estimate the magnitude, energy, and frequency content of the shock wave transmitted to the head region from each exposure. The results from each exposure will be tabulated and time stamped so that the cumulative exposure for each individual will be calculated.

To augment the pressure measurements recorded on each individual, the research team will make a site visit to each test location to deploy additional instrumentation to aid in the interpretation of each individual's pressure data. During these visits the research team will use additional pressure gauges positioned inside the structure while the breaching exercises are being conducted. These additional gauges will be used to assist in the explanation of any pressure anomalies observed in the individual's pressure data.

Supporting all of the electronic data collected, video recording of field exercises will be made using wireless camera systems in and around the breaching area to enhance the precision in determining physical relations between study subjects, features in the environment, and distance from blast. Videos collected for data analysis purposes will be used in briefings to training group commanders to

demonstrate the relationship between the characteristics of the exposure event and the exposure levels. Videos will be used only if individuals in the video are "blurred" or otherwise de-identified.

Symptomology
Subjects will complete a Symptom Questionnaire (Appendix B) daily at multiple time points before, during, and after training, as per subject availability. This questionnaire will be used to assess the presence of symptoms consistent with brain injury (e.g. headaches, ringing in ears, forgetfulness, etc.). It includes 32 items rated by the subject on a 5-point Likert Scale (0-4; 0 = not experienced at all; 4 = a severe problem) and a constant vs. intermittent choice. The questionnaire also includes space for the subject to report other symptoms they are experiencing.

Neuropsychological Measures
Subjects will perform the ANAM4 TBI Battery daily at multiple time points before, during, and after training, as per subject availability. The ANAM4 TBI battery is specifically designed, based on empirical data and experience, to be sensitive to TBI and to be administrable within approximately 20 minutes. This test battery is administered on computer, which allows it to be administered to large groups with multiple workstations, and it is designed to easily accommodate repeated administration, by sampling from a large pool of items for each administration. The ANAM4 TBI Battery includes the following 8 tests, with the neuropsychological qualities assessed listed in brackets:

- Stanford Sleepiness Scale [Self-Assessment Fatigue (state/trait)]
- Mood Affect Score [Vigor (high energy level), Happiness (positive disposition), Depression (dysphoria), Anger (negative disposition), Fatigue (low energy level), Anxiety (anxiety level), Restlessness (motor agitation)]
- Simple Reaction Time [Basic Neural Processing (speed/efficiency)]
- Code Substitution [Associative Learning (speed/efficiency), Visual Search, Sustained Attention, Working Memory]
- Procedural Reaction Time [Processing Speed (Choice RT/Rule Adherence)];
- Mathematical Processing [Working Memory]
- Matching to Sample [Visual Spatial Memory]
- Code Substitution (Delayed) [Retention]

In addition to the ANAM, subjects will also perform the ImPACT Version 2.0 and the Defense Automated Neurobehavioral Assessment (DANA). ImPACT will be conducted before and after training, as per subject availability. Version 2.0 of ImPACT is a computer administered neuropsychological test battery that has been shown to be sensitive to the acute effects of concussion and can be administrable within approximately 25 minutes. It consists of six individual test modules that measure aspects of cognitive functioning including attention, memory, reaction time, and processing speed. This test can also accommodate multiple administrations, albeit separated by several days to avoid interference, by sampling from additional versions of the individual modules. The ImPACT 2.0 includes the following 6 modules, with the neuropsychological qualities assessed listed in brackets:

- Word Memory [Immediate and delayed memory for words]
- Design Memory [Immediate and delayed memory for designs]
- X's and O's [Attention, concentration, working memory, reaction time]
- Symbol Match [Visual processing speed, learning and memory]
- Color Match [Focused attention, response inhibition, reaction time]
- Three Letters [Attention, concentration, working memory, visual-motor speed]

DANA will be conducted before and after training as well as periodically during training, as per subject availability. DANA is a behavioral assessment tool developed for DOD use in field settings to reflect personnel impairment and level of functioning. In the current study, its 2 principal uses will be a 40-minute exhaustive assessment and as a 5-minute surveillance assessment. The 40-minute assessment includes the following 16 tests (in order of execution): Simple Reaction Time, Verbal Learning Test (Learning), Code Substitution (Learning), Verbal Learning Test (Recall, short delay), Procedural Reaction Time, Spatial Processing, Code Substitution (Recall), Choice Reaction, Sternberg Memory Search, Verbal Learning Test (Recall, long delay), Simple Reaction Time, Combat Exposure Scale, Patient Health Questionnaire-9, Pittsburgh Sleep Quality Index, PTSD CheckList-Military, and Deployment Stress Inventory. The 5-minute assessment includes the following 3 tests (in order of execution): Simple Reaction Time, Procedural Reaction Time, and Choice Reaction. The 40-minute assessment can be used twice and would be used in a before and after training paradigm in the proposed work. The 5-minute assessment can be used repeatedly, in rapid succession, without limit on number of administrations; the 5-minute assessment would be used in a daily paradigm in the proposed work.

