BLOOD-BASED BIOMARKER ALTERATIONS IN ATHLETES FOLLOWING SUB-CONCUSSIVE AND CONCUSSIVE IMPACTS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The College of Graduate Studies

(Interdisciplinary Studies)

THE UNIVERSITY OF BRITISH COLUMBIA

(Okanagan)

September 2017

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BLOOD-BASED BIOMARKER ALTERATIONS IN ATHLETES FOLLOWING SUB-CONCUSSIVE AND CONCUSSIVE IMPACTS

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the degree of Doctor of Philosophy.

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Abstract

Sport-related concussion affects an estimated 1.6 – 3.8 million people in the United States annually, and it has been suggested many more go unreported due to lack of knowledge regarding symptoms. Current diagnostic measures are based on subjective criteria, which rely heavily on self-reporting by the individual who has suffered the injury. Further, it is possible for symptoms of a concussion to manifest up to 72-hours post-injury, making it even more difficult to form a mechanistic link between trauma and symptoms. Recently, there has been an increased concern for the deleterious physiological effects of repetitive, sub-concussive impacts. An objective, quantifiable measure of the pathophysiological effects of both sub-concussive and concussive impacts is essential for concussion diagnosis, and could aid in tracking the pathophysiological processes underlying acute and repetitive mild traumatic head trauma.

Three prospective cohort studies form the backbone of this dissertation. Study 1 examined plasma concentrations of total tau (T-tau), along with serum concentrations of neurofilament light (NF-L), A disintegrin and metalloproteinase domain-containing 10- (ADAM10) and caspase 3-cleaved fragments of tau (Tau-A and Tau-C, respectively) before and after a season of athletic competition in the following sports: American football, ice hockey, rugby, soccer, and cross country running. Study 2 examined NF-L before and after an acute bout of soccer heading. To complete the dissertation, Study 3 involved the quantification of these biomarkers at preseason and at 6- and 14-days following a concussion in American football, ice hockey, and soccer players.

Serum NF-L appeared to be more sensitive to the effects of repetitive, sub-concussive impacts (i.e. studies 1 and 2) than a single, traumatic event resulting in a concussion (i.e. study

3), where it did not change. T-tau, Tau-A, and Tau-C were unaffected by both repetitive sub-concussive impacts and impacts resulting in a concussion. Thus, it appears that serum NF-L may be a sensitive biomarker of axonal damage in repetitive sub-concussive and concussive contexts.

Lay Summary

Sport-related concussions are a major health concern, with an estimated 1.6 – 3.8 million occurring annually. Concern has also arisen regarding the long-term negative effects of repetitive, sub-concussive head impacts (i.e. impacts to the head that do not cause a concussion). Currently, health care professionals rely mainly on symptoms reported by the athlete to determine if a concussion is present, which is largely subjective. This can lead to many unrecognized concussions, which can place athletes in danger of suffering more serious injuries should they return-to-play too soon and suffer a subsequent injury before having fully recovered.

The key goals of this dissertation are to identify an objective marker of the damage associated with both repetitive sub-concussive impacts and a concussion. The results suggest that a molecule released from damaged neurons called neurofilament light (or "NF-L") is a sensitive marker for the pathophysiological processes associated with head trauma. No changes were noted for tau, Tau-A, and Tau-C.

Preface

The study presented in Chapters 3 and 5 was approved by the clinical research ethics board (CREB) of the University of British Columbia (H14-02996). The study presented in Chapter 4 was approved by CREB at the University of British Columbia (H14-00368).

A version of the literature review on fluid-based biomarkers (Chapter 1, Section 4) is currently in the process of being drafted for submission to *PLoS ONE*, an open access scientific journal published by the Public Library of Science (PLOS). I was responsible for gathering the relevant sources for the manuscript, along with selecting the appropriate publications, writing, and editing of the manuscript. Jonathan D. Smirl was also heavily involved in reviewing and editing the first draft, and I hope he continues his efforts as our collaboration will produce a quality document.

A version of the study in Chapter 3 was selected for a poster presentation at the 5th International Consensus Conference on Concussion in Sport in October 2016 (Berlin, Germany). The poster was entitled: *"Serum Neurofilament Light Concentration Increases Following a Season of American Football."*. Authors included: Colin Wallace, Henrik Zetterberg, Kaj Blennow, Kim Henriksen, Kelsey Bryk, Michael Jakovac, Alexander D. Wright, Jonathan D. Smirl, and Paul van Donkelaar. I was responsible for the blood collection, sample processing, sample shipment, data analysis, writing and formatting of the abstract, poster design and build. A version of this chapter is currently being drafted into a manuscript for submission to the journal *PLoS ONE*, an open access journal published by Public Library of Science. Authors will remain the same for the manuscript version of the chapter.

With respect to Chapter 4, I was responsible for the majority of blood sample collection and processing. I was fully responsible for the shipment of samples along with data analysis. Co-investigators on this project included Kevin Bouliane, Joel Burma, Jill Dierijck, Sarah Markson, Maggie McLeod, Jonathan McNulty, Jason Purpur, Jonathan D. Smirl, and Alexander D. Wright.

A version of the study presented in Chapter 5 was accepted for an oral presentation at the International Brain Injury Association's Eleventh World Congress on Brain Injury 2016 (The Hague, Netherlands). The presentation was entitled: *"Assessment of Blood-Based Biomarker Concentrations and Executive Function Pre- and Post-Concussion."* Authors included: Colin Wallace, Kelsey Bryk, Alexander D. Wright, Kaj Blennow, Kim Henriksen, Henrik Zetterberg, and Paul van Donkelaar. I was fully responsible for blood collection, sample processing, sample shipment, data analysis, writing and formatting of the abstract, presentation design and build.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

αSMA – alpha smooth muscle actin

Aβ – amyloid beta

ADAM10 – A Disintegrin and Metalloproteinase 10

AE – athlete exposure

BBB – blood-brain barrier

BCSFB – blood-cerebrospinal fluid barrier

BDP – breakdown product

CHI – closed head injury

CNS – central nervous system

CP – choroid plexus

C-tau – cleaved tau

CTE – chronic traumatic encephalopathy

CuPLA – cumulative peak linear acceleration

CuPRA – cumulative peak rotational acceleration

DAI – diffuse axonal injury

DLB – dementia with Lewy bodies

EAA – excitatory amino acid (e.g. glutamate)

ECF – extracellular fluid

EEG – electroencephalogram

ELISA – enzyme linked immunosorbent assay

FIFA – Fédération Internationale de Football Association

fMRI – functional magnetic resonance imaging

FTD – frontotemporal dementia

GFAP – glial fibrillary acidic protein

HIC – head impact criterion

ICH – intracerebral hemorrhage

IQR – interquartile range

ISF – interstitial fluid

iTBI – inflicted traumatic brain injury

kDa – kilo Daltons

LLOQ – lower limit of quantification

LOC – loss of consciousness

MAP – microtubule-associated protein

MBP – myelin basic protein

MOI – mechanism of injury

mRNA – messenger RNA

MS – multiple sclerosis

mmTBI – mild and moderate TBI

moTBI – moderate TBI

mTBI – mild traumatic brain injury (commonly referred to as a concussion)

NFL – National Football League

NF-L – neurofilament light

NFT – neurofibrillary tangle

NPV – negative predictive value

NSE – neuron specific enolase

nTBI – noninflicted traumatic brain injury

PET – positron emission tomography

pNF-H – phosphorylated neurofilament heavy

PLA – peak linear acceleration

PNS – peripheral nervous system

PPV – positive predictive value

PRA – peak rotational acceleration

p-tau – phosphorylated tau

RTP – return-to-play

SRC – sports-related concussion

sTBI – severe traumatic brain injury

Tau-A – ADAM10-cleaved tau protein

Tau-C – caspase-3-cleaved tau protein

TBI – traumatic brain injury

T-tau – total tau

VaD – vascular dementia

Acknowledgements

I offer my heartfelt thanks to Dr. Paul van Donkelaar for allowing me to pursue a career in the field of biomarker research. I am indebted to you for your support, patience, and guidance throughout my time in your lab.

My gratitude is also extended to Dr. Jonathan Little and Dr. Henrik Zetterberg for their supervision, and affording me the opportunity to engage with their students, technicians, and colleagues. This lead to many conversations, even more questions (mostly from me), and further collaborative efforts.

Dr. Jonathan Smirl has been an incredible addition to the lab, and a source of much needed guidance as this document progressed. Dr. Smirl's commitment to his work is a source of inspiration, and I cannot thank him enough for his help throughout my PhD.

I have had the honour of working with a number of incredible people throughout the past 5 years. Thank you to especially Kevin Bouliane, Kelsey Bryk, Joel Burma, Tanis Burnett, Jill Dierijck, Mike Kennefick, Kit Marsden, Maggie McLeod, Jason Purpur, and Nicole Strachan. Thank you all for making this experience a memorable one.

To the staff and faculty of the Human Kinetics and Athletics departments at the University of British Columbia, including, but certainly not limited to, Dr. Philip Ainslie, Dr. Gord Binsted, Nicole Carlos, Dr. Brian Dalton, Dr. Gregory duManoir, Dr. Neal Eves, Terry Hill, Audrey Kirby, Kevin Phillips, Jennifer Rhodes, and Jeff Thorburn. Thank you all for always opening your door to me whenever I needed guidance or a place to let off steam.

Special thanks to my family including my wife Emily, my son Jayden, my daughter Brooklyn, and my parents, Kieran and Joyce. Your encouragement and belief in me gives me

strength each and every day. I cannot thank you enough for your support, both morally (everyone) and financially (mom and dad!)

Dedication

This entire process may not have happened without the love and support of my wife, Emily Wallace. I am incredibly lucky to have met you, and I am grateful to past-Colin for having the courage to call you up and ask you on a date. I knew, after that night, it was going to be a long time before you got rid of me! Here we are, almost 16 years later, and I still smile when I wake up next to you. Our two beautiful children are incredibly lucky to have you as their Mom and their best friend. I am in awe of your patience, your capacity to love, and your dedication to friends and family. I cannot say this enough, I love you so very much.

To my son Jayden, and my daughter Brooklyn. You two are the best things to ever happen to your Mom and I. I love you both so, so much. Jayden, I love how you ask me to do jigsaw puzzles with you, how you ask me to play trains with you, and how you ask me to play chase or hide and seek with you. I hope that never stops; however, I know one day you'll grow up and the opportunity to do a jigsaw puzzle with you won't come around as often for me. Please know that I cherished every second of those times when you were a kid. Brooklyn, your sense of humour has me in stitches most of the time! You are hilarious, compassionate, and the daughter I always wanted. I see so much of Mommy in you, and that makes me happy, as I know you'll grow up and continue to be the incredible person you already are! I love playing hide and seek with you, making you Nutella and toast in the mornings, and reading stories to you at night. I pretty much have every Pinkalicious book memorized! Thank you for being you, and please don't ever change!

To my parents, thank you so very, very much. For your moral support, your financial support, and every other type of support you've given me over the years. Whether it was a swift

kick in the rear to get me motivated, or listening to me rant (when I actually did come upstairs or call to let you know how the last two weeks of my life went.) I know I can always call you and get the guidance I need to make the best decision possible for my family and myself. I question my abilities a lot, maybe too much, but in athletics and academia, that comes with the territory. Those nights where I've been in bed wondering if I could do what I needed to do the next day, I would inevitably think of the confidence you both have in me, and I'd drift off to sleep, thinking "I've got this." Thank you for that piece of mind, even if you didn't know you were providing it. Your voices have always been stronger than those in my head telling me I can't do it. I can't thank you enough for providing those voices.

To Lynda North, Will North, Rick and Monique North, and the rest of the North family, thank you for always welcoming us into your homes, for visiting ours, and for all the jokes, good food, drinks, and music shared between them. I hope, as soon as this degree is concluded, we are able to visit much more often, so we have more opportunities to enjoy all the aforementioned fun stuff (i.e. food, drinks, music).

Thank you, to everyone who helped me along the way. Truly, thank you.

Chapter 1: Introduction

1.1 Background and Significance

Researchers and clinicians alike are striving to identify optimal objective test protocols in order to truly assess both neurocognitive decline and pathophysiological changes that occur as a result of a concussion, or after a series of sub-concussive impacts. As it stands, there are no objective, *in vivo* methods of determining if an individual has suffered a concussion. A sensitive test for these conditions would be invaluable to the medical community. Advances in research will allow us to recognize pathophysiological changes, that may lead to future treatment options to limit neurological damage. By implementing a treatment protocol in the early stages following brain trauma, health care professionals may be able to stave off symptoms, allowing the individual to live a longer, healthier life. As it stands, this is not possible, and until researchers discover an objective test, concussions remain one of the most complicated injuries sports medicine professionals encounter.

1.2 Goals and Specific Aims

The overall goals of this dissertation are to examine both the acute and chronic pathophysiological effects of concussion through plasma and serum sample analysis for the identification of an objective fluid biomarker indicating axonal trauma. Current literature on fluid-based biomarkers focuses on the identification of these proteins through the use of CSF; however, this method of testing includes a host of challenges, and is invasive to the patient. Further, not all athletes or individuals can be tested at similar time points post-injury, and so this

dissertation will examine time points that differ from those in the literature for biomarkers of axonal disruption in order to extend the current temporal profile for each.

This particular dissertation includes three specific aims:

***Aim 1*:** To examine concentration changes in serum and plasma-based biomarkers to detect indicators of axonal disruption following a season of sub-concussive impacts in rugby, soccer, ice hockey, and American football players when compared with non-contact athletes (i.e. cross-country runners). ***Aim 2*:** To determine whether an acute bout of soccer heading results in increased serum and plasma concentrations of biomarkers of axonal disruption. ***Aim 3*:** To examine concentration changes in serum neurofilament light, ADAM10-cleaved tau (Tau-A), and caspase 3-cleaved tau (Tau-C), along with plasma total tau at preseason baseline, and again at 6- and 14-days post-concussion. The goal is to extend the current temporal profile of these proteins and their derivatives post-concussion.

1.3 Hypotheses

1.3.1 Chapter 3, Study 1 (Blood-Based Biomarkers and Sub-Concussive Impacts)

Hypothesis 1: Athletes engaged in contact-sport participation will experience an increase in blood-based biomarker concentration compared to cross country runners.

Hypothesis 2: The increase in blood-based biomarker concentration in American football players will be positively correlated with head impact metrics including cumulative number of hits sustained over the course of a season, cumulative peak linear acceleration, and cumulative peak rotational acceleration.

1.3.2 Chapter 4, Study 2 (The Effect of an Acute Bout of Soccer Heading on Blood-Based Biomarkers of Axonal Disruption)

Hypothesis: An acute bout of soccer heading will lead to an increase in blood-based biomarker concentration 1-hour post-intervention compared to sham and control trials.

1.3.3 Chapter 5, Study 3 (Blood-Based Biomarkers of Axonal Disruption Show No Change at 6- and 14-Days Post-Concussion)

Hypothesis 1: A concussion will result in an increase in neurofilament light, Tau-A, and Tau-C concentration at 6- and 14-days post-concussion.

Hypothesis 2: Total tau concentration will be no different from baseline than at 6- and 14-days post-concussion.

1.4 Literature Review

1.4.1 Concussion

Concussion, a mild form of traumatic brain injury (TBI), is the term used to describe contact to the head or body resulting in a disturbance to brain tissue, and subsequent clinical symptoms[1]. In recent years, the long-term ramifications associated with concussions have seen an increase in publicity, and have commonly been described as a global health concern. Faul *et al.* (2011) reported an estimated 1.7 million TBIs from all causes (sport-related, motor vehicle accidents, falls, etc.) report to the emergency department each year in the United States, with the vast majority of these injuries falling into the mild category[2]. However, there are many more individuals who sustain a mild TBI (mTBI) and may not visit an emergency department (ED) for treatment. As such the estimate provided by Faul *et al*., (2011) likely underestimates the overall

incidence rates. Other avenues for diagnosis and recovery for concussion sufferers include: hospital outpatient settings, physician offices, military facilities, and professional, semi-professional, amateur, collegiate, and high school sports teams with the appropriate medical personnel[3]. A further unknown is the number of individuals who sustain a concussion but do not seek medical care. When all of these populations are taken into consideration, it has been estimated that the number of purely sport-related TBIs that occur in the USA each year is closer to 3.8 million[4].

A 2008 survey in the United States reported that an average of approximately 60 million children and adolescents participated in organized athletics each year[5], and a 2010 survey in Canada found, on average, 7.2 million Canadians aged 15 and older participated in athletics each year[6]. Assuming there is a fairly equal concussion rate between both the USA and Canada, we can extrapolate and estimate there are approximately 400,000 sport-related concussions in Canada each year, although Canadian data are not available[7]. Another common mechanism for concussion reporting is to quantify the number of injuries per athlete exposure (AE: practice or game situations). Sports with the highest reported incidence rates of concussion per 1000 AE for males include rugby (2.50-5.86), American football (0.33-0.61), ice hockey (0.41), and soccer (0.17-0.49), while sports involving females with the highest reported incidence rates include ice hockey (0.91), soccer (0.13-0.63), and basketball (0.16-0.43)[8-13].

Although the estimated number of sport-related TBIs occurring each year appears to be high, it is believed that many more concussions go undetected[4]. Indeed, multiple surveys involving athletes (including athletes immediately following an injury to those who have years after recovery), have found under-reporting of symptoms is an extremely common practice within athlete populations[14-17]. Results from a 2004 study by McCrea *et al.* revealed 52.7% of

concussions during a season of high-school football went unreported[18]. Players in this study presented reasons such as thinking the injury was not serious enough and/or simply not knowing they had suffered a concussion. Kroshus *et al*. found nearly 48% of male and female collegiate contact and collision sport athletes reported continuing to play while experiencing concussive symptoms following an impact[14]. Over 25% of the athletes sampled in this study reported experiencing pressure to keep playing following a head impact from their coach, a teammate, fan or parent. Antiquated terminology associated with mTBI's may also influence an athlete's depiction of the number of their reported concussions. In a survey of 520 high school athletes by McLeod *et al.,* only 44 (8.5%) reported suffering a concussion at some point in their athletic history[19]. In contrast, 130 (25.0%) reported having had their bell rung or being dinged, even though the terms "bell-ringer" and "being dinged" are commonly associated with concussions and have long-since been dropped from medical vernacular when referring to a head injury[20].

Table 1.1 – Traumatic Brain Injury Classifications[21]

Criteria	Mild	Moderate	Severe
Structural Imaging	Normal	Normal or abnormal	Normal or abnormal
Loss of consciousness	0 – 30 minutes	>30 min and <24 hours	>24 hours
Alteration of consciousness / mental state	A moment up to 24 hours	>24 hours. Severity based on other criteria	
Post-traumatic amnesia	0 – 1 day	>1 and <7 days	>7 days
Glasgow Coma Scale (best available score in first 24 hours)	13 – 15	9 – 12	<9

Under-reporting of the symptoms associated with concussions may also be a result of confusion in the exact signs and symptoms of this injury, due in part to the variation in diagnostic criteria from multiple sources. The Management of Concussion/mTBI Working Group gives the following criteria (Table 1.1) for a concussion: (i) alteration of consciousness

less than or equal to 24 hours; (ii) loss of consciousness (LOC) of 0 – 30 minutes; (iii) post-traumatic amnesia less than or equal to 24 hours; (iv) normal structural imaging. The Glasgow Coma Scale (GCS) was developed in 1974 and is designed to grade the severity of impaired consciousness in patients with traumatic brain injuries[22]. The scale consists of 15 points, 4 for Best Eye Response, 5 for Best Verbal Response, and 6 for Best Motor Response (Figure 1.1). The GCS is the most widely used tool to assess the neurological status of a patient[23,24]. From the GCS, TBI is separated into mild (13 – 15), moderate (9 – 12), and severe (<9) categories. In contrast to these relatively strict criteria, the Consensus Statement on Concussion in Sport[25] defines a concussion as an injury to the head that results in one or more of the following: (i) one or more of 22 symptoms (including somatic, cognitive, and emotional) listed in the Sport Concussion Assessment Tool, 5th Edition (SCAT5); (ii) physical signs such as LOC or amnesia; (iii) behavioural changes; (iv) cognitive impairment; (v) sleep disturbance. While these are all possible common features of a concussion, they are not mandatory for a clinical diagnosis. Ergo, it is possible for an individual to suffer a concussion and not experience amnesia, LOC, or a measureable cognitive impairment. Indeed, commonly reported symptoms of a concussion include headache, dizziness, and irritability[25]. Interestingly, a minor headache (1 – 2 on the 7 point Likert scale found in the SCAT5) may be an individual's sole complaint. Another obstacle in detecting a concussion is this symptom may not manifest and present for minutes to hours after the concussive event has occurred[25]. Further, many of the symptoms listed in the SCAT5, such as nausea, dizziness, fatigue, and irritability, can exist in a variety of complex disease processes and can be outcomes from an intense practice and/or game where the athlete received no body or head contact. Taken all together, it is apparent the clinical diagnosis of a concussion

can be difficult due to the subjective nature of the self-reported symptoms in the current diagnostic system.

GLASGOW COMA SCALE (GCS)[3]

Time of assessment			
Date of assessment			
Best eye response (E)			
No eye opening	1	1	1
Eye opening in response to pain	2	2	2
Eye opening to speech	3	3	3
Eyes opening spontaneously	4	4	4
Best verbal response (V)			
No verbal response	1	1	1
Incomprehensible sounds	2	2	2
Inappropriate words	3	3	3
Confused	4	4	4
Oriented	5	5	5
Best motor response (M)			
No motor response	1	1	1
Extension to pain	2	2	2
Abnormal flexion to pain	3	3	3
Flexion / Withdrawal to pain	4	4	4
Localizes to pain	5	5	5
Obeys commands	6	6	6
Glasgow Coma score (E + V + M)			

Figure 1.1 – Glasgow Coma Scale[26] (Reprinted with permission)

Regardless of the reason, athletes who do not report symptoms and return-to-play (RTP) too soon can put themselves at risk for possibly disastrous consequences. In 2006, 13-year-old Zackery Lystedt, from Maple Valley, Washington, was allowed to RTP in an American football match following a concussion without the clearance from a physician. At the end of the game, Zackery collapsed on the field, and soon after underwent emergency life-saving brain surgery. This event inspired the passing of the first RTP law in the United States, House Bill 1824, otherwise known as the Zackery Lystedt Law. This law requires youth athletes to be removed

from practice or play at the time of a suspected concussion or head injury, and medical clearance from a physician in order to RTP following a suspected concussion. After the state of Washington adopted the Lystedt Law, the number of documented concussions more than doubled[27]. This increase is thought to reflect increased awareness of the injury, along with its signs and symptoms.

Following the implementation of concussion legislation in Washington state, an overall improvement in concussion knowledge and awareness was documented in coaches. Of the 270 coaches surveyed for a study by Chrisman et al. in 2014, 91% received education on concussions in multiple formats (i.e. written, video, slide presentation, test, or in person)[28]. Unfortunately, only 34.7% of athletes and 16.2% of parents received the same training. Lack of knowledge in reporting signs and symptoms of a concussion may result in an athlete returning to play too soon. If a second concussive injury is sustained prior to recovery from the initial insult, prolonged postconcussion syndrome may occur[29]. In extreme cases, permanent disability and even death are possible outcomes of second impact syndrome[30]. It is important for coaches, parents, officials, and athletes alike to be aware of the signs and symptoms of a concussion, as the brain is in a vulnerable state following the injury.

1.4.2 Neurometabolic and Structural Physiology of Concussion

The vulnerable state of the brain following a concussion exists due to specific microstructural events that occur immediately following the onset of injury. When an axon is stretched less than 15-20% of its resting length, mechanoporation occurs in the absence of axon tearing[31,32]. Due to this mechanical disruption of neuronal membranes an ionic flux occurs at the cellular level[31,33,34]. Along with a sodium (Na^+) influx, potassium (K^+) channels open resulting

in a notable K^+ efflux[33-36]. This K^+ efflux can, if great enough, induce a spreading depression of neuronal activation[35]. Following K^+ efflux, glutamate, an excitatory amino acid (EAA), is released unsystematically throughout the cortical tissue[33,34,36,37], resulting in a widespread depolarization and further increase of K^+ efflux, along with an intracellular increase of calcium (Ca^{2+}) ions[32,37].

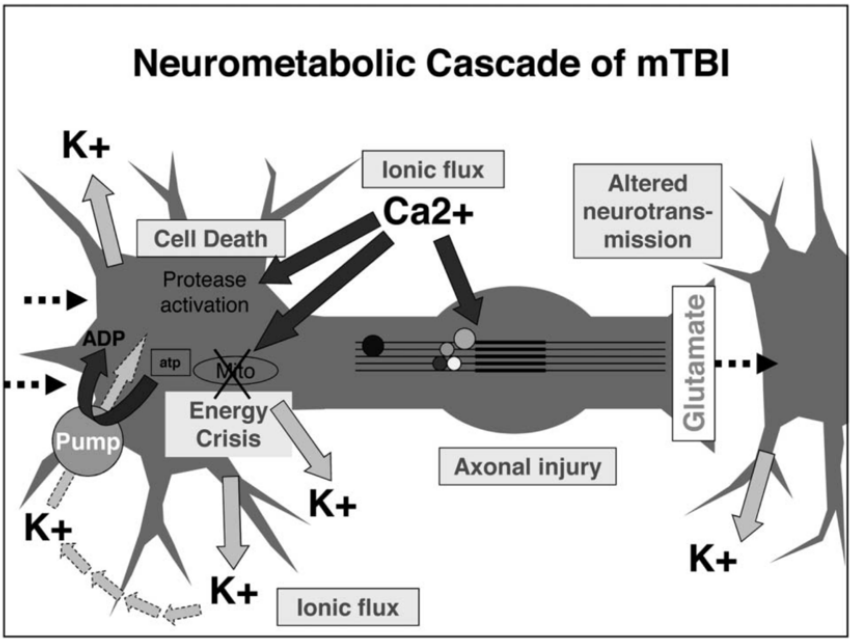

Figure 1.2 – Neurometabolic Cascade of mTBI[34] (Reprinted with permission)

This process can damage the local cellular environment, impairing the neurons and is commonly known as excitotoxicity. An increase in intracellular Ca^{2+} has been shown to occur when the neuron strains are <5%, leading to axon degeneration[38]. In response to this intracellular Ca^{2+} flux, which is primarily derived from intracellular stores, Ca^{2+} is shuttled into the mitochondria, that can result in organelle pathology in the form of secondary axotomy[32,34,37]. Immediately following a concussion, this ionic flux and subsequent depolarization may cause voltage- or ligand-gated ion channels to open, resulting in a *"spreading depression-like state"* that is thought to be an underlying mechanism in both the neurocognitive impairments and

symptomology present[34]. As a result of these ionic imbalances, ATP-requiring membrane pumps exhibit a transient hyperactivity, leading to hyperglycolysis[34].

