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Investigating in vivo Brain Metabolite Levels in Concussed Female Athletes and a Murine Model of Repetitive Closed Head Injury

Amy L. Schranz
The University of Western Ontario

Supervisor
Bartha, Robert
Robarts Research Institute

Graduate Program in Medical Biophysics
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Abstract

After a concussion there is a complex cascade of events, termed the neurometabolic cascade, that includes changes in ion flux, neurotransmission, and cellular energetics. How this pathophysiological process translates into cognitive deficits remains poorly understood. Magnetic resonance spectroscopy (MRS) provides a non-invasive technique that allows for the quantification of brain metabolites that are involved in these processes, including glutamate and glutamine, which are involved in neurotransmission. Moreover, female athletes are underrepresented in studies on concussion, limiting our knowledge and understanding of sex differences. The overall goal of this thesis was to examine metabolite changes using MRS in female athletes before and after concussion, with the added goal of quantifying glutamate and glutamine separately. The second objective was to replicate metabolite changes in an animal model of concussion, to position future studies to probe the reasons for these changes, and to explore whether these changes represent potential therapeutic targets.

MRS was acquired from the prefrontal WM of female athletes (contact and non-contact sports) and sedentary women at 3T to explore metabolite differences between groups and changes after concussion. In addition, an animal model of repeated closed head impacts was studied at 9.4T, in an effort to replicate the findings observed in humans.

In the contact athlete cohort, reduced glutamine and glutamine/total creatine (Gln/Cr) were found following concussion, and after a season of play in non-concussed athletes. In the non-contact athlete cohort, metabolite levels did not change over the course of a season, and they did not differ from age matched sedentary women, except for a small difference in *myo*-inositol. Most interestingly, glutamine levels were significantly elevated in contact athletes compared to sedentary and non-contact groups, suggesting that sub-concussive impacts may have a long-term effect on brain metabolite levels. Furthermore, the large difference in glutamine levels between contact and non-contact athletes has implications in study design in regards to control groups versus test-retest paradigm.

In the final study, we used a murine model (C57BL6) of repeated closed head injury to investigate metabolite level changes post-injury. Elevated Gln/Cr was observed 10-weeks post-injury, suggesting that the model may be appropriate to study sub-concussive injury.

Together, these studies suggest that there exists a cumulative effect on the brain from sub-concussive impacts in contact sports, that manifests as elevated glutamine levels. Moreover, concussion in the same cohort of athletes results in reduced glutamine levels. Further work aimed at replicating these findings in animal models will be crucial to understanding the effects of cumulative impacts and concussion.

Lay Summary

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MRS was acquired from the prefrontal white matter of female athletes (contact and non-contact sports) and sedentary women to explore metabolite differences between groups and changes after concussion. In the non-contact athlete cohort, metabolite levels did not change over the course of a season, and they did not differ from age matched sedentary women, except for a small difference in *myo*-inositol. Most interestingly, glutamine levels were significantly *elevated* in these contact athletes compared to sedentary and non-contact groups, suggesting that sub-concussive impacts may have a long-term effect on brain metabolite levels. Moreover, *reduced* glutamine levels were found following concussion in the contact cohort.

In the final study, we used a mouse model of repeated closed head injury to investigate metabolite level changes post-injury. Elevated Glutamine/Creatine was observed 10-weeks post-injury, suggesting that the model may be appropriate to study sub-concussive injury.

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Keywords

Mild traumatic brain injury, magnetic resonance imaging, magnetic resonance spectroscopy, concussion, sub-concussion, metabolites, glutamine

Abbreviations

AD	Axial diffusivity
ADP	Adenosine diphosphate
AFP	Adiabatic full passage
ASL	Arterial spin labeling
ATP	Adenosine Triphosphate
BBB	Blood brain barrier
^{13}C	Carbon-13
Ca^{2+}	Calcium
CBF	Cerebral blood flow
CHESS	Chemical shift selective
CHI	Closed head impact
Cho	Total choline
Cl^-	Chlorine
Cr	Total creatine
CRLB	Cramer-Rao Lower Bound
CV	Coefficient of variation
DAI	Diffuse axonal injury
DAMPs	Damage-associated molecular patterns

DTI	Diffusion tensor imaging
ECC	Eddy current correction
FA	Fractional anisotropy
FDG	Fluorodeoxyglucose
fMRI	Functional magnetic resonance imaging
FOV	Field of view
GCS	Glasgow Coma Scale
GABA	γ-aminobutyric acid
Gln	Glutamine
Glu	Glutamate
GLUT	Glucose Transporter
Glx	Glutamate + Glutamine
GM	Grey matter
GS	Glutamine synthetase
GSH	Glutathione
^{1}H	Proton
ImPACT	Immediate Post-Concussion Assessment and Cognitive Testing
K^{+}	Potassium
Lac	Lactate
LASER	Localization by Adiabatic Selective Refocusing
MD	Mean diffusivity
MET	Metabolic equivalent
MRS	Magnetic resonance spectroscopy
Myo	*Myo*-inositol
Na^{+}	Sodium

NAA	*N*-acetyl aspartate
NOS	Reactive nitrogen species
^{31}P	Phosphorus-31
PCr	Phosphocreatine
PET	Positron emission tomography
PRESS	Point Resolved Spectroscopy Sequence
QUALITY	Quantification improvement by converting lineshapes to the lorentzian type
RD	Radial diffusivity
RF	Radio frequency
ROS	Reactive oxygen species
SCAT	Sport Concussion Assessment Tool
SNR	Signal to noise ratio
SPECT	Single photon emission computed tomography
T_1	Longitudinal relaxation
T_2	Transverse relaxation
Tau	Taurine
TBI	Traumatic Brain Injury
TCA	Tricarboxylic acid
TE	Echo time
TR	Repetition time
VAPOR	Variable pulse powers and optimized relaxation delays
WM	White matter
X/Cr	Metabolite ratio relative to creatine, X= Metabolite

Co-Authorship Statement

The following thesis contains three manuscripts that are in preparation or have been published in scientific journals. Amy Schranz contributed significantly to this work, including aspects of study design, participant recruitment, animal handling, data acquisition, analysis and interpretations, drafting, revising and approval of the manuscripts. The content of this thesis reflects the efforts of the entire concussion team at Western University and work with other students. In particular:

Dr. Robert Bartha: Overall project and study design, MR spectroscopy acquisition design, interpretation of data, provided feedback on all manuscripts, supervision and guidance, and is responsible for published work as senior author on all publications. Patrick McCunn was a peer PhD student responsible for the analysis and interpretation of the DTI data and provided feedback on Chapter 4.

Dr. Ravi S. Menon: Overall project and study design, MR image acquisition design, provided feedback on Chapters 2. Dr. Kathryn Y. Manning: peer PhD student responsible for the analysis of the DTI data and provided feedback on Chapter 2.

Dr. Gregory A. Dekaban: Overall project design, and provided feedback on Chapters 2 and 3. Christy Barreira was the study coordinator and provided feedback on Chapter 2, and Kevin Blackney was a peer MSc student responsible for the analysis of hematology data (reported elsewhere) and provided feedback on Chapter 2.

Dr. Arthur Brown: Overall project design, and provided feedback on all Chapters. Kathy Xu: Research associate in the Dr. Brown lab responsible for animal handling, inducing the injury paradigm, and sacrificing the animals, and provided feedback on Chapter 4.

Dr. Lisa Fischer: Overall project design, patient evaluation, and provided feedback on Chapters 2 and 3. Tatiana Jevremovic was part of the clinical team and designated a co-author in Chapter 2. Dr. Timothy Doherty, Dr. Douglas Fraser and Dr. Jeff Holmes were all part of the concussion team at Western University and involved in aspects of study design, data acquisition (Dr. Jeff Holmes, balance data, reported elsewhere), interpretation, and provided feedback on Chapters 2 and 3.

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Chapter 1

1 Introduction

1.1 Prevalence and Clinical Definition

Traumatic brain injury (TBI) imposes a healthcare burden of $60 billion annually in the United States alone[1] and an estimated 57 million people worldwide have been hospitalized with one or more TBIs[2]. The severity of TBIs is typically classified as mild, moderate, to severe, with "concussion" and "mild TBI" often being used interchangeably[3]. Furthermore, the National Collegiate Athletic Association Injury Surveillance Program has reported the concussion rate among student-athletes in 25 different sports to be 4.47 per 10,000 Athlete-Exposures (defined as one athlete participating in one practice or competition) overall, with some sports (e.g. hockey, football, wrestling) as high as 20 concussions per 10,000 Athlete Exposures[4]. Additionally, Hirschhorn et al. (2018)[5] found that the most commonly injured body part among emergency transport incidents (ETIs) in both collegiate and high school players to be the head and face, followed by the neck, with concussion as a frequent diagnosis at both levels. Although concussions can occur in many situations, including motor vehicle accidents, domestic violence, and slips and falls, athletes participating in contact sports have a high risk of sustaining a concussion due to the nature of the activity[1].

Concussion can be defined as a complex pathophysiological process affecting the brain, induced by traumatic biomechanical forces that may be caused by a direct blow to the head, face, neck, or elsewhere on the body with an impulsive force transmitted to the head[6,7]. Traditionally, concussion is considered a subset of traumatic brain injury (TBI) and is less severe than impacts that cause cranial fractures and intracranial hemorrhage. Clinical symptoms can include physical, cognitive, emotional, and sleep disturbances. Onset of symptoms is typically rapid, short-lived, and the mechanism of impairment is usually a functional disturbance rather than structural injury. Additionally, brain loading patterns of stress and strain vary throughout the brain depending on factors including external forces, geometry, tissue properties and bony architecture of the skull, such that clinical symptoms may vary from person to person[6].

Although an athletic trainer is often the first health care provider on the scene to provide initial care[5], diagnosis of concussion is made through clinical assessment by a sport medicine physician based on mechanism of injury and symptomatology[8]. In sports, this diagnosis is often made with the aid of clinical tests such as the Sport Concussion Assessment Tool (SCAT) or Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT). The SCAT is a standardized tool that integrates the Glasgow Coma Scale (GCS) and Maddocks score[9]. The GCS assesses visual, verbal and motor response on a 15-point scale, with concussed athlete's scoring 14-15, and more severe TBI's scoring lower, while the Maddocks score consists of simple questions such as "Who scored last in this match" to assess short-term memory. Furthermore, the SCAT requires the athlete to rank 22 different symptoms (including headache, neck pain, nausea, dizziness, blurred vision, etc.) on a scale of 0-6, followed by questions and tasks that specifically assess orientation, immediate memory, concentration and balance. The ImPACT is a computerized test that also assesses 22 symptoms (on a 7-point scale) and employs neurocognitive tests that evaluate verbal and visual memory, processing speed, reaction time and impulse control[10]. However, these assessments are limited since they are subjective, and rely on athletes to voluntarily report symptoms that are often delayed. Additionally, athletes with musculoskeletal injuries have been found to have impaired neurocognitive test results similar to those of concussed athletes, demonstrating the lack of specificity to concussion[11]. Furthermore, the proper testing environment needed for these tests in addition to the training required to properly review the results (e.g. ideally by a neuropsychologist), are impractical for schools and organizations[12]. Therefore, an objective biomarker that could be diagnostic and/or prognostic is greatly needed for concussion, especially in sports where the decision to remove the athlete and return to play is time sensitive.

1.2 Concussion and Clinical Practice

1.2.1 Motivation and Overview

After a concussion there is a complex cascade of events, termed the neurometabolic cascade, that includes changes in ion flux, neurotransmission, and cellular energetics[13]. Although magnetic resonance spectroscopy provides a technique that is able to measure

several different metabolites that may be associated with this process, glutamate and glutamine are hardly ever reported separately, because their signals overlap and are confounded by macromolecules, reducing quantification precision and reliability[14]. However, it is important to quantify these metabolites separately due to their involvement in neurotransmission, and several metabolic pathways is the central theme throughout this thesis.

In order to appreciate the complex pathophysiological process of concussion, this section begins by introducing the basics of the central nervous system, including neurotransmission, the metabolic demands of the brain, and traumatic brain injury, before describing the biochemical changes associated with the neurometabolic cascade. Then an overview of medical imaging in concussion follows, before describing the basics of magnetic resonance and the metabolites that are measurable by magnetic resonance spectroscopy, and their relevance to concussion.

This thesis then uses magnetic resonance spectroscopy to explore metabolite changes in the prefrontal white matter of female contact athletes before and after concussion, with the added goal of quantifying glutamate and glutamine separately. With the findings from these female human studies, an animal model of repeated closed head impacts is developed to replicate these findings, to position future studies to probe the reasons for these changes, and how to use these changes as potential therapeutic targets.

1.2.2 Concussion Management and Recovery

Every concussion is unique and it is therefore necessary to have an individualized approach to concussion management and return to play as well as return to learn. First and foremost, it is important that the athlete is removed from play and not allowed to return to play the same day. Studies have shown that athletes that return to play the same day are at greater risk of having a recovery period longer than 21 days[15]. While it is recommended that athletes reduce their physical and cognitive activity acutely after a concussion, studies have shown that some physical activity is beneficial, so long as it does not exacerbate symptoms[12]. Alternatively, it has been suggested that subthreshold aerobic exercise (progressive exercise to the point of symptom exacerbation) may improve patient outcomes

after concussion and post-concussion syndrome, by aiding in the restoration of autonomic function[16].

Return to play is usually accomplished by following the graduated stepwise program updated by the Berlin Concussion in Sport Group[6], which starts with (1) symptom-limited activity, then moves the athlete into (2) light aerobic exercise, (3) sport-specific exercise, (4) non-contact training drills, (5) full-contact practice, and last (6) return to sport. If at any of these stages symptoms are triggered, the athlete falls back to the previous step. Ideally, the progression through these stages is monitored by a health care provider or athletic trainer[12]. Depending on the nature of the injury, athletes may have also experienced cervical strain, vestibular injuries or oculomotor dysfunction (resulting in prolonged dizziness or balance deficits), which would likely require physical therapy and rehabilitation during the recovery stage as well.

There are no current medications that treat concussion specifically, however, the recommendation of medications is common among primary care, emergency department, and sports medicine physicians[12] to treat sleep disturbances and somatic, emotional, or cognitive symptoms[17]. Acetaminophen and nonsteroidal anti-inflammatory medications are the most commonly used[12]. Melatonin, an endogenous hormone produced by the pineal gland from serotonin, can be prescribed for sleep disturbances, as well as Trazodone, a serotonin reuptake inhibitor. Antidepressants such as amitriptyline are commonly used to treat posttraumatic headaches as well as more severe emotional disturbances[17].

1.2.3 Female Concussion

Estrogens and androgens can influence neuronal and glial structure and function in the brain, due to the distribution of receptors throughout the cerebral cortex and other brain regions[18], providing a neurobiological foundation for potential sex differences in brain injury. How these neurobiological differences influence brain injury following concussion, however, is not well understood. Females tend to report a greater number of symptoms, present with a different collection of symptoms, and take longer to recover[19]. It has been proposed that these disparities could be due to the neck musculature and head/neck stability, making females more susceptible to injury[20]. It has also been suggested that

changes in hormonal levels in females, due to the menstrual cycle, can impact recovery outcomes[20]. For example, the reduction of progesterone after concussion may impact recovery outcomes, with a greater reduction reflecting worse outcome[20].

Even though sex may be a risk factor for concussion or influence injury severity, there exists a disparity between male and female studies in the concussion and TBI literature. Males have dominated the majority of TBI cases, with greater hospitalization rates that were attributed to differences in societal roles and risk-taking behavior[20]. Furthermore, the majority of studies on concussion have been male dominated due to the high rates of concussions in American football[19]. However, females actually suffer more concussions in sports where males and females compete equally, such as soccer or basketball (9.5% versus 6.4%)[19]. Therefore, it is clear that more studies focused on female concussion are greatly needed, to better understand the clinical significance of sex differences in concussion.

1.3 The Human Brain

1.3.1 Basics of the Central Nervous System: Key Components

Understanding the neurobiological effects of concussion requires a brief introduction to the central nervous system. The nervous system can be divided into two broad cell types: nerve cells, or neurons, and glial cells[18]. Neurons and glia contain the same organelles found in all cells, however the localization of organelles and general morphology of these cell types differ. Generally, neurons are composed of a cell body, axons, and dendrites. Dendrites, which can be highly branched, are the primary location for synaptic input from the axon terminals of other neurons[18], while an axon is an extension of the cell body that carries electrical signals known as action potentials[18].

Neurons are specialized cells for electrical signaling over long distances[18]. These signals transition from one neuron to the next through synapses, or synaptic transmission. A synapse consists of a presynaptic and postsynaptic neuron, and usually there is no physical continuity between these two components, but rather an elegant transmission of molecules from the presynaptic neuron to receptors on the postsynaptic cell through

extracellular space known as the synaptic cleft. The number of synaptic inputs to a given neuron vary throughout the nervous system from 1 to 100,000[18].

Glia, like neurons, have complex processes extending from their cell bodies, but do not serve the same purposes as axons and dendrites. They do not participate directly in synaptic transmission or electrical signalling but provide supportive functions that are imperative to maintaining the neurons signalling abilities, in addition to providing a scaffold during neurodevelopment and aiding neurons after injury[18]. In the mature brain, glia can be further subdivided into astrocytes, oligodendrocytes, and microglia. Astrocytes help maintain the chemical environment for neuronal signalling, oligodendrocytes produce and maintain the lipid-rich myelin around axons (Schwann cells in the periphery), and microglia are immune cells involved in regulating neuronal development, innate immune response and wound healing[21].

Together, an ensemble of neurons (and surrounding glia) are organized into neural circuits, which process specific information, setting the foundation for sensation, perception, movement, and behavior[18]. The basic constituents of a neural circuit are afferent neurons, efferent neurons, and interneurons. Afferent neurons carry information from the periphery to the central nervous system while efferent neurons carry information away, and interneurons act locally within the circuit, evident by their short axons[18]. Neural circuits that process similar types of information make up a neural system. Neural systems can be broadly divided into sensory systems (visual, auditory), motor systems, and associational systems[18]. Nerves cells are further organized in the brain into regions rich in neuronal cell bodies called gray matter and regions rich in axons called white matter. The segregation of the brain into gray and white matter is a ubiquitous feature of the vertebrate anatomy[22]. In the human brain, there are approximately 32.8 billion total cells in gray matter with a glial to neuron ratio of about 3:2, whereas of the 44.4 billion cells in white matter the majority are glial cells[23].

1.3.2 Neurotransmission

As a function of cortical firing rate, the amount of glutamate that is packaged into vesicles, released, and then recycled through glial cells is proportional across species[24]. The intricate

activity of neurotransmitters and their receptors are the basis for nerve cell communication[18]. Neurons generate electrical signals based on the flow of ions across their plasma membranes. When at rest, neurons generate a constant voltage across their membrane, called the resting membrane potential, typically -40 to -90 mV[18]. The resting membrane potential is primarily due to the selective permeability of potassium ions (K^+) caused by K^+-permeable membrane channels, resulting in more K^+ inside the neuron than outside[18]. Electrical signals produced by neurons are caused by responses to stimuli, which then change the resting membrane potential, resulting in an action potential. During an action potential the membrane becomes transiently permeable to sodium (Na^+) by the opening of Na^+ channels, allowing Na^+ influx, causing the membrane potential to depolarize. This rise in Na^+ permeability is followed by a transient rise in membrane K^+ permeability by the opening of K^+ channels, allowing K^+ efflux, repolarizing the neuronal membrane[18]. The Na^+-K^+ ATPase restores the resting membrane potential by actively moving Na^+ out, and K^+ into the cell. Local depolarization causes neighbouring Na^+ channels to open and continue the generation of the action potential. Myelination of an axon further speeds up action potential conduction by acting as an electrical insulator[18].

While action potentials allow the transmission of a signal along an axon, communication from one neuron to the next is made possible by synapses, the functional contact between neurons. There are two general classes of synapses in the human brain: electrical and chemical. In electrical synapses, the presynaptic and postsynaptic neurons are actually linked by gap junctions, and ionic current is allowed to flow passively through the pores created by these gap junctions[18]. However, the majority of synapses throughout the central nervous system are not electrical, but chemical. In chemical synapses, neurotransmitters are packaged into membrane-bound organelles called synaptic vesicles within the presynaptic terminal. The arrival of an action potential leads to the opening of calcium (Ca^{2+}) channels causing a rapid influx of Ca^{2+}. The rapid influx of Ca^{2+} allows the synaptic vesicles to fuse with the plasma membrane, releasing the neurotransmitters into the synaptic cleft. The neurotransmitters diffuse across the synaptic cleft to where they bind to their receptors on the postsynaptic neuron causing channels to open or close, depending on the nature of the neurotransmitter and receptor, resulting in an increased or decreased probability that the neuron will fire an action potential.

