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Effects of Interval Exercise on Commonly Studied Fluid Biomarkers for Sport-related Concussion in Serum and Plasma

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Effects of Interval Exercise on Commonly Studied Fluid Biomarkers for Sport-related Concussion in Serum and Plasma

by

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Abstract

High intensity interval exercise has been shown to increase blood levels of commonly studied fluid biomarkers for SRC. If true, the potential diagnostic or prognostic applications of these markers for SRC may be limited due to exercise being implicit in sport. This thesis examines the effects of interval exercise on serially collected plasma levels of t-tau, GFAP, NFL, and UCH-L1 in healthy young adults (7 females; 3 males), and differences in biomarker levels between plasma and serum matrices. The first study showed small and short-lived decreases in plasma NFL and GFAP immediately following interval exercise. The second study demonstrated differences between plasma and serum concentrations of t-tau, NFL, and GFAP. Together, these results suggest exercise should be considered prior to the clinical validation of these biomarkers for diagnosis or prognosis of SRC and highlights the need to harmonize analytical methodologies across research investigations aiming to develop objective measures of SRC.

Keywords: sport-related concussion (SRC), interval exercise, blood biomarkers, total tau (t-tau), glial fibrillary acidic protein (GFAP), neurofilament light chain (NFL), ubiquitin c-terminal hydrolase-L1 (UCH-L1).

Preface

The study undertaken for this thesis was approved by the University of Calgary Conjoint Health Research Ethics Board (REB21-0768). This work sought to explore the effects of a single bout of varying intensity interval exercise on fluid biomarkers commonly studied for sport-related concussion in plasma and serum matrices.

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Dedication

I dedicate this thesis to my wife (Madison), and my parents (Kym and Jared) for their boundless support in my life and academic journey.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
BBB	Blood brain barrier
CNS	Central nervous system
CSF	Cerebral spinal fluid
CISG	Concussion in Sport Group
CRT5	Concussion recognition tool 5
ELISA	Enzyme-linked immunosorbent assay
GFAP	Glial fibrillary acidic protein
GFR	Glomerular filtration rate
HIIT	High intensity interval training
iNOS	Inducible nitric oxide synthase
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IR	Incidence rate
ISF	Interstitial fluid
mTBI	Mild traumatic brain injury
MICT	Moderate intensity continuous training
MIIT	Moderate intensity interval training
NFL	Neurofilament light chain
NO	Nitric oxide
PNS	Peripheral nervous system
p-tau	Phosphorylated tau
PA	Physical activity
ROS	Reactive oxygen species
s100B	S 100 calcium-binding protein b
SIMOA	Single-molecule array
t-tau	Total tau
TBI	Traumatic brain injury
TNF-α	Tumor necrosis factor alpha

Chapter 1: Introduction

1.1 Background

The benefits of engaging in physical activity (PA) throughout an individual's lifespan are well established, specifically in the context of overall health. In adults, regular PA has been shown to delay age-associated cognitive decline through a variety of mechanisms including increases in cerebral perfusion, synaptic plasticity, and neurogenesis (Bailey et al., 2013; Brugniaux et al., 2014; Cabral et al., 2019). PA has been positively associated with many physical indicators of health (e.g., bone health, cardiovascular fitness, muscular strength, adiposity), including cognitive health (e.g., psychological distress and quality of life), in children and adolescents (Poitras et al., 2016). Sport is the main source of PA in youth and persists as a main contributor to PA through adulthood (Statistics Canada, 2019). The benefits of sport in developing youth also include positive effects on social health outcomes in team sport settings (e.g., higher self-esteem, increased social skills, fewer depressive symptoms) (Eime et al., 2013; McKee et al., 2014).

Despite numerous health benefits of sport, a major concern regarding sport participation is an increased risk of injury and the associated burden. It is estimated that 1 in 3 youth seek medical attention annually for a sport-related injury and 1 in 3 working adults miss at least one day of work due to sport-related injuries (Black et al., 2021; Conn et al., 2003; Emery & Pasanen, 2019). Sport-related injury can occur through traumatic or overuse mechanisms resulting in broad ranges of physiological disruptions (Patel et al., 2017). It is common in injury prevention literature to separate sport-related injuries into two general categories for investigation including musculoskeletal injuries (e.g., any disruption to typical functions regarding the muscular or skeletal systems) and brain injuries (Mrazik et al., 2016; Patel et al.,

2017). Due to the delicate nature of a brain injury, there has been an exponential increase in sport-related concussion research over the past 20 years.

1.1.1 Sport-related concussion.

Sport-related concussion (SRC) is a mild traumatic brain injury (mTBI) induced by biomechanical forces resulting in the rapid of onset of neurological impairment that manifests as a vast range of clinical signs and symptoms (McCrory et al., 2017). SRC incidence rates (IR) vary by sport given the different goals, mechanisms, formats, and rules governing play (Pfister et al., 2016). For example, estimated SRC IRs (/1000 athlete exposures) in youth team collision sports such as rugby (4.18; 95% CI 2.50-5.86), ice hockey (IR=1.20; 95% CI 1.00-1.31), and American football , (IR=0.53; 95% CI 0.40-0.67) are significantly higher than non-contact sports such as baseball (IR=0.06; 95% CI 0.04-0.08) (Pfister et al., 2016). SRC remains a vital target for all levels of prevention to reduce the significant injury burden in youth and adults (McCrory et al., 2017). Moving upstream towards primary prevention through sport policy change, equipment, and/or training programs has the greatest potential to reduce injury incidence rates and potential long term consequences of SRC (Emery et al., 2017). However, research targeting secondary and tertiary prevention strategies remains vital to inform comprehensive concussion diagnosis and prognosis with the understanding all concussions cannot be eliminated.

Current assessment and treatment strategies following SRC are reliant on subjective clinical measures and clinician judgement. A major research focus in these secondary and tertiary prevention domains is the identification of objective methods for concussion diagnosis or prognosis to augment clinical decisions, ultimately improving patient outcomes and reducing recovery time. Blood biomarkers have emerged as a potential objective measure to supplement the current subjectively reliant diagnosis and prognosis of SRC.

1.1.2 Blood biomarkers for sport-related concussion.

Blood biomarkers are measurable molecules contained within blood of the human circulatory system that are indicative of a physiological state (Zetterberg & Blennow, 2016). In specific context of injury and disease, blood biomarkers can be useful objective measures of injury or disease progression and severity while providing insight on the underlying pathophysiology. Protein biomarkers produced in the central nervous system (CNS), specifically the brain, are suitable targets for objective measures of brain injury and disease. Some of the most commonly studied blood biomarkers for SRC include structural components of neurons or supporting glial cells like tau protein, neurofilament-light chain (NFL), ubiquitin C-terminal hydrolase L1 (UCH-L1), and glial fibrillary acidic protein (GFAP) (McCrea et al., 2017; Zetterberg & Blennow, 2016). Most research in the field to date is designed towards establishing possible associations between concussion and these protein biomarkers of injury for diagnostic purposes. As discussed in detail in chapter two, the characterization of these blood biomarkers in non-injured states has largely been ignored. Blood biomarkers in healthy individuals may also be influenced by exercise, an important variable considering the athletic context of which SRC occurs (Di Battista, Moes, et al., 2018; Shahim et al., 2018).

1.1.3 The effect of exercise on CNS derived blood biomarkers.

Few studies have examined the effects of exercise on blood biomarker levels. Some studies have shown that some CNS derived blood biomarkers were elevated after single bout of high intensity interval exercise in healthy individuals (Di Battista et al., 2018; Shahim et al., 2018). As such, the role of exercise and the mechanisms involved in the presentation of CNS derived blood biomarkers may need to be accounted for when considering blood biomarkers as an objective measure of SRC. However, due to the lack of rigorous studies investigating the

effects of exercise on biomarker concentrations, the mechanisms involved are not well understood. Previous studies have suggested an increase in blood brain barrier (BBB) permeability following exercise, postulating hyperacute increases in reactive oxygen species (ROS) or transient tight junction shrinkage due to water loss from perspiration might play a role (Koh & Lee, 2014; Roh et al., 2017). Entirely theoretical in the current state, this knowledge gap necessitates further investigation into the role of exercise on blood biomarkers commonly associated with concussion.

1.2 Research Rationale

Given the significant public health burden of SRC it is critical to develop objective measures of diagnosis and prognosis to increase diagnostic certainty, reduce time to recovery, and develop targeted treatment strategies. The mechanisms involved in the presentation of CNS derived blood biomarkers post-SRC and their role in the underlying pathophysiology is not yet fully understood. Before objective blood biomarker measures for SRC can be clinically validated and implemented, it is necessary to disentangle any potential influences on blood biomarkers. For this reason, this thesis targets the largely unknown effects of interval exercise on CNS derived blood biomarker levels in heathy individuals, as exercise is inherently present in many sport contexts where the injury occurs.

1.3 Purpose

The objective of this thesis is to examine the effects of interval exercise on commonly studied fluid biomarkers associated with sport-related concussion in healthy young adult plasma and serum. The first study explores differences in the plasma biomarker concentrations of total tau protein (t-tau), NFL, UCH-L1, and GFAP at ten serial time points following differing intensity interval exercise bouts (control, moderate intensity interval exercise [MIIT], high

intensity interval exercise [HIIT]), and the second study seeks to understand if the concentrations of these blood biomarkers differ between sample matrices (i.e., plasma vs. serum) across the aforementioned interval exercise intensities.

1.3.1 The specific primary objectives include the following:

1.3.1.1 Chapter 3 (Effects of Interval Exercise on Commonly Studied Blood Biomarkers Associated with Sport-related Concussion).

1. To examine if a single interval exercise bout of varying intensity (moderate and high) is associated with significant changes in concentrations of plasma t-tau, NFL, UCH-L1, and GFAP biomarkers across serial timepoints following exercise.
2. To explore if sex, age, and sleep in the night prior to exercise influence the potential changes in plasma t-tau, NFL, UCH-L1, and GFAP biomarkers across serial timepoints.

1.3.1.2 Chapter 4 (Commonly Studied Blood Serum and Plasma Biomarkers for Sport-related Concussion: Does matrix matter?).

1. To examine if a single interval exercise bout of varying intensity (moderate and high) results in differential correlations between plasma and serum concentrations of t-tau, NFL, UCH-L1, and GFAP biomarkers.
2. To examine the correlation between plasma and serum concentrations of t-tau, NFL, UCH-L1, and GFAP biomarkers in individuals across all sampling timepoints.

1.4 Summary of Thesis Format

Chapter two is a review of the relevant literature examining the current state of blood biomarker research in the area of SRC. Chapter three compares blood biomarker concentrations

in plasma at serial time points following differing intensity interval exercise in healthy young adults (ages 18-26) over a three-month data collection period. Chapter four examines correlation of the blood biomarkers between plasma and serum matrices after differing intensity interval exercise utilizing the same study as Chapter 3. Finally, chapter five summarizes and discusses findings from chapters three and four while commenting on future directions of research leading to the development of objective blood-based measures for SRC diagnosis and prognosis.

Chapter 2: Literature Review

2.1 Traumatic Brain Injury and SRC

Traumatic brain injury (TBI) is a disruption in normal brain function caused by external biomechanical forces transmitted directly or indirectly to the head (Najem et al., 2018). TBI is a markedly heterogenous injury that has been separated into three classes of clinical severity as measured by the Glasgow Coma Scale (GCS); mild, moderate, and severe (Teasdale & Jennett, 1974). The GCS is a cumulative score of three scaled tests (visual, verbal, and motor) between 3 and 15; 3-8 is severe TBI, 9-12 is moderate TBI, and 13-15 is mild TBI (mTBI) (Maas et al., 2008). Recognized as one of the leading causes of death and disability worldwide, TBIs across the GCS severity scale pose a significant public health and economic burden (Najem et al., 2018; Public Health Agency of Canada, 2020). In the United States, TBI has contributed up to 61,000 total deaths in the emergency department (ED) alone. (Centers for Disease Control, 2020; Fu et al., 2016). TBI has been labelled as "the silent epidemic" by virtue of a significant number of cases going unrecognized or unreported (Rusnak, 2013). Specifically, many who suffer mTBI may not seek medical attention due to a general public misunderstanding of the injury caused by inefficiencies in education practices (Merz et al., 2017). The underestimation of TBI cases likely occurs most often within the mTBI classification group due to numerous cases with minor symptoms going unreported, underdiagnosed, or misdiagnosed (Buck, 2011). The significant burden of concussion, combined with the generally unknown burden on those that go unrecognized, necessitates investigations aimed at identifying and implementing targets for all levels of prevention across the spectrum of TBI severity.

Sport-related concussion (SRC) is an mTBI occurring in sport environments and is clinically defined under four criteria by the 5th international consensus statement on concussion

in sport (McCrory et al., 2017). These include: 1) force transmitted directly or indirectly to the head; 2) rapid onset of cognitive impairment that typically resolves spontaneously; 3) negative findings on neuroimaging techniques (e.g., traditional magnetic resonance imaging, computed tomography scan) indicating a functional, rather than structural, disturbance; and 4) with or without loss of consciousness (McCrory et al., 2017). It has been estimated 1 in 450 Canadians (ages 12+) report SRC or "other brain injury" as their most significant injury associated with disability in the previous year (Gordon & Kuhle, 2020). Additionally, ineffective education modalities for athletes and ruminating internal and external pressures experienced by athletes to continue playing through injuries are thought to play causal roles in those who sustain a SRC but don't seek medical attention (Carroll-Alfano, 2017; Kroshus et al., 2015). For example, a study investigating SRC reporting in NCAA division 1 athletes found they were motivated to hide their concussion symptoms despite a considerable knowledge of the potentially severe risks and consequences of the injury (Conway et al., 2020). Lack of injury reporting as attributed to a fear of loss of athletic standing, interpersonal pressures, and downplaying of symptom severity (Conway et al., 2020). These results suggest that even with robust concussion education and knowledge translation, athletes will continue to hide symptoms of SRC, especially in elite high school and college aged athletes attempting to climb the professional ranks of their sport. Considering the majority of recognized and unrecognized SRC's occur in youth and young adults, identifying prevention strategies and objective measures for diagnosis or prognosis are of top priority (Daneshvar et al., 2011) .

2.2 Current Standards for Sport-related Concussion Diagnosis and Prognosis

The importance of unrecognized and unreported cases of SRC is highlighted by the current notion that SRC is one of the most complex injuries to diagnose, assess, and manage in

sports medicine (McCrory et al., 2017). Due to injury heterogeneity and lack of a true understanding regarding complexities of the underlying pathophysiology, there are currently no perfect tests or markers for SRC diagnosis or prognosis, restricting clinicians to utilize subjective assessment and self-report of cognitive functioning symptoms (McCrory et al., 2017). Nonetheless, the International Consensus on Concussion in Sport through the efforts of the Concussion in Sport Group (CISG) have outlined the current standard of practice for management of the injury beginning with sideline assessment immediately following identification of a suspected concussion (McCrory et al., 2017).

Identifying suspected SRC relies on the known signs and symptoms in the physical (e.g., headache, nausea, dizziness), behavioral (e.g., irritability, sadness, emotional lability), cognitive (e.g., difficulty concentrating, difficulty remembering, confusion), and sleep (e.g., sleeping more/less than usual, trouble falling asleep, drowsiness) domains (Purcell, 2014). Usually these signs and symptoms appear immediately following a significant impact anywhere on the body with force transmitted to the head in collision sport settings, but can also present in the hours to days following an a injury without significant impact (Purcell, 2014). This makes it extremely difficult to detect certain SRCs, adding to the unknown number of unreported SRCs. Though this subjective reliance in identifying SRCs may result in unidentified injuries, helpful resources exist for parents, coaches, and players to help assist in SRC identification. For example, the CISG's Concussion Recognition Tool (version 5; CRT5) outlines the signs and symptoms of a suspected SRC, in addition to steps for removal from play and subsequent medical attention for use by anyone in attendance of a sporting event (BJSM Publishing Group, 2017b). Once a suspected SRC has been identified, sideline testing by a trained professional (e.g., athletic therapist,

physiotherapist) is essential to the proper assessment and subsequent management of the injury (McCrory et al., 2017).

Sideline assessment following suspected SRC is comprised mainly of brief neuropsychological batteries for attention, concentration, and memory, and symptom inventories (Purcell, 2014). The Sport Concussion Assessment Tool version 5 (SCAT5) is currently the most rigorous and well-established tool for SRC sideline assessment by trained medical professionals (McCrory et al., 2017). It includes examination of observable signs/symptoms, memory assessment in the form of Maddocks Questions, GCS score, cervical spine assessment, post-concussion symptom inventory checklist, cognitive screening involving orientation, immediate memory, and concentration domains, a brief neurological screen, and a balance test utilizing the modified balance error scoring system (MBESS) (BJSM Publishing Group, 2017a). The SCAT5 is useful in differentiating concussed from non-concussed athletes immediately following SRC (within 3-5 days of injury), but diagnosis remains extremely difficult requiring both a significantly knowledgeable physician and cooperative patient (McCrory et al., 2017; Tator, 2013). Understandably, the difficulty in proper diagnosis of SRC holds true moving into the appropriate prognosis and treatment.

The innate heterogeneity of SRC makes it impossible to postulate a one-size-fits-all approach to treatment or prediction of injury sequelae. SRC can be considered a "snowflake injury", meaning like two snowflakes, no two SRC's are identical. Traditionally, the only known treatment for concussion was rest – both physical and cognitive – until symptoms spontaneously resolved (Tator, 2013). However, individuals with persistent post-concussion symptoms (defined as failure to recovery within the typical clinical recovery time frame; greater than 4 weeks for youth, and greater than 2 weeks for adults) would not experience a spontaneous resolution of

symptoms and be stuck in a perpetual “rested” state thought to exacerbate their symptoms in a vicious cycle of non-recovery (Leddy et al., 2018; McCrory et al., 2017). Moving away from the “rest is best” dogma, symptom limited exercise is being researched and trialed with early signs of success for treatment of SRC (C. C. Giza et al., 2018; Leddy et al., 2019). Today, the CISG recommends a brief period of complete physical and cognitive rest following SRC for 24-48 hours, followed by a stepwise 6 stage return to play protocol involving symptom-limited gradual increases in cognitive and physical activity (McCrory et al., 2017). Additionally, the heterogenous nature of SRC requires individualized rehabilitation approaches targeting specific areas of impairment in the psychological, vestibular, and cervicogenic domains to minimize time to recovery (McCrory et al., 2017).

Like the difficult nature of SRC diagnosis and treatment, accurately predicting individual SRC injury sequalae remains a challenge. This is partially due to the heterogeneity of the injury but is also a product of a reliance on subjective measures. The absence of objective markers creates a distinction between clinical recovery (currently subjective) and physiological recovery (objective) of SRC. The only currently available predictors of recovery are pre-injury risk factors in youth such as a history of concussions and sex, among others (Zemek et al., 2016). Current research has yet to establish a physiological time window for SRC recovery with these pre-injury factors alone, but evidence suggests physiological recovery may extend beyond clinical recovery assessed using subjective methods (Kamins et al., 2017; McCrory et al., 2017). This relative void in the ability to predict SRC sequalae may be filled by investigating and implementing objective measures of the injury, such as blood biomarkers.

2.3 Blood Biomarkers

A biomarker is a measurable indicator of a biological state, particularly as it pertains to the contraction, presence, or stage of disease (Rifai et al., 2006). Specifically, fluid biomarkers are measurable molecules indicative of a biological state contained within human fluids, such as, but not limited to, cerebrospinal fluid (CSF), blood, saliva, urine, and tears (Rifai et al., 2006). Fluid biomarkers of cellular damage or metabolic dysfunction present a unique snapshot of the human physiological state that allows objective identification and understanding of the pathophysiological consequences of various human diseases and injuries.

CSF and blood biomarkers have their own unique advantages and disadvantages in the context of identifying and predicting brain disease and injury states. CSF freely communicates with the brain interstitial fluid that surrounds the brain parenchyma, so biochemical changes in the brain are often adequately represented in the CSF (Rifai et al., 2006). This direct reflection of brain metabolites allows the most robust inference into the brain state for disease identification or prediction. For example, various neurodegenerative CSF biomarkers, like tau and neurofilament light chain (NFL), have consistently shown strong associations with Alzheimer's disease diagnosis and the associated cognitive impairment outcomes, suggestive of their role in the pathophysiological consequences of the disease (Blennow & Zetterberg, 2018; Olsson et al., 2016). However, access to human CSF requires lumbar puncture which is an invasive sampling technique that has been shown to produce transient headaches in up to 20% of patients (Duits et al., 2016). Considering headache is the most common symptom of concussion, routine lumbar puncture may not be the best fluid sampling technique in concussed individuals as it may exacerbate present symptoms potentially leading to a prolonged recovery (McCrory et al., 2017).

In contrast, the circulatory system carrying blood throughout the body remains separated from the brain by the blood brain barrier (BBB) and relies on the multiple mechanisms for clearance of brain metabolites into the bloodstream (Hladky & Barrand, 2019). These mechanisms include transport across the BBB, perivascular efflux, or transport via the glymphatic system (Hladky & Barrand, 2019; Iliff et al., 2012; Jessen et al., 2015). Glymphatic clearance appears to be modulated by subarachnoid CSF entering and exiting the brain via paravascular space, clearing the brain parenchyma of extracellular waste and metabolites through lymphatic drainage (Iliff et al., 2012). In contrast, perivascular efflux is thought to contribute to the clearance of smaller, water-soluble metabolites along blood vessel walls not part of the vascular lumen (Hladky & Barrand, 2019). Though these two mechanisms are well known brain waste clearance pathways, elimination of metabolites from the brain parenchyma primarily occurs through the highly restrictive BBB (Kadry et al., 2020). As these metabolites pass from the network of neurons and supporting cells comprising the brain, through the CSF and BBB into the blood, detectable concentrations in blood are diluted by both the blood:CSF volume ratio and the restricting passage nature of the BBB (Janigro et al., 2021; Zetterberg & Blennow, 2016). Accordingly, the concentrations of biomarkers in blood are not as robust a reflection of the brain state for disease identification or prediction compared to CSF. For example, typical total tau (t-tau) concentrations in CSF of those 21 to 50 years old are less than 300 pg/mL, while serum t-tau values for healthy collegiate athletes typically ranges between 0.4 pg/mL to 4.4 pg/mL (Asken et al., 2018; Sjögren et al., 2001). This drastic dilution hinders the mechanistic link between the biomarker and its role in the physiological sequalae of injury, highlighting the need for ultra-sensitive detection methods and advanced understanding the physiological mechanisms related to the presentation of these biomarkers in blood. Furthermore, blood contains more proteolytic

activity than CSF, adding a degree of uncertainty surrounding the time course of detection post injury (Zetterberg & Blennow, 2016). In the context of biomarkers for diagnosis and prognosis, this has major implications with respect to how long after injury samples are taken. Presumably there is degradation of the potential biomarkers over time, limiting the time window to study potential associations between the concentration of the biomarker and the injury. Fortunately, blood biomarkers of axonal and glial damage appear to present in the acute hours to days following injury, allowing a feasible window of opportunity for research utilizing serial sampling aimed at understanding the temporal presentation of biomarker concentrations for diagnostic utilization (Gul et al., 2017). Finally, compared to CSF lumbar puncture, venous blood sampling is a less invasive procedure with some blood biomarkers showing promise in the detection of acute and delayed effects of various forms of TBI due its pathological disruption of the BBB (Zetterberg & Blennow, 2016).

2.4 Blood Brain Barrier

The BBB presents unique challenges to the detection of brain biomarkers in blood, as the strict regulatory barrier between the CNS and the peripheral circulatory system functions largely to control the interorgan transport of molecules between the CNS and the periphery (Abbott et al., 2010). The mechanistic action of the highly regulatory BBB is largely regulated by a layer of specialized endothelial cells held together by restrictive tight junctions, surrounded by a matrix of mural cells, astrocytes, pericytes, microglia, and oligodendrocytes that form the neurovascular unit (NVU) (Daneman & Prat, 2015). The restrictive nature of the BBB is essential to prevent toxins, pathogens, larger or polar molecules, and blood cells from passively entering the CNS, while maintaining the unique and tightly controlled chemical composition in a microenvironment specific to optimize neuronal activity (Sweeney et al., 2019). Although the restrictive BBB is

essential for basic physiological function, it presents obstacles to interventions of the brain state via peripheral modalities. Despite significant advances in knowledge of the BBB structure, medical and nano technologies, and drug development; many CNS disease states remain under-treated due to a lack of effective intravenous drugs able to cross the BBB (Y. Chen & Liu, 2012). Despite this challenge, the BBB allows for investigation into the relationships between peripheral marker concentrations of disease and the disease in question as BBB breakdown or dysfunction is often modulated by such disease (Chodobski et al., 2011). Alterations in BBB permeability, for example, can be a pathological consequence of hypoxic, ischemic, or inflammatory insults arising from TBI, Alzheimer's, or Multiple Sclerosis (Hawkins & Davis, 2005). In specific reference to TBI, it is likely that an influx of inflammatory cytokines and proinflammatory mediators into the traumatized brain parenchyma contributes to increased BBB permeability allowing greater concentrations CNS injury markers to be detected in blood (Chodobski et al., 2011). However, prior research has indicated exercise might affect BBB permeability, which has specific implications when investigating peripheral markers of SRC in the acute stage post-injury (Małkiewicz et al., 2019).

2.4.1 The effects of exercise on blood brain barrier.

The current understanding related to the role of exercise on BBB permeability is complex and multi-mechanistic, involving various metabolic pathways, hormones, and inflammatory responses (Małkiewicz et al., 2019). Although there are a multitude of potential exercise effects on BBB permeability, this section will focus on inflammation given its often overlooked role in the effects of SRC on the BBB (Giza et al., 2018).

Factors related to inflammation in those who engage in a sedentary lifestyle result from physical inactivity and obesity. Obesity is characterized by low-grade systemic inflammation,

which is frequently associated with oxidative stress, both of which are hypothesized to transiently increase BBB permeability (Hotamisligil, 2006; Houstis et al., 2006; Varatharaj & Galea, 2017). For example, elevated blood levels of pro-inflammatory cytokines like interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) have been found in obese patients with type 2 diabetes, but also in patients with other forms of brain disease (Gironès et al., 2004; Rajkovic et al., 2014). This suggests pro-inflammatory cytokines inducing a stress response in various conditions can potentially increase BBB permeability. Important components in this regard are reactive oxygen species (ROS), which can have devastating consequences to physiological functions of the brain at high levels (Cobley et al., 2018). It is hypothesized the increase in BBB permeability is a function of pro-inflammatory cytokines inducing a specific nitric oxide synthase (iNOS) to increase nitric oxide (NO) production at a 1000-fold greater rate than the typical Ca^{2+} dependent NO production essential for vasculature maintenance and neuronal communication (Lubos et al., 2008). This inflammation mediated systemic increase in NO is said to increase BBB permeability through mechanisms that are not fully understood, but is likely linked to the increased flux in ROS (Lubos et al., 2008).

Comparatively, regular bouts of moderate intensity continuous training (MICT) are thought to reinforce anti-oxidative capacity, reduce oxidative stress, and have anti-inflammatory properties, which implies MICT strengthens the BBB and may reduce its permeability (Calverley et al., 2020; Małkiewicz et al., 2019). One investigation found both moderate aerobic and resistance exercise training reduced the systemic inflammatory state of obese patients with type-2 diabetes as a function of reduced TNF-α levels, while another found that a 4-month regular and moderate intensity exercise training program reduced levels of IL-6 and TNF-α in elderly women, suggesting regular exercise reduces release of pro-inflammatory cytokines associated

with systemic inflammation (Abd El-Kader, 2011; Chupel et al., 2018). Beyond the anti-inflammatory effects of MICT, it is also found to improve endothelial function by increasing blood flow and improving bioavailability of metabolites, contributing to the strengthening effects of regular exercise on BBB integrity (Di Francescomarino et al., 2009). Given this ample evidence, it is likely that regular, moderate intensity physical exercise has protective effects on the BBB. However, it appears that these protective effects of exercise are entirely dependent on the length and intensity of the exertion period and intensity.

Effects of high intensity exercise on the BBB in the context of inflammation are much less clear than physical inactivity related obesity and regular bouts of moderate intensity exercise. Compared to low-intensity treadmill running, blood concentrations of s 100 calcium-binding protein B (s100B) and ROS (in the form of NO) have been found elevated in those who performed high-intensity treadmill running (Roh et al., 2017). As previously mentioned, a delicate balance of NO has important implications regarding vasculature function and neuronal communication (Lubos et al., 2008). However, increased ROS has been consistently linked to BBB disruptions, suggesting increases in NO might transiently increase BBB permeability (Michinaga & Koyama, 2019; Morris et al., 2011). Additionally, applicability of s100B as a marker for BBB permeability is heavily debated, with some evidence suggesting an increase in s100B is a reliable indicator of increasing BBB permeability, and some not; an argument magnified by the extra-cranial release of s100B (Blyth et al., 2009; Kleindienst et al., 2010; Koh & Lee, 2014). This muddies the link between high intensity exercise and BBB permeability specific to s100B. Considering the results of increased NO and s100B together, it is possible that high intensity exercise may increase blood brain barrier permeability (Koh & Lee, 2014). Moreover, it has been hypothesized that hyperosmolarity of extracellular fluid caused by loss of

hypotonic sweat during prolonged periods of high intensity exercise, especially in warmer environments, causes water to move from the CNS to the periphery, resulting in a shrinkage of endothelial cells lining the BBB contributing to the increase in permeability (Watson et al., 2006).

In contrast, high intensity interval exercise (HIIT) has been found to potentiate systemic cerebrovascular adaptation and neuroprotection, though much more research regarding mechanisms and specifics of HIIT program is required (Calverley et al., 2020). Nonetheless, the potential for high intensity exercise to acutely disrupt the BBB has major implications regarding CNS derived blood biomarkers applications for SRC. If the BBB is indeed disrupted by high intensity exercise due to one (or a combination of) the potential mechanisms discussed above, it is possible that some of the most promising biomarkers for TBI including glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase L1 (UCH-L1), tau, and NFL might be elevated in the blood as a result of exercise during sport and not the injury itself. Further knowledge of this relationship is required before potential diagnostic or prognostic assessments of SRC can include objective blood biomarkers , especially in the acute stages of injury.

2.4.2 The effects of sleep on blood brain barrier.

Adding to the complexity of potential differential effects on cerebrovascular hemodynamics by varying forms of exercise, PA, and sleep duration and quality likely modulate such effects of a designated exercise session. Sleep and the BBB are heavily intertwined; the BBB actively participates in the modulation of sleep-wake activities, and sleep-wake disturbances can have negative consequences on global BBB functioning (Pan & Kastin, 2017). The key to understanding the significant role of the BBB modulating sleep-wake rhythm lies within the notion that endogenous oscillators in both the CNS and periphery work in unison to establish a

biological circadian rhythm in the face of external stimuli (Pan & Kastin, 2017). Various hormonal, inflammatory cytokine, and amino acid CSF:serum profiles have been associated with specific circadian rhythm timepoints, suggesting the BBB allows peripheral circadian regulation factors to reach their CNS targets in a highly regulated manner (Pan & Kastin, 2017). In a cyclical relationship, it appears sleep duration and quality may also modulate the regulatory mechanisms of the BBB specific to sleep-wake rhythms (Pan & Kastin, 2017). For example, BBB research in mice models has elicited results suggestive of increased BBB permeability due to chronic sleep restriction and sleep fragmentation (He et al., 2014; Pan & Kastin, 2017). As mechanistic BBB investigations are mainly undertaken in animal models due to its complicated nature, more investigation into the interplay of BBB and sleep-wake rhythms in humans is required before links can be established. Fortunately, a potential marker of BBB permeability disruption has been investigated in humans (matrix metalloproteinase – 7: MMP7), though its utility is still to be determined (Nichols et al., 2021). The potential increase in BBB permeability due to sleep restriction or fragmentation might have significant implications in the presentation of CNS derived biomarkers in blood and cannot be ignored.