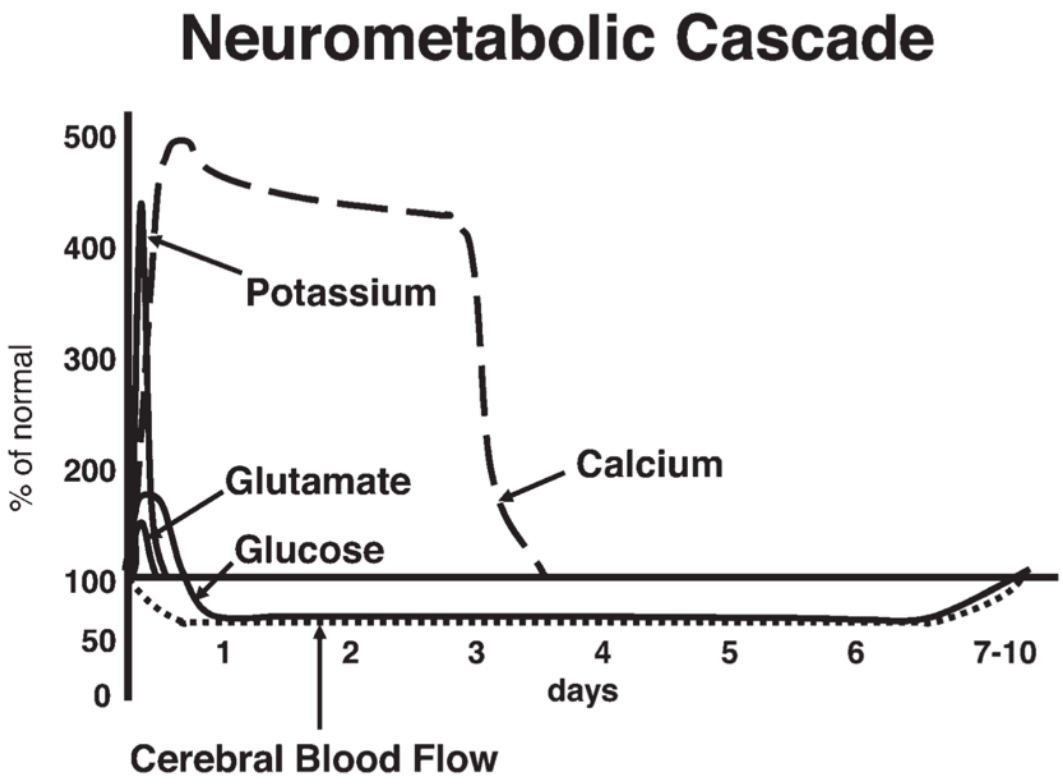

Figure 1.3 – Neurometabolic Cascade[34] (Reprinted with permission)

In addition, it has been speculated there is mitochondrial dysfunction present following a concussion, that results in a mismatch between the supply and demand of energy sources within the brain[33,34,37]. This energy crisis has been associated with neurocognitive impairments, which has a deleterious effect on synaptic neuroplasticity[34,39]. Combined, these are some of the speculated reasons why the brain is especially vulnerable to a second injury while recovering from a previous concussion[34,39].

Calcium-dependent, non-lysosomal cysteine proteases, known as calpains, are involved in fundamental processes such as synaptic plasticity and cell signalling; however, they also play a pathological role in excitotoxicity[40,41]. Following an axonal injury and the subsequent change

in intracellular Ca^{2+} concentration, calpains migrate into the plasma membrane from the cytosol, and proteolytic processes begin. One particular cytoskeletal protein, αII-spectrin, found along the intracellular edge of the plasma membrane, is targeted by calpain and caspase-3[32]. This proteolytic activity results in the cleavage of αII-spectrin derivatives, otherwise known as spectrin breakdown products (SBDP), including calpain derived N-terminal fragment (SNTF), and C-terminal fragments SBDP150 and SBDP145, along with caspase-3 derived SBDP150i and SBDP120[42,43]. Following a TBI in both human and rat models, CSF concentration of αII-spectrin cleaved protein fragments is elevated compared to non-injured subjects[44-47].

Within the context of a sport-related concussion, SNTF has also shown promise as a viable biomarker of injury. In a 2015 prospective study, elevated serum SNTF concentrations were reported in Swedish professional ice hockey players who suffered a concussion during a league game[48]. Elevated concentrations were found within 1 hour post-injury, and remained elevated for up to 144-hours post-concussion. These values had returned to pre-season baseline levels when all players returned to play. Indeed, these are promising results, and validate further investigation into the diagnostic and prognostic capabilities of SNTF. However, αII-spectrin is not a CNS-specific protein; it is also a structural, cytoskeletal component of human plasma membranes, and is found in a variety of locations throughout the body including red blood cells (RBCs), muscle, and peripheral neurons[49,50]. The ubiquitous nature of αII-spectrin has added doubt to its reliability in being a biomarker for objectively diagnosing brain injuries. A CNS-specific protein has the potential of being relied on as a more sensitive and specific measure of CNS axonal injury.

Tau protein is a microtubule-associated protein (MAP) that is predominantly found in CNS cells, primarily in thin, unmyelinated axons[51]. Tau functions to promote microtubule

assembly, and it is a major contributor to the structural integrity and stabilization of the microtubule[52,53]. Following a sport-related concussion, plasma tau concentrations have been shown to display similar dynamics to serum SNTF, with levels increased when compared to non-injured controls up to 144 hours after a mTBI[54]. Repetitive head trauma during amateur boxing matches have also revealed augmented CSF levels of tau, indicating chronic, sub-concussive impacts may also cause axonal trauma[55].

Another family of proteins, neurofilaments, are enriched in the CNS, primarily in large, myelinated axons and aid in resisting stretch injury[51,56]. Neurofilaments are intermediate filaments with three subgroups, neurofilament light (NF-L), neurofilament medium (NF-M), and neurofilament heavy (NF-H)[57], and all but NF-M have been examined for their diagnostic and prognostic capabilities. Proteolytic activity following intracellular Ca^{2+} increase also results in the compaction of cytoskeletal neurofilament components, and has been found following a TBI in humans and rats[58]. For example, NF-H has been shown to increase following a bout in amateur Olympic boxing, remaining at elevated levels for up to six days[59]. NF-L has been studied more extensively, and has shown to increase in a variety of populations exposed to head trauma[55,60-62]. CSF NF-L levels increase following a concussion and remain elevated for up to 36 weeks post-injury[63]. Serum and CSF NF-L levels have also been shown to increase following a series of sub-concussive impacts[55,64,65]. Furthermore, CSF levels of NF-L are elevated in individuals suffering from post-concussion syndrome (PCS)[66]. In addition to its diagnostic properties, NF-L has also displayed prognostic abilities with respect to TBI[67,68], giving promise for its use as a reliable biomarker for CNS axonal damage.

From an objective diagnostic TBI biomarker perspective these are certainly encouraging results, and should provide guidance for future research. With respect to NF-L, it is imperative

future studies include similar time-points to those sampled in research on tau protein (and other biomarkers), starting with as close to time of injury as possible and extending until clinical recovery and return-to-play. As it stands, there exists only one case study publication examining NF-L concentrations post-concussion. Longitudinal cohort studies with larger sample sizes are required in order to truly assess the diagnostic and prognostic properties of the neurofilament family of proteins.

In summary, to date no single biomarker has emerged as a widely used objective clinical tool that is able to specifically examine brain damage following a concussion. Reasons for this include limitations in sensitivity, specificity, and standardized quantification across multiple laboratories and studies.

1.4.3 The Need for Objective Markers of Concussion

Currently, clinicians are reliant on subjective measurements to identify an individual with a concussion including: reaction time (assessed through numerous neurocognitive testing paradigms[69,70]), memory[69], and self-reported symptom scores on a 7-point Likert scale[1,71]. Although these computer and paper-based assessment tools are widely used[72-74], there has been much debate as to their clinical efficacy in accurately identifying both whether an individual has suffered a concussion, and when complete physiological recovery has occurred[71,75,76]. The accuracy of any computer or paper-based tool is a product of its sensitivity and reliability, both of which are susceptible to a number of outside influences, such as practice effects or players purposely underperforming on baseline assessments (i.e., sandbagging) and rely heavily on the conditions under which the test was administered[76,77]. An ideal objective biomarker would

enable the accurate identification of a concussion, potentially predict the duration of clinical outcome and be able to track an individual's recovery.

Detecting the extent of axonal injury is important, particularly in contact sport athletes. Conventional structural neuroimaging techniques, such as computed tomography (CT) and magnetic resonance imaging (MRI), of the brain following a concussion are unremarkable, and are only employed when the structural integrity of the skull is in question or the possibility of a hemorrhage is present[1]. Repetitive sub-concussive and concussive events have been linked to white matter abnormalities on diffusion-tensor magnetic resonance (MR) imaging[78], and certain neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS)[79,80], and chronic traumatic encephalopathy (CTE)[81,82]. CTE is a neurodegenerative disease with a clinical onset later in life, typically years after recovery from brain trauma. Although the relationship between repetitive concussion and CTE is unclear, there is a growing body of research hypothesizing the physiological and mechanistic link between the two[82-85].

Following a mild axonal injury there is an increased expression of Na^+ channels. One potential mechanism is the pathologic increase in intracellular Ca^{2+}, seen after consecutive mild traumatic axonal injuries (i.e. 3% strain in the axon) spaced 24 hours apart, resulting in degeneration of the affected axons[38]. This is of particular relevance for athletes who participate in sports involving repetitive, sub-concussive impacts, such as American football. Head impacts experienced by defensive linemen occur up to 1.98 times more frequently than defensive skill players (e.g. cornerbacks)[86]. When expanded to a full season, offensive and defensive linemen in American football sustain a greater frequency of head impacts when compared to other positions[87].

Taken further, dementia in retired National Football League (NFL) players over the age of 50 occurs 5 times more frequently than the national average for men in that particular age group[88]. Researchers at Boston University have examined the donated brains of former players and have confirmed the presence of CTE in over 90% of the cases[89]. In 2013, researchers reported the presence of CTE in 3 of 6 brains of former Canadian Football League (CFL) players[90]. As it stands, there are currently no objective tools that are able to quantify structural changes due to the presence of CTE *in vivo*; the diagnosis ultimately occurs during post-mortem examination.

An objective biomarker of axonal damage due to concussive and sub-concussive impacts would provide valuable insight for healthcare professionals into the neuronal health of contact sport athletes and the broader population. The identification of an objective biomarker not only has the potential to identify individuals who have suffered a concussion, but also opens the door for targeted medical approaches to the immediate management and recovery of these individuals.

1.4.4 Fluids Examined

Identification of objective markers of CNS injury and degeneration has been attempted through the analysis of both CSF and blood. CSF is a plasma-like, modified filtrate of the blood produced in the ventricles, specifically the choroid plexus (CP), of the brain. The CSF provides mechanical support to the brain, along with immunological protection, nutrients, and astroglial molecules to neurons of the CNS. CSF produced in the lateral ventricles follows a path through the third ventricle to the fourth ventricle. The fourth ventricle contains a bifurcation allowing the CSF to flow to the central portion of the brain and spinal cord through the central canal, or it can flow laterally into the subarachnoid space. The CSF is ultimately collected through

subarachnoid granulations and is reabsorbed into the venous system. Each day, approximately 400 – 500mL of CSF is produced and reabsorbed, maintaining an intracranial volume consistently at approximately 150mL within the cranial ventricular system[91,92]. Sampling of CSF can be done in outpatients, through the intervertebral space between two lumbar vertebrae, typically L3 and L4 or L4 and L5[93,94]. A 2010 prospective study reported a low incidence (i.e. 2.6%) with just 28 mild, post-lumbar puncture headaches astroglia in 1089 cases[94]. This was the only reported complication following the procedure, reaffirming the safety of the lumbar puncture procedure as a sampling method for trained individuals.

Safe sampling of CSF from the lumbar region allows access to a fluid in direct contact with the brain parenchyma. Thus, the CSF should provide candid information on any modifications to the biochemical status of the brain. These modifications are also subject to low proteolytic activity, increasing their sensitivity to detection methods. One particular disadvantage is the volume of CSF; approximately 150mL is present at any given point in time[91]. This limited volume of fluid makes sampling at multiple time points within a short period of time difficult. Furthermore, lumbar puncture is considered more invasive than a blood draw; it requires the placement of a spinal catheter by a skilled physician in order to safely perform the procedure. For these reasons, the ability to establish the temporal profile associated with each CSF specific biomarker is limited. The time profile is essential information for the construction of biomarker panels for CNS-injury, as it is highly unlikely an individual will be present at the clinic when an injury occurs.

Blood samples on the other hand can be collected at multiple time points in a relatively short period of time, given that there is approximately 4 – 5L in the average human adult[95]. Additionally, a blood sample can be separated into either serum or plasma that enables further

comparison to be made. Blood is also more accessible than CSF, with the easiest point of access through a vein in the antecubital fossa, preferably the median cubital vein. Serum does not contain fibrinogens responsible for blood clotting; however, both serum and plasma contain proteins that can be quantified through a variety of analytical techniques. The sensitivity of these techniques is an ever-evolving process, as molecules of interest may exist in the blood at extremely low concentrations (i.e. femtogram per milliliter or 0.00000000000001g /mL). This can occur for a variety of reasons, including the fact that blood does have a relatively high proteolytic rate when compared to CSF. Proteins found in the blood are constantly degrading due to enzymatic activity, which can occur even after blood has been obtained through a venous sample. Furthermore, these proteins in the blood derived from the brain parenchyma must cross specific neural barriers in the brain before they reach the bloodstream. Therefore, the ability to measure them in the blood depends on their safe passage across these neural barriers and the sensitivity of the assessment tools (for more detail refer to Methods, Section 2.2).

1.4.5 Neural Barriers of the Brain

Neural signaling within the CNS is a complex process, and so the microenvironment, surrounding both dendritic and synaptic regions, requires stringent regulation. This is achieved through the combined efforts of three restrictive barriers: (i) the blood-brain barrier (BBB); (ii) the blood-CSF barrier (BCSFB); and (iii) the avascular arachnoid epithelium, otherwise known as the arachnoid barrier (AB)[96,97].

With a surface area of approximately $20m^2$ within the human brain[98], and the multitude of protective mechanisms, the BBB is the primary structure responsible for homeostasis of the internal cerebral environment. This is accomplished through numerous communication

pathways between neurons, astrocytes, pericytes, and endothelial cells. The result is the transcellular passage of specific proteins both into and out of the interstitial fluid (ISF), providing an optimal setting for synaptic and axonal function.

Astrocyte presence has a direct influence on the molecular transport capabilities of the endothelium; an increase in astrocytic expression leads to an increase in tight junction density. Astrocytes also provide cerebral microenvironment protection through a metabolic barrier involving a variety of enzymes aimed at metabolizing potentially harmful substances attempting transcellular movement. In the presence of astrocytes, endothelial cells also express an increased density of alkaline phosphatase and Na^+/K^+ ATPase, responsible for removing phosphate groups from molecules and maintaining cellular resting potential, respectively[99]. This metabolic barrier is strengthened with the presence of pericytes, located in the basal lamina and encircling the capillaries. These cells are thought to serve a variety of functions, such as the formation and stabilization of the BBB during embryogenesis, regulation of vascular permeability, and phagocytic activity[100,101]. Through communication with endothelial cells and neurons, both pericytes and astrocytes influence cerebrovascular autoregulation and capillary blood flow in response to metabolic demands and ionic shifts. Following simulated ischemia and TBI, pericytes increase their expression of α-smooth muscle actin (αSMA), a major constituent of the contractile apparatus of smooth muscle cells[99]. Given the multitude of functions in which pericytes are involved, it stands to reason that they possess stem cell-like qualities. Indeed, pericytes have been found to form bone nodules, chondrocytes, and adipocytes[102,103]. The pluripotentiality of pericytes provides the BBB with an evolving defense mechanism, capable of responding to conditions of stress.

The signal transduction pathways of the BBB are governed by the interaction between the various components of the BBB. The physical barrier of cerebral endothelial cells of the BBB differ from those of non-neural structures including reduced caveolae presence, the existence of tight junctions between the cells, and increased density of mitochondria. Caveolae are membrane-bound, with openings directed either to the luminal or abluminal direction. Channels connecting both luminal and abluminal spaces can be formed by the joining of adjacent caveolae; however, this only occurs in the cerebral endothelium following breakdown of the BBB[99]. Proteins in the plasma required for the maintenance of cell homeostasis are selected by cerebral endothelial cells and deposited into caveolae, followed by receptor-mediated and receptor-independent transcytosis (the transport of macromolecules across the interior of a cell), endocytosis (the taking in of matter by a living cell by invagination of its membrane to form a vacuole), or potocytosis (receptor-mediated endocytosis in which small molecules are transported across the plasma membrane of a cell). The movement of the caveolae within the cell is mediated by a number of actin cytoskeleton-related proteins, including spectrin, that contribute to membrane integrity[44]. SNTF has been shown to increase in concentration in the blood samples from concussed hockey players when compared to controls[48], indicating there is a possible BBB membrane breakdown with this injury. Due to the decreased density of caveolae in the cerebral endothelium, there are fewer occurrences of endo- and transcytosis as compared with the peripheral endothelium. Molecular transport is further regulated by the presence of the pentalaminar tight junction (Figure 1.4), located at the apical end of adjacent endothelial cells, forcing most molecules to pass transcellularly from the luminal to the abluminal membrane, or vice versa.

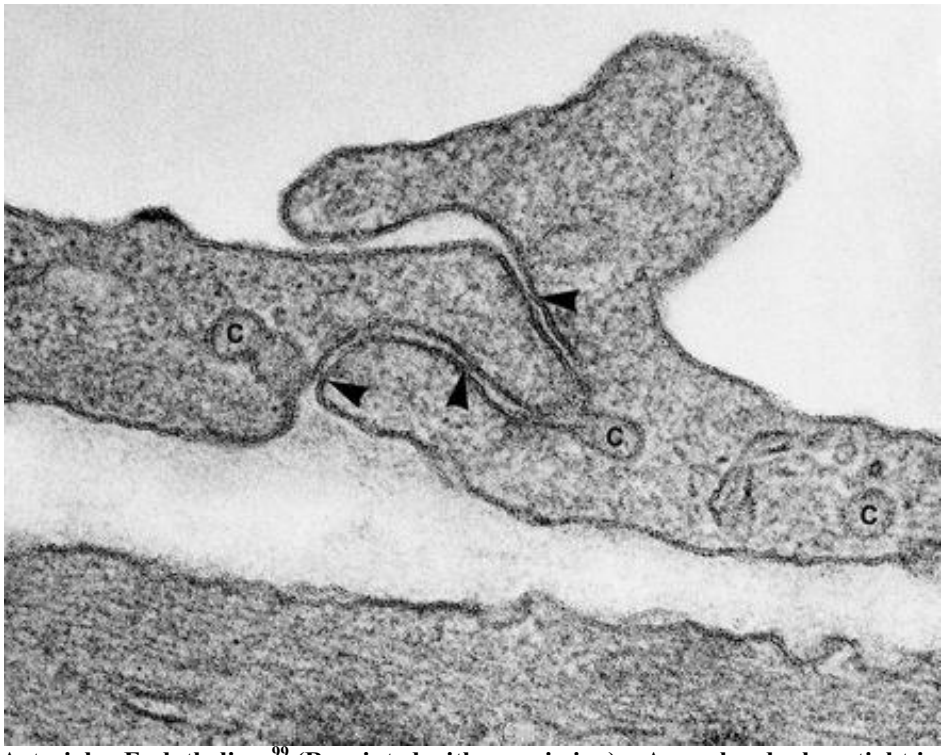

Figure 1.4 – Arteriolar Endothelium[99] (Reprinted with permission) – Arrowheads show tight junctions along interendothelial space. Caveolae (C) are also pictured.

The second restrictive barrier (BCSFB), is present primarily in two distinct areas of the brain: i) at the arachnoid blanket; and ii) the CP in the third, fourth, and lateral ventricles. At the arachnoid blanket, CSF reabsorption is the dominant function, whereas CSF secretion predominantly occurs at the CP. The human CP is responsible for a turnover rate of 400 – 500 mL/day of CSF, with regulation occurring from a sequence of compartments and, ultimately, tight junctions along the apical surface of the choroidal epithelium[91,92]. These tight junctions restrict small proteins such as microperoxidase, that has a molecular weight of 2 kDa. The mediation of protein concentration occurs further in the interstitial fluid of the brain through phagocytosis by fibroblast and macrophage activity, and through lysosome activity at the

epithelial cell level[104]. Due to the strict membrane permeability regulation of protein transport, CSF protein concentration is up to 3 orders of magnitude lower than in plasma[99].

Further protection is provided by the arachnoid barrier, comprised of cells containing tight junctions, that comes in direct contact with the CSF deep to the arachnoid mater in the subarachnoid space[97]. The arachnoid barrier is avascular and has a small surface area, as such very little exchange between the blood and CSF takes place at this site[105]. Taken together, these three restrictive barriers, including the BBB, BCSFB, and AB all function to maintain an optimal environment for CNS function.

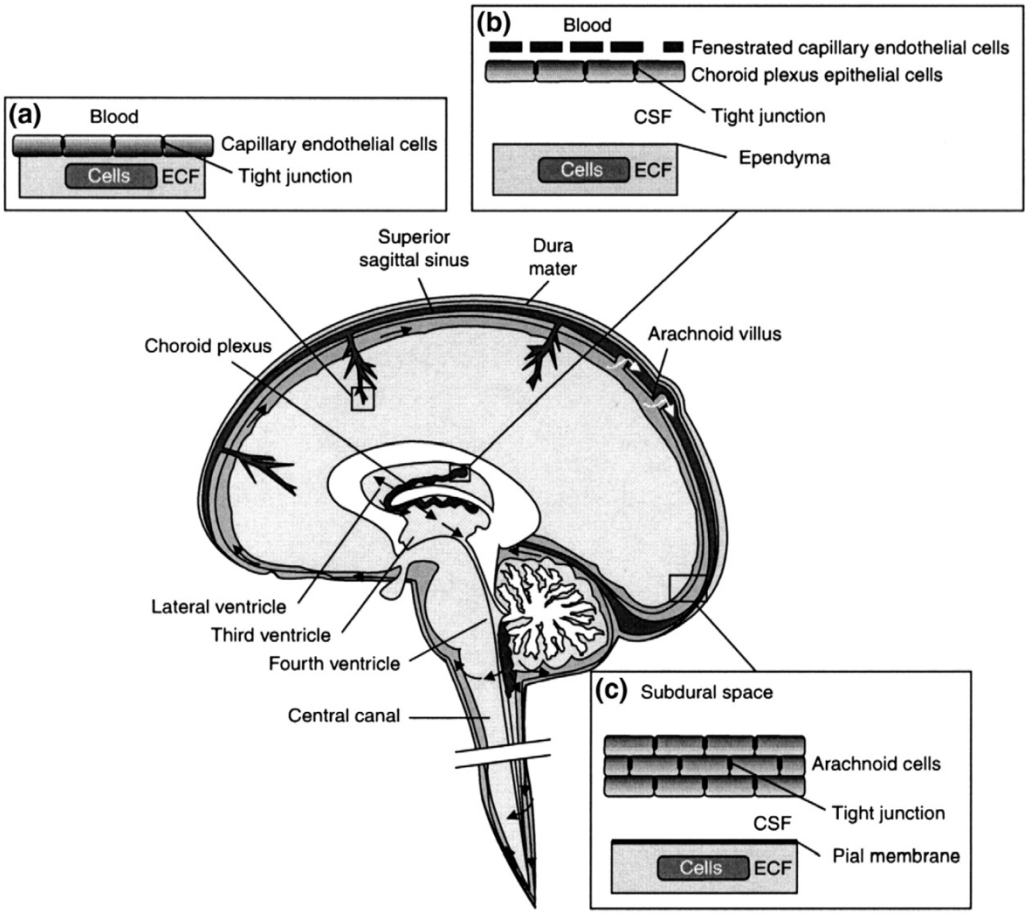

Figure 1.5 – The Barriers of the Brain[105] (Reprinted with permission) – (a) The BBB; (b) the BCSFB; (c) the arachnoid barrier

1.4.6 Astrocytes

Despite the complex organization of the nervous system, it can be divided into two fundamental cell types, neurons (excitable nervous cells) and neuroglia (non-excitable or supporting nervous cells). Glia are present in the CNS, and it is estimated glial cells are either equal to or greatly exceed the number of neurons[106,107]. The neuroglia can be subdivided into two major classifications; the macroglia (i.e. astrocytes, oligodendrocytes, ependymal cells, radial glia, Schwann cells, satellite cells, and enteric glial cells) and microglia. For the purpose of this thesis, I will focus on the role of the astrocytes.

Astrocytes are non-excitable cells that communicate with neurons through Ca^{2+} pathways, and compose ~20-40% of the total number of glial cells[106,108]. These cells play a wide variety of roles crucial to the optimal development and function of neural communications. With respect to neuronal development, astrocytes provide molecular boundaries that guide axonal development[109]. Additionally, they aid in the release of molecular signals that enable synapses to develop[110], and the loss of astrocytes during neuronal development can lead to demyelination[111]. Cerebral blood flow is heavily influenced by astrocytic end-feet as they help form the BBB and release various compounds that affect blood vessel diameter[107]. Due to their presence surrounding virtually every synapse, astrocytes also play a vital role in maintaining the homeostasis of neurons by helping control pH and neurotransmitter migration both from the neurons and blood[107].

1.4.7 Fluid-Based Biomarkers

This ability to detect proteins at low concentrations (i.e. pg/mL) in plasma[54], serum[61,68], and CSF[55] following an injury or in a disease state has resulted in the increased interest in

biomarker research. With respect to concussions, a variety of blood- and CSF-based biomarkers have been examined for their potential diagnostic and prognostic capabilities. In order for a fluid biomarker to be clinically relevant, several criteria must be met: 1) it must reflect the pathophysiological processes which present following a concussion, 2) they need to be found exclusively within the CNS in detectable quantities, 3) alterations in biomarker concentrations need to correlate with injury severity, 4) following the injury the biomarkers must appear rapidly in biofluids, 5) they need to be resistant to protease activity, and 6) the change in concentration only occurs following damage within the cerebral tissue itself and does not under other circumstances (such as during other physiological stressors like exercise bouts)[51,58,112]. The age of the individual can also have an influence on biomarker concentration, and as such this additional factor must also be considered when interpreting results. Furthermore, the biomarker should be able to cross the BBB such that changes in either serum or plasma concentrations are able to be quantified and tracked. Serum and plasma collections are preferred by subjects, as these fluids are more readily available (antecubital venous puncture vs CSF lumbar puncture) and are present in much greater quantities (~5000 mL blood volumes vs ~150 mL CSF). Despite the extensive research in this area, to date no single biomarker has emerged as sensitive and specific enough to clinically detect and track recovery following a concussion[25]. As a result, it is likely a broader panel of select proteins and other metabolites will be needed in order to accurately assess cerebral damage and recovery following a concussive event. For the purposes of this dissertation, I will discuss the proteins that have received most of the attention including myelin basic protein (MBP), neuron specific enolase (NSE), S100β, glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase L1 (UCHL1), NF-L, and tau. The following literature

review will place particular attention on their specific locations within the CNS, and the rationale underlying both the scientific and clinical interest their potential diagnostic capabilities.

1.4.7.1 Myelin Basic Protein

MBP is the second most abundant protein in myelin, representing 30% of the total content in the CNS, although it is also present in the PNS[113-115]. This biomarker came into prominence due to the mechanistic breakdown of myelin in demyelinating diseases such as multiple sclerosis (MS)[116,117]. Research has revealed patients with MS have elevated CSF concentrations of MBP[118] as compared to levels found in matched-control subjects. Combining the research from the MS populations with the notion both white and grey CNS matter are susceptible to traumatic biomechanical forces during head injury, it stands to reason the biofluid concentration of MBP will likely increase following a CNS insult.

Indeed, elevated MBP concentrations are found in serum of patients with CNS trauma compared to uninjured controls, with higher levels present in patients with an unfavourable outcome (i.e. death or severe brain injuries)[119,120]. Taken further, Thomas *et al*. (1984) demonstrated increased serum MBP concentrations in patients with brain trauma when compared with both uninjured controls and patients with spinal or peripheral nerve damage[121]. These findings suggest MBP is likely not only specific to CNS trauma, the extent the biofluid is expressed in the serum is directly associated with the extent of the brain parenchyma trauma. In 2005, Berger *et al*. further extended the previous research by demonstrating elevated MBP levels in patients with intracerebral hemorrhage (ICH) compared to those with no ICH[122]. They also revealed peak concentrations occurred ~16 hours post-injury[122]. In a more recent study, MBP within the CSF was found to be significantly elevated in paediatric patients compared to

uninjured controls[123]. However, it should be noted in this study the control group (8-months-old) was drastically younger than the TBI group (7-years-old), which may have impacted the accuracy of these findings. Further examination into MBP concentration and age within just those patients present in the TBI group revealed patients >1 year had higher MBP levels than those <1 year[123].