Although there are over 100 different neurotransmitters in the human brain, the main excitatory neurotransmitter in the brain is glutamate, and the main inhibitory neurotransmitter is γ-aminobutyric acid (GABA)[18]. Glutamate is a nonessential amino acid that does not cross the blood brain barrier, and therefore must be synthesized in the brain from local precursors. These precursors include tricarboxylic acid (TCA) cycle intermediates (α-ketoglutarate), and predominantly glutamine by the mitochondrial enzyme glutaminase[18]. Glutamate is then packaged into synaptic vesicles by vesicular glutamate transporters. Once released into the synaptic cleft, glutamate is rapidly removed by excitatory amino acid transporters (EAATs, a family of Na^+-dependent co-transporters), present on some presynaptic terminals and primarily on glial cells, specifically astrocytes[18]. Once in the astrocyte, glutamate is converted to glutamine by glutamine synthetase[25]. Glutamine is then transported back to the neuron, where it can be converted to glutamate. The sequence of these events is better known as the glutamate-glutamine cycle, see Figure 1.1. GABA on the other hand, while most commonly found in local circuit interneurons, is primarily synthesized from glutamate by the glutamic acid decarboxylase (GAD) enzyme and can also be synthesized from glutamine and pyruvate[18]. GABA is then similarly packaged into vesicles, released into the synaptic cleft, and removed by Na^+-dependent co-transporters found on neurons and astrocytes. GABA is then converted to succinate by GABA transaminase and succinic semialdehyde dehydrogenase (SSADH), a TCA cycle intermediate[18]. These tightly coupled glutamatergic and GABAergic systems are better known as the GABA-glutamate-glutamine cycle, and is depicted in Figure 1.1.

Glutamatergic neurotransmission is excitatory due to the postsynaptic receptors that glutamate binds. There are metabotropic glutamate receptors (mGluR) that can excite or inhibit, and therefore are actually quite varied in their physiological roles[18]. However, the vast majority of glutamate receptors are ionotropic receptors (AMPA, NMDA, and Kainate) that always produce excitatory responses due to the influx of Na^+ and K^+, and sometimes Ca^{2+}, when activated[18]. Activation of these receptors can also result in excitotoxicity, the ability of prolonged glutamate synaptic transmission to destroy neurons. Evidence of excitotoxicity causing neuronal damage after brain injury has been observed during studies of ischemia and epilepsy[18,26]. GABAergic neurotransmission on the other

hand, is inhibitory because activation of these receptors causes an influx of Cl^-, which hyperpolarizes the membrane and therefore transiently inhibits an action potential[18].

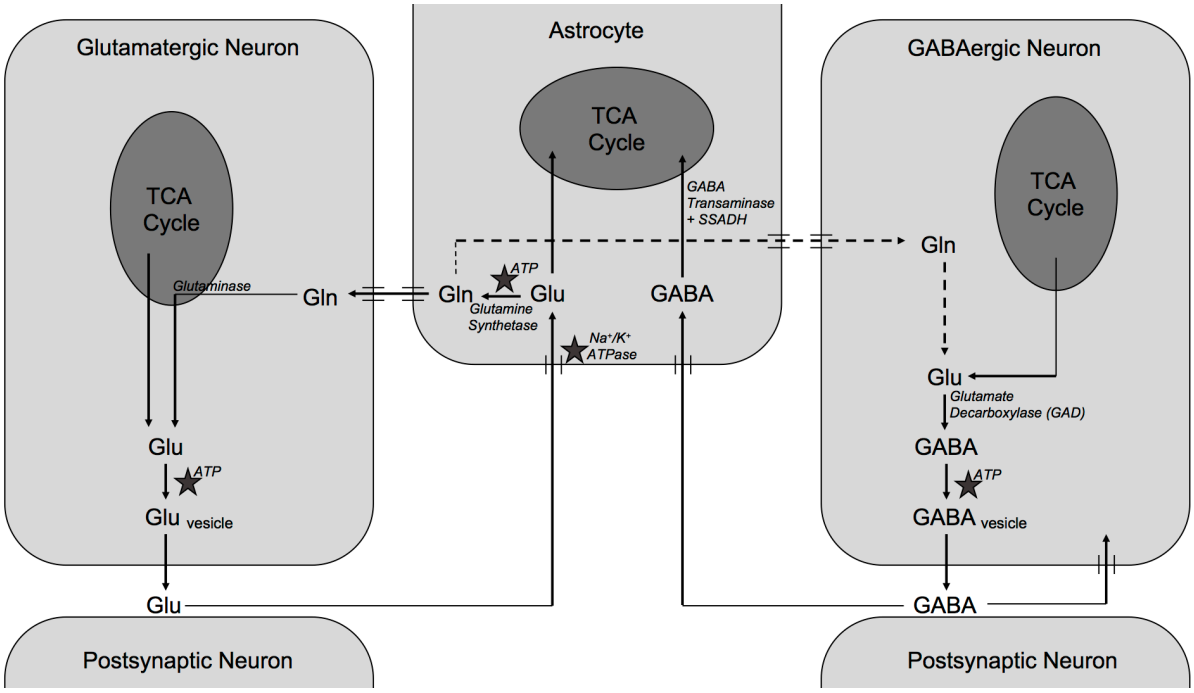


Figure 1.1: Illustration of the GABA-Glutamate-Glutamine Cycle. Glutamate is packaged into synaptic vesicles and released into the synaptic cleft. Glutamate is rapidly removed by excitatory amino acid transporters, present on some presynaptic terminals and primarily on glial cells, specifically astrocytes. Once in the astrocyte, glutamate is converted to glutamine by glutamine synthetase. Glutamine is then transported back to the neuron, where it can be converted to glutamate. The sequence of these events is better known as the glutamate-glutamine cycle. GABA on the other hand, is primarily synthesized from glutamate by the glutamic acid decarboxylase (GAD) enzyme and can also be synthesized from glutamine and pyruvate. GABA is then similarly packaged into vesicles, released into the synaptic cleft, and removed by Na^+-dependent co-transporters found on neurons and astrocytes. GABA is then converted to succinate by GABA transaminase and succinic semialdehyde dehydrogenase (SSADH), a TCA cycle intermediate. These tightly coupled glutamatergic and GABAergic systems are better known as the GABA-glutamate-glutamine cycle.

1.3.3 Metabolic demands of the human brain

The principal nutrients that support biosynthesis in mammalian cells are glucose and glutamine[27]. Although the regulation of glutamine through signalling pathways is still being elucidated[27], the brain utilizes 20% of the body's total glucose and oxygen to generate ATP to fuel physiologic functions[28]. Glucose is also a key substrate in forming the second messenger inositol, as well as glutamate and GABA for neurotransmission, as discussed earlier[29]. It is generally accepted that glucose is the obligatory energy substrate for the brain[30,31], and crosses the blood brain barrier through glucose transporters, primarily GLUT1. Even though neurons possess the glucose transporter, GLUT3, several studies suggest that glucose is mostly consumed by glial cells while neurons depend on glucose metabolites released from the glia[32]. Further studies have suggested other useful energy substrates for the brain, including lactate and acetate[32]. In astrocytes, after glycolysis, pyruvate can easily be converted into lactate, and is potentially a major source to support axonal function[33]. Although controversial, it has also been proposed that this lactate is transported to neurons where it serves as the primary metabolic fuel, known as the astrocyte-neuron lactate shuttle hypothesis[32,34]. Acetate, which can be metabolized to acetyl CoA to enter the TCA cycle, has been shown to be metabolized *in vitro* to glutamate and aspartate by neurons, and to glutamine by astrocytes[35]. *In vivo* studies have found acetate to be rapidly incorporated into glutamine, GABA, glutamate, and aspartate, with the greatest amount metabolized to glutamine[32]. The role of other substrates over glucose is particularly important in pathological conditions where glucose supply is disrupted or limited.

The human brain is highly energy demanding, as ATP is consumed through non-signaling and signaling processes in the brain[36]. Non-signaling processes include housekeeping mechanisms and maintaining the resting potential, while signaling processes include the neural firing rate, action potential conduction, synaptic transmission, neurotransmitter recycling, and calcium activity[24]. The calculated average neuronal firing rate in the resting awake human is approximately 1.15Hz, much lower than the rat (approximately 4.3Hz)[24]. Experimental studies have observed brain energy metabolism from glucose oxidation to correlate with signaling processes[37,38]. Several studies

investigating energy budgets did not include glial activity[39,40], even though it has been reported that astrocytes and oligodendrocytes have active calcium responses to spiking processes[41,42]. It has been reported that the nonsignaling costs of glial cells are three times higher than neurons, and nearly half of glial metabolism is due to calcium signaling[24]. Furthermore, Yu et al. (2018)[24] estimated energy budgets for gray matter by taking into account neuronal and glial energy demands, and validated their findings with *in vivo* PET and ^{13}C magnetic resonance spectroscopy (MRS). They found that 70% and 30% of the energy demand in gray matter is related to signaling and non-signaling processes respectively, while in white matter the cost is approximately 20% and 80%. These results matched well with other studies, for example, a recent *in vivo* ^{13}C-MRS study in rat and human brain suggested 70-75% and 25-30% of the energy in the awake state supports signaling and nonsignaling functions respectively in gray matter[43]. The main difference between gray and white matter energy demand stems from the cost of neuronal signaling in the cerebral cortex, and the higher biosynthesis turnover demands in white matter[24].

1.3.4 Traumatic Brain Injury

Traumatic brain injury (TBI) begins not as a disease but as an event that causes traumatic impact to the brain[44]. The primary injury is the result of immediate mechanical damage from direct or indirect contact to the brain. This damage can be due to, for example, acquired brain injury, a stroke, an aneurysm, hemorrhaging, or axonal shearing. Next, secondary injury evolves over minutes to days after the primary injury due to a cascade of metabolic, cellular and molecular events[3]. These include glutamate excitotoxicity, disrupted calcium homeostasis, mitochondrial dysfunction, increased free radicals, inflammation, and diffuse axonal injury (DAI)[3].

More specifically, when the brain is damaged, damage-associated molecular patterns (DAMPs) are passively released by injured cells. DAMPs, which can include proteins, ATP, and DNA, bind pattern recognition receptors (PRR) on microglia and astrocytes to initiate innate immune activation[45] through the production of cytokines and chemokines[3]. These molecules will further activate other receptors that in turn initiate inflammatory signaling cascades and the recruitment of immune cells (leukocytes) to the site of damage[3,45]. Studies have shown leukocyte recruitment to contribute to the

development of BBB breakdown, edema formation, and potentially contribute to secondary damage[3]. As a result, several studies have investigated the therapeutic potential of blocking leukocyte migration and adhesion in animal models of TBI[46,47]. However, the extent to which leukocyte recruitment is harmful to brain parenchyma remains controversial.

Taken together, it appears that TBI is a multimodal disease process with primary and secondary injury mechanisms that cause structural and functional damage. These deficits include cognitive, behavioral, emotional and physical, resulting in diminished quality of life[3]. These deficits can include communication problems, mood disorders, and cognitive impairment even six months after mild TBI[3]. Post traumatic seizures, although more prevalent following severe TBI, can follow mild and moderate TBIs[3]. Furthermore, the vast majority of studies done of TBI in the literature have examined severe or penetrating TBI, leaving mild TBI less well characterized.

1.4 Concussion

1.4.1 The Neurometabolic Cascade

During a concussive injury, the brain may experience linear and rotational accelerations, with dynamic shear, tensile, and compressive strains within the tissue[48]. As depicted in Figure 1.2, these stresses can result in mechanoporation, leading to altered ion flux, including calcium influx, and glutamate release immediately after injury[13,49,50]. Glutamate release can interact with immune receptors and trigger a series of events resulting in synaptic injury and cellular damage[51]. Higher intracellular calcium can lead to phosphorylation of neurofilaments, resulting in loss of axonal structural integrity[13,52], and interruption of axonal transport[48], producing diffuse axonal injury (DAI). Subsequently, the shift in intracellular and extracellular ions requires ATP ionic pumps to restore balance, leading to an acute depletion of intracellular energy reserves[13,53]. Additionally, this increased demand for energy is accompanied by reduced cerebral blood flow[54], further prolonging the mismatch between energy demand and supply. This reduced cerebral blood flow has been sustained for up to 2 weeks post-concussion[55]. Furthermore, the sequestration of calcium into mitochondria can result in mitochondrial dysfunction, worsening the cellular energy crisis[13]. The extent to which these events are sustained

appears to vary, from calcium influx lasting 3-5 days, to reduced cerebral blood flow as long as 7-14 days[13].

Fluorodeoxyglucose (FDG) PET and single photon emission computed tomography (SPECT) studies have characterized changes in glucose and oxygen consumption, and cerebral blood flow (CBF) after concussion and TBI[56]. Generally, hypermetabolism has been found acutely after concussion, followed by reduced glucose metabolism in various brain regions. Additionally, a reduced cerebral metabolic rate of oxygen has been observed, indicating diminished oxidative metabolism[57,58]. Although global CBF often appears unchanged, reduced regional CBF has been found in several studies (ranging from frontal and temporal cortex to cerebellum and brainstem), which is consistent with functional deficits in attention, working memory, verbal fluency, and processing speed[56].

Activation and infiltration of microglia have been well documented in TBI[59,60], and more recent studies suggest that inflammation is also triggered in concussion[61,62]. Madathil et al., (2018)[62] observed robust microglial activation after single and repeated impacts using a rodent closed-head concussive impact injury model, that resolved by 72 hours after a single impact but persisted in rodents with repeated impacts. Even though these inflammatory processes are currently being studied in concussion and TBI, their role in injury severity is largely unknown. Additionally, techniques such as carbon-13 (^{13}C) and phosphorus-31 (^{31}P) NMR spectroscopy may be useful in identifying shifts in cerebral metabolism, intracellular pH, free magnesium, and cellular bioenergetic status[63] after concussion. In fact, Bartnik and colleagues (2007)[64] found reduced glutamine labeling up to 24-hrs post-injury in a fluid percussion injury rodent model using ^{13}C NMR, suggesting reduced oxidative metabolism after injury. Additionally, Cernak and colleagues (2004)[63] found a decline in free magnesium and reduced bioenergetic status 4-hours after an impact-acceleration induced severe diffuse axonal injury in rats using ^{31}P NMR, suggesting increased energy demand in response to attempts to restore ionic balance. These studies can provide insights into neuronal and glial energetics after injury, due to the location of specific metabolic enzymes, such as glutamine synthetase, which is exclusively found in

glial cells[65]. By combining these findings on inflammation, cerebral energetics, and other disturbances after concussion, it is possible to build a more detailed picture of the metabolic changes post-concussion and move towards identifying an objective biomarker for diagnosis and prognosis.

1.4.2 From Clinical Practice to Areas of Active Research: A Historical Perspective of Imaging Concussion

Although the notion of closed head injury dates as far back as Ancient Greek medicine, the entity of concussion was first described as an abnormal transient physiologic state without gross brain lesions by the Arabic physician, Rhazes around 900 AD[66,67]. It wasn't until 1280 that concussion was first discussed as a separate entity from penetrating brain wounds by the modern physician Lanfrancus[67,68]. Over the next few centuries, the clinical description would be further refined and the term concussion would come into widespread use. Following the development of the microscope in 1694, the focus shifted to a pathophysiological approach to understanding the well documented clinical entity of concussion through animal models and case reports[67,69,70], resulting in several new physiologic theories focusing on functional rather than structural injuries. This led to two broad schools of thought: structural versus functional brain injury, which have both led to several hypotheses and studies aimed at exploring diffuse axonal injury, mechanoporation, and subsequent events following concussion.

It wouldn't be until 1895, with the discovery of the x-ray, that the era of medical imaging would commence, from the introduction of nuclear medicine and PET in the 1950's, to the advent of CT and MRI in the 1970's[71]. CT and MRI became commonly used in hospitals throughout the 80's and 90's, with CT traditionally used to rule out a more serious TBI injury such as structural lesions, fractures or intracranial hemorrhages[72]. In the clinical setting, CT is beneficial due to low cost and greater speed compared to an MRI, but convention structural neuroimaging by CT and MRI is rather insensitive to concussion[72]. From the end of the 1980's into the 21st century, fluorodeoxyglucose (FDG) PET and single photon emission computed tomography (SPECT) studies characterized changes in glucose and oxygen consumption, and cerebral blood flow (CBF) after concussion and TBI[56]. More recently, over the last two decades,

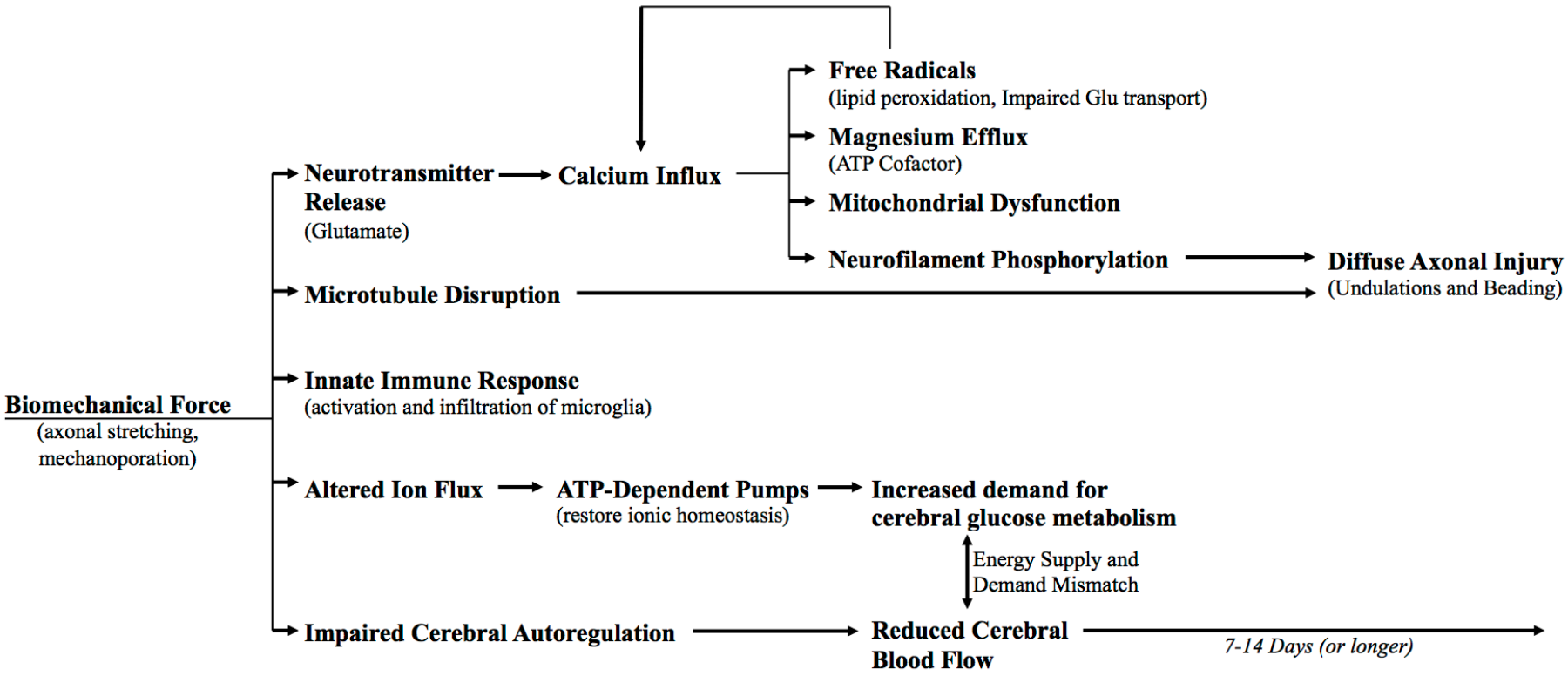


Figure 1.2: Flow Chart of the Neurometabolic Cascade after Concussion

thanks to the advancement of novel ligands, PET has been used to explore activated microglia and tauopathies after concussion and TBI to investigate neuroinflammation and neurodegenerative changes.

Alongside the advancement of concussion with PET imaging, several different MRI techniques that were developed throughout the 1990's were also investigated, including arterial spin labeling (ASL), diffusion tensor imaging (DTI), and functional MRI (fMRI). ASL is a perfusion technique that allows the assessment of CBF[73]. Similar to what was found with PET, some ASL studies observed regional decreases in CBF[55,74] that were associated with changes in processing speed, learning and memory, executive function and verbal fluency[74]. Furthermore, studies have found reduced regional CBF to persist in athletes that take longer to recover[75]. However, other studies have found significantly elevated regional CBF[76]. These discrepancies are likely due to the heterogenous nature of the injury, timing of data acquisition relative to the concussion, and the specific brain region.

The MRI technique, diffusion tensor imaging (DTI), is a type of diffusion weighted imaging that quantifies an elliptical diffusion tensor within a voxel. Radial (RD) and axial diffusivity (AD) describe the magnitude of diffusion along the radial and long axes of the ellipsoid, respectively. The mean diffusivity (MD) represents the average magnitude of water diffusion, while fractional anisotropy (FA), describes the directionality of water diffusion on a scale of 0 (isotropic) to 1 (maximum anisotropy). Human studies using DTI have found an acute period of high FA with low RD, followed by decreases and increases in FA and RD respectively[77,78], while other studies have found the exact opposite[79]. Changes in AD and MD have also been observed[80]. Alterations in these diffusion parameters may reflect neuronal swelling, edema[77,81], or axonal pathology[80]. Furthermore, decreased FA in frontal and temporal regions relative to healthy controls has been observed several months to years following concussion, indicating a loss of myelin and potentially degenerative changes[81,82]. However, a study by Ilvesmaki et al., (2014)[83] found no differences in DTI metrics between mTBI patients and healthy controls. Future research directions include the application of multi-compartmental models of diffusion and

multi-shell imaging. More information on these techniques can be found in Delouche et al., 2016[84].