Accelerometer-based activity monitors have been implemented in research to quantify daily physical activity and sleep, allowing analysis into the effects of PA and sleep on outcomes (John & Freedson, 2012). With recent advancements in technology, ActiGraphs are reasonably accurate and reliable activity trackers (John & Freedson, 2012). Due to the varying effects of different exercise and sleep on the BBB, ActiGraphs were utilized as part of this thesis to quantify physical activity and sleep surrounding each exercise condition. This is an essential part of this investigation as pre- or post-condition exercise and sleep likely play a role on the presentation of potential SRC biomarkers, implied by the discussion above.

2.5 Blood Biomarkers Under Study

All blood biomarkers under investigation in this thesis play structural and functional roles within neurons and supporting cells of the CNS. These include glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase L1 (UCH-L1), total tau, and neurofilament light chain (NFL) (Figure 2.1). These four markers are commonly seen in the literature within the context of investigating objective methods for concussion diagnosis or prognosis and are the focus concerning exercise effects on CNS derived blood biomarkers in this thesis (Zetterberg & Blennow, 2016).

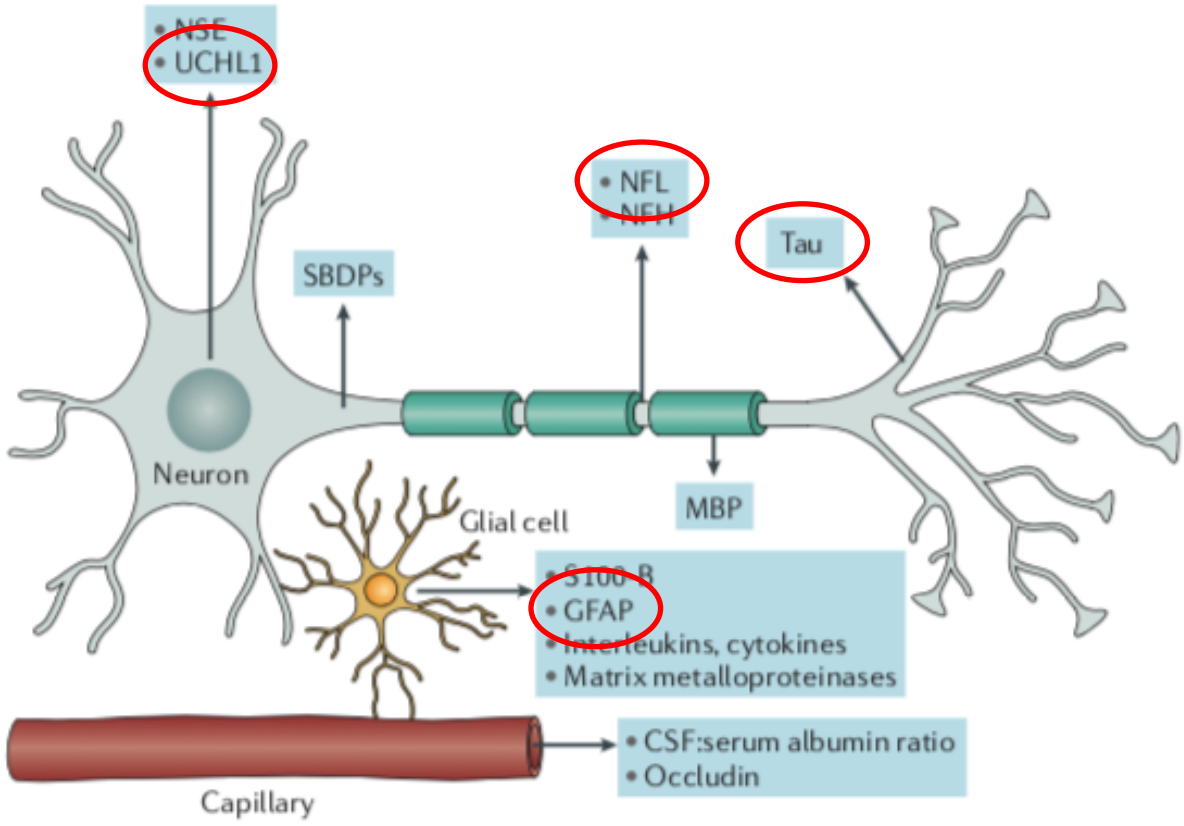

Figure 2.1. Neuronal schematic of SRC biomarkers

Commonly researched neuronal and glial biomarkers for SRC. UCH-L1 is a marker of neuronal injury, GFAP is a marker of gliosis, NFL and Tau are markers of axonal injury. Adapted by permission from Springer Nature: Nature Reviews Neurology (Fluid biomarkers for mild traumatic brain injury and related conditions, Zetterberg & Blennow), copyright (2016) (Appendix A).

2.5.1 GFAP.

Glial fibrillary acidic protein (GFAP) is the principal protein component of intermediate filaments found withing mature astrocytes of the CNS (Eng et al., 2000; Zetterberg & Blennow, 2016). It is hypothesized damage/destruction of neurons in the CNS due to injury or disease causes rapid production of astrocytes near the site of damage, termed reactive astrogliosis, which is characterized by rapid synthesis of GFAP and intermediate filaments (McMahon et al., 2015). As a function of astrocytes lining the abluminal wall of cerebral vessels, some astrocytes may release GFAP into circulation upon BBB damage (Hermann & ElAli, 2012). Accordingly, GFAP is a well-established marker of brain disease, injury, or gliosis, and presents in increased blood concentrations due to various neurological diseases and trauma (Olsson et al., 2011). Studies examining GFAP after severe TBI have found increasing levels in CSF is associated with increased mortality, while other studies have suggested elevated serum GFAP is observed across the entire TBI spectrum with the ability to predict positive CT scans in concert with clinical information (Czeiter et al., 2012; McMahon et al., 2015; Welch et al., 2016). Recently, GFAP was FDA approved along with UCH-L1 to help identify the necessity of a CT scan for adult mTBI patients in the ED who might have intracranial lesions (Bazarian et al., 2018). Importantly, elevated GFAP has been detected within 1 hour of TBI, reaching peak levels within the first 24-48 hours post injury (Gul et al., 2017; McCrea et al., 2020; Papa et al., 2016). Moreover, a study examining multiple blood biomarkers in National Collegiate Athletic Association (NCAA) athletes found an area under the curve (AUC) of 0.71 (95% CI, 0.64-0.78) for the ability of GFAP and UCH-L1 to differentiate concussed athletes from contact sport controls (McCrea et al., 2020). This gives merit to the potential abilities of GFAP to be utilized in concert with other

biomarkers for sideline or ED assessment within the hours immediately following SRC, though the literature is inconsistent concerning acute elevations after SRC. (Meier et al., 2017).

To date, a single study has examined the effects of high intensity interval exercise (HIIT) on blood biomarkers associated with brain injury, and the authors were unable to statistically evaluate GFAP due to a significant number samples reading below the limit of detection (GFAP concentrations greater than 9.38 pg/mL in less than 20% of sample) (Di Battista, Moes, et al., 2018; Quanterix, 2022). As the majority of blood GFAP is thought to originate from the CNS, high intensity exercise should elicit increased GFAP in the blood only if the BBB is disrupted; a theory strengthened by findings of exercise-induced increases in neurotrophic factors promoting GFAP expression and astrogliosis in the CNS (Krityakiarana et al., 2010). Clearly, the current state of research into GFAP as an objective marker of SRC is comprehensive, but little knowledge exists of how exercise affects the presentation of GFAP in blood of healthy individuals which is a necessary step to disentangle the complex relationship between exercise, SRC, and GFAP.

2.5.2 UCH-L1.

Ubiquitin C-terminal hydrolase L-1 (UCH-L1) is a cytoplasmic protein abundantly found in neurons comprising both the CNS and the peripheral nervous system (PNS) (Chen et al., 2010; Zetterberg & Blennow, 2016). In injured or diseased states of the brain, increased UCH-L1 expression functions to remove excessive, misfolded, or oxidized proteins (Mondello et al., 2012). Increased expression of UCH-L1 has also been linked to the pathogenesis of various neurodegenerative diseases like Alzheimer's and Parkinson's disease (Setsuie & Wada, 2007). Despite its apparent lack of specificity to the CNS, UCH-L1 is another well-established candidate biomarker for various forms of TBI (Zetterberg & Blennow, 2016). Multiple studies

examining severe TBI have observed elevated blood and CSF concentrations of UCH-L1 after trauma, while investigation into concussion have also observed elevated serum levels of UCH-L1 in patients with concussion compared to non-head related trauma controls (Brophy et al., 2011; Diaz-Arrastia et al., 2013; Papa et al., 2012). Because of these promising and relatively consistent findings, serum UCH-L1 was FDA approved along with GFAP to help identify the necessity of a CT scan for adult concussion patients who might have intracranial lesions (Bazarian et al., 2018).

Although the progression of UCH-L1 research in TBI is reasonably promising for the potential diagnostic or prognostic utilization of UCH-L1 for SRC, the effects of exercise alone on biomarker presentation should not be ignored. In fact, studies evaluating UCH-L1 as a biomarker for SRC have mixed results, with some studies showing an increase in UCH-L1 compared to non-injured and non-contact athlete controls in the early acute phase post-injury, and some showing no significant changes in UCH-L1 (Asken et al., 2018; Meier et al., 2020; Shahim et al., 2020). It is likely that these mixed results are mainly a function of varying methodologies and immunoassay techniques to detect the biomarker, but it is also possible levels of UCH-L1 may be affected by both the exercise itself and the SRC. UCH-L1 has also been found to play a significant role in the maintenance, structure, and function of the neuromuscular junction (NMJ), which is known to be highly adaptable and plastic in response to various exercise training (F. Chen et al., 2010; Deschenes, 2019). Though the specific relationship between exercise effects on UCH-L1 in the NMJ is not completely known, it stands to reason presentation of UCH-L1 in the blood might be influenced by exercise. As there are currently zero studies in the broader literature directly examining this relationship, it is a vital gap to address before an objective modality utilizing UCH-L1 can be implemented for SRC.

2.5.3 Tau.

Tau is a microtubule-associated protein that is highly expressed in thin unmyelinated axons of the cerebral cortex within the CNS and found in neurons of the PNS (Trojanowski et al., 1989; Zetterberg & Blennow, 2016). It functions mainly to regulate and organize the complicated cytoskeletal network within axons, modulating signal pathways associated with axon extension or shortening (Shahani & Brandt, 2002). Though phosphorylation of tau is a normal process, hyperphosphorylation appears to be the mechanism linking tau protein to various tauopathies associated with neurofibrillary tangles, which are staples of many neurogenerative diseases like Alzheimer's and other dementias (Shahani & Brandt, 2002). Multiple forms of tau biomarkers can be measured in the blood or CSF including total tau (t-tau) and phosphorylated tau (p-tau) (Rubenstein et al., 2017). As the name suggests, t-tau assays do not discriminate between various phosphorylated forms of tau and unphosphorylated tau, or differentially spliced isoforms of tau, giving a measurement of all tau forms, while p-tau assays measure various forms of phosphorylated tau specific to the epitope in question (Zetterberg & Blennow, 2016). Due to the role of tau in pathology of many neurogenerative diseases, various forms of tau have been extensively studied as potential biomarkers across the severities of TBI (Zetterberg & Blennow, 2016). Numerous investigations into severe TBI have shown that various forms of tau are increased after injury compared to controls, and this increase is associated with poorer outcomes (Ost et al., 2006; Zemlan et al., 2002). Similarly, investigations of tau as a potential biomarker of concussion have shown increases compared to controls (Bulut et al., 2006; Olivera et al., 2015). However, the literature concerning tau in the SRC context is mixed, with some studies showing increasing tau differentiating SRC from controls and predicting symptoms and recovery, to no significant differences in tau concentrations between

injury groups or relation to clinical outcomes (Di Battista, Rhind, et al., 2018; Gill et al., 2017; Shahim et al., 2018). These conflicting results are predominantly affected by differing methodologies for tau analysis, such as fluid type (CSF vs. serum vs. plasma), form of tau, and assay type, but are also potentially confounded by the role of exercise on tau presentation.

Unlike the previous biomarkers discussed, there has been some investigation into the role of exercise in healthy individuals on t-tau concentrations. One study found significant elevations in plasma t-tau 1 hour after a *"friendly game of hockey"* compared to pre-game, which normalized after 12 hours (Shahim et al., 2018). These results suggest acute increases in tau may be directly influenced by exercise; though a *"friendly game of hockey"* is hardly controlled exercise and allows for additional confounders, such as potential repeated concussive impacts during gameplay, to muddy this interpretation. Nonetheless, there lies significant implications in terms of the complicated relationship between t-tau presentation, SRC, and exercise. Another investigation found an increase in plasma t-tau pre- to post- HIIT session, which attenuated after 2 weeks of performing the HIIT condition 3 times per week (Di Battista et al., 2018). Importantly, these results replicate the notion that acute increases in t-tau might be influenced by a single bout of exercise. The conflicting results regarding t-tau as a potential biomarker for SRC, while considering the potential for exercise alone to influence the presentation of this biomarker, reveals the complex nature of t-tau as a potential objective marker for SRC. Further understanding how t-tau in blood is influenced by exercise alone in healthy individuals is a step in the right direction towards untangling these complicated associations, with the ultimate goal of determining if t-tau could be a suitable objective marker for sideline assessment of SRC.

2.5.4 NFL.

Neurofilament light chain (NFL) is a structural intermediate filament protein of the neuronal cytoplasmic cytoskeleton, functioning in radial axon growth, dendritic arborization, and neural plasticity, and in contrast with tau is highly expressed in the large, myelinated axons of both the CNS and PNS (Friede & Samorajski, 1970; Zetterberg & Blennow, 2016). In the CNS, hyperphosphorylated neurofilaments like NFL associate with hyperphosphorylated tau to form the neurofibrillary tangles that characterize many neurodegenerative diseases (Lépinoux-Chambaud & Eyer, 2013). In the PNS, hyperphosphorylated NFL appears to play a role in the progressive neurodegeneration of motor neurons and loss of related muscle movements seen in patients with amyotrophic lateral sclerosis (Lépinoux-Chambaud & Eyer, 2013). As an established marker of various neurodegenerative diseases, NFL is a well-studied biomarker for various TBI's. In severe TBI, both CSF and blood concentrations of NFL have shown acute elevations compared to controls, with predictive ability of worse 12-month clinical outcomes (Shahim, Gren, et al., 2016; Zetterberg et al., 2006). A similar increase in NFL acutely following SRC has been seen, along with mixed results regarding correlation with clinical outcomes (Guedes et al., 2020; Nimer et al., 2015). Like the other biomarkers, the association between NFL and SRC is unclear. One study shows no changes in serum NFL after SRC compared to controls, another shows biphasic spikes in serum NFL 1 hour and 10 days post SRC, and another showed serum NFL was increased 1 hour and 1 month after performing 40 soccer headers in 20 minutes compared to controls (Shahim, Tegner, et al., 2016; Wallace, Smirl, et al., 2018; Wallace, Zetterberg, et al., 2018).

Beyond contributions from long motor neurons of the PNS discussed later, it is likely that high intensity exercise may also play a role in NFL presentation in the blood, as NFL is

constantly released from axons in low levels under normal conditions (Gaetani et al., 2019). A single study to date has examined this specifically; the same investigation that found acute elevations in tau after a *"friendly game of hockey",* found no changes in NFL pre to post game (Shahim et al., 2018). Considering the potential increase in BBB permeability due to acute high intensity exercise, it stands to reason small spikes in serum NFL might be seen after a high intensity exercise bout. As this is the only known study analyzing NFL after exercise in healthy individuals, more investigation into the effects of a controlled exercise bout is required before an objective modality utilizing NFL for SRC can be validated.

2.6 Serum vs. Plasma Matrix Considerations

When considering blood biomarkers as objective tools for examination of the physiological state, it is important to consider what component of whole blood said biomarker is expected to be maintained within. The two most commonly used matrices to study blood biomarkers include serum and plasma (Yu et al., 2011). Serum is obtained from the liquid portion of centrifugated blood after coagulation, resulting in a medium excluding blood cells and the related coagulation factors (Yu et al., 2011). Plasma is obtained from the liquid portion of centrifugated blood treated with an anticoagulant, like EDTA, resulting in a medium excluding only blood cells (Yu et al., 2011). As such, potential blood biomarkers directly or indirectly involved in coagulation processes or blood cell structure may have differential presentations between serum and plasma, though it is a generally held view that most metabolites are highly correlated between matrices (Yu et al., 2011).

In specific context of the biomarkers under examination of this thesis, two studies were found analyzing differences in blood concentrations between matrices as the primary outcome (Huebschmann et al., 2020; O'Connell et al., 2019). Results from these investigations suggest

measured NFL levels to be highly equivalent between matrices, but measured t-tau levels to be consistently lower in serum relative to plasma, and measured GFAP levels to be highly correlated between serum and plasma (Huebschmann et al., 2020; O'Connell et al., 2019). With serum historically being viewed as the gold standard for blood biomarker analysis, UCH-L1 and NFL have mainly been analyzed in serum, while results for t-tau and GFAP have been reported in both serum and plasma (Plebani et al., 2020; Zetterberg & Blennow, 2016). To my knowledge, this study will be the first to analyze correlation in blood biomarker concentrations between matrices following exercise in serially collected samples, allowing correlational analysis at the individual level. This will allow future investigations to target specific matrices for specific biomarkers ultimately reducing the great costs associated with collecting and assaying multiple matrices.

2.7 SIMOA

Advancements in immunoassay technology have greatly improved biomarker detection sensitivity, allowing detection of biomarkers at significantly lower levels than in the past (Wilson et al., 2016). This biomarker detection at low levels may serve to establish clinically relevant indicators of injury or disease. Specifically, newer single-molecule array technology (SIMOA) allows detection at the femtogram level, which is approximately 900x more sensitive than conventional enzyme-linked immunosorbent assay (ELISA) technology (Kuhle et al., 2016). Briefly, this digital technology works to restrict diffusion of florescent product molecules of the substrate-enzyme reaction by confining individual labelled immunocomplexes with the substrate to femtolitre-sized wells, allowing thousands of single-molecule signals within the array to be counted simultaneously (Wilson et al., 2016). Additionally, SIMOA allows for multiplexing, which enables detection of up to ten biomarkers in a single assay (Wilson et al., 2016).

The ability of SIMOA to utilize multiplex assays confers several advantages and disadvantages over singleplex assays. A main advantage lies within the greatly reduced costs of assaying for multiple biomarkers in one machine run. This is a result of requiring significantly less volumes for analyte detection when a single run can be performed to determine multiple analyte concentrations opposed to one run per analyte, as well as the reduced time and resources associated with one machine run. However, multiplex assays require multiple reagents to target each biomarker, so interactions between reagents combined in the multiplex may result in optical cross talk – defined as a signal from one well optically scattering into neighboring wells, potentially resulting in incorrect classification of an active bead or falsely elevated average enzyme bound outputs (Rissin et al., 2013). This effect is mitigated in singleplex assays that include only one reagent. Fortunately, SIMOA technology utilizes a computational method for active image processing correction to reduce the impact of optical cross talk in multiplex assays, and has been found to preserve sensitivity of measurements achieved using singleplex assays (Rissin et al., 2013).

Though SIMOA has greatly improved sensitivity on a fully automated platform with multiplexing capabilities, there remain some assay limitations. Antibody specificity remains a vital consideration when determining biomarker concentrations, especially if the biomarker is to be utilized in clinical practice (Drucker & Krapfenbauer, 2013). Antibody specificity can be reduced by the three dimensional conformation of the antigen, use of monoclonal or polyclonal antibodies, and poor correlation between synthetic antibody lots, leading to biased biomarker concentration estimates (Bordeaux et al., 2010). Additionally, assay interference via endogenous or exogenous substances and test variability can contribute to biased concentration estimates (Dahlin et al., 2004; Kramer et al., 2016). Unfortunately, these limitations will likely exist

beyond the ongoing technological advancements in assay techniques due to the highly complex and variable properties accompanying physiological metabolite detection. However, the progression towards more sensitive assays (such as SIMOA) allows researchers to examine associations between much smaller biomarker concentrations and various outcomes than possible before, progressing our understanding of objective markers of disease and injury.

2.8 Limitations of Current Literature and Future Directions

Beyond the limitation in SRC biomarker research that this project is seeking to address – a generally absent understanding of how exercise alone affects the presentation of these blood biomarkers; SRC biomarker research is plagued with another major limitation: a largely unaddressed or ignored lack of biomarker specificity to the CNS. In reference to the biomarkers under investigation in this thesis, this is most prominent in the cases of UCH-L1, Tau, and NFL (F. Chen et al., 2010; Gu et al., 1996; Lépinoux-Chambaud & Eyer, 2013; Morris et al., 2011; Takami et al., 2007). Gene expression of UCH-L1 is highest in brain tissue, but also appears biased in adrenal, ovary, and testis tissues (Figure 2.2) (NCBI, 2022d). Owing to this extracranial gene expression, prominent peripheral sources of UCH-L1 include neurons of the PNS, where it is thought to be highly responsible for function of the NMJ, and endothelial and smooth muscle cells, where it is shown to partially attenuate vasculature remodeling (F. Chen et al., 2010; Takami et al., 2007). As both vasculature remodeling and the neuromuscular junction can be affected by exercise and repeated non-head trauma sustained in a variety of sports, careful consideration of theses complex relationships in the context of SRC must be considered when assessing the diagnostic or prognostic applicability of UCH-L1 for SRC. Tau is an especially multifaceted biomarker as it pertains to peripheral sources. It has been found to be a master regulator of various signalling pathways and organelle organization within neurons of the PNS

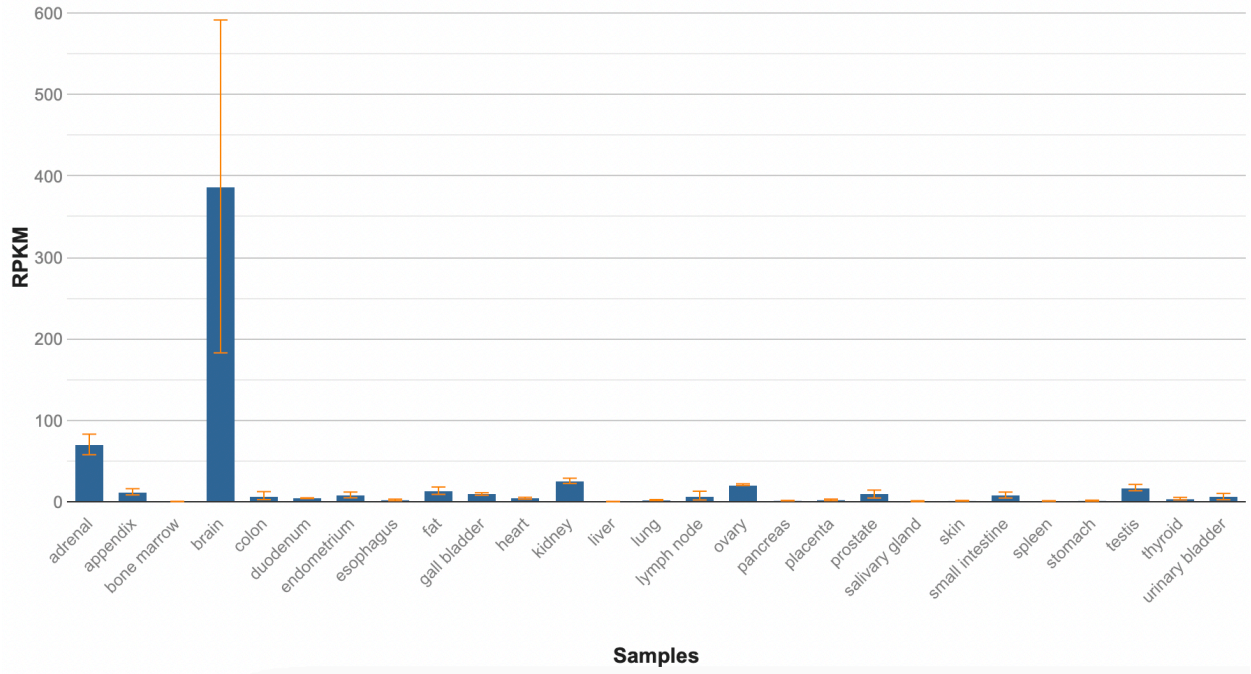

Figure 2.2. UCH-L1 tissue specific gene expression

Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) gene expression from various tissues in the body. Note the main source for UCH-L1 is the brain, however there are peripheral sources that can result in expression of this gene which include the adrenal glands, kidneys, adipose tissue, ovaries (in females) and testis (in males). Retrieved from National Library of Medicine (NLM) at https://www.ncbi.nlm.nih.gov/gene/7345 (NCBI, 2022d).

and is expressed at relatively high levels in the heart, kidney, testis, adrenal gland, stomach, and fat tissues (Figure 2.3) (Morris et al., 2011; NCBI, 2022b). The extensive extracranial sources of tau cannot be ignored when examining its clinical utility as a biomarker for SRC, especially considering the potential role of exercise on t-tau presentation. Similar to tau, NFL plays a significant role in neurons of the PNS where it modulates cytoplasmic skeleton organization of long motor neurons, but extracranial gene expression appears limited to adrenal, esophagus, and kidney tissues (Figure 2.4) (Lépinoux-Chambaud & Eyer, 2013; NCBI, 2022c). GFAP appears to be the most CNS specific biomarker owing to its exclusive gene expression in astrocytes (Figure 2.5), though extra-cranial protein expression has been seen in male and female specific tissues (Figure 2.6) (NCBI, 2022a; The Human Protein Atlas, 2022). Although these peripheral sources

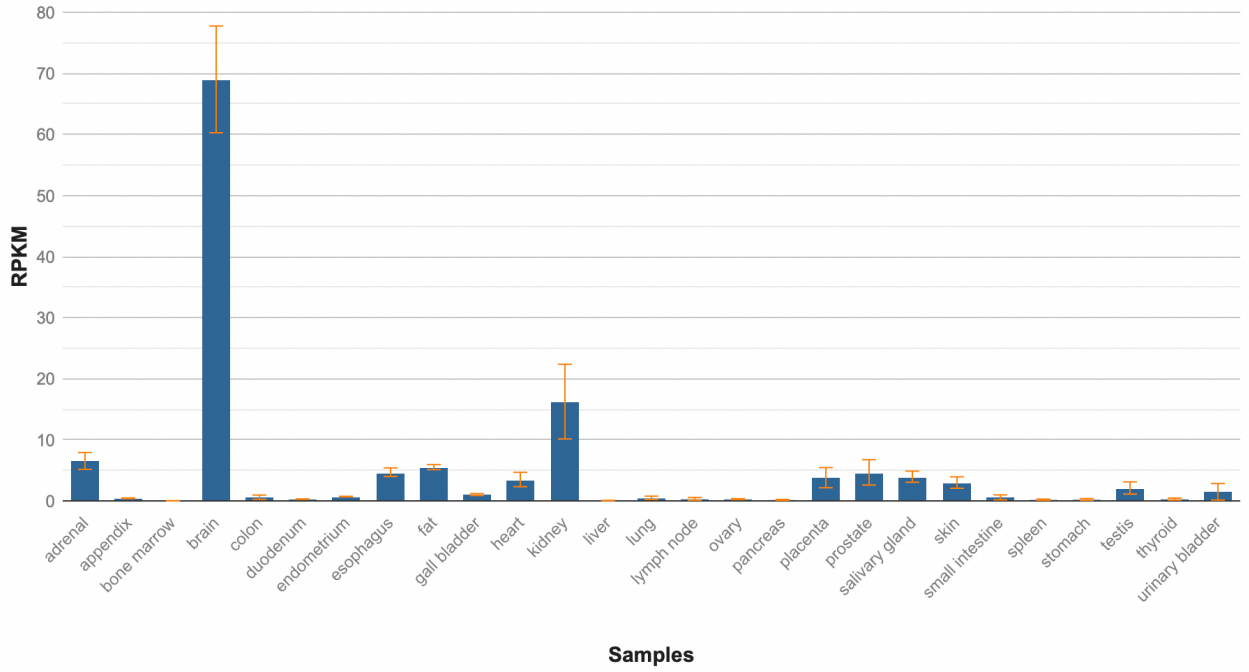

Figure 2.3. Tau tissue specific gene expression

Tau gene expression from various tissues in the body. Note the main source for tau is the brain, however there are peripheral sources that can result in expression of this gene which include the adrenal glands, kidneys, esophagus, adipose tissue, heart, placenta (in females), prostate and testis (in males), saliva, skin, and bladder. Retrieved from National Library of Medicine (NLM) at https://www.ncbi.nlm.nih.gov/gene/4137 (NCBI, 2022b).

of commonly studied TBI biomarkers cannot be ignored, the repeated findings of increased biomarkers distinguishing various severities of TBI from controls and potential predictive ability of post injury outcomes is promising. However, further research is necessary to disentangle these complicated relationships between peripheral sources, exercise, and the injury, especially as it pertains to investigating potential objective diagnostic or prognostic modalities for clinical or sideline assessment of SRC. Beyond the two major limitations already discussed, other limitations in SRC biomarker literature include inconsistent assay methodologies and blood sampling and processing techniques, small sample sizes with a lack of control groups, insufficient female representation, and a consistent lack of control for diet and sleep. It is important that future research considers these factors when working to establish a clear physiological link between the injury and the biomarker in both males and females, so large-

scale studies with standardized procedures can be implemented to investigate the utility of the biomarkers in the context of SRC diagnosis and prognosis.