See Appendix B for examples and additional descriptions of the ANAM4 TBI Battery, ImPACT Version 2.0, and DANA.

mTBI Biomarker Analysis

Blood samples will be collected daily at multiple time points before, during, and after training, as per subject availability. No greater than 10ml of blood per collection will be collected via venipuncture to the volunteer's extremity (e.g., antecubital vein) by a military phlebotomist or other individual certified to draw blood, with the exception of an additional 10 ml drawn on the first and the last days of sample collection (i.e., an additional 20 ml). Samples will be separated into aliquots and frozen. Each aliquot will be labeled with the volunteer's unique identifier (no identifiable information will be recorded on the sample labels). The samples will be stored temporarily at the study site before transport to the following laboratories to be assayed: Banyan Biomarkers in Alachua, Florida; James J. Peters VA Medical Center/Mount Sinai School of Medicine in Bronx, NY; National Institute of Nursing Research, National Institutes of Health, Bethesda, MD. Banyan Biomarkers is the established leader in discovery of innovative brain injury biomarkers, and will analyze serum samples for a panel of biomarkers that may include:

- UCH-L1: A biomarker of cell body injury
- SBDP150: Biomarker of axonal injury and cellular necrosis
- SBDP120: Biomarker of axonal injury and cellular apoptosis
- MAP-2: A persistent biomarker of dendritic injury
- GFAP: A biomarker of glial injury
- sICAM-1: A biomarker of vascular damage, and
- s100β: A well-established benchmark biomarker for brain injury

Biomarker levels in serum samples obtained from study subjects will be determined by standard 96-well microtiter plate based Enzyme-Linked Immunosorbent Assay (ELISA) technology. This ELISA format employs a biomarker-specific capture antibody attached to the surface of the microtiter plate well. An aliquot of the serum sample is mixed with buffer and applied to the microtiter well for 60-90 minutes to allow for binding of the biomarker to the capture antibody. After washing of the plate to remove all unbound material a secondary antibody is added, which is also specific for the biomarker, but typically which binds to a different portion (epitope) of the biomarker molecule. The resulting tri-molecular complexes or sandwiches are then detected via an enzymatic reaction that involves horse radish peroxidase (HRP). This enzyme may be directly attached to the detection antibody through

conjugation, or indirectly via a biotin-streptavidin bridge, or through attachment of a tertiary antibody that carries this enzyme. The enzymatic reaction involves the turn-over of a substrate that results in formation of a color, fluorescence, or chemiluminescence, whereby the amount of substrate turn-over is directly proportional to the number of biomarker molecules trapped in the sandwich. Measurement of the amount of color, fluorescence or luminescence that is generated and comparison to a calibration curve allows accurate quantization of the biomarker with a lower level of detection that varies between 0.1 and 1.0ng/ml in serum. The precision (intra- and inter-assay coefficients of variation) may vary between 10% and 25%, which then determines the lower limit of quantization.

In addition to serum-based biomarkers assessed by Banyan, serum and other blood components will be assayed by other collaborators listed above. Epigenetic analyses will be performed for modulation of gene expression mediated by DNA methylation in response to neurological insult and analysis of autoimmune- or inflammation-based responses and broad mircoRNA arrays will be assessed as markers of neurological insult.

See Appendix B for details of the Banyan Biomarkers standard operation procedure for serum collection and storage. Once analyzed, the blood samples will be destroyed. Note that collection of cerebrospinal fluid (CSF) is described in this appendix but will not be executed in this protocol. Collection and storage of other blood components (peripheral blood mononucleated cells) will be by parallel methods but with difference in collection container (e.g., green top vacutainer v red top or tiger top vacutainer). Analysis of serum and other blood components will also be open to other collaborating laboratories, based on new collaborator findings and pilot data.

Vestibular System Assessment
Subjects will undergo vestibular testing using the Portable BioSway Device (Biodex Medical Systems Inc., Shirley NY) daily at multiple time points before, during, and after training, as per subject availability. The Clinical Test for Sensory Integration of Balance (CTSIB) helps to determine which sensory system (visual, vestibular, or somatosensory) a person relies on to maintain balance. It provides a generalized assessment of how well a patient can integrate various senses with respect to balance and compensate when one or more of those senses are compromised. It is administered by: 1) manipulating the support surface (firm vs. foam); 2) visual conditions (eyes open vs. eyes closed); and 3) vestibular system sway reference by using the computerized sway platform, while an individual is asked to maintain their standing balance. A 3" Airex® Indexed Foam Pad is used as the compliant surface for the unstable support surface. The CTSIB requires subjects to complete four 30 sec tests.

- Condition 1 – Eyes open firm surface: Baseline: Incorporates visual, vestibular and somatosensory inputs
- Condition 2 – Eyes closed firm surface: Eliminate visual input to evaluate vestibular and somatosensory inputs.
- Condition 3 – Eyes open on a dynamic surface used to evaluate somatosensory interaction with visually input.
- Condition 4 – Eyes closed on dynamic surface: used to evaluate somatosensory interaction with vestibular input

See Appendix B for a detailed description of the Portable BioSway Device and standard operating procedure for its use.

Eye-Tracking Test
Subjects will perform a Smooth Pursuit Eye Movement (SPEM) task using the head mounted,

Portable Eye-Tracking Device (Brain Trauma Foundation) daily at multiple time points before, during, and after training, as per subject availability. The SPEM task requires the subject to visually track a target stimulus, a red circle of 0.2 º diameters, which follows a circular clockwise trajectory with a radius of 7º and at a speed of 0.4 Hz. The red circle takes exactly 2.5 seconds to complete a revolution, or cycle. A circular pursuit task was chosen because it allows for the recording of both horizontal and vertical components concurrently, enabling a greater amount of data to be acquired in a shorter amount of time. The signals representing eye and target movements will be simultaneously processed during the testing trials by a proprietary "attention-detection algorithm" to produce the "attention score", which will represent the subject's eye movement variability on a 1-100 scale, with 100 representing near-to-zero variability (a perfect score) and 1 representing very high variability. The attention score, subject identifier, testing date/time and other inputted information will be saved automatically on an irremovable storage card in a handheld control tablet for future recall.

See Appendix B for a detailed description of the Portable Eye-Tracking Device and standard operating procedure for its use.

Sleep/Wake Actigraphy
Subjects will wear a wrist-worn device called an actigraph (ReadiBand, Fatigue Science, Honolulu, HI, or comparable alternate product) throughout the course of data collection before, during, and after training, as per subject availability. The actigraph records wrist movements, which are subsequently processed through a sleep-scoring algorithm to determine sleep/wake amounts. Alternate devices (e.g., Actiheart, CamNtech, Boerne, TX) can supplement the movement record with a heart rate monitor record, improving sleep/wake assessments by calculating activity energy expenditure in free-living conditions. In an example, low level exercise may yield a motion record similar to sitting in a rocking chair or riding in an automobile but the types of activities here can be expected to have bearing on derived sleep/wake measures.