Early fMRI studies found hyperactivation in frontoparietal brain regions in concussed patients relative to healthy controls under moderate processing loads, and hypoactivation for lower processing loads[85,86]. Hypoactivation in the dorsolateral prefrontal cortex and other brain regions during working memory tasks has also been reported after concussion in several studies[87–90], as well as associations between hypoactivation and clinical measures such as symptom severity[91]. Furthermore, studies have found different areas of activation and/or hypoactivation depending on the mode of injury[91]. To date, several resting state (RS) fMRI studies have also found changes in connectivity in the default mode network (DMN), intrinsic connectivity networks (ICN), and other networks using seed-based approaches or independent component analysis (ICA)[91,92].

1.5 Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) is a magnetic resonance-based method that can be used to detect and quantify specific metabolites in a brain region of interest. Quantifying metabolite changes following concussion could aid in identifying altered neurotransmission from the secondary chemical cascade of ion flux that occurs after receiving a hit to the head.[13] In this section, the basic principles of MRS are outlined, followed by the acquisition and post-processing of MRS data, and finally a description of the metabolites that can be measured and their relevance to concussion, in humans and animal models.

1.5.1 Magnetic Resonance Principles

NMR is the study of the magnetic properties and energies of nuclei. In NMR, only nuclei with nonzero nuclear spin are detectable (For example; ^{1}H, ^{31}P, ^{13}C)[93]. This is because nuclei with a nonzero spin quantum number have a nonzero spin angular momentum

$$\mu = \gamma L, \qquad \text{(Eq. 1.1)}$$

where L is the spin angular momentum, γ is the gyromagnetic ratio, an intrinsic property of each nuclei (^{1}H γ = 42.58 MHz/T), and μ is the magnetic moment. The spin angular momentum (L) is quantized and given by[94]

$$L = \frac{h}{2\pi}\sqrt{I(I+1)}, \qquad \text{(Eq. 1.2)}$$

where h is Planck's constant and I is the spin quantum number, which can only be an integral or half-integral. By substituting (Eq. 1.1) into (Eq. 1.2) we show that the magnetic moment is also quantized:

$$\mu = \gamma \frac{h}{2\pi}\sqrt{I(I+1)}. \qquad \text{(Eq. 1.3)}$$

Nuclear magnetic resonance occurs when a nucleus with a magnetic moment, μ , is placed in an external magnetic field (B_0). The magnetic field will exert a torque on the magnetic moment, leading to precession of the nucleus about B_0. The frequency of this precession is known as the Larmor frequency (ν) and is given by:

$$\nu = \left(\frac{\gamma}{2\pi}\right) B_o. \qquad \text{(Eq. 1.4)}$$

It is convention that the z-direction is set along B_0, and a magnetic moment in a magnetic field will have potential energy (E) given by:

$$E = -\mu_z B_0. \qquad \text{(Eq. 1.5)}$$

In quantum mechanics the direction of angular momentum is specified by the quantum number, *m,* which can have I(I+1) values given by I, I-1, I-2, …, -I. The component of the magnetic moment in the z direction is given by:

$$\mu_z = \gamma \left(\frac{h}{2\pi}\right) m, \qquad \text{(Eq. 1.6)}$$

where μ_z is the component of the magnetic moment in the z direction. By substituting (Eq. 1.6) into (Eq. 1.5) we show that the energy levels are also quantized:

$$E = -\gamma \frac{h}{2\pi} m B_0. \qquad \text{(Eq. 1.7)}$$

In a magnetic field, for a ^{1}H of spin I=1/2 [94], there are only two energy levels (m = -1/2 and +1/2), and the energy difference (ΔE) is given by:

$$\Delta E = \gamma \left(\frac{h}{2\pi}\right) B_0. \qquad \text{(Eq. 1.8)}$$

The two spin states m= +1/2 (parallel to B_0), and m= -1/2 (antiparallel to B_0) are referred to as the α and β spin states respectively. In a macroscopic sample there are many spins that distribute themselves among these two orientations according to the Boltzmann Equation[94]. Due to energy differences between these spin states, as shown by Equation 1.8, there will also be a difference in the population of these spin states, given by:

$$\left(\frac{n_\alpha}{n_\beta}\right) = e^{\Delta E/kT} = e^{h\nu/kT}, \qquad \text{(Eq. 1.9)}$$

where n is the number of spins the low energy state (α) and high energy state (β), k is the Boltzmann constant and T is the absolute temperature.

The total net magnetic moment, the magnetization (M), of a macroscopic sample is the sum over all the individual magnetic moments, μ. Due to the population difference in z, there will be a net component of M parallel with B_0 along the +z axis, known as the longitudinal magnetization, M_0. At thermal equilibrium, the amplitude of this magnetization is given by:

$$M_0 = \left(\frac{\gamma h}{2\pi}\right)^2 \left(\frac{n B_0}{4kT}\right), \qquad \text{(Eq. 1.10)}$$

where $n = n_\alpha + n_\beta$, the total number of ^{1}H nuclear spins in the macroscopic sample.

MRI scanners are designed to detect magnetization that is perpendicular to the main magnetic field (in the transverse plane). However, at thermal equilibrium the spins have no phase coherence in the transverse plane, and the net longitudinal magnetization, M_0, is a static vector[94]. Therefore, the net magnetization must be rotated onto the transverse plane for detection. This is achieved by applying a second magnetic field, B_1, in the transverse plane, oscillating in the radio frequency (RF) MHz range. While this RF pulse (B_1) is applied, the magnetization will precess about B_0 and B_1, resulting in a rotation of the magnetization towards the transverse plane. If the B_1 field is applied long enough, M_0 will be excited onto the transverse plane (90º excitation) or even inverted to the -z axis (180º excitation). Following the RF pulse, M_0 will only experience B_0 and will precess around it at the Larmor frequency, *v*, inducing an electromotive force (emf) in the receiver coil, giving rise to the detected NMR signal. This signal is an alternating current with frequency equal to the Larmor frequency, that decays exponentially due to transverse and longitudinal relaxation. The Fourier Transform of this signal produces a Lorentzian lineshape in the frequency domain[94].

1.5.2 Chemical Shift and J-Coupling

If the frequency of nuclear spins were solely determined by Equation 1.4, then *in vivo* ^{1}H MRS would have little value, since every ^{1}H nucleus in all metabolites would precess at the same frequency, determined by their identical gyromagnetic ratios. Fortunately, this is not the case. In fact, in addition to the gyromagnetic ratio and magnetic field, the resonance frequency of a given nucleus depends on the chemical environment of the nucleus, also known as the chemical shift. The chemical shift arises because electrons surrounding the nucleus effectively shield the nucleus from the external magnetic field, ultimately reducing the magnetic field that is experienced by the nucleus[94]. The effective magnetic field (B_{eff}) experience by the nucleus is given by:

$$B_{eff} = B_0(1 - \sigma), \quad \text{(Eq. 1.11)}$$

where σ is the shielding coefficient in units of parts per million (ppm), and is independent of B_0. Therefore, within the same molecule, each ^1H that is in a different chemical environment will precess at a slightly different frequency, resulting in a distribution of resonance frequencies, which produces a fingerprint for the given metabolite.

Chemical shifts are generally not expressed in units of Hz, since this would make them dependent on the magnetic field strength. Alternatively, chemical shifts (δ) are expressed in terms of ppm defined as:

$$\delta = \frac{f - f_{ref}}{f_{ref}} \times 10^6, \quad \text{(Eq. 1.12)}$$

where f is the frequency of the nucleus under investigation and f_{ref} is the frequency of a reference compound. Proton MRS commonly uses sodium 3-trimethylsilyl-propionic acid (TSP) as a chemical shift reference[94].

In addition to the chemical shift phenomenon, nuclei with magnetic moments can influence each other through electrons in covalent bonds, resulting in more complex spectral patterns, known as spin-spin coupling or J-coupling. J-coupling results in slight frequency shifts, that appears as splitting of the peaks. For example, in the case of glutamine, there is a single methine proton and 4 methylene protons, in a total of 3 different chemical environments (Figure 1.3A). Without J-coupling, this would produce 3 different peaks resonating at slightly different frequencies due to the chemical shift (Figure 1.3B). However, in reality a peak is split according to the spin orientation of the non-identical spin (or spins) it is coupled to. In the example of glutamine, the methine proton (^2CH) is split into three sub-peaks with relative areas in a 1:2:1 ratio (Figure 1.3C) because the spins of the coupled $^3CH_2$ protons have three possible spin configurations, resulting in different energy states: (up, up) one way, (up, down) two ways, and (down, down) one way. In general, a coupled nucleus will be split by n magnetically equivalent nuclei into

n+1 peaks, and the amplitudes of these peaks are given by Pascal's triangle (1:1, 1:2:1, 1:3:3:1, etc.)[94]. The frequency difference between these peaks is given by the scalar coupling constant (Hz), and is independent of the applied external magnetic field. Furthermore, the scalar coupling constant rapidly decreases with increasing number of chemical bonds and can typically be ignored for four or more bonds.

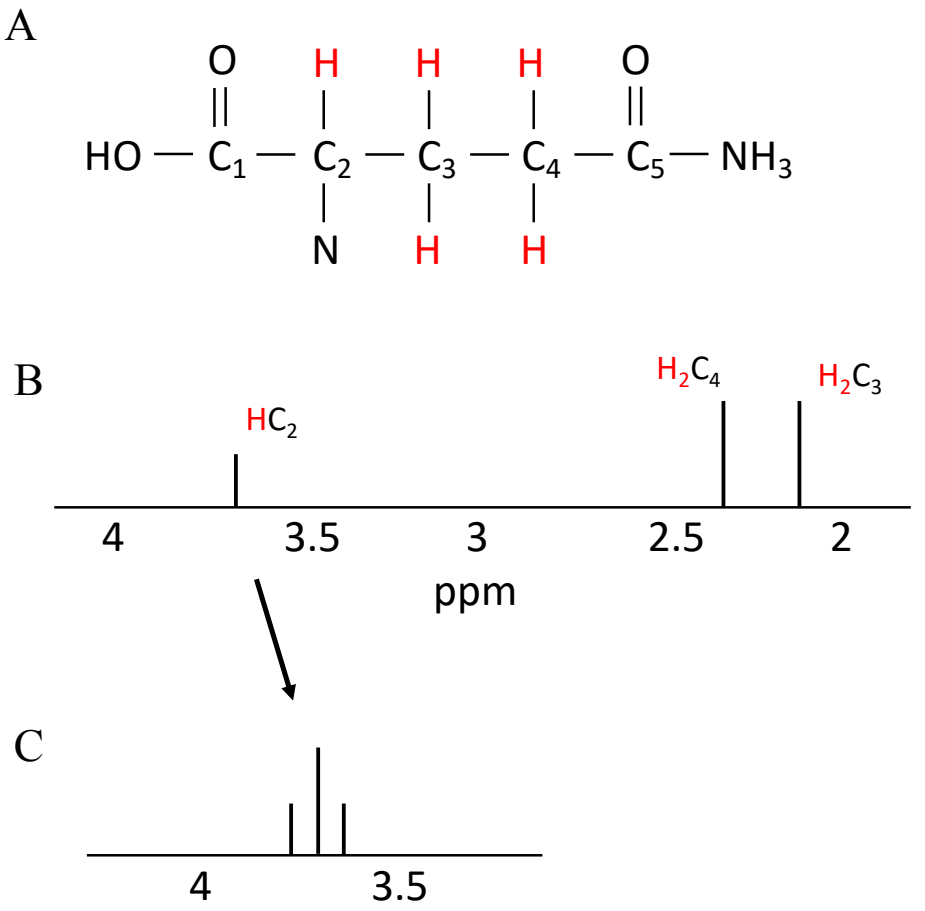


Figure 1.3: (A) Structure of Glutamine molecule, with MRS visible protons in red (B) MRS spectrum of glutamine according to chemical shift, and (C) splitting of glutamine methine group according to J-coupling

1.5.3 ^{1}H MRS Acquisition

Single voxel acquisition is the magnetic resonance spectroscopy technique used in this thesis. To acquire MRS data from a specific region of the brain, a voxel is placed in the

region of interest by an MRI technician using sagittal, axial, and coronal images to guide the voxel placement (see Figure 1.4). Saturation bands are placed near the boundaries of the voxel to reduce signal from the surrounding tissue. The signal detected by the MR scanner is localized to the voxel by using gradients that selectively excite three orthogonal planes, where the intersection of these planes defines the voxel of interest. This is achieved by using the Point Resolved Spectroscopy Sequence (PRESS), which uses a slice-selective excitation (90º) combined with two slice-selective refocusing pulses (180º). Signal from outside the voxel of interest is either not excited (by the 90º pulse), or not refocused (by the 180º pulse). Furthermore, most MRS studies of sport concussion have utilized a short echo time (TE < 50 ms), which makes metabolite quantification more challenging due to the overlapping broad macromolecule resonances that are most prominent within the 2-4 ppm region of the spectrum. To avoid this complication, a long echo time (TE = 135 ms) was chosen for the work presented in this thesis.

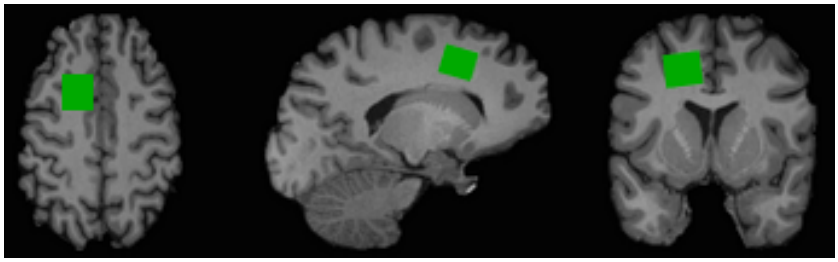
The most abundant compound in mammalian tissue is water, and as a result the NMR signal is dominated by the water resonance at 4.7 ppm[94]. This becomes problematic when trying to detect metabolites with concentrations that are 10 000 times lower than that of water. Therefore, to properly resolve the metabolite signals it is important that the water signal is suppressed during acquisition. This is often achieved by chemical shift selective (CHESS) water suppression, which uses a frequency selective RF pulse to excite the water followed by strong crusher gradients to dephase the magnetization in the transverse plane, ideally leaving the metabolite resonances unperturbed.

Although the above techniques are used for the majority of this thesis, different acquisition methodology is used in Chapter 4. Specifically, MRS spectroscopy data were acquired on an Agilent 9.4 Tesla small-bore MRI scanner (Santa Clara, CA, USA). Magnetic resonance spectroscopy was acquired using Localization by Adiabatic Selective Refocusing (LASER), a localization method that uses adiabatic excitation and refocusing RF pulses. This sequence, excites the entire sample with a nonselective adiabatic half passage pulse (90º), followed by three pairs of adiabatic full passage (AFP, 180º) pulses in the presence of magnetic field gradients to achieve slice-selective refocusing. Pairs of AFP

are used to refocus the phase that accumulates across the slice when a single AFP is applied along with a slice select gradient.

Water suppression was achieved by variable pulse powers and optimized relaxation delays (VAPOR), which uses seven frequency selective RF pulses interspersed with optimized T_1 recovery delays. Moreover, a short echo time of TE = 18 ms was used, which required the addition of an extra step to remove macromolecule signals, before the spectrum could be fitted. This was achieved by acquiring a macromolecule only spectra interleaved with the acquisition of the full spectrum using a single-inversion preparation pulse. This subject specific macromolecule spectrum was then subtracted from the full spectrum, leaving behind only the metabolites.

A



B

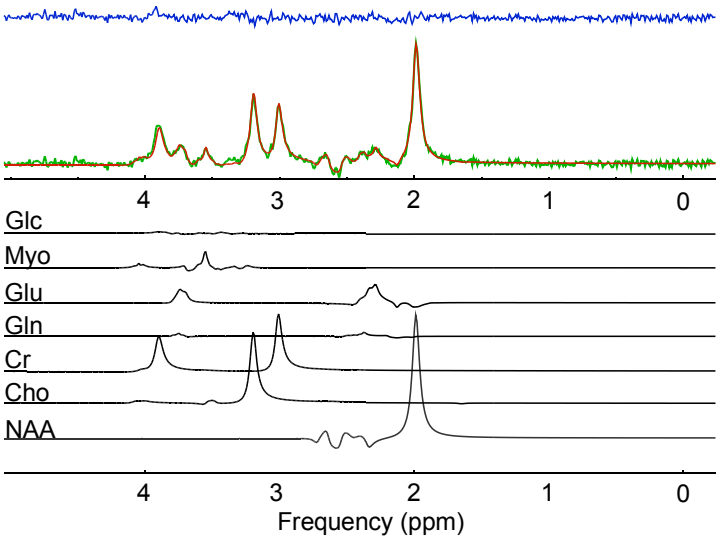


Figure 1.4: (A) From left to right; axial, sagittal, and coronal views of a T_1-weighted anatomical image with the spectroscopy voxel overlaid in green in the prefrontal region. (B) Spectrum acquired (green) from the voxel in A, reconstructed spectrum (red), the residual after fitting (blue), and the individual prior knowledge components of the spectrum shown in black. Glc = Glucose; Myo = *Myo*-inositol; Glu = Glutamate; Gln = Glutamine; Cr = Creatine; Cho = Choline; NAA = *N*-acetyl aspartate; ppm = parts per million.

1.5.4 ^{1}H MRS Post Processing

Lineshape distortions can be induced from time dependent, spatially dependent, and time and spatially dependent B_0 inhomogeneities. Popular methods to correct the lineshape use a reference signal, which is typically the unsuppressed water signal from the MRS voxel. The two methods employed in this thesis are QUALITY (QUAntification improvement by converting Lineshapes to the lorentzian TYpe) deconvolution[95] and ECC (Eddy Current Correction)[96]. QUALITY deconvolution divides the time-domain signal by the unsuppressed water time-domain signal, and effectively removes spectral distortions and restores the Lorentzian lineshape. However, the T_2 of water is shorter than the T_2 of most metabolites, resulting in division by near-zero water signals towards the end of the time-domain signal. By employing ECC towards the end portion of the time-domain signal, a subtraction of just the phases of the two signals, the division of near-zero values can be avoided. This combined spectroscopic lineshape correction is known as QUECC[97], and is the technique used in this thesis. Once the lineshapes are restored, the spectrum is then fitted to a prior knowledge template. Spectra were reconstructed using linear combinations of metabolite lineshapes obtained from spectra of in vitro metabolites solutions that were acquired under controlled temperature and pH.

The concentration of a metabolite is determined from the area of the resonance peak(s) because the area is directly proportional to the number of nuclei resonating at that frequency. In order to calculate the absolute tissue concentration of the metabolites from the measured signals, several assumptions must be made. First, we must assume that water densities and signal relaxation times of GM, WM, and CSF in the voxel can be reliably estimated and do not change significantly among groups[98]. Second, we assume that volume

fractions are accurately measured, and this is done in this thesis by segmentation of the T_1-Weighted MPRAGE anatomical image (for Chapter 4, the animal study, a 2D fast spin-echo anatomical image was segmented for tissue/CSF partial volume correction). Third, we assume the relative proton density of water, compared to that of pure water to be 0.82, 0.73 and 1.0 for GM, WM, and CSF, respectively. The relative proton density of metabolites in GM, WM and CSF are not known and therefore assumed to be 1.