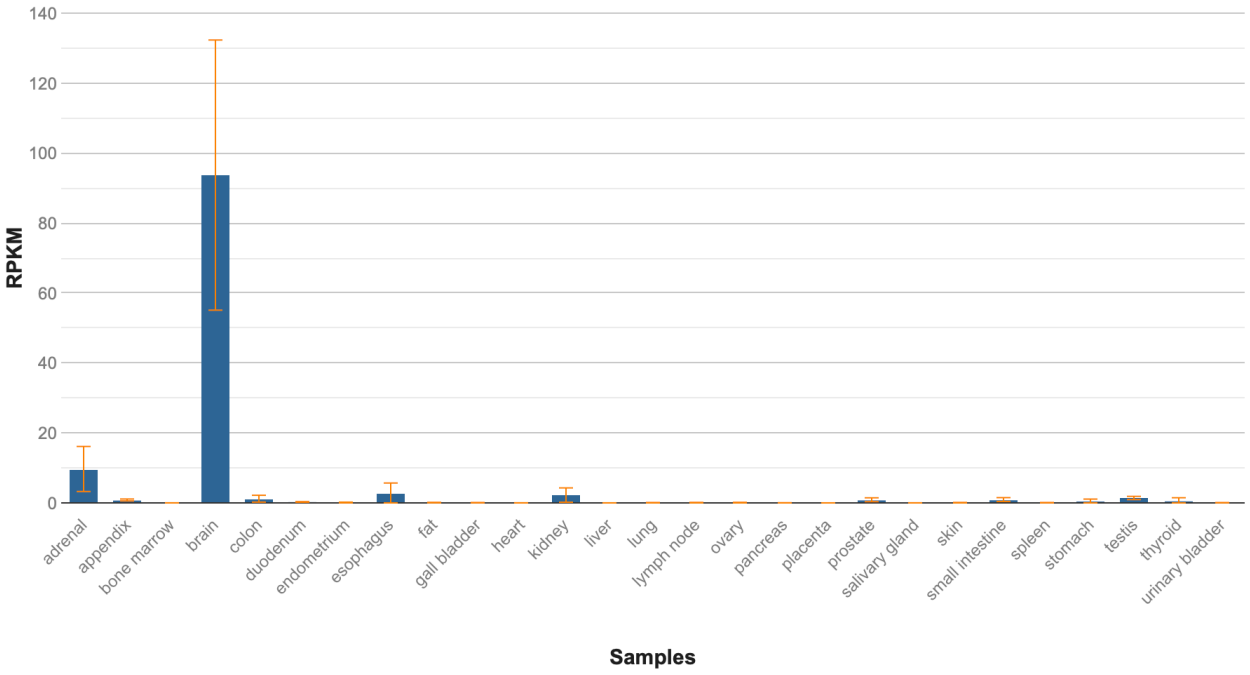

Figure 2.4. NFL tissue specific gene expression

Neurofilament light chain (NFL) gene expression from various tissues in the. Note the main source for NF-L is the brain, however there are peripheral sources that can result in expression of this gene which include the adrenal glands, kidneys, and esophagus. Retrieved from National Library of Medicine (NLM) at https://www.ncbi.nlm.nih.gov/gene/4747 (NCBI, 2022c).

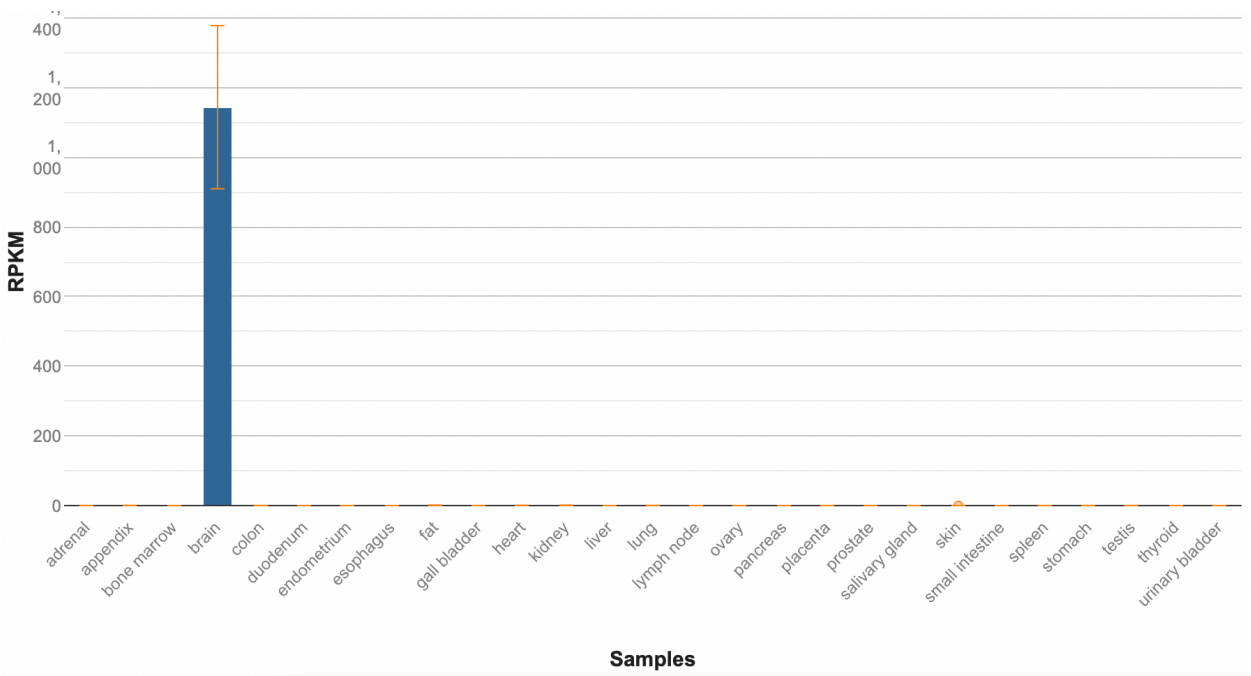

Figure 2.5. GFAP tissue specific gene expression

Glial fibrillary acidic protein (GFAP) gene expression. Note the main source for NFL gene expression is the brain. Retrieved from National Library of Medicine (NLM) at https://www.ncbi.nlm.nih.gov/gene/2670 (NCBI, 2022a).

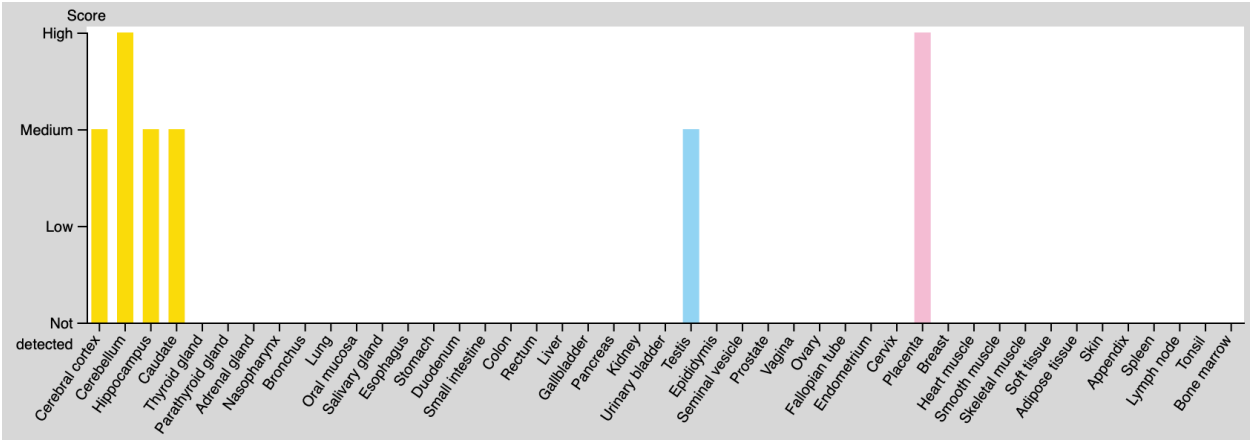

Figure 2.6. GFAP tissue specific protein expression

Glial fibrillary acidic protein (GFAP) protein expression. Note the main source for GFAP protein expression is the brain, but also in the testis (males) and placenta (females). Image credit: Human Protein Atlas (proteinatlas.org), available from https://www.proteinatlas.org/ENSG00000131095-GFAP/tissue Retrieved from (The Human Protein Atlas, 2022; Uhlén et al., 2015).

2.9 Conclusion

As exercise is inherent in sport, understanding the role exercise plays in the presentation of CNS derived blood biomarkers is vital before objective diagnostic or prognostic tests for SRC utilizing these markers can be developed. The highly controlled study design undertaken as part of this thesis and resulting findings will lay the groundwork for future investigation of these important associations, ultimately propelling the field forward in the hunt for objective markers to decrease the burden of SRC in all athletes.

Chapter 3: Effects of Interval Exercise on Commonly Studied Blood Biomarkers Associated with Sport-related Concussion

3.1 Introduction

Sport-related concussion (SRC) is a form of mild traumatic brain injury (mTBI) that results in a broad range of clinical signs and symptoms (e.g., headache, dizziness, balance impairments) (McCrory et al., 2017). SRC poses a significant public health burden as it is estimated that 1 in 450 Canadians report SRC or "other brain injury" as their most significant injury associated with disability in the previous year (Gordon & Kuhle, 2020). The estimated prevalence of SRC is likely an underestimation due to the propensity by athletes to hide concussion symptoms or refrain from seeking medical attention for fear of loss of athletic standing or interpersonal pressures (Conway et al., 2020). Furthermore, without a "gold standard" objective tool or measure to support the diagnosis of SRC, clinicians must rely heavily on subjective symptom reports and clinical measures which may further obscure SRC diagnosis (McCrea et al., 2017). More objective measures of brain injury, such as fluid biomarkers, have been extensively researched in recent years with the goal of becoming clinically useful in supplementing diagnostic and prognostic assessments and aiding the decision for targeted treatment strategies throughout recovery. Despite considerable progress in the field, this research remains in its infancy and both the analytical and clinical validation of fluid biomarkers of SRC requires further investigation.

Some of the leading blood biomarkers for SRC detection have functional or structural roles in neurons and supporting cells of the neurovascular unit. Tau is a microtubule-associated protein highly expressed in thin unmyelinated axons of the cerebral cortex. Tau functions to regulate and modulate signaling pathways within the complex axonal cytoskeletal network

(Shahani & Brandt, 2002; Trojanowski et al., 1989). Neurofilament-light chain (NFL) is a structural intermediate filament protein of the neuronal cytoplasmic cytoskeleton modulating neural plasticity, radial axon growth, and dendritic arborization (Friede & Samorajski, 1970). Glial fibrillary acidic protein (GFAP) is the principal protein component of intermediate filaments in astrocytes playing major roles in synaptic plasticity and reactive gliosis (Middeldorp & Hol, 2011). Ubiquitin c-terminal hydrolase L-1 (UCH-L1) is a cytoplasmic protein abundantly found in neurons and primary function is to remove excessive, misfolded, or oxidized proteins in injured or diseased brain states (Mondello et al., 2012). These four blood biomarkers have shown promising results regarding their ability to identify and predict severe TBI outcomes (Brophy et al., 2011; Czeiter et al., 2012; Ost et al., 2006; Shahim, Gren, et al., 2016). In the context of complicated mild TBI, defined as mild by clinical indicators with complications of positive abnormalities on neuroimaging techniques, GFAP and UCH-L1 were recently approved by the Food and Drug Administration (FDA) in the United States to identify whether a CT scan is warranted in adults with acute concussion at risk of intracranial lesions, demonstrating their potential as tools to supplement clinical decisions in complicated mTBI (Bazarian et al., 2018). However, results are mixed concerning the ability of these fluid biomarkers to accurately distinguish mTBI or sport-related concussion from various controls (non-contact controls, non-athlete controls, musculoskeletal injury controls, non-head trauma injury controls) (Di Battista, Rhind, et al., 2018; McCrea et al., 2020; Shahim et al., 2018, 2020). This is likely due to a combination of factors including inconsistent sampling, processing, and assay methodologies across studies (Zetterberg & Blennow, 2016). Additionally, biological factors pertaining to the effects of high intensity exercise and sleep may also influence the concentration of these biomarkers, neither of which have been adequately investigated to date.

The main route of passage for central nervous system (CNS) biomarkers to reach the circulatory system is through the highly regulatory and restrictive blood brain barrier (BBB) (Iliff et al., 2012). It has been postulated the BBB may be transiently affected by high intensity interval exercise, allowing CNS derived molecules to "leak" into the general circulation (Koh & Lee, 2014). It is also possible changes in arterial pulse characteristics due to exercise might induce CSF clearance, as arterial pulse rate has been shown to correlate with CSF flow in mice (Mestre et al., 2018; Wang et al., 2018). If this is true, the association between blood biomarkers and CNS injury would be confounded by the influence of exercise, creating potential complications to their utility in SRC management given the inherent nature of exercise in sport. Additionally, sleep and the endogenous circadian rhythm of the BBB regulate it's permeability, introducing another potential confound to concentration of CNS derived biomarkers in blood (Cuddapah et al., 2019). There are few studies specifically examining the influence of exercise on blood biomarkers of CNS injury, and none investigating the potential role of sleep in this relationship. One investigation examining brain injury biomarkers after a single bout of high intensity exercise found elevated concentrations of 10 of 12 biomarkers examined (including plasma total tau (t-tau)) (Di Battista, Moes, et al., 2018). A second study found increases in plasma t-tau, but not serum NFL from baseline after a *"friendly game of hockey"* (Shahim et al., 2018), however no quantification of exercise was performed. Both studies above demonstrated elevated biomarker concentrations after exercise, supporting the theory that acute permutations in BBB permeability may exist post-exercise. However, both investigations only had two sampling timepoints (pre- and post-condition) and failed to control for a variety of important factors, including sex, sleep, and history of concussion. Therefore, the purpose of this exploratory study was to investigate the effects of a single bout of moderate and high intensity

interval exercise on plasma t-tau, NFL, GFAP, and UCH-L1 serially in healthy adults without a history of diagnosed concussion, accounting for the potential influence of age, sex, and sleep.

3.2 Methods

3.2.1 Study design.

In a randomized crossover cohort design, participants completed two different intensity interval exercise conditions (moderate intensity interval training [MIIT], and high intensity interval training [HIIT]) and a control condition separated by approximately 28 days. A 28-day period between conditions was chosen to allow physical recovery, baseline stabilization of blood, and an attempt for female participants with regular menstrual cycles to complete all three conditions within the same phase of their cycle (Carmichael et al., 2021). Ten blood samples were taken serially for each condition (pre-condition, during condition, and 0, 1, 2 , 4 , 6 , 8, 24 , and 48 hours post-condition), with the pre-condition draw occurring between 07:45 and 08:15 on the morning of each condition day. Each participant completed all conditions and blood sampling in the Cerebrovascular Concussion Lab at the University of Calgary (UCalgary, Alberta, Canada). See Figure 3.1 for study design flowchart.

3.2.2 Participants.

Participants were recruited at UCalgary via word of mouth and recruitment posters displayed in the Faculty of Kinesiology. Exclusion criteria were any prior concussion diagnosis, metabolic disease, or daily smoking/vaping. Participants were included if deemed fit to participate in the peak power cycle ergometer protocol and subsequent interval intensity conditions by passing all general health questions on the Physical Activity Readiness Questionnaire (2021 PAR-Q+) (Appendix B) (Warburton et al., 2011), and engaging in exercise (including sport) three or more times per week. University students provided written informed

consent to participate in this study (Appendix C) under the University of Calgary's Conjoint Health and Research Ethics Board (REB21-0768). All participants were required to fill out a COVID-19 screening checklist (Appendix D) before attending the university on all study days, in-line with the University of Calgary COVID-19 guidelines during the time of data collection.

3.2.3 Procedures.

3.2.3.1 Peak power cycle ergometer protocol.

On the first study day, all participants completed the peak power cycle ergometer protocol on a Corival cpet bicycle ergometer (Lode BV, Groningen, Netherlands), fitted with a wireless Polar H10 heart rate monitor (Polar, Kempele, Finland). The peak power protocol was completed 3-5 days prior to the first condition day to pinpoint individualized power outputs for the MIIT and HIIT conditions. Two power-based ramp protocols were programmed on the ergometer's digital display, necessary to adjust for individual fitness levels while targeting completion of the peak protocol in the range of 8 to 12 minutes for all participants (Yoon et al., 2007). Both protocols included a 2-minute warmup at 50 watts for minimal resistance cycling. Following the 2-minute warmup, a ramp increase in watts (20 watts/min for protocol 1; 30 watts/min for protocol 2) was implemented and completed until failure by the participant to sustain cadence equal to or above 80 revolutions per minute or stopped by the study team. Participants were blinded to the purpose of the peak power protocol and instructed to focus on completing the test to volitional exhaustion. The protocol was chosen based on each participants height, weight, and self-reported physical fitness ability. One of ten participants completed protocol 2 (male; 25 years old), the other nine completed protocol 1.

3.2.3.2 Cycle ergometer interval conditions

Following completion of the peak power cycle ergometer protocol, participants completed three different 30-minute exercise intensity interval conditions separated by approximately 28 days in a randomized order: 1) *Control* (30-minutes seated); 2) *MIIT* (5 minute warmup at 50 watts, 10 x 1 minute intervals at 60% achieved peak power separated by 1 minute complete rest, 5 minute cooldown at 50 watts); and 3) *HIIT* (5 minute warmup at 50 watts, 10 x 1 minute intervals at 100% achieved peak power separated by 1 minute complete rest, finishing with a minimum 5 minute cooldown). Participants abstained from exercise, caffeine, and alcohol for at least 12 hours before each exercise condition. Participants were not instructed to sustain cadence within a specific range, only to focus on keeping their feet moving during intervals to maximize effort. To further isolate effects of exercise on biomarker presentation during condition days, participants were instructed to eat the same breakfast at home and were provided with 2 vanilla nutrition shakes in lab (Kirkland signature, Washington, United States) to consume between timepoints 2 and 4 hours-post condition, and water, and Gatorade (Gatorade, Illinois, United States) as needed to ensure adequate hydration.

3.2.3.3 Blood collection and processing

Two methods were used to collect plasma blood samples at all ten serial timepoints after completion of a pre blood draw questionnaire (Appendix E). For timepoints between pre-condition to 8 hours post-condition, a 22-guage IV catheter (Protectiv® winged, Smiths Medical, Minnesota, United States) was inserted and secured into a peripheral vein of the antecubital fossa of each participant by the phlebotomist on the morning of each condition day. A short (2') microclave IV connector was connected to the IV catheter for ease of access. At each blood sampling timepoint, a VAMP adult closed blood sampling system (Edwards Lifesciences,

California, United States) was attached to the microclave IV connector. Briefly, this sampling device allows direct closed access to the vein via a small (5ml) flexure mechanism and the peripheral IV catheter for filling vacutainer blood collection tubes without necessitating a clearing volume (Appendix F) (Edwards Lifesciences, 2022). Following collection into a 10 mL plasma vacuum tube (lavender top, ethylenediaminetetraacetic acid [K_2EDTA] additive), the VAMP system was detached and the peripheral IV was flushed with isotonic saline to prevent clotting until the next draw using sterile technique. Following the 8-hour post-condition draw the IV catheter was removed from the participant. For the 24- and 48-hour timepoints, a single-use 21-gauge butterfly needle was used by a trained phlebotomist with sterile technique to collect plasma in the same K_2EDTA vacutainer. Blood draws at each serial timepoint were attempted on all participants barring two unforeseen events. One participant (male; 26 years old) missed their 48-hour post-MIIT blood draw due to COVID-19 symptom presentation, and one participant (female; 22 years old) missed blood draws at the 2 hours through 8 hours post-control timepoints due to an interstitial IV line.

Following plasma collection, samples were processed and frozen within 2 hours according to the study processing protocol (Appendix G). All plasma samples were centrifuged within 30 minutes of collection at room temperature (23ºC) for 10 minutes at 1300g. Following centrifugation, 500 µl supernatant aliquots were pipetted into 1.2 ml cryovials using aseptic technique. All 500 ul plasma specimens were frozen at -80° C until analysis.

3.2.3.4 SIMOA

Single Molecule Array (SIMOA; Quanterix, Massachusetts, United States) technology was used to acquire blood biomarker concentrations of t-tau, NFL, GFAP, and UCH-L1 (neurology 4-plex assay B [N4PB]; Quanterix) at the Djavad Mowafaghian Centre for Brain

Health at the University of British Columbia (British Columbia, Canada). Briefly, this technology allows ultra-sensitive detection of various biomarkers at the femtogram level – approximately 900x more sensitive than a standard ELISA assay (Kuhle et al., 2016; Quanterix, 2022). Samples were run in duplicate and biomarker concentrations were calculated using calibration curves resulting from eight known concentration calibrators run on the same plate as specified by Quanterix. The mean of duplicates was used as the final biomarker concentration in subsequent analyses. UCH-L1 was dropped from analysis due to a significant proportion (>30%) of replicates falling below the lower limit of quantification (9.38 pg/mL) or the lower limit of detection (1.90 pg/mL) (Quanterix, 2022); this was expected given the known insensitivity of this assay to blood UCH-L1, especially in adults. The lower limit of quantification for t-tau is 0.125 pg/mL, NFL is 0.500 pg/mL, and GFAP is 9.38 pg/mL (Quanterix, 2022).

3.2.3.5 Physical activity and sleep monitoring

Physical activity and sleep were monitored prior to and following each condition utilizing waist (GT3x) and wrist (GT9x) worn ActiGraphs, respectively (ActiGraph Corporation, Florida, United States). Participants were provided one GT3x and one GT9x ActiGraph (ActiGraph Corporation, Florida, United States) along with instructions for use starting 48 hours prior to each condition and ending at the 48-hour post-condition timepoint. Briefly, ActiGraphs are accelerometry-based monitors that have been utilized in research as reasonably accurate and reliable monitors of physical activity and sleep (John & Freedson, 2012). The GT3x included a strap to wear on the waist during waking hours to measure physical activity, and the GT9x included a strap to wear on the wrist during sleeping hours to measure sleep. All participants received daily morning text messages as a reminder to remove the GT9x wrist ActiGraph and wear the GT3x waist ActiGraph for the day. Additionally, each participant was sent a link to

complete an in-house sleep survey held within a secure online database (REDCap; Vanderbilt University) (Appendix H). The completed sleep surveys were used to validate sleep time measured by the GT9x. Upon completion of the 48-hour post condition blood draw, participants returned both ActiGraphs. GT3x and GT9x offline data was uploaded to ActiGraph processing software ActiLife (ActiGraph Corporation, Florida, United States) for processing. Waist worn GT3x data was manually validated in 15 second epochs to include only wear time during waking hours, and physical activity metrics including minutes spent in sedentary, light, moderate, vigorous, and moderate and vigorous physical activity (MVPA) were obtained using Freedson's energy expenditure algorithm (Freedson et al., 2011). Wrist worn GT9x data was manually validated in 60 second epochs with the sleep survey to include only wear time during sleeping hours, and sleep metrics including total sleep time were obtained using Cole-Kripke's sleep algorithm for adults (Quante et al., 2018).

3.2.4 Statistical Analyses

Statistical analyses were completed using STATA 17 (StataCorp, Texas, United States) and R studio (version 1.4.1060). No power calculations were performed to determine necessary sample size due to the exploratory nature of this study. The crossover design was chosen to better isolate effects of time, condition, and their interaction whereby each participant was randomized to each condition at a different time. As such, demographic, and outcome variables were presented and analyzed using means and standard deviations. Biomarker concentrations were obtained as the mean concentration from duplicate runs on a single sample. Individual plots for each biomarker including all three conditions were obtained to describe general trends and individual variation (Figures 3.4 - 3.6). Cohen's *d* effect sizes were used to determine the relative magnitude of differences between conditions in total sleep time (minutes) the night before each

condition and total time (minutes) spent in moderate to vigorous physical activity (MVPA) the day before each condition. The Friedman test was used to examine differences in biomarker concentrations across timepoints by conditions with alpha set at 0.05 to determine significant differences. A constrained baseline analysis approach was adopted using mixed effects models adjusting for small number of clusters with restricted maximum likelihood estimation and the Kenward-Roger method for computing degrees of freedom (Hooper et al., 2018) to further assess differences in biomarker concentrations across timepoints by condition considering the influence of age, sex, and total sleep time (α=0.05). To mitigate potential over-fitting of the mixed models along with the intent to analyze sex, age, and total sleep time, MVPA was excluded from the final models. Mixed model results are reported with beta coefficients, 95% confidence intervals, and p values.

3.3 Results

Overall and by sex participant characteristics by condition are shown in Table 3.1. Seven female and three male participants with a mean age of 23 ± 2.62 years and mean BMI of 24.32 ± 3.27 kg/m^2 completed all three conditions (Figure 3.1). The mean total sleep time in the night before the control condition (456.11 ± 102.55 minutes) showed a negligible magnitude difference when compared to the night before MIIT (455.40 ± 83.06 minutes; Cohen's *d* = 0.01 [negligible]) and HIIT (459.00 ± 41.62 minutes; Cohen's *d* = -0.04 [negligible]). Minutes spent in MVPA on the day prior to control (34.36 ± 27.04) had a negligible magnitude difference when compared to the night before MIIT (36.02 ± 27.27 minutes; Cohen's *d* = -0.09 [negligible]) and HITT (37.60 ± 22.06 minutes; Cohen's *d* = - 0.17 [negligible]). Mean plasma t-tau, NFL, and GFAP concentrations (pg/ml) by condition with standard deviations are shown in Table 3.2.

Friedman test revealed no differences by condition across timepoints for plasma t-tau (Q (2) = 2.33, p = 0.31), plasma NFL (Q (2) = 2.03, p = 0.36), and plasma GFAP (Q (2) = 2.93, p = 0.23).

Table 3.1. Participant (n=10) characteristics by condition.

Sex	Characteristic	Control	MIIT	HIIT
All	Age (years)	23 (2.62)	-	-
	BMI (kg/m^2)	24.32 (3.27)	-	-
	Max HR (bpm)	98.70 (10.99)	160.80 (13.01) *effect size = 5.16 [large]*	189.70 (8.37) *effect size = 9.32 [large]*
	Sleep (minutes)	456.11 (102.55)	455.40 (83.06) *effect size = 0.01 [negligible]*	459.00 (41.62) *effect size = -0.04 [negligible]*
	MVPA (minutes)	34.36 (27.04)	36.02 (27.27) *effect size = -0.09 [negligible]*	37.60 (22.06) *effect size = -0.17 [negligible]*
Female (n=7)	Age (years)	22.71 (2.56)	-	-
	BMI (kg/m^2)	22.80 (1.48)	-	-
	Max HR (bpm)	100.86 (9.77)	164.86 (11.33) *effect size = 6.05 [large]*	190.14 (6.64) *effect size = 8.35 [large]*
	Sleep (minutes)	468.33 (111.92)	466.71 (79.50) *effect size = 0.02 [negligible]*	468.57 (46.52) *effect size = 0.00 [negligible]*
	MVPA (minutes)	29.29 (10.46)	23.00 (5.12) *effect size = 0.78 [moderate]*	29.61 (4.79) *effect size = -0.08 [negligible]*
Male (n=3)	Age (years)	23.67 (3.21)	-	-
	BMI (kg/m^2)	27.87 (3.80)	-	-
	Max HR (bpm)	93.67 (14.22)	151.33 (13.58) *effect size = 4.15 [large]*	188.67 (13.43) *effect size = 6.86 [large]*
	Sleep (minutes)	431.67 (97.00)	429.00 (102.90) *effect size = 0.03 [negligible]*	436.67 (15.28) *effect size = -0.07 [negligible]*
	MVPA (minutes)	44.50 (49.20)	66.42 (35.89) *effect size = -0.51 [moderate]*	56.25 (37.10) *effect size = -0.27 [small]*

Means (standard deviations) presented with Cohen's d effect sizes compared to control. All Cohen's d effect sizes negligible. BMI is body mass index in kg/m^2. Sleep and moderate to vigorous physical activity (MVPA) indicate minutes spent in each variable the night and day prior to condition days, respectively.

The constrained baseline mixed model revealed no significant differences in t-tau comparing MIIT and HIIT to control. However, an effect of time was found whereby a significant decline in t-tau concentration was shown throughout all three conditions at timepoints 6- and 8-hours post-condition compared to pre-condition (6; β = -1.050, 95%CI: -1.875 - -0.225, p = 0.013. 8; β = -1.141, 95%CI: -1.966 - -0.315, p = 0.007) (Figure 3.2). Increasing age leading to significant increases in t-tau at all timepoints (β = 0.128, 95%CI: 0.007 - 0.249, p = 0.040), with no

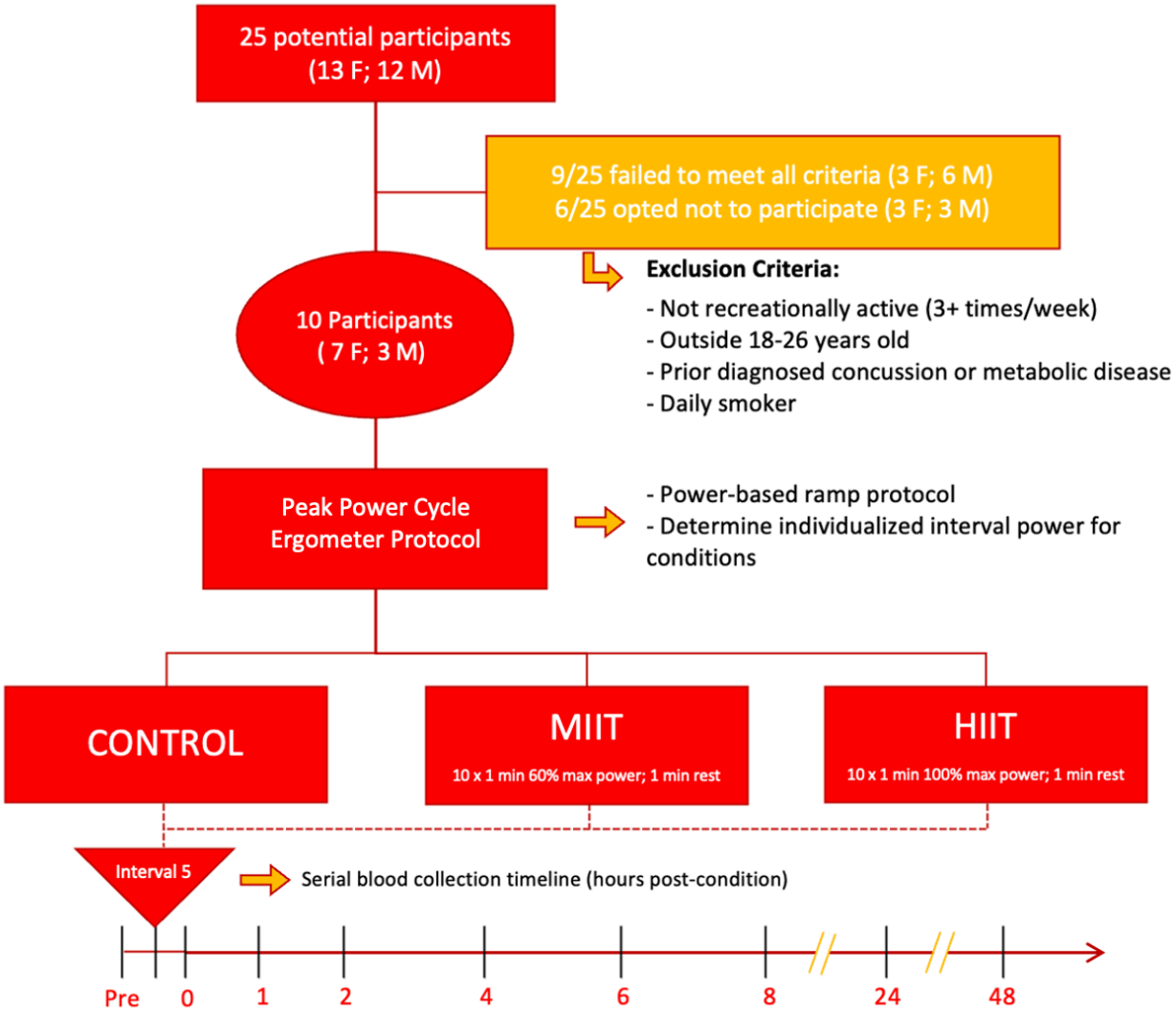

Figure 3.1. Study design flowchart

Participants completed all three conditions separated by one month in a random order. Conditions include 1) control with no exercise; 2) moderate intensity interval training (MIIT); and 3) high intensity interval training (HIIT). Blood collected via IV line on each condition day pre-condition, during the rest stage after interval 5 of each condition, and 0, 1, 2, 4, 6, and 8 hours following each condition. Single use butterfly needle used for 24- and 48-hour post condition blood collection.

significant effect of sex or sleep. For NFL, the mixed model revealed significant decreases in concentration immediately following both MIIT (β = -1.002, 95%CI: -1.852 - -0.152, p = 0.021) and HIIT (β = -1.414, 95%CI: -2.263 - -0.564, p = 0.001) compared to control (Figure 3.2). Additionally, higher total sleep resulted in significant decreases in NFL concentration at all

timepoints (β = -0.003, 95%CI: -0.005 - -0.001, p = 0.012), but no effects of age or sex were found. Lastly, the mixed model revealed significant decreases in GFAP concentrations

Table 3.2. Plasma biomarker concentrations.