Contrasting the previous research, Mondello *et al*. conducted a study in 2016 that added a layer of skepticism regarding the clinical utility of MBP in diagnosing an individual with a TBI. In their study, there were no increases in MBP concentration in TBI patients compared to uninjured controls[124]. In sum, it appears MBP may play a role in detecting paediatric patients under 7 years old with ICH. Its role and clinical ability to detect mTBI in older populations has yet to be explored, but it is worth considering given its abundance in the cortical white matter. However, since this biomarker has been demonstrated to be present in both the CNS and PNS, it doesn't meet all of the criteria previously outlined for being the ideal biomarker for mTBI detection. Therefore, there is a need to consider the applicability of other biomarkers for this role, such as NSE.

1.4.7.2 Neuron Specific Enolase

Originally named 14-3-2 protein, NSE is a glycolytic enzyme highly localized in neurons and neuroendocrine cells of the PNS and CNS[125,126]. Although the name implies it is specific to neurons, research has demonstrated it is also located in oligodendrocytes, thrombocytes, and erythrocytes[127]. It is the latter that is most concerning when considering the diagnostic utility of NSE in detecting a concussion as the presence of NSE in erythrocytes complicates identifying where the trauma occurred (central or peripheral). This concern is especially important when

investigating athletes, as some athletic endeavors, such as running, can cause hemolysis[128]. Since these additional factors can potentially release NSE into the bloodstream from non-neuronal sources, this can lead to peripheral contributions to the increase in concentration in a sample, thus limiting the diagnostic capabilities in detecting and tracking concussions.

As with many other biomarkers, NSE has been found in elevated concentrations in various disease states, specifically in the CSF of those with Creutzfeldt-Jakob Disease (CJD)[129], and metastatic lung cancer[130]. Following a major head injury, a significant elevation in both CSF and serum NSE concentrations relative to control samples has been reported, although this finding was far from universal as less than half (i.e. 47%) of patients had elevated NSE levels[131]. NSE levels were also compared between controls and patients with a minor head injury, and no differences were noted[131]. Unfortunately, the Ross *et al* (1996) study did not report the dichotomizing factors separating the minor and major head injury groups. Furthermore, CSF NSE levels were not predictive of either clinical outcomes or injury severity. In slight contrast to the aforementioned findings, another investigation revealed elevated NSE levels in non-survivors of sTBI when compared to survivors[132]. However it should be noted, NSE was augmented only at the 48-hour time point, once again highlighting the need to track the time-course associated with each fluid biomarker[132]. There were no differences at all other time points within this investigation, which included at time of admission to a neuro-critical care unit, along with 24, 72, and 96-hours following the initial sample. Skogseid *et al*. (1992) similarly found elevated NSE serum concentrations, following a moderate to severe head injury. Interestingly, the authors discovered a positive correlation between maximum NSE levels and injury severity (r=0.72)[133]. Additional researchers have shown NSE to be elevated in those with a TBI compared to controls[134]. In this particular study, NSE concentration was a strong predictor of death in those

with sTBI; with a cutoff level of 257.90 ng/mL having 86.66% sensitivity and 100% specificity[134]. Most of these studies enrolled TBI cohorts with a wide age-range (<7 years to >59 years), including children, which could explain the contradictory findings with respect to NSE levels and injury severity[131,133,134].

The temporal profile of NSE also needs to be considered. Serum NSE levels are significantly increased following a TBI 2-days following injury when compared to injured controls (i.e TBI in the absence of CT abnormalities) (p=0.025), but there were no significant differences in NSE levels 1-day post-injury (p=0.088)[135]. As was previously mentioned, these findings have been reported in a more recent study[132]. A 2002 study by Berger *et al*. found elevated CSF NSE levels in children following inflicted TBI (iTBI) and non-inflicted TBI (nTBI) (i.e. 24.29 ng/mL in both groups compared to 10.15 ng/mL for controls), but no correlation was found with GCS score or mechanism of injury (MOI)[136]. Similar findings in children were reported when serum levels were analyzed; patients with both iTBI and nTBI were found to have elevated NSE concentrations, but these were not correlated with GCS scores upon admission to hospital[122].

As was previously mentioned (refer to section 1.4.1), a GCS score of 13 – 15 categorizes individuals with a mTBI. When examining NSE concentrations as a diagnostic tool for concussions the results from the literature are less promising, as findings to date do not distinguish injured patients from uninjured controls[54,137]. Moreover, it appears exercise itself can cause an increase in peripheral NSE concentration, likely due to the contributions from erythrocyte NSE sources[138]. Following a friendly hockey game (i.e. a professional Swedish Hockey League game in which no player sustained a concussion), serum NSE concentrations were elevated compared to samples taken at both 1-hour and 144-hours following a concussion[54].

Similar results were seen in a sample of soccer players from the Swedish Elite Soccer League; pre- and post-game analysis, in which samples were drawn within 15 minutes of the game ending, reveal a significant increase in NSE, with no correlation between the number of headers performed during the game and protein level[139].

In summary, it appears for those with sTBI NSE may prove to be a useful addition when examining the extent of injury provided measures are made at 48-hours post-injury. Although it needs to be noted, certain additional factors must be considered when interpreting test results, including the mechanism of injury, the age of the individual, and the activity the person was engaged in when they sustained the injury. As exercise causes an increase in serum NSE concentration, the clinical utility of the protein in detecting if a TBI occurred during a game/practice in athletes likely cannot be established. Consequently, further investigation into other potential objective biomarkers for this specific population is warranted.

1.4.7.3 S100β

The S100 family of proteins help regulate intracellular Ca^{2+} levels, stimulate neurite outgrowth and the development of new astrocytes, and protect neurons from apoptosis in the event of glucose deprivation[140-142]. Two of the most widely studied biomarkers, with respect to traumatic brain injury, are S100β and GFAP; both of which are associated with astrocytic processes. First isolated in 1965[143], S100β is a member of the S100 calcium-binding protein family and in the CNS, is localized to astroglia. Elevated CSF S100β concentration has been found in individuals with spinal cord tumours, ischemic CNS disorders, subarachnoid hematomas[144], and those suffering from CJD[129,145]. Serum levels of S100β are also elevated in patients who have suffered a stroke[146], giving promise to its applicability as a biomarker of brain

damage. Based on these findings S100β was thought to be a CNS-specific protein; however, S100β mRNA has also been discovered in other body tissues, including the colon, skin, and fat cells[142,147].

There is a large volume of published studies describing the effect TBI has on S100β concentration in both serum and CSF; indeed, it is the most widely studied biomarker to date[148,149]. Early research investigated the possible role of S100β as a marker for detecting TBI in both children and adults. When compared to healthy, uninjured controls and to patients with a peripheral fracture serum S100β levels were significantly increased at approximately 6-hours post-injury in children with nTBI (0.016 ng/mL and 0.026 ng/mL, respectively, and at approximately 23-hours post-injury in children with iTBI (same values)[122]. A 2010 study by Honda *et al.,* reported serum levels of S100β levels are elevated in those with a TBI compared to uninjured controls[150]. Furthermore, for adults, the severity of injury as assessed through CT scans[151-154] and clinical outcomes[151] have been associated with a serum S100β concentrations. Consistent with these results, Romner *et al*. (2000) found a significant negative correlation in serum S100β level and GCS in adults[154]. Building upon the earlier investigation, Mondello and colleagues similarly found negative correlations between serum S100β levels and both GCS and Glasgow outcome scale (GOS) following paediatric TBI[124]. Collectively, the aforementioned studies outline the potential role of S100β in the clinical decision for a patient to undergo a CT scan. Indeed, it has been suggested that including S100β concentration measurement could be an adjunct to the clinical assessment process thus reducing total CT scans performed by up to 30%[155].

In the aforementioned studies, blood samples were drawn relatively close to the time of injury (i.e. 0.25 – 30.0 hours). This timeframe is important for S100β quantification, as its half-life in blood is thought to range from 25 – 120 minutes[122,156,157]. Blood has a high proteolytic activity, therefore it is important to consider the temporal profile of any potential biomarker, as the time the blood sample was drawn following injury becomes vital in determining quantification.

1.4.7.4 Glial Fibrillary Acidic Protein

GFAP was discovered in 1971 and is a cytoskeletal protein found in astrocytic cells of both grey and white matter[99,158]. This protein was first discovered in human brain tissue of patients suffering from severe fibrous gliosis[158]. Within the CNS, it is the principle intermediate filament found in astrocytes[159] and occupies ~15% of the total astrocytic cell body volume[99]. Although GFAP is primarily found in astrocytes of the CNS[142], it is also present in various other body tissues including satellite cells of peripheral ganglia, Schwann cells, the enteric nervous system, and mesenchymal stem cells of many cell types throughout the body[107,160,161].

Most of the research in the last 40 years has examined GFAP levels in serum and CSF as a marker for a variety of conditions and disorders outside of head trauma. Elevated GFAP concentrations were found in individuals who had experienced a stroke[146,162], in patients with AD and CJD[145], and individuals suffering from meningitis and cerebral infarctions[163] compared to control subjects. AD brains are characterized by the presence of neuritic plaques and amyloid beta (Aβ) and GFAP messenger RNA (mRNA) levels that have shown a strong positive correlation with the presence of these deposits in AD brains[164,165]. Only recently has the

diagnostic role of GFAP been expanded from these clinical disorders and been examined within the context of TBI diagnosis.

Early research into the sensitivity and specificity of GFAP with respect to severe TBI (sTBI) garnered promising results. Pelinka *et al*. (2004) measured the concentration of GFAP in serum at multiple time point intervals in patients admitted to trauma centres. Time point intervals across 22-days included: <12 hours, 12-36 hours, 37-60 hours, 61-84 hours, and 85-108 hours. Increased GFAP concentrations were found in non-survivors of TBI compared to those who survived the injury across all time point intervals, and were associated with increased levels of intracranial pressure[166]. In the same study, patients admitted with multiple traumas without a co-morbid TBI were also enrolled, and GFAP levels remained significantly lower across all time point intervals when compared to TBI patients[166]. A number of researchers have also discovered positive correlations, in both children and adults, between serum GFAP concentration and TBI severity[124,150,167-171]. Serum GFAP concentration has also been shown to be prognostic regarding clinical outcome following a TBI[167,168,170,172,173].

Given the aforementioned high proteolytic activity in blood, it is possible protein modifications follow specific and brief time-course profiles upon release from the brain tissue and subsequent movement from CSF into the bloodstream. Therefore, by examining biofluids for both whole proteins and their breakdown products, a more accurate representation of neuronal damage may be obtained. Recently, investigators have examined the effect of mTBI on serum concentrations of GFAP and breakdown products of the protein (GFAP-BDP). Papa *et al*. (2012) found serum concentrations of GFAP-BDP to be higher and detectable within 4-hours following injury in mTBI patients with CT detected intracranial lesions as compared to lesion-free patients[174]. Metting and colleagues (2012) also report higher concentrations of serum GFAP

in mTBI patients with abnormal CT findings[175]. The findings from these studies could impact initial screening protocols for patients with suspected head trauma upon admission to the ED. For example, radiographic evaluation is typically used to determine injury severity in this population; however, there is concern regarding the risk of ionizing radiation exposure present in CT scans. Consistent with the S100B findings (refer to section 1.4.7.3) GFAP-BDP levels within 24-hours of injury was thought to reduce CT scans performed by up to 30%[176]. The combined S100B and GFAP data suggest, utilizing these biomarkers as screening tools could prevent unnecessary radiation exposure to many ED patients. These findings reveal the potential for the clinical application of GFAP (and S100B), as a protein that could have good specificity for brain injury and reduce the burden of unnecessary CT scans on the healthcare system.

It has also been demonstrated CSF GFAP concentration increases following a boxing match[55]. In this study, GFAP concentration was higher in boxers compared to control subjects at two time points following a bout: 1-6 days (~100% greater) and; at least 14-days (~50% greater). In a follow-up study, this same group also examined serum GFAP concentration in boxers, but for each group and time point concentrations fell below the minimum detection limit (150 pg/mL)[177]. These findings highlight the importance of obtaining multiple samples at various time points following injury, especially with serum and plasma samples in order to limit proteolytic modifications. Despite the researchers' thorough methodology, this study was missing a key variable, namely the number and magnitude of head impacts were not recorded[177]. Therefore, it is speculative to purely suggest this data provides a conclusive link between elevated GFAP concentration and head trauma. Indeed, the augmented concentration could be due simply to the strenuous exercise associated with a boxing match, as exercise in and of itself

has been shown to increase other biomarkers associated with CNS trauma including S100β and neuron specific enolase (NSE)[138,139,178,179].

1.4.7.5 Ubiquitin C-Terminal Hydrolase L1

UCHL1, also referred to as neuronal-specific protein gene product 9.5 (PGP9.5), was initially detected in the human brain by Jackson and Thompson in 1981[180]. Similar to several of the other biomarkers mentioned in this section, only recently have the diagnostic aspects of this biomarker with respect to brain injury been explored. This neuron-specific protein is highly enriched in the CNS, but, consistent with S100β and GFAP, it is also present in other regions of the body as trace amounts can also be found in the large intestine, kidney, ovaries, and testes[180]. Despite these small extracranial locations, it should be noted the vast majority of UCHL1 is found as soluble brain protein (constitutes up to 5% of total)[181,182]. The function of UCHL1 is to add or remove ubiquitin from proteins that are to be metabolized[181,182].

As is the same with most biomarkers of brain damage to date, UCHL1 was first examined in the context of sTBI in CSF. Papa and colleagues found elevated UCHL1 concentrations at various time points between 6 – 168 hours following injury (44.2±7.9 ng/mL) compared to uninjured controls (2.7±0.7 ng/mL)[183]. The peak UCHL1 concentration occurred within the first 24-hours[183]. Within the sTBI group, UCHL1 concentration ~24-hours post-injury also appeared to be related to the severity of GCS rating upon admission. Furthermore in this study, UCHL1 identified patients in the sTBI group who were more likely to experience post-injury complications[183].

As was previously stated, serum and plasma are the preferred fluids to examine following injury. A prospective study by Mondello *et al*. (2011) examined both UCHL1 and GFAP in

those with sTBI upon their hospital admission with subsequent follow-up measures performed every 6-hours for the first 24-hours of the hospitalization period[184]. Results from this study revealed GFAP and UCHL1 concentrations were higher in non-survivors (1.6±0.22 ng/mL) than in survivors (0.65±0.07 ng/mL). Not only did UCHL1 and GFAP predict clinical outcome and identify more serious injury, specific neuroradiological findings were associated with each biomarker. GFAP levels were higher in those with mass lesions on CT scan, while UCHL1 levels were higher in those with more diffuse injury. These findings highlight how CNS-specific biomarkers may be used to assess specific molecular events in the brain following injury. A 2012 follow-up study by Papa and colleagues examined serum concentrations of UCHL1 in those with mTBI and moTBI compared to uninjured and injured (without CNS trauma) control subjects[185]. Serum UCHL1 levels were elevated in TBI compared to controls, with group differences noted within 1-hour post-injury. Furthermore, UCHL1 levels at 1-hour were increased in the mTBI group (GCS score of 15: no clinical symptoms) and the uninjured controls but also an additional group of trauma patients who presented to the ED with no CNS injury. UCHL1 was also shown to predict CT scan abnormalities and the need for neurosurgical intervention when levels exceeded 0.09 pg/mL. In addition, plasma samples obtained within 48-hours from TBI subjects reveal higher UCHL1 concentrations than uninjured controls, with the highest levels found in those with more serious head trauma[169,186].

Unfortunately, these promising findings for UCHL1 are not universal. In another study where TBI subjects were separated into mild, moderate, and severe based on the GCS, no differences were observed between any of the TBI groups[187]. There were, however, increases noted in both the moTBI and sTBI groups when compared to control subjects[187]. Further contrasting the results of the 2012 study by Papa *et al*.[185], the results from the Berger *et al.*

study[187] did not reveal a relationship between biomarker concentrations and abnormal CT scans. A possible explanation for these discrepancies is the study by Berger *et al*. only enrolled children ranging in age from 1 week to 12.4 years[187], whereas the Papa *et al.* study enrolled participants with an average age of 39±15 years. Throughout childhood the brain is undergoing volumetric and structural changes[188,189], and as such, it is possible the CNS proteome is still developing and undergoing changes that may impact certain protein biofluid concentrations throughout the developmental spectrum. These contradictory findings have important implications for developing standardized collection and analytical methods for quantification of protein biomarkers in each biofluid. As highlighted in the results from aforementioned studies, age of the subject, injury severity, and the temporal profile of each biomarker must be considered when interpreting results in a clinical setting with respect to TBI.

1.4.7.6 Neurofilaments

The NF family of proteins are intermediate filaments found in large-caliber, myelinated axons, that can extend into subcortical layers, and consist of three isoforms: i) neurofilament light (NF-L); ii) neurofilament medium (NF-M); and iii) neurofilament heavy (NF-H)[190,191]. Each intermediate filament consists of one NF-L subunit and either a NF-M or NF-H subunit[57]. Together, they are highly expressed in the CNS and account for the vast majority of cytoskeletal proteins, making them ideal candidates for the detection of neuronal and, more specifically, axonal damage[51,192]. NFs function to increase the strength of axons and dendrites, that helps to maintain cell shape[57].

Consistent with the previous biomarkers, NF levels in both CSF and serum are elevated in certain disease states. CSF levels of a phosphoform of NF-H have been shown to be elevated

in individuals with progressive MS, with the degree of elevation correlating with three clinical scales designed to assess MS disability[193]. Tortelli *et al*. (2012) examined CSF NF-L levels and found them to be elevated in ALS and NF-L levels were correlated with progression of the disease[194]. Taken together, these results suggest NF proteins are useful markers of CNS neurodegeneration in clinical conditions. Expanding upon this notion, Skillbäck *et al*. examined CSF NF-L concentrations in various dementia types, and revealed elevations in individuals with either frontotemporal dementia (FTD) or vascular dementia (VaD)[60]. CSF NF-L levels were associated to both poor survival and disease severity in these populations, indicating the potential use for NF quantification as a diagnostic tool of axonal degeneration and damage[60].

Serum levels of hyperphosphorylated NF-H (pNF-H) correlate with injury severity and clinical outcome in children with TBI[195]. In this particular study, pNF-H concentration on days 2, 3, and 4 following injury were higher for those individuals with a Glasgow Outcome Scale (GOS) score of 1 (i.e. death) at 6-month follow up. The pediatric patients with CT scans revealing a diffuse axonal injury (DAI) reported higher serum pNF-H levels. Contrary to the previous studies investigating GFAP and UCHL1 across the aging spectrum[185,187], adults with moTBI and sTBI revealed similar results to the pediatric population. Namely, serum unphosphorylated NF-H concentrations at 24- and 72-hours post-injury were also elevated in patients with sTBI as compared to moTBI[196]. NF-H levels at 24-hours are also associated with outcome as assessed by the GOS, indicating that NF-H concentration can be used to predict patient outcome across the aging spectrum.

Serum NF-L levels have been shown to predict severity of injury and clinical outcome[67,68]. Compared to uninjured controls, patients with sTBI had markedly greater levels of NF-L from the day of admission and remained elevated at 12-days post-injury[68]. Moreover, non-

survivors had higher levels than survivors at 24-hours post-injury, suggesting there is a relationship between NF-L level at 24-hours post-injury and GOS at 12-months follow-up. Of note, S100β concentrations were also measured in serum, with no correlation found between initial levels and clinical outcome.

Turning our attention toward sub-concussive impacts, a bout of Olympic boxing is associated with increased concentrations of CSF NF-L and NF-H, with levels remaining elevated for two weeks following a bout[55,59]. Also, a greater increase in CSF NF-L is seen in boxers who report a high number of head impacts during a fight[64]. Collectively, these results indicate axonal damage can be caused by sub-concussive impacts to the head, and continued axonal degeneration that persists following a boxing bout despite the absence of a concussion. A case study examining CSF NF-L from a boxer knocked out in a fight showed elevated levels at 16-days following the bout and remained augmented for 28-weeks, with normalization finally occurring at 36-weeks[63]. Interestingly, the subject did not report symptoms related to concussions (e.g. headache, dizziness, blurred vision) either immediately upon regaining consciousness, nor when examined at the hospital. A CT scan was performed and revealed no structural abnormalities. These results suggest CSF NF-L levels may be used as a more sensitive objective marker of acute axonal injury even in the absence of self-reported clinical symptomology and diagnostic imaging abnormalities. Further to this, serum NF-H concentration has been found to be higher in those with mTBI compared to uninjured healthy controls at both 1- (p=0.00001) and 3-days (p=0.0001) following injury[197]. Consistent with the previously reported findings, NF-H levels were also able to distinguish between those with CT abnormalities and those without in the mTBI group[197]. It should be noted the group identified by CT abnormalities had significantly

more subjects with a GCS of 13 compared to the CT- negative group (55.5% and 12.5%, respectively), which could influence the results.

In addition to these data, serum NF-L has also been found to increase as a result of the contact experienced in American football players over the course of a season[65]. Further to this point, there was a greater increase noted in starters (+6.5 pg/mL) compared to non-starters (+0.36 pg/mL)[65]. An intriguing finding was that there were two notable points within the season where substantial increases in NF-L were observed. The first came upon completion of preseason training camp as compared to NF-L levels pre-camp, which corresponded to an initial period of exposure to contact and intensity, the second came following the start of conference play. These results suggest NF-L levels are sensitive to sub-concussive impacts; however, in contrast, Zetterberg *et al*. (2007) found no increase in CSF NF-L 7-10 days following an acute bout of soccer heading[62]. The major differences in these two studies are the extent of impact exposure, with American football players experiencing more impacts, greater impact magnitude, and the data for the American football was obtained across an entire season of play that greatly increases the potential extent of head trauma one is likely to experience as compared to a single session involving heading a soccer ball. Overall, these results indicate NF-L is a sensitive marker for axonal injury following sTBI, mTBI, and a season of sub-concussive impacts in American football.

The increase in NF-L concentration following an mTBI is an important discovery. Currently, health care professionals heavily rely on subjective measures to diagnose and manage a concussion. An objective biomarker such as NF-L would enable medical personnel to detect axonal damage without relying on the athlete self-reporting their symptoms accurately. Previous research has demonstrated athletes have been known to purposefully under-report concussions

and their associated symptoms in order to remain on the playing field[18]. Removal from play in the presence of a concussion will limit the incidence of second-impact syndrome (SIS), that occurs when an athlete sustains a second head injury before fully recovering from the first injury. The second impact may be of less magnitude than the first, and can result in collapse and respiratory failure[30].

To date, there has been little research examining NF concentrations following exercise. Therefore, it is difficult to truly ascertain if elevated levels in blood following mTBI are due to physical exertion or axonal injury. However, NF-L has been suggested as the most sensitive and specific biomarker of axonal injury[51] and, with a half-life of approximately 3 weeks[198], more research is warranted in order to truly discover the diagnostic and prognostic capabilities of the NF family of proteins following mTBI. It is also important to note that NF-L is expressed in peripheral nerves; injury to peripheral nerves may thus influence its blood concentrations. Nevertheless, the higher concentration in CSF and the robust correlation of blood with CSF NF-L concentrations seen in a large number of studies[199-202], suggest that most of the NF-L detectable in blood is CNS derived.

1.4.7.7 Tau

Tau protein concentration in CSF and blood has been one of the most extensively examined biomarkers[54,55,64,203]. This biomarker has received its fair share of media publicity (i.e. "*Concussion"* (2015) starring Will Smith) due to its increased presence in the brains in the form of neurofibrillary tangles (NFTs) of individuals who have been diagnosed with CTE[204]. Six CNS-specific isoforms of tau ranging from 352 to 441 amino acids in length, with molecular weights ranging from 45 – 67 kilodaltons (kDa), are present in humans; the smallest isoform is

present in fetal development, while the remaining 5 appear in adulthood[205,206]. These 5 adult isoforms likely have specific physiological roles during development, as they are differentially expressed across the aging spectrum. Tau is a microtubule-associated protein (MAP); along with MAP1A, MAP1B, MAP2, MAP3, and MAP4, it acts to stabilize microtubules through its binding affinity with tubulin, and plays a role in microtubule assembly through the formation of cytoplasmic extensions including both dendrites and axons[52,53,205]. Tau also interacts with the neural plasma membrane, indicating it may play a role in cell shape[207]. Indeed, tau's putative roles in the formation and further development of specific neuronal structures establish the protein as an ideal candidate for detection analysis following nervous system trauma.

Furthermore, tau may have received increased attention due to the accepted tenet it is a neuronal CNS-specific protein. Although the majority of research examining the structure, function, and location of tau molecules has focused on murine models, an additional isoform of tau has been identified in the human PNS[208]. This high-molecular-weight isoform of tau, referred to as "big tau" has an approximate size of 100 kDa[53,206,208,209]; 50% greater than the 441 amino acid length isoform. Big tau has been identified in the glial cells of the CNS in certain disease states, in the PNS, in human fibroblast cells, the enteric nervous system of the human intestine, and in various rat tissues, including the heart, skeletal muscle, lung, kidney, testis, adrenal gland, stomach, pancreas, and liver[205,210-214].

Tau phosphorylation is a normal occurrence, and is thought to influence the binding of tau to the microtubule[53]. Hyperphosphorylation of tau can also occur, and is a key event in the development of certain pathological conditions including AD, in which axonal breakdown occurs[53,215,216]. Axonal trauma also leads to the proteolytic cleavage of tau by enzymes such as A Disintegrin and Metalloproteinase 10 (ADAM10), calpain-1, and caspase-3, resulting in

fragments of tau[114,217,218]. These processes lead to numerous tau polypeptides that can be quantified including: i) total tau (T-tau)[54]; ii) phosphorylated tau (P-tau), that can include tau molecules phosphorylated at specific sites such as Threonine 181 (noted in the literature as P-tau_{181}) and hyperphosphorylated tau[219]; iii) and enzyme-cleaved tau fragments (C-tau), including, but not limited to Tau-A (ADAM10-cleaved tau) and Tau-C (caspase-3-cleaved tau)[220].

Although one isoform is present in the PNS of humans, it would appear that tau is predominantly located in the CNS, specifically in thin unmyelinated axons of cortical neurons[112]. As a result, there has been extensive interest surrounding the potential TBI- and neuropathological-diagnostic capabilities associated with this protein[112]. As was previously stated, hyperphosphorylated tau deposits in the form of NFTs are the pathophysiological hallmark of CTE[221]. Elevations in CSF and serum T-tau, P-tau, and tau fragments have been demonstrated in individuals with AD[215,216,218,222]. Certain P-tau polypeptides can distinguish between those with AD and other forms of dementia. Specifically, P-tau_{181} has been shown to distinguish AD patients from those with dementia with Lewy bodies (DLB)[223], while P-Tau_{231} can differentiate between AD and FTD[224]. CJD patients can be distinguished from both AD and FTD patients by the quantification of T-tau[225]. One possible explanation is the relatively low presence of NFT and hyperphosphorylated tau in those with CJD. Additionally, it has been shown that both CSF and serum levels of T-tau are elevated in those with CJD compared to those with AD, other rapidly progressive forms of dementia, and healthy controls[226]. An intriguing finding from this study was no significant difference in serum T-tau between AD patients and controls.