The fully relaxed signal (*S*) from water or any metabolite in the spectroscopy voxel will be proportional to the number of moles (*n*) of the molecule in the voxel:

$$\frac{S_{metab\_relaxed}}{S_{H20\_relaxed}} = \frac{n_{metab}}{n_{H2O}}. \quad \text{(Eq. 1.13)}$$

Provided that the metabolite(s) and water come from the same volume of the voxel, we can solve for the metabolite concentration (M):

$$[M] = \frac{S_{metab\_relaxed}}{S_{H20\_relaxed}}[H_2O]. \quad \text{(Eq. 1.14)}$$

Note that to obtain the fully relaxed signal described above, we must correct for T_1 and T_2 relaxation $\left(R_{T_1}R_{T_2}\right)$, number of signal averages (NA), number of protons (ρ), and any gain/scaling factors (G):

$$[M] = \frac{S_{metab}/\left(R_{T_1}R_{T_2metab}\right)(NA)(\rho)(G)}{S_{H20}\ /\left(R_{T_1}R_{T_2water}\right)(NA)(\rho)(G)}[H_2O]. \quad \text{(Eq. 1.15)}$$

To correct the water signal for T_1 and T_2 related signal loss, we directly apply the following to our signal:

$$\begin{aligned} R_{T_1}R_{T_2water} = {} & f_{GM}\alpha_{GM}\left(1 - e^{-TR/T_{1GM}}\right)\left(e^{-TE/T_{2GM}}\right) \\ & + f_{WM}\alpha_{WM}\left(1 - e^{-TR/T_{1WM}}\right)\left(e^{-TE/T_{2WM}}\right) \\ & + f_{CSF}\alpha_{CSF}\left(1 - e^{-TR/T_{1CSF}}\right)\left(e^{-TE/T_{2CSF}}\right), \end{aligned} \quad \text{(Eq. 1.16)}$$

which describes relaxation of the transverse magnetization, where f_x denotes the volume fraction of x = GM, WM, or CSF in the spectroscopic voxel, α_x denotes the relative proton density, and TR/TE represent the repetition and echo time of the spectroscopy experiment (2000/135 ms for human experiments, and 3250/18.9 ms for mouse experiments in this thesis). To correct the metabolite signal for T_1 and T_2 related signal loss, a similar approach is taken, except that no correction is applied for the CSF compartment, because metabolites are assumed to be exclusively found in tissue:

(Eq. 1.17)

$$R_{T_1}R_{T_2 metab} = \frac{f_{GM}}{f_{GM}+f_{WM}}\left(1-e^{-TR/T_{1GM}}\right)\left(e^{-TE/T_{2GM}}\right) + \frac{f_{WM}}{f_{GM}+f_{WM}}\left(1-e^{-TR/T_{1WM}}\right)\left(e^{-TE/T_{2WM}}\right).$$

Since the relative proton densities of metabolites are assumed to be 1, they are omitted from the equation. By substituting (Eq. 1.16) and (Eq. 1.17) into (Eq. 1.15), the final equation for the corrected metabolite concentration with respect to the entire volume of the voxel is as follows:

(Eq. 1.18)

$$[M] = \underbrace{\frac{S_{metab}}{S_{H2O}}}_{\text{Ratio of measured signals}} \cdot \underbrace{\left(\frac{f_{GM}\alpha_{GM}\left(1-e^{-\frac{TR}{T_{1GM}}}\right)\left(e^{-\frac{TE}{T_{2GM}}}\right)+f_{WM}\alpha_{WM}\left(1-e^{-\frac{TR}{T_{1WM}}}\right)\left(e^{-\frac{TE}{T_{2WM}}}\right)+f_{CSF}\alpha_{CSF}\left(1-e^{-\frac{TR}{T_{1CSF}}}\right)\left(e^{-\frac{TE}{T_{2CSF}}}\right)}{\frac{f_{GM}}{f_{GM}+f_{WM}}\left(1-e^{-\frac{TR}{T_{1GM}}}\right)\left(e^{-\frac{TE}{T_{2GM}}}\right)+\frac{f_{WM}}{f_{GM}+f_{WM}}\left(1-e^{-\frac{TR}{T_{1WM}}}\right)\left(e^{-\frac{TE}{T_{2WM}}}\right)}\right)}_{\text{Correction for partial volume and relaxation}} \cdot \underbrace{\left(\frac{NA_{H2O}}{NA_M}\cdot\frac{\rho_{H2O}}{\rho_M}\cdot\frac{G_{H2O}}{G_M}\right)}_{\text{Correction for number of averages, nuclei, and gain}} \cdot \underbrace{(55.14M)}_{\text{Concentration of pure water}}$$

Finally, Equation 1.18 can be multiplied by $(f_{GM}+f_{WM})^{-1}$ to determine the concentration of the metabolite in brain tissue alone, and this is how the absolute concentrations are reported in this thesis.

1.5.5 ^{1}H MRS Metabolites and findings in Concussion Studies

There are several metabolites of interest that are measurable by *in vivo* ^{1}H MRS. Table 1.1 outlines these metabolites, including their main functions within the brain and relevance to concussion. Briefly, the main metabolites found in human ^{1}H MRS include *N*-acetyl aspartate (NAA), total choline (Cho), total creatine (Cr), glutamate (Glu), glutamine (Gln), and *myo*-inositol (Myo). Several different changes in these metabolites, as well as different directions in these changes, have been observed after sport concussion. This section outlines the main function of each of these metabolites, and introduces the current findings in sport concussion (Table 1.2) and the timing of these changes (Table 1.3).

The MRS creatine peak incorporates signals from both creatine and phosphocreatine (total creatine), which is used as a marker of energy metabolism, due to their primary role as an intracellular buffer for ATP[99,100]. Creatine is primarily synthesized in the liver and transported to the brain[99], although it is also suggested that the brain is able to endogenously synthesize its own creatine[100]. Furthermore, creatine plays an important role in energy metabolism through interconversion to the high energy phosphorylated analogue, phosphocreatine (PCr)[100]. The *N*-phosphoryl group is transferred from PCr to ADP, yielding ATP[100]. Moreover, creatine and PCr allow for the shuttle of high-energy phosphates from mitochondria to sites of utilization in the cytoplasm[100]. Therefore, the Cr measured by MRS may be sensitive to the energy supply and demand mismatch that can occur after a concussive injury. The MRS signal of total creatine (Cr) produces two singlets at 3.03 and 3.93 ppm[94]. In human studies, no change in Cr has been observed after concussion, however elevated Cr has been reported in the motor cortex of contact athletes at the beginning of season compared to non-contact athletes, followed by reduced Cr during the season[101]. Moreover, creatine is commonly used as the denominator when calculating metabolite ratios in the MRS literature. Therefore, to compare to the literature, metabolite ratios are reported in this thesis in addition to absolute quantification, as already discussed.

NAA is one of the highest concentrated free amino acids in the brain[102], and is commonly regarded as a marker of neuronal health and integrity, as it is synthesized in neurons and its spatial distribution in GM has been shown to be correlated with the density

of neuronal cells[99]. Although the exact function of NAA is not fully understood, it is implicated in acetyl-CoA and aspartate storage, a source for lipid synthesis in oligodendrocytes, a potential osmolyte, and involved in NAAG synthesis[103,104]. NAA is synthesized in the mitochondria of neurons from acetyl coenzyme A and aspartate, where it is then transported along axons to oligodendrocytes, except for a small amount that is converted with glutamate to NAAG[102]. In oligodendrocytes, NAA is catabolized to aspartate and acetate, where the acetate can then be used in the synthesis of fatty acids, such as myelin[102,103]. Studies of diseases in which there is a loss of neurons or axons, such as infarctions, brain tumors, and multiple sclerosis, have presented with decreased NAA[105]. The NAA MRS signal is composed of a singlet at 2.01 ppm, as well as smaller resonances at 2.49, 2.67, and 4.38 ppm[94]. In the case of concussion and TBI, a decrease in NAA has the potential to aid in the assessment of irreversible and temporary neuronal damage[99]. In human studies, mostly decreases in NAA and NAA/Cr have been reported[106–110], with a single study reporting elevated NAA/Cr[111] in the WM of the frontal lobes.

The choline signal is made up of choline, glycerophosphocholine and phosphocholine. Choline is an essential nutrient that is involved in neurotransmitter synthesis (acetylcholine), lipid transport (lipoproteins), and cell membrane signaling (phospholipids)[112]. The majority of choline, however, is converted to phosphatidylcholine, the predominant phospholipid in cell membranes[112]. The most prominent signal produced from total choline is at 3.2 ppm[94]. Quantification of the MRS choline peak can be used to infer changes in membrane turnover rate such as demyelination after injury[113], likely due to the degradation of myelin phospholipids[114]. Elevated Cho/Cr[115] has been observed after sport concussion, as well as reduced Cho[80]. Additionally, changes in Cho have been observed in retired athletes[116–118].

Mentioned previously, glutamate is a nonessential amino acid that is synthesized in the brain from local TCA cycle precursors (α-ketoglutarate), but predominantly from glutamine[18]. Glutamate is an excitatory neurotransmitter, and up to 90% of neurons excrete glutamate during neurotransmission[99]. Moreover, there is evidence of glutamatergic signaling in the white matter as well[119], although the role of WM signaling is less well

understood. In regards to the spectral pattern of glutamate, it is complex with a resonance at 3.75 ppm and multiplets from 2.04-2.35 ppm[94].

As demonstrated in Figure 1.1, glutamine is important in recycling neurotransmitters, as part of the tightly coupled GABA-glutamate-glutamine cycle. Additionally, glutamine is involved in brain nitrogen homeostasis[120], and there is also evidence suggesting that the metabolite is immunomodulatory in models of infection and trauma[121]. Glutamine is structurally similar to glutamate, resulting in a resonance at 3.76 ppm, and multiplets from 2.12-2.46 ppm (Figure 1.3)[94]. In more severe head injuries, studies have shown glutamine synthetase (GS) activity, the enzyme that converts glutamate to glutamine, to be reduced due to increased oxidative stress, and that this inhibition in GS can lead to reduced glutamine[122,123], suggesting that glutamine levels could change post-concussion. However, most studies to date do not differentiate between glutamate and glutamine, but instead report the sum of these metabolites as Glx. In human studies of sport concussion, reductions in Glx/Cr have been observed[108,109], as well as increases[124].

Myo-inositol is a simple isomer of glucose that is synthesized in the brain from glucose-6-phosphate[125]. *Myo*-inositol was initially found in astrocytes and assumed to be a useful marker of glial proliferation[35,125]. However, *myo*-inositol is also involved in many biochemical and signaling pathways including synthesis of inositol-containing phospholipids and as a component of the secondary messenger system, the phosphoinositide pathway[125], which has been shown to be activated immediately following experimental rodent TBI[126]. *Myo*-inositol has resonances at 3.52, 3.61, and 4.05 ppm, as well as a resonance at 3.27 ppm that is obscured by the Cho resonance. An increase[109] and decrease[127] in Myo/Cr has been reported 6 months post-concussion, as well as elevated Myo in retired athletes[116,118].

Table 1.1: Summary of ^{1}H MRS visible metabolites

Metabolite	Function	Where it is synthesized	Relevance to Concussion	Other Notes
***N*-acetyl aspartate (NAA)**	Marker of neuronal health and integrity (*Bittsansky et al., 2012*) Storage substance for acetyl-CoA and aspartate, regulator of protein synthesis, source of acetyl group for lipid synthesis, catabolic product of NAAG, and osmolyte (*Moffett and Namboodiri 2006*)	Synthesized in the mitochondria of Neurons (*Bittsansky et al., 2012*) Exogenous sources of NAA do not cross the blood brain barrier, but NAA can be exported out of the brain via astrocytes (can be detected in blood and urine) (*Karaman et al., 2011; Prokesch et al., 2016*)	Is a sensitive tool to assess irreversible and temporary neuronal damage. (*Bittsansky et al., 2012*) Has been found to decrease after several studies of concussion (*see Table 1.2*)	Studies of diseases in which there is a loss of neurons or axons (infarctions, brain tumors, multiple sclerosis) confirm a decrease in NAA (*Barker and Lin 2006*). Decreases in NAA also reflect neuronal dysfunction, which can be reversible, opposed to neuronal death (*Bittsansky et al., 2012*)
Choline (Cho) *Glycerophosphocholine (GPC), Phosphocholine (PC) and a small amount of choline itself*	Neurotransmitter synthesis (acetylcholine), cell-membrane signaling (phospholipids) and lipid transport (lipoproteins) Major fate of choline is conversion to phosphatidylcholine, the predominant phospholipid in cell membranes (Zeisel et al., 2009)	Essential Nutrient; can be acquired from diet (crosses blood brain barrier) and de novo synthesis from phosphatidylethanolamine	Can detect changes in memrane turnover rate, such as demyelination after injury (Davie et al., 1993) Elevated levels have been found in retired athletes with a history of concussion (*see Table 1.2*)	Active demyelination causes an increase in tCho (*Davie et al., 1993*); likely due to the degradation of myelin phospholipids to GPC, or as a result of inflammation (*Brenner et al., 1993*).
Creatine (Cr) *Creatine and Phosphocreatine*	Phosphorylated to phosphocreatine which can be used as energy storage and can create ATP from ADP without oxygen consumption (*Bittsansky et al., 2012*)	Synthesized in Liver and transported to the brain (*Bittsansky et al., 2012*)	May be sensitive to energy supply and demand mismatch seen after a concussive injury	Since creatine is synthesized in the liver, its brain concentration would decrease during liver damage (*Barker and Lin 2006*).
Glutamate (Glu)	Is an excitatory neurotransmitter Up to 90% of neurons excrete glutamate during their excitation. (*Bittsansky et al., 2012*)	Synthesized predominantly from glutamine, but also from TCA cycle intermediates (*Purves et al., 2012*)	Could detect changes in the glutamate-glutamine cycle, as a result of altered neurotransmission	Most studies to date do not differentiate between glutamate and glutamine, but instead report the sum of these metabolites as Glx *At 3 T, glutamate and glutamine can be reliably separated (Provencher 1993; Srinivasan et al., 2005)*
Glutamine (Gln)	Most abundant amino acid in the CNS. Serves as a precursor to glutamate and as energy fuel (*Albrecht et al., 2019*)	Synthesized in astrocytes from glutamate and ammonia (*Albrecht et al., 2019*)	Could detect changes in the glutamate-glutamine cycle, as a result of altered neurotransmission	
GABA	Main inhibitory neurotransmitter in the brain (*Purves et al., 2012*)	Synthesized predominantly from glutamate, but can also be synthesized from glutamine and pyruvate (*Purves et al., 2012*)	Increased GABA/Cr has been observed 2 weeks post-concussion in the frontal region of the brain (*Friedman et al., 2017*)	Studies investigating GABA after concussion tend to specifically use MEGA-PRESS (*see Table 1.2*)
***Myo*-Inositol (Myo)**	Glial marker, osmolyte, synthesis of inositol-containing phospholipids, and component of phosphoinositide pathway (*Kim et al., 2005*)	Synthesized in brain de novo from glucose-6-phosphate and transported from blood (*Kim et al., 2005*)	Believed to represent glial proliferation (*Harris et al., 2012*) Phosphoinositide pathways have been implicated in TBI studies (*Lyeth et al., 1996; Chen et al., 2012*)	Primary source of *myo*-insoitol in neurons is from recycling of phosphoinositide pathway constituents (*Kim et al., 2005*)

Table 1.2: Review of MRS in Sport Concussions

Lab	ROI	Type of MRS	Study Designs	Sex	Sport	Main Finding	References
London, UK	Right Lentiform Nucleus	1.5 T STEAM (TR/TE/TM = 2270/270/12 ms)	Followed Ex-Boxers with Parkinsonian syndrome (n=3), idiopathic Parkinson's Disease (n=6), Age Matched Controls (n=6)	M	Ex-Boxers	↓ [NAA] in ex-boxers vs. Controls	Davie et al. 1995 *J of Neurology, Neurosurgery, and Psychiatry*
Catania and Rome, Italy	WM of Frontal Lobes (Bilateral)	3 T PRESS (TR/TE = 2000/144 ms) **Vagnozzi et al. 2010 used 1.5 and 3 T* (one centre used CSI with TE = 135 ms instead)	Mixed Concussed Athletes (n=13) scanned at 3, 15 and 30 DPI were compared to age-matched control volunteers (not specified as athletes or not)	M/F	Mixed; Skiing, soccer, kickboxing, rugby	↓ NAA/Cr at 3 and 15 DPI Compared to controls	Vagnozzi et al. 2008 *Neurosurgery*
			Concussed Athletes (n=40) scanned at 3, 15, 22, and 30 DPI, and heathy control subjects (n=30)	M/F	Mixed; soccer, rugby, horse riding, boxing, basketball, kickboxing, skiing, bike riding	↓ NAA/Cr and /Cho at 3 and 15 DPI Compared to controls **Values did not differ across MRI centres*	Vagnozzi et al. 2010 *Brain*
			Concussed Athletes (n=11) sanned at 3, 15, 30, 45 DPI, and healthy control subjects (n=11)	M/F	Mixed; Soccer, Skiing, Boxing, Baskteball, Rugby, Kickboxing	↑NAA/Cr at 3 and 15 DPI; ↓NAA/Cr at 30 DPI; ↓NAA/Cho at 3, 15, 30 DPI; ↑Cho/Cr 3, 15 DPI	Vagnozzi et al. 2013 *J Head Trauma Rehabil*
Montreal, Canada	Bilateral DLPFC, Hippocampus, M1	3 T PRESS (TR/TE = 1500/30 ms)	Concussed (n=12) to non-concussed (n=12) athletes scanned 1-6 DPI	M	Mixed	↓Glu/Cr and NAA/Cr 1-6 DPI	Henry et al. 2010 *J Neurotrauma*
	Bilateral DLPFC and M1		Concussed football players (n=10) and non-concussed (n=10) athletes scanned 1-6 DPI and at 6-months	M	American Football	↓Glu/Cr (M1) , NAA/Cr (M1&DLPFC) 1-6 DPI ↑Myo/Cr (M1)	Henry et al. 2011 *BMC Neurology*
	Corpus Callosum	**Chamard 2012 used TE=35 ms*	Hockey players were scanned pre- and postseason; concussed athletes were scanned 72hrs, 2 weeks and 2 months post injury	M/F	Varsity Hockey	↓NAA/Cr in non-concussed female athletes Post-Season	Chamard et al. 2012 *Neurosurg Focus*
	Bilateral DLPFC, M1, Hippocampus		Female athletes with concussion (n=10) and control female athletes (n=10) were scanned 7 months post-concussion	F	Mixed	↓Myo/Cr Hippocampus and M1 (7 Months)	Chamard et al. 2013 *Brain Injury*
	Dominant M1	*De Beaumont 2013 used TR=1200 ms*	Former university-level athletes (ages 51-75) with and without a history of concussion	M	Mixed	↓Glu; Concussed group had exacerbated declines in Glu compared to control	De Beaumont et al. 2013 *BMC Neurology*
	Bilateral PFC and MTL		Former athletes who sustained their last sports concussion >3 decades (n=15) prior to testing were compared with those with no history of TBI (n=15)	M	Hockey or Football	↑Myo , ↓Cho (L MTL) ↑Cho (R PFC)	Tremblay e al. 2013 *Cerebral Cortex*
	Bilateral DLPFC, M1, Hippocampus		Female concussed athletes (n=11) and female control athletes (n=10) were scanned 7-10 DPI and at 6 months	F	Mixed	↑Glx/Cr in concussed 6 months in DLPFC (trend) and M1 ↓NAA/Cr 6 months in controls athletes	Chamard et al. 2014 *J Neurotrauma*
	Left M1	3 T MEGA-PRESS (Edit off spectra summed to look at other metabolites)	Football players with a concussion history (n=16) and football players without (n=14) were scanned at a single session	M	Football	No changes but GABA and Glx not correlated in experimental group	Tremblay et al. 2014 *Clin Neurophysiol*
	Left M1 and PFC	3 T MEGA-PRESS, TE=68 ms (Edit off spectra summed to look at other metabolites)	Nonathletes (n=24), contact athletes (n=24), non-contact athletes (n=24) came in for a single session	M/F	Contact (Rugby, Soccer); Non-contact (Swimming)	↑Myo in contact (M1) ↓Glx in contact (DLPFC)	Lefebvre et al. 2018 *J Neurotrauma*
Pennsylvania, USA	Corpus Callosum	3D CSI (TR/TE=1510/135 ms; NA=1)	Concussed Athletes (n=28) and athletes with no history of concussion (n=20) were scanned with a week to 3 weeks of injury (were scanned 1, 2 or >3 weeks post injury)	M/F	Mixed	↓NAA/Cr and /Cho in genu (Greatest alterations observed in those recovering from 1st concussion)	Johnson et al. 2012 *J Neurotrauma*
Ohio, USA	Anterior cingulate gyrucs, Left DLPFC and Thalamus	3 T PRESS (TR/TE=3000/144 ms)	Concussed pediatrics subjects and control group (not specified as athletes)	M/F	Mixed; soccer, footbal, wrestling	no changes in NAA at any of the time points in any ROIs	Maugans et al. 2012 *Pediatrics*
Loma Linda, California	Corpus Callosum and Parietal WM	3 T MRSI 9 x 8 x 6 cm volume covering corpus callosum and midbrain; PRESS (TR/TE=1700/144 ms, NA = 1)	Concussed pediatric subjects (n = 15) and control group (n = 15, not specified if athletes)	M/F	Mixed; soccer, football, softball	↓NAA/Cr and /Cho in corpus callosum and in parietal WM region	Bartnik-Olson et al. 2014 *J Neurotrauma*

DPI, days post injury; DLPFC, dorsolateral prefrontal cortex; M1, motr cortex; PFC, prefrontal cortex; MTL, medial temporal lobe; AC, anterior commissure; PC, posterior commisure; PCG, posterior cingulate gyrus; ACG, anterior cingulate gyrus