Biomarker (pg/mL)	Timepoint									
	Before	During	After	1	2	4	6	8	24	48
Control										
t-tau	3.28 (1.74)	3.07 (0.77)	3.09 (0.88)	2.38 (0.99)	2.38 (0.76)	2.25 (0.52)	2.09 (0.32)	1.92 (0.57)	2.33 (0.54)	2.37 (0.69)
NFL	5.12 (2.79)	5.01 (2.74)	5.58 (3.19)	5.53 (2.89)	4.24 (1.12)	4.76 (2.54)	5.16 (2.97)	5.48 (3.22)	5.26 (2.49)	4.99 (2.10)
GFAP	48.42 (19.11)	52.33 (26.10)	52.07 (21.71)	57.59 (26.14)	62.21 (32.37)	55.91 (24.14)	59.95 (28.67)	65.47 (34.39)	55.4 (29.93)	62.61 (27.1)
MIIT										
t-tau	3.53 (2.06)	2.65 (0.92)	2.71 (1.21)	2.71 (0.94)	2.56 (0.85)	2.27 (0.95)	2.17 (0.58)	2.35 (0.68)	2.96 (1.11)	2.86 (0.95)
NFL	5.39 (1.98)	3.89 (1.04)	4.41 (2.06)	5.92 (2.75)	5.75 (2.40)	5.23 (2.42)	5.45 (2.92)	5.01 (1.71)	5.12 (2.26)	4.87 (2.03)
GFAP	54.03 (28.95)	50.3 (22.55)	41.4 (23.15)	61.85 (30.39)	67.41 (37.00)	63.47 (43.82)	62.31 (30.05)	65.75 (38.13)	54.56 (27.22)	58.05 (29.58)
HIIT										
t-tau	3.00 (1.09)	2.58 (1.56)	3.00 (1.19)	2.72 (0.61)	2.33 (0.86)	2.32 (0.68)	2.04 (0.56)	1.86 (0.43)	2.67 (0.97)	2.42 (1.03)
NFL	5.07 (1.86)	4.32 (1.73)	3.99 (2.01)	5.33 (1.98)	5.41 (2.68)	4.97 (2.14)	4.73 (1.75)	4.97 (2.14)	5.22 (2.35)	5.77 (2.3)
GFAP	45.33 (24.17)	47.11 (25.82)	35.19 (15.97)	53.64 (28.66)	65.47 (29.39)	58.36 (27.32)	53.63 (26.09)	63.77 (34.07)	52.15 (15.85)	57.11 (32.26)

Values presented as means (standard deviations) in pg/mL. Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints before and after are referring to immediately before and immediately following each condition. Timepoint during refers to rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. Biomarkers include plasma total tau (t-tau), plasma neurofilament-light chain (NFL), and plasma glial fibrillary acidic protein (GFAP).

immediately following both MIIT (β = -14.750, 95%CI: -27.154 - -2.345, p = 0.020) (Figure 3.2) and HIIT (β = -20.956, 95%CI: -33.358 – -8.554, p = 0.001) (Figures 3.2 - 3.3), but no effects of age, sex, or sleep were found. See appendix I and J for mixed model visualization figures by sex. Biomarker concentrations for each individual across serial timepoints by condition are displayed in Figures 3.4 – 3.6. For all biomarkers, the range in concentrations across serial timepoints by condition appears variable at the individual level.

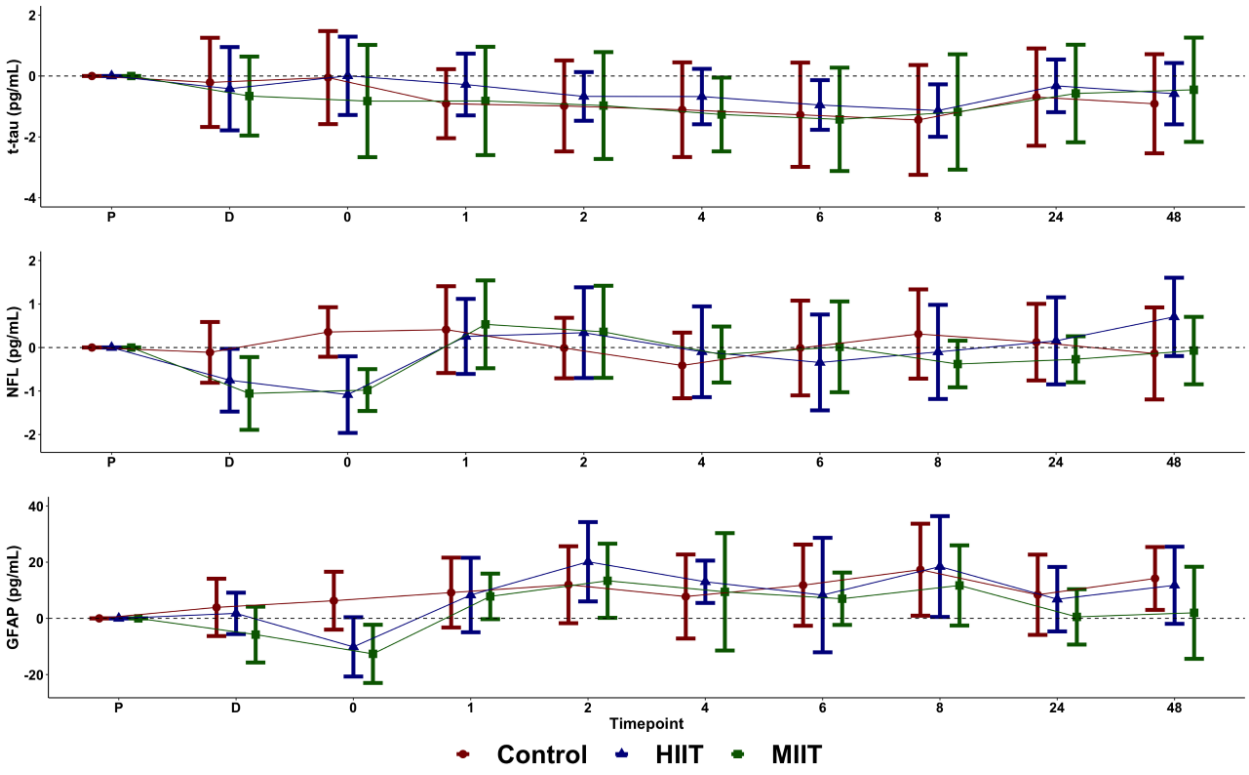

Figure 3.2. Mean biomarker differences by condition at each timepoint compared to pre-condition.

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. Biomarkers include plasma total tau (t-tau), plasma neurofilament-light chain (NFL), and plasma glial fibrillary acidic protein (GFAP). See Appendix F for presentation by sex.

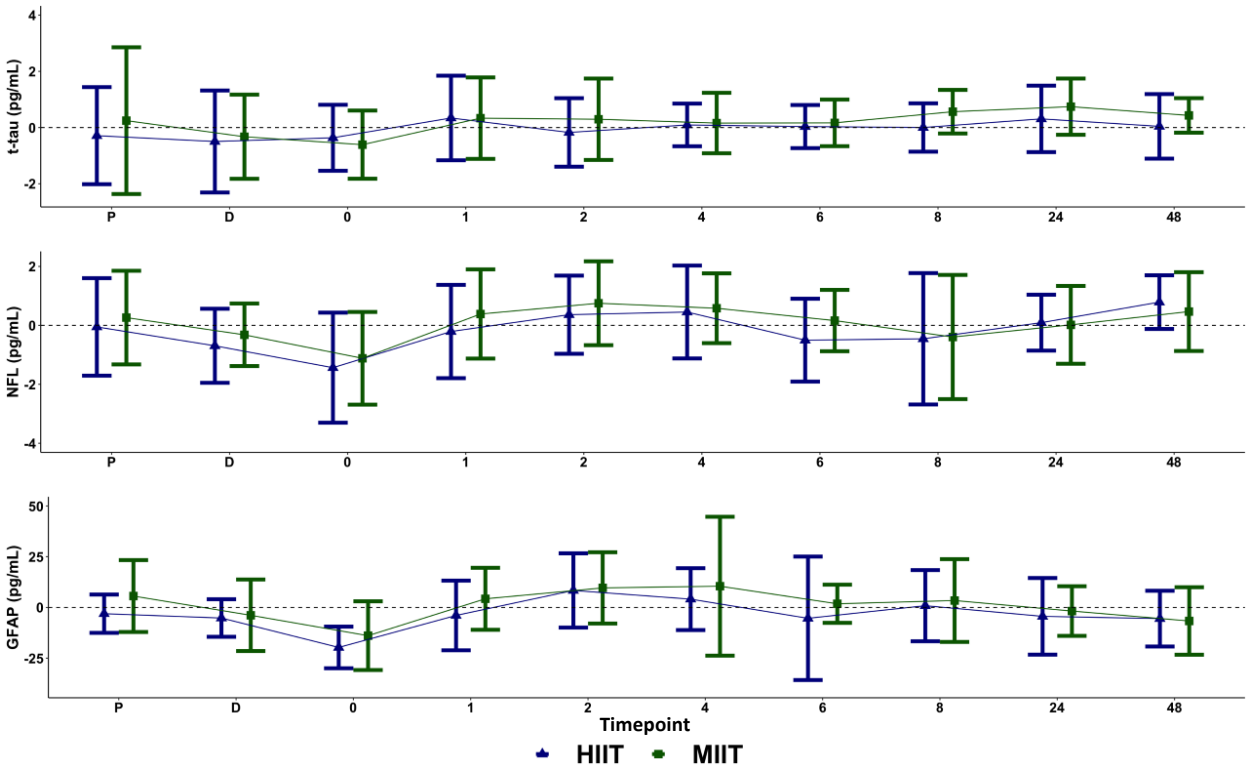

Figure 3.3. Mean biomarker differences following MIIT and HIIT compared to control at each timepoint.

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. Biomarkers include plasma total tau (t-tau), plasma neurofilament-light chain (NFL), and plasma glial fibrillary acidic protein (GFAP).

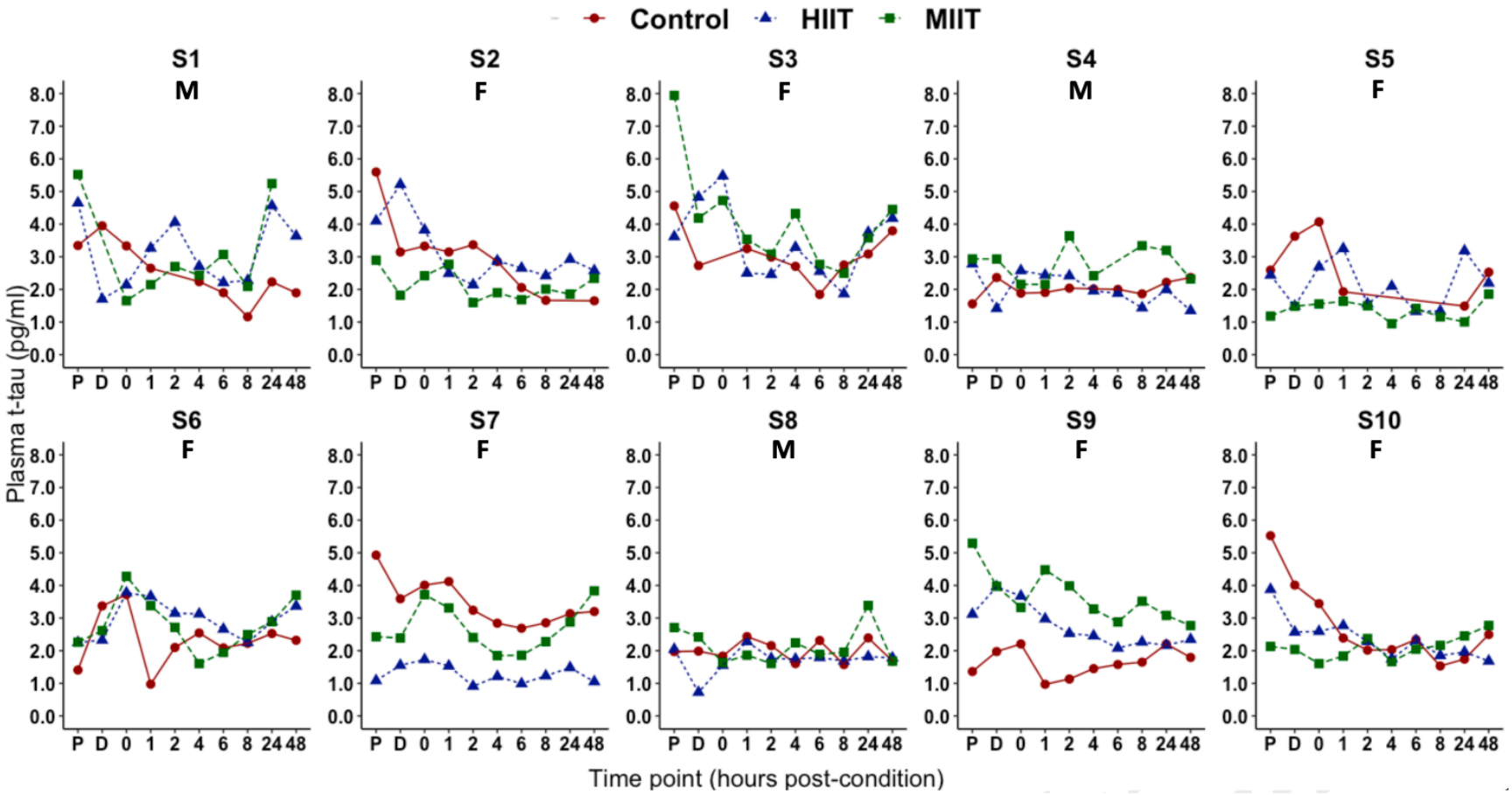

Figure 3.4. Plasma t-tau by individuals.

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. Biomarkers include plasma total tau (t-tau), plasma neurofilament-light chain (NFL), and plasma glial fibrillary acidic protein (GFAP). S# denotes a single participant, while M and F denote male and female, respectively.

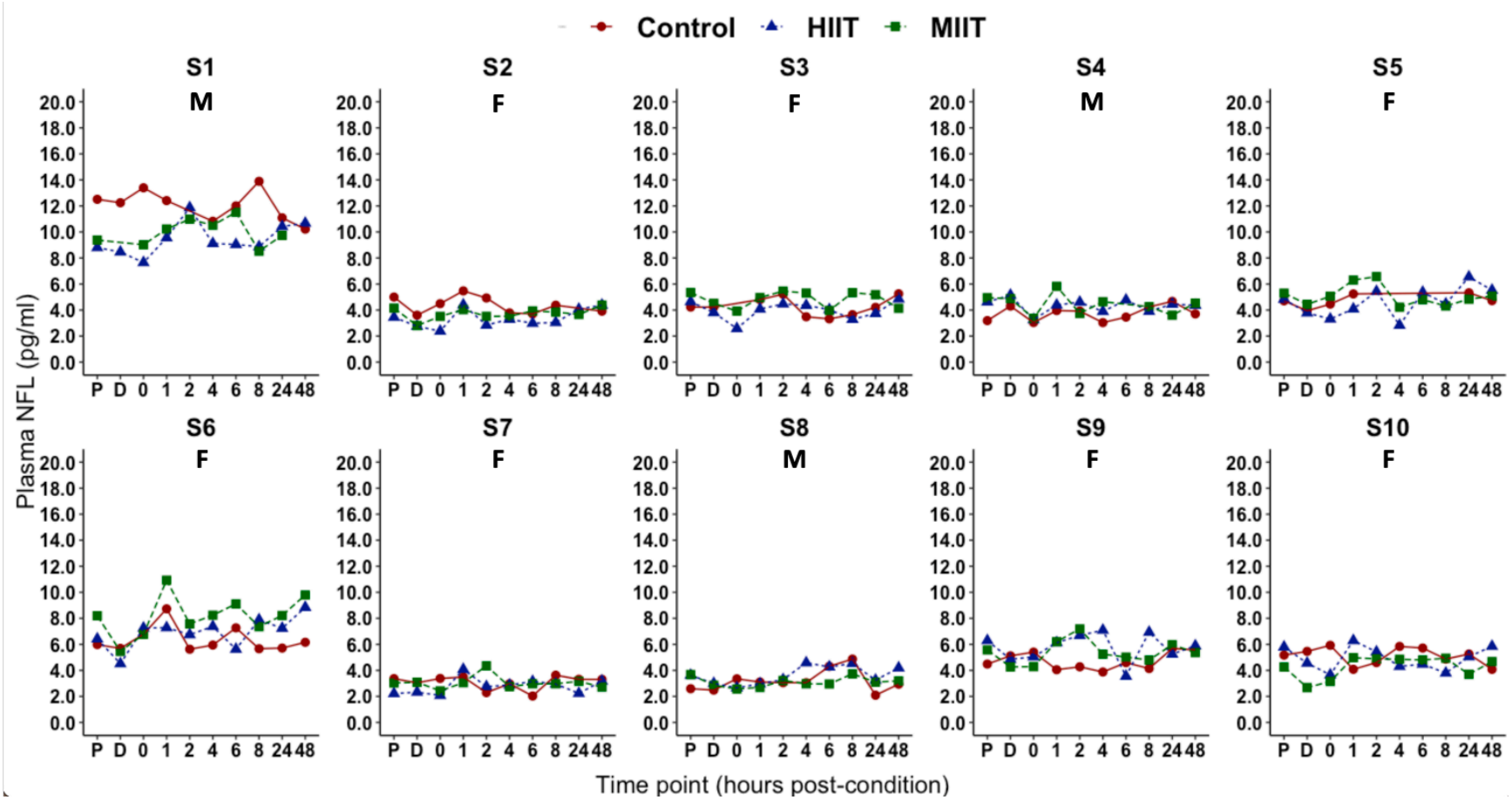

Figure 3.5. Plasma NFL by individuals.

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. Biomarkers include plasma total tau (t-tau), plasma neurofilament-light chain (NFL), and plasma glial fibrillary acidic protein (GFAP). S# denotes a single participant, while M and F denote male and female, respectively.

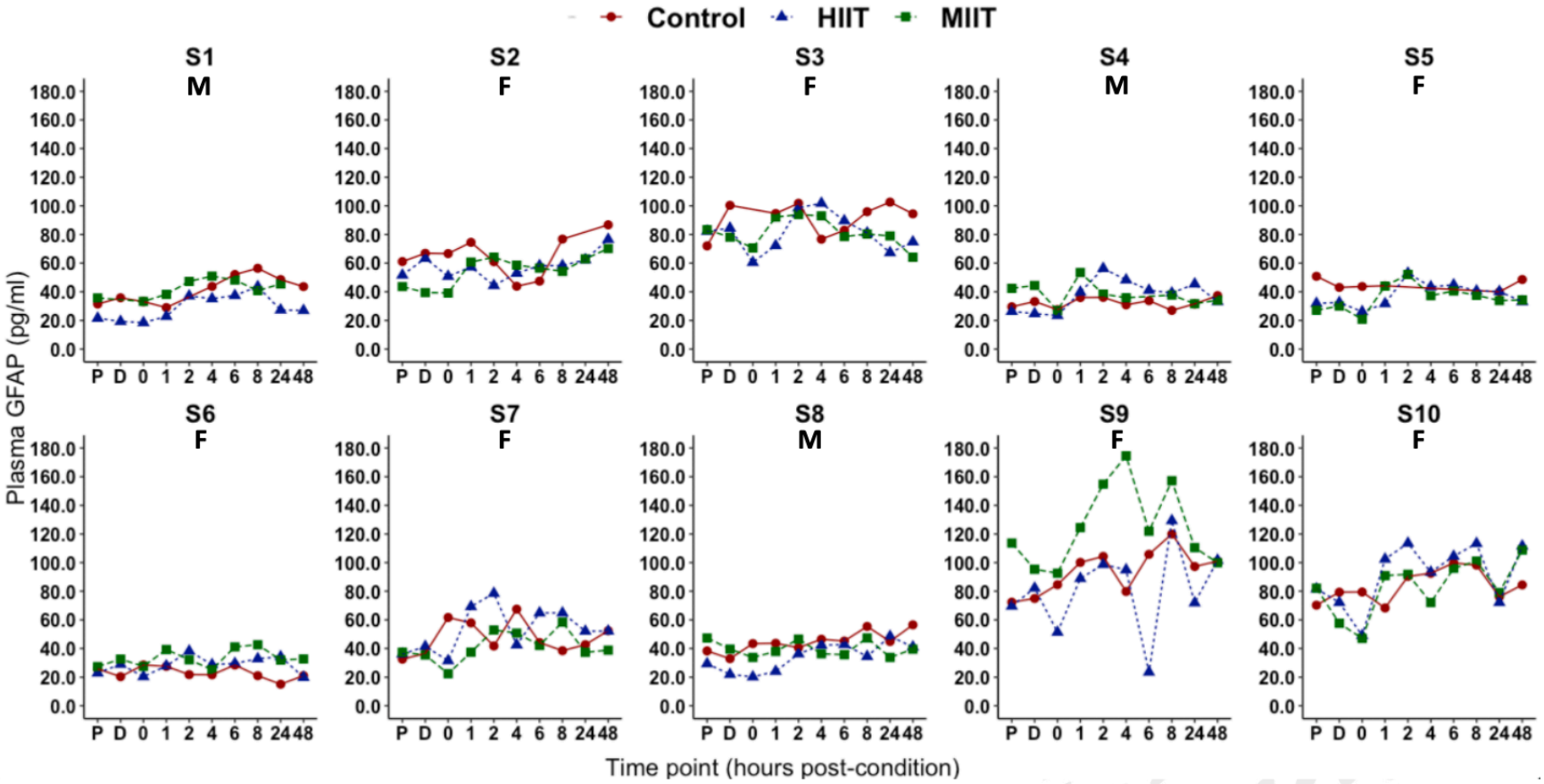

Figure 3.6. Plasma GFAP by individuals.

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. Biomarkers include plasma total tau (t-tau), plasma neurofilament-light chain (NFL), and plasma glial fibrillary acidic protein (GFAP). S# denotes a single participant, while M and F denote male and female, respectively.

3.4 Discussion

This study revealed an acute bout of MIIT and HITT may transiently affect some of the leading CNS derived blood biomarkers associated with SRC in healthy adults. For plasma t-tau no effect of exercise was seen, however a small decline in t-tau concentration throughout the day across conditions was statistically significant at the 6- and 8-hours post-condition timepoints (Figure 3.2). Additionally, increasing age resulted in significant increases in t-tau concentration at all timepoints. In plasma NFL results showed a small but significant concentration decline immediately following both MITT and HITT that appeared to stabilize to control levels by the 1-hour post-condition timepoint (Figure 3.2). This effect appeared modulated by sleep, where more total sleep in the night before each condition lead to significantly decreased plasma NFL concentration at all timepoints. Results for plasma GFAP were like NFL, where a small but significant concentration decline immediately following both MITT and HITT was found and appeared to stabilize to control levels by the 1-hour post-condition timepoint (Figure 3.2 – 3.3). However, there was not a statistically significant effect of age or sleep on GFAP.

3.4.1 t-tau.

Figure 3.4 displays plasma t-tau concentration for individual participants at each timepoint across all three exercise conditions. Some participants had a narrower range of values compared to others across conditions. Specifically, S8's (male) range including all conditions between 0.72 and 3.39 pg/ml is narrower than S1's (male) range between 1.16 and 5.52 pg/ml. Likewise comparing females, S3's range including all conditions between 1.84 and 7.95 pg/ml is wider than S4's range between 1.35 and 3.64 pg/ml. Though this highlights the importance of considering inter-individual variability in the context of t-tau as a potential blood biomarker for

SRC, variation at these low levels in a sample of healthy young adults may not be clinically relevant in the context of future applications for SRC diagnosis or prognosis.

Although the mixed model reveals no effects of exercise conditions on plasma t-tau presentation, a decrease in t-tau throughout the day is seen in all conditions with significant decreases at the 6- and 8-hours post-condition timepoints (Figure 3.2). These results directly conflict with findings from a prior study showing a significant increase in t-tau following HIIT (Di Battista, Moes, et al., 2018). However, findings do align with those from Shahim et al. (2018) showing no changes in t-tau following a *"friendly game of hockey"*. Contradicting findings are likely due to methodological inconsistencies in processing and assay techniques used across studies to determine biomarker concentrations. Di Battista et al. (2018) used standard ELISA technique via MesoScale (MesoScale Diagnostics LLC, Maryland, United States) and Shahim et al. (2018) used *"novel immunoassays using digital array technology (Quanterix)"*. Inconsistent findings might also be due the timing of sampling post exercise, where Di Battista and colleagues (2018) sampled immediately following exercise, but Shahim and colleagues (2018) sampled 1-hour post-exercise, which is why the present study adopted a serial sampling approach. Nonetheless, this highlights the need for future research in blood biomarkers of SRC to strive for collaborative, standardized study design, sample collection protocols, and assay methodologies across studies to eliminate potential measurement variability in results.

Decreases in t-tau concentrations at 6- and 8- hours post conditions could have significant implications. Sample timing during the day should be taken into consideration in the context of potential use of t-tau as a blood biomarker for SRC diagnosis or prognosis. T-tau decline throughout the day may be a product of diurnal metabolite and waste clearance in healthy adults, and if t-tau declines throughout the day in concussed individuals, blood samples taken towards

the end of the day may not accurately reflect CNS injury compared to blood samples taken earlier in the day. This necessitates further research into the potential diurnal variation in plasma t-tau levels in concussed individuals before t-tau can be clinically validated as a marker for SRC.

A significant effect of age was found whereby increasing age was associated with increasing plasma t-tau levels at all timepoints. A novel finding in this younger adult sample, this relationship also requires future investigation to disentangle the possible influence of age as a confounding variable on the effects of exercise on these biomarkers, especially considering the majority of SRC's occur in youth and young adulthood when development of the brain is still ongoing (Giedd et al., 2015; Purcell, 2014). Though sex appeared insignificant in the mixed model, it's plausible the small male sample size was underpowered to detect a potential difference. As it has been shown females have higher levels of circulating t-tau and worse outcomes following SRC compared to males (Gill et al., 2017; Koerte et al., 2020), it remains vital to consider biomarker concentrations may vary between males and females, requiring further research with larger groups by sex.

3.4.2 NFL.

Figure 3.5 displays plasma NFL concentration for individual participants at each timepoint across all three conditions. Eight of the ten participants have values within a narrow range of approximately 2 to 6 pg/ml across all conditions, while S1 (male) ranges from 7.65 to 13.89 pg/ml and S6 (female) ranges from 4.51 to 10.93 pg/ml. Like t-tau, inter-individual variability of NFL as a potential blood biomarker for SRC is important to consider, but variation at these low levels in a sample of healthy young adults may not be clinically relevant.

Results showing no significant differences in plasma NFL concentration 1-hour following MIIT and HIIT compared to control (Figure 3.2) agree with findings of a prior study showing no

changes in NFL 1-hour after a *"friendly game of hockey"* (Shahim et al., 2018). However, the present study revealed a novel finding immediately following both MIIT and HIIT where plasma NFL levels were significantly lower than control and appear to stabilize to control levels by the 1-hour post-condition timepoint (Figure 3.2). This finding could have implications regarding the potential use of NFL as an objective marker for SRC in the minutes to hours following injury. If exercise transiently decreases plasma NFL levels in a concussed athlete and blood is sampled within minutes to an hour of exercise/injury, it is possible their NFL levels may be lower than expected for a concussed individual due to an exercise-induced decrease. This could lead to misinformed diagnoses or prognoses, suggesting timing of sampling following injury/exercise should be considered before clinical validation of NFL as a biomarker for SRC. However, blood NFL has been shown to have greater correlation with brain injury in the weeks to months following injury compared to the hours to minutes following injury (Czeiter et al., 2012; Shahim et al., 2020), so it's likely this transient decrease following exercise may be clinically irrelevant. Considering NFL is constantly released from axons under normal conditions (Gaetani et al., 2019), these results also suggest interval exercise might temporarily halt or reduce the speed of this process. Future research is required to better understand this mechanism and the interplay between exercise and concussion as it pertains to this marker of brain injury.

In addition to the significant decrease in NFL following both MIIT and HIIT, the mixed model revealed more sleep in the night prior to each condition resulted in significantly decreased plasma NFL levels at all timepoints. This finding highlights the importance of considering the glymphatic system when studying these CNS derived biomarkers as objective measures of SRC, as sleep has been shown to accelerate neurotoxic waste product clearance (Xie et al., 2013). In line with this theory, if an individual sleeps for a longer period of time it is expected there would

be more “waste” metabolites in the blood. These results showing an inverse relationship between sleep and plasma NFL conflict with this theory and requires further investigation to expose potential mechanisms. Additionally, although there was no effect of sex seen, potential sleep differences between the sexes should not be ignored. It has been shown though women subjectively report more disrupted and inefficient sleep than men, objective measures reveal healthy women have better sleep quality than men (Mong & Cusmano, 2016). This highlights a potentially important consideration regarding blood biomarker presentation and requires further investigation in light of the current null findings. It is also important to note sleep fragmentation and chronic sleep restriction have been shown to increase blood brain barrier permeability in mice, which in theory would also result in elevated concentrations of CNS derived blood biomarkers by an alternate mechanism if true in humans (He et al., 2014; Pan & Kastin, 2017). As these sleep disturbances are a common symptom of concussion, the interplay between sleep, exercise, and concussion, along with their effects on plasma NFL requires further research to disentangle this extremely complex injury sequalae.