Following sTBI, T-tau concentration, assessed by microdialysis, is increased in patients who have suffered a focal TBI compared to those with DAI[227]. What is more, tau deposits seem

to be located in specific areas of the brain, mainly pericontusional regions following sTBI[228]. Further to this, Zemlan *et al*. (2002) found CSF levels of C-tau to be elevated more than 40,000 times in sTBI patients when compared to neurologic and non-neurologic control groups[229]. High levels of CSF T-tau also have been demonstrated to correlate with clinical outcome, specifically high T-tau levels relate to a more unfavourable outcome 1-year post-injury[203]. Taken together, these studies display promise for tau as a biomarker of TBI.

However, early investigation into the role of tau as a biomarker of mTBI yielded mixed results[55,64,177,230-232]. In paediatric patients, serum tau levels are 2.5x higher in those who have suffered minor head trauma compared to controls[230]. Furthermore, Bulut *et al*. (2006) report serum tau levels to be elevated in those with high risk mTBI (307±246 pg/mL) compared to those with low risk mTBI (77±61 pg/mL)[231]. However, when all mTBI groups were pooled, there were no differences in tau levels between the mTBI group and uninjured, healthy controls. Conversely, Kavalci *et al*. (2007) report serum tau levels are unable to distinguish between CT-positive and CT-negative mTBI groups[232]. By contrast, Shahim *et al*. (2014) demonstrated plasma T-tau levels were higher in the first hour post-concussion (5.8 pg/mL) when compared to preseason control values (4.5 pg/mL)[54].

Expanding these findings to the realm of sub-concussive impacts Neselius *et al*. have demonstrated CSF and plasma T-tau are elevated at 1-6 days following an amateur boxing bout[55,177]. Contrasting these findings, Zetterberg *et al*. (2006) reported P-tau in boxers during the same 1-6 days following a bout when compared with control subjects (i.e. family members with no history of boxing participation or brain trauma)[64]. The elevation in T-tau could potentially be due to exercise, as it has been reported plasma T-tau level is 30% higher 1-hour following a friendly hockey game (i.e. in the absence of concussion)[54]. Therefore, it appears as though

exercise potentially affects circulating T-tau concentration, but more research is required before firm conclusions can be made on this topic.

Thus, it would appear a number of factors must be taken into consideration when assessing the diagnostic proficiency of tau in the context of mTBI. Namely, the phosphorylated state of tau protein, the individual's preinjury activity, and the time at which the blood sample is drawn following the injury. Very rarely are multiple tau polypeptides quantified collectively within a singular study in the literature. Instead, most studies focus on one specific form of tau. T-tau is measured with assays that are unable to distinguish between different isoforms, nor between phosphorylated and unphosphorylated molecules[51]. Phosphorylated tau (p-tau) is measured with assays that distinguish it from unphosphorylated tau and are specific to a phosphorylated epitope[112], whereas tau fragments are quantified using assays specific to a particular fragment[218]. Therefore, the full spectrum of tau does not appear to have been quantified in a singular study.

Chapter 2: Methods

2.1 Collection of Blood Samples

The ability to collect and analyze blood samples for the presence of a disease state provides us with the opportunity to diagnose and provide treatment for a variety of conditions, including neurological syndromes[60]. In order for this to occur, proper phlebotomy procedures are essential to prevent hemolysis and preserve the target analyte.

The collection process began with the participant seated comfortably in a private, temperature controlled room and the arm supported, from which the sample was obtained. Comfort of the practitioner performing the venipuncture was also considered, with all the equipment needed for the procedure within easy reach. Samples were collected from the antecubital fossa as it is easily identified and stabilized during venipuncture[233]. The participant was requested to keep the arm straight during the procedure, as any movement may stop the flow of blood into the Vacutainer tube. Following a thorough explanation of the procedure, the following steps were performed:

1. Wash hands with antibacterial soap
2. Gloves adorned before touching the participant.
3. Place a tourniquet 7 – 8 centimetres proximal to the antecubital fossa.
4. Clean the area with an alcohol wipe for 30 seconds in a circular fashion.
5. Identify the vein from which the sample will be drawn.
6. Apply traction with the thumb onto skin 2 – 3 centimetres distal to the insertion site.
7. Inspect the needle to ensure the bevel is oriented upwards
8. Insert the appropriate size needle at a 15 – 30° angle, depending on the size and location of the vein.

a. For the studies contained within this dissertation, a 21-gauge needle was used. This gauge needle was selected to reduce the occurrence of red blood cell lysis as the vacuum tube fills[233].

9. Depress the vacutainer blood tube into the needle.
10. Upon first release of blood from the vein into the Vacutainer tube, release the tourniquet.
11. Once the tube has completely filled remove it from the needle. If a second tube is to be used, place it in the needle compartment.
12. Place a cotton ball over the needle entry site and remove the needle. When the needle is removed apply pressure on the insertion site with the cotton ball.
13. Place a strip of medical tape on the cotton ball to keep it in place.
14. Dispose of sharps into appropriate sharps container.
15. Invert the Vacutainer tubes 4 times to mix ethylenediaminetetraacetic acid (EDTA) (plasma samples) or silica (serum) with blood sample.

Following collection, serum samples sat for 30 – 60 minutes at room temperature to be allowed to clot thereby resulting in high quality serum samples[234,235]. Samples for plasma collection were placed immediately in a container with crushed ice so as to prevent clotting. There are no consistently recommended procedures for centrifugation to obtain optimal T-tau, NF-L, Tau-A, and Tau-C concentrations in plasma and serum; however, centrifugation procedures for biomarkers of neurological conditions have been proposed at 2000g for 10 minutes[236] at 4°C. As such, all samples were spun at these settings for consistency. Samples were then aliquoted into two separate 500 μL tubes and stored at -80°C until shipment. One 500 μL serum aliquot was shipped on dry ice through Fed Ex to Nordic Bioscience (Herlev,

Denmark) for Tau-A and Tau-C analysis. Three 500 μL aliquots (2 serum; 1 plasma) were sent on dry ice through Fed Ex to the University of Gothenburg (Gothenburg, Sweden) for T-tau and NF-L analysis. Samples were drawn from local university-aged athletes who were all free from concussion in the 6 months prior to blood sample collection. A prospective cohort study design was used for all 3 studies included in this dissertation. Samples were collected prior to the season for each sport. Post-season samples were collected within 1-week of the season ending. Post-concussion samples were drawn at 6- and 14-days post-injury.

2.2 Measurement of Biomarker Concentrations

The *gold-standard* assay in numerous scientific fields is the enzyme-linked immunosorbent assay (ELISA)[237], primarily due to its heterogeneous characteristic (i.e. reactants can be used in a variety of combinations to target specific proteins). This has allowed for the development of the elements necessary for successful protein, metabolite, or antibody detection, including specific monoclonal antibodies and peptide antigens.

Fundamentally, the traditional ELISA process begins with the adsorption of proteins (typically, either an antigen or antibody form) to a solid phase (usually a microtiter plate well). A common configuration of a microtiter plate contains 96 wells, measuring approximately 5 mm deep by 8 mm in diameter, arranged in a 12 x 8 format. The ELISA process has been developed into three basic systems: direct; indirect; and sandwich. For the purposes of this dissertation, the sandwich ELISA method was used.

Figure 2.1 – ELISA 96-well Microplate

The sandwich ELISA is used for a number of reasons, which include: i) when the protein of interest competes directly with other proteins in concentration to bind to the solid phase; ii) when there is a low concentration of the protein of interest in solution; or iii) when the antigen has a low affinity for binding to the solid phase. This first antibody, bound to the 96-well plate, is used to capture the antigen of interest in order for it to be quantified. Once the antigen is bound to the capture antibody, the detection antibody is added. The detection antibody can be identical to the capture antibody, or an alternate antibody reacting to either the same or a different epitope. The protein is added in buffer and allowed to passively attach to a capture antibody (that is bound to the plate). Following an incubation period, the excess protein is washed away and a specific antibody (linked to an enzyme) is added. A wash stage follows in order to remove the unbound reagents. A specific substrate is added (with or without a colourless chromophore - depending on the protein of interest), and then an incubation period takes place. The process is then stopped and the colour is read by a spectrophotometer, that provides the concentration of the protein of interest.

In order for fluid-based biomarkers to be clinically relevant, the ability to measure them at very low concentrations is required. Blood-based biomarkers of Alzheimer's disease (AD)[238,239] and sport-related concussion[54] have been shown in pico- to sub-femtomolar ranges. Traditional (analog) ELISAs typically quantify proteins above picogram range[240], limiting their diagnostic and prognostic use in certain neurological conditions. Although two of the proteins of interest for this thesis are relatively large (i.e. tau, 45 – 65 kDa[205], and NF-L, 70 – 200 kDa[241]), an ultra-sensitive method of detection is necessary to quantify them. This is due to the low concentration of plasma tau and serum NF-L, even following diffuse traumatic axonal injury[54,61]. Quanterix (Lexington, MA, USA) have developed a platform based on single molecule assay (Simoa) analysis.

The Simoa process begins with the attachment of capture antibodies to paramagnetic beads (2.7 μm diameter). These beads are then added to a dilute solution of molecules (Figure 2.2, Step A). The target analyte is then introduced to the solution and will bind to the capture antibody (Figure 2.2, Step A), after which a detection antibody is introduced specific to the target analyte (Figure 2.2, Step B). An enzyme conjugate (Figure 2.2, Step B) is then added, followed by an enzyme substrate (Figure 2.2, Step C). The final solution is introduced to an array assembly on a Simoa disc containing 216,000 femtoliter-sized wells (each 4.25 μm width, 3.25 μm depth) constructed to hold no more than one bead per well. The wells are then sealed with oil and imaged to determine analyte concentrations. If a target analyte has been captured, the resultant substrate will emit a fluorescent product that is detected (Figure 2.3). Two fluorescence images are compared; the first taken prior to the addition of substrates, and the second after the substrates have been introduced. By comparing the two images, it is possible to detect any

increase in signal, which confirms the presence and concentrations of the target analyte bound to a bead.

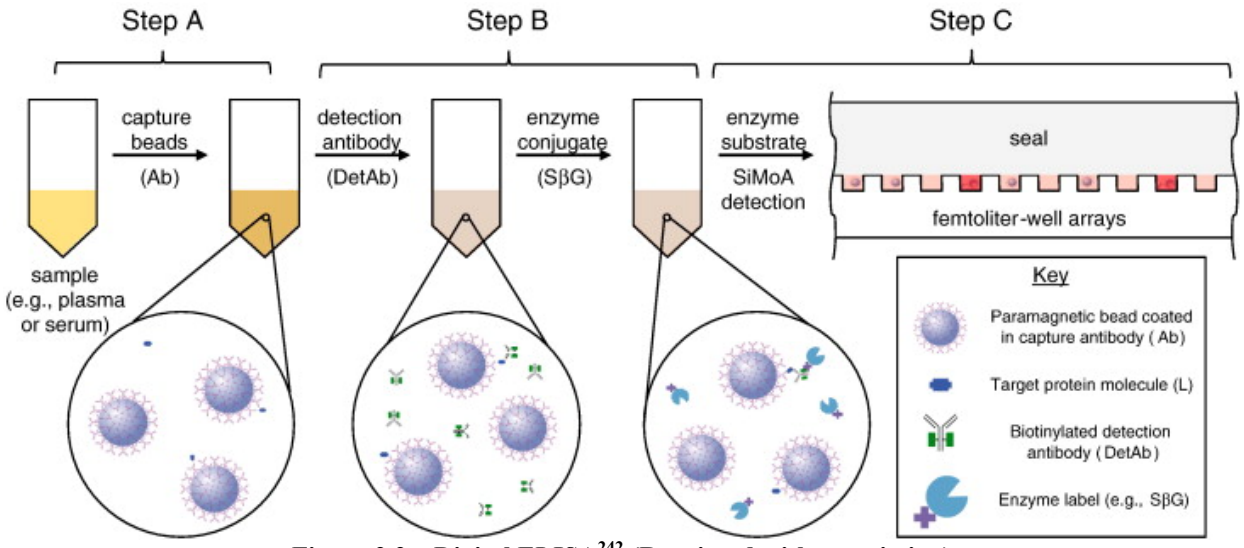

Figure 2.2 – Digital ELISA[242] (Reprinted with permission)

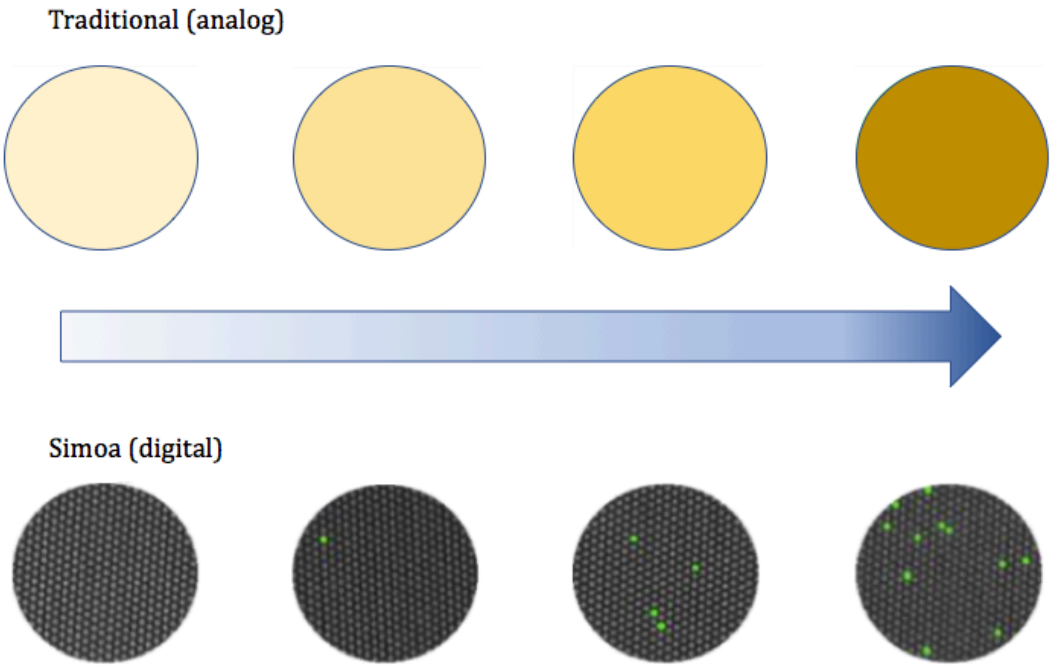

Figure 2.3 – Comparison between traditional and Simoa platforms (adapted from Simoa White Paper 1.0 available at: http://www.quanterix.com/sites/default/files/resources/Simoa%20White%20Paper%201.0%20final_0.pdf)

When measuring the concentration of any analyte through ELISA, the limit of detection (LOD) and lower limit of quantification (LLOQ) are important, as they represent the smallest concentration that are reliably measured. More specifically, the LOD represents the lowest concentration of analyte likely to be distinguishable from the limit of blank (LOB) (that represents the highest apparent analyte concentration expected to be found when a sample containing no analyte is tested). Therefore, the LOD is higher than the LOB. The LLOQ represents the lowest concentration of target analyte that can be accurately measured. LOD and LLOQ values for the target analytes in this dissertation can be found in Table 2.1.

Table 2.1 – ELISA Assay Characterizations

Analyte	Platform	LOD	LLOQ
Tau-A	Traditional ELISA	2.9 ng/mL	11.9 ng/mL
Tau-C	Traditional ELISA	1.3 ng/mL	10.4 ng/mL
NF-L	Simoa	0.29 pg/mL	2.7 pg/mL
T-tau	Simoa	0.02 pg/mL	1.22 pg/mL

Both Tau-A and Tau-C were detected using traditional ELISA methods, whereas T-tau and NF-L were detected on the Simoa platform. The development of this novel, ultrasensitive digital assay format allows for the quantification of molecules in both serum and plasma at very low concentrations. At 0.02 pg/mL, the LOD for T-tau is more than 1000-fold more sensitive than traditional ELISAs for this specific analyte. As outlined in the literature review, T-tau has been quantified and found to be increased (in CSF) following both sub-concussive[55,64,65] and concussive[63] head trauma; however, tau concentration in serum has been reported as ten times lower than in CSF[243]. This disparity between biofluids demonstrates a need for more sensitive measures of quantification for certain proteins. More recently, Meso Scale Diagnostics (Rockville, MD, USA) have developed an assay platform that has been shown to detect tau in serum and plasma at much lower levels than the Simoa platform, with a LOD of 6 fg/mL

(femtograms per milliliter: 0.000000000000001 g/mL) and a LLOQ of 21 fg/mL. Indeed, the ability to detect proteins at such small concentrations has already yielded promising results. The development of more sensitive protein quantification platforms may pave the way for the discovery of novel diagnostic assays of brain tissue trauma from both sub-concussive and concussive events.

Chapter 3: Study #1 – Blood-Based Biomarkers and Sub-Concussive Impacts

3.1 Background

Research on concussions has seen an exponential increase in recent years. In the United States alone, it has been estimated 3.8 million sport-related traumatic brain injuries (TBI) occur each year[4]. Collision sport athletes, such as football, rugby, and ice hockey players, are at an increased risk for sustaining a concussion due to the nature of their sport[244]. Rugby players are largely unprotected on the field, with scrum caps worn by only some players. Rugby (2.50-5.86) is associated with a higher concussion incidence rate than American football (1.2)[13,245,246]. Although concussions have received a great deal of interest, there is growing concern over the possible deleterious neurophysiological and neurocognitive effects associated with sub-concussive impacts[65,139,247].

Repetitive, sub-concussive head impacts are thought to be associated with progressive neurodegeneration, and may potentially lead to certain neurological disorders such as chronic traumatic encephalopathy (CTE)[83]. CTE is a progressive neurodegenerative disease characterized by symptoms of aggression, irritability, short- and long-term memory loss, and increased suicidal thoughts[221]. Of particular concern with respect to sub-concussive impacts is the world's most popular sport, soccer. According to the Fédération Internationale de Football Association (FIFA), 270 million people are actively involved in soccer globally[248]. Soccer is unique in that the ball is purposefully directed during game play by the player's head. A 2014 case report documented a retired professional soccer player who developed CTE[249], and repetitive head impact exposure in former National Football League players positively correlates with plasma total tau concentration in later-life[250]. If the act of soccer heading does indeed cause

axonal disruption and increase one's risk of developing CTE, an objective biomarker to detect the degree of damage within the brain parenchyma would be an invaluable tool to the medical community.

The blood-based biomarkers of focus in this study are NF-L and two enzyme-specific cleaved fragments of tau protein, A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10)-cleaved tau (Tau-A), and caspase-3-cleaved tau (Tau-C). Neurofilament intermediate proteins including NF-L, neurofilament medium chain (NF-M), and neurofilament heavy chain (NF-H), compose approximately 50% of the neuronal cytoskeleton[251]. These proteins function to stabilize the microtubule by maintaining both its shape and size, thereby allowing an uninterrupted conduction of nerve impulses along the neuron[252]. Current research on neurofilament proteins and their potential as a fluid-based biomarker of brain damage has found increased concentrations in cerebrospinal fluid (CSF) following a bout of amateur boxing without concussion[55,59]. Tau is a microtubule-associated protein (MAP) primarily responsible for stabilizing microtubules within neuronal cells in the central nervous system (CNS)[53,209,253]. To date, research on tau as a biomarker for neurodegenerative disease and neuronal injury has examined its presence in blood as a whole protein using assays that do not discriminate between phosphorylated and non-phosphorylated forms[54,226]. Depending on the isoform, the approximate half-life of tau can range from 0.5 – 12 hours[254,255], which may explain the relatively quick return to baseline values compared to NF-L.

Serum NF-L has been shown to increase over the course of a season of American football[65]; however, to our knowledge, head impact metric calculations have not been explored in conjunction with NF-L changes. Also, we sought to replicate the findings of increased serum

NF-L as a result of a season of football by Oliver *et al*. (2016); as such, we compared pre- and post-season NF-L levels for American football players.

The primary goal of this study was to evaluate the change in biomarker concentration due to a season of athletic competition in both contact and non-contact sport athletes. To our knowledge, no study has examined NF-L, Tau-A, and Tau-C at preseason and post-season time points across a variety of sports. Consequently, we examined serum NF-L and plasma levels of Tau-A, and Tau-C from pre- to post-season in American football, ice hockey, rugby, soccer, and cross country (running) athletes. It was hypothesized that NF-L, Tau-A, and Tau-C concentrations would be significantly increased in all contact sport athletes from pre- to post-season.

3.2 Methods

3.2.1 Participants

Fifty-six university-aged athletes were recruited for this study from the following sports: American football (n=13; all male), ice hockey (n=10; all male), rugby (n=9; 4 female, 5 male), soccer (n=15; 7 female, 8 male), and cross country (n=9; 5 female, 4 male) (Detailed demographics are presented in Table 3.1). All subjects were between the ages of 17 years and 36 years, and had not sustained a concussion in the 6 months prior to sample collection. Written informed consent was obtained from all participants prior to the start of data collection. This study was approved by a sanctioned Research Ethics Board at the University of British Columbia.

3.2.2 Procedures

Pre- and post-season blood samples were collected. Initial collections occurred during preseason training camps immediately before a training session and approximately 24 hours following the most recent bout of exercise. All post-season samples were collected as soon as possible following the completion of each athlete's respective competitive season (median 3 days, range 1 – 7) following the completion of the competitive season. This study was part of a larger project investigating the effects of acute concussions on blood biomarkers; however, all athletes sustaining a concussion during the season were excluded from the current results. The definition of concussion was taken from the consensus statement on concussion from the 5th International Conference on Concussion in Sport. This definition states: *a concussion is an injury caused by a direct blow to the head, face, neck, or elsewhere on the body with an impulsive force transmitted to the head resulting in impaired neurologic function and acute clinical symptoms*[25].

Blood samples were obtained through venipuncture using a 21-gauge BD Eclipse blood collection needle and collected into BD K2EDTA ethylenediaminetetraacetic acid (EDTA) coated plasma tubes, and BD silica spray-coated serum tubes. Samples were then centrifuged at 2000g, for 10 minutes, and at 4^{0}C within 60 minutes of collection, aliquoted, then stored at -80^{0}C. Plasma samples were shipped on dry ice from Kelowna, British Columbia, Canada to the Department of Psychiatry and Neurochemistry at Sahlgrenska University Hospital in Mölndal, Sweden. Plasma NF-L was measured with a novel immunoassay using the Simoa HD-1 Analyzer (Quanterix Corp). Due to the novelty of the assay, at the time a lower limit of quantification (LLOQ) had not yet been defined. Serum samples were shipped to Nordic

Bioscience (Herlev, Denmark) to be analyzed for Tau-A (LLOQ – 13.6 ng/mL) and Tau-C (LLOQ – 5.18 ng/mL).

A subset (n=7) of American football players wore the xPatch sensor (X2 Biosystems, Seattle, WA, USA). The sensors were affixed to the mastoid process posterior to the right ear with an adhesive patch. Head impacts were recorded on the sensor when it measured a peak linear acceleration greater than 10 g. Following each game, the data were downloaded using the Head Impact Monitoring System software (X2 Biosystems, Seattle, WA, USA). From this software, the following head impact metrics were used: total number of head impacts (CuHIT), cumulative peak linear acceleration (CuPLA) reported in g's, and cumulative peak rotational acceleration (CuPRA) reported in rad/s^2. Recent work advocates a link between both the number and severity of sub-concussive impacts over the course of a season and the development of pathological neurophysiological changes[256-258].

3.3 Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics (v. 23, IBM Corp). All graphics were generated using R Studio (v. 1.0.143, Foundation for Open Access Statistics). Group differences for Tau-A, and Tau-C were examined by use of a 5 (sport) by 2 (time) repeated measures mixed ANOVA. Due to the non-normality of the NF-L data, separate pairwise comparisons were run for each team. Football and cross country data were analyzed using Wilcoxon signed rank tests, while ice hockey, soccer, and rugby data were analyzed with independent T-tests. Pearson correlation coefficients were used for the analysis of correlation between NF-L change and each head impact metric of interest, including CuPLA, CuPRA, and

CuHIT. Data are presented as mean ± SD and significance was set *a priori* at $p<0.05$ for all calculations.

3.4 Results

Demographic data on the subjects in each test group is presented in Table 3.1. There was a main effect for age between the teams ($p<0.01$). The cross country team included two athletes >30 years of age; however, preseason (4.48, 8.30 pg/mL) and post-season (4.97, 7.86 pg/mL) NF-L concentrations were similar to those seen in athletes <30 years of age[66,259]. As such, these subjects were included in all analyses.

There were no differences from pre- to post-season in NF-L concentration for soccer ($p=0.27$), rugby ($p=0.34$), ice hockey ($p=0.63$) or cross country athletes ($p=0.31$); however, serum NF-L levels increased 19.9% in football players from pre- to post-season ($p=0.03$, effect size = 0.42) (Figures 1 – 5). There were no changes from pre- to post-season for tau, Tau-A, and Tau-C. Within the football cohort that wore the xPatch, positive correlations were found between NF-L changes and CuHIT ($p<0.05$, $R^2=0.61$), CuPLA ($p<0.05$, $R^2=0.62$), and CuPRA ($p<0.05$, $R^2=0.61$) (Figures 3.6 – 3.8, respectively). The average CuHIT, CuPLA, CuPRA for all 7 players were 276(±231.6), 10441.56(±9060.46) *g*, and 1840604(±1578960) rad/s^2, respectively.

3.5 Discussion

The key finding from this study was that only American football is associated with an increase in NF-L from pre- to post-season. Consistent with the work of Oliver *et al*. (2016), who found a significant increase in NF-L from pre- to post-season in American football players[65], the

current findings revealed a ~20% increase in NF-L over the course of a season of American football (Δ = 1.56±2.20 pg/mL) (Figure 3.1). Increased CSF NF-L levels are also found following a bout of amateur boxing, and were positively correlated with the number of head impacts sustained[64]. The increase in NF-L concentration found in the current study correlated with CuPLA (R^2=0.62, p<0.05), CuPRA (R^2=0.61, p<0.05), and the total number of hits sustained in games during the season (R^2=0.61, p<0.05). Of note, Oliver *et al* (2016) found the increases in NF-L concentration corresponded to points in the season where the number of head impacts likely increased, including the start of the regular season, the start of conference play and playoff games[65]. Taken together, it appears the sub-concussive impacts sustained during a season of American football leads to an increase in serum NF-L.

NCAA male hockey players sustain ~350 head impacts per season during organized play with 95% of these impacts below 43.7g PLA and 4764 rad/s^2 PRA[260]. American football players in the current study sustained, on average, 276 head impacts per season, with an average of 37.8g PLA and 6668.9 rad/s^2 PRA. These values are from games only, and do not include full contact practices that occurred twice per week for the 10-week season. The difference in athlete exposure to head impacts could explain the difference in findings between these sports. Further, the number of head impacts per season in 9- and 10-year-old junior rugby players has been reported as 46 and 116, respectively[261,262]. PLA and PRA for both 9-year-olds (i.e. median – 15g, 10,434 rad/s^2) and 10-year-olds (i.e. mean – 22g, 4041 rad/s^2) were well below the values reported in the current study for American football players, which may explain the differences between these two sports with respect to serum NF-L increase.

NF-L and tau fragments were selected as the biomarkers of interest for this study due to increased concentrations found in CSF and blood following sports-related concussion[54,63], and in

populations with neurodegenerative disease[60,217,218]. Interestingly, plasma T-tau levels 1 hour post-concussion correlate with the time to resolution of post-concussion symptoms with 9.5 pg/mL predicting a recovery longer than 6 days[54]. In a case study at 2 weeks following a knockout loss in boxing, CSF NF-L levels were found to remain elevated, compared to normal reference values, for up to 36 weeks following the injury[63]. Serum NF-L levels remain elevated, compared to controls, 10-12 days following a sTBI[68]. These results suggest both NF-L and T-tau are sensitive markers of neuronal damage.