Table 1.2: Review of MRS in Sport Concussions Continued

Lab	ROI	Type of MRS	Study Designs	Sex	Sport	Main Finding	References
Indiana, USA	DLPFC, M1	3 T PRESS (TR/TE=1500/30 ms)	Contact (n=34) and non-contact (n=10) athletes were scanned prior to and during their active season	M	Contact (High School Football); Non-contact (swimming, track and field, tennis)	↑Myo (DLPFC), Glx and Cr (M1) in contact at baseline; ↓Glx and Cho (M1), Cr (DLPFC & M1)	Poole et al. 2014 and 2015 *Developmental Neuropsychology*
			Collision athletes were scanned at pre-season, twice during the season, and at post-season (4-8 weeks) and 20-24 weeks post-season; Non-contact athletes were scanned twice during their season, 5-18 weeks apart	M/F	Collision (Male Football, Female Soccer); Non-contact (cross-country, swimming, track and field, basketball, softball)	Males: ↓Glx/Cr, ↑Cho/Cr (DLPFC) during season Females: ↑Glx/Cr (M1) in season and post-season	Bari et al. 2018 *Brain Imaging and Behavior*
New Mexico, USA	Above Lateral Ventricles, Parallel to AC&PC	3 T PRESS (TR/TE=1500/40 ms)	MMA Fighters were scanned in season and at a 1 year follow up; Healthy controls were scanned in season and at 3-6 months follow up	M	Mixed Martial Arts Fighters	↓NAA at 1 year follow up	Mayer et al. 2015 *J Neurotrauma*
Boston, USA	PCG	3 T L-COSY	Former NFL athletes (n=5) and male healthy controls (professionals with no history of concussion, n=5) were examined	M	Football	↑Cho, Glx, Phenylalanine, Fucose	Lin et al. 2015 *Alzheimer's Research & Therapy*
		3 T PRESS (TR/TE=2000/30 ms)	Retired soccer players (no history of concussion) were examined in comparison to age matched controls (non-contact)	M	Soccer	↑Cho/Cr, Myo/Cr	Koerte et al. 2015 *J Neurotrauma*
	Corpus Callosum (Splenium)	3T PRESS (TR/TE=2000/35 ms) *(Same Dataset at Chamard et al. 2012)*	Pre- and post-season scans of male and female varsity ice hocey athletes	M/F	Varsity Hockey	↓NAA (M/F), ↓Cho (M/F trend)	Panchal et al. 2018 *Frontier in Neurolgy*
	ACG and PCG, Left Parietal WM	3T PRESS (TR/TE=2000/35 ms)	Retired NFL Players, symptomatic vs asymptomatic	M	NFL Football	↓NAA (PWM)	Alosco et al. 2019 *Brain Imaging and Behavior*
Seattle, USA	PCG, Frontal Lobe	3 T MEGA-PRESS, TE=68 ms	Pediatric sport concussion compared to healthy controls (admitted to emergency for non-head related injuries)	M/F	Physical education, weight lifting, football, soccer, lacrosse, ultimate frisbee, skateboarding	↑GABA/Cr (Frontal), ↓GABA/Cr (PCG, trend)	Friedman et al. 2017 *American Academy of Neurology*
Newcastle, Australia	PCG, Parietal WM	3 T PRESS (TR/TE=2000/40 ms)	Retired Rugby Players and Matched Controls (No History of contact play or concussion)	M	Rugby	↓Glutathione (PCG)	Gardner et al. 2017 *Int J Sports Med*
Toronto, Canada	Left and Right Motor Cortex *(Values averged together)*	3 T STEAM (TR/TE/TM = 2000/30/10 ms)	Non-contact, contact, and collision athletes recruited from varsity teams (n = 65) were scanned during preseason	M/F	Mixed	↓NAA/Cr in collision compared to non-contact	Churchill et al. 2017 *Frontiers in Neurology*
London, Canada	Prefrontal White Matter	3 T PRESS (TR/TE=2000/135 ms)	Concussed Hockey players recruited from the banting division (n = 17) were scanned 24-72 hours and 3 months post-concussion, compared to non-concussed hockey players (n=26)	M	Hockey	↓Cho 3 months after concussion compared to controls	Manning et al. 2017 *Neuroloy*

DPI, days post injury; DLPFC, dorsolateral prefrontal cortex; M1, motr cortex; PFC, prefrontal cortex; MTL, medial temporal lobe; AC, anterior commissure; PC, posterior commisure; PCG, posterior cingulate gyrus; ACG, anterior cingulate gyrus

Table 1.3: Review of MRS Timepoints in Sport Concussions

	NonConcussed Group / Control Group					Concussed Group							
Reference	**In Seaon**	**During**	**Off Season**	**Retired**	**Non Athletes**	**Baseline**	**24-72 Hours**	**2 Weeks**	**1 Month**	**2 Months**	**3 Months**	**6 Months**	**Retired**
Davie et al. 1995 *J of Neurology, Neurosurgery, and Psychiatry*	-	-	-	IPD and Age matched		-	-	-	-	-	-	-	↓[NAA]
Vagnozzi et al. 2008 *Neurosurgery*	-	-	-	-	*"Control Volunteers"*	-	↓NAA/Cr (3 DPI)	↓NAA/Cr (15 DPI)	(30 DPI)	-	-	-	-
Vagnozzi et al. 2010 *Brain*	-	-	-	-	*"Control Volunteers"*	-	↓NAA/Cr (3 DPI)	↓NAA/Cr (15 DPI)	(22 and 30 DPI)	-	-	-	-
Vagnozzi et al. 2013 *J Head Trauma Rehabil*	-	-	-	-	*"Control Volunteers"*	-	↑NAA/Cr, Cho/Cr ↓NAA/Cho (3 DPI)	↑NAA/Cr, Cho/Cr ↓NAA/Cho (15 DPI)	↑NAA/Cr, Cho/Cr (30 DPI)	(45 DPI)	-	-	-
Henry et al. 2010 *J Neurotrauma*	-	No History of Concussion	-	-	-	-	↓Glu/Cr (M1), ↓NAA/Cr (M1&DLPFC) (1-6 DPI)		-	-	-	-	-
Henry et al. 2011 *BMC Neurology*	-	Nonconcussed Athletes	(18 months later)	-	-	-	↓Glu/Cr (M1), ↓NAA/Cr (M1&DLPFC) (1-6 DPI)		-	-	-	↑Myo/Cr (M1)	-
Chamard et al. 2012 *Neurosurg Focus*	Pre-Season	-	↓NAA/Cr (Females) Post-Season	-	-	Pre-Season	(72 Hours)	(2 weeks)	-	(2 months)	-	-	-
Chamard et al. 2013 *Brain Injury*	-	Female Athlete Control Group	-	-	-	-	-	-	-	-	-	↓Myo/Cr (M1 and Hippo) (7 Months)	-
De Beaumont et al. 2013 *BMC Neurology*	-	-	-	Mixed Athletes	-	-	-	-	-	-	-	-	↓Glu
Tremblay e al. 2013 *Cerebral Cortex*	-	-	-	No History of TBI	-	-	-	-	-	-	-	-	↑Myo , ↓Cho (L MTL) ↑Cho (R PFC)
Chamard et al. 2014 *J Neurotrauma*	-	↓NAA/Cr (2nd scan; 6 months)	-	-	-	-	-	(9 DPI)	-	-	-	↑Glx/Cr to 9 DPI (6 months)	-
Tremblay et al. 2014 *Clin Neurophysiol*	-	Glx and GABA do not correlate in experimental group	-	-	-	-	-	-	-	-	-	-	-
Lefebvre et al. 2018 *J Neurotrauma*	-	↑Myo in contact (M1) ↓Glx in contact (DLPFC)	-	-	*Non-athletes*	-	-	-	-	-	-	-	-
Johnson et al. 2012 *J Neurotrauma*	-	Normal Volunteer Student-Athletes with no mTBI History	-	-	-	-	-	↓NAA/Cr, /Cho (1-3 Weeks Post Injury)		-	-	-	-
Maugans et al. 2012 *Pediatrics*	-	-	-	-	"Healthy Controls"	-	(24-72 Hours)	(2 Weeks)	(1 Month or Greater)		-	-	-
Bartnik-Olson et al. 2014 *J Neurotrauma*	-	-	-	-	"Healthy Controls"	-	-	-	-		↓NAA/Cr, /Cho (2-12 months post-injury)		

IPD, idiopathic parkinson' disease; DPI, days post injury; Hippo, hippocampus; DLPFC, dorsolateral prefrontal cortex; M1, motr cortex; PFC, prefrontal cortex; MTL, medial temporal lobe; AC, anterior commissure; PC, posterior commisure; PCG, posterior cingulate gyrus; ACG, anterior cingulate gyrus; F, female; M, male

Table 1.3: Review of MRS Timepoints in Sport Concussions Continued

	NonConcussed Group / Control Group					Concussed Group							
Reference	**In Seaon**	**During**	**Off Season**	**Retired**	**Non Athletes**	**Baseline**	**24-72 Hours**	**2 Weeks**	**1 Month**	**2 Months**	**3 Months**	**6 Months**	**Retired**
Poole et al. 2014 and 2015 *Developmental Neuropsychology*	↑Myo (DLPFC), Glx and Cr (M1) in contact	↓Glx and Cho (M1), Cr (DLPFC & M1)	-	-	-	-	-	-	-	-	-	-	-
Bari et al. 2018 *Brain Imaging and Behavior*	Pre-Season	↓Glx/Cr, ↑Cho/Cr (M, DLPFC) ↑Glx/Cr (F, M1)	↑Glx/Cr (F, M1)	-	-	-	-	-	-	-	-	-	-
Mayer et al. 2015 *J Neurotrauma*	Baseline	↓NAA (2 Scans, 1 year apart) from baseline	-	-	*Healthy Controls (no changes)*	-	-	-	-	-	-	-	-
Lin et al. 2015 *Alzheimer's Research & Therapy*	-	-	-	-	*Healthy Controls (No History of concussion)*	-	-	-	-	-	-	-	↑Cho, Glx
Koerte et al. 2015 *J Neurotrauma*	-	-	-	-	*Retired (Non-Contact)*	-	-	-	-	-	-	-	↑Cho/Cr, Myo/Cr
Panchal et al. 2018 *Frontier in Neurolgy*	Pre-Season	-	↓NAA (M/F), ↓Cho (M/F trend)	-	-	-	-	-	-	-	-	-	-
Alosco et al. 2019 *Brain Imaging and Behavior*	-	-	-	-	*Asymptomatic Retired NFL Players*	-	-	-	-	-	-	-	↓NAA (PWM)
Friedman et al. 2017 *American Academy of Neurology*	-	-	-	-	Admitted to emerg for non-head related injuries	-	-	↑GABA/Cr (Frontal), ↓GABA/Cr (PCG, trend) (12 DPI)	-	-	-	-	-
Gardner et al. 2017 *Int J Sports Med*	-	-	-	-	*No History of contact play or concussion*	-	-	-	-	-	-	-	↓Glutathione (PCG)
Churchill et al. 2017 *Frontiers in Neurology*	↓NAA/Cr in collision vs non-contact	-	-	-	-	-	-	-	-	-	-	-	-
Manning et al. 2017 *Neuroloy*	-	Male Hockey Control Group	-	-	-	-	(24-72 hrs DPI)	-	-	-	↓Cho	-	-

IPD, idiopathic parkinson' disease; DPI, days post injury; Hippo, hippocampus; DLPFC, dorsolateral prefrontal cortex; M1, motr cortex; PFC, prefrontal cortex; MTL, medial temporal lobe; AC, anterior commissure; PC, posterior commisure; PCG, posterior cingulate gyrus; ACG, anterior cingulate gyrus; F, female; M, male

The spectroscopy acquired from mice in this thesis (Chapter 4) additionally quantifies taurine (Tau), lactate (Lac), and glutathione (GSH). Tables 1.4, 1.5, and 1.6 highlight single and repetitive closed head impact MRS findings from the literature, as well as the timing of these findings. The exact function of taurine is not known but it has been proposed to be an osmoregulator, play a role in modulating neurotransmission[94], and cell volume regulation[128]. Taurine has two triplets at 3.25 and 3.42 ppm, with the former overlapping with the total choline peak[94]. Animal models have found elevated Tau[129], and reduced Tau/Cr[128]. Lactate is the end product of anaerobic glycolysis[94], and has been shown to increase in more severe cases of TBI, due to hypoxic/ischemic conditions[130,131]. The lactate signal gives rise to a doublet resonance at 1.31 ppm and a quartet at 4.10 ppm[94]. Finally, glutathione is an antioxidant that is primarily located in astrocytes and its singlet at 3.77 ppm overlaps with glutamate. Reduced GSH has been observed in retired athletes with a history of concussion[132].

Moreover, Pascual et al. (2007)[129] observed elevated Tau, Glu, GABA, NAA, Myo, and Cr after injury using a weight drop model. Although, this study had a high mortality rate, indicating more severe injury than concussion. However, a later study by Singh et al. (2017)[130] also found elevated Glu, Myo, and Cr in a milder weight drop model. Additionally, Singh et al. (2017)[130] and Lyons et al. (2018)[133] both observed reduced NAA, Cr, and Cho, while only Singh et al. (2017) found reduced Gln and GABA. Another study found reduced Tau/Cr[128], and reduced NAA/Cr[107] in male rats after using a weight drop model. Moreover, even fewer studies to date have investigated the effects of repeated head injury[134,135]. Therefore, investigating concussion or the effects of repetitive impacts with MRS in animal models, remains relatively unexplored. Investigating repetitive CHI will help to delineate how the timing between impact and concussion history may affect an individual's metabolic profile.

Table 1.4: Review of Single and Repetitive Closed Head Impact MRS Studies

Single:	Reference	ROI	MRS / Study Design	Sex, Animal	Impact	Main Finding
	Pascual et al. (2007) *J Neurotruma*	Whole Brain, *ex vivo*	Divided rats into sham, 4 hours, 1, 2, and 3 Days post impact 8.4 T, ex vivo NMR	M, Rats	Weight Drop Model 450g/2m (High Mortality Rate)	3 Time course Patterns: (1) ↑Tau, Threonine, Gly at 24hrs (2) ↑Glu, GABA, Ala 24-48 hrs, (3) ↑NAA, Myo, Cr at 48 hrs
	Signoretti et al. (2009) *Mol Cell Biochem*	Whole Brain, *ex vivo*	Using HPLC* at 2, 6, 24, 48, and 120 hours post mTBI (n=6 in each group), compared to sham control *(*High-performance liquid chromatograhy on deproteinized whole brain extracts)*	M, Rats	Weight Drop Model 450g/1m Impact: Central portion of the skull	↓ Cr and NAA at 2, 6, 24, and 48 hours in mTBI group
	Schnieder et al. (2015) *J Neurotrauma*	Tissue Punches, *ex vivo* *(PFC, Hippocampus, Amygdala)*	Divded mice into sham or mTBI groups and were sacrificed before (naïve) or after a fear conditioning paradigm MRS: 11.7 T High-resolution magic angle spinning MRS	M, Mice	Closed Head Impactor (5mm diameter tip; 5 m/s, 1 mm depth, 100 ms dwell time) Impact: Midpoint between lambda and bregma	↑GABA/Cr in PFC at days 8 in mTBI ↓GABA/Cr in dorsal hippocampus and ↑Glu/Cr in ventral hippocampus at 25days Changes may be reflective of dysregulated excitatory and inhibitory neurotransmission (overlearning of conditioned fear and delayed extinction)
	Singh et al. (2016) *NMR in Biomedicine*	Cortex (1.7 x 4 x 3 mm) Hippocampus (2 x 4 x 3 mm)	Divided rats into sham, mTBI + repeat anesthesia, and mTBI no anesthesia MRS: 7 T PRESS (TR/TE=2500/20 ms, NA=512)	M, Rats	Weight Drop Model 450g/25cm Impact: Sagittal midway of brain	↓Tau/Cr in cortex 5 days post mTBI with or without anesthesia compared to control. ↓Glu/Cr in Injury combined with anesthesia in rat cortex
	Singh et al. (2017) *NMR in Biomedicine*	Hippocampus (2 x 4 x 3 mm)	Divided rats into mild and moderate injuries (baseline imaging) for *in vivo* MRS: same as Singh et al. 2016 *(Also divided another group of rats in control, mild and moderate for in vitro MRS)*	M, Rats	Weight Drop Model 450g/25cm (mild) and 450g/50cm (moderate) Impact: Sagittal midway of brain	↑Cho, Myo (Hippo) in moderate injury, no changes in mTBI using *in vivo* MRS (*several changes observed using in vitro, see Table 1.5*)
	Lyons et al. (2018) *J Neurotrauma*	Bilateral dorsal hippocampus (2 x 5.23 x 1.2 mm)	Divided mice into sham, 3 and 28 days post injury MRS: 7 T PRESS (TR/TE=1500/135 ms, NA=400); 2nd experiment used LASER (TR/TE=2500/21 ms, NA=200)	M/F Mice	Closed Head Impactor (5mm flat steel tip; 5m/s, 1mm depth, 100 ms dwell time) Impact: Midline impact (mediolateral 0mm, anteroposterior 1.5mm)	↓NAA, Cr, Cho at 3 Day post injury compared to sham
Repetitive:	**Reference**	**ROI**	**MRS / Study Design**	**Sex, Animal**	**Impact**	**Main Finding**
	Vagnozzi et al. (2005) *Neurosurgery*	Whole Brain, *ex vivo*	2 Repetitive impacts at 3 or 5 days apart (Group 1 and 2), single mild (Group 3), single severe (Group 4) and sham (Group 5) *(Using HPLC)*	M, Rats	Weight Drop Model 450g/1m (mild); 450g/2m (severe) Impact: Midline between bregma and lambda	↓NAA in all groups compared to sham (Reductions in 3-rmTBI and severe TBI were lower than sham mild/5day)
	Fidan et al. (2018) *ASN Neuro*	Hippocampi (2 x 2 x 2 mm)	Divided rat into sham, single mTBI, and rmTBI 7 T, PRESS (TR/TE=1800/40ms, NA=576)	M, Rats	Closed Head Impactor Model: 4 ms, 1 mm depth, 50 ms duration Impact: 1.8mm caudal to bregma, 3mm left of midline	rmTBI Group: ↓NAA/Cr (contra. & ips.; vs sham & mTBI), ↓Cho/Cr & Lip/Cr (ips.) ↑Myo/Cr (contra. & ips.) mTBI Group: ↓NAA/Cr (ips.), ↑Myo/Cr (contra. & ips.)

Tau, taurine; Gly, glycine; Glu, glutamate; Ala, alanine; NAA, N-acetyl aspartate; Myo, myo-inositol; Cr, creatine; mTBI, mild traumatic brain injury; rmTBI, repeated mTBI; PFC, prefrontal cortex; Hippo, hippocampus; contra, contralateral; ips, ipsilateral

Table 1.5: MRS Imaging Timepoints in Single Closed Head Injuries

Reference	Groups	Before CHI	2 Hours	3 Hours	4 Hours	1 Day	2 Days	3 Days	5 Days	1 Week	2 Weeks	1 Month
Pascual et al. (2007) *J Neurotruma*	Sham (n=4)	-	-	-	-	-	-	-	-	-	-	-
	Head Injured (n=4 at each time)	-	-	-	(9 Hours)	↑Tau,Thr, Gly, Glu, GABA, Ala	↑Glu, GABA, Ala, NAA, Myo, Cr	(3 days)	-	-	-	-
Signoretti et al. (2009) *Mol Cell Biochem*	Sham (n=6)	-	-	-	-	(Sacrificed)	-	-	-	-	-	-
	Head Injured (n=6 at each time)	-	↓NAA, Cr	-	↓NAA, Cr (6 Hours)	↓NAA, Cr	↓NAA, Cr	-	(120 Hours)	-	-	-
Schnieder et al. (2015) *J Neurotrauma*	Sham (n=12/10)	-	-	-	-	-	-	-	-	n=12 Sacrificed (Naïve)	Fear Conditioning Days 14-24	n=10 Sacrificed
	Head Injured (n = 10/6)	-	-	-	-	-	-	-	-	↑GABA/Cr in PFC (Naïve)		↓GABA/Cr in dhippo, ↑Glu/Cr in vhippo
Singh et al. (2016) *NMR in Biomedicine*	Sham Anesthesia (n=7)	/	-	-	/	/	-	/	/	-	-	-
	mTBI + Anesthesia (n=7)	/	-	-	/	/	-	/	↓Tau/Cr (Cortex)	-	-	-
	mTBI Only (n=7)	-	-	-	-	-	-	-	↓Tau/Cr (Cortex)	-	-	-
Singh et al. (2017) *NMR in Biomedicine* (n=5 at each timepoint)	in vivo mTBI	Yes	-	-	(4 Hours)	(Day 1)	-	-	(Day 5)	-	-	-
	in vivo moTBI	Yes	-	-	(4 Hours)	(Day 1)	-	-	↑Cho, Myo (Hippo)	-	-	-
	in vitro sham	-	-	-	-	-	-	-	-	-	-	-
	in vitro mTBI	-	-	-	↓BCAA, Gln ↑Cr, Glu, Myo, Tau	↓BCAA, Gln, Succ ↑Cr, Glu, Myo, Tau	-	-	↓BCAA, Gln, Suc	-	-	-
	in vitro moTBI	-	-	-	↓NAA, Succ ↑Acetate	↓BCAA, NAA, Succ, Glu ↑Myo, GABA	-	-	↓BCAA, GABA ↑Cr, Glu, Myo, Tau, Lac	-	-	-
Lyons et al. (2018) *J Neurotrauma*	Sham (n=13)	-	-	-	-	-	-	-	-	-	-	-
	mTBI (n=15)	-	-	-	-	-	-	↓NAA, Cr, Cho	-	-	-	(28 Days)

/ Timing of sham, if specified in study

- Groups not assessed at this timepoint

Ala, alanine; BCAA, branched chain amino acids; CHI, closed head impact; Cho, choline; Cr, creatine; Gln, glutamine; Glu, glutamate; Gly, glycine; dhippo, dorsal hippocampus; vhippo, ventral hippocampus; mTBI, mild traumatic brain injury; moTBI, moderate TBI; Myo, *myo*-inositol; NAA, *N*-acetyl aspartate; PFC, prefrontal cortex; Tau, taurine; Thr, theronine

Table 1.5: MRS Imaging Timepoints in Single Closed Head Injuries Continued

		Hit Paradigm						Imaging Paradigm		
Reference	**Groups**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Before CHI**	**2 Days**	**1 Week**
Vagnozzi et al. (2005) *Neurosurgery*	Sham (n=6)	/			/			-	(2 Days)	-
	Single Mild (n=6)	×						-	↓NAA	-
	Single Severe (n=6)	×						-	↓NAA (to sham and mild/5day)	-
	rmTBI 3 (n=6)	×			×			-	↓NAA (to sham and mild/5day)	-
	rmTBI 5 (n=6)	×					×	-	↓NAA	-
Fidan et al. (2018) *ASN Neuro*	Sham (n=8)	/	/	/				-	-	-
	mTBI (n=9)	/	/	×				-	-	↓NAA/Cr (ips.), ↑Myo/Cr (contra. & ips.)
	rmTBI (n=10)	×	×	×				-	-	↓NAA/Cr (contra. & ips.; vs sham & mTBI), ↓Cho/Cr & Lip/Cr (ips.) ↑Myo/Cr (contra. & ips.)