3.4.3 GFAP.

Figure 3.6 displays plasma GFAP concentration for each individual participant at all timepoints across the three conditions. Compared to the rest of the sample, S9’s (female) range across all conditions between 23.51 and 174.57 pg/ml is staggeringly wide. It’s possible this intra-individual variability across conditions may be explained by the influence of menstrual cycle phase as S9 was the only female participant in the study to complete all three conditions at three different self-report menstrual cycle phases due to scheduling conflicts and an irregular period (control = menses; MIIT = ovulation; HIIT = luteal), though this is speculation. Nonetheless, this necessitates future research regarding differential effects of female

reproductive cycle phases on the presentation of these biomarkers as significant differences in cardiometabolic blood biomarkers have been shown across the menstrual cycle (Schisterman et al., 2014).

Like NFL, the mixed model revealed small but significant decreases in GFAP immediately following both MIIT and HIIT compared to control (Figures 3.2 - 3.2), with the differences disappearing by the 1-hour post-condition timepoint. Again, this may have implications regarding the potential use of GFAP as an objective marker for SRC in the minutes to hours following injury. The potential extent of this implication may be more pervasive than in the case of NFL, as elevated GFAP has been detected within 1 hour of brain injury and peak levels reached within the first 24-48 hours (Gul et al., 2017; Meier et al., 2020; Papa et al., 2016). As an acute marker of injury, if blood is sampled in the minutes to hours following SRC for diagnostic or prognostic applications in the future, it's possible the influence of the inherent exercise on GFAP may lead to misinformed results. However, the small decline seen at low levels in healthy individuals may be clinically irrelevant in a concussed population where the expected GFAP levels are much higher. Regardless, if transient exercise-induced decreases in GFAP occur following SRC, timing of sampling remains critical to consider before clinical validation of these biomarkers for diagnostic or prognostic use in the context of injury.

3.4.4 Potential factors affecting biomarker presentation.

As it was not the primary outcome, mechanisms relating to the findings in the present study cannot be elucidated. However, there are a few potential factors related to exercise and the effects they might have on the presentation of CNS biomarkers in the blood that remain important considerations. Exercise has shown to increase cerebral blood flow, which suggests markers exiting the brain through perivascular efflux or lymphatic drainage pathways should

increase (Smith & Ainslie, 2017). Though contradictory to the current findings, this increase in cerebral blood flow should not be ignored in future research aimed to highlight mechanisms of CNS biomarker presentation. Another potential mechanism could be increased degradation of biomarkers in the blood as a result of inflammatory proteases that are up-regulated following exercise (Suhr, 2019), which is in-line with the current results though theoretical in the current state. Adding to these potential mechanisms are the effects of glomerular filtration rate (GFR) and hydration (Poortmans, 1984). Renal plasma flow is reduced during exercise relative to the exercise intensity as a result of sympathetic activation, which may influence biomarker presentation acutely following exercise as less proteins are filtered by the kidneys, resulting in increased markers in the blood (Poortmans, 1984). This is further modulated by hydration status, which is influenced by exercise as water is lost through sweat, specifically at higher intensities (Poortmans, 1984). In summary, many potential factors relating to exercise could influence the presentation of CNS biomarkers in the blood and future research elucidating these mechanisms will greatly enhance our understanding of the results presented herein.

3.4.5 Limitations.

The present study is not without limitations. Though the randomised crossover cohort study design was used to eliminate individual variability between groups allowing for a smaller sample size, the possibility of insufficient power to detect significant differences is recognized. To limit this potential in the baseline constrained mixed model analysis, restricted maximum likelihood estimation and the Kenward-Roger method for computing degrees of freedom were implemented. Another limitation is the potential non-specificity of the biomarkers to the CNS. Though these biomarkers are commonly referred to as CNS-specific due to the majority of gene transcription and translation occurring in the CNS, extra-cranial sources of t-tau and NFL remain

prominent (Lépinoux-Chambaud & Eyer, 2013; Morris et al., 2011). Specifically, t-tau sources may include the heart, kidneys, testis, adrenal glands, stomach, and adipose tissue, and NFL sources may include adrenal glands, esophagus, and kidneys (NCBI, 2022b, 2022c). Without in-vivo labelling it is impossible to pinpoint the source of these biomarkers, rendering potential mechanisms regarding BBB disruption difficult to infer. Though this study intended to analyze sex differences, the inclusion of only three male participants made this difficult beyond including sex as a covariate in the mixed model analysis. As initial evidence suggests sex differences regarding concussion risk and outcomes exists (Koerte et al., 2020), this remains a vital target for future research. Finally, the assay technique and use of means of replicates for analysis may introduce bias in the results. Though SIMOA technology is marketed as an ultra-sensitive method to obtain biomarker concentrations, the comparatively small concentrations of these injury markers in a population of healthy young adults results in a high amount of noise in the measurements. This is especially true in the case of UCH-L1, where over 30% of the replicates were below the lower limit of quantification. Though a significant majority of replicates for t-tau, NFL, and GFAP were well above the lower limits of quantification, some replicate values were markedly different. Inclusion of all replicate means minimizes potential bias that might occur due to dropping means of replicates with a larger spread. Future analyses should consider the inclusion of both replicate concentrations in a modelling approach to eliminate this potential bias and better characterize the associations regarding biomarker concentrations.

3.5 Conclusion

A single bout of moderate and high intensity interval exercise appears to transiently affect some blood biomarkers associated with SRC (e.g., NFL and GFAP), but not t-tau in healthy young adults. However, this small and transient decline seen in a sample of healthy

young adults may be clinically irrelevant for future objective diagnostic or prognostic applications for SRC including these markers. Regardless, given these conflicting results with currently existing literature, the importance of methods harmonization and standardization across fluid biomarker research must be stressed to better characterize these plasma biomarker concentrations in the context of exercise and brain injury. Future research should use larger sample sizes including both sexes for blood biomarker analysis in the immediate minutes to hours following exercise in those with a history of concussion to inform the association between exercise and injury on these plasma biomarkers. This will enable more robust characterization of the interplay between exercise and injury, moving the field one step closer to clinical validation of objective physiological markers of brain injury to aid in the diagnosis and prognosis of SRC.

Chapter 4: Commonly Studied Blood Serum and Plasma Biomarkers for Sport-related Concussion: Does matrix matter?

4.1 Introduction

Sport-related concussion (SRC), defined as a form of mild traumatic brain injury (mTBI) resulting in a broad range of clinical signs and symptoms (e.g., headache, dizziness, balance impairments), relies on subjective reporting and interpretation of such symptoms for diagnosis and predicting injury recovery (McCrory et al., 2017). Blood biomarkers have recently emerged as a potential avenue for objective tests to supplement clinical decision making in the context of SRC. The two matrices used to assay biomarker levels are plasma and serum, which are liquid portions of whole blood obtained by different post-draw biochemical processes (Yu et al., 2011). Plasma is the liquid portion of whole blood that has not clotted, obtained via centrifugation of a blood-filled plasma collection tube containing an anticoagulant (e.g., ethylenediaminetetraacetic acid [EDTA], heparin, citrate) (Vignoli et al., 2022). Serum is the liquid portion of whole blood that has clotted, obtained by centrifugation of blood-filled serum tubes that are allowed to clot for a period of time (typically 30 minutes or more) (Vignoli et al., 2022). As such, fibrin clots formed during coagulation and the related coagulation factors are separated from serum and held within the blood clot (Yu et al., 2011). Additionally, platelets release various proteins and metabolites (e.g., proinflammatory cytokines, sphingolipids) into serum during coagulation (Schnabel et al., 2010; Yatomi et al., 1997). Heavily studied disease states like Diabetes Mellitus and Myocardial Infarction hold general consensus on the suitable matrix to examine various biomarkers associated with said disease – serum troponin assays for Myocardial Infarction and plasma glucose assays for Diabetes Mellitus (Lima-Oliveira et al., 2018; Sacks et al., 2002). Due to the relatively early nature of blood biomarker research for SRC, potential matrix differences

amongst some of the commonly studied blood biomarkers for SRC have not been adequately addressed to date.

Some of the leading protein biomarkers for SRC (e.g., tau, neurofilament light [NFL], and glial fibrillary acidic protein [GFAP]) serve functional or structural roles in neurons and astrocytes, which are supporting cells of the neurovascular unit (Zetterberg & Blennow, 2016). One investigation comparing serum and plasma levels of tau and NFL measured by enzyme-linked immunosorbent assays found NFL to be comparable between matrices, but tau to be significantly reduced in serum compared to plasma (O'Connell et al., 2019). It remains unclear how biochemical changes due to clot activation may or may not affect the presentation of these biomarkers between matrices at the processing level, though it has been postulated tau is trapped in the fibrin-platelet matrix during clotting due to a propensity to aggregate (O'Connell et al., 2019). Nonetheless, analysis of total tau (t-tau), NFL, and GFAP following SRC has been performed in both serum and plasma with mixed results (Di Battista, Rhind, et al., 2018; Guedes et al., 2020; McCrea et al., 2020). Due to the chemical composition differences between plasma and serum, comparisons between studies analyzing biomarkers for SRC in different matrices remains a challenge. Additionally, it is unknown if metabolic changes following exercise might differentially affect the presentation of these biomarkers across matrices. This necessitates a push towards standardization of blood processing methodologies across studies to better characterize the effect of injury in a single matrix and requires careful consideration of the potential differences across matrices by injury and exercise. To this end, the present study aimed to describe and compare plasma and serum concentrations of t-tau, NFL, and GFAP measured by the highly sensitive SIMOA platform (Quanterix, 2022) in healthy individuals across differing intensity exercise bouts completed on a cycle ergometer.

4.2 Methods

4.2.1 Study design.

Participants completed three different intensity interval conditions separated by approximately 28 days (control, moderate intensity interval training [MIIT], and high intensity interval training [HIIT]) in a randomized crossover cohort design. A 28-day period between conditions was chosen to allow physical recovery, baseline stabilization of blood, and an attempt for female participants with regular menstrual cycles to complete all three conditions within the same phase of their cycle (Carmichael et al., 2021). One serum and one plasma blood sample were serially for each exercise condition (pre-condition, during condition, and 0, 1, 2, 4, 6, 8, 24,and 48 hours post-condition). The first samples (pre-condition) for all participants were collected between 7:45am and 8:15am on the morning of each condition to control for potential circadian rhythm influence. Each participant completed all conditions and blood sampling in the Cerebrovascular Concussion Lab at the University of Calgary (Alberta, Canada).

4.2.2 Participants.

Participants were recruited at the University of Calgary via word of mouth and recruitment posters displayed in the Faculty of Kinesiology. Exclusion criteria included: any prior concussion diagnosis, metabolic disease, or daily smoking/vaping. Participants were included if deemed fit to participate in the peak power cycle ergometer protocol and subsequent interval intensity conditions by passing all general health questions on the Physical Activity Readiness Questionnaire (PAR-Q+) (Appendix B) (Warburton et al., 2011), and engaging in exercise (including sport) three or more times per week. Ten university students provided written informed consent (Appendix C) to participate in this study under the University of Calgary's Conjoint Health and Research Ethics Board (REB21-0768). All participants were required to fill

out a COVID-19 symptom questionnaire (Appendix D) before attending the university on all study days, in-line with the University of Calgary COVID-19 guidelines during the time of data collection.

4.2.3 Procedures.

4.2.3.1 Peak power cycle ergometer protocol.

On the first study day, all participants completed the peak power cycle ergometer protocol on a Corival cpet bicycle ergometer (Lode BV, Groningen, Netherlands), fitted with a wireless Polar H10 heart rate monitor (Polar, Kempele, Finland). The peak power protocol was completed 3-5 days prior to the first condition day to pinpoint individualized power outputs for the MIIT and HIIT conditions. Two power-based ramp protocols were programmed on the ergometer's digital display, necessary to adjust for individual fitness levels while targeting completion of the peak protocol in the range of 8 to 12 minutes for all participants (Yoon et al., 2007). Both protocols included a 2-minute warmup at 50 watts for minimal resistance cycling. Following the 2-minute warmup, a ramp increase in watts (20 watts/min for protocol 1; 30 watts/min for protocol 2) was implemented and completed until failure by the participant to sustain cadence equal to or above 80 revolutions per minute or stopped by the study team. Participants were blinded to the purpose of the peak power protocol and instructed to focus on completing the test to volitional exhaustion. The protocol was chosen based on each participants height, weight, and self-reported physical fitness ability. Nine of ten participants completed protocol 1 and one participant completed protocol 2 (male; 25 years old).

4.2.3.2 Cycle ergometer interval conditions.

Following completion of the peak power cycle ergometer protocol, participants completed three different 30-minute exercise intensity interval conditions separated by

approximately 28 days in a randomized order: 1) *Control* (30-minutes seated); 2) *MIIT* (5 minute warmup at 50 watts, 10 x 1 minute intervals at 60% achieved peak power separated by 1 minute complete rest, 5 minute cooldown at 50 watts); and 3) *HIIT* (5 minute warmup at 50 watts, 10 x 1 minute intervals at 100% achieved peak power separated by 1 minute complete rest, 5 minute cooldown at 50 watts). Participants abstained from exercise, caffeine, and alcohol for at least 12 hours before each exercise condition. Participants were not instructed to sustain cadence within a specific range, only to focus on keeping their feet moving during intervals to maximize effort. To minimize potential effects of diet on condition days, participants were instructed to eat the same breakfast at home on all three condition days and were provided with 2 vanilla nutrition shakes in lab (Kirkland signature, Washington, United States) to consume between timepoints 2 and 4 hours-post condition, and water and Gatorade as needed (Gatorade, Illinois, United States) to ensure adequate hydration.

4.2.3.3 Blood collection and processing.

Two methods were used to collect plasma and serum blood samples at all ten serial timepoints following completion of a pre blood draw questionnaire (Appendix E). For timepoints between pre-condition to 8 hours post-condition, a 22-guage IV catheter (Protectiv® winged, Smiths Medical, Minnesota, United States) was inserted and secured into a peripheral vein of the antecubital fossa of each participant by a trained phlebotomist on the morning of each condition day. A short (2') microclave IV connector was connected to the IV catheter for ease of access. At each blood sampling timepoint, a VAMP adult closed blood sampling system (Edwards Lifesciences, California, United States) was attached to the microclave IV connector. This sampling device allows direct closed access to the vein via a small (5ml) flexure mechanism and the peripheral IV catheter for filling vacutainer blood collection tubes without necessitating a

clearing volume (Appendix F) (Edwards Lifesciences, 2022). Following collection first into a 10 mL serum vacuum tube (red top, no additive), then into a 10 mL plasma vacuum tube (lavender top, ethylenediaminetetraacetic acid [K_2EDTA] additive), the VAMP system was detached and the peripheral IV was flushed with isotonic saline to prevent clotting until the next draw using sterile technique. Following the 8-hour post-condition draw the IV catheter was removed from the participant. For the 24- and 48-hour timepoints, a single-use 21-gauge butterfly needle was used by a trained phlebotomist with sterile technique to collect plasma and serum in the same vacutainers.

Following plasma and serum collection, samples were processed and frozen within 2 hours according to the study processing protocol (Appendix G). All plasma samples were centrifuged within 30 minutes of collection at room temperature (23ºC) for 10 minutes at 1300g. Serum samples were left to clot at room temperature (23ºC) for a minimum of 30 minutes prior to centrifugation for 10 minutes at 1300g. Following centrifugation, 500 µl supernatant serum and plasma aliquots were pipetted into 1.2 ml cryovials using aseptic technique. All 500 µl plasma specimens were frozen at -80° C until analysis.

4.2.3.4 SIMOA.

Single Molecule Array (SIMOA; Quanterix, Massachusetts, United States) technology was used to acquire blood biomarker concentrations of t-tau, NFL, and GFAP (neurology 4-plex assay B [N4PB]; Quanterix) at the Djavad Mowafaghian Centre for Brain Health at the University of British Columbia (British Columbia, Canada). Briefly, this technology allows ultra-sensitive detection of various biomarkers at the femtogram level – approximately 900x more sensitive than a standard ELISA assay (Kuhle et al., 2016; Quanterix, 2022). Samples were run in duplicate and biomarker concentrations were calculated using calibration curves resulting

from eight known concentration calibrators run on the same plate as specified by Quanterix. The mean of both replicates run from a single sample was used as the final biomarker concentration in subsequent analyses except for the descriptive coefficient of variation analysis. All sample reads in plasma and serum were above the lower limit of quantifications, which is 0.125 pg/mL for t-tau, 0.500 pg/mL for NFL, and 9.38 pg/mL for GFAP (Quanterix, 2022).

4.2.4 Statistical Analyses.

Statistical analyses were run using STATA 17 (StataCorp, Texas, United States) and R studio (version 1.4.1060). No power calculations were performed to determine necessary sample size due to the exploratory nature of the study. Individual plots showing plasma and serum concentrations across conditions at each timepoint were obtained to describe general trends and individual variation. Spearman rank correlation coefficients (ρ) were calculated to assess correlation between serum and plasma concentrations of t-tau, NFL, and GFAP across conditions at group and individual levels, and interpreted by strength of linear relationship outlined by Chan (Chan, 2003). Relative bias was calculated using plasma concentrations minus serum concentrations to determine magnitude and direction of potential differences. Bland-Altman plots were used to describe agreement between parallelly drawn plasma and serum concentrations across all conditions. Descriptive analysis of coefficient of variations (CV's) for the mean biomarker values of replicates was implemented to pragmatically assess the precision of each analyte measurement across sex and conditions.

4.3 Results

Seven female and three male participants with a mean age of 23 ± 2.62 years and mean BMI of 24.32 ± 3.27 kg/m^2 completed all three conditions (Figure 4.1). Obtaining a serum and plasma sample at each serial timepoint was achieved on all participants across conditions barring

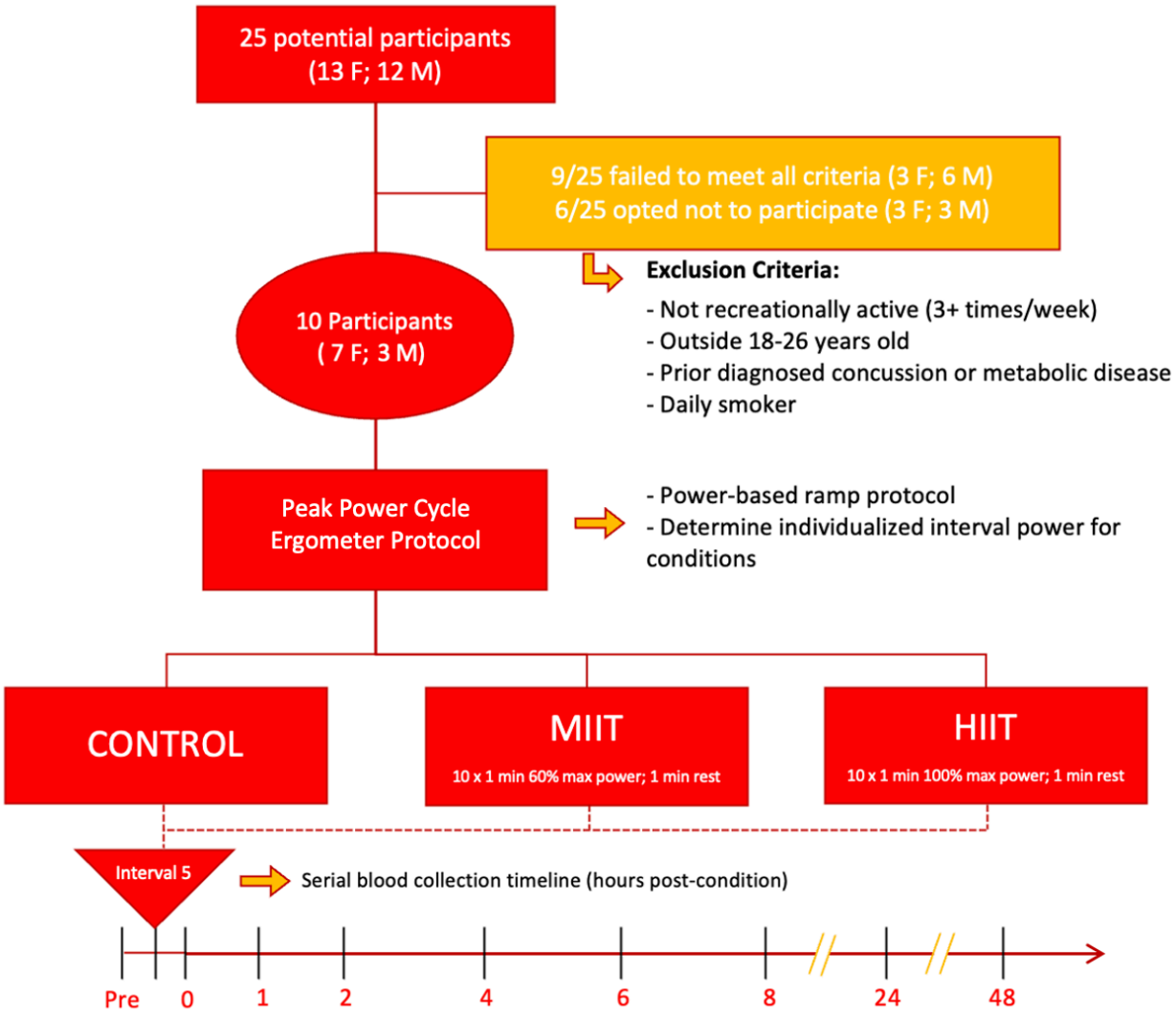

Figure 4.1. Study design flowchart.

Participants completed all three conditions separated by one month in a random order. Conditions include 1) control with no exercise; 2) moderate intensity interval training (MIIT); and 3) high intensity interval training (HIIT). Blood collected via IV line on each condition day pre-condition, during the rest stage after interval 5 of each condition, and 0, 1, 2, 4, 6, and 8 hours following each condition. Single use butterfly needle used for 24- and 48-hour post condition blood collection.

two unforeseen events. One participant (male; 26 years old) missed their 48-hour post-MIIT blood draw due to COVID-19 symptom presentation, and one participant (female; 22 years old) missed blood draws at the 2 hours through 8 hours post-control timepoints due to an interstitial

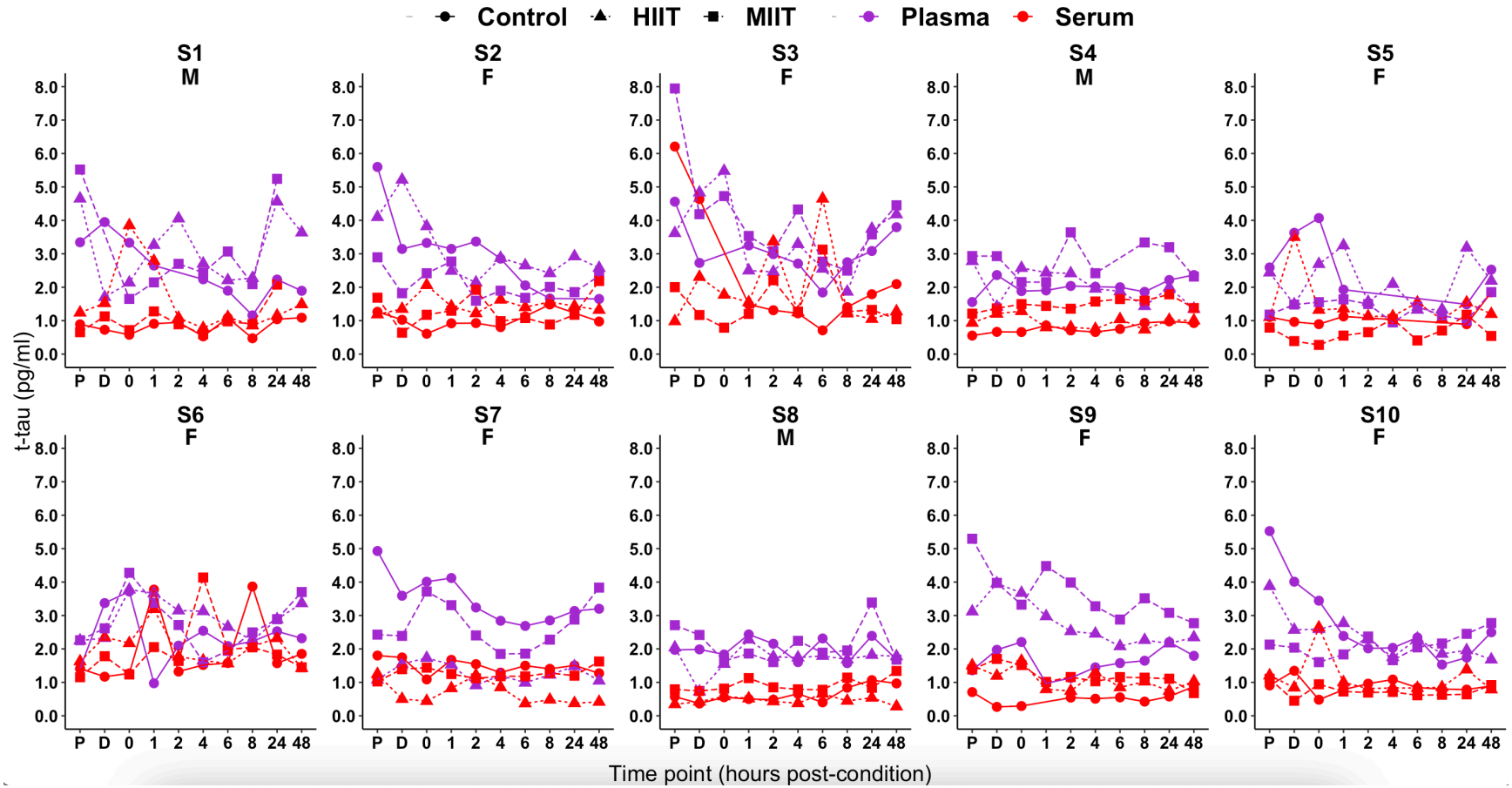

Figure 4.2. Plasma and serum t-tau by individuals.

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. S# denotes a single participant, while M and F denote male and female, respectively.

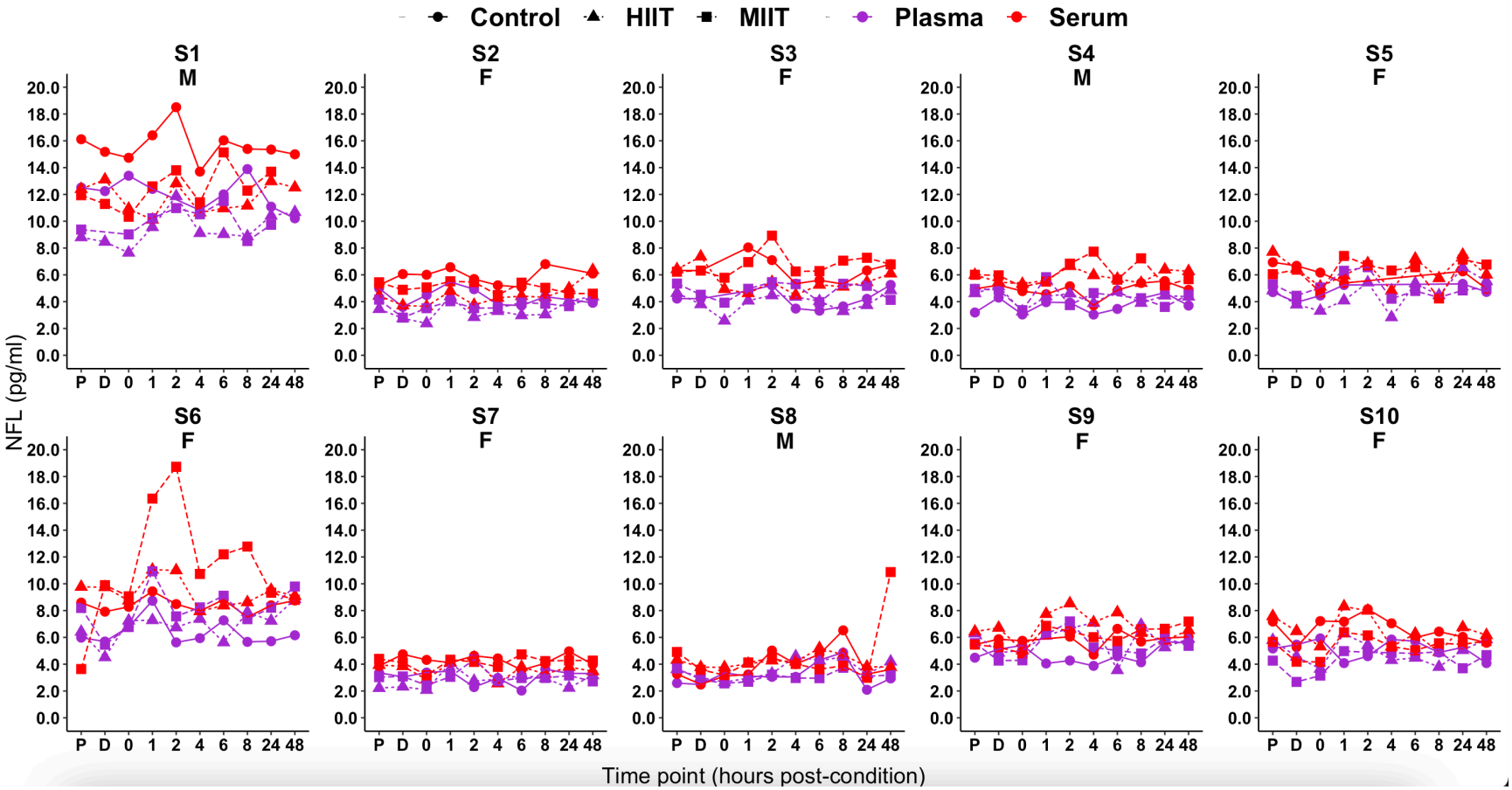

Figure 4.3. Plasma and serum NFL by individuals.

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. S# denotes a single participant, while M and F denote male and female, respectively.