See Appendix B for a detailed description of the ReadiBand Actigraph Device and standard operating procedure for its use.

Phase B: Hospital Assessments

Detailed descriptions of the procedures to be conducted during Phase B appear in the NINDS protocol attached as Appendix A.

Neuropsychological Measures
Subjects will perform a series of neuropsychological tests as well as paper-and-pencil and computer tests of executive function, emotional function, language, memory, intelligence and other cognitive abilities (e.g. California Verbal Learning Test, Delis-Kaplan Executive Function System Sorting Test, and Booklet Category Test).

Note: Questionnaires or interviews related to history of abuse, sexual behaviors, or drug/alcohol abuse will not be included as part of this study.

Blood Components Analysis
Subjects will be asked to provide a single 20ml blood sample to be sent to the following laboratories where they will be assayed in the same way as described for Phase A: Banyan Biomarkers in Alachua, Florida; James J. Peters VA Medical Center/Mount Sinai School of Medicine in Bronx, NY; National Institute of Nursing Research, National Institutes of Health, Bethesda, MD.

Neuroimaging
Subjects will undergo multiple neuroimaging sessions during their visit at NINDS using routine, microstructural, and functional imaging techniques to achieve maximal sensitivity and specificity for the detection of TBI. Structural imaging procedures will include magnetic resonance imaging (MRI) to look for possible brain lesions, diffusion tensor imaging (DTI) to evaluate microstructural disruption of white matter, perfusion imaging to look at microscopic levels of cerebral blood flow, and susceptibility weighted imaging (SWI) to detect microhemorrhages within the brain.

Subjects will also be asked to participate in an imaging procedure that involves the use of Gadolinium, which is a contrast agent that enhances blood vessels in MRI for detecting disruptions of the blood-brain barrier. Only subjects that meet specific screening criteria for safe use of this compound will be eligible for this procedure (see Potential Risks section below). Subjects will be specifically screened for prior allergic reactions and for risk of decreased renal function according to NIH policies. Eligible subjects who agree to participate in this procedure will have an angiocatheter placed by an intravenous (IV) nurse in the NIH radiology department. The angiocatheter will be placed in the upper extremity, and be of a sufficient size to accommodate power injection. Following contrast administration, dynamic susceptibility contrast (DSC) imaging and standard structural imaging will be performed.

Functional magnetic resonance imaging (fMRI) will be used to measure changes hemodynamic signals related to neural activity in response to cognitive and emotional stimulation using experimental paradigms such as the N-Back and Task Switching tasks.

Vestibular and Auditory Assessments
Subjects will undergo balance testing using computerized dynamic posturography (CDP) with a SMART EquiTest System (NeuroCom International, Inc.). The CDP allows for the objective quantification and differentiation among the wide variety of possible sensory, motor, and central adaptive impairments to balance control. Tests may include the Sensory Organization Test (SOT), which is used to identify which sensory system (vestibular, visual, or somatosensory) is abnormally used to control balance; the Limits of Stability test (LOS), which is used to identify problems with voluntary motor control of balance; and Dual Tasking Posturography (DTP), which is used to assess the interaction between cognition and the control of balance. Subjects will also undergo balance testing using the Five Times Sit to Stand test (FTSST) and the Dynamic Gait Index (DGI), and the self-reported Dizziness Handicap Inventory (DHI).

In addition, subjects will undergo three tests of specifically designed to identify vestibular dysfunction and distinguish disorders of the peripheral and central vestibular systems. These tests include Sinusoidal Harmonic Acceleration (SHA), which examines the vestibulo-ocular reflex and its response to rotations at a variety of stimulus frequencies; the caloric irrigation subtest of videonystagmography (VNG), which examines horizontal semicircular canal function; and Vestibular Evoked Myogenic Potentials (VEMP), which is used to evaluate the vestibulo-colic response. They represent a diagnostic extension of the functional assessments conducted during posturography. Finally, subjects will undergo tests of auditory functioning including pure-tone threshold assessment and tympanometry.