While the findings from both the current and aforementioned studies reached statistical significance[54,63,65], the clinical implications of these findings remain unknown. Normal median and interquartile range (IQR) CSF values for NF-L have been documented at 187 (94) pg/mL for those <30 years of age, and 274 (66.5) pg/mL for those 30 to <40 years age[263]; however, these values were determined from a relatively small sample size (i.e. <30 age group – n=17; 30 to <40 age group – n=15). Average values at the end of a season of American football, including those from our study (9.4 – 13 pg/mL), are well below those seen 24 hours following TBI (216 pg/mL)[65,68]. One of the challenges with analyzing plasma or serum samples for biomarkers of CNS damage is the blood-brain barrier (BBB). The BBB is a stringent filter separating the brain tissue from the peripheral circulation. It functions to both protect cerebral tissue from potentially damaging molecules in blood, and limiting the molecules that are able to pass from the CSF into the bloodstream[264]. Due to the selective permeability of the BBB, many protein molecules are typically unable to directly pass from the CSF into the circulating blood. It has been postulated a sufficient biomechanical force capable of causing a concussion could cause a disruption in the BBB. Indeed, BBB disruption has been shown to result from a TBI[265,266].

Caspase-3 and ADAM10 are proteolytic enzymes with the capability of cleaving tau protein into fragments. Once cleaved, Tau-A and Tau-C fragments then possess the capability to pass through the intact BBB, from the CSF into the blood stream. Tau-A was of great interest in this particular project as its concentration in serum has been shown to have an inverse correlation to scores on the Mattis Dementia Rating Scale (MDRS) in Alzheimer's disease (AD) patients[217]. Tau-C was examined primarily due to the role caspase enzymes play in the formation of NFTs. de Calignon *et al*. (2010) found a correlation between caspase activation in Tg4510 (tau transgenic) mice and the presence of NFTs in brain tissue[267]. Previous research has identified a correlation between the number of NFTs within brain tissue to the degree of dementia in AD[268].

Furthermore, normal reference interval values for biomarkers of brain injury have not yet been examined, which poses a sizable barrier in translating the research into applicable clinical knowledge. Given the nature of the BBB, NF-L concentration in serum is expected to be lower in blood by a large margin. Indeed, Shahim *et al*. (2016) found CSF and serum NF-L levels to be positively correlated, with CSF values 2 orders of magnitude greater than those in serum[68]. The brain is bathed in CSF, and so any biochemical changes in the brain will be reflected in CSF. However, the use of this fluid to detect protein levels reflecting neuronal death is not void of challenges. Obtaining a CSF sample can prove difficult due to the invasive nature of the lumbar puncture procedure. This method also restricts the number of draws that can be measured, given that the body has a limited supply of CSF. The ability to identify subtle changes in the brain through neuroimaging, and correlate these changes with fluid-based, and more specifically blood-based, biomarker concentration changes from pre- to post-injury may lead to an accurate diagnostic tool for identifying an individual with a concussion.

It has been reported soccer players engage in purposeful heading of a ball traveling at 72 – 89 km/h, and sometimes up to 115 km/h (71.5 mph)[269]. On average, players head the ball approximately 6 times per game[270]. When these values are extrapolated across a 51-game competitive season in the professional leagues in Europe, and coupled with practice sessions, a player can easily exceed 1,000 headers per year. The practice of heading in a soccer setting has come under intense scrutiny, with suggestions of playing soccer is an increased risk for developing amyotrophic lateral sclerosis (ALS)[79]. In a 1999 study, researchers found that amateur soccer players, not all of whom reported suffering a concussion during their career, scored significantly worse on a battery of neuropsychological tests than control athletes[271]. A 1989 study reported that soccer players experienced higher rates of electroencephalographic (EEG) abnormalities, along with cerebral atrophy, a sign of CTE[272].

Post-mortem evaluations of American football players confirmed to have had CTE showed cerebral atrophy as early as stage 2 (of 4)[221]. Seventy percent of soccer players examined in the Tsyvaer *et al*. study (1989) exhibited neurological deficits, including all players classified as those whose position naturally exposes them to more opportunities to head the ball[272]. However, a study examining the effect of 10-20 controlled headers on CSF levels of NF-L, T-tau, glial fibrillary acidic protein, and S100β did not reveal any abnormalities[62], suggesting an acute bout of soccer headers may not be sufficient enough to affect cerebral integrity. It was suggested there was sufficient time between headers and CSF sampling, allowing the brain time to move through the proper stages of healing, thus limiting the damage to cerebral tissue[62]; however, the repeated exposures experienced throughout a career may eventually overwhelm the window of healing. As such, performing >1000 soccer headers per season over the course of a career could possibly lead to cerebral damage.

Tau fragments have been proposed as biomarkers of neuronal damage and disease progression, specifically in Alzheimer's disease (AD)[217,218]. Also, the blood-brain barrier (BBB) is highly restrictive and, when intact, does not allow for the passage of large molecules[99]. To date, there is no evidence of BBB disruption following a sports-related concussion[64]. Further to this, proteolytic cleavage of tau occurs from the interaction with a variety of proteases, including calpains and caspases[273]. If there is a transient disruption to the BBB leading to leakage of tau from CSF to blood, it could quickly succumb to proteosomal activity, as tau values have been shown to return to baseline values within 1 week post-concussion[54].

The deleterious effects of repeated concussions have been well documented. An individual who sustains a concussion can experience a wide range of symptoms including, but not limited to, dizziness, irritability, nausea, vomiting, fatigue, low-energy, and headache[25]. The majority (80 – 90%) of individuals who suffer a concussion find these symptoms resolve within a 7 – 10 day period[1]. Less is known about the consequences of repetitive sub-concussive impacts on long-term neurological health.

In order to clinically interpret the results from this study, more research in this area is needed. Currently, the clinical model for concussion diagnosis relies strongly on subjective tools, making it possible for some concussions to go undetected. Conventional imaging techniques such as magnetic resonance imaging (MRI) and computed tomography (CT) are typically unable to detect a concussion; however, improvements in imaging techniques have allowed for detectable changes in recent years. Indeed, a 2016 study found that a concussion in hockey leads to a decrease in myelin water fraction using a novel myelin water imaging modality[274]. There were, however, no changes noted in those hockey players who did not sustain a concussion from pre- to post-season, suggesting this technique is not sensitive enough to detect

trauma from repetitive sub-concussive impacts. It is imperative that reference intervals be built across the entire age spectrum in order to establish clinical significance of any fluid-based biomarker.

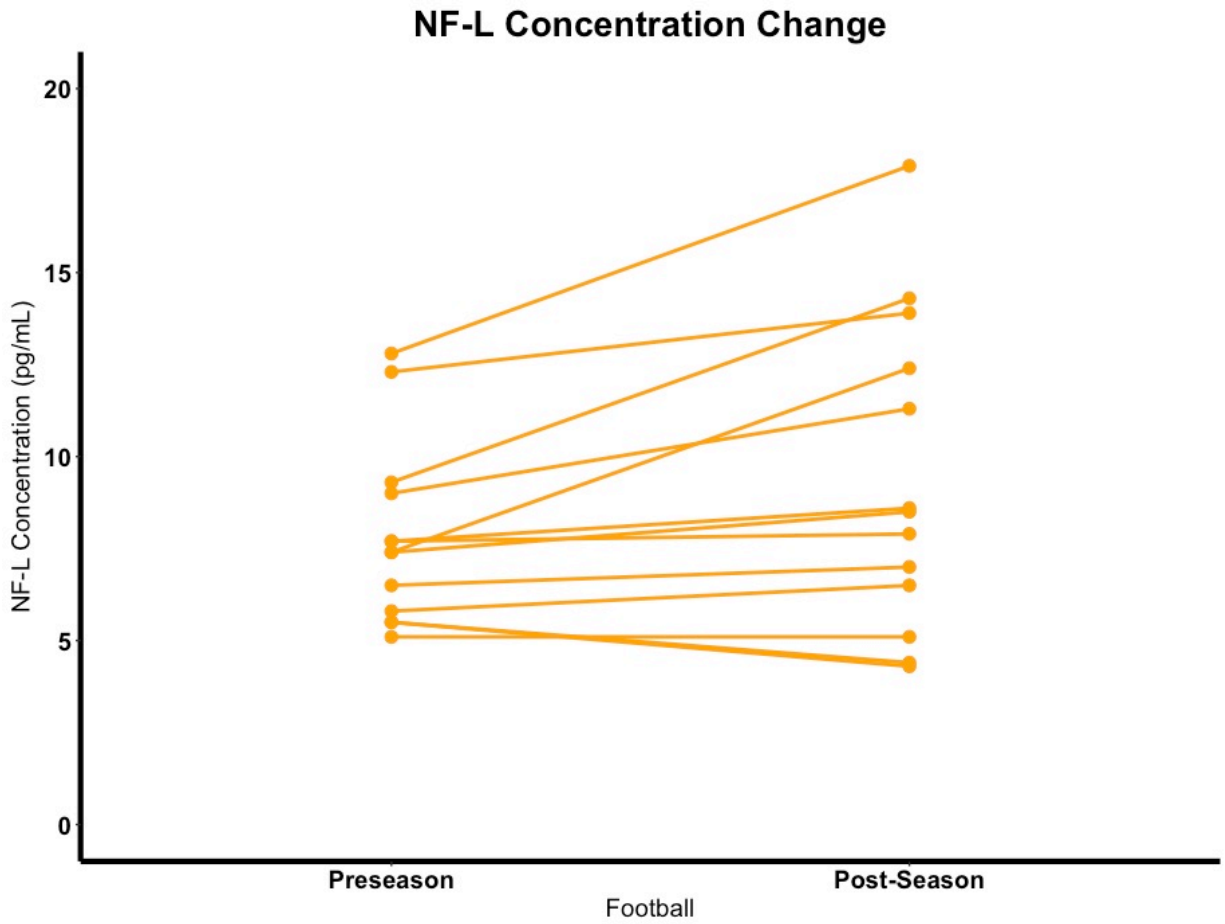

Figure 3.1 – Neurofilament Light Concentration Change from Pre- to Post-Season in American Football Players

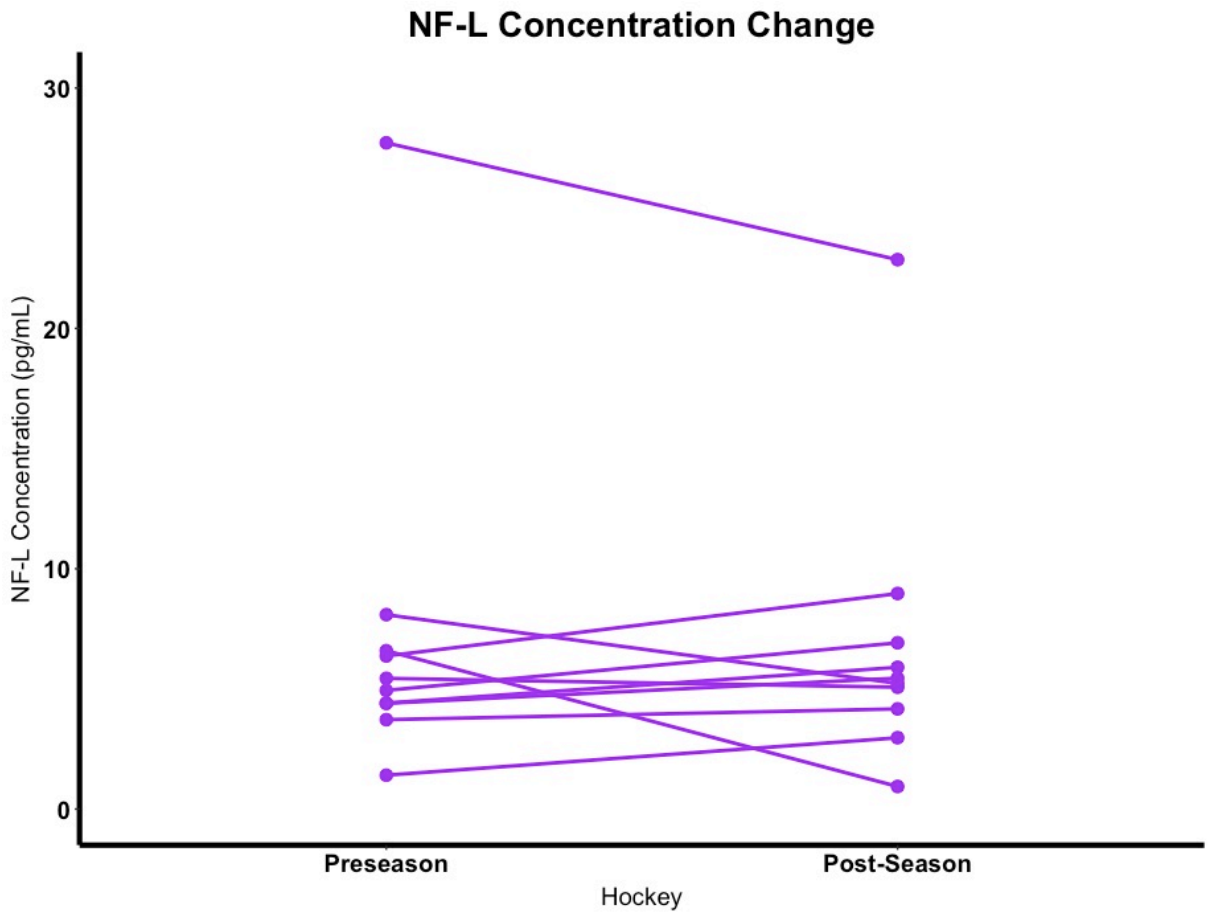

Figure 3.2 – Neurofilament Light Concentration Change from Pre- to Post-Season in Hockey Players

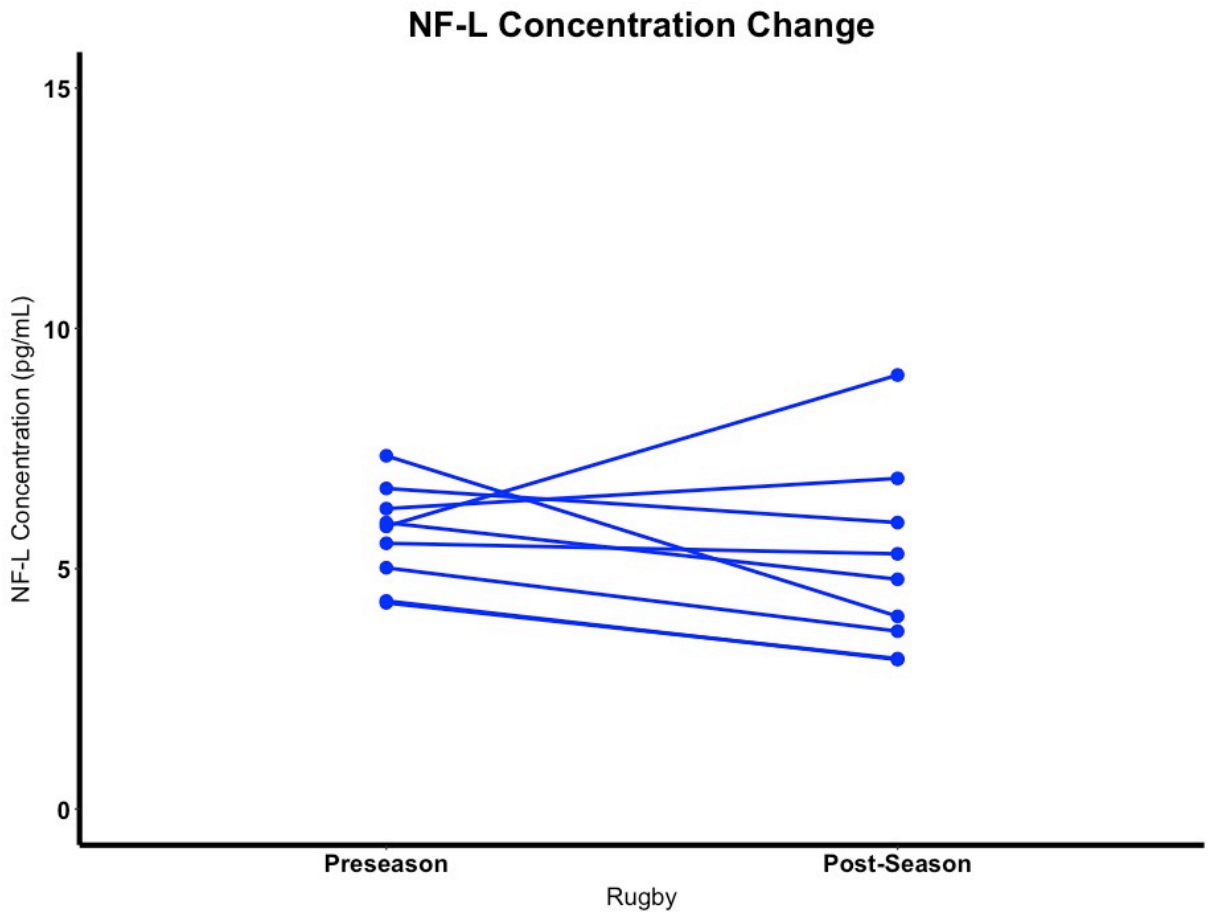

Figure 3.3 – Neurofilament Light Concentration Change from Pre- to Post-Season in Rugby Players

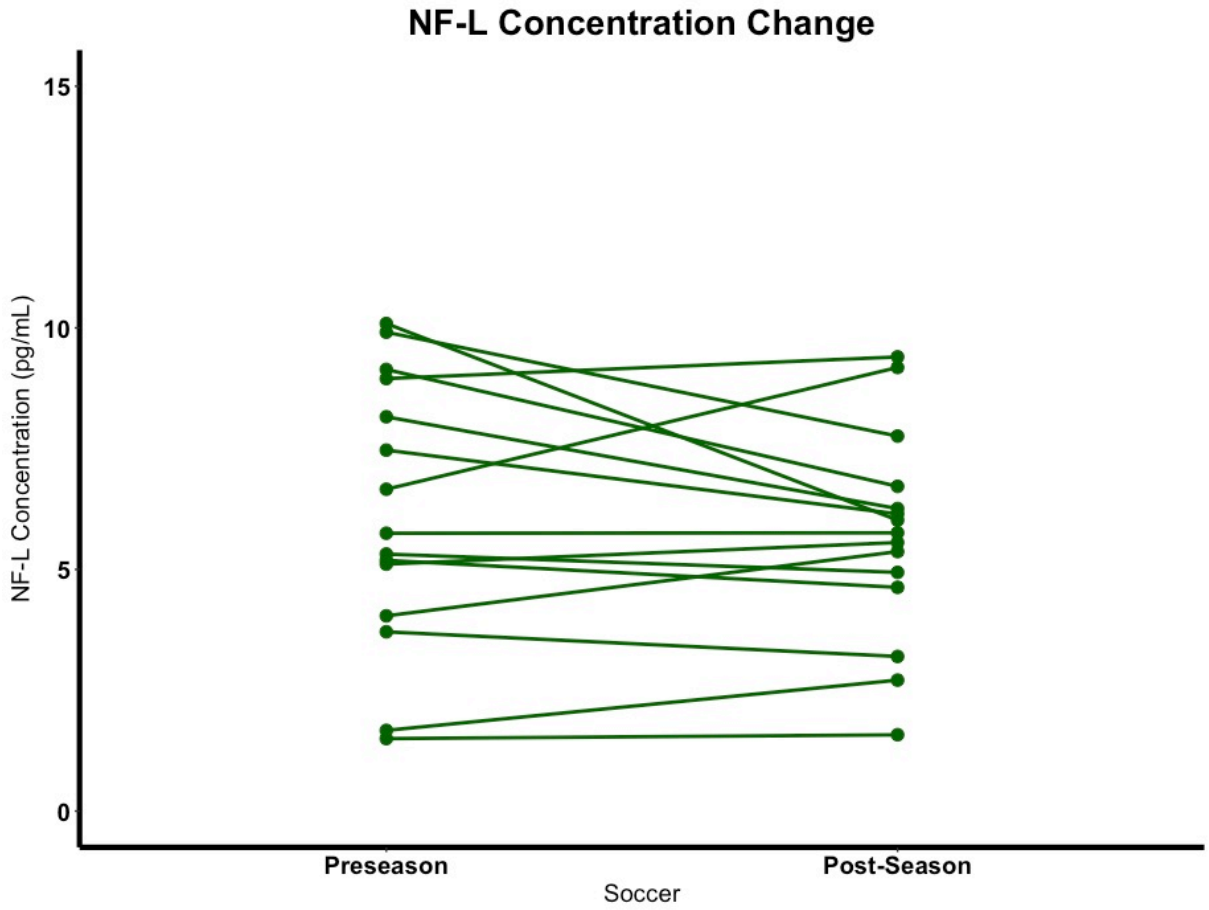

Figure 3.4 – Neurofilament Light Concentration Change from Pre- to Post-Season in Soccer Players

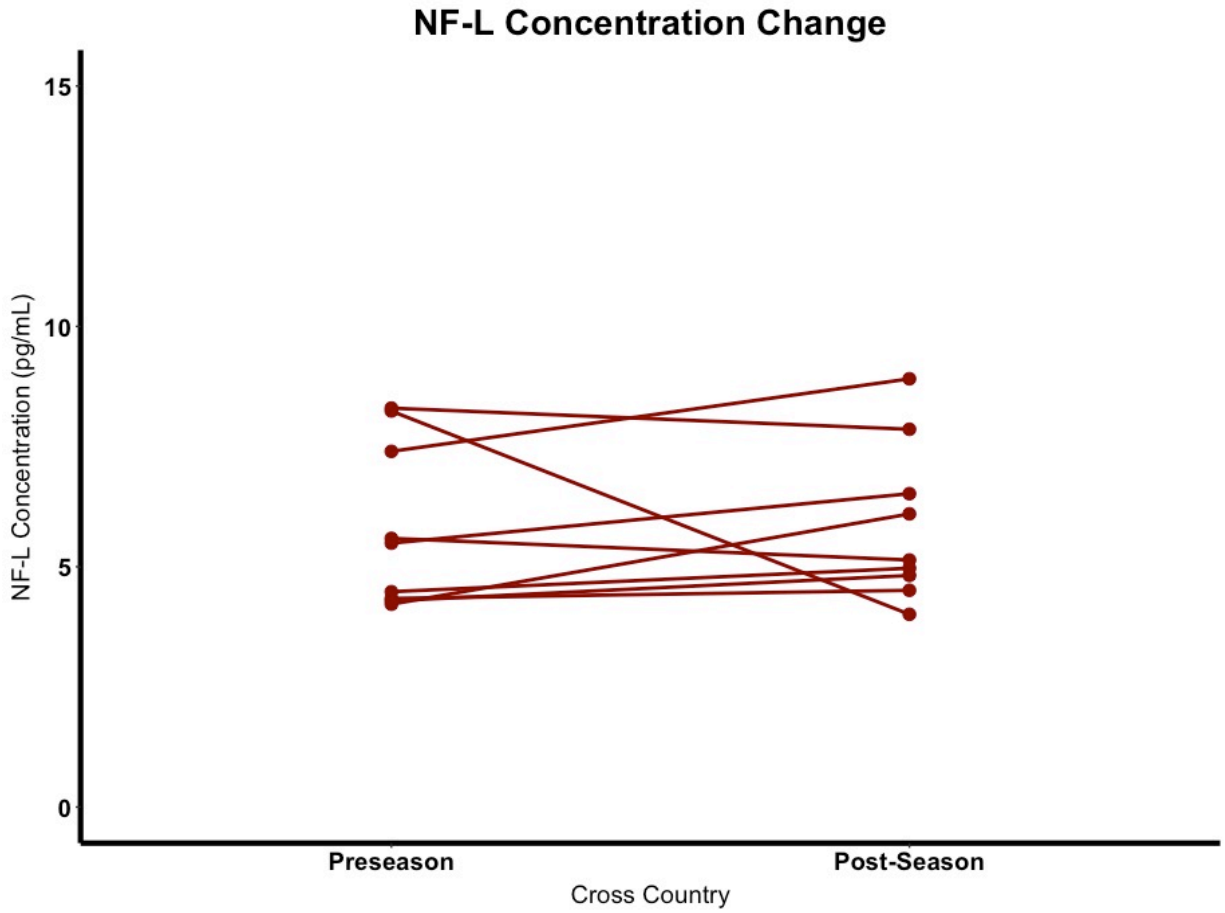

Figure 3.5 – Neurofilament Light Concentration Change from Pre- to Post-Season in Cross Country Runners

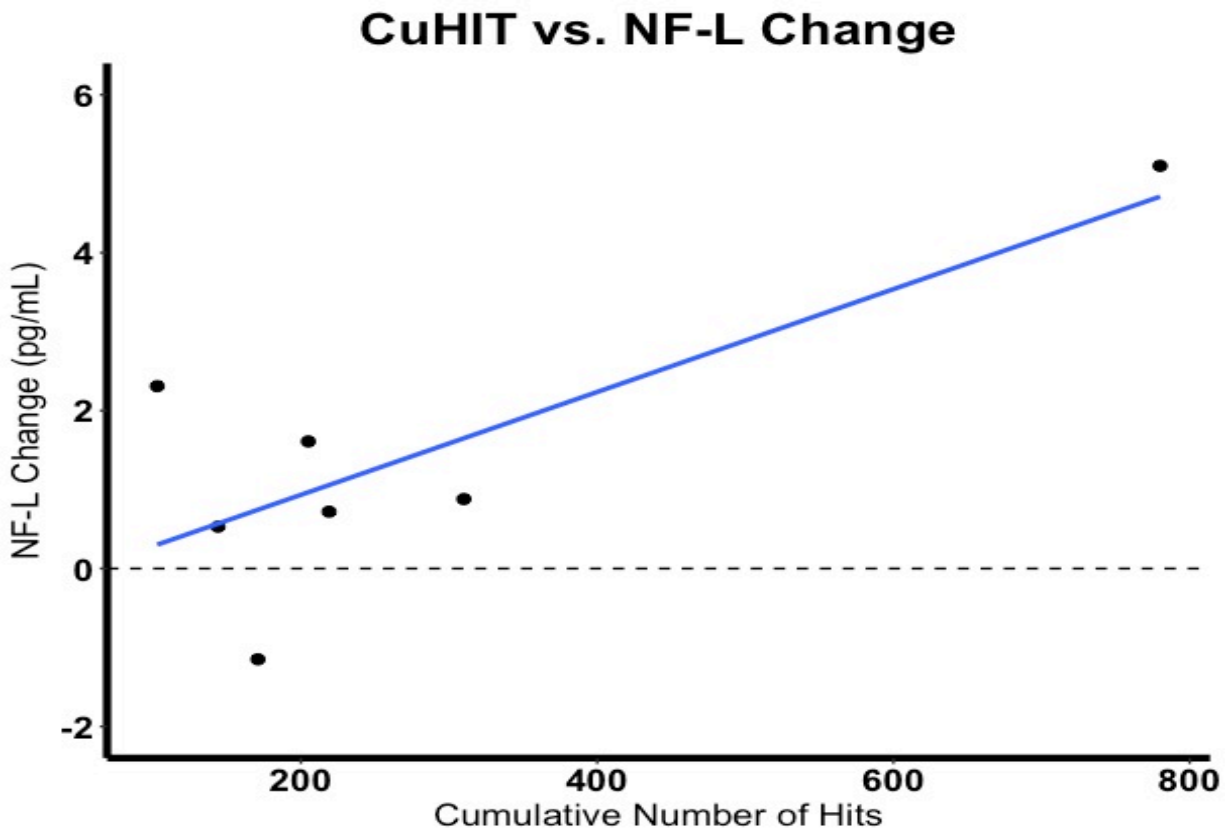

Figure 3.6 – Cumulative Number of Hits and Neurofilament Light Change Over a Season of American Football