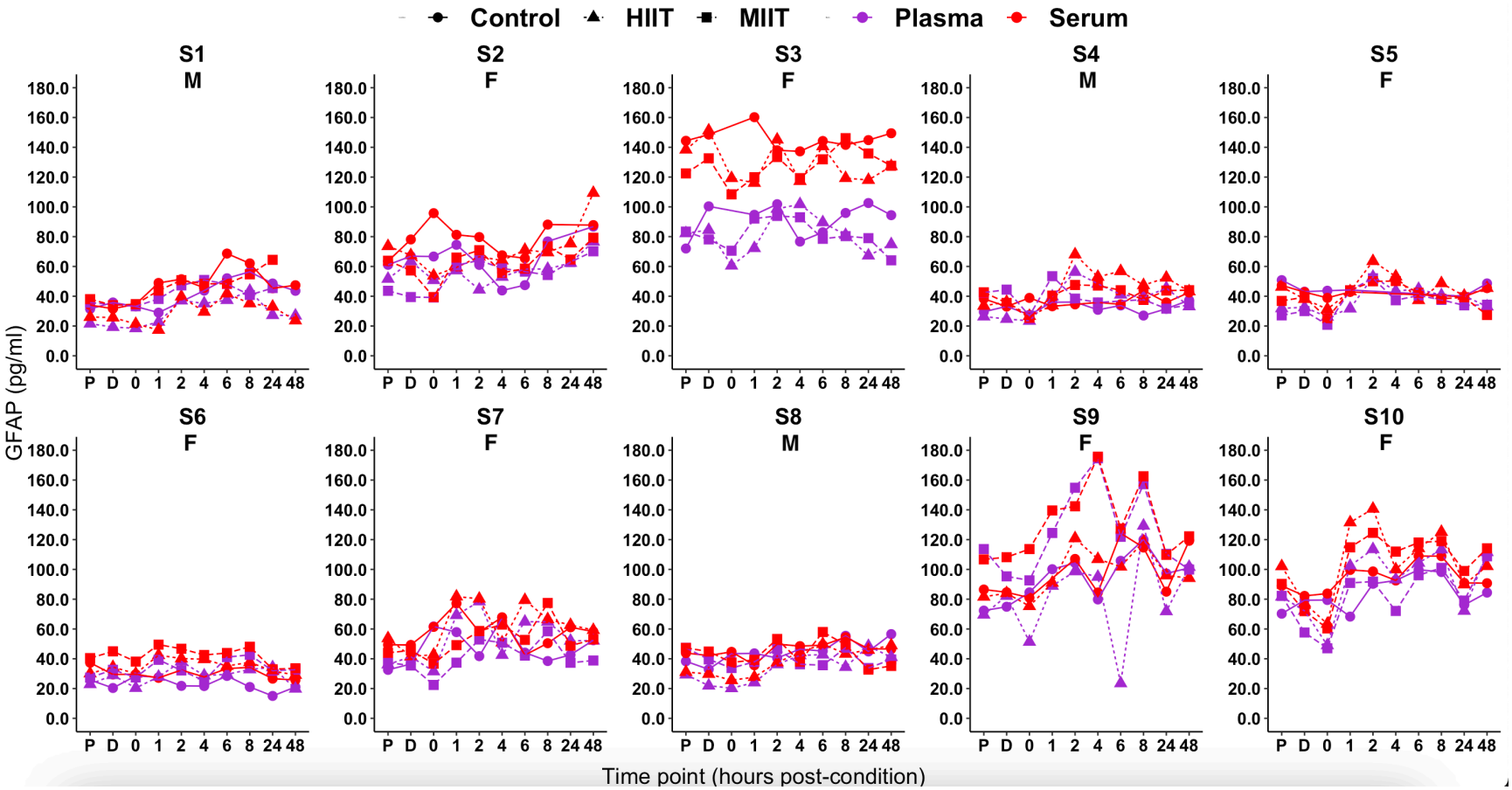

Figure 4.4. Plasma and serum GFAP by individuals.

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. S# denotes a single participant, while M and F denote male and female, respectively.

IV line. Individual plots displaying plasma and serum biomarker concentrations across conditions are shown in Figures 4.2 – 4.4. Spearman correlation coefficients (ρ) and the accompanying p-values for each biomarker by condition are shown in Table 4.1. Significant but fair rank correlations were seen between serum and plasma t-tau across conditions (control, ρ = 0.41, $p < 0.001$; MIIT, $\rho = 0.40$, $p < 0.001$; HIIT, $\rho = 0.51$, $p < 0.001$). Significant and very strong rank correlations were shown between serum and plasma NFL across conditions (control, $\rho = 0.87$, $p < 0.001$; MIIT, $\rho = 0.80$, $p < 0.001$; HIIT, $\rho = 0.88$, $p < 0.001$), and between serum and plasma GFAP across conditions (control, $\rho = 0.92$, $p < 0.001$; MIIT, $\rho = 0.89$, $p < 0.001$; HIIT, $\rho = 0.92$, $p < 0.001$).

Table 4.1. Spearman rank correlations between plasma and serum by condition.

Biomarker	Condition	Correlation (ρ)	p-value
t-tau	Control (n=93)	0.41	<0.001
	MIIT (n=99)	0.40	<0.001
	HIIT (n=100)	0.51	<0.001
NFL	Control (n=93)	0.87	<0.001
	MIIT (n=99)	0.80	<0.001
	HIIT (n=100)	0.88	<0.001
GFAP	Control (n=93)	0.92	<0.001
	MIIT (n=99)	0.89	<0.001
	HIIT (n=100)	0.92	<0.001

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT).

Table 4.2 displays Spearman correlation coefficients and the accompanying p-values for each participant in all conditions to compare with group results. Relative bias between plasma and serum for each biomarker across conditions was calculated and graphically displayed in Figure 4.1, and relative bias by sex and condition was calculated and is shown in Table 4.3. Overall, plasma t-tau concentrations were 48.73% greater than serum t-tau concentrations, while plasma NFL concentrations were 33.33% less than serum NFL concentrations, and plasma

Table 4.2. Spearman rank correlations between plasma and serum by participant.

Biomarker	Participant – Sex	Correlation (ρ)	p-value
t-tau	S1 – M (n=27)	0.08	0.709
	S2 – F (n=29)	0.04	0.827
	S3 – F (n=29)	-0.04	0.843
	S4 – M (n=29)	**0.50**	**0.006**
	S5 – F (n=26)	0.29	0.156
	S6 – F (n=30)	-0.24	0.209
	S7 – F (n=30)	**0.75**	**<0.001**
	S8 – F (n=30)	-0.039	0.836
	S9 – F (n=29)	**0.78**	**<0.001**
	S10 – F (n=30)	0.23	0.214
NFL	**S1 – M (n=27)**	**0.78**	**<0.001**
	S2 – F (n=29)	**0.71**	**<0.001**
	S3 – F (n=29)	**0.61**	**<0.001**
	S4 – M (n=29)	**0.61**	**<0.001**
	S5 – F (n=26)	**0.46**	**0.020**
	S6 – F (n=30)	**0.37**	**0.045**
	S7 – F (n=30)	0.29	0.121
	S8 – F (n=30)	**0.62**	**<0.001**
	S9 – F (n=29)	**0.46**	**0.014**
	S10 – F (n=30)	**0.60**	**<0.001**
GFAP	**S1 – M (n=27)**	**0.84**	**<0.001**
	S2 – F (n=29)	**0.73**	**<0.001**
	S3 – F (n=29)	**0.48**	**0.010**
	S4 – M (n=29)	**0.52**	**0.004**
	S5 – F (n=26)	**0.54**	**0.005**
	S6 – F (n=30)	**0.66**	**<0.001**
	S7 – F (n=30)	**0.86**	**<0.001**
	S8 – F (n=30)	**0.71**	**<0.001**
	S9 – F (n=29)	**0.82**	**<0.001**
	S10 – F (n=30)	**0.80**	**<0.001**

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Significant ($p<0.05$) correlations bolded. S# denotes a single participant, while M and F denote male and female, respectively.

GFAP concentrations were 22.22% less than serum GFAP concentrations. Bland-Altman plots showing agreement between plasma and serum biomarker concentrations are shown in Figure 4.2. A mean difference of 1.36 pg/mL (95% LOA: -0.77, 3.49) was seen for t-tau in plasma compared to serum, while a mean difference of -1.54 pg/mL (95% LOA: -4.16, 1.07) was seen

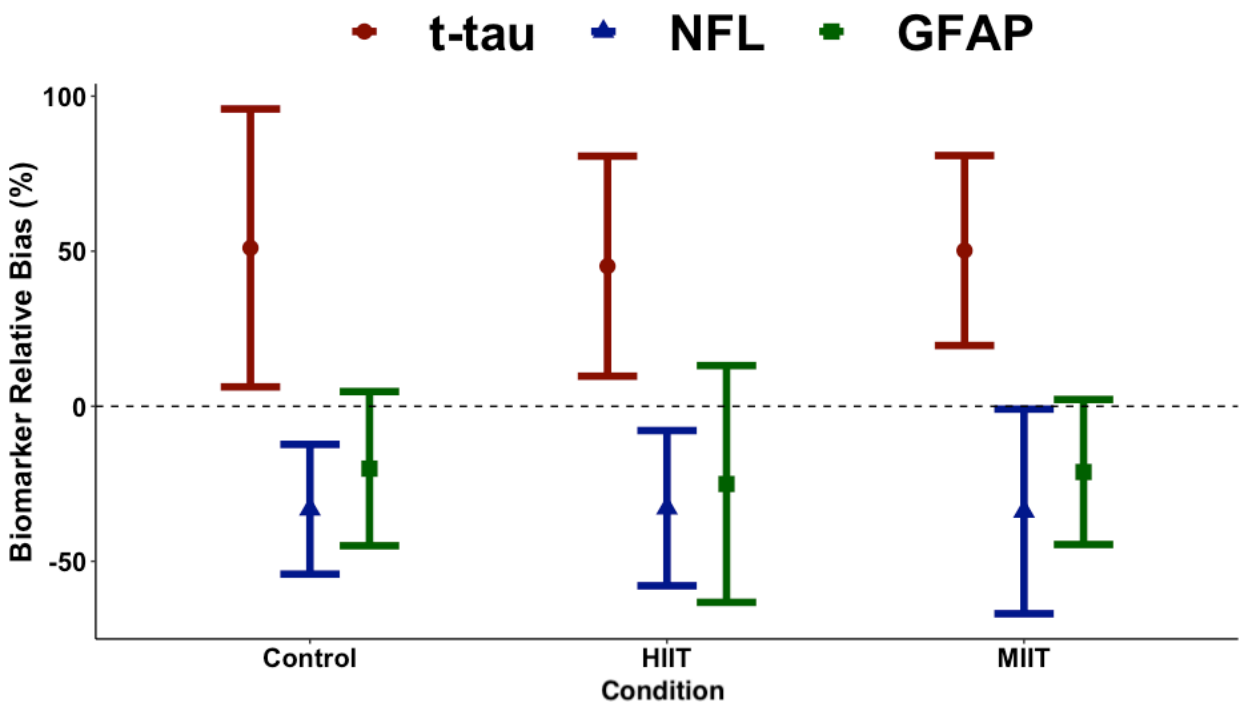

Figure 4.1. Relative bias of each biomarker between plasma and serum across conditions.

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). All relative bias calculations performed using plasma – serum. Point estimate (mean bias) with standard deviation bars shown.

for NFL in plasma compared to serum, and GFAP revealed a mean difference of -11.80 pg/mL (95% LOA: -43.46, 19.86) comparing plasma to serum. Descriptive coefficient of variation analysis by sex and condition is presented in Table 4.4. Overall, 5.17% of plasma t-tau replicate means had a CV greater than twenty percent compared to 12.33% in serum t-tau. For NFL and GFAP, a lower percentage of replicate mean CV's greater than twenty percent was obsereved in serum (NFL, 3.77%; GFAP, 5.82%) compared to plasma (NFL, 10.34%; GFAP, 14.48%).

Table 4.3. Relative bias between plasma and serum by conditions and sex.

Biomarker	Variable	Group	Relative bias	% Absolute relative bias
t-tau	TOTAL		0.4873	48.73%
	Sex	Male (n=86)	0.5738	57.38%
		Female (n=203)	0.4507	45.07%
	Condition	Control (n=92)	0.5105	51.05%
		MIIT (n=97)	0.5021	50.21%
		HIIT (n=100)	0.4516	45.16%
NFL	TOTAL		-0.3333	33.33%
	Sex	Male (n=86)	-0.3123	31.23%
		Female (n=203)	-0.3423	34.23%
	Condition	Control (n=92)	-0.3323	33.23%
		MIIT (n=97)	-0.3392	33.92%
		HIIT (n=100)	-0.3287	32.87%
GFAP	TOTAL		-0.2220	22.22%
	Sex	Male (n=86)	-0.1167	11.67%
		Female (n=203)	-0.2666	26.66%
	Condition	Control (n=92)	-0.2013	20.13%
		MIIT (n=97)	-0.2120	21.20%
		HIIT (n=100)	-0.2507	25.07%

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). All relative bias calculations performed using plasma – serum.

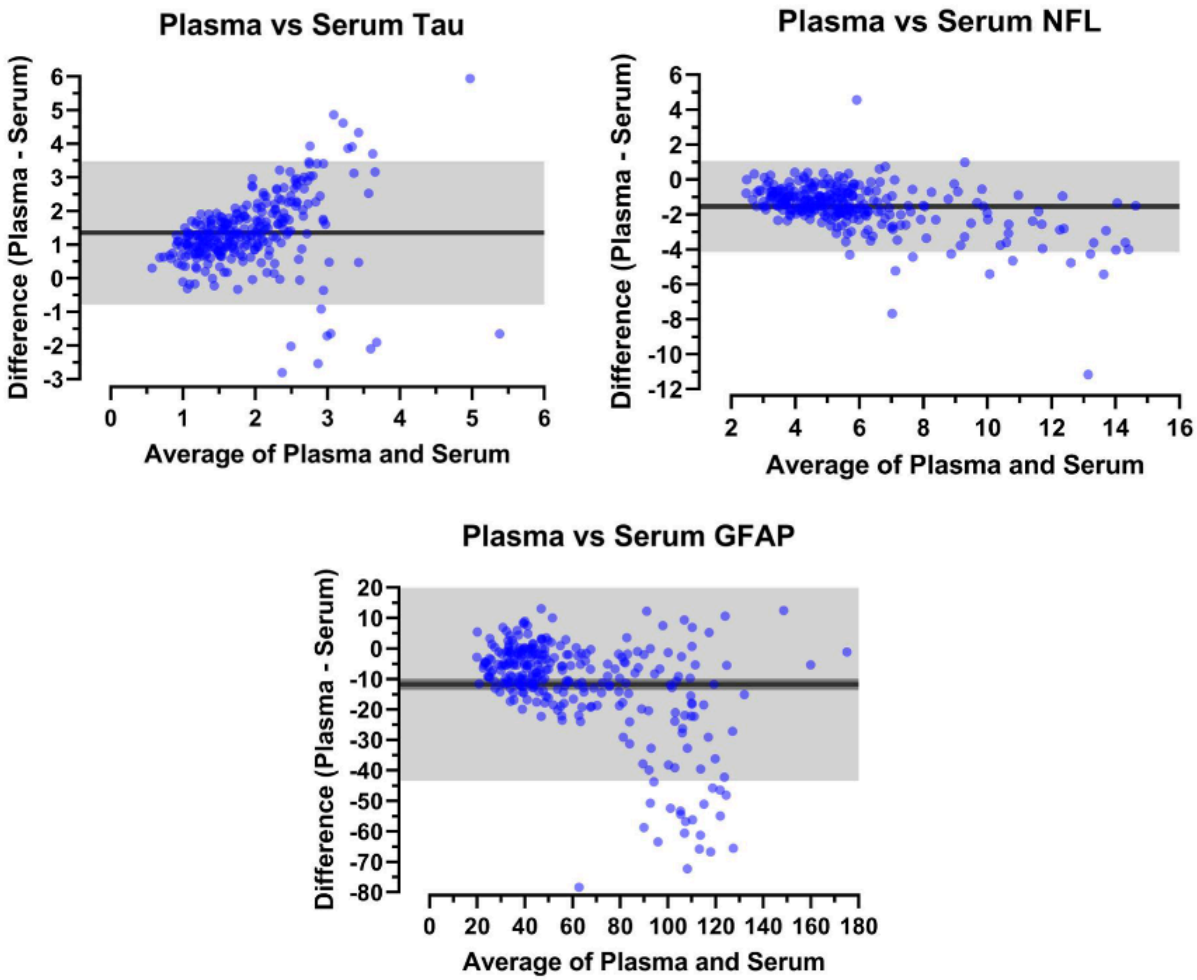

Figure 4.2. Bland-Altman plots displaying agreement between plasma and serum biomarker levels.

All calculations performed using plasma – serum. Axes units are biomarker concentrations in pg/mL.

Table 4.4. Descriptive coefficient of variation analysis comparing plasma and serum replicate means by sex and condition.

Biomarker	Variable	Group	# CV's > 20%		% CV's > 20%	
			Plasma	Serum	Plasma	Serum
t-tau	TOTAL		15	36	5.17%	12.33%
	Sex	Male	6	16	6.98%	17.98%
		Female	9	20	4.41%	9.85%
	Condition	Control	6	15	6.45%	16.13%
		MIIT	5	12	5.15%	12.12%
		HIIT	4	9	4.00%	9.00%
NFL	TOTAL		30	11	10.34%	3.77%
	Sex	Male	9	5	10.47%	5.62%
		Female	21	6	10.29%	2.96%
	Condition	Control	8	3	8.60%	3.23%
		MIIT	11	4	11.34%	4.04%
		HIIT	11	4	11.00%	4.00%
GFAP	TOTAL		42	17	14.48%	5.82%
	Sex	Male	17	8	19.77%	8.99%
		Female	25	9	12.25%	4.43%
	Condition	Control	13	5	13.98%	5.38%
		MIIT	15	2	15.46%	2.02%
		HIIT	14	10	14.00%	10.00%

Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). CV = coefficient of variation

4.4 Discussion

Results show differences exist between plasma and serum concentrations of the biomarkers under examination, and a single interval exercise bout of varying intensity does not appear to influence these differences (Figure 4.1). In t-tau a significant but fair ρ across conditions suggests concentrations in plasma may not be comparable to concentrations in serum, a finding strengthened by an overall relative bias of 48.72% higher concentrations in plasma than serum. In NFL and GFAP significant and very strong ρ across conditions suggests concentrations of these biomarkers in plasma are rank comparable to serum, but 33.33% lower NFL and 22.22% lower GFAP in plasma than serum, along with mixed individual Spearman

results and wide 95% limits of agreement from Bland-Altman analysis suggests further interpretation on the comparability of these matrices in the context of research and future clinical use is required.

4.4.1 t-tau.

Figure 4.2 displays plasma and serum t-tau concentrations across sampling timepoints by condition. Though some individual plasma and serum t-tau concentrations overlap across conditions at specific timepoints (e.g., S3 and S6), a clear pattern is seen where plasma concentrations are consistently higher than serum concentrations. This is confirmed by the 48.73% overall increase in plasma t-tau compared to serum t-tau, with a narrow range across exercise conditions between 45.16% - 51.05% (Table 4.3). Within the context of Spearman's ρ being fair across all conditions, these relative bias results suggest descriptive and inferential statistics to examine biomarker associations would not be the same between plasma and serum, and exercise appears to have no effect on these potential differences. Additionally, Bland-Altman analysis (Figure 4.2) showing comparatively wide 95% limits of agreement between plasma and serum t-tau at -0.77 to 3.49 pg/mL is in-line with these results, adding credence to the notion serum and plasma t-tau measurements should not be viewed as interchangeable. There also appears to be a larger bias in males (57.38%) than females (45.07%), though it's likely the smaller male sample size might skew this bias considering S1 appears more variable than S4 and S8 (Figure 4.2). Additionally, more replicate means had CV's greater than 20 percent in serum (12.33%) than in plasma (5.17%); a pattern that remained true across sex and conditions (Table 4.4), suggesting measurements in plasma are more precise than measurements in serum.

Taken together, this highlights a crucial research need to analyze t-tau in a single matrix across studies so comparisons can be made to better characterize the effects of injury on this

marker. A heavily studied candidate biomarker for Alzheimer's disease (AD) and in-line with the current results, t-tau has previously shown to be more accurately measured in plasma compared to serum (O'Connell et al., 2019). Additionally, plasma t-tau has shown to be elevated in AD patients compared to cognitively normal controls, lending credence to the use of plasma when examining brain disease and injury associations with t-tau (Mattsson et al., 2016; Mielke et al., 2018). However, investigations examining t-tau in the sport-related concussion literature continue to measure t-tau in both plasma and serum (Asken et al., 2018; Di Battista, Rhind, et al., 2018; McCrea et al., 2020). In this regard, consensus concerning the appropriate matrix to study t-tau in the context of future clinical diagnostic or prognostic use for SRC is warranted. Among considering precision of measurement as an accurate reflection of the physiological state, ease and time required to process should also be considered in this context. Plasma does not require time to allow for clotting, which would effectively reduce processing time for diagnostic or prognostic tests (Lima-Oliveira et al., 2018). Though minimal, the absence of clotting time would allow development of faster turn-around diagnostic or prognostic modalities, having the largest impact on future sideline testing modalities. Considering this and the results of the present study, it is suggested blood measurements of t-tau should be analyzed in plasma across investigations moving forward towards development of objective markers for SRC.

It is important to note the developing shift from investigations examining associations between brain injury or disease and t-tau to phosphorylated tau (p-tau), specifically in investigations of Alzheimer's disease (AD). Though t-tau has been shown elevated in those with AD compared to cognitively normal controls (Mielke et al., 2018), plasma t-tau has shown weak correlation with cerebrospinal fluid (CSF) t-tau suggesting the majority of plasma t-tau comes from peripheral sources (Z. Chen et al., 2019; Fossati et al., 2019). In contrast, p-tau appears

more specific to AD pathology as post-translational phosphorylation can result in misfolding and subsequent formation of toxic oligomers (Mankhong et al., 2022). The apparent association between plasma p-tau and AD pathology suggests the influence of extracranial sources may be mitigated when analyzing p-tau compared to t-tau, warranting future research regarding associations between plasma p-tau and SRC.

4.4.2 NFL.

Plasma and serum NFL concentrations across sampling timepoints by condition are displayed in Figure 4.3. Though it appears plasma and serum NFL concentrations overlap at timepoints across conditions in all participants, serum concentrations generally appear higher than plasma concentrations in contrast with t-tau. This is confirmed by the 33.33% overall increase in serum NFL compared to plasma NFL, with narrow ranges across exercise conditions. Spearman correlation results revealing very strong correlations across exercise conditions (Table 4.3) suggests descriptive and inferential statistics to examine biomarker associations would be the same or similar between plasma and serum across exercise conditions. In-line with the present group results, prior investigations comparing plasma and serum found NFL to be highly equivalent and comparable across matrices (O'Connell et al., 2019). However, the current crossover cohort study design enabled individual analysis of Spearman's ρ across conditions to describe comparability of plasma and serum at the individual level, which demonstrates inconsistent results (Table 4.2).

Some individuals showed moderately strong Spearman correlation between serum and plasma across exercise conditions while others showed fair Spearman correlation, and none showed very strong Spearman correlation between serum and plasma (Table 4.2). These mixed results reveal serum and plasma NFL correlation appears to vary in healthy individuals,

suggesting comparability between matrices might be different from one individual to another. Comparatively wide 95% limits of agreement between plasma and serum NFL at -4.16 to 1.07 pg/mL (Figure 4.2) further suggests significant matrix differences that question interchangeability of matrices, despite very strong rank correlation results. As such, group Spearman results showing very high correlation across conditions should be interpreted with caution. Considering the correlational variability at the individual level between plasma and serum and wide 95% limits of agreement, it is unlikely these matrices can be considered interchangeable for NFL analysis in a healthy population. Therefore, consensus regarding the appropriate matrix used to study NFL in the context of SRC is warranted.

The descriptive CV analysis revealed more replicate CV's above twenty percent in plasma (10.34%) than in serum (3.77%), suggesting measurement of serum NFL to be slightly more precise than measurements of plasma NFL (Table 4.4). However, this pragmatic assessment is unable to pinpoint the physiological determinants relating to this potential precision difference, so it is unclear which matrix is a more accurate reflection of the physiological state. Research in the AD domain has historically measured plasma NFL opposed to serum NFL despite the higher serum concentrations than plasma (Li & Mielke, 2019). Importantly, rates of change in plasma NFL have been found to correlate with rates of change in cognition and established CSF markers of AD, suggesting plasma to be a suitable candidate matrix for assessing NFL in brain disease or injury states (Mattsson et al., 2019; Mielke et al., 2019). Additionally, although the descriptive CV analysis shows NFL measurements in serum might be more precise than in plasma, the future application of this biomarker involving diagnostic or prognostic use for SRC will likely involve a panel of biomarkers. In this case, it would be pragmatic to analyze a single matrix with respect to time and ease of sampling

limitations, provided each biomarker is reflective of the physiological state in such matrix. Should t-tau be included in such panel of biomarkers, it would be prudent to analyze plasma NFL with the understanding plasma t-tau may be a better reflection of the physiological state than serum t-tau (O'Connell et al., 2019). In combination with results showing plasma NFL correlating with rates of cognitive decline in AD (Mattsson et al., 2019; Mielke et al., 2019), it is suggested investigations analyzing NFL in the context of SRC should use plasma in an effort to harmonize matrix analyses across SRC investigations to better characterize the effects of injury on this marker.

4.4.3 GFAP.

Figure 4.4 displays plasma and serum GFAP concentrations at all timepoints across conditions. Like NFL, plasma and serum GFAP concentrations overlap at timepoints across conditions in all participants except one (S3), but serum concentrations appear generally higher than plasma concentrations (Figure 4.4). Relative bias results show an overall increase in serum GFAP of 22.22% compared to plasma, which remained similar across conditions (control = 20.13%, MIIT = 21.20%, HIIT = 25.07%) (Table 4.2). Females showed a higher bias at 26.66% compared to males at 11.67%, though future research is required to understand this potential difference as the current study is limited in male participants (n = 3) (Table 4.2). Considering these results with very strong Spearman's ρ across all conditions (Table 4.1) suggests descriptive and inferential statistics to examine GFAP associations would be the same or similar between plasma and serum across exercise conditions. However, individual analysis of Spearman's ρ across conditions to describe comparability of plasma and serum at the individual level demonstrates inconsistent results (Table 4.2).

In reference to Table 4.2, four individuals had very strong (> 0.80) Spearman correlation between plasma and serum, three individuals had moderately strong (> 0.60) Spearman correlation, and three individuals had fair (> 0.30) Spearman correlation (Chan, 2003). Like with NFL, this individual variation in correlation between plasma and serum GFAP suggests these matrices may not be interchangeable for GFAP analysis in a healthy population. Additionally, the Bland-Altman plot (Figure 4.2) revealed another comparatively wide 95% limits of agreement between plasma and serum GFAP at -43.46 to 19.86 pg/mL, questioning interchangeability of matrices despite very strong rank correlation results. As such, consensus regarding the appropriate matrix used to study GFAP in the context of SRC is necessary.

Descriptive CV analysis revealed less replicate CV's above twenty percent in serum (5.82%) than in plasma (14.48%), suggesting measurement of serum GFAP is slightly more precise than measurements of plasma GFAP (Table 4.4). Still, this pragmatic interpretation is unable to identify the physiological determinants relating to this potential precision difference, so it is unclear which matrix is a more accurate reflection of the physiological state. A prior investigation comparing serum and plasma GFAP following mTBI in older adults found despite increased concentrations in serum compared to plasma, the matrices were highly correlated and both able to discriminate those with and without intracranial CT abnormalities following mTBI (Huebschmann et al., 2020). This suggests both plasma and serum to be suitable candidate matrices for assessing GFAP in brain disease or injury states. However, it remains important to consider the future application of biomarkers for diagnostic or prognostic use for SRC will likely involve a panel of biomarkers. In this sense it is pragmatic to analyze a single matrix with respect to time and ease of sampling limitations, provided GFAP is reflective of the physiological state in said matrix. Should t-tau be included in such panel of biomarkers, it would be logical to

analyze plasma GFAP with the understanding plasma t-tau may be a better representation of the physiological state than serum t-tau (O'Connell et al., 2019). However, future research is necessary to establish which matrix is the most accurate reflection of blood GFAP concentrations, moving towards synchronization of matrix analyses across SRC investigations to better characterize the effects of injury on this marker.

4.4.3 Limitations.

There remain some limitations in the present study. Though descriptive CV analysis of replicate means allows pragmatic assessment of the precision of SIMOA measurements in each matrix, it does not allow inference into physiological underpinnings relating to the presentation of a biomarker in each matrix. Additionally, this study intended to analyze sex differences, but only three male participants made this difficult beyond descriptive differences. Finally, the assay technique and use of means of replicates for Spearman's analysis may introduce bias in the results. Though SIMOA technology is marketed as an ultra-sensitive method to obtain biomarker concentrations, the comparatively small concentrations of these injury markers in a population of healthy young adults may introduce noise in the measurements. In this sample all replicates for t-tau, NFL, and GFAP were above the lower limits of quantification, but some replicate values were still markedly different. Future investigations should consider modelling analyses using both replicate concentrations to minimize the potential bias introduced using means of replicates.

3.5 Conclusion

A single bout of moderate and high intensity interval exercise does not appear to influence differences between plasma and serum in any of the biomarkers under study in this healthy young adult sample. Total tau showed fair rank correlation across exercise conditions with plasma concentrations consistently higher than serum concentrations, while GFAP and NFL

showed very strong rank correlation across exercise conditions with serum concentrations slightly higher than plasma concentrations. These results alone agree with a prior investigation showing t-tau consistently lower in serum than plasma, and NFL and GFAP appearing highly equivalent between serum and plasma (Huebschmann et al., 2020; O'Connell et al., 2019). However, the individual analysis revealed varying correlations between plasma and serum NFL and GFAP in a healthy young adult sample, and the Bland-Altman analysis showed comparatively wide 95% limits of agreement between plasma and serum concentrations of all biomarkers, suggesting these matrices should not be viewed as interchangeable in a healthy adult population. This highlights a crucial need for researchers studying blood biomarkers of SRC to come to a consensus on a single matrix to analyze each neuro injury biomarker across studies to better characterize these markers in the context of exercise and injury. Only then can the interplay of injury and exercise be understood as a step forward to the clinical validation of these objective markers of injury. Future research should target SRC populations to better understand potential matrix differences following injury. This will enable more robust characterization injury differences between matrices, moving the field one step closer to clinical validation of objective physiological markers for the subjectively reliant diagnoses and prognoses accompanying sport-related concussion.

Chapter 5: Conclusions and Future Directions

Given the heavy reliance on subjective symptom reporting and clinical judgement for diagnosis and prognosis of sport-related concussion (SRC), there have been increased research efforts to establish objective markers of injury to supplement clinical decisions. However, the effects of interval exercise on some of the most common biomarkers studied for SRC have not been adequately addressed considering the inherent nature of exercise in sport. Therefore, the objectives of this thesis were to: 1) review and discuss the relevant literature regarding commonly studied blood biomarkers of traumatic brain injury (TBI), specifically mTBI and SRC, and the potential influence of exercise; 2) explore the effects of varying intensity interval exercise bouts on t-tau, NFL, GFAP, and UCH-L1 concentrations in serially collected plasma samples; and 3) explore potential differences between plasma and serum concentrations of these blood biomarkers following exercise.