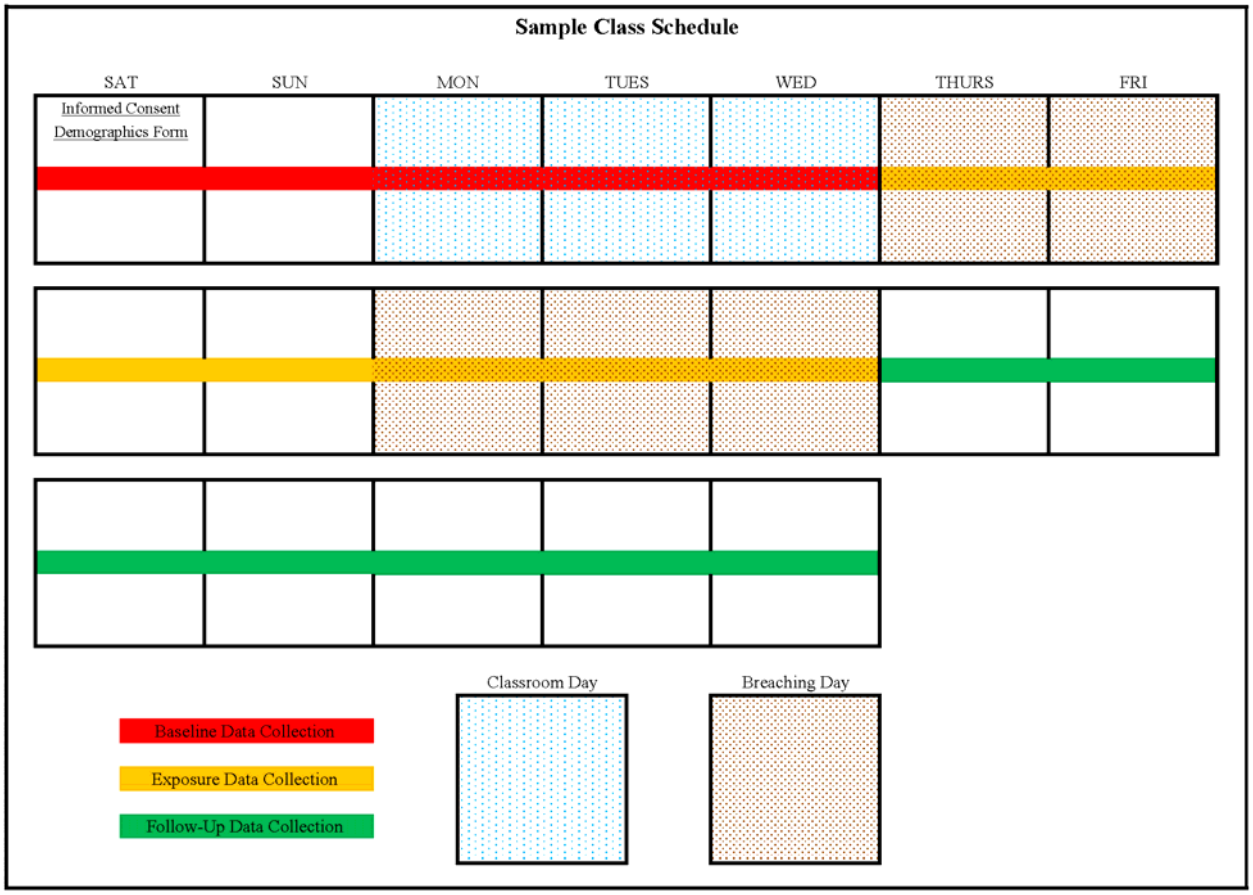
Polysomnography
Subjects that have a significant sleep disturbance as indicated by self-report or actigraphy data collected during Phase A (average total sleep time and/or sleep continuity are two standard deviations from age-appropriate norms), will undergo a sleep assessment using one night of polysomnographic (EEG) recording to rule out the presence of overt sleep disorders (e.g., obstructive sleep apnea, periodic leg movements during sleep, etc.). Electrodes will be applied over the head for the EEG

recording, around both eyes to monitor eye movements, around the chest and abdomen to monitor respiration, and on both legs to monitor leg movement. Analyses of polysomnographic recording includes total sleep time, sleep efficiency, latency to sleep onset and REM (rapid eye movement) sleep, and sleep architecture with ratios of various sleep stages (stages 1, 2, 3, and REM sleep).

Companion Interview
The companion interview will consist of surveys which include demographic information, measurements for companions' stress, and self-rated health, as well as questionnaires that ask about the subject including the presence of symptoms, depressed mood, physical function, and self-care. Furthermore we will ask caregivers to complete some of the same questionnaires as the subject in order to compare responses. For "Off-site" companions, once informed consent has been obtained, study personnel will mail the Companion Questionnaire Battery for completion. Companions will be instructed to contact study personnel with any questions or concerns regarding the questionnaires or if they chose to withdraw their consent to participate in the project.

6.3.5 Study Time Line

Phase A
This diagram illustrates a hypothetical schedule of data collection adapted to a typical evolution of an explosive entry course.



6.4 Statistical Consideration

6.4.1 Primary Endpoints

The primary endpoints of this study will be the data collected from the neuropsychological tests, blood components analysis, neuroimaging sessions, vestibular assessments, and sleep analysis. These measures were selected based on their known sensitivity to brain injury and are expected to demonstrate significant differences when comparing the experimental groups. The outcome of this research effort will be documentation of findings and recommendation to mitigate operational risk. Results will be presented to military commands engaged in breaching as well as prepared as manuscripts for publication.

6.4.2 Data Analysis

Data will be analyzed by repeated measures ANOVA with both within-subject factors (degree of exposure) and between-subjects factors (Breachers vs. Artillery Controls vs. Unexposed Controls) followed by a priori planned post-hoc tests. Post-hoc tests will use a standard correction for number of comparisons within an analysis (e.g. Bonferonni or Geiser-Greenhouse procedures). Note that if the distribution of data within a group on any single variable is skewed or non-normal, either non-parametric tests will be used to analyze the data or the data will be normalized using a standard transformation such as a log-normal transformation. Any subjects from Phase A data collection who endorse exclusion criteria for Phase B will be considered separately in analysis.

6.4.3 Safety Monitoring

Safety monitoring will be in place primarily due to the use Gadolinium as a MRI enhancing contrast agent during Phase B of the study. Gadolinium contrast imaging presents some moderate risks (see Potential Risks section below for complete list). The primary risk is to people with kidney disease as they may have a serious reaction to gadolinium contrast called “nephrogenic systemic fibrosis” which has resulted in a very small number of deaths. Careful screening of subjects for abnormal kidney function will be the primary process for mitigating this risk. Subjects that have diabetes, kidney disease or liver disease will undergo a blood test to assess kidney function within 4 weeks before any MRI scan with gadolinium contrast and those whose kidney function is not normal will not receive gadolinium for a research MRI scan.

6.4.4 Sample Size Estimation

The goal for Phase A of this protocol is to recruit up to 250 subjects (150 breachers, 50 artillery controls, and 50 unexposed controls). Multiple training sites will need to be visited to meet the primary goal of this study, which is the collection of data from a significant number of individuals with chronic exposure to blast in order to develop a time-course of acute signal changes during breacher training. In order to maximize the efficient use of resources during visits to training facilities, we will also collect data from individuals who do not meet criteria for experienced individuals. Therefore, the number above was estimated based on the expected ratio of experienced individuals (operators and instructors) to inexperienced trainees that typically appear in breacher training courses.