/ Timing of sham, if specified in study

× Timing of closed head impacts

contra, contralateral; Cho, choline; Cr, creatine; ips, ipsilateral; Lip, lipids; Myo, *myo*-inositol; NAA, *N*-acetyl aspartate; rmTBI, repeated mild traumatic brain injury

1.5.6 Animal Models of TBI and Concussion

As previously discussed, the effect of concussion on the brain has been largely studied using MRS in athletes participating in contact sports, and various changes in metabolites have been observed. The large variability in metabolite findings across the literature is likely due to the heterogeneous nature of concussion (e.g. impact site, force of impact, timing of imaging post impact, concussion history, sex, diet, etc.)[136]. Animal models can be utilized to minimize many confounding variables, allowing exploration of the mechanisms and relevance of specific metabolite changes after concussion[137].

To date, the majority of MRS animal models have studied moderate to severe traumatic brain injuries using fluid percussion injury[138–140], controlled cortical impact[141–144], or blast injuries[145,146], rather than mild TBI or concussion. Few animal studies to date have investigated a closed head impact (CHI) model of concussion, and these are summarized in Table 1.4 and Table 1.5. The majority of these single CHIs have used a weight drop model of 450g[107,128–130] with drop heights from 25-100 cm characterized as mild injuries, and >100 cm as moderate to severe, with high mortality rates. Closed-head controlled cortical impact models have also been used with 5mm diameter tips at 5-4 m/s at a depth of 1 mm being characterized as mild[133,134,147].

1.6 Thesis overview and objectives

This introductory chapter provided an overview of concussion and the human brain, including the neurometabolic cascade of events that occur at the molecular level following a concussion. Next, the main principles of in-vivo magnetic resonance spectroscopy were provided, including the acquisition and data analysis, followed by descriptions of the different brain metabolites measured by this technique, and their relevance in concussion. The overall objective of this thesis was to use MRS to explore metabolite changes in the prefrontal white matter of female contact athletes before and after concussion, then to replicate these findings in an animal model of concussion, to position future studies to probe the reasons for these changes, and how to use these changes as potential therapeutic targets.

Chapter 2 addresses the first objective by studying a team of female varsity rugby athletes over 5 seasons, collecting data at the beginning and end of season, as well as 24-72 hours, 3 months, and 6 months post-concussion. This study found a reduction in glutamine levels in female rugby athletes after concussion, and after a season of play.

Chapter 3 further explores the nature of this glutamine change by studying non-contact athletes and sedentary women, in comparison to contact athletes. This study demonstrated that glutamine changes, among other metabolite changes, exist in contact athletes likely as a result of the accumulation of sub-concussive impacts.

Chapter 4 translates these findings to an animal model that investigates metabolite changes in mice after repeated head injury, and similarly finds changes glutamine that suggest that the model used might be appropriate as a model of sub-concussive injury.

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Chapter 2

2 Reduced brain glutamine in female varsity rugby athletes after concussion and in non-concussed athletes after a season of play

Permission to reuse this article has been obtained. Please see Appendix B.

Schranz AL, Manning KY*, Dekaban GA, Fischer L, Jevremovic T, Blackney K, Barreira C, Doherty T, Fraser D, Brown A, Holmes J, Menon RS, Bartha R. *Reduced Brain Glutamine in Female Varsity Rugby Athletes after Concussion and in Non-Concussed Athletes after a Season of Play*. Human Brain Mapping 2018; 39(4):1489-1499. doi: 10.1002/hbm.23919

**Dr. Kathryn Y. Manning analyzed the DTI data and contributed to those written sections.*

The purpose of this study was to use non-invasive proton magnetic resonance spectroscopy (MRS) and diffusion tensor imaging (DTI) to monitor changes in prefrontal white matter metabolite levels and tissue microstructure in female rugby players with and without concussion (ages 18-23, n = 64). Evaluations including clinical tests and 3T MRI were performed at the beginning of a season (in-season) and followed up at the end of the season (off-season). Concussed athletes were additionally evaluated 24-72 hours (n = 14), three months (n = 11), and six months (n = 8) post-concussion. Reduced glutamine at 24-72 hours and three months post-concussion, and reduced glutamine/creatine at three months post-concussion were observed. In non-concussed athletes (n = 46) both glutamine and glutamine/creatine were lower in the off-season compared to in-season. Within the MRS voxel, an increase in fractional anisotropy (FA) and decrease in radial diffusivity (RD) were also observed in the non-concussed athletes, and correlated with changes in glutamine and glutamine/creatine. Decreases in glutamine and glutamine/creatine suggest reduced oxidative metabolism. Changes in FA and RD may indicate neuroinflammation or re-myelination. The observed changes did not correlate with clinical test scores suggesting these imaging metrics may be more sensitive to brain injury and could aid in assessing recovery of brain injury from concussion.

2.1 Introduction

A concussion is a brain injury caused by forces applied to the head or another part of the body, causing the brain to experience rapid rotational and translational accelerations [1]. Momentum from such forces can cause axonal stretching, cell membrane disruption, dysregulation in ion fluxes and uncontrolled neurotransmitter release [2,3]ultimately leading to mitochondrial oxidative dysfunction [4], inflammation and edema [5].

The National Collegiate Athletic Association Injury Surveillance Program has reported the concussion rate among student-athletes in 25 different sports to be 4.47 per 10,000 Athlete-Exposures (defined as one athlete participating in one practice or competition) overall, with some sports as high as 20 concussions per 10,000 Athlete Exposures [6]. It is likely that the true incidence is even higher as many concussions are not reported [6]. Although concussions can occur in many situations, including motor vehicle accidents, domestic violence, and slips and falls, athletes participating in contact sports have a high risk of sustaining a concussion due to the nature of the activity.

Currently, concussion diagnosis in sport is made clinically through assessment by a physician [7] based on symptomatology, often with the aid of the Sport Concussion Assessment Tool III (SCAT3) [8]. Such assessments are limited since they rely on athletes to voluntarily report symptoms that are often delayed. The difficulty in identifying when a concussion is sustained during a sporting event, and making the decision to remove the athlete from the event increases an athlete's risk for sustaining multiple concussions in a short period of time. Multiple concussions can induce second impact syndrome, which has been associated with rapid brain swelling, herniation and, in severe cases, death [9]. Repetitive head trauma in sports may also be linked to chronic traumatic encephalopathy, a neurodegenerative disease characterized by a specific distribution of phosphorylated tau in the brain [10]. Chronic traumatic encephalopathy has been found in individuals that have sustained multiple concussions [10] as well as in individuals without a history of concussion [11].

Axons are vulnerable to biomechanical stretching, which in concussion can lead to undulations and beading [12]. However, this diffuse axonal injury (DAI) is not easily

discernable with conventional CT or MRI [12]. Diffusion tensor imaging (DTI) provides a means of detecting DAI [13]. Along with DAI, a secondary chemical cascade of ion flux and altered neurotransmission follows, which can result in mitochondrial dysfunction and altered metabolism [13]. These events have the potential to manifest as a decrease in *N*-acetyl aspartate (NAA), an amino acid marker of neuronal integrity and mitochondrial function measured by proton (^{1}H) magnetic resonance spectroscopy (MRS) [14]**.** MRS can also measure choline (Cho), creatine (Cr), glutamate (Glu), glutamine (Gln), and *myo*-inositol (Myo). Metabolites such as glutamate and glutamine may also be altered following concussion since both are involved in neurotransmission and oxidative metabolism [15,16].

Over the past decade the effect of concussion on the brain has been studied by MRS in athletes participating in various sports including football [17], boxing [18], hockey [19] and others [20,21]. These studies have shown changes in metabolite levels (primarily decreases in NAA/Cr) in multiple brain regions, in both white and grey matter. Brain regions studied have included the motor cortex [22], dorsolateral prefrontal cortex [17,23], corpus callosum [24], and the white matter of the frontal lobes [25]. However, the majority of previous studies were cross-sectional, comparing concussed groups to control groups rather than longitudinally examining athletes prior to and after concussion. In addition, control groups were often athletes from other contact sports. Interestingly, several recent studies have reported metabolite changes in the brains of non-concussed athletes participating in contact sports suggesting such athletes are not ideal controls. For example, Chamard and colleagues (2012)[26] found decreased NAA/Cr in the corpus callosum in females *without* concussion after a season of hockey. Furthermore, Poole and colleagues (2014)[23] investigated changes in metabolite levels in grey matter in the motor cortex and dorsolateral prefrontal cortex in high school football players without concussions throughout a single season. Decreases in choline and creatine, and an increase in the sum of glutamate and glutamine (Glx) were found in the motor cortex (1-3 months into the season). Decreases in creatine and *myo*-inositol were subsequently found in the dorsolateral prefrontal cortex. These studies underscore the importance of utilizing *pre-concussion scans* in athletes to better assess metabolic changes post-concussion.

Additionally, most studies to date have focused on male athletes. Therefore, studies in female athletes are urgently needed. The objective of the current prospective cohort study was to measure changes in prefrontal white matter metabolite levels using a rigorous MRS protocol in female varsity rugby players after a season of play and up to six months following concussion. Based on previous literature, it was predicted that *N*-acetyl aspartate would decrease after a season of play in the non-concussed players. Immediately post-concussion it was hypothesized that *N*-acetyl aspartate would decrease, and *partially* recover by six months. It was also hypothesized that decreased *N*-acetyl aspartate would be associated with altered tissue diffusion after concussion, but not after a season of play in the non-concussed players. Additionally, it was predicted that changes in the imaging measures would persists beyond clinical assessments.

2.2 Materials and Methods

2.2.1 Participants

This study was approved by the University of Western Ontario's Health Sciences Research Ethics Board. Informed consent was obtained from each player prior to the start of each season. All participants in this study were university level athletes (21 ± 1.5 years old) recruited from a women's varsity rugby team over the course of four seasons. Forty-eight athletes participated in this study, 24 athletes played a single season, 14 played two consecutive seasons, nine played three consecutive seasons, and one played two seasons, interspersed with a season of no play.

The rugby season ran from September to the end of October, with training beginning at the end of August. August through September is referred to as the in-season time point throughout the study, and January through March is referred to as the off-season. There was an average of 14.5 games in a full rugby season, with four contact practices per week throughout the season. From October to April players continued to participate in tournaments and a weekly practice. For the detailed training schedule from August to April, please see Table 1. There were 20 documented concussions in 15 different athletes over the four seasons the team was followed. Only an athlete's first concussion was used in the analysis. Athletes were evaluated for a potential concussion

when self-reporting symptoms, or symptoms were noticed by the trainers, and a similar protocol for return to play was followed as outlined in McCrory et al. (2017)[27]. Participants included in the non-concussed group were concussion free for at least 10 months prior to their in-season scan, and were not diagnosed with a concussion while participating in this study. Participants with data from multiple seasons were treated as separate data sets. Each athlete was evaluated at the beginning of the season (in-season), and followed up at the end of season (off-season), after their last tournament (160 $\pm$ 39 days). Athletes that were concussed, and available to participate were additionally evaluated 24-72 hours post-concussion, and then again at three and six months post-concussion. Not all concussed athletes attended their scheduled visits or were available to participate at each follow-up time point if they had moved away. Please see Supplementary Table 2.1 for the complete concussion timeline on these 15 athletes. Each evaluation consisted of two clinical assessments, magnetic resonance imaging and MRS, and blood collection (data to be reported elsewhere). In total, we acquired 63 spectra at the beginning of season, 56 at the end of season (49 paired sets), 14 at 24-72hours post-concussion, 11 at three-months post-concussion, and eight at six-months post-concussion.

Table 2.1: Rugby Players Training Schedule

	Contact Practices *1 session=2hrs*	Weight Training *1 session=2hrs*	Light Practice	Games	Notes
August	2/day	-	-	3-4	2-Week Pre-Season Training Camp
September-October	4/week	1/week	-	1/week	Regular Season
November	-	-	-	1/day*	*National Championships (4 days)
December-April	1/week	3-4/week	3-4/week	1/month†	Contact practices focus on technical skill, light contact compared to regular season †1 day tournament per month (January-March)

2.2.2 Clinical Scores

Two standard clinical tests were used in this study. The first was the Sport Concussion Assessment Tool III (SCAT3)[8]. The SCAT3 is a standardized tool used for evaluating injured athletes (age 13 and older) on the sidelines. For the complete SCAT3 tests and procedures please see SCAT3 (2013). Briefly, it integrates the Glasgow coma scale (GCS) with the Maddocks Score, and includes a brief cognitive and physical evaluation. The GCS assesses an athlete's visual, verbal, and motor responses on a 15-point scale, with concussed individuals usually scoring around 14-15, and lower scores requiring medical attention. The Maddocks score consists of five simple questions to assess short-term memory. The cognitive and physical evaluation consists of questions to assess the athlete's orientation, concentration, balance and coordination, as well as short-term memory. The SCAT3 also documents the athlete's background information (medication, concussion history, etc.), as well as scores 22 different symptoms the athlete may be experiencing on a scale of 0 to 6, with a symptom severity score out of 132 (maximum points multiplied by number of symptoms). The second clinical test employed was the Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT)[28]. ImPACT is a computerized concussion evaluation system used by clinicians, and consists of four different sections. The first section asks for the athlete's demographic information and medical history. The second section records concussion history, current medication, and rates 22 symptoms on a 7-point scale. The third section consists of neurocognitive tests to evaluate the athlete, and the final section displays the athlete's results. The results are broken into six different composites, verbal memory, visual memory, processing speed, reaction time, impulse control, and total symptoms.

2.2.3 Magnetic resonance imaging acquisition

Siemens 3 T Magnetom Tim Trio and Prisma Fit MRI Scanners (Erlangen, Germany), both using a 32 channel head coil, were used for data acquisition. Anatomical images were acquired using a sagittal T_1-weighted magnetization-prepared rapid acquisition gradient echo sequence (TE/TR=2.94/2300 ms, flip angle=9^o, matrix size 256 x 256, FOV=256 mm x 240 mm, number of slices=160, slice thickness=1.22 mm). A rapid T2-

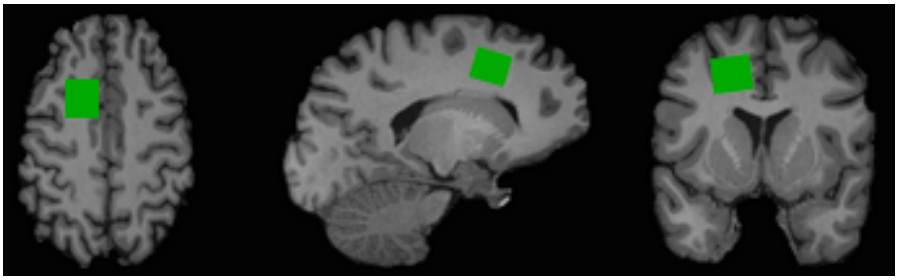
weighted FLAIR image was acquired to guide the placement of a 6 cm^3 (2 cm x 2 cm x 1.5 cm) voxel in the prefrontal white matter region of the brain (Fig. 1A) for the acquisition of the spectroscopy data (slices=50, TE/TR=139/15000 ms, slice thickness=3 mm, turbo factor=38, matrix size 256 x 256, FOV=256 mm, inversion time=2850 ms). Water suppressed (number of acquisitions = 192) and unsuppressed (number of acquisitions = 8) spectroscopy data were acquired using the PRESS (point resolved spectroscopy) pulse sequence (TE/TR = 135/2000 ms, dwell time = 833 μs, number of points = 1024). A long echo-time was chosen in the current study to reduce the error associated with quantification of the macromolecule baseline at shorter echo times. Improper quantification of the macromolecule baseline can greatly bias the measurement of glutamate and glutamine. A spin-echo echo-planar diffusion tensor imaging sequence (TE/TR = 79/7200 ms, matrix size = 98 x 98, FOV = 200 mm x 200 mm, number of slices = 64, slice thickness = 2 mm, $b_1 = 0$, $b_2 = 1000$ s/mm^2, gradient directions = 64, IPAT acceleration factor = 3) was used to examine tissue microstructure.

2.2.4 Magnetic resonance spectroscopy analysis

Spectra were processed and analyzed as previously described [29,30]. Spectra with a signal to noise ratio (SNR) less than 50 or water linewidth greater than 12 Hz were not included in the analysis. Signal to noise ratio was measured as the NAA peak height divided by the standard deviation of the noise. Briefly, spectra were lineshape corrected by combined QUALITY (Quantification improvement by converting lineshapes to the lorentzian type) deconvolution and eddy current correction [31] then fitted in the time domain using a Levenberg-Marquardt minimization routine [29] using prior knowledge of metabolic lineshapes (Fig. 1B). Analysis software created in our laboratory in the IDL (version 5.4 Research Systems Inc., Boulder, CO, USA) programming language was used to model the spectra using prior knowledge acquired from *in vitro* spectra obtained from aqueous solutions of metabolites at pH=7.0 prior to the study [29]. In the current study, we report absolute metabolite levels using unsuppressed water from the voxel as an internal standard as previously described in detail [29,30]. The calculation of absolute metabolite levels incorporated a correction to account for tissue partial volume (grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) voxel fractions) obtained by

segmenting the T_1-weighted anatomical images using the FMRIB (Functional MRI of the Brain) Software Library (FSL) tools (FMRIB, Oxford). The metabolite levels were also corrected for T_1 and T_2 relaxation related signal loss. The same relaxation time constants were used in all groups and were obtained from the literature from measurements made at 3 T. To allow for comparison to other studies, and to eliminate the uncertainty associated with partial volume correction from influencing the results, metabolite ratios relative to Cr were also calculated. The reproducibility of voxel placement within subjects, between time points was assessed by calculating the relative fraction of gray matter, white matter and CSF in the voxels, and by registering the follow-up anatomical images to the baseline images to quantify the voxel overlap using the Dice index [32].

A



B

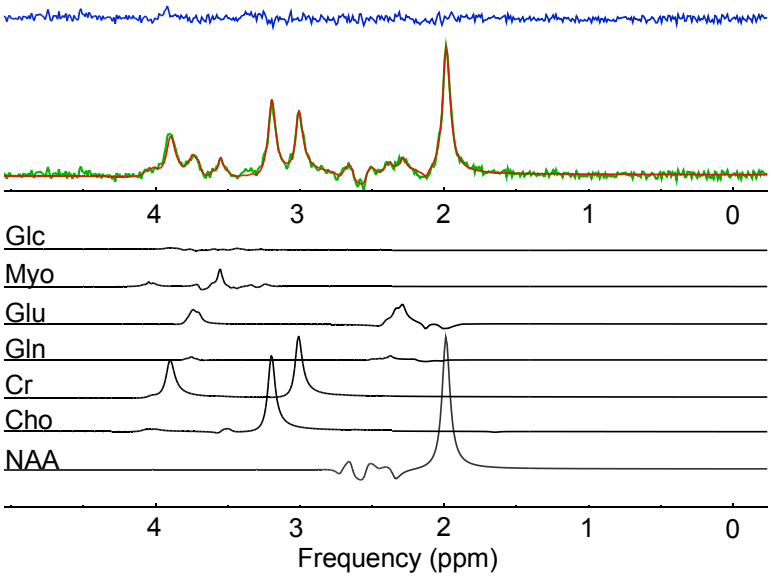


Figure 2.1: (A) From left to right; axial, sagittal, and coronal views of a T_1-weighted anatomical image with the spectroscopy voxel overlaid in green in the prefrontal region. (B) Spectrum acquired (green) from the voxel in A, reconstructed spectrum (red), the residual after fitting (blue), and the individual prior knowledge components of the spectrum shown below in black. Glc = Glucose; Myo = *Myo*-inositol; Glu = Glutamate; Gln = Glutamine; Cr = Creatine; Cho = Choline; NAA = *N*-acetyl aspartate; ppm = parts per million.