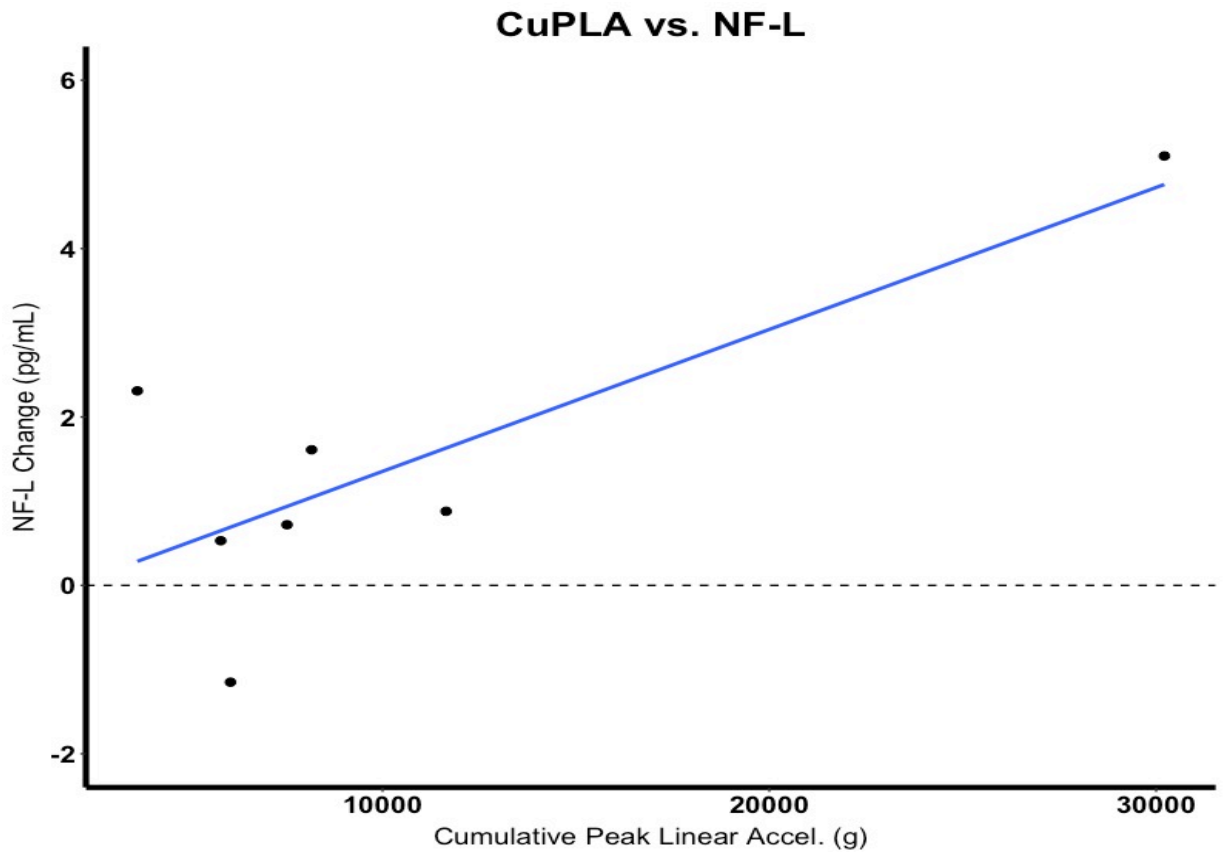

Figure 3.7 – Cumulative Peak Linear Acceleration and Neurofilament Light Change Over a Season of American Football

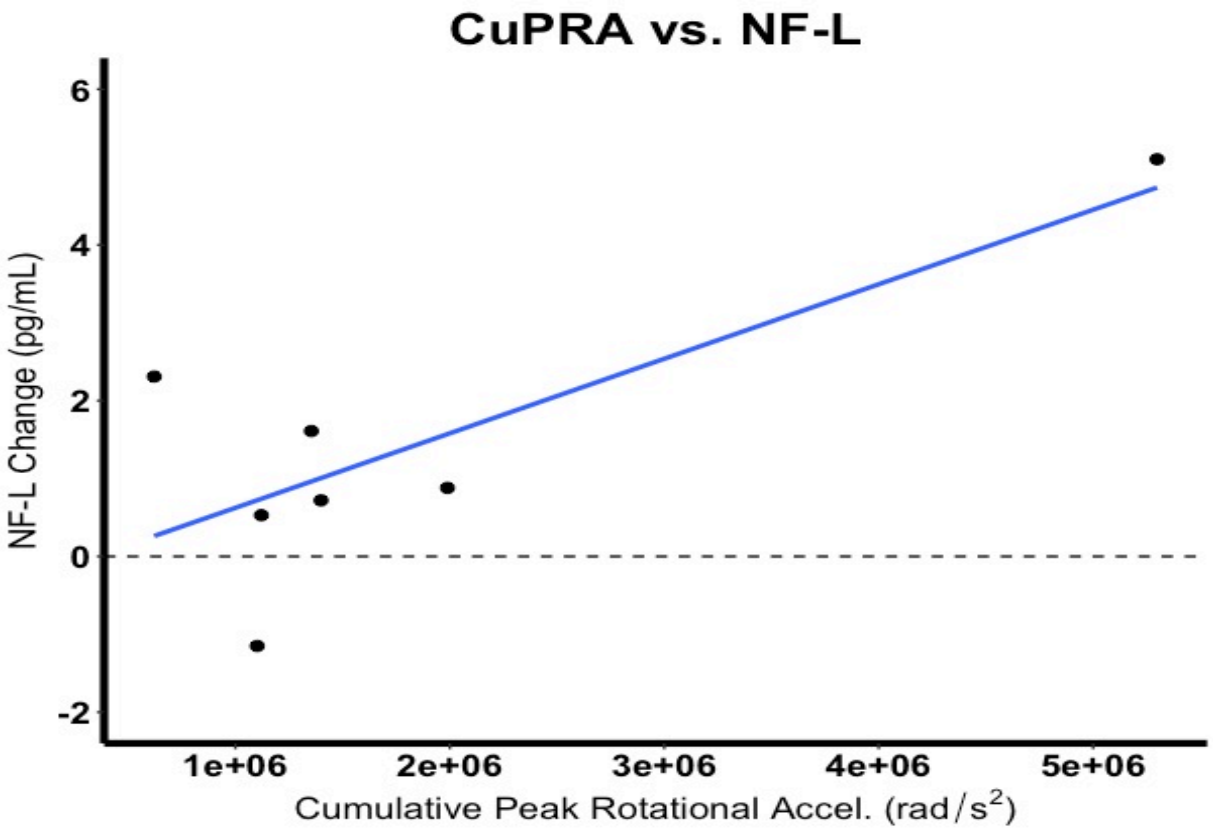

Figure 3.8 – Cumulative Peak Rotational Acceleration and Neurofilament Light Change Over a Season of American Football

Chapter 4: Study #2 – The Effect of an Acute Bout of Soccer Heading on Blood-Based Biomarkers of Axonal Disruption

4.1 Background

With 270 million active players in the world, soccer is one of the most popular sports on the planet[248]. Soccer is unique to other sports, as the gameplay enables players to use their head to contact the ball, and the number of headers performed in a given season can vary based on position and typically range from 6 – 12 per game[269,275]. The act of heading a soccer ball in and of itself is a complex skill, as it requires precision accuracy and can be performed while the player is in a variety of positions (e.g. standing, walking, running forward, running backward, jumping, diving), or be challenged either in the air or on the ground by an opponent. As such, soccer participation has been known to lead to head and neck injuries, including concussions[269,276]. Concussion rates in collegiate competition for soccer have been reported at 0.49 per 1000 athlete exposures (AE) for men, and 0.92 – 1.8 per 1000 AE in women[8,12]. For men's sports, only American football (0.47 – 0.64 per 1000 AE), and ice hockey (0.54 per 1000 AE) have a higher reported concussion incidence rate[12]. Women's soccer, on the other hand, is associated with the highest concussion rate in collegiate athletics[8,12]. Sideline concussion assessment is commonly conducted using the Standardized Concussion Assessment Tool (5th edition), which includes cognitive screening tools, a balance examination, and symptom evaluation[25]. Following a bout of soccer heading that included 5 headers in 10 minutes, only a slight increase in symptoms was reported (i.e. 1 symptom by 6 out of 16 participants).

Although the rate of concussive injury is high compared to other sports, the media has placed soccer under intense scrutiny recently with respect to the potential negative, long-term effects of heading the ball. This focus was initially triggered in 2002 by the death of Jeff Astle,

and his subsequent diagnosis of CTE. Astle, who played 20 seasons of soccer in Europe, was a prolific header of the ball. Typically, headers occur on punts (72 km/h) or on a goal kick (89 km/h)[269]. Over the course of a professional playing career, the number of headers during match play is estimated at 2000, with some players performing even more[275]. This does not take into account the years of soccer involvement prior to becoming a professional, nor does it take into account headers occurring during practice. Combining games and practices, the number of self-reported headers can be in excess of 1000 per year in some athletes[78]. When extrapolated over the course of a career, this results in a massive exposure to sub-concussive impacts.

Exposure to this number of head impacts throughout a playing career has been proposed as a potential cause for cerebral atrophy[270], attentional deficits[78,277-279], along with white matter[78] and electroencephalogram (EEG)[272] abnormalities. It is worth noting many of these studies were conducted in the "heavy ball" era (i.e. prior to the late 1980s), when a leather ball was used. A leather ball soaks up more moisture during game play, as such it can be much heavier than the modern soccer ball, that can weigh 420 – 445 grams. Interestingly, this was attributed to the cognitive decline and, ultimately, the death of Jeff Astle. A bout of soccer heading has also been associated with balance errors[280] and cognitive dysfunction[247], although these findings have been widely refuted[281-287].

In 2004, Stålnacke *et al*. found serum S100β levels to be elevated immediately following a soccer game, and the increase seen was positively correlated with the number of headers performed[139]. In contrast to these findings, Zetterberg *et al*. (2007) examined serum and cerebrospinal fluid (CSF) S100β following a bout of soccer heading and found CSF S100β to be elevated in controls who did not perform any headers, compared to the groups performing headers[62]. S100β has also been found to increase following non-contact sport, including

swimming, running, and basketball[138,178,179], casting doubt on S100β as a sensitive marker of central nervous system injury.

There is a dearth of literature describing the pathologic changes of repetitive head impacts in soccer players. In recent years, two separate case studies have been published, each describing chronic traumatic encephalopathy (CTE) in retired professional soccer players[249,288]. CTE is a neurodegenerative disease with marked tau deposits in the form of neurofibrillary tangles (NFT), cerebral atrophy in stages 3 and 4 (of 4)[221]. CTE can lead to behavioural and mood symptoms along with cognitive impairment, and is only diagnosed by post-mortem autopsy[82]. Although concussion history was not reported, it was postulated repetitive head impacts may have been a major contributing factor in the development of the disease[249,288]. The accumulation of repetitive impacts may cause the disruption of axonal projections in the brain[221]. It appears that, in order to form a mechanistic link between repetitive heading and acute axonal disruption, a sensitive method to detect axonal disruption is needed, as current neuropsychological and balance testing paradigms fall short.

Therefore, we decided to investigate whether heading the ball would result in a change in blood-based biomarker concentration. Specifically, we examined both plasma total tau (T-tau) and serum neurofilament light (NF-L) as they are both proposed to be indicators of axonal damage. T-tau has been shown to increase following a concussion, with the highest levels seen 1 hour post-injury[54]. NF-L has yet to be examined within the same time construct following a concussion; however, NF-L is increased 1 hour following a severe traumatic brain injury (sTBI)[68]. Secondly, we had athletes complete the symptom checklist of the SCAT3 (as it was the current version at the time of the study). The SCAT3 is the current clinical gold standard concussion diagnostic tool, and the symptom checklist is comprised of 22 symptoms, including

somatic, cognitive, and neurobehavioural, that the participant ranks on a 0 – 6 Likert scale (0 – no symptom, 6 – most severe). Our hypotheses for this project were:

i) there would be an increase in T-tau and NF-L following an acute bout of soccer heading; and

ii) an increase in both the total number of symptoms (TS) (0 – 22) and symptom severity (SS) (0 – 132) score would be reported by participants following the acute bout of soccer heading.

4.2 Methods

A total of 11 male participants consented to participate in this study, all of whom were current or former university varsity soccer players with at least 5 years playing at a highly competitive level. Every player was free from concussion for 6+ months prior to testing.

Table 4.1 – Subject Demographics Included for NF-L and Tau Analysis

	Heading	Control	Sham	*p*-Value
NF-L	11	9	8	
	22.7±3.8	23.4±3.9	23.5±4.1	0.65
Tau	9	9	8	
	23.0±4.2	23.4±3.9	23.5±4.1	0.44

The study protocol consisted of three separate testing days, each with its own distinct condition. There were 2 less participants included in the tau analysis for the Heading condition as values were below 1.2 pg/mL, the lower limit of quantification (LLOQ). On each testing day participants completed two blood draws; one prior to the condition, and the second at 1-hour post-condition, as T-tau has been shown to peak at this time point following a concussion[54]. SCAT3 symptom evaluations were also completed before and after each condition. Subjects were instructed to assign a value to each symptom based on their current state at time of testing.

The three randomized conditions were as follows, with each visit separated by a minimum of 1 week:

i) Heading – 40 approved headers with 30 seconds separating each trial

ii) SHAM – 40 approved sham trials (i.e. contact to the body other than the head)

iii) CTRL – control session (the athlete was brought to the field/gym for 20 minutes, then returned to the laboratory)

The soccer ball used was a Fédération Internationale de Football Association (FIFA) regulation size 5 ball inflated to a regulation pressure of 13.0 psi, and was propelled from a JUGS soccer machine (JUGS International, Tualatin, Oregon, USA; Fig 1). Initial launch velocity was read with a radar gun (INSERT MODEL HERE). For interventions i) and ii), the participant stood approximately 25m from the machine and redirected the ball to a member of the research team standing 10m from them on either side (Figure 4.2). For conditions i) and ii), the soccer ball was propelled from the JUGS machine at 79 (SD 3.39) km/h initial velocity. This speed was chosen to match the typical velocity of a punt, corner kick, or goal kick[269].

Blood sampling was completed as detailed in the Methods section (2.1 and 2.2) of this dissertation.

Figure 4.1 – JUGS Machine

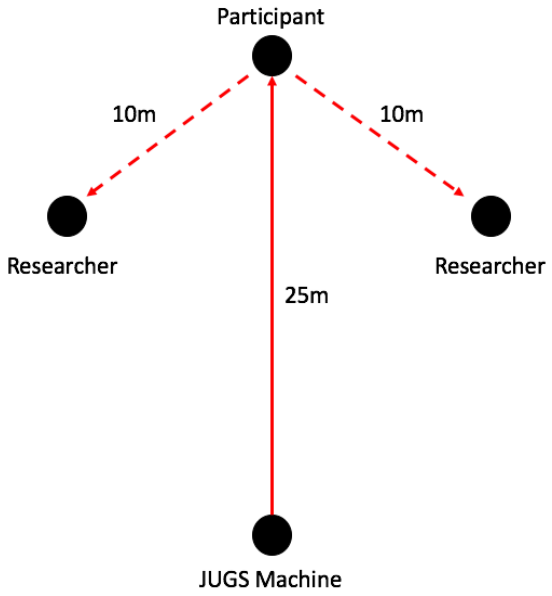

Figure 4.2 – Study Setup

4.3 Statistical Analysis

Multiple 3 (condition) by 2 (time) repeated measures ANOVA models were performed based on the following dependent variables: NF-L concentration, SCAT3 total symptoms reported (SCAT3 TS), and SCAT3 symptom severity score (SCAT3 SS) (Table 4.2). False discovery rate post-hoc analyses were conducted when $p<0.05$ to determine condition by time interactions. All statistics were run in R Studio (v 1.0.143, Foundation for Open Access Statistics), and presented as mean ± SD.

4.4 Results

Analysis of variance revealed no significant differences were found between conditions for both NF-L and tau concentration, as $p>0.05$ for both (Table 4.1). Interestingly, main effects of condition by time were present for both the total number of symptoms reported ($p=0.009$) and symptom severity score ($p=0.017$), as both increased significantly following the Heading condition compared to both CTRL and SHAM (Table 4.2). Post-hoc analysis revealed

participants reported a greater number of symptoms following the Heading condition (7.4±6.8) compared to both CTRL (2.4±3.3) and SHAM (1.8±2.9). Participants also reported more severe symptoms following the Heading condition (11.25±12.6) compared to both CTRL (2.6±3.9) and SHAM (2.0±3.1) conditions. These results suggest soccer heading can cause an increase in both the total number of symptoms reported and the severity of those symptoms listed on the SCAT3 even in the absence of NF-L and T-tau concentration changes (Table 4.2).

Interestingly, we noticed a trend in some participants to show greater variability in NF-L throughout the study duration (n=5). Upon further exploration, it was noted the participants who experienced a dramatic increase in NF-L concentration from 1st visit to their 2nd visit completed the Heading condition first. In order to gather further information into the effect a bout of soccer heading has on serum NF-L concentration, the order of visits was chronologically sorted, with T1 representing NF-L concentration prior to the first intervention and T2 representing NF-L concentration prior to the second intervention. Average time between T1 and T2 was 31.57 (± 23.0) days. Average NF-L concentrations for T1 and T2 were 8.52±5.30 pg/mL and 35.0±29.92 pg/mL, respectively, while average T-tau concentrations for T1 and T2 were 2.89±1.33 pg/mL and 4.33±2.08 pg/mL, respectively (Figure 4.3). Wilcoxon sign tests were run for both NF-L and T-tau, and results showed a significant increase in NF-L (p=0.02) from T1 to T2 (Figure 4.3), whereas T-tau showed no statistical change (p>0.05).

Table 4.2 – Pre- and Post-Intervention Values

	Heading	Control	Sham	*p*-value
NF-L (pg/mL)				
Pre	14.5±17.8	32.6±42.8	15.4±11.6	0.71
Post	16.1±17.7	26.2±42.1	15.9±11.9	
T-tau (pg/mL)				
Pre	2.86±1.05	4.37±2.65	3.90±2.50	0.23
Post	3.38±1.81	2.75±1.78	3.51±1.64	
SCAT3 TS[1]				
Pre	2.1±2.6	3.6±4.8	3.0±3.0	0.009*
Post	7.4±6.8	2.4±3.3	1.8±2.9	
SCAT3 SS[1]				
Pre	2.8±3.6	4.9±7.3	3.8±3.8	0.017*
Post	11.25±12.6	2.6±3.9	2.0±3.1	

All data reported as mean±SD

[1] SCAT3 TS: total # of symptoms present (sum of symptoms reported with severity >0)
SCAT3 SS: total severity of symptoms present (sum of all Likert scale rankings)
Conditions: Heading; CTRL – control; SHAM – sham

4.5 Discussion

The main findings from this study include the following: i) serum NF-L and plasma T-tau are unchanged at 1 hour following a bout of soccer heading when compared to sham and control conditions; ii) serum NF-L appears to increase at approximately 1 month following an acute bout of soccer heading (although more investigation is needed), and iii) plasma T-tau remains unchanged at 1 month following an acute bout of soccer heading.

It has previously been shown S100β is increased in male soccer players following participation in a competitive game[139]; however, given the fact that S100β is found in peripheral tissue, the increase reported could be due to physical exertion. A 2015 study examining plasma S100β levels before and at 1 – 1.5 hours post-heading, in which 14 players headed a soccer ball travelling at varying speeds from 30 – 50 mph (~50 – 80 km/h), found no changes[289]. Furthermore, the contrasting results from the S100β studies are not entirely unexpected. S100β has previously been shown to increase following a variety of athletic events in which no head

trauma has occurred, including swimming[178], hockey[54], running[138], and basketball[179]. Interestingly, in the study by Otto *et al*. (2000), the increase in S100β was seen following a 25 km race, but not a 10,000m race or two minutes of sprinting[138]. The authors surmised this increase could be due to axial vibration of the brain during each step and astroglial destruction coupled with the ~25000 steps needed to complete 25 km could be the underlying cause of elevated S100β levels. In addition, in a 2003 study by Stålnacke *et al*., the level of increase in S100β correlated with the number of jumps performed while playing basketball, which was the source of most acceleration/deceleration actions[179].

The source of S100β related to the increase in blood in the aforementioned studies is unclear. S100β has been shown to increase in serum in the absence of neurological trauma as a marker of BBB disruption, suggesting an increase in peripheral S100β does not necessarily equate to a neuronal damage occurrence[290]. Moreover, S100β levels in both CSF and serum have previously been shown to be unaffected 7 – 10 days following an acute bout of soccer heading[62]. This could be due to the fact that S100β has a relatively short half-life (i.e. 25 – 120 minutes)[156,157]. As such, future studies on S100β should include sampling as close to the intervention as possible to truly gauge the dynamics of the protein.

T-tau and NF-L were selected as potential biomarkers for this study given their status as proposed biochemical markers of brain damage[112]. CSF T-tau levels have been shown to be unaffected 7 – 10 days following an acute bout of soccer heading[62]. T-tau, like S100β, has a short half-life in plasma (i.e. 0.5 – 12)[254,255], therefore for accurate results the sample collection time points play a crucial role in detecting concentration differences. As such, we sought to examine its dynamics 1 hour following an acute bout of soccer heading. From the results of the

current study, it appears both plasma T-tau and serum NF-L levels are not altered immediately following an acute bout of soccer heading. In the 2007 study by Zetterberg *et al.*[62], participants headed the ball either 10 or 20 times and the velocity of the ball was not recorded. Also, the sensitivity of the detection method due to limitations of the technology at the time only allowed for quantification of the protein only above a concentration of 125 pg/mL, which none of the samples contained. The advancement of ultrasensitive detection methods now allows for a much lower limit of quantification in serum, approximately 2.7 pg/mL[68]. In the current study, 40 headers were performed in 20 minutes at speeds that mimic those seen in a game. When the interventions were ordered chronologically in the subset of participants who completed header trials on their first visit, NF-L concentrations were increased (8.52±5.30 to 35.0±29.92 pg/mL) at approximately 1 month (median 22, IQR 15 – 43 days) following a bout of soccer heading (p=0.04).

Soccer heading has received criticism for exposing players to repetitive, sub-concussive impacts. Researchers and health care professionals alike fear the repetitive nature of these impacts can have negative long-term effects, mimicking the effects seen following multiple concussions. The US Youth Soccer Organization has banned the act of heading the ball for players 10 years of age and younger, while players aged 11 and 12 are allowed to head the ball during practices and games up to a maximum of 25 headers per week. With this rule change, the goal is to reduce the number of concussions sustained along with reduce the volume of sub-concussive impacts an individual is exposed to throughout their career, particularly at a young age as the brain is still maturing.

This delayed time course for the release of NF-L is consistent with previous literature on NF-L following repetitive head trauma experienced in boxing[55] and across a season of American

football[65]. The NF-L increase during boxing was positively correlated with the number of head impacts sustained when examined 1 – 6 days following the bout[64,291]. A season of sub-concussive impacts experienced during American football has been associated with an increase in serum NF-L, with sharper increases immediately following times during the season in which an increase in the number and severity of head impacts were most likely to occur [65]. Serum NF-L dynamics tend to be much slower than that of tau; in a 2016 study involving sTBI patients that included sampling every day from admission to hospital (i.e. day 0) to the final sample collection (i.e. day 12) the highest levels were measured at 12 days[68]. It appears as though serum NF-L concentrations remain elevated for up to 70 days following head trauma.

As was expected, the SCAT-TS (p=0.009) and the SCAT-SS (p=0.017) were higher following the acute heading condition than those reported following either the sham or control conditions (Table 4.2). Immediately following the Heading condition, 64% of participants reported feeling a headache (no headache scores were reported prior), with severity scores ranging from 1 – 4. Only one study to date has examined symptom scores before and after an acute bout of soccer heading[289]. Of the 16 participants tested in the prior study, only 6 reported an increase in symptoms following randomly assigned interventions where 5 headers were preformed when the ball was travelling at either: 30 mph (n=1), 40 mph (n=2) and 50 mph (n=3)[289]. Certainly, the data from the current study suggest more research is needed into the potentially deleterious effect a bout of soccer heading has on neurological integrity.

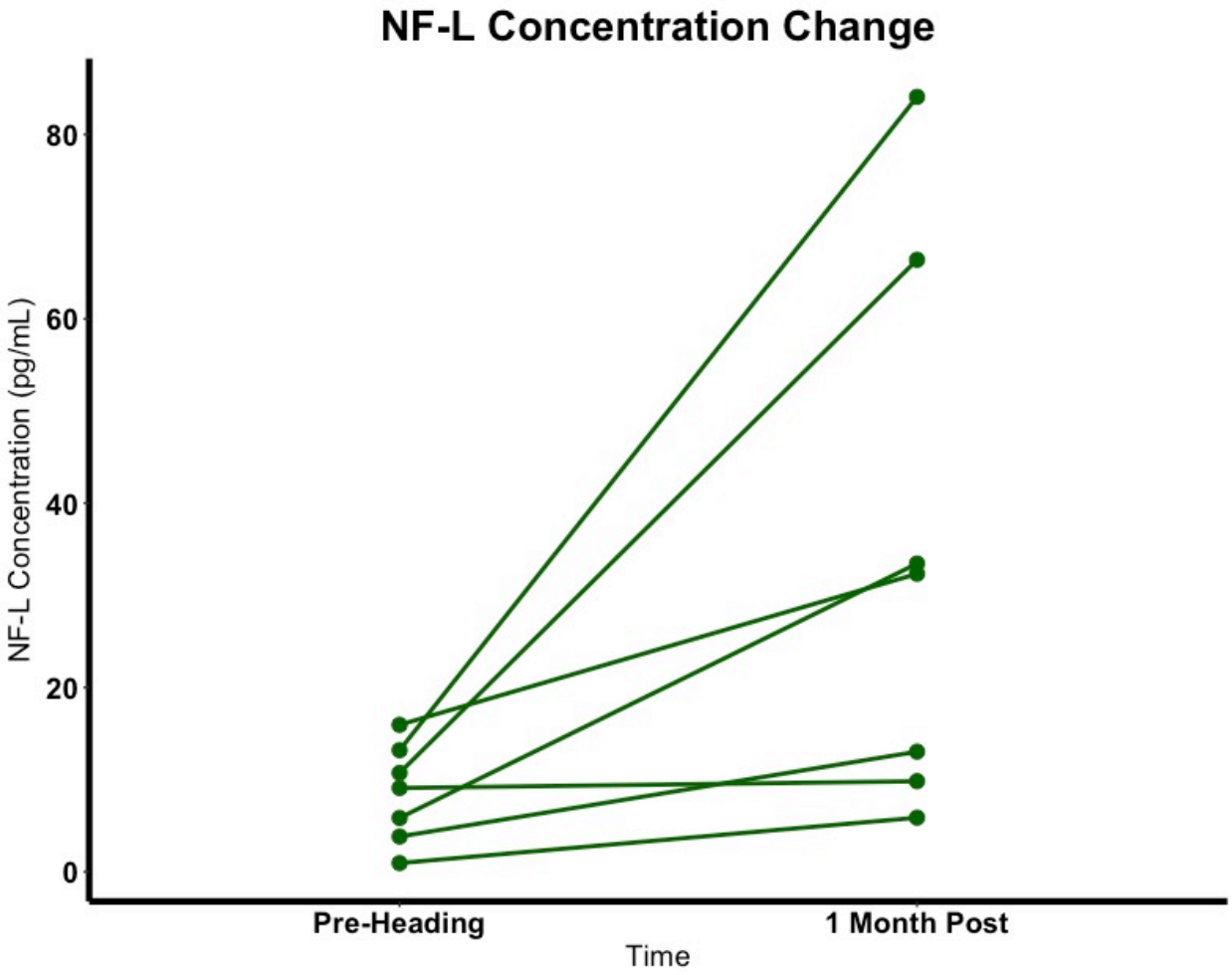

Figure 4.3 – Neurofilament Light Concentration by Time

Chapter 5: Study #3 – Blood-Based Biomarkers of Axonal Disruption Show No Change at 6- and 14-Days Post-Concussion

5.1 Background

With approximately 1.6 – 3.8 sports-related TBIs occurring in the U.S. alone, there is great concern for the short-term and long-term deleterious effects of this injury[4]. Although sports such as American football, ice hockey, rugby, boxing, lacrosse, and soccer are associated with an increased risk of sustaining a concussion[12,244,292-294] a concussion can, theoretically, occur in any sport, even those without player-to-player contact. Indeed, concussive injuries occur at a rate of 1.4 injuries per 10,000 AE in cheerleading[12], while concussions during women's gymnastics occur at a rate of 0.7 – 2.65 injuries per 10,000 AE[12,293].