The goal for Phase B of this protocol is to recruit a minimum of 60 subjects (20 breachers, 20 artillery controls, and 20 unexposed controls) to travel to NIH for the hospital based assessments. The number of subjects to be included in this protocol was determined from consideration of the main objective of detecting a chronic exposure effect, typically reported as an effect on cognitive ability (esp., "memory difficulty"), and the previously observed effect size among the cohort of interest (Carr et al., unpublished manuscript). The use of the same computer-based testing paradigm in both the completed study and the

proposed study affords a straightforward estimation of sample size. In the more difficult and more sensitive of the 2 computer-based tests involving demand of memory, Code Substitution Delayed, the mean difference in accuracy (percent correct) at baseline between the experienced group and members of the more naïve group matched according to IQ, age, and blast history, was 6.6%. With a standard deviation of 8.46 and 6.26 for each of these 2 groups and an intergroup correlation of .33, the resultant large effect size (.76) yields an estimate of 16 subjects needed per group to re-detect this difference at baseline (Erdfelder et al., 1996). This effect size is consistent with related literature on concussion and military populations (Warden et al., 2001). In the protocol proposed, considerable effort will be expended to carefully select and support research volunteers so attrition is not expected. However, to accommodate some attrition, error, and data loss, the requested sample size for this protocol will be 25% above the minimum required, so 20 subjects per group are requested.

6.5 Reporting Adverse Events

6.5.1 Expected Adverse Events from Research Risks and Reporting

Potential Risks

Risks associated with the testing procedures are mitigated by having qualified medical personnel on the team to supervise safety procedures. A risk of loss of anonymity due to data being linked to the subject's identity applies to both phases of the study, however, this risk is mitigated by the confidentiality procedures (subject coding) described below. The confidentiality of active duty military service members may not be able to be maintained as their chain of command may request information obtained during our study (e.g. copies of consent forms, copies of questionnaires, raw or processed data). In addition, there may be circumstances where reporting to the chain of command may be required (e.g. violations of UCMJ, abuse, etc.). As with all research subjects, active duty service members can choose not to answer sensitive questions.

Phase A

- The significant risk of being exposed to explosives and repeated blasts that will occur during the field assessment phase of this protocol is not different than the subjects' level of risk during routine explosive entry training. This protocol will have a minimal effect on their training regimen and will be conducted during previously scheduled training events.
- There is minimal risk due to the addition of sensors to subjects' helmets, but the light weight of this equipment (15 oz.) is not a significant burden.
- During blood draw, the subject may experience some discomfort at the site of needle entry and there is a risk of bruising. There is a remote risk of fainting or local infection. These risks are mitigated by having trained military and civilian medical personnel conduct the blood draws.
- There is a small risk of falling off of the BioSway apparatus. This will be mitigated by having a member of the research team supervising vestibular tests.
- The neuropsychological tests, eye-tracking, and sleep/wake actigraphy are not expected to pose any risk to the subjects.

Phase B

Risks associated with the hospital phase of this protocol are described in detail in the NINDS protocol (Appendix A). The following is a summary of these risks:

- There is some risk in the transport of volunteers to Bethesda, MD, but this risk is not greater than that most people encounter every day.
- During blood draw, the subject may experience some discomfort at the site of needle entry and there is a risk of bruising. There is a remote risk of fainting or local infection. These risks are mitigated by having trained military and civilian medical personnel conduct the blood draws.

- The neuropsychological tests and questionnaires may be frustrating or stressful. Subjects may refuse to answer any question or stop a test at any time and for any reason.
- All vestibular and auditory tests are standard clinical practices and present only minimal risk to the subject including some sensation of dizziness or nausea.
- During the sleep assessment, there is a risk of discomfort during the application and removal of the EEG electrodes.
- There is a small risk of emotional discomfort from performance of the functional neuroimaging tasks; however, this risk is mitigated by explaining the nature of these tasks to the subject and giving them the option of stopping a test at any time.
- The MRI scanning procedures in this protocol present some risk to volunteers in the case of any unsecured metal in the strong magnetic field, of unprotected exposure to the MRI noise environment, and of potential discomfort from lying supine for an hour in a movement-restricted environment. These risks, however, are present for any MRI procedure and are well demonstrated to be successfully mitigated by standard protections offered in metal safety, hearing conservation, patient screening, and patient monitoring. If participants have a question about any metal objects being present in their body, they should inform the staff. If there is uncertainty about the presence of metal, we will obtain plain radiographs before performing MRI. These studies are considered part of standard care before MRI. There is a risk to operational readiness from incidental clinical findings; however, subjects are informed beforehand of this possibility.
- Gadolinium contrast imaging presents some moderate risks. The risks of the IV catheter placement include bleeding, infection, or inflammation of the skin and vein with pain and swelling. Symptoms from the contrast infusion are usually mild and may include coldness in the arm during the injection, a metallic taste, headache, and nausea. In an extremely small number of patients, more severe symptoms have been reported including shortness of breath, wheezing, hives, and lowering of blood pressure. Subjects will not receive gadolinium-based contrast agents if they previously had an allergic reaction to them. Subjects will be asked about such allergic reactions before a contrast agent is administered. People with kidney disease are at risk for a serious reaction to gadolinium contrast called “nephrogenic systemic fibrosis” which has resulted in a very small number of deaths. Subjects that have diabetes, kidney disease or liver disease will undergo a blood test to assess kidney function within 4 weeks before any MRI scan with gadolinium contrast. Subjects will not receive gadolinium for a research MRI scan if their kidney function is not normal.