2.2.5 Diffusion tensor imaging analysis

Raw DTI data were eddy-current corrected and conservatively brain extracted using FSL tools (FMRIB, Oxford). A diffusion tensor was fitted at each voxel to create maps of fractional anisotropy (FA), mean diffusivity (MD), radial diffusivity (RD) and axial diffusivity (AD). These maps were then registered and transformed to standard space using the standard Montreal Neurological Institute (MNI) 2 mm atlas [33]. The MRS voxel varied slightly from person to person and from scan to scan. To ensure alignment to the preprocessed and registered DTI data, a slightly smaller volume (1cm × 1cm × 1cm) located in MNI space at x = 36, y = 68 and z = 51 that only included WM (GM and CSF masked) was used for analysis (Supplementary Fig. 1). The alignment with the MRS voxel and DTI volume was visually inspected for all datasets. The FA, MD, RD, and AD values were extracted from this voxel and correlated with the MRS-derived metabolite levels.

2.2.6 Statistical analyses

All statistical analyses were performed using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software, San Diego California USA). Metabolite levels were compared between the in-season and off-season in the non-concussed group (n=49) using a repeated measures two-tailed Student's t-test with an alpha value of 0.05. In the concussed group, data were not available for all athletes at all time points, eliminating the possibility of a repeated measures ANOVA. Alternatively, a one-way ANOVA was used to compare

each metabolite across all time points. Metabolites that changed over time were further examined to determine differences between the in-season and the 24-72 hours post-concussion (N=14), in-season and the three-months post-concussion follow up (N=11), and in-season and the six-months post-concussion follow up (N=8) using t-tests with Tukey's multiple comparisons correction. Additionally, the metabolites that changed over time were also examined using a repeated measures two-tailed Student's t-test with an alpha value of 0.05 in all available athletes to confirm differences between the mentioned time points.

The association between metabolite changes and clinical scores (i.e. SCAT3 and ImPACT), as well as between metabolite changes and DTI were examined with two-tailed Spearman correlations. An alpha value of 0.05 was used. Additionally, the effect of concussion history was assessed at the in-season time point using a two-tailed Student's t-test with an alpha value of 0.05. Athletes with no concussion history (Never concussed) were compared to those with a concussion history (Ever concussed). Finally, metabolite changes across two consecutive seasons (four time points, first and second in-season and off-season scans) were evaluated using a repeated measures ANOVA in non-concussed athletes (N=7 using absolute metabolite levels, N=8 using metabolite ratios). Metabolites that changed over time were then further examined using Tukey's multiple comparisons test with an alpha value of 0.05 to determine differences between an athlete's first in-season, first off-season, second in-season and second off-season time points.

2.3 Results

2.3.1 Concussion history

In the non-concussed group (n = 47), 28 reported no prior concussion history, 12 individuals reported having 1-2 prior concussions, 1 reported having 3 prior concussions, and 6 individuals did not provide a concussion history. In the concussed group, three reported no prior concussions, seven reported having 1-2 prior concussions, and one did not provide a concussion history. At the in-season time point no significant differences were found in any metabolite concentrations or DTI metrics between athletes with and

without a previous history of concussion. Supplementary Figure 2.2 compares imaging metrics as a function of concussion history for NAA ($p = 0.47$), Gln ($p = 0.11$), FA ($p = 0.07$) and RD ($p = 0.18$) values.

2.3.2 Quality assurance measures

The spectroscopy voxel was placed in the prefrontal region with mean (± standard deviation) tissue content: GM 20% ± 8%, WM 77% ± 9%, and CSF 3% ± 2%. The tissue composition of the voxel did not significantly change within subjects between time points. The voxel overlap between in-season and follow-up scans was 46% on average using the Dice similarity coefficient [32]. For all in-season spectra (n = 54), the average full-width at half maximum of the water peak was 6.7 Hz and the average signal to noise ratio was 92. Additionally, all spectra were visually inspected prior to statistical analyses for artefacts. No spectra were eliminated from the analysis due to artefacts or insufficient quality (SNR<50 or water linewidth > 12 Hz). Cramér-Rao Lower Bounds (CRLB) were calculated for all metabolites but not used to eliminate spectra to avoid bias selection [34]. For all in-season spectra (n = 54), the average CRLB for *N*-acetyl aspartate, choline, creatine, glutamate, glutamine, and myo-inositol were 0.65%, 1.4%, 1.4%, 4.6%, 35.5% and 7.9% respectively.

2.3.3 Participants

A total of N = 49 paired metabolite ratio data sets were collected in the non-concussed group. Absolute quantification could not be performed on two athletes due to missing data, leaving N = 47 paired data sets. One additional individual ended the scan prior to the acquisition of the DTI, leaving a total of N = 46 paired DTI data sets. Absolute quantification could not be performed on one 24-72 hours post-concussion data set due to incomplete data acquisition, leaving N = 13 data sets for that time point.

2.3.4 Clinical Data

The complete results of the SCAT3 and ImPACT will be reported in a subsequent manuscript focused on resting state fMRI changes in the rugby players. In the subset of 46 non-concussed rugby players with complete imaging data in the study, ImPACT

verbal memory ($p = 0.046$), ImPACT visual motor speed ($p = 0.0003$), and SCAT3 concentration ($p = 0.047$) all increased in the off-season compared to in-season. In the concussed group the SCAT3 symptom score ($p < 0.0001$) and symptom severity score ($p = 0.0008$) were higher 24 hours post-concussion compared to the in-season and returned to the in-season values at 3 months post-concussion (Supplementary Figure 2.3).

2.3.5 MRI data

No changes in *N*-acetyl aspartate were found in the concussed group (Figure 2.2A) or non-concussed group (Figure 2.2B). However, glutamine was significantly lower in the concussed group (Figure 2.2C, $F = 3.52$, $p = 0.02$), with a 52% decrease in the mean observed 24-72 hours post-concussion ($p = 0.04$) and a 56% decrease 3 months post-concussion ($p = 0.03$) compared to the initial in-season level. The repeated measures t-test using the subset of subjects with both baseline and post-concussion data yielded the same changes in glutamine 24-72 hours post-concussion ($N = 6$, $p = 0.02$) and 3 months post-concussion ($N = 5$, $p = 0.03$). A 21% decrease in glutamine was also observed in the *non-concussed* group in the off-season compared to the in-season (Figure 2.2D, $p = 0.01$). When examining metabolite ratios, a change in the Gln/Cr ratio was also found in the concussed group (Figure 2.2E, $F = 3.45$, $p = 0.03$), with a 58% decrease observed 3 months post-concussion ($p = 0.03$) relative to the initial in-season value. The repeated measures t-test using the subset of subjects with both baseline and post-concussion data showed a 48% decrease in Gln/Cr 24-72 hours post-concussion ($N = 6$, $p = 0.04$) and the same reduction 3 months post-concussion ($N = 5$, $p = 0.04$). A 25% decrease in Gln/Cr in the non-concussed group was observed in the off-season compared to in-season (Figure 2.2F, $p = 0.005$). No other metabolite changes were found.

No significant changes in FA within the MRS region of interest (ROI) were found in the concussed group (Figure 2.3A), however a small 3.8% increase was observed within the voxel in the non-concussed group in the off-season compared to the in-season (Figure 2.3B, $p = 0.01$). No significant changes in RD were found in the concussed group (Figure 2.3C), although a small 2.1% decrease in RD was observed in the non-concussed group in the off-season compared to the in-season (Fig. 3D, $p = 0.048$). No other significant changes were observed.

In a sub-set of athletes that played two consecutive seasons, Gln was significantly altered (Supplementary Figure 2.4A, F = 4.7, $p = 0.047$), with a 60% decrease in the mean observed at the second off-season in comparison to the first in-season ($p = 0.03$) and a 54% decrease in the mean observed at the second off-season in comparison to the first off-season ($p = 0.02$). Additionally, Gln/Cr was significantly altered (Supplementary Figure 2.4B, F = 4.8, $p = 0.04$), with a 54% decrease in the mean observed at the second off-season in comparison to the first in-season ($p = 0.002$) and first off-season ($p = 0.02$). Within the ROI, FA significantly changed (Supplementary Figure 2.4C, F = 6.4, $p = 0.02$) with an increase observed at the second off-season relative to the first in-season ($p = 0.006$). Similarly, RD significantly changed (Supplementary Figure 2.4D, F = 5.1, $p = 0.04$) with an increase observed at the second off-season relative to the first in-season ($p = 0.015$).

In the non-concussed group, a negative correlation was found between the change in Gln/Cr and the change in FA (Figure 2.4A, $p = 0.01$, r = -0.39) within the ROI. Additionally, a positive correlation was observed between the change in Gln/Cr and the change in RD (Figure 2.4B, $p = 0.002$, r = 0.45). Similar correlations were observed between Gln and FA ($p = 0.008$, r = -0.38, not shown), and Gln and RD ($p = 0.002$, r = 0.44, not shown) within the ROI. No correlations were found between changes in metabolite levels and clinical measures.

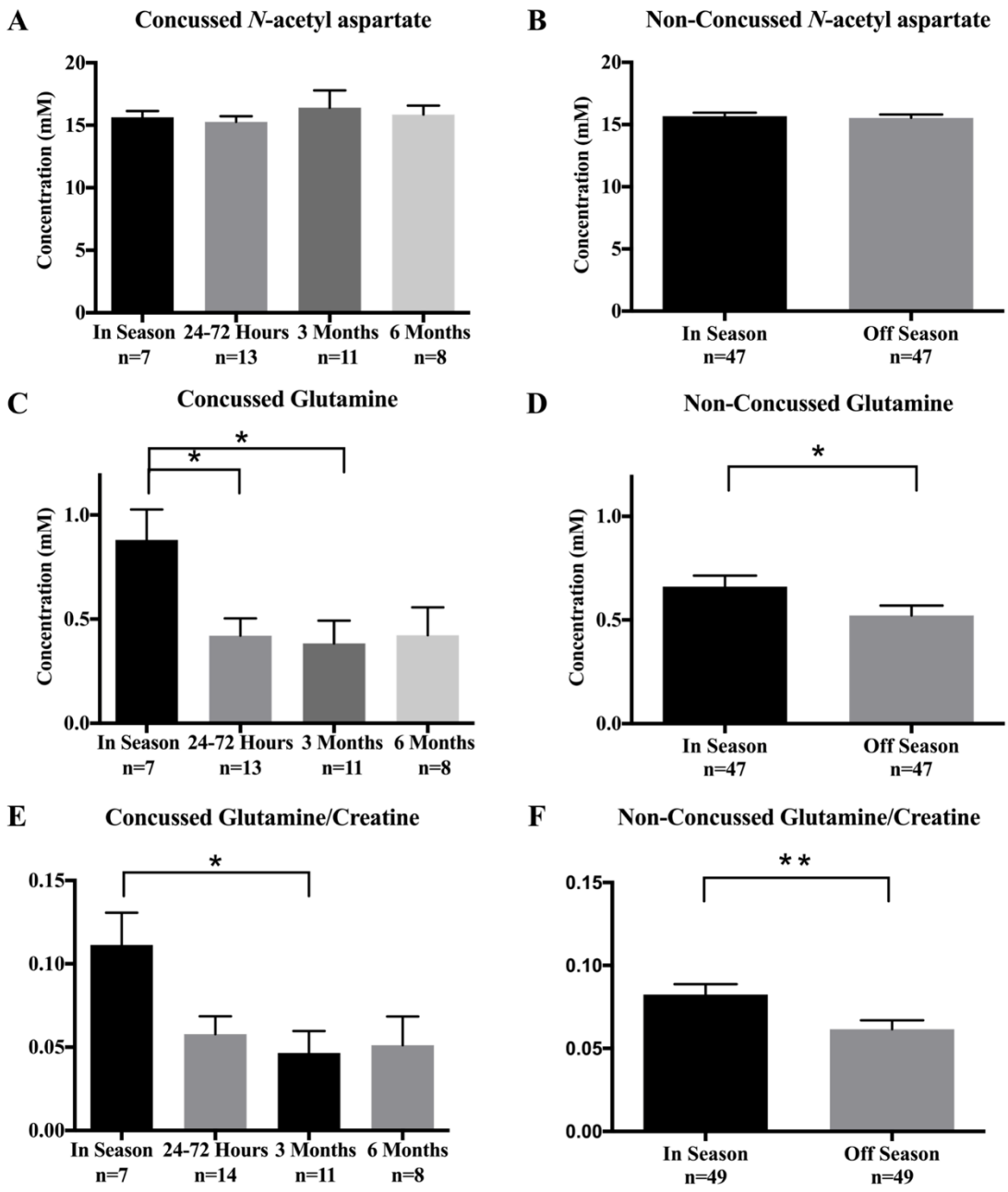


Figure 2.2: Bar graphs showing the mean concentration of *N*-acetyl aspartate (NAA), glutamine (Gln) and the glutamine/creatine ratio (Gln/Cr) in the concussed and non-concussed groups. Standard error of the mean (SEM) is represented by vertical bars. NAA levels did not change in the concussed (A) or non-concussed group (B). Gln levels (C) decreased in the concussed group ($F = 3.52$, $p = .02$) by 52% at 24-72 hours ($p = .04$) and by 56% at 3 months ($p = .03$). Gln levels in the non-concussed group (D) decreased

by 21% (p = .01). Gln/Cr (E) decreased in the concussed group (F = 3.45, p = .03) by 58% at 3 months (p = .03), and in the non-concussed group (F) by 25% (p = .005).

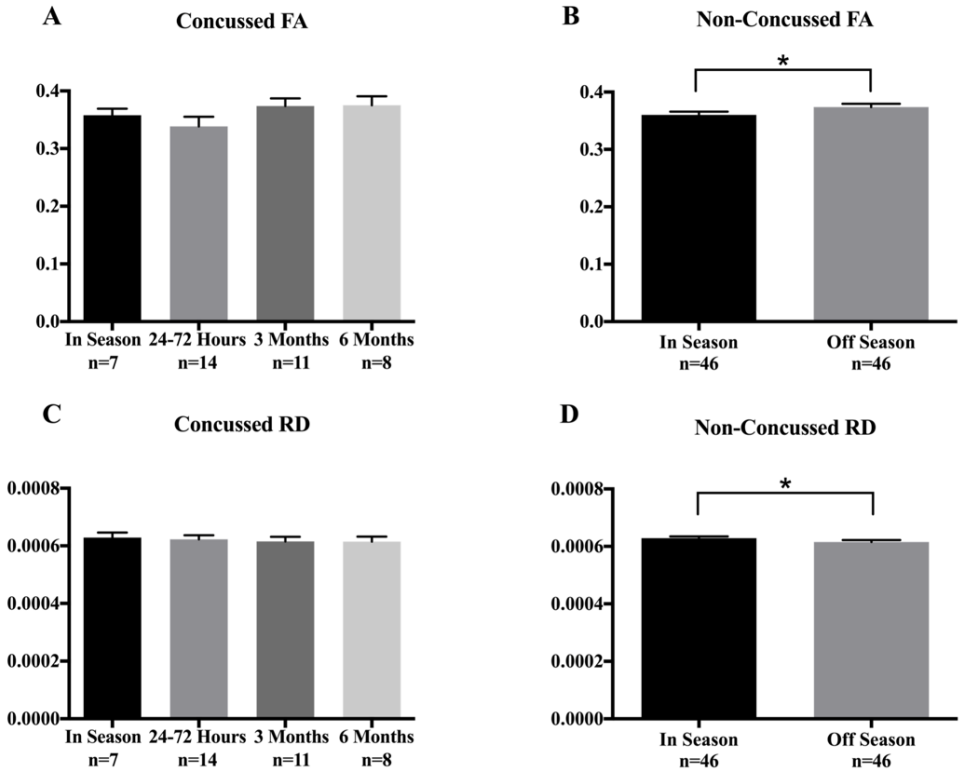


Figure 2.3: Bar graphs showing the mean Fractional Anisotropy (FA) and Radial Diffusivity (RD) values in the concussed and non-concussed groups found within the spectroscopy voxel. Standard error of the mean (SEM) is represented by vertical bars. Mean FA values in the concussed group (A) did not change (F = 1.41, p = .26), but increased (B) in the non-concussed group (p = .01). RD values in the concussed group (C) did not change (F = 0.14, p = .93), but decreased (D) in the non-concussed group (p = .05).

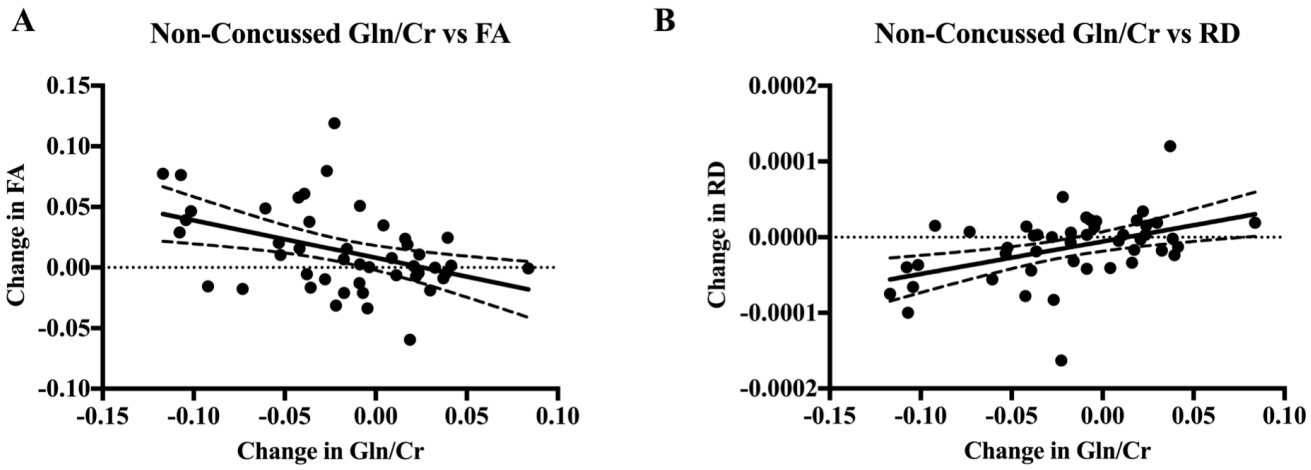


Figure 2.4: Correlations between the change in Gln/Cr and the (A) change in FA (p = .01, r = -.39), and (B) change in RD (p = .002, r = .45), plotted with 95% confidence bands.

2.4 Discussion

The purpose of this study was to examine white matter metabolite levels using MRS in female rugby players after a season of play and following concussion. Based on previous studies, it was originally hypothesized that NAA would decrease in both groups, however no changes in NAA were found. Rather, reduced Gln and Gln/Cr were observed in both the concussed and non-concussed groups. Within the same ROI, FA increased and RD decreased in the non-concussed group, and both changes correlated with the change in Gln and Gln/Cr. These trends were also observed in the sub-set of athletes that played two consecutive seasons concussion free.

2.4.1 *N*-acetyl aspartate and previous studies

In the current study, levels of NAA and Myo remained stable across all time points in both groups. This result was unexpected since previous studies have shown changes in these metabolites [35,36]. The lack of change observed in the current study may be due to a number of factors including differences in concussion severity between the current study and previous reports [36], differences in the age and sex of study participants [21,23], and differences in the timing and location of measurements. For example, a previous report examining more severe cases of traumatic brain injury [37], indicates that decreased NAA

and increased Myo may be indicative of a more severe head injury. Additionally, other studies have examined different regions of the brain [19,36] or voxels primarily in the grey matter [22]. Increases in Myo have also been reported within the first two weeks of brain injury [37]. Such changes may have been missed in the current study because we did not examine the same time points post-concussion. Changes in Myo, Cho, Cr and Glx have also been reported in males and in different age cohorts [21,23] compared to the current cohort. The methodological differences between studies makes it difficult to draw conclusions between potential sex differences in regards to concussion [38]. However, a meta-analysis by Dougan et al. (2014)[39] presented evidence that more severe deficits in neuropsychological functioning were seen acutely post-concussion (1-10 days) in female athletes in comparison to males. Alternatively, the study by Reynolds et al. (2017)[40] found no differences in the number or severity of head impacts between males and females in collegiate soccer. Taken together, these studies suggest that potential sex related differences are likely due to a range of physiological and metabolic differences, and not necessarily differences in the number or severity of head impacts [38].