5.1 Chapter Summaries

5.1.1 Chapter 2: Literature review.

The relevant literature concerning the current state of research in biomarkers for SRC and the potential effects of exercise were reviewed in Chapter 2. SRC was defined as an mTBI according to the 5th international consensus statement on concussion in sport (McCrory et al., 2017), along with estimates of SRC injury rates and burden in youth and adults (Black et al., 2021; Gordon & Kuhle, 2020). Current standards of SRC clinical care were outlined, highlighting the emphasis on subjective symptom reporting and interpretation, lending credence to the ongoing investigations of objective blood biomarkers for SRC (McCrory et al., 2017; Zetterberg & Blennow, 2016). Blood biomarkers were defined as measurable indicators of a biological state in blood and reviewed in relation to cerebrospinal fluid markers and the

restrictive nature of the BBB (Daneman & Prat, 2015; Rifai et al., 2006). The blood brain barrier (BBB) was also reviewed in the context of SRC, exercise, and sleep, due to the potential influence these factors may have on CNS derived biomarkers into circulation (Chodobski et al., 2011; Pan & Kastin, 2017; Roh et al., 2017). Blood biomarkers total tau (t-tau), NFL, GFAP, and UCH-L1 were highlighted as commonly studied markers showing promise in the ability to distinguish SRC from various controls despite mixed results (Di Battista, Rhind, et al., 2018; McCrea et al., 2020; Papa et al., 2012; Shahim, Gren, et al., 2016; Welch et al., 2016). Additionally, two studies specifically examining the effects of exercise on some of these markers were discussed (Di Battista, Moes, et al., 2018; Shahim et al., 2018). Serum and plasma were defined as distinctive matrices due to differences in post-draw biochemical processes, and considered in the context of the blood biomarkers under investigation in this thesis (Plebani et al., 2020; Yu et al., 2011). Finally, the limitations pertaining to blood biomarker research for SRC were discussed, specifically highlighting the potential influence of exercise and non-CNS derived biomarker sources contributing to the variation in blood. Overall, the current literature suggests blood biomarkers may serve as objective measures to supplement clinical decisions regarding SRC diagnosis or prognosis in the future, however further work is required to overcome numerous limitations.

5.1.2 Chapter 3: Effects of interval exercise on commonly studied blood biomarkers associated with sport-related concussion.

Chapter 3 examined the effects of varying intensity interval exercise bouts (control, moderate intensity interval training [MIIT], and high intensity interval training [HIIT]) on a panel of biomarkers (t-tau, NFL, GFAP, UCH-L1) in serially collected plasma samples using a randomized crossover cohort study design. In a sample of 10 healthy university students (ages

19-26), there were no significant differences across serial timepoints comparing MIIT and HIIT conditions to the control condition for plasma t-tau. However, a significant effect of time on t-tau was found throughout all three conditions at 6- and 8-hours post-condition compared to pre-condition (6 hours: β = -1.050, 95%CI: -1.875 - -0.225, p = 0.013; 8 hours: β = -1.141, 95%CI: -1.966 - -0.315, p = 0.007), suggesting plasma t-tau concentrations decline throughout the day. Additionally, significant increases in plasma t-tau were seen at all timepoints with each increasing year of age (β = 0.128, 95%CI: 0.007 - 0.249, p = 0.040). In NFL and GFAP, a significant effect of MIIT (NFL: β = -1.002, 95%CI: -1.852 - -0.152, p = 0.021; GFAP: β = -14.750, 95%CI: -27.154 - -2.345, p = 0.020) and HIIT (NFL, β = -1.414, 95%CI: -2.263 - -0.564, p = 0.001; GFAP, β = -20.956, 95%CI: -33.358 – -8.554, p = 0.001) was observed in the sampling timepoint immediately following exercise conditions compared to the control condition, suggesting a short-lived decrease in these plasma biomarkers immediately following interval exercise that returned to control levels by the 1-hour post condition timepoint. Results for NFL also revealed a significant effect of sleep, where greater total sleep (minutes) in the night prior to all conditions resulted in decreased NFL concentrations at all timepoints (β = -0.003, 95%CI: -0.005 - -0.001, p = 0.012). UCH-L1 was dropped from statistical analysis due to a large proportion (>30%) of replicates falling below the lower limits of quantification (9.38 pg/mL) or detection (1.90 pg/mL) (Quanterix, 2022).

5.1.3 Chapter 4: Commonly studied blood serum and plasma biomarkers for sport-related concussion: Does matrix matter?

Using data from the same randomized crossover cohort study as in Chapter 3, Chapter 4 explored differences between plasma and serum concentrations of t-tau, GFAP, NFL, and UCH-L1 after varying intensity interval exercise bouts in serial samples. In the same sample, plasma t-

tau showed significant but fair Spearman rank correlations with serum t-tau across the three conditions at serial timepoints (control, $\rho = 0.41$, $p < 0.001$; MIIT, $\rho = 0.40$, $p < 0.001$; HIIT, $\rho = 0.51$, $p < 0.001$), suggesting plasma concentrations may not be comparable to serum concentrations. Plasma NFL and GFAP showed significant Spearman rank correlations with their serum counterparts across the three conditions at serial timepoints (NFL, control, $\rho = 0.87$, $p < 0.001$; MIIT, $\rho = 0.80$, $p < 0.001$; HIIT, $\rho = 0.88$, $p < 0.001$; GFAP, control, $\rho = 0.92$, $p < 0.001$; MIIT, $\rho = 0.89$, $p < 0.001$; HIIT, $\rho = 0.92$, $p < 0.001$), suggesting plasma and serum concentrations are comparable. However, individual Spearman results for NFL varied across individuals from fair to moderately strong and varied across individuals from fair to very strong for GFAP. This inter-subject variability in Spearman correlations between these markers in serum and plasma suggests these matrices should not be viewed as interchangeable and highlights the importance of harmonizing biomarker analysis across studies in a single matrix to better characterize the associations of injury and exercise on these markers. UCH-L1 was dropped from statistical analysis due to a large proportion (>30%) of replicates falling below the lower limits of quantification (9.38 pg/mL) or detection (1.90 pg/mL) (Quanterix, 2022).

5.2 Limitations

The importance of addressing the current limitations in the field of blood biomarkers for SRC cannot be understated. While this thesis project attempted to address the potential influence of exercise on these injury markers in healthy individuals, the added variation by non-CNS derived sources of these biomarkers remains a major limitation. If these biomarkers sources are predominantly extra-cranial, an association between brain injury and blood presentation of these markers could not be established. This is especially true in the cases of t-tau, NFL, and UCH-L1 where extra-cranial sources are known (F. Chen et al., 2010; Gu et al., 1996; Lépinoux-

Chambaud & Eyer, 2013; Morris et al., 2011). Adding to this associative uncertainty, proteins in blood are broken down by proteases and filtered by the renal system, so measured biomarkers at any given time may be more reflective of these biological filtering processes than brain injury, especially at low concentrations in healthy individuals. Additionally, the influences of sleep and sex on these blood biomarkers has not been adequately addressed to date. A significant effect of a single night of sleep prior to conditions on the presentation of NFL (Chapter 3) highlights an important variable to study in future investigations of biomarkers for SRC, especially considering sleep/wake disturbances are a common symptom of concussion (McCrory et al., 2017). Moreover, the limited number of males in the current study as opposed to females made analysis of sex differences difficult. As evidence suggests physiological differences exist between males and females in the context of concussion (e.g., females higher risk of sustaining concussion, females may have worse outcomes compared to males), sex differences in biomarker presentation following injury and exercise requires further exploration (Koerte et al., 2020).

The assay technique and use of means of replicates for analysis may introduce bias in the results. Although SIMOA is marketed as an ultra-sensitive method to obtain biomarker concentrations, the comparatively small concentrations of these injury markers in a population of healthy young adults results in a high amount of noise in the measurements. This is especially true in the case of UCH-L1, where over 30% of the replicates were below the lower limit of quantification. Though a significant majority of replicates for t-tau, NFL, and GFAP were well above the lower limits of quantification, some replicate values were markedly different. Future analyses should consider the inclusion of both replicate concentrations in a modelling approach to eliminate this potential bias and better characterize the associations regarding biomarker concentrations.

Considering results from Chapter 4 showed differences between plasma and serum concentrations of these biomarkers, the first step towards addressing these limitations remains harmonization of sampling and processing methodologies across studies in this field. This will allow comparisons between studies examining associations of blood biomarkers and SRC to enable more robust characterization of the effect of injury and exercise on these markers, contributing towards development of objective tests to supplement clinical diagnosis and prognosis.

5.3 Future Directions and Key Messages

Beyond continuing to address the limitations in blood biomarker research for SRC mentioned above, this thesis project emphasises additional avenues for exploration of the effects of exercise on these markers in different age and injury groups. The current sample included only healthy adult individuals without a prior history of diagnosed concussions to explore the effects of exercise on biomarkers commonly studied in SRC. In Chapter 3, results showed short-lived decreases in NFL and GFAP immediately following exercise, and a decline in t-tau throughout the day across conditions. It's plausible the interplay between injury and exercise might result in blood biomarker changes not seen in the present study comprising only healthy individuals. In the context of NFL and GFAP, this provides an avenue for immediate next steps to examine the effects of interval exercise and on these markers in a sample with a history of concussion, and potentially a group with concussion considering modifications to the intensity of exercise. In-line with current results, these future investigations should target two or three sampling timepoints in the minutes to hours following exercise, reducing the costs associated with ten serial sampling timepoints in the present study. For t-tau, an immediate next step could be determining healthy and injured reference ranges at specific times throughout the day to mitigate the potential impact

diurnal variation may have when sampled at different times of day following injury. This is key in the context of current results suggesting timing of day should be taken into consideration when sampling to obtain t-tau concentrations. Another important next step is to examine how sex hormones may influence these associations, as estradiol and progesterone have shown to possess anti-inflammatory and BBB strengthening properties (Bazarian et al., 2010). Additionally, these effects may be different in a youth sport population compared to young adults due to growth and development considerations (Giedd et al., 2015) and future research considering the youth population is recommended.

Research examining the effects of exercise on these markers in injured and youth populations is necessary before inferences can be made regarding how these effects may be important to consider in future objective diagnostic or prognostic tests for concussion. Regardless, conjecture can be made within context of the current results. The absence of a significant association between exercise and t-tau concentrations in the current sample suggests sampling blood for SRC diagnosis or prognosis in the future could be performed immediately following the injury without apprehension of the influence of timing and levels of exercise on t-tau presentation. However, the decline in t-tau throughout a single day suggests samples taken towards the end of day may not be an accurate reflection of t-tau compared to the beginning of the day. As such, timing of sample collection should be considered in future applications for SRC including t-tau as a marker. In NFL and GFAP, the brief decline in concentrations immediately following interval exercise may have implications if these injury markers were found to be elevated in the minutes to hours following injury. In this case, a transient decrease in NFL or GFAP immediately following SRC (and the inherent exercise) might result in artificially lower concentrations that could lead to a misdiagnosis if this drop crosses cut-off points yet to be

established. Although NFL and GFAP have been found elevated in concussed individuals in the minutes to hours following injury (Gul et al., 2017; Shahim, Gren, et al., 2016), peak GFAP levels are seen in the 24-48 hours following injury (Papa et al., 2016), and NFL has shown biphasic peaks at 1 hour and 10 days post-SRC (Shahim, Gren, et al., 2016). Considering this in context with current results, sampling NFL for SRC diagnosis or prognosis in the future may require careful attention of the timing and levels of exercise if sampled immediately following injury and exercise, but it would be expected the transient decrease in GFAP following exercise would not influence the 24-48 hour post-injury peak. Again, with the lack of evidence in both youth and injury groups these conclusions are mostly speculative in the current state.

Results from Chapter 4 showing fair rank correlation between serum and plasma concentrations at the group level for t-tau, and at the individual level for NFL and GFAP, highlights an important key message for future research in biomarkers for SRC. It is currently difficult to make comparisons across studies in this field due to inconsistent assay methodologies, particularly the use of either plasma or serum matrices for biomarker assays. Although Spearman correlation results suggest some of these biomarkers might be comparable, consensus and harmonization of methodologies across studies is vital to further understanding the mechanisms and associations of these biomarkers in the context of injury and exercise. This will enable much more robust characterization of the associations between injury and the biomarkers, ultimately moving the field towards clinical validation of biomarkers to supplement diagnostic or prognostic decisions. Further, common methods and fluid biomarker outcomes in the field will facilitate opportunities for combining data across studies (e.g., meta-analyses).

5.4 Conclusions

This thesis project demonstrates interval exercise has a transient effect in healthy individuals on some blood biomarkers previously associated with SRC, which may be important to consider in the future applications of objective biomarkers for SRC diagnosis or prognosis. Future research is necessary to disentangle the effects of injury and exercise on these markers in a youth and adults, considering the influence of sex and sleep. Methodology harmonization across investigations remains vital, specifically consensus of matrices used to assay certain biomarkers. This will enable better characterizations of the associations between injury, exercise, and the biomarkers, hopefully leading to the clinical validation of objective biomarkers to supplement clinical SRC diagnosis and prognosis.

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APPENDIX B: 2021 PAR-Q +

2021 PAR-Q+

The Physical Activity Readiness Questionnaire for Everyone

The health benefits of regular physical activity are clear; more people should engage in physical activity every day of the week. Participating in physical activity is very safe for MOST people. This questionnaire will tell you whether it is necessary for you to seek further advice from your doctor OR a qualified exercise professional before becoming more physically active.

GENERAL HEALTH QUESTIONS

Please read the 7 questions below carefully and answer each one honestly: check YES or **NO.**	**YES**	**NO**
1) Has your doctor ever said that you have a heart condition ☐ **OR** high blood pressure ☐?	☐	☐
2) Do you feel pain in your chest at rest, during your daily activities of living, **OR** when you do physical activity?	☐	☐
3) Do you lose balance because of dizziness **OR** have you lost consciousness in the last 12 months? Please answer **NO** if your dizziness was associated with over-breathing (including during vigorous exercise).	☐	☐
4) Have you ever been diagnosed with another chronic medical condition (other than heart disease or high blood pressure)? **PLEASE LIST CONDITION(S) HERE:** _____	☐	☐
5) Are you currently taking prescribed medications for a chronic medical condition? **PLEASE LIST CONDITION(S) AND MEDICATIONS HERE:** _____	☐	☐
6) Do you currently have (or have had within the past 12 months) a bone, joint, or soft tissue (muscle, ligament, or tendon) problem that could be made worse by becoming more physically active? Please answer **NO** if you had a problem in the past, but it **does not limit your current ability** to be physically active. **PLEASE LIST CONDITION(S) HERE:** _____	☐	☐
7) Has your doctor ever said that you should only do medically supervised physical activity?	☐	☐

If you answered NO to all of the questions above, you are cleared for physical activity.
Please sign the PARTICIPANT DECLARATION. You do not need to complete Pages 2 and 3.

- Start becoming much more physically active – start slowly and build up gradually.
- Follow Global Physical Activity Guidelines for your age (https://www.who.int/publications/i/item/9789240015128).
- You may take part in a health and fitness appraisal.
- If you are over the age of 45 yr and NOT accustomed to regular vigorous to maximal effort exercise, consult a qualified exercise professional before engaging in this intensity of exercise.
- If you have any further questions, contact a qualified exercise professional.

PARTICIPANT DECLARATION

If you are less than the legal age required for consent or require the assent of a care provider, your parent, guardian or care provider must also sign this form.

I, the undersigned, have read, understood to my full satisfaction and completed this questionnaire. I acknowledge that this physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if my condition changes. I also acknowledge that the community/fitness center may retain a copy of this form for its records. In these instances, it will maintain the confidentiality of the same, complying with applicable law.

NAME _____ DATE _____

SIGNATURE _____ WITNESS _____

SIGNATURE OF PARENT/GUARDIAN/CARE PROVIDER _____

If you answered YES to one or more of the questions above, COMPLETE PAGES 2 AND 3.

Delay becoming more active if:

- You have a temporary illness such as a cold or fever; it is best to wait until you feel better.
- You are pregnant - talk to your health care practitioner, your physician, a qualified exercise professional, and/or complete the ePARmed-X+ at www.eparmedx.com before becoming more physically active.
- Your health changes - answer the questions on Pages 2 and 3 of this document and/or talk to your doctor or a qualified exercise professional before continuing with any physical activity program.

 1 / 4

01-11-2020

APPENDIX C: PARTICIPANT CONSENT FORM

UNIVERSITY OF CALGARY
CONSENT TO PARTICIPATE IN RESEARCH

TITLE: Effects of exercise on potential blood biomarkers of sport-related concussion

SPONSOR: The University of Calgary

FUNDER: University of Calgary Kinesiology startup funds and SHRed Concussions

INVESTIGATORS:

Primary Investigator: Dr. Jonathan Smirl, PhD

Co-Investigators: Dr. Carolyn Emery PhD; Dr. Chantel Debert MD; Dr. Cheryl Wellington PhD; Dr. Olivia Galea PhD (Post-doctoral Scholar); Joel Burma MSc (PhD Student); Lauren Miutz MSc (PhD Candidate); Courtney Kennedy HBK (MSc Student); Joseph Carere BK (MSc Student)

Contact Information: (780) 263-1373 or cerebrovascular.lab@ucalgary.ca

INTRODUCTION

Dr. Jonathan Smirl, and associates from the Faculty of Kinesiology and Cummings School of Medicine at the University of Calgary are conducting a research study.

This consent form is only part of the process of informed consent. It should give you the basic idea of what the research is about and what your participation will involve. Please ask if you would like more information about something not mentioned here. Take the time to read this carefully and to understand the associated information. You will receive a copy of this form for your records.

You were identified as a possible participant in this study because you identified as a young healthy individual who:

1) are between the ages of 18-25 years,
2) have no history of diagnosed concussion(s),
3) and are able to perform a maximal exercise protocol. Your participation in this research study is voluntary.

The aims of this study are:

1) Explore biomarker changes over time after different intensity exercise bouts
2) Explore differences between sexes in biomarker changes over time after exercise

WHY IS THIS STUDY BEING DONE?

This study is exploring how commonly studied blood biomarkers of concussion are affected by exercise alone in healthy individuals. If you agree to take part, you will perform a maximal exercise test at the start of testing during a 1-hour lab session. You will be invited to come back on three other days that take roughly 9 hours each, separated by ~4 weeks, and 6 days for a brief (15 minutes) blood draw. On the longer study days, you will perform one of the three exercise conditions. These conditions will either be rest, moderate intensity interval biking, or high intensity interval biking. Blood samples will be taken before, during, immediately after, and 1 hour, 2 hours, 4 hours, 6 hours, and 8 hours after each exercise condition via an IV line implanted on the morning of testing that will be removed after the 8-hour blood draw. During these three condition days you will be provided a comfortable workstation in the lab where you can work remotely or rest. Two meal replacement drinks, Gatorades, and water will be provided on each condition day. You will be required to remain in the lab for the duration of the condition days and restroom access will be provided. On the two days following each condition day, you will be invited to return to the lab at the same time as exercise start for 24- and 48-hour after exercise single sample blood draws. You will also be provided two ActiGraphs before each condition day to monitor your activity. One will be for use on your wrist during sleep. The other will be for the waist during the day to measure activity. These will be worn for two days before and two days after each exercise bout day.

~~During each exercise bout, you will be attached to 3 imaging devices. These are safe and have been used in previous studies. These include:~~

~~1) Electrocardiogram – Measures heart rate~~
~~2) End-tidal system – Measures the air you breath out~~
~~3) Finometer – Measures blood pressure~~

Currently, there is an underrepresentation of females in research studies. This study is seeking to reduce this gap and help increase the number of studies that include female participants.

HOW MANY PEOPLE WILL TAKE PART IN THIS STUDY?

About 20 people will take part in this study in Calgary, Alberta, Canada. There will be equal number of male and female participants (10 male, 10 female).

WHAT WILL HAPPEN IF I TAKE PART IN THIS STUDY?

If you volunteer to participate in this study, the researcher will ask you to do the following:

Please note all of the aspects of the study are completely voluntary. You are welcome to stop at any time. You are welcome to opt out of any individual parts of the study, you do not wish to do.

Visit 1:

- Fill out a COVID-19 pre-screening questionnaire to ensure you are safe to enter the laboratory. Your temperature will also be recorded with a non-touch, infrared forehead thermometer.
- You will answer some question about your previous medical history. These include: whether you have had any illnesses, injuries, or take medication. This information will remain confidential and will not be shared.
- You will fill out a questionnaire that asks about diet, personal characteristics, and exercise. No information on the questionnaires will be audio or video recorded.
- You will fill out a questionnaire (PARQ+) ensuring your fitness to complete maximal exercise testing. If health concerns arise regarding this questionnaire, you will not continue with the study.
- Perform a maximal exercise test on a bike. You will breathe into a mouthpiece to determine oxygen and carbon dioxide levels.
- Be given Actigraphs and instructed for use in the two days before and two days after each exercise condition.
- This visit will take about 60 minutes to complete.

Please note the following will be completed in ~9 hours.

Visits 2, 5 and 8:

- Fill out a COVID-19 pre-screening questionnaire to ensure you are safe to enter the laboratory. Your temperature will also be recorded with a non-touch forehead thermometer.
- You will answer some question about your previous medical history. These include: whether you have had any illnesses, injuries, or take medication. This information will remain confidential and will not be shared.
- You will fill out questionnaires that ask about diet, personal characteristics, exercise, and your health. No information on the questionnaires will be audio or video recorded.
- An intravenous blood sampling line will be inserted by a trained medical professional. This may cause some discomfort for individuals.
- During the exercise bout you will be asked to wear a small blood pressure monitor around your finger. Wear a heart rate monitor (i.e., electrocardiogram with 3-electrodes) located near your collar bone and belly button. This is safe and not painful. If body hair is present, a small 2x2cm region will be shaved to allow for the electrode to be on the skin with no interference.
- You will also be asked to breathe into a mouthpiece that will monitor your exhaled carbon dioxide and oxygen levels. This is safe and not painful. It can be taken out in a matter of seconds if you feel uncomfortable. A nose clip will be worn to ensure all breathing is through your mouth. Perform exercise bout. You will complete one of three exercise/control bouts on each of these visits. Each exercise bout day will be separated

by one month. The three exercise conditions are as follows. A control bout of sitting quietly at rest on the bike for 30 minutes. A 30 minute moderate intensity exercise bout including a 5 minute warmup, ten 1 minute on/1 minute off intervals, and a 5 minute cool down. A 30 minute high intensity exercise bout including a 5 minute warmup, ten 1 minute on/1 minute off intervals, and a 5 minute cool down.

- Provide blood samples before, during, immediately after, and 1 hour, 2 hours, 4 hours, 6 hours, and 8 hours after each condition. Each blood sample will take <2 minutes. When the blood samples are not being performed, a desk will be provided for you to use.
- Please note the timing of each assessment is approximate. These tasks could be extended a few minutes to ensure sufficient data. In most cases, the suggested time will be accurate.
- All protocols will take place in the University of Calgary, Kinesiology Building Block-B, Room 306 (Exercise) and Room 3318 (IV lines).

Please note the following will be completed in ~15 minutes.

Visits 3, 4, 6, 7, 9, and 10:

- Fill out a COVID-19 pre-screening questionnaire to ensure you are safe to enter the laboratory. Your temperature will also be recorded with a non-touch forehead thermometer.
- Provide blood samples at 24 and 48 hours after each condition by single use butterfly needles.
- This will take place in the University of Calgary, Kinesiology Building Block-B Room 3318.
- On the 48-hour blood draw visit you will return the Actigraphs.

HOW LONG WILL I BE IN THIS STUDY?

Participation will take a total of about 30-hours to complete.

- You will be in this study over the course of 2 months. Each exercise or control day will be seperated by ~4 weeks.
- The first visit is for the maximal exercise test (~1 hour) and ActiGraph pickup.
- The 3 exercise days (visits 2, 5, and 8) will take about 9 hours. The exercise will only take 30 minutes, and the blood draws are expected to take <2 minutes each. This is a total of about 1 hour study participation over the course of the 9-hour day. During the rest of the time in lab you will be given work or rest space in the lab.
- The 6 follow up (24/48 hours after exercise/control bout) blood sample days (visits 3, 4, 6, 7, 9 and 10) will take about 15 minutes for a single blood draw. You will come to the Cerebrovascular Concussion Laboratory for a total of 10 visits to the lab. Each day will have different time commitments as explained above.
- There will be a total of three needle pokes per condition. One IV and two follow up butterfly needle pokes, for a total of 9 pokes across the entire study duration. Trained

phlebotomists will ensure these needle pokes are as quick and painless as possible by following proper phlebotomy technique.

ARE THERE ANY POTENTIAL RISKS OR DISCOMFORTS THAT I CAN EXPECT FROM THIS STUDY?

- You may have an increased exposure during COVID-19 with travel to the university and exposure to other people. However, the risk is similar as other activities such as going grocery shopping. The research team will take all necessary precautions including:
    - Screening people attending in-person appointments,
    - Using PPE for both research staff and participants (e.g., masks, gloves, etc.),
    - Using hand sanitized for both research staff and research participants,
    - Using single use research apparatuses where possible,
    - Physical distancing measures,
    - Regular sanitization of surfaces and multi-use equipment between patients/participants.

- As the conditions and maximal exercise test are physical in nature, they may entail a slight risk or be uncomfortable if you are unfit or do not typically perform exercise. The risk of a cardiac event (heart attack, dysrhythmias, etc.) in a mixed subject population (healthy low risk and unhealthy high risk patients together) is approximately 6:10,000. The risk decreases in a previously healthy (i.e., moderately active) population, so it is highly unlikely you might experience a cardiac event. If this rare event does occur, there will be a physician on call in the building assess you. You may also experience increased awareness of breathing, muscle pain and/or fatigue, increased sweating, or a general feeling of fatigue or nausea, all of which are not unexpected consequences of exercise and are only expected to present for a brief period. You may also experience discomfort from the breathing device during the exercise tests. This device can be removed at a moment's notice. Two experienced experimenters will always be present during the test. At least one individual will have a current CPR certification. You will always have a lab member nearby so you can communicate any discomforts/concerns. This is to reduce the rare case that you become dizzy, lightheaded, and faint throughout the testing procedures. Exercise will be performed on a bike which will reduce the risk of falling.

- You may feel distressed about needles used for blood draws. Experienced study personal will ensure proper technique to reduce participant discomfort. The total blood draw volume over the entire study duration is expected to be 540 mL, which is just over a typical blood donation volume. On exercise days, you will provide approximately 144 mL of blood. On the 24 and 48 hour follow up visits, you will provide approximately 36 mL of blood. This equals approximately 180mL of blood over a 48 hour period after each condition. Each condition will be separated by 1 month to allow blood to stabilize to

baseline and mitigate any potential risks associated with significant blood volume changes.

- You have the right to signal you want to stop testing at any time.

ARE THERE ANY POTENTIAL BENEFITS IF I PARTICIPATE?

There will be no direct benefit to you from participating in this study. This study may help the researchers learn more about concussion biomarkers. It will understand how biological sex impacts this relationship. This will increase the number of future studies with female participants.

WHAT OTHER CHOICES DO I HAVE IF I CHOOSE NOT TO PARTICIPATE?

You are free to choose not to participate in the study. If you decide not to take part in this study, there will be no penalty to you.

CAN I STOP BEING IN THE STUDY?

Yes. You can decide to stop at any time. Tell the researchers if you are thinking about stopping or decide to stop.

WITHDRAWAL OF STUDY DATA

If you decide you no longer wish to participate you have to option to also withdraw your data from the study. If you decide you wish to withdraw your data, this data will be withdrawn from the study and confidentially removed from our database. However, all data will be retained for a minimum of 5 years in accordance with the ethical review board guidelines. After the retention period, the data from the withdrawn subjects will be destroyed.

WILL I BE PAID FOR PARTICIPATING, OR DO I HAVE TO PAY FOR ANYTHING?

You will be financially compensated for the cost of parking in lot 10 or 11 at UCalgary ($8 per day / total $80 throughout the study). You are responsible for covering any other associated costs (e.g. time lost from work, transportation, etc.).

WILL INFORMATION ABOUT ME AND MY PARTICIPATION BE KEPT CONFIDENTIAL?

The researchers will do their best to make sure that your private information is kept confidential. Information about you will be handled as confidentially as possible, but there is always the potential for an unintended breach of privacy. The research team will handle data according the Data Management Plan as outlined below:

- No identifiable information about you will be kept with the research data.

- All identifiable information about you will be replaced with a code. A master list linking the code and your identifiable information will be kept separate from the research data.
- Only the investigators responsible for this study, the research team members directly supervised by a study investigator, and the team statistician who will help analyze the data.
- The University of Calgary and the Conjoint Health Research Ethics Board will have access to this information.
- Confidentiality will be protected by using only study identification numbers in the database.
- Any results of the study, which are reported, will in no way identify study participants. No direct link is made between your information and your data
- All research data and records will be maintained in a secure location at the University of Calgary. Only authorized individuals will have access to it.
- All research data and records will be stored electronically on a secure computer and/or network with double encryption and/or password protection.
- De-identified blood samples will be shipped to Dr. Cheryl Wellington's lab at UBC for assessment, after which they will be destroyed.

HOW LONG WILL INFORMATION FROM THE STUDY BE KEPT?

- The researchers intend to keep the research data and records indefinitely for future research.
- Data collected for this study may be shared with other researchers for future studies that are unknown at this time. Any data shared with other researchers, will not include your name or other personal identifying information.

Any future use of this research data is required to undergo review by a Research Ethics Board.

WHAT OTHER THINGS SHOULD I CONSIDER BEFORE PARTICIPATION?

RESEARCHER CONFLICTS OF INTERESTS

No members on the research team have a personal financial interest that may impact the outcomes of the study.

USE OF DATA FOR FUTURE RESEARCH

As mentioned above, data collected for this study may be shared with other researchers for future studies that are unknown at this time. Any data shared with other researchers, will not include your name or other personal identifying information.

CONTACT FOR FUTURE RESEARCH

University of Calgary researchers may contact me in the future to ask me to take part in other research studies.

❑ YES
❑ NO

IF I SUFFER A RESEARCH-RELATED INJURY, WILL I BE COMPENSATED?

There is minimal risk for harm with the participation in this study. It is important that you tell the researchers if you believe that you have been injured because of taking part in this study.

In the unlikely event that you suffer injury as a result of participating in this research, no compensation will be provided to you by the University of Calgary or the researchers. However, you still have all your legal rights. Nothing said in this consent form alters your right to seek damages.

WHOM MAY I CONTACT IF I HAVE QUESTIONS ABOUT THIS STUDY?

The Research Team:

You may contact PI: Dr. Jonathan Smirl at (403) 220-8426 or Linden Penner at (780) 263-1373. The laboratory group can also be contact via email at concussion.lab@ucalgary.ca. Please feel free to reach out with any questions or concerns about the research or your participation in this study.

Conjoint Health Research Ethics Board (CHREB):

If you have any questions concerning your rights as a possible participant in this research, please contact the Chair, Conjoint Health Research Ethics Board, University of Calgary at 403-220-7990.

HOW CAN I FIND OUT ABOUT THE STUDY RESULTS?

If you wish to find out more about the results of the study, please contact the laboratory email at concussion.lab@ucalgary.ca

WHAT ARE MY RIGHTS IF I TAKE PART IN THIS STUDY?

Taking part in this study is your choice. You can choose whether or not you want to participate. Whatever decision you make, there will be no penalty to you.

- You have a right to have all of your questions answered before deciding whether to take part.
- If you decide to take part, you may leave the study at any time

HOW DO I INDICATE MY AGREEMENT TO PARTICIPATE?

Your signature on this form indicates that you have understood to your satisfaction the information regarding your participation in the research project and agree to take part in the study. In no way does this waive your legal rights nor release the investigators or involved institutions from their legal and professional responsibilities.