6.5.2 Reporting Serious and Unexpected Adverse Events to the IRB

Serious Adverse Events: The PI will report all serious adverse events (SAE) and unanticipated problems involving risk occurring in subjects enrolled in this DOD protocol to the NMRC Office of Research Administration (ORA) within 24 hours. Formal reporting of all adverse events and unanticipated problems will be completed within 5 days using the NMRC ORA IRB Form 3. Serious adverse events will be reported even if the PI believes that the adverse events are unrelated to the protocol.

The WRAIR Division of Human Subjects Protection (DHSP) will be copied on all such reports for acknowledgment. A summary of all serious or unexpected side effects also must be included in the Annual Progress Report.

6.5.3 Medical Care for Research-Related Injury

No compensation will be provided for injuries that are a direct result of being in this study. It will be explained to subjects in the consent forms that this is not a waiver or release of their legal rights and that they should discuss this issue thoroughly with the principal investigator before they enroll in this study.

For Phase A, military service members as well as civilians will be treated at a Military Treatment Facility in accordance with MRMC Command Policy Memorandum 2010-10, Medical Care for Research-Related Injury. DOD healthcare beneficiaries (e.g. active duty military, military spouse or dependent), are entitled to medical care for injuries within the DOD healthcare system, as long as they remain a DOD healthcare beneficiary. This care includes but is not limited to free medical care at a military treatment facility. Non-DOD healthcare beneficiaries are also entitled to free medical care for their injury at a military treatment facility. It cannot be determined in advance which military treatment facility will provide care. If non-DOD healthcare beneficiaries get care for research-related injuries outside of a military treatment facility, the subject or their insurance will be responsible for medical expenses.

For Phase B, the NIH Clinical Center will provide short-term medical care for any injury resulting from participation in research at that site. In general, no long-term medical care or financial compensation for research-related injuries will be provided by the National Institutes of Health, the Clinical Center, or the Federal Government. However, subjects have the right to pursue legal remedy if they believe that their injury justifies such action.

6.5.4 Subject Withdrawal from Participation

Subjects may withdraw from participating in the study at any time with no consequences. If a subject withdraws during Phase A, the research team will stop data collection from that subject immediately and it will not affect their ability to complete the training program. If a subject withdraws during Phase B, the research team will stop data collection from that subject immediately and arrangements will be made for their return home. Subjects who withdraw early from either Phase will be asked if we are permitted to retain data collected up to that point. Should the subject request, their individual data will be excluded. Subjects will be compensated for the time and/or procedures they completed as outlined in the compensation section above.

The principal investigators may terminate participation in this study if continued participation is considered to be detrimental to the subject's health, if the subject fails to cooperate with the study, or if the military mission requires it. The same rights and procedures described above apply when the investigators terminate participation.

6.6 Human Biological Specimens/Tissue

Procedures for the collection and use of blood samples are described in this protocol. Blood samples will be sent to collaborating laboratories and destroyed once analyzed. Details of procedures used for samples can be reviewed in Appendix B. Collection of blood samples will be highlighted in the Informed Consent form and described to the subject before consent is obtained.

6.7 Subject Confidentiality Protection

All subjects will be assigned a 4-digit identifier (e.g. subject #7264) generated from a random number generator during the informed consent process. This ID# will be stored with the subject's name and research group assignment (i.e., breacher, control 1, or control 2) in a password protected record at NMRC. All other data records will be labeled only with the subject ID#, vice identifying information.

The coded data from this project will be stored in locked and password-protected facilities. All data from this project will be subject to review by blinded external reviewers. With appropriate authorization to release, all aspects of this study and the de-individualized data may appear in open publication.

NINDS and CNRM follow similar subject confidentiality procedures, which are described in detail in Appendix A.

Auditing authorities for the Navy and Army, CNRM, Uniformed Services University, Henry M Jackson Foundation, and NIH may request to review study documents, which could affect the confidentiality of subjects' identity and research records. Specifically, the Department of the Navy Human Research Protections Program (DON HRPP) and the United States Army Medical Research and Materiel Command (USAMRMC) Human Research Protection Office could perform an audit of the files, which could include the consent forms.

6.7.1 Certificate of Confidentiality

This study does not include a Certificate of Confidentiality. Subject confidentiality will be secured using the procedures described in this protocol. As described in the section above on potential risks, the confidentiality of active duty military service members may not be able to be maintained as their chain of command may request information obtained during our study (e.g. copies of consent forms, copies of questionnaires, raw or processed data). In addition, there may be circumstances where reporting to the chain of command may be required (e.g. violations of UCMJ, abuse, etc.). As with all research subjects, active duty service members can choose not to answer sensitive questions.

6.7.2 HIPAA Authorization

This study will include the collection of "Identifiable Protected Health Information" as well as the following personal identifiers: name, address, age, telephone number, e-mail address, social security number. Therefore, in accordance with the requirements of the Health Insurance Portability and Accountability Act (HIPAA) and DOD HIPAA regulations 6025.LL-R, subjects will need to sign a HIPAA Authorization form (see Appendix F).

a. Confidentiality of research source documents

Data in this study will be stored by study identification code, vice other identifying information. The key that links data code to the individual's information will be stored separately from the data, according to the description in the paragraph below. This stored key will be the only means to identify subjects' data for the duration of storage and will be accessible only by the principal investigators.

Coded hardcopies of data will be stored in locked cabinets in a locked office at NMRC/WRAIR (Building 503, room 2W109) and at ARA's facility in Littleton, CO (10720 Bradford Rd., Suite 102). Data will be accessible only by study lead investigators. Electronic data will be kept in 2 forms. 1) PC-compatible files in various software formats (e.g. MS Excel, E-Prime, Presentation, ASCII text, MS Word; 2) neuroimaging files will be kept in the following Unix/Linux-compatible software formats: AFNI, ANALYZE, DICOM, NIFTI. The PC-compatible files will be stored on a computer at NMRC/WRAIR (Building 503; room 2W109) with access limited to study personnel via DOD Common Access Card-enabled logon policy and user account privilege. NMRC/WRAIR computer data are backed up per DOD requirements. Neuroimaging files will be kept on a non-networked computer with Linux operating system at NMRC/WRAIR (Building 503; room 2W109), with access limited to study personnel via physical access to the room and username/password logon requirements.