So far, the majority of fluid-based biomarker research in the context of TBI has taken place in a hospital emergency department setting and involved mild TBI (mTBI), moderate TBI (moTBI), and severe TBI (sTBI) patients[174,185,295]. A 2012 observational study involving 108 individuals with mTBI and moTBI found that glial fibrillary acidic protein breakdown product (GFAP-BDP) elevations at 2.5 hours post-injury in those with TBI compared to uninjured controls[174]. A 2012 prospective cohort study found ubiquitin carboxyl-terminal hydrolase 1 (UCHL1) levels to be higher in those with mTBI and a Glasgow Coma Scale score of 15 at 1 hour post-injury compared to uninjured controls[185]. To date, only one study has examined NF-L levels in the context of sport-related concussion. Shahim *et al*. (2017) found serum NF-L in players from the Swedish Hockey League to be elevated post-concussion compared to controls, with the highest levels seen at 144 hours post-injury[61]. Elevated total tau (T-tau) levels were also discovered in Swedish professional hockey players as early as 1 hour post-concussion when compared to uninjured controls, and they remained elevated at 144 hours post-injury[54].

Following a bout of amateur boxing, serum neurofilament light (NF-L) is also elevated compared to controls, and levels were also able to distinguish between those boxers who received severe head impacts (i.e. >15 punches to the head) than those with mild head impacts (i.e. <15 punches)[61]. A 2014 case-study involving a boxer who was knocked-out during a bout showed elevated CSF levels at 2 weeks that only returned to normal reference values at 36 weeks post-injury[63]. Oliver *et al*. (2016) found serum NF-L levels to increase over the course of a NCAA American football season in starters when compared to non-starters and swimmers[65], findings that were repeated in Chapter 3 of this dissertation.

Athletes with access to immediate medical assistance, such as those in a varsity athletic or professional sport team setting, notionally have the opportunity for a more rapid diagnosis of injury. This provides the prospect of rapid medical attention, potentially limiting recovery time, and a reduced risk of further injury due to an immediate removal from play[296,297]. Unfortunately, not all sporting events have on-site medical coverage, and so a concussion can go unreported. Time is an important factor when identifying potential blood-based biomarkers of concussion[54]. Individuals may not immediately seek medical attention, and this, coupled with the fact the vast majority of proteins identified as potential biomarkers have a half-life <12 hours, exploration into time points greater than 144 hours is warranted.

Thus far, T-tau and NF-L have shown the most promise, mostly due to the fact they are highly, though not exclusively, expressed in the CNS[112]. Fragments of tau protein have also shown promise as markers of cognitive dysfunction in Alzheimer's disease (AD)[217,218] patients and may be used to predict symptom duration following a concussion[298].

It is imperative an objective method of concussion detection be developed that is sensitive, measureable, reliable, repeatable, quick, and cost-effective[112]. With respect to blood-

based biomarker concentration, a significant, and sustained, increase in the biomarker is required in order to truly distinguish between concussed and non-concussed individuals. The goals of this prospective cohort study were to determine the profile of serum NF-L at 1- and 2-weeks post-concussion, to extend the temporal profile of plasma T-tau past the currently known 144 hours[54], and to examine serum tau breakdown products Tau-A and Tau-C to possibly extend the temporal profile of tau even further.

5.2 Methods

Three local contact-sport teams (2 ice hockey, 1 American football) were recruited for this study. In total, 48 hockey players (aged 18.4±1.1 years) and 94 American football (aged 19.7±1.5 years) participated in this study. Initial samples were drawn from all 142 athletes during their respective preseason training camp. All samples were drawn prior to practice and all athletes had not participated in exercise at least 12 hours prior to sample collection. Post-concussion samples were then collected at 6 (±0.79) and 14 (±0.51) days. Samples were taken, prepared and analyzed as per the Methods section (Chapter 2) of this dissertation.

A one-way repeated measures ANOVA was run for each biomarker of interest, including NF-L, T-tau, Tau-A, and Tau-C.

Table 5.1 – Blood Concentrations of Neurofilament Light, Total Tau, Tau-A and Tau-C preseason vs. 6- and 14-days following concussion

Biomarker	# of Participants	Concentration			*p*-Value
		Preseason	6 days	14 days	
NF-L	12	8.05±3.03	9.05±3.42	7.88±3.86	0.50
T-tau	9	2.34±0.71	2.82±1.11	2.82±1.38	0.35
Tau-A	6	13.02±2.86	12.25±4.22	15.85±7.06	0.23
Tau-C	6	9.78±3.80	10.62±3.32	11.87±4.44	0.25

All values given as mean±SD
Biomarker concentrations reported as pg/mL (NF-L & T-tau) and ng/mL (Tau-A & Tau-C)

5.3 Results

In total, 142 athletes provided preseason blood samples, and 12 reported a concussion. Concussion diagnosis was made by each team's medical personnel. Eleven of the 12 athletes had detectable levels of NF-L at 6 days, while all 12 athletes had detectable levels at 2-weeks post-concussion. 9 players had detectable T-tau levels at all time points, while only 6 players had detectable levels of both Tau-A and Tau-C at all time points. None of the athletes reported or were found to have sustained an orthopedic injury at the time of concussion or during the post-concussion follow up collections.

There were no significant differences in biomarker concentration from pre- to post-injury across all time points. NF-L (p=0.05), T-tau(p=0.35), Tau-A(p=0.23), and Tau-C (p=0.25) levels are shown across time in Figures 5.1 – 5.4, respectively.

5.4 Discussion

Although this study involved a small cohort of concussed athletes, the key finding was that both T-tau and NF-L appeared to be unchanged at 6-days post-concussion elite junior hockey and football players. Contrasting the results from the current study are found in a 2014 study involving Swedish professional ice hockey players, where T-tau levels were elevated 6-days post-concussion (median 6.5, range 0.93 – 88.0 pg/mL) when compared to uninjured baseline controls (median 4.5, 0.06 – 22.7 pg/mL)[54]. Median (range) for the same time points in our study were 3.21 (0.86 – 4.09) pg/mL (6-days post-concussion) and 2.48 (1.35 – 3.30) pg/mL (baseline) (Figure 5.2). One possibility is the concussed and control groups may have been different even in the absence of concussion. As baseline and post-concussion samples were not from the same individuals, the variability in biomarker concentration from person to person

could have driven the significance in the study by Shahim *et al*. (2014)[54]. Future studies should include pre- and post-injury samples from the same individuals, and more sampling time points throughout the season for athletes to truly assess normal ranges, which will create reference intervals for each participant in the study.

Another possibility for the lack of differences between groups is the high protease activity occurring in the blood. The half-life of tau has been estimated at 0.5 – 12 hours in plasma and serum[254,255]; as such, sampling time points extending beyond this window may fail to identify any transient increases in concentration, which was noted in this study (Table 5.1, Figure 5.2). Wallerian degeneration is a process in that the severed portion of an axon degenerates following stretch injury in peripheral nerves[299,300]. This process has also been shown to occur in the central nervous system[301] and can extend as far as 12 weeks post-injury[302]. For this reason, samples were analyzed for T-tau at 14-days post-concussion, and fragments of tau, specifically Tau-A and Tau-C. Tau-A is negatively correlated with cognitive function in AD patients[217], and caspase-3, the enzyme generating the Tau-C fragment, is activated following TBI[303]. Following a concussion, serum Tau-A concentration is elevated at both 1 hour and 12 hours in hockey players with post-concussion symptoms lasting longer than 10 days[298]. In the same study, it was discovered that serum Tau-C levels are elevated immediately following a concussion when compared to uninjured controls, with no change in concentration up to 144 hours post-injury[298]. Similarly, no change in Tau-A was seen over time. In this study, neither Tau-A nor Tau-C showed an increase in concentration at 6 and 14 days following concussion. Similarly, T-tau also remained stable through these time points, which could explain the stability of both fragments post-concussion in this cohort.

Concussion is heterogeneous in nature; symptoms and severity can vary greatly between individuals[25]. It is possible that the concussions sustained by athletes in this cohort were mild in nature, and, as such, there was limited axonal damage. Symptoms experienced following concussion could have been brought on by ionic flux, decreased cerebral blood flow, and energy imbalance seen following axonal insult[34].

Interestingly, there was no increase in NF-L, T-tau, Tau-A, and Tau-C at 6 and 14 days following concussion in this study. With respect to NF-L and T-tau, these results are in contrast to previous concussion research[54,61]. Possible causes for this include the prospective cohort design of the study (limiting variability between subject samples), a high protease activity in blood coupled with extended time points, and the injuries experienced were mild in nature, limiting axonal disruption. In order for T-tau, NF-L, and tau fragments Tau-A and Tau-C to be validated as true markers of neuronal damage, future studies should involve a prospective cohort design with multiple time points throughout the season so as to truly ascertain the dynamics of each protein following sport-related concussion.

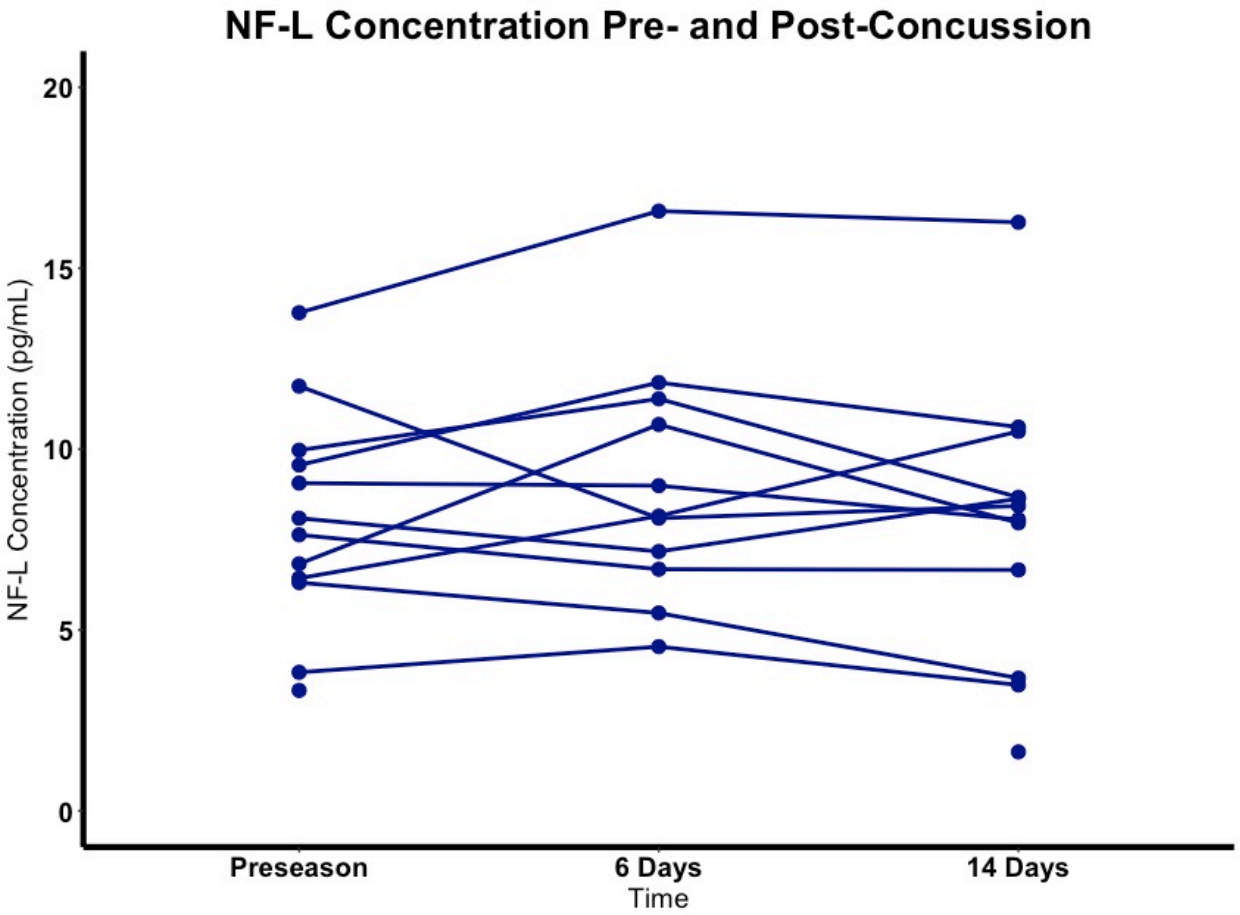

Figure 5.1 – Neurofilament Light Concentration Preseason, 6-days, and 14-days Post-Concussion

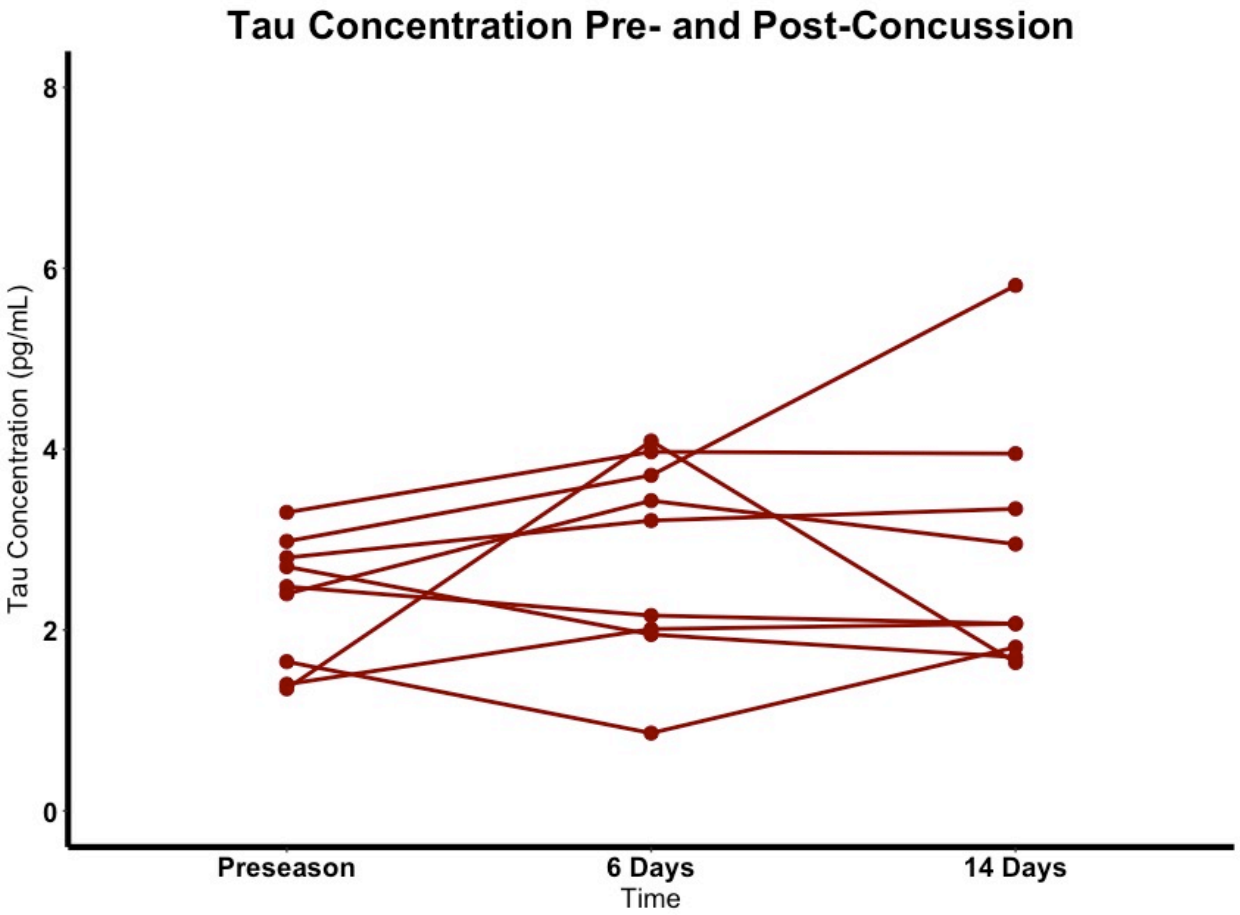

Figure 5.2 – T-Tau Concentration Preseason, 6-days, and 14-days Post-Concussion

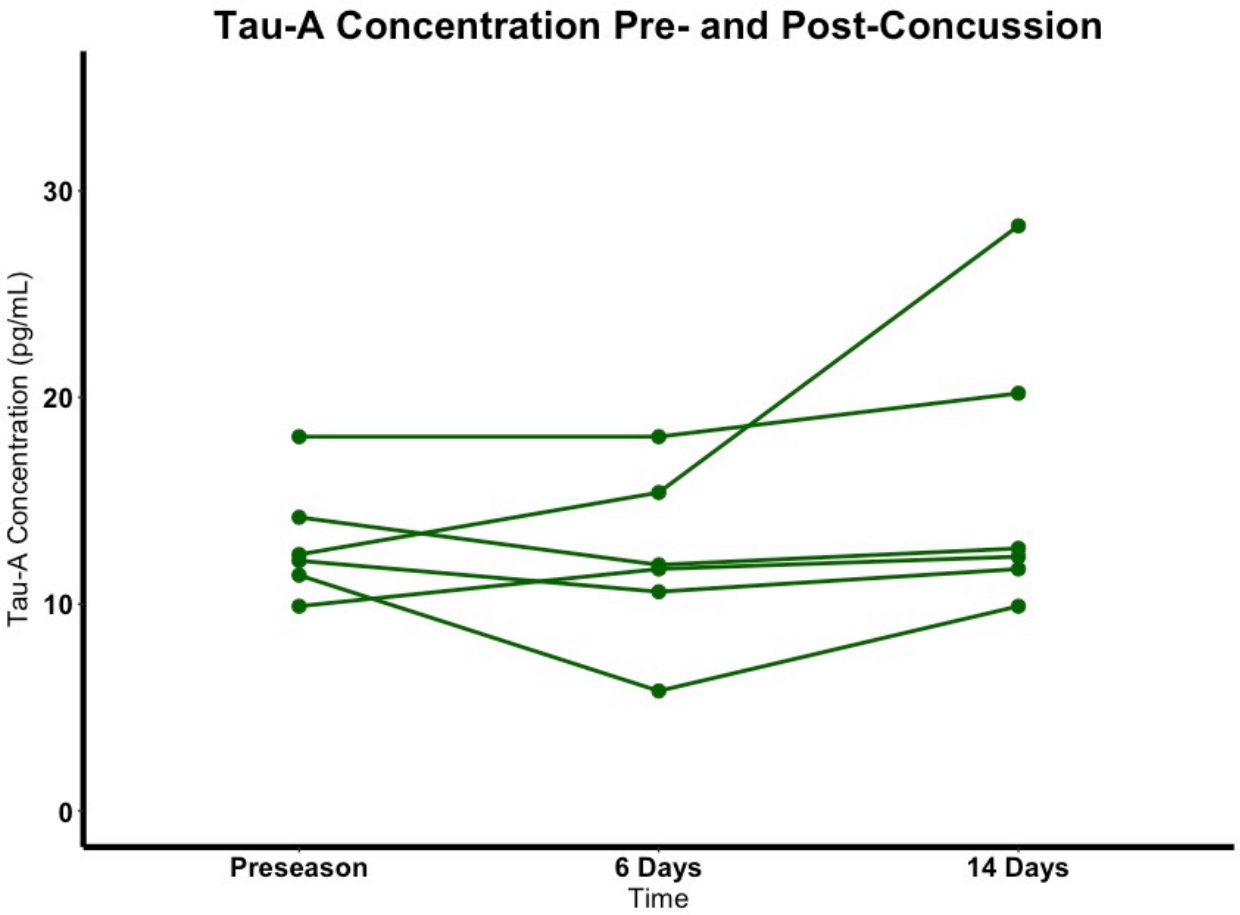

Figure 5.3 – Tau-A Concentration Preseason, 6-days, and 14-days Post-Concussion

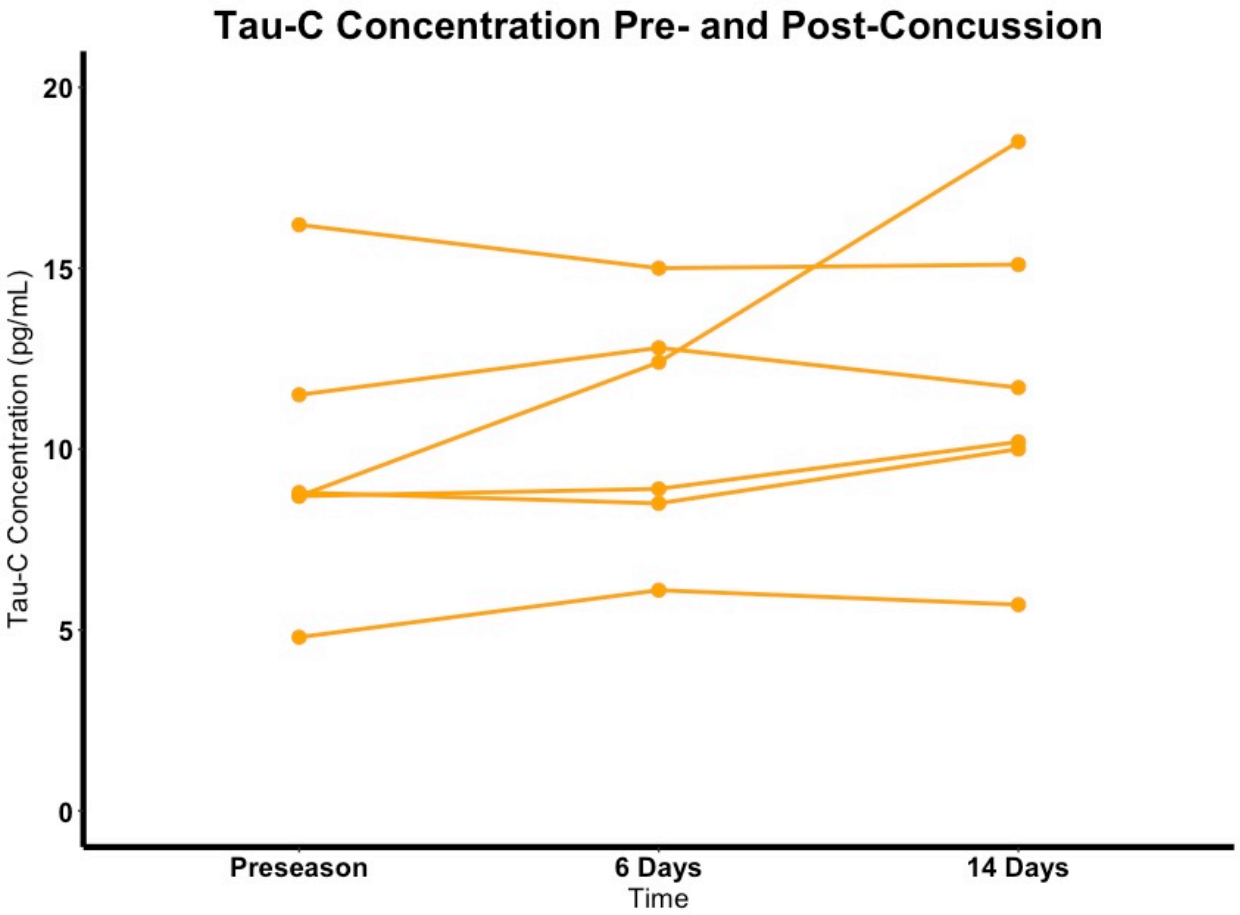

Figure 5.4 – Tau-C Concentration Preseason, 6-days, and 14-days Post-Concussion

Chapter 6: Conclusion

6.1 Summary

The following section outlines the contributions from this dissertation to the volume of research on blood-based biomarkers of sport-related concussion (SRC). Researchers in this field strive to identify the neurochemical and molecular alterations present as a result of this injury. To date, several candidate biomarkers have been proposed and include proteins of both neuronal (e.g. NF-L, tau) and glial (e.g. S100β, GFAP) origin. The vast majority of biomarker research with respect to concussion involves concentration comparisons for these, and other, proteins between concussed and non-concussed control groups. This dissertation builds upon the existing literature through three studies that are among the first to examine biomarker concentration change using a prospective cohort study design. This allowed for the quantification of within-subject biomarker alterations both pre- and post-intervention/injury, providing true insight into the effects of both sub-concussive and concussive impacts on the neurochemical profiles of NF-L, T-tau, Tau-A, and Tau-C.

The key finding from Chapters 3 and 4 is the increase in concentration of serum NF-L following a season of participation in American football (Chapter 3) and an acute bout of soccer heading (Chapter 4). With respect to Chapter 3, the increase in NF-L seen in American football players correlated with several head impact metrics, including CuPLA, CuPRA, and the cumulative number of head impacts sustained during the season. Athletes participating in other contact sports, including rugby, ice hockey, and soccer, experienced no increase in serum NF-L concentrations, nor did cross country runners. Contrastingly, it appears that T-tau, Tau-A, and Tau-C concentrations are unaffected by repetitive sub-concussive impacts, as no changes from

pre- to post-season were observed. No change was seen in cross country runners, along with ice hockey, rugby, and soccer players, indicating not all sports appear to impact the biomarkers examined in this thesis in similar fashions.

The increase in serum NF-L following a bout of soccer heading (Chapter 4) did not occur at 1-hour following the heading intervention; rather, the increase was noted at ~1-month post-heading. This delayed increase in serum NF-L following head trauma extends the findings by Shahim *et al*. (2017)[61], in that they revealed NF-L levels following concussion reached peak values at 144-hours post-injury, which were higher than uninjured controls. Heading in the context of high performance soccer can reach astonishingly high levels. Combining both games and practices, many players self-report experiencing ~1000 headers per year, with some reporting in excess of 5000 headers per year[78]. Expanded over the course of a playing career, this can equal upwards of 10,000 – 50,000 sub-concussive head impacts from heading a soccer ball. With in-game speeds of up to 89 km/h[269], the possibility of long-term deleterious effects should not be ignored. However, the findings from Chapter 4 involve a small sample size, as such further research is required in order to truly determine a mechanistic link between soccer heading and axonal disruption.