SIGNATURE OF STUDY PARTICIPANT

_____

Name of Participant

_____ _____

Signature of Participant Date

SIGNATURE OF PERSON OBTAINING CONSENT

_____ _____

Name of Person Obtaining Consent Contact Number

_____ _____

Signature of Person Obtaining Consent Date

SIGNATURE OF THE WITNESS

_____

Name of Witness

_____ _____

Signature of Witness Date

A copy of this consent form has been given to you to keep for your records and reference.

APPENDIX D: COVID-19 SCREENING CHECKLIST

Daily Screening Checklist - to be completed prior to arriving at the Faculty of Kinesiology for Human Research Testing

Name_____ **Phone number** _____ **Date** _____

Non-Invasive Digital Temperature Screening (>37.5 °C) _____

FACULTY OF KINESIOLOGY SCREENING CHECKLIST	YES	NO
1. Do you currently have any of the below symptoms:		
Fever		
Cough		
Shortness of Breath/Difficulty Breathing		
Sore throat		
Chills		
Painful Swallowing		
Runny Nose/Nasal Congestion		
Feeling Unwell/Fatigued		
Nausea/Vomiting/Diarrhea		
Unexplained loss of appetite		
Loss of sense of taste or smell		
Muscle/Joint aches		
Headaches		
Conjunctivitis (Pink Eye)		
2. Have you, or anyone in your household, travelled outside of Canada in the last 14 days?		
3. Have you had close unprotected* contact (face-to-face contact within 2 metres/6 feet) with someone who is ill with cough and/or fever?		
4. Have you or anyone in your household been in close unprotected contact in the last 14 days with someone who is being investigated or confirmed to be a case of COVID-19?		

If you have answered "yes" to any of the above questions, please do not go to the Faculty of Kinesiology or any associated research site(s). Please use the AHS Online Assessment Tool to determine if testing is recommended.
https://myhealth.alberta.ca/Journey/COVID-19/Pages/COVID-Self-Assessment.aspx

UNIVERSITY OF CALGARY
FACULTY OF KINESIOLOGY

This information is collected under the authority of section 33(c) of the *Freedom of Information and Protection of Privacy Act* and will be used and disclosed solely for the purposes of pre-screening research participants to the Faculty of Kinesiology complex and associated community research sites during the COVID-19 pandemic. The completed record will be kept on site at the Faculty of Kinesiology for a period of fourteen (14) days at which time it will be destroyed. For questions, please contact the Associate Dean Research in the Faculty of Kinesiology at reimer@ucalgary.ca.

APPENDIX E: PRE BLOOD DRAW QUESTIONNAIRE

Please complete the following questions to the best of your ability by circling or writing the answer that applies to you. If you have any questions, please ask study personnel.

Sex and Exercise:

1. What is your biological sex? Male Female Other Prefer not to respond

If you indicated **FEMALE**:

a) When was the start of your first menstrual cycle? (Year/Month) _____

b) Have you been regular over the last 12 months? Yes No

If **YES**, how long is your cycle? (Days)

_____

c) When was the start of your most recent cycle? (Day/Month/Year) _____

d) Are you currently using any intervention for birth control? (Including birth control pills, injectables, and/or IUD and other methods)

Yes No

If **YES**, what type? (Type/Brand)

_____

2. Have you exercised in the last 24 hours? Yes No

If you indicated **YES**:

a) What type of exercise? Aerobic Anaerobic Both

b) Did you complete the exercise in the last: 3 hours 6 hours 12 hours 24 hours

Medications:

1. Are you taking any prescription oral/inhaled/injectable/topical medications on a regular basis?

Yes No

If **YES**, please list in this format: medication/use; medication/use

_____

2. Are you taking over the counter medications as needed such as Advil, Aleve, or Tylenol?

Yes No

If **YES**, please list in this format: medication/use; medication/use

_____

If **YES**, How much and how often have you taken it over the last week? (Dose/Frequency)

_____

3. Are you taking protein powder, creatine, probiotic, or vitamins? Yes No

If **YES**, please list in this format: name; name

_____

4. Are you taking any performance enhancing substances? Yes No

If **YES**, please list in this format: name; name

_____

Other Questions:

1. Have you had any caffeine today? Yes No

If **YES**, how much caffeine? (e.g. 2 cups of coffee, 1 energy drink)

_____

2. To your knowledge, have you been diagnosed with COVID-19? Yes No

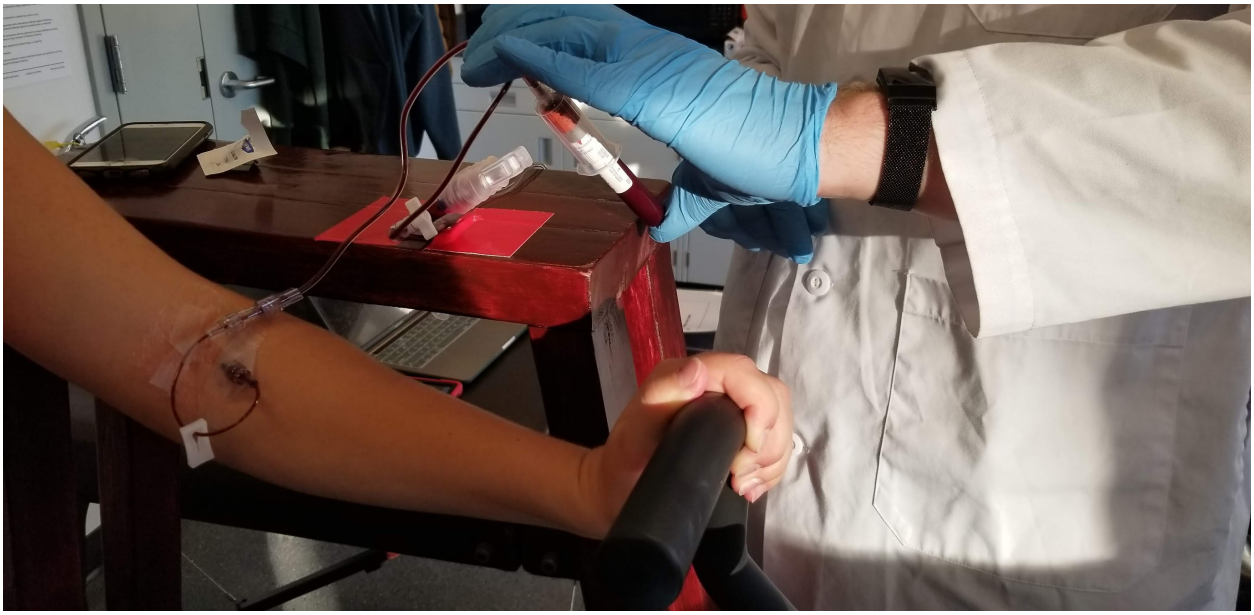
If **YES**, when? (Day/Month/Year)

_____

3. What time did you wake up this morning? _____

4. What did you eat for breakfast? _____

APPENDIX F: VAMP ADULT BLOOD SAMPLING SYSTEM



VAMP adult sampling system in use with a participant exercising on the cycle ergometer. The researcher is filling the vacutainer through the closed sampling system. Note the closed valve beside the flexure system opposite to the inserted IV catheter. When this valve is opened, the flexure system pulls up to draw and contain a clearing volume, then the valve is closed to allow sampling in a closed system. After the sample is drawn, the valve is opened and the clearing volume is pushed back into the participant. Excess blood in the line is then flushed with isotonic saline and disconnected.

APPENDIX G: BLOOD COLLECTION & PROCESSING PROTOCOL

Guidelines for Phlebotomy Room Health & Safety

Before blood can be safely collected for the study, a number of protocols and safety issues must be addressed in full. With strict adherence to the safety protocols and procedures detailed here, blood may be safely and securely collected. If any of these requirements are absent, then blood collection may not proceed.

Contact Information:
In the event of any incident happening in the lab (e.g., participant feels unwell, accidental needle stick, broken glass/biohazard spill, the provincial study coordinator must be informed, and the project coordinator/primary site investigator **must** be contacted. See Fainting Protocol in participant flow section. Additional adverse events will be handled as per phlebotomy standard procedures at each study site.

Primary site Investigator or coordinator: Carolyn Emery (403-510-1454); Research Coordinator - Shane Esau (403-220-3113)

UBC: Cheryl Wellington or Ian Pike (604-999-8041); Research Coordinator - Jennifer Smith and Liam Tapsell

UManitoba: Kelly Russell (204-480-1312) ;Research Coordinator - Allison Poppel

Laval: Claude Goulet (418-656-2131, 403870); Research Coordinator - David Lapperriere

CHEO: Roger Zemek (613-219-3849); Research Coordinator - Lauren Dawson

York or Bloorview: Nick Reed or Alison MacPherson (416-434-3430); Research Coordinator - Cheryl Beech

UAlberta: Martin Mracik (780-492-8052); Research Coordinator - George Frost

Montreal Children's Hospital of the McGill University Health Center: Isabelle Gagnon (514-398-4400 ext 099057); Research Coordinator – Joanna Mazza

Western University: Lisa Fischer (519-661-3011); Research Coordinator – Stacey Wanlin

Safety Controls

Appropriate phlebotomy chairs: Size and positioning of phlebotomy chair are important to a successful blood draw. Ensure the participant is seated in a stationary (non-rolling) chair prior to venipuncture.

Washable surfaces: When performing a collection, ensure that there is no cloth or towels on hand. Some phlebotomists prefer to use cloth to prop a participant's arm, but this poses a significant health risk, as the material is easily contaminated, and notoriously difficult to sanitize.

Safety-engineered needles: BD UltraTouch push button blood collection butterfly needles are preferred (BD 367365 (21G x 0.75", 12" tubing) for superior participant comfort and safety. BD

Eclipse Blood Collection needles (BD 3686751 (21G x 1.25 in), BD 368651 (22G x 1.25 in) are also acceptable phlebotomy needles for this study. Alternative needle to Ultratouch is catalogue # 367281 (21 G x .75 in. BD Vacutainer® Safety-LokTM Blood Collection Set with 12 in. tubing which has a safety shield, just not in a push button format)

Once the blood collection is complete, immediately snap the cover guard over the needle, and place in a yellow sharp-resistant biohazard bucket.

Puncture-resistant sharps containers: Sharps containers must be placed in each phlebotomy room. Needles should be disposed of here, immediately after completion of the blood draw. (This means before labeling tubes, bandaging patient, or doing anything other than placing gauze over the open wound.)

Hand hygiene facilities: Each phlebotomy room contains hand sanitizer, a hand washing station, and paper towels. It is required that workers wash hands before each new participant, and between every participant

Any spills: cover/wipe up and clean with bleach solution. Refer to biosafety protocol for spills/contact with human fluid.

Disposal: After blood collection, you must ensure that yellow biohazard bins are picked up when full according to your institution's guidelines.

Administrative controls

Blood Collection Kits will be provided or assembled for each study site:

- Freezerwork labels will be created and sent to each phlebotomy site with pre-determined participant codes that will be match to unique study ID (i.e. based on institution site)
- Disposable non-latex tourniquet (one tourniquet used per participant and discarded after use)
- Disinfecting 70% alcohol pad
- Sterile gauze or cotton balls
- Band-Aid
- Vacutainer for needle attachment
- Needles: one 21g, one 22g needle, one 21g butterfly. *Choose appropriately sized needle and save the unopened needle for another draw.
- BD Vacutainer blood collection tubes (1 Red top non-additive tube and 1 Lavender EDTA tube)
- Eppendorf screw-cap tubes (10 for serum aliquots, 10 for plasma aliquots)
- Plastic transfer pipettes (1 for serum, 1 for plasma)

Standard Operating Procedure

A. Collecting specimens

- Disinfect working area with alcohol or disinfectant spray.

- Introduce yourself and identify participant (confirm name and date of birth as it appears on the form/requisition). Ensure subject ID code is on the requisition (and matches labels attached).
- Ensure participant is comfortable and explain procedure.
- Prepare tubes in order of draw, first serum (red top) then plasma (lavender top).
- Ensure equipment is positioned within reach before procedure has begun.
- Wash hands and don latex-free gloves (latex may cause allergic reaction in subject).
- Assess and select appropriate vein (assess median cubital vein first). Participant may have a preference for which arm/vein.
- Position the arm downwards and apply tourniquet a few (3-4) inches above the puncture site.
- Palpate vein even when visible.
- Cleanse site with 70% alcohol pad and allow skin to dry.
- Place needle on vacutainer containing first tube. Use BD Eclipse Blood Collection needles (BD 3686751 (21G x 1.25 in, or BD 368651 (22G x 1.25 in).
- Immobilize or anchor the vein with non-needle hand during procedure. Do NOT encourage participant to make a fist (this may result in hemolysis).
- Puncture the skin and vessel as quickly and smoothly as possible at a 15-30o angle, with the needle bevel facing up.
- Gently push the blood collection tube stopper (lid) to connect with the needle utilizing lip of vacutainer to help push the tube on and off; blood should flow freely. The tube can be pre-assembled with the vacutainer prior to insertion (tube wedged on, so long as seal remains unbroken).
- When collection is complete, gently release tourniquet; place clean cotton ball or gauze at the site and gently remove the needle.
- Apply pressure to the site to prevent formation of a haematoma – DO NOT allow subject to bend their elbow.
- Dispose of the needle in appropriate sharps container immediately – DO NOT recap needles. Dispose of all visibly contaminated materials, including gloves, bandages and cotton balls into an autoclavable biohazard bag. All other waste can go into regular garbage.
- Invert all blood tubes 5-10x, each with one complete turn of the wrist 180 degrees and back.
- Record collection date, time, and record collector's initials on requisition. Label tube with subject ID#, date and time of collection (24 hour clock).
- Disinfect the area using the same cleaning products used prior to initial collection.
- Offer juice/cookie to subject.

B. Processing Specimens

- Serum: Allow blood to clot at room temperature for a minimum of 30 min prior to centrifuging.
- Plasma: Specimen can be centrifuged immediately (after proper inversion).
- If specimens need to be transported to another location for centrifugation, place specimens on ice in a dedicated cooler with a lockable lid in a secondary container with an absorbent liner and sealable lid. Mark the secondary container on two sides with the name and contact information of the principal investigator at the relevant institution. An individual certified in the Transportation of Dangerous Goods (Class 6.2 – Infectious Substances) will transport samples to a University laboratory.

- Centrifuge each specimen for 10 minutes @ 1300g. If clots are present in supernatant (serum), try to extract or rim the clot using a wooden applicator stick (do NOT remove the entire clot as it could affect the testing phase). Re-centrifuge the specimen and then try to separate the serum again. If plasma contains a fibrin strand, use another wooden applicator stick to remove it before separating plasma from the cells.
- Collect supernatant from each specimen as defined below.

- Set necessary aliquot tubes (EppendorfXXX) in rack.
- Take one blood tube at a time, remove rubber top; set aside.
- Collect 500 ul aliquot of serum per sample. Keep tip away from blood portion of sample. You should expect to collect at least 8-9 aliquots per tube.
- Dispose pipette into biohazard bag. Replace cap on blood tube.
- Cap aliquots. Add the appropriate Freezerworks sticker label supplied with the kit.
- Discard blood collection tubes into biohazard bag.
- Use a new pipette tip for the next sample.
- Place aliquots in -80 freezer until ready to ship on dry ice to Cheryl Wellington's laboratory at UBC.

Dr. Cheryl Wellington, c/o Jasmine Gill
Djavad Mowafaghian Centre for Brain Health
University of British Columbia
2215 Wesbrook Mall
Vancouver BC Canada V6T 1Z3
Email: wcheryl@mail.ubc.ca or jasmine.gill@pathology.ubc.ca Tel: 604-827-3769 or 250-809-5375

*If any clarification is required for the processing procedure, please contact Jasmine by e-mail or phone as provided above.

Personal Protective Equipment

- Phlebotomists should have a white lab coat on for each and every subject.
- Workers are expected to wash hands before/between every subject (even when changing gloves between subjects). Hand sanitizer is available and should be used liberally.
- Lab-rated safety goggles should be worn during sample processing.
- Full-length pants and closed-toe shoes should be worn every session.

Worker's Qualification & Miscellaneous

1. Worker Qualification

a. Workers must be a certified phlebotomist. This may occur through a laboratory assistant (MLA) or nursing program, EMT specialization, or from individual courses tailored to the required techniques. Certifications should be on file with project coordinators.
b. As per University requirements, workers should be certified in Biosafety courses 1 & 3, as well as the WHMIS safety course, and Laboratory Safety course.
c. Workers must be absent of illness or any flu-like symptoms to be eligible to perform venipuncture blood collection.
d. Workers must be up to date on immunizations, including hepatitis B, measles, mumps, rubella, pertussis, and diphtheria. Of this list, only hepatitis B is not standard for most Canadians.

2. Participant Evaluation

a. Prior to beginning the procedure, check that subject is comfortable and feeling well.
b. Ask subject if they have a preferred arm, or any medical issues that would preclude one arm from use. Adjust venipuncture site accordingly.
c. If subject is at all tense, or seems apprehensive, remind them the study is completely voluntary, and they may continue the study without completing the blood collection portions.

Complications

In the event that a participant has a negative reaction, immediately inform the project coordinator for the night, and inform the provincial project head, Carolyn Emery (403-510-1454) and Cheryl Wellington (604-827-3769). The most common event is faintness during or after the collection. In the event that the participant feels faint while sitting in the chair, have them rest their head on the chair's arm rest. Ask someone to grab a cold compress (i.e. paper towel dampened with cold water) and place it on the back of the participant's neck. If the chair has no arm rest, have the participant lower their head between the knees to recirculate blood to their head and proceed with the cold compress on the back of the neck. Have the participant lie down for no less than 15 minutes, and insist that they have some of the juice/cookies available with the study materials. This reaction disqualifies them from further participating in the current study session and any future study phlebotomy and must be reported as an adverse event.

APPENDIX H: ACTIGRAPH SLEEP SURVEY

Confidential

Actigraph Sleep Log

Please complete the survey below based on previous day/night.

The SHRed team is here to support you, if you have any questions, please email us at SHREDactigraph@ucalgary.ca

UUID

_____

Name: [participant_detail_arm_1][actg_name]

Date form completed

This is hidden from the survey and auto-populates with the date and time the survey was opened, based on the users device time and date

_____

Today's Date

_____

1:00pm = 13:00
5:00pm = 17:00
9:00pm = 21:00
2:00pm = 14:00

6:00pm = 18:00
10:00pm = 22:00
3:00pm = 15:00

7:00pm = 19:00
11:00pm = 23:00
4:00pm = 16:00

8:00pm = 20:00
12:00pm = 24:00

Last night I went to bed at

_____

Last night, How long did it take you to fall asleep?

_____
(Minutes)

Last night, Did you take any medication to help you sleep?

◯ Yes ◯ No

Medication 1 - Name of medication?	_____
Medication 1 - Dosage	_____
Medication 1 - Was this medication prescribed?	_____
Did you take a second medication?	◯ Yes ◯ No
Medication 2 - Name of medication?	_____
Medication 2 - Dosage	_____
Medication 2 - Was this medication prescribed?	_____
How many times did you wake-up last night?	_____
I woke-up this morning at:	_____
This morning, I got out of bed at:	_____
Rate the quality of your sleep from 1-10	◯ 1 (Worst Sleep Ever) ◯ 2 ◯ 3 ◯ 4 ◯ 5 ◯ 6 ◯ 7 ◯ 8 ◯ 9 ◯ 10 (Best Sleep Ever)
Did you take a nap yesterday?	◯ Yes ◯ No
How many naps did you take?	◯ 1 ◯ 2 ◯ 3 ◯ 4 ◯ 5 ◯ 6
Approximately when was your last nap?	_____
Did you take the Actigraph off yesterday?	◯ Yes ◯ No

Time 1: Actigraph taken off at	_____
Time 1: Actigraph put back on at	_____
Did you take the actigraph off a SECOND time?	◯ Yes ◯ No
Time 2: Actigraph taken off at	_____
Time 2: Actigraph put back on at	_____
Did you take the actigraph off a THIRD time?	◯ Yes ◯ No
Time 3: Actigraph taken off at	_____
Time 3: Actigraph put back on at	_____

What COLOUR Actigraph did you wear during the DAY yesterday?

◯ Red ◯ Black

Where did you wear your actigraph during the DAY yesterday?

◯ Waist ◯ Wrist

What COLOUR Actigraph did you wear during the NIGHT yesterday?

◯ Red ◯ Black

Where did you wear your actigraph during the NIGHT yesterday?

◯ Waist ◯ Wrist

Just a friendly reminder to please wear the

Red Actigraph on your WAIST during the DAY

Black Actigraph on your WRIST at NIGHT

Please use the textbox to provide any comments or notes about wearing the Actigraphs yesterday or about completing this form.	_____

Think about all the vigorous activities that you did in the last 24 hours. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. Based on the last 24 hours, how much time did you spend doing vigorous physical activity on activities like running, fast bicycling, playing games or sports like hockey or basketball?
Please answer in this format: hours; minutes (e.g. for 2 hours and 15 minutes you would put -> 2; 15)

_____
(e.g. 2; 15)

Think about all the moderate activities that you did in the last 24 hours. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

2. Based on the last 24 hours, how much time did you spend doing moderate physical activity on activities like bicycling, rollerblading or skateboarding at a regular pace, light swimming, or playing sports like golf?
Please answer in this format: hours; minutes (e.g. for 2 hours and 15 minutes you would put -> 2; 15)

_____
(e.g. 2; 15)

Think about all the light activities that you did in the last 24 hours. Light activities refer to activities that take little physical effort and make you breathe normal rate. Think only about those physical activities that you did for at least 10 minutes at a time.

3. Based on the last 24 hours, how much time did you spend doing light physical activity on activities like light walking from place to place, shower/bathing, making to eat and doing dishes?
Please answer in this format: hours; minutes (e.g. for 2 hours and 15 minutes you would put -> 2; 15)

_____
(e.g. 2; 15)

The last question is about the time you spent being sedentary based on the last 24 hours. Sedentary activities refer to activities that require little to no movement.

4. Based on the last 24 hours, how much time did you spend being sedentary, like time spent sitting at home and at school, visiting with friends and reading or lying watching television or playing video games?
Please answer in this format: hours; minutes (e.g. for 2 hours and 15 minutes you would put -> 2; 15)

_____
(e.g. 2; 15)

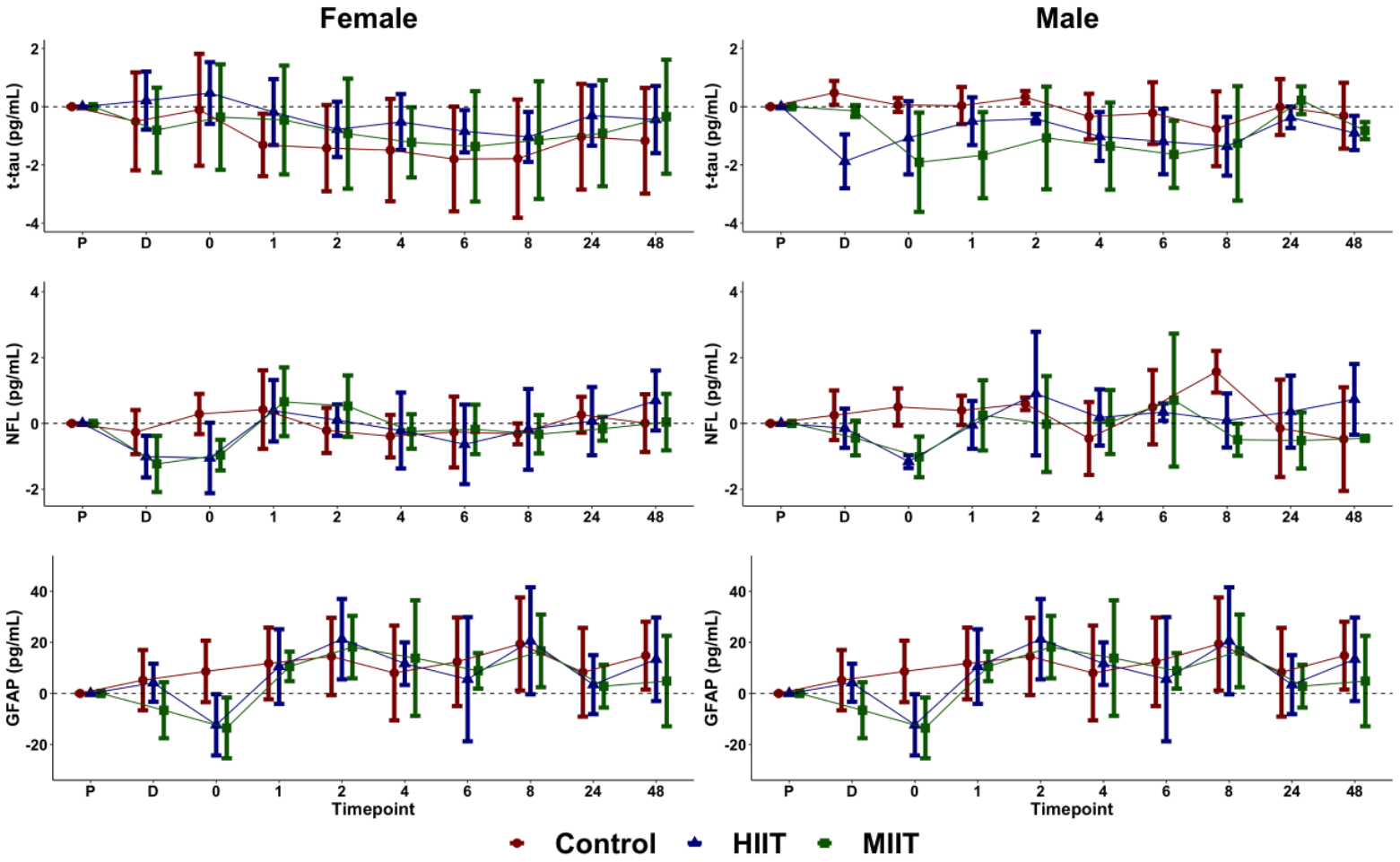
Coordinator/RA comments

_____

How/when was this log completed?
Prospectively - Paper = Completed on paper by participant but entered into the redcap by study staff
Retrospectively - On their own = They completed the log using the survey link but Completion Date/Time ≠ Today Date
Retrospectively - Researcher Assisted = Research spoke with participant to complete the logs after the fact

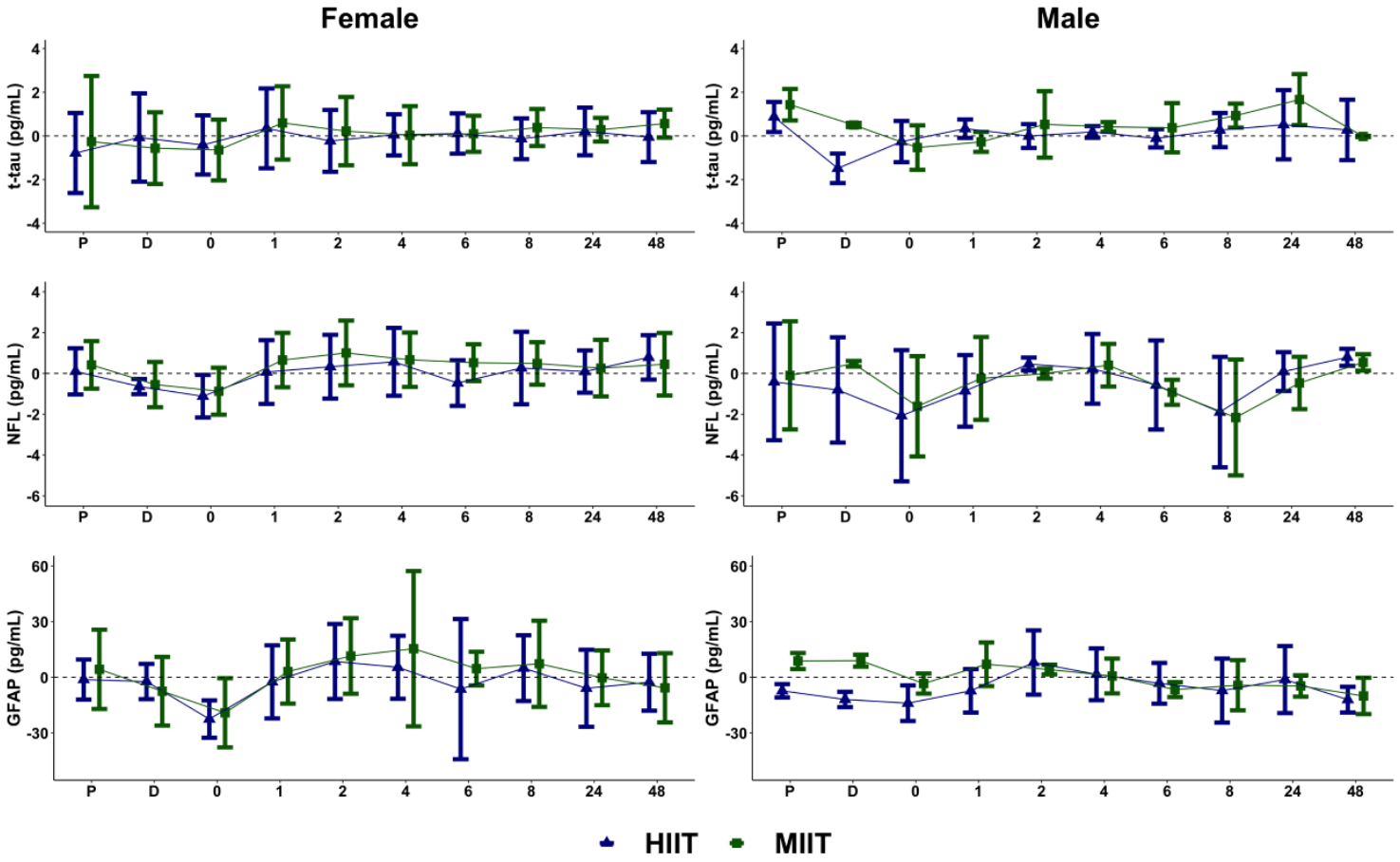
◯ Prospectively-Survey Link
◯ Prospectively-Paper
◯ Retrospectively-On Their Own
◯ Retrospectively-Researcher Assisted

APPENDIX I: MEAN DIFFERENCE ACROSS TIMEPOINTS COMPARED TO PRE TIMEPOINT BY SEX



Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. Biomarkers include plasma total tau (t-tau), plasma neurofilament-light chain (NFL), and plasma glial fibrillary acidic protein (GFAP). Means with standard deviation bars shown. Female n = 7, male n = 3.

APPENDIX J: MEAN DIFFERENCE ACROSS TIMEPOINTS COMPARED TO CONTROL BY SEX



Conditions are control with no exercise, moderate intensity interval training (MIIT), and high intensity interval training (HIIT). Timepoints P and 0 are referring to immediately before and immediately following each condition. Timepoint D refers to the rest stage following interval 5 of MITT and HITT or halfway through control. Timepoints 1 through 48 denotes hours post-condition. Biomarkers include plasma total tau (t-tau), plasma neurofilament-light chain (NFL), and plasma glial fibrillary acidic protein (GFAP). Means with standard deviation bars shown. Female n = 7, male n = 3.