Details for the protection of coded data stored at NINDS and CNRM are described in Appendix A.

b. Storage and destruction of the research source documents

Upon completion of the study, data will be archived but available for future study. Data in this study will be stored by study identification code, vice other identifying information, but the key that links data by code to the individual's information will also be stored (separately) so data will be identifiable for the duration of their storage. The rationale for retaining subject identity to data is because these data may be used in a future investigation, and that investigation could include individuals from this protocol, for examination of longitudinal effect of exposure to blast, an important research question for this protocol. That investigation could not be performed without retaining data identity.

c. Sharing of research data

Data exchange with study partners will be with de-identified data and in a sample of at least 5 subjects rather than on an individual basis. The primary institutions for execution of work in this protocol and the storage of protocol data are NMRC, WRAIR, NINDS, and ARA. NMRC holds United States Department of Health and Human Services (DHHS) FWA Number FWA00000152; WRAIR holds DHHS FWA Number FWA00000015; NINDS holds DHHS FWA Number FWA00005897; and ARA holds DHHS FWA Number FWA00014065.

6.8 Reporting Protocol Deviations, Amendments, and Continuing Reviews

Any protocol deviations during the course of the study will be promptly reported to the NMRC IRB and sponsor, as well as the WRAIR DHSP for acknowledgment.

All amendments to research documents (protocol, consent forms, etc.) will be submitted for approval to the NMRC IRB and WRAIR DHSP. Amendments will include a memorandum outlining the changes, clean copies of the changed research documents, as well as copies with the changes marked. Annual continuing review reports outlining study progress and a study closeout report upon completion of the research will also be provided to the NMRC IRB and WRAIR DHSP.

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8. FACILITIES/ORGANIZATIONS TO BE USED

8.1 Collaborators (see Appendix C for the Research Collaborative Agreement):

- Naval Medical Research Center
- Walter Reed Army Institute of Research
- National Institute of Neurological Disorders and Stroke
- Applied Research Associates, Inc.
- University of Virginia

8.2 Performance Sites (see Appendix G for letters of approval from performance sites):†

- United States Army Special Operations Command
- 75th Ranger Regiment
- John F. Kennedy Special Warfare Center and School
- Forced Entry Tactical Training
- United States Army Engineer School

† Individual sites will be added to the protocol as each collaboration is formalized (reference pg. 24)

9. ROLES AND RESPONSIBILITIES OF EACH INVESTIGATOR AND COLLABORATOR

	NAME	DEGREE	INSTITUTION	ROLE
1	LCDR Peter Walker	PhD	NMRC	LEAD INVESTIGATOR, Neurocognitive Investigator
2	MAJ Angela Yarnell	PhD	WRAIR	LEAD INVESTIGATOR, Neurocognitive Investigator
3	CAPT Eric Wasserman	MD	NINDS	LEAD INVESTIGATOR, Neurocognitive Investigator, Consenter
4	Lee Ann Young	MA	ARA	LEAD INVESTIGATOR, Engineer
5	CPT Matthew LoPresti	PhD	WRAIR	Neurocognitive Investigator, Neuroimaging
6	MAJ Walter Carr	PhD	WRAIR	Neurocognitive Investigator, Consenter
7	Thomas Baker	PhD	WRAIR	Neurocognitive Investigator, Consenter
8	Gary Kamimori	PhD	WRAIR	Neurocognitive Investigator, Consenter
9	CPT Angela Yarnell	PhD	WRAIR	Neurocognitive Investigator, Consenter
10	Tracy Doty	PhD	WRAIR	Neurocognitive Investigator, Neuroimaging
11	Tim Walilko	PhD	ARA	Engineer
12	James Stone	MD PhD	UVA	Neuroimaging
13	Yvonne Allard	BA	WRAIR	Research Assistant
14	Nora Prindle	BA	WRAIR	Research Assistant
15	Jessica Kim	BS	WRAIR	Research Assistant
16	SGT Sharae Murray		WRAIR	Research Assistant
17	SPC George Adams		WRAIR	Research Assistant
18	LT Jacob Norris	PhD	NMRC	Neurocognitive Investigator, Consenter
19	Carmen Contreras-Sesvold	MS	NMRC	Neurocognitive Investigator, Consenter
20	Elena Polejaeva	BS	WRAIR	Research Assistant, Consenter
21	Kristine Dell	BA	WRAIR	Research Assistant, Consenter, Research Contact

22	John Butman	MD, PhD	NIH	Neuroradiologist, Neuroimaging
23	Leighton Chan	MD, MPH	NIH	Neurocognitive Investigator, Vestibular Testing
24	Christiane Zampieri-Gallagher	PhD	NIH	Vestibular Testing
25	Carmen Brewer	PhD	NIH	Vestibular and Auditory Testing
26	John Dsurney	PhD	NIH	Neurocognitive Investigator
27	MAJ Jeffrey Lewis	MD, PhD	NINDS	Neurocognitive Investigator, Consenter
28	Michael Tierney	MA	NINDS	Neurocognitive Investigator, Consenter
29	Kristine Knutson	MA	NINDS	Neuroimaging
30	CPT Aaron M. Smith	Psy.D	WRNMMC	Neurocognitive Investigator, Consenter
31	CDR John Hughes	MD	NMRC	Research Monitor
32	Richard McCarron	PhD	NMRC	Neurocognitive Investigator
33	Thomas Balkin	PhD	WRAIR	Neurocognitive Investigator
34	Laura Coombs	PhD	ACR	Neuroimaging
35	Corrina Lathan	PhD	AnthroTronix	Data Analysis of Non-human Subjects Data