Thus, the combined results from Chapters 3 and 4 suggest serum concentration of NF-L is sensitive to the effects of repetitive, sub-concussive impacts, and these effects differ between sports. American football players enrolled in the experiments described in this dissertation experienced higher CuPLA, CuPRA, and a greater number of head impacts than has previously been reported in both ice hockey (179 – 347 hits per season) and rugby (46 – 116 hits per season)[260-262]. Collectively, it appears serum NF-L concentration increases can be quantified with exposure to multiple sub-concussive impacts at small time gaps between each, or over the

course of a season of repetitive sub-concussive impacts with an increase in PLA and PRA magnitudes.

Lastly, none of the biomarkers examined in Chapter 5 of this dissertation (i.e. serum NF-L, Tau-A, and Tau-C; plasma T-tau) changed in concentration when examined at 1- and 2-weeks following a concussion. The 6- and 14-day time points were chosen to extend the temporal profile of T-tau and NF-L following concussion. Secondary axotomy is a process of axonal degeneration that occurs in axons not severed at the time of injury[304]. The half-life of tau has been documented at 0.5 – 12 hours in blood[254,255]; however, unmyelinated axons are more susceptible to secondary axotomy than their myelinated counterparts[305], which is why T-tau, Tau-A, and Tau-C were examined at these time points. Although the results from the study in Chapter 5 suggest no presence of secondary axotomy and Wallerian degeneration in both myelinated and unmyelinated axons following concussion, further research is needed with larger prospective data sets to confirm these findings.

6.2 Strengths and Weaknesses

As with any investigation, there are some inherent strengths and weaknesses associated with its design and implementation. The current thesis is no exception to this construct and the following sub-section addresses these respective areas.

The greatest strength of this study (employing a prospective cohort design) is also a cause of the major weakness to the study (small sample sizes). The data presented in this study was collected over 3 years, and from multiple teams. The athletes and medical staff for each team were relied upon to notify the research team when a concussion occurred. If this happened, travel, work, and school schedules had to be accommodated, along with the coordination of

transport to the university for post-injury testing sessions. Participation in each of the studies included in this dissertation was voluntary, and as such, players were not required to provide post-injury samples even if they had provided a preseason sample. Also, there were concussed individuals who declined to provide follow-up samples for comparison. For baseline testing, although we typically had approximately 70-80% of the players from all teams volunteer, the majority of the concussions occurred in the 20-30% of players who did not participate in baseline testing, as such we were unable to collect prospective data on these individuals.

We currently did not have the necessary equipment (i.e. Tau-A and Tau-C assays and Simoa platform) for analyte quantification on campus. Therefore, we were reliant upon collaborations with other institutions in performing these assessments. However, this also lead to a strength, as the samples run were performed at the same institutions that perform the same assessments for many of the other results published in the field. This is also a strength when comparing the findings to the broader literature as it enables direct comparisons across the currently published studies in the field.

As previously mentioned blood samples were obtained from antecubital venous punctures (refer to Methods: Chapter 2 for details) instead of sampling CSF fluids (that do not require the analytes to cross the BBB). The venous samples were performed to increase subject comfort and participation in the investigation and provide data sets that would be more applicable to game-time assessments.

The time points from the current study were selected based on subject availability (time of day) and follow-up points (1-week and 2-weeks) to extend the current findings in this field[54]. However, it is still unknown what the complete temporal and diurnal profiles are for these analytes and more research is needed to establish these profiles.

Lastly, a concussive injury likely results in multiple biomarkers being affected concurrently. Assessing a panel of biomarkers may provide the necessary sensitivity and specificity for providing clinical diagnosis regarding both when a concussion has occurred and (possibly more importantly) when physiological recovery from this traumatic injury has been reached.

Collectively, after completing the research chapters presented in this thesis and understanding their respective strengths and weaknesses has provided insight into the direction I envision for my future career in the biomarker and concussion fields, which are outlined below.

6.3 Future Directions

Given the half-life of many protein biomarkers, the full spectrum of each has not yet been quantified. Biomarker research following mTBI has only truly taken off in the past 10 years, and as such more work is needed in order to unmask the diagnostic and prognostic capacities of the proteins investigated in this thesis as well as those yet to be identified. Additionally, standardization of procedures is necessary to enable comparisons between research paradigms, as various factors (e.g. sampling time-points following injury) can have profound influences on the concentration of a given protein.

One such factor is the biofluid to be analyzed; as CSF has a much lower protease activity when compared to blood[243]. However as reviewed previously, CSF volume (~150 mL) is a small fraction of the total blood volume (~5000 mL) in an adult, this would obviously limit the number of samples able to be obtained post-injury. Additionally, sampling time points need to be established for each biomarker, that will enable researchers and clinicians to optimize the biofluid of choice with respect to protein half-life. As previously mentioned, the half-life of

proteins can range from minutes (e.g. S100β[122]) to weeks (e.g NF-L[198]), and beyond (e.g. pNF-H is completely resistant to protease activity[196]). Future biomarker literature should clearly state specific post-injury sampling time-points as a guide to future research. This will allow for objective validation of each biomarker as a tool for TBI detection and enable informed clinical decision making processes.

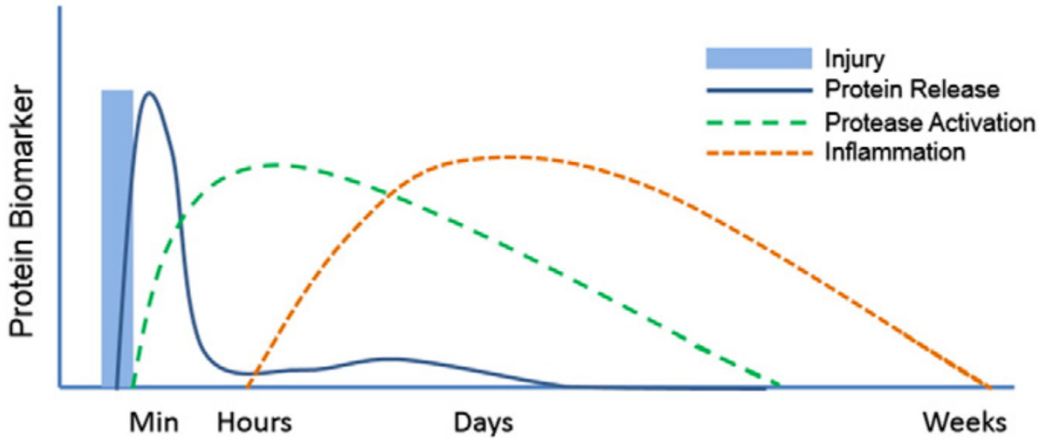

Figure 6.1 – Biomarker Time Course Following Injury[149] (Reprinted with permission)

Due to the high proteolytic activity in blood, when venous or arterial samples are being obtained, each biomarker must be studied for potential influence from breakdown products (BDPs). To date only tau (in the form of Tau-A and Tau-C), GFAP, and spectrin have been examined for BDPs; additional research is required for all other biomarkers in order to truly assess the dynamics of each protein following TBI. Multiple freeze-thaw cycles must also be taken into consideration when selecting a target protein. Stability of amyloid beta (1-42) (Aβ42), a protein found in higher concentration in the CSF of AD patients compared to controls, has been shown to be affected by multiple freeze-thaw cycles[306]. Furthermore, as detection technology becomes more sensitive and the identification of novel biomarkers is evolving, it may be

beneficial to store biofluid samples in multiple, small aliquots, so as to allow future exploratory research via previously collected samples.

There is also a need for specific reference intervals for each individual protein of interest that takes into consideration such elements as age, gender, and pre-injury status (e.g. exercising at the time of injury). As it stands, the research to date has mainly compared injured individuals with uninjured controls, often with wide disparity in age ranges between groups. As the research in this field grows and established reference values are determined for various demographic groups. Without these critical normative comparison values, it is extremely difficult for researchers and clinicians to determine the pathological extent of injury in clinical populations. For example, healthy normal individuals have been shown to have large variances in both their NF-L and GFAP levels. NF-L in those under the age of 30 has been found to have an interquartile range (IQR) of close to 200 ng/L, while GFAP in the same age group has an IQR of approximately 350 ng/L[263]. Because of this, when possible, future research should focus on a within-subjects design with multiple baseline values in order to establish typical ranges (under controlled conditions) for each subject. Furthermore, quantification of multiple baseline values will help give individualized data aimed to minimize the possible effect of diurnal and between-day variations for the biomarker of interest. However, this form of research is not feasible for all investigations due to the extensive volume and time constraints this would place on data collections. If completed, it will provide more robust results that in turn would reflect the biomarker concentration change due to the injury. Speaking to this, it is imperative diurnal variation studies be conducted in a longitudinal fashion across the aging spectrum in order to determine the influence of circadian rhythm on protein concentration.

Over the course of my career, I aim to address these gaps in the literature, help to build the necessary reference intervals for biomarkers showing clinical promise and relevance, and find new and more specific biomarkers or biomarker combinations that will enable clinical diagnoses of both the occurrence of, and recovery from, a SRC.

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Appendix

Appendix A

Sport Concussion Assessment Tool – 3rd Edition

SCAT3™

Sport Concussion Assessment Tool – 3rd Edition

For use by medical professionals only

Name

Date/Time of Injury:
Date of Assessment:

Examiner:

What is the SCAT3?[1]

The SCAT3 is a standardized tool for evaluating injured athletes for concussion and can be used in athletes aged from 13 years and older. It supersedes the original SCAT and the SCAT2 published in 2005 and 2009, respectively[2]. For younger persons, ages 12 and under, please use the Child SCAT3. The SCAT3 is designed for use by medical professionals. If you are not qualified, please use the Sport Concussion Recognition Tool[1]. Preseason baseline testing with the SCAT3 can be helpful for interpreting post-injury test scores.

Specific instructions for use of the SCAT3 are provided on page 3. If you are not familiar with the SCAT3, please read through these instructions carefully. This tool may be freely copied in its current form for distribution to individuals, teams, groups and organizations. Any revision or any reproduction in a digital form requires approval by the Concussion in Sport Group.

NOTE: The diagnosis of a concussion is a clinical judgment, ideally made by a medical professional. The SCAT3 should not be used solely to make, or exclude, the diagnosis of concussion in the absence of clinical judgement. An athlete may have a concussion even if their SCAT3 is "normal".

What is a concussion?

A concussion is a disturbance in brain function caused by a direct or indirect force to the head. It results in a variety of non-specific signs and/or symptoms (some examples listed below) and most often does not involve loss of consciousness. Concussion should be suspected in the presence of **any one or more** of the following:

- Symptoms (e.g., headache), or
- Physical signs (e.g., unsteadiness), or
- Impaired brain function (e.g. confusion) or
- Abnormal behaviour (e.g., change in personality).

SIDELINE ASSESSMENT

Indications for Emergency Management

NOTE: A hit to the head can sometimes be associated with a more serious brain injury. Any of the following warrants consideration of activating emergency procedures and urgent transportation to the nearest hospital:

- Glasgow Coma score less than 15
- Deteriorating mental status
- Potential spinal injury
- Progressive, worsening symptoms or new neurologic signs

Potential signs of concussion?

If any of the following signs are observed after a direct or indirect blow to the head, the athlete should stop participation, be evaluated by a medical professional and **should not be permitted to return to sport the same day** if a concussion is suspected.

Any loss of consciousness?	Y	N
"If so, how long?"		
Balance or motor incoordination (stumbles, slow/laboured movements, etc.)?	Y	N
Disorientation or confusion (inability to respond appropriately to questions)?	Y	N
Loss of memory:	Y	N
"If so, how long?"		
"Before or after the injury?"		
Blank or vacant look:	Y	N
Visible facial injury in combination with any of the above:	Y	N

1 Glasgow coma scale (GCS)

Best eye response (E)	
No eye opening	1
Eye opening in response to pain	2
Eye opening to speech	3
Eyes opening spontaneously	4
Best verbal response (V)	
No verbal response	1
Incomprehensible sounds	2
Inappropriate words	3
Confused	4
Oriented	5
Best motor response (M)	
No motor response	1
Extension to pain	2
Abnormal flexion to pain	3
Flexion/Withdrawal to pain	4
Localizes to pain	5
Obeys commands	6
Glasgow Coma score (E + V + M)	of 15

GCS should be recorded for all athletes in case of subsequent deterioration.

2 Maddocks Score[3]

"I am going to ask you a few questions, please listen carefully and give your best effort."

Modified Maddocks questions (1 point for each correct answer)

What venue are we at today?	0	1
Which half is it now?	0	1
Who scored last in this match?	0	1
What team did you play last week/game?	0	1
Did your team win the last game?	0	1
Maddocks score		of 5

Maddocks score is validated for sideline diagnosis of concussion only and is not used for serial testing.

Notes: Mechanism of Injury ("tell me what happened"?):

Any athlete with a suspected concussion should be REMOVED FROM PLAY, medically assessed, monitored for deterioration (i.e., should not be left alone) and should not drive a motor vehicle until cleared to do so by a medical professional. No athlete diagnosed with concussion should be returned to sports participation on the day of injury.

BACKGROUND

Name: _____ Date: _____

Examiner: _____

Sport/team/school: _____ Date/time of injury: _____

Age: _____ Gender: ☐ M ☐ F

Years of education completed: _____

Dominant hand: ☐ right ☐ left ☐ neither

How many concussions do you think you have had in the past? _____

When was the most recent concussion? _____

How long was your recovery from the most recent concussion? _____

Have you ever been hospitalized or had medical imaging done for a head injury? ☐ Y ☐ N

Have you ever been diagnosed with headaches or migraines? ☐ Y ☐ N

Do you have a learning disability, dyslexia, ADD/ADHD? ☐ Y ☐ N

Have you ever been diagnosed with depression, anxiety or other psychiatric disorder? ☐ Y ☐ N

Has anyone in your family ever been diagnosed with any of these problems? ☐ Y ☐ N

Are you on any medications? If yes, please list: ☐ Y ☐ N

_____

SCAT3 to be done in resting state. Best done 10 or more minutes post excercise.

SYMPTOM EVALUATION

3 How do you feel?

"You should score yourself on the following symptoms, based on how you feel now".

	none	mild		moderate		severe	
Headache	0	1	2	3	4	5	6
"Pressure in head"	0	1	2	3	4	5	6
Neck Pain	0	1	2	3	4	5	6
Nausea or vomiting	0	1	2	3	4	5	6
Dizziness	0	1	2	3	4	5	6
Blurred vision	0	1	2	3	4	5	6
Balance problems	0	1	2	3	4	5	6
Sensitivity to light	0	1	2	3	4	5	6
Sensitivity to noise	0	1	2	3	4	5	6
Feeling slowed down	0	1	2	3	4	5	6
Feeling like "in a fog"	0	1	2	3	4	5	6
"Don't feel right"	0	1	2	3	4	5	6
Difficulty concentrating	0	1	2	3	4	5	6
Difficulty remembering	0	1	2	3	4	5	6
Fatigue or low energy	0	1	2	3	4	5	6
Confusion	0	1	2	3	4	5	6
Drowsiness	0	1	2	3	4	5	6
Trouble falling asleep	0	1	2	3	4	5	6
More emotional	0	1	2	3	4	5	6
Irritability	0	1	2	3	4	5	6
Sadness	0	1	2	3	4	5	6
Nervous or Anxious	0	1	2	3	4	5	6

Total number of symptoms (Maximum possible 22) _____

Symptom severity score (Maximum possible 132) _____

Do the symptoms get worse with physical activity? ☐ Y ☐ N

Do the symptoms get worse with mental activity? ☐ Y ☐ N

☐ self rated ☐ self rated and dinician monitored

☐ clinician interview ☐ self rated with parent input

Overall rating: If you know the athlete well prior to the injury, how different is the athlete acting compared to his/her usual self?

Please circle one response:

no different	very different	unsure	N/A

Scoring on the SCAT3 should not be used as a stand-alone method to diagnose concussion, measure recovery or make decisions about an athlete's readiness to return to competition after concussion. Since signs and symptoms may evolve over time, it is important to consider repeat evaluation in the acute assessment of concussion.

COGNITIVE & PHYSICAL EVALUATION

4 Cognitive assessment

Standardized Assessment of Concussion (SAC)[4]

Orientation (1 point for each correct answer)

What month is it?	0	1
What is the date today?	0	1
What is the day of the week?	0	1
What year is it?	0	1
What time is it right now? (within 1 hour)	0	1
Orientation score		of 5

Immediate memory

List	Trial 1		Trial 2		Trial 3		Alternative word list		
elbow	0	1	0	1	0	1	candle	baby	finger
apple	0	1	0	1	0	1	paper	monkey	penny
carpet	0	1	0	1	0	1	sugar	perfume	blanket
saddle	0	1	0	1	0	1	sandwich	sunset	lemon
bubble	0	1	0	1	0	1	wagon	iron	insect
Total									

Immediate memory score total of 15

Concentration: Digits Backward

List	Trial 1		Alternative digit list		
4-9-3	0	1	6-2-9	5-2-6	4-1-5
3-8-1-4	0	1	3-2-7-9	1-7-9-5	4-9-6-8
6-2-9-7-1	0	1	1-5-2-8-6	3-8-5-2-7	6-1-8-4-3
7-1-8-4-6-2	0	1	5-3-9-1-4-8	8-3-1-9-6-4	7-2-4-8-5-6
Total of 4					

Concentration: Month in Reverse Order (1 pt. for entire sequence correct)

Dec-Nov-Oct-Sept-Aug-Jul-Jun-May-Apr-Mar-Feb-Jan	0	1
Concentration score		of 5

5 Neck Examination:

Range of motion Tenderness Upper and lower limb sensation & strength

Findings: _____

6 Balance examination

Do one or both of the following tests.

Footwear (shoes, barefoot, braces, tape, etc.) _____

Modified Balance Error Scoring System (BESS) testing[5]

Which foot was tested (i.e. which is the **non-dominant** foot) ☐ Left ☐ Right

Testing surface (hard floor, field, etc.) _____

Condition

Double leg stance:	Errors
Single leg stance (non-dominant foot):	Errors
Tandem stance (non-dominant foot at back):	Errors

And / Or

Tandem gait[6,7]

Time (best of 4 trials): _____ seconds

7 Coordination examination

Upper limb coordination

Which arm was tested: ☐ Left ☐ Right

Coordination score of 1

8 SAC Delayed Recall[4]

Delayed recall score of 5

INSTRUCTIONS

Words in *Italics* throughout the SCAT3 are the instructions given to the athlete by the tester.

Symptom Scale

"You should score yourself on the following symptoms, based on how you feel now".

To be completed by the athlete. In situations where the symptom scale is being completed after exercise, it should still be done in a resting state, at least 10 minutes post exercise.

For total number of symptoms, maximum possible is 22.

For Symptom severity score, add all scores in table, maximum possible is 22 x 6 = 132.

SAC[4]

Immediate Memory

"I am going to test your memory. I will read you a list of words and when I am done, repeat back as many words as you can remember, in any order."

Trials 2 & 3:

"I am going to repeat the same list again. Repeat back as many words as you can remember in any order, even if you said the word before."

Complete all 3 trials regardless of score on trial 1 & 2. Read the words at a rate of one per second. **Score 1 pt. for each correct response.** Total score equals sum across all 3 trials. Do not inform the athlete that delayed recall will be tested.

Concentration

Digits backward

"I am going to read you a string of numbers and when I am done, you repeat them back to me backwards, in reverse order of how I read them to you. For example, if I say 7-1-9, you would say 9-1-7."

If correct, go to next string length. If incorrect, read trial 2. **One point possible for each string length.** Stop after incorrect on both trials. The digits should be read at the rate of one per second.

Months in reverse order

"Now tell me the months of the year in reverse order. Start with the last month and go backward. So you'll say December, November … Go ahead"

1 pt. for entire sequence correct

Delayed Recall

The delayed recall should be performed after completion of the Balance and Coordination Examination.

"Do you remember that list of words I read a few times earlier? Tell me as many words from the list as you can remember in any order."

Score 1 pt. for each correct response

Balance Examination

Modified Balance Error Scoring System (BESS) testing[5]

This balance testing is based on a modified version of the Balance Error Scoring System (BESS)[5]. A stopwatch or watch with a second hand is required for this testing.

"I am now going to test your balance. Please take your shoes off, roll up your pant legs above ankle (if applicable), and remove any ankle taping (if applicable). This test will consist of three twenty second tests with different stances."

(a) Double leg stance:

"The first stance is standing with your feet together with your hands on your hips and with your eyes closed. You should try to maintain stability in that position for 20 seconds. I will be counting the number of times you move out of this position. I will start timing when you are set and have closed your eyes."

(b) Single leg stance:

"If you were to kick a ball, which foot would you use? [This will be the dominant foot] Now stand on your non-dominant foot. The dominant leg should be held in approximately 30 degrees of hip flexion and 45 degrees of knee flexion. Again, you should try to maintain stability for 20 seconds with your hands on your hips and your eyes closed. I will be counting the number of times you move out of this position. If you stumble out of this position, open your eyes and return to the start position and continue balancing. I will start timing when you are set and have closed your eyes."

(c) Tandem stance:

"Now stand heel-to-toe with your non-dominant foot in back. Your weight should be evenly distributed across both feet. Again, you should try to maintain stability for 20 seconds with your hands on your hips and your eyes closed. I will be counting the number of times you move out of this position. If you stumble out of this position, open your eyes and return to the start position and continue balancing. I will start timing when you are set and have closed your eyes."

Balance testing – types of errors

1. Hands lifted off iliac crest
2. Opening eyes
3. Step, stumble, or fall
4. Moving hip into > 30 degrees abduction
5. Lifting forefoot or heel
6. Remaining out of test position > 5 sec

Each of the 20-second trials is scored by counting the errors, or deviations from the proper stance, accumulated by the athlete. The examiner will begin counting errors only after the individual has assumed the proper start position. **The modified BESS is calculated by adding one error point for each error during the three 20-second tests. The maximum total number of errors for any single condition is 10.** If a athlete commits multiple errors simultaneously, only one error is recorded but the athlete should quickly return to the testing position, and counting should resume once subject is set. Subjects that are unable to maintain the testing procedure for a minimum of **five seconds** at the start are assigned the highest possible score, ten, for that testing condition.

OPTION: For further assessment, the same 3 stances can be performed on a surface of medium density foam (e.g., approximately 50 cm x 40 cm x 6 cm).

Tandem Gait[4,7]

Participants are instructed to stand with their feet together behind a starting line (the test is best done with footwear removed). Then, they walk in a forward direction as quickly and as accurately as possible along a 38mm wide (sports tape), 3 meter line with an alternate foot heel-to-toe gait ensuring that they approximate their heel and toe on each step. Once they cross the end of the 3m line, they turn 180 degrees and return to the starting point using the same gait. A total of 4 trials are done and the best time is retained. Athletes should complete the test in 14 seconds. Athletes fail the test if they step off the line, have a separation between their heel and toe, or if they touch or grab the examiner or an object. In this case, the time is not recorded and the trial repeated, if appropriate.

Coordination Examination

Upper limb coordination

Finger-to-nose (FTN) task:

"I am going to test your coordination now. Please sit comfortably on the chair with your eyes open and your arm (either right or left) outstretched (shoulder flexed to 90 degrees and elbow and fingers extended), pointing in front of you. When I give a start signal, I would like you to perform five successive finger to nose repetitions using your index finger to touch the tip of the nose, and then return to the starting position, as quickly and as accurately as possible."

Scoring: 5 correct repetitions in < 4 seconds = 1

Note for testers: Athletes fail the test if they do not touch their nose, do not fully extend their elbow or do not perform five repetitions. **Failure should be scored as 0.**

References & Footnotes

1. This tool has been developed by a group of international experts at the 4th International Consensus meeting on Concussion in Sport held in Zurich, Switzerland in November 2012. The full details of the conference outcomes and the authors of the tool are published in The BJSM Injury Prevention and Health Protection, 2013, Volume 47, Issue 5. The outcome paper will also be simultaneously co-published in other leading biomedical journals with the copyright held by the Concussion in Sport Group, to allow unrestricted distribution, providing no alterations are made.

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3. Maddocks, DL; Dicker, GD; Saling, MM. The assessment of orientation following concussion in athletes. Clinical Journal of Sport Medicine. 1995; 5(1): 32–3.

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6. Schneiders, A.G., Sullivan, S.J., Gray, A., Hammond-Tooke, G. & McCrory, P. Normative values for 16-37 year old subjects for three clinical measures of motor performance used in the assessment of sports concussions. Journal of Science and Medicine in Sport. 2010; 13(2): 196–201.

7. Schneiders, A.G., Sullivan, S.J., Kvarnstrom. J.K., Olsson, M., Yden. T. & Marshall, S.W. The effect of footwear and sports-surface on dynamic neurological screening in sport-related concussion. Journal of Science and Medicine in Sport. 2010; 13(4): 382–386

ATHLETE INFORMATION

Any athlete suspected of having a concussion should be removed from play, and then seek medical evaluation.

Signs to watch for

Problems could arise over the first 24–48 hours. The athlete should not be left alone and must go to a hospital at once if they:

- Have a headache that gets worse
- Are very drowsy or can't be awakened
- Can't recognize people or places
- Have repeated vomiting
- Behave unusually or seem confused; are very irritable
- Have seizures (arms and legs jerk uncontrollably)
- Have weak or numb arms or legs
- Are unsteady on their feet; have slurred speech

Remember, it is better to be safe.
Consult your doctor after a suspected concussion.

Return to play

Athletes should not be returned to play the same day of injury.
When returning athletes to play, they should be **medically cleared and then follow a stepwise supervised program,** with stages of progression.

For example:

Rehabilitation stage	Functional exercise at each stage of rehabilitation	Objective of each stage
No activity	Physical and cognitive rest	Recovery
Light aerobic exercise	Walking, swimming or stationary cycling keeping intensity, 70% maximum predicted heart rate. No resistance training	Increase heart rate
Sport-specific exercise	Skating drills in ice hockey, running drills in soccer. No head impact activities	Add movement
Non-contact training drills	Progression to more complex training drills, eg passing drills in football and ice hockey. May start progressive resistance training	Exercise, coordination, and cognitive load
Full contact practice	Following medical clearance participate in normal training activities	Restore confidence and assess functional skills by coaching staff
Return to play	Normal game play	

There should be at least 24 hours (or longer) for each stage and if symptoms recur the athlete should rest until they resolve once again and then resume the program at the previous asymptomatic stage. Resistance training should only be added in the later stages.

If the athlete is symptomatic for more than 10 days, then consultation by a medical practitioner who is expert in the management of concussion, is recommended.

Medical clearance should be given before return to play.

Scoring Summary:

Test Domain	Score		
	Date:	Date:	Date:
Number of Symptoms of 22			
Symptom Severity Score of 132			
Orientation of 5			
Immediate Memory of 15			
Concentration of 5			
Delayed Recall of 5			
SAC Total			
BESS (total errors)			
Tandem Gait (seconds)			
Coordination of 1			

Notes:

CONCUSSION INJURY ADVICE

(To be given to the **person monitoring** the concussed athlete)

This patient has received an injury to the head. A careful medical examination has been carried out and no sign of any serious complications has been found. Recovery time is variable across individuals and the patient will need monitoring for a further period by a responsible adult. Your treating physician will provide guidance as to this timeframe.

If you notice any change in behaviour, vomiting, dizziness, worsening headache, double vision or excessive drowsiness, please contact your doctor or the nearest hospital emergency department immediately.

Other important points:

- Rest (physically and mentally), including training or playing sports until symptoms resolve and you are medically cleared
- No alcohol
- No prescription or non-prescription drugs without medical supervision. Specifically:
  - No sleeping tablets
  - Do not use aspirin, anti-inflammatory medication or sedating pain killers
- Do not drive until medically cleared
- Do not train or play sport until medically cleared

Clinic phone number

Patient's name _____

Date/time of injury _____

Date/time of medical review _____

Treating physician _____

Contact details or stamp