----------ROLE DEFINITIONS----------

- LEAD INVESTIGATOR .....Primary responsibility for IRB compliance, documentation, reporting, data storage
- Consenter .....Administration of informed consent
- Site Coordinator ……..Primary responsibility for coordinating data collection at performance sites for Phase A (identified in Delegation Log for each site)
- Engineer .....Design, measurement, and analysis of blast
- Neurocognitive Investigator .....Administration and analysis of neurocognitive testing
- Neuroimaging .....Design, execution, and analysis of neuroimaging data, subject screening/MRI safety
- Neuroradiologist .....Interpretation of neuroimaging results
- Vestibular and Auditory testing…...Administration and analysis of vestibular and auditory tests
- Research Assistant……...Assist with data collection and analysis
- Research Contact.....Primary contact for Phase B subjects
- Research Monitor .....Primary responsibility for overseeing safety of subjects

10. TIME REQUIRED TO COMPLETE THE RESEARCH (INCLUDING DATA ANALYSIS)

Study Duration = 5 years

11. APPENDICES

APPENDIX A – Experienced Breacher Study (EBS), National Institute of Neurological Disorders and Stroke (NINDS), Protocol # 12-N-0065, Version 6.0

- NINDS IRB Approval Letter
- Protocol and Consent Forms
- EBS Test Battery Forms

APPENDIX B – Questionnaires/Procedure Descriptions

- Eligibility Checklist
- Demographics Form and Head Injury Questionnaire
- Combat Exposure Checklist
- Symptom Questionnaire
- Automated Neuropsychological Assessment Metrics (ANAM4), TBI Battery
- Immediate Post-concussion Assessment and Cognitive Test (ImPACT), Version 2.0
- Defense Automated Neurobehavioral Assessment (DANA)
- Banyan Biomarker standard operating procedure (BANDITS)
- Portable BioSway Device product description and standard operating procedure
- Portable Eye-Tracking Device product description and standard operating procedure
- Fatigue Science ReadiBand Actigraph Device product description and standard operating procedure
- National Institutes of Health MRI Safety Questionnaire and Standard of Practice: MRI Contrast Policy
- National Institutes of Health Radiology Department MRI Safety Questionnaire

APPENDIX C – Research Collaborative Agreement

APPENDIX D – Consent Forms

- Phase A: Field Assessments
- Phase B: Hospital Assessments (Primary Subjects)
- Phase B: Hospital Assessments (Companions)

APPENDIX E – Supervisor Permission Form

APPENDIX F – HIPAA Authorization Form

APPENDIX G – Performance Site Approval Letters

- United States Army Special Operations Command
- 75th Ranger Regiment
- John F. Kennedy Special Warfare Center and School
- Forced Entry Tactical Training
- United States Army Engineer School

APPENDIX H – Research Monitor Addendum

APPENDIX I – Delegation of Roles and Responsibilities Log and Best Practice Recommendations

APPENDIX J – Communication to subjects for re-consent request

Appendix C

Permission for Use of Figures

February 28, 2018

Permission granted by Jessica M. Gill, PhD to use the following figures in Katie Edwards' dissertation manuscript:

1) Figure 1 on page 274 of the paper:

Gill, J., Merchant-Borna, K., Lee, H., Livingston, W. S., Olivera, A., Cashion, A., . . . Bazarian, J. J. (2016). Sports-Related Concussion Results in Differential Expression of Nuclear Factor-kappaB Pathway Genes in Peripheral Blood During the Acute and Subacute Periods. *J Head Trauma Rehabil, 31*(4), 269-276. doi:10.1097/htr.0000000000000191

2) Figures 4 on page 1581 and Figure 5 on page 1582 of the paper:

Merchant-Borna, K., Lee, H., Wang, D., Bogner, V., van Griensven, M., Gill, J., & Bazarian, J. J. (2016). Genome-Wide Changes in Peripheral Gene Expression following Sports-Related Concussion. *J Neurotrauma, 33*(17), 1576-1585. doi:10.1089/neu.2015.4191

3) Figure 1 on page 92 of the paper:

Gill, J., Motamedi, V., Osier, N., Dell, K., Arcurio, L., Carr, W., . . . Yarnell, A. (2017). Moderate blast exposure results in increased IL-6 and TNFalpha in peripheral blood. *Brain Behav Immun*. doi:10.1016/j.bbi.2017.02.015

2-28-2018

Supplement:

NanoString Methods

A subset of genes examined in RNAseq data were selected to validate gene expression changes by assaying 50 ng of mRNA using a direct digital detection system (Nanostring Technologies, Seattle, WA). A panel was designed for each pathway to include 50 markers of interest, plus a total of 10 reference/housekeeping genes for data normalization, including *ABCF1, ALAS1, DECR1, GAPDH, GUSB, HPRT1, IPO8, PGK1*, and *TBP* (these genes are also noted in Table 3-2 and 3-3 of the dissertation manuscript). Care was taken to ensure that reference genes selected met the following criteria: 1) not dysregulated in the RNA-seq data for the same samples; 2) not clearly implicated in traumatic brain injury, blast exposure, or a similar condition; and 3) no published evidence that this is an unstable reference gene in human blood. Probes for the 50 genes of interest and the housekeeping genes were designed and manufactured by Nanostring Technologies. Briefly, probes for marker and reference RNAs were multiplexed and assayed using the nCounter Digital Analyzer. Samples were randomly assigned to plates to avoid run-order bias. In an effort to control for plate-to-plate variations and drift, one sample was used as an internal control. We also validated the result with 50 genes from each network (100 genes total) using NanoString technology, which showed congruent finding with RNA sequencing data.