2.4.2 Reduced glutamine and Gln/Cr

A large and significant reduction in Gln and Gln/Cr was found in both concussed athletes and non-concussed athletes in the off-season. The absolute Cr concentration did not significantly change, implying the decrease in Gln/Cr was due to the decrease in Gln. To our knowledge, no other human study has found significant changes in Gln post-concussion, since most ^{1}H-MRS studies do not quantify Gln alone, but instead quantify the sum of Glu and Gln as Glx. Past studies have found increases [35,37] and decreases [23] in Glx that could have been caused by changes in Gln. In the current study a decrease in Gln/Cr was observed, but Glu/Cr and Glx/Cr remained stable. The decrease in Gln and Gln/Cr observed in the days and months post-concussion in the current study is consistent with studies of rodent models using Carbon-13 spectroscopy that have found reduced Gln levels up to 24-hours post-injury [41]. A previous cross-sectional study of 13-14 year old hockey players using the same methodology as the current study also found a statistical trend towards reduced glutamine levels in the concussed athletes three months after concussion [42]. Since Gln is a by-product of glucose metabolism these results suggest that

the reduction in Gln may be the result of reduced glucose metabolism [41]. Another possible explanation for the observed change is that concussion and repetitive *sub-concussive* hits over the course of the season could alter oxidative metabolism in the brain, causing a shift in the glutamate-glutamine cycle, a major pathway for Gln in the brain. Specifically, the release of Glu and increased oxidation of Glu through the tricarboxylic acid cycle post-concussion [43] may decrease the amount of Glu that is converted to Gln, causing an overall decrease in Gln.

2.4.3 Non-concussed brain changes

In the non-concussed group, we also found a significant increase in FA and decrease in RD in the off season. These results suggest alterations in the white matter microstructure within the MRS ROI. Similar changes have been associated with neuroinflammation [44], as well as re-myelination [45]. However, neuroinflammation seems an unlikely explanation since no significant increase in Myo, a marker of increased glial cell activity, was observed.

Past studies have suggested that similar changes in DTI metrics may be the result of *sub-concussive* hits or undiagnosed concussions [46,47]. Additionally, a study by McAllister and Colleagues (2013)[48] that measured the effect of head impacts on diffusivity measures in athletes in contact versus non-contact sports found significant group differences in MD and FA, as well as a relationship between the magnitude and timing of head impacts and changes in white matter diffusion measures in various brain regions. Another study by Chamard and Colleagues (2012)[26] examined female varsity hockey players and found a decrease in NAA/Cr in the corpus callosum *in non-concussed players* and also attributed these changes to *sub-concussive* impacts. Furthermore, there have been several studies that have examined non-contact athletes, and found no changes in ^{1}H MRS or brain structure over time in athletes [23,49]. To our knowledge, no other study has examined associations between DTI and ^{1}H MRS measures within the same tissue region. Although small, significant correlations were found between the change in Gln/Cr and the change in FA and RD, as well as between the change in Gln and change in FA and RD. Additionally, the same changes in Gln, FA and RD were observed in the sub-set of athletes that played two consecutive seasons, further suggesting a relationship

between these metrics. These correlations suggest a potential relationship between alterations in metabolite levels and white matter integrity. Remyelination during the off-season is consistent with the observations of decreased Gln, increased FA, and decreased RD. The metabolic demand in oligodendrocytes increases during myelin synthesis [50]. Remyelination in the off-season could produce a shift in oxidative metabolism to favor lipid formation [50], leading to an overall decrease in the Gln substrate. Therefore, different mechanisms may be responsible for the decrease in Gln observed in the non-concussed and concussed groups. In the concussed group, the reductions in Gln with no change in DTI metrics suggests altered oxidative metabolism as described above. Whereas in the non-concussed athletes, the association between the change in Gln/Cr and the change in FA and RD over the course of a season are consistent with changes in tissue microstructure suggesting a remyelinating recovery process.

2.4.4 Clinical correlations

Consistent with previous studies [51] concussed players reported significantly more symptoms 24-72 hours post-concussion compared to in-season. At 3 months post-concussion these symptoms had recovered while the reduction in Gln and Gln/Cr persisted. This persistent reduction in Gln suggests that metabolic tissue changes due to concussion extend well beyond clinical recovery. It is possible that the recovery of Gln levels was delayed because the athletes continued to participate in contact sports. Examination of Gln levels in non-concussed athletes that played multiple seasons demonstrated a partial recovery between seasons suggesting Gln levels can recover. We found no association between Gln or Gln/Cr levels and clinical measures consistent with Mayer and colleagues (2015)[52] who found no association longitudinally between decreased NAA, and self-reported symptoms in MMA Fighters. Additionally, Sasaki and colleagues (2014)[44], who reported similar DTI findings to this study in hockey players, also reported no association with ImPACT or SCAT2 scores. However other studies have reported associations [22,46] between MRI and clinical data. It should be noted that in contrast to the concussed group, the non-concussed group had increased verbal memory, visual motor speed, and concentration scores in the presence of MRI changes. Although

the MRI changes correlated with each other, these metrics also did not correlate with the clinical changes.

2.4.5 Limitations and strengths

There are several limitations to the current study. For one, the placement of the voxel was performed manually at the time of the scan, limiting the reproducibility of voxel placement within subjects. However, the tissue partial volume did not differ, and the voxel was well within the prefrontal region. The effect of small shifts in voxel position is likely minimal since the affected white matter likely extends beyond the voxel. Although the athletes studied are at a greater risk of head injury, the number and magnitude of impacts throughout the season were not directly monitored, making it difficult to relate the observed changes directly to impact severity. Recording impacts in future studies will facilitate comparisons between studies. Additionally, in the non-concussed group the time between the off-season scan and the last game played was on average 97 ($\pm$ 25) days. However, weekly contact practices continued up to, and past, the off-season scan (Table 2.1), making it difficult to attribute changes to neuroinflammation or a recovery mechanism. There are also many WM tracts that overlap in the prefrontal region of the brain, limiting the interpretation of the DTI changes found within the ROI. Additionally, athlete compliance for attending concussion follow up scans was moderate, making it difficult to achieve high power in the concussed group. Due to the resultant low sample size, the reduced glutamine must be considered exploratory at this point. However, further research efforts directed at validating reduced glutamine post-concussion is warranted. Greater conspicuity may be achieved with a larger sample size, and use of spectral editing or ultra high-field MRI [53]. There was also no explicit control group in this study. A future study is now needed with the same experimental design to investigate what changes may be found in female athletes from a non-contact sport or non-athletes. Such a study will help elucidate the mechanism behind the unexpected metabolite and microstructure changes observed in the non-concussed group. Additionally, future work should include a similar study design with male rugby athletes to better elude to how differences in sex may affect outcome from concussion.

There are many strengths to this current study. First, we chose to study the prefrontal white matter bordering the cortex because past studies have shown this region to be susceptible to changes following concussion [22,23]. In addition, Bayly and colleagues (2005)[1] found that regions bordering grey and white matter experience the greatest shear forces during mild acceleration due to differences in tissue stiffness. Pre-concussion scans were also used. Repeated measures additionally detected the small change in Gln/Cr at 24-72 hours post-concussion in the concussed group. Since these metrics can vary greatly from subject to subject, an individual's pre-concussion measures can help identify small changes regardless of the inter-subject variability. Without the concussed athletes in-season data for comparison, the changes in the concussed group would not have been observed when compared against the non-concussed athletes in-season levels. For a biomarker to be clinically relevant for making the decision to return an athlete to play it must be sensitive to the structural, metabolic, or functional changes in the brain due to a concussion independent of symptoms. This highlights the importance of having pre-concussion measures before the start of a sports season, for self-comparison, rather than simply a comparison to an age matched control group.

2.5 Conclusion

To conclude, reduced Gln and Gln/Cr were found in the prefrontal region of the brain following concussion and in the off-season in non-concussed female varsity athletes. Within the same tissue region, increases in FA and decreases in RD were found in the non-concussed athletes. The decrease in Gln and Gln/Cr may suggest a reduction in oxidative metabolism, and the changes in FA and RD suggest neuroinflammation or re-myelination, both of which have been previously reported in concussion. The absence of any correlations with the clinical data in the presence of changes in metabolite levels and DTI measures demonstrates the insensitivity of current symptomatic diagnosis and emphasizes the need for alternative methods.

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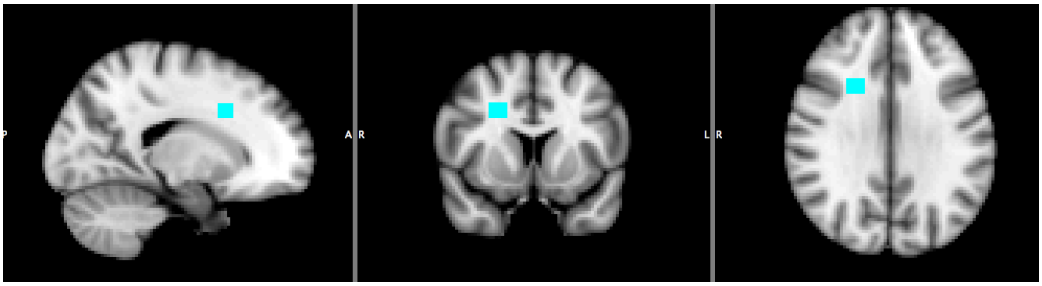
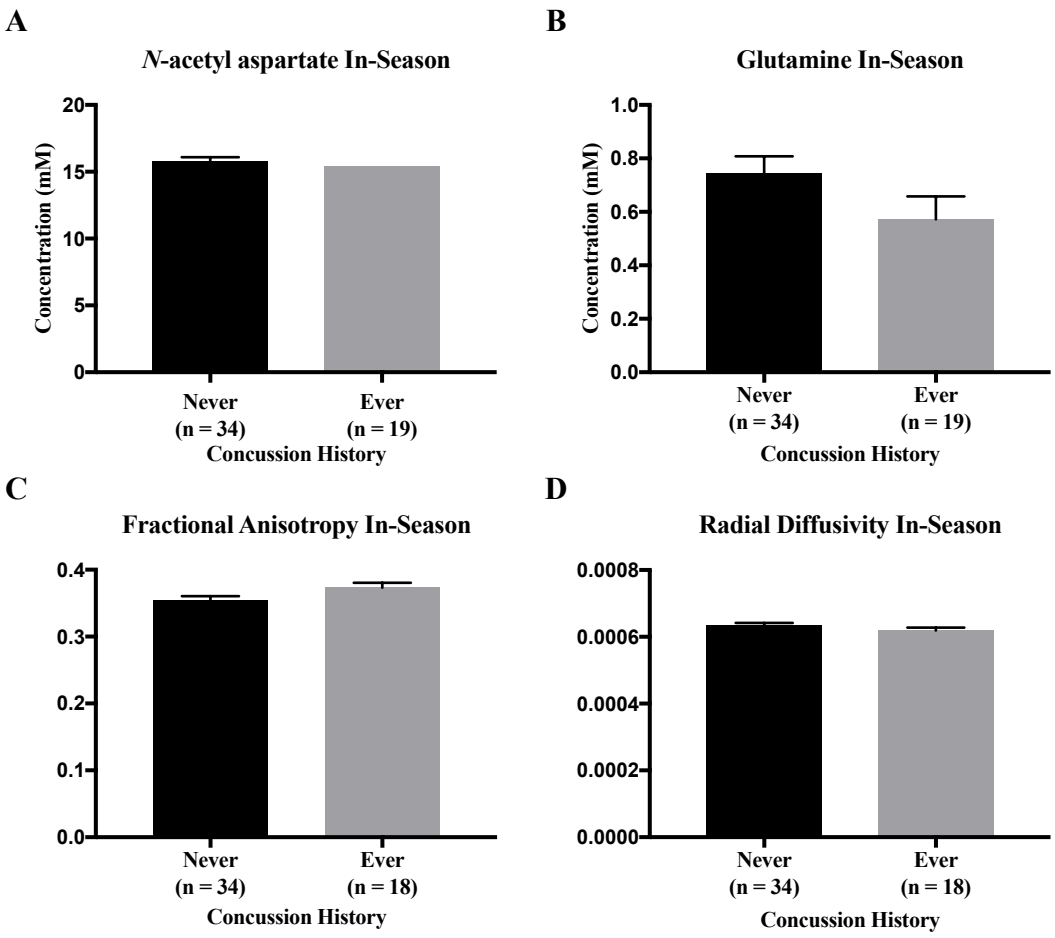
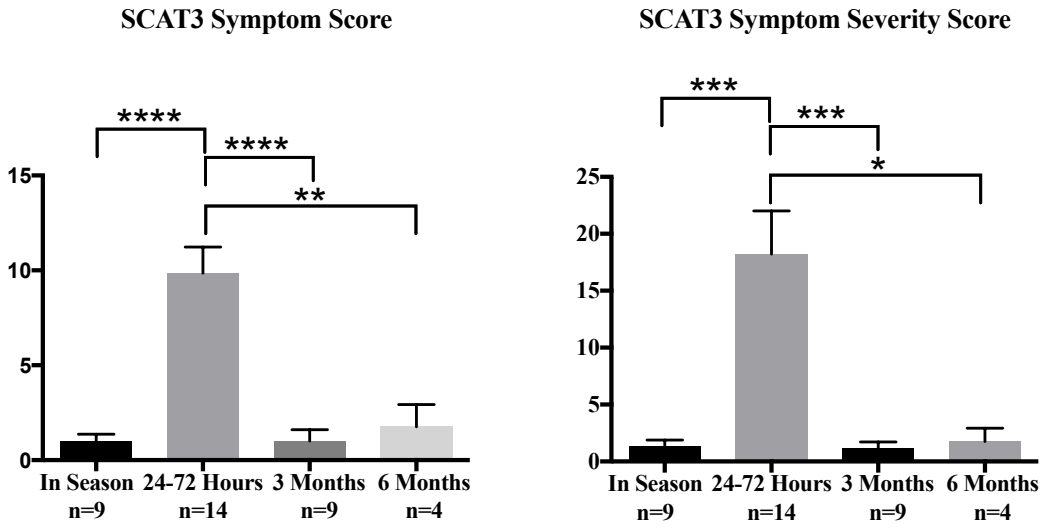
2.7 Supplemental Material



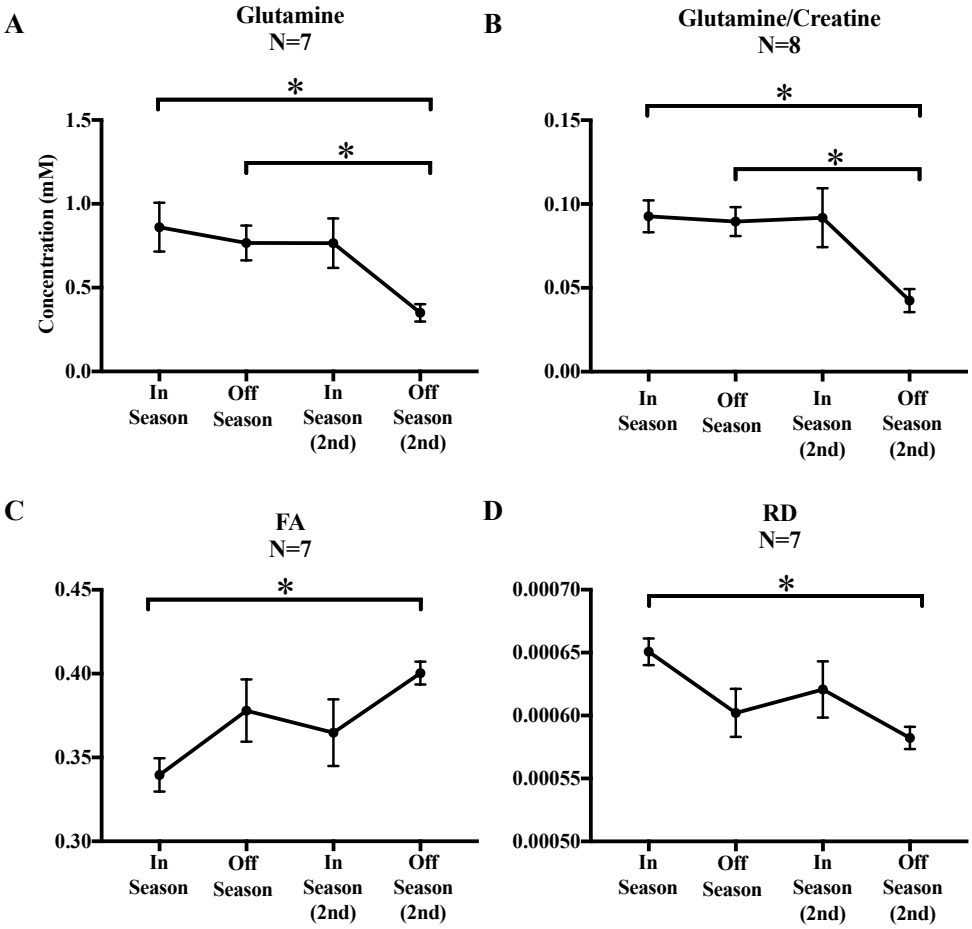
Supplementary Figure 2.1: From left to right; sagittal, coronal, and axial views of the mask used in MNI space at x = 36, y = 68 and z = 51 to extract FA, MD, RD, and AD values



Supplementary Figure 2.2: (A) *N*-acetyl aspartate ($p = 0.47$) and (B) glutamine ($p = 0.11$), (C) fractional anisotropy ($p = 0.07$), and (D) radial diffusivity ($p = 0.18$) of Never concussed and Ever concussed players at the in-season time point. Standard error of the mean (SEM) is represented by vertical bars.



Supplementary Figure 2.3: Bar graphs showing the mean SCAT3 Symptom Score and SCAT3 Symptom Severity Score in the concussed group. Standard error of the mean (SEM) is represented by vertical bars. The mean SCAT3 Symptom Score (A) significantly increased ($F = 17.6$, $p < 0.0001$) post-concussion ($p < 0.0001$), then recovered from post-concussion by 3 ($p < 0.0001$) and 6 ($p = 0.002$) months. The mean SCAT3 Symptom Severity Score (B) significantly increased ($F = 9.7$, $p = 0.0001$) post-concussion ($p = 0.0008$), then recovered from post-concussion by 3 ($p = 0.0007$) and 6 ($p = 0.02$) months.



Supplementary Figure 2.4: Line graphs showing the mean concentration of Glutamine (Gln) and Glutamine/Creatine (Gln/Cr), and Fractional Anisotropy (FA) and Radial Diffusivity (RD) in the same non-concussed (N = 7) athletes playing two consecutive seasons (Except Gln/Cr, N = 8). Standard error of the mean (SEM) is represented by vertical bars. Mean Gln levels (A) decreased (F = 4.7, $p = 0.047$) by 60% from the first in-season to the second off-season ($p = 0.03$), and by 54% from the first off-season to the second off-season ($p = 0.02$). Mean Gln/Cr levels (B) decreased (F = 4.8, $p = 0.04$) by 54% from the first in-season to the second off-season ($p = 0.02$), and from the first off-season to the second off-season ($p = 0.02$). The mean FA value (C) changed over time (F = 6.4, $p = 0.02$), with an increase between the first in-season and second off-season ($p = 0.006$), and the mean RD value (D) changed over time (F=5.1, P=0.04), with a decrease between the first in-season and second off-season (P=0.015).

Supplementary Table 2.1: Rugby Timeline of Concussed Athletes

		Athlete 1	Athlete 2	Athlete 3	Athlete 4	Athlete 5	Athlete 6	Athlete 7	Athlete 8	Athlete 9	Athlete 10	Athlete 11	Athlete 12	Athlete 13	Athlete 14	Athlete 15
	August															
Season 1	September	***In Season***	***Concussion***	In Season	In Season	In Season	In Season									
	October	***Concussion***														
	November															
	December		***3 Month***													
	January	***3 Month***														
	February															
	March			***Off Season***		Off Season	Off Season									
	April															
	May				Off Season											
	June		6 Month													
	July		Concussion	***Concussion***												
	August				***In Season***				***Concussion***							
Season 2	September			Concussion		***In Season***	***In Season***	***In Season***								
	October		3 Month		***Concussion***	Concussion	***Concussion***	***Concussion***								
	November								***3 Month***							
	December															
	January			3 Month	***3 Month***	***3 Month***	***3 Month***	***3 Month***								
	February		6 Month						***6 Month***							
	March															
	April			6 Month	***6 Month***	***6 Month***	***6 Month***	***6 Month***								
	May															
	June															
	July															
	August		In Season	In Season				In Season		In Season						
Season 3	September										***Concussion***					
	October		Concussion													
	November									***Concussion***						
	December										***3 Month***					
	January		3 Month							Concussion	Concussion					
	February															
	March															
	April		6 Month								3 Month					
	May									3 Month						
	June															
	July															
	August													***Concussion***		
Season 4	September									6 Month					***Concussion***	***Concussion***
	October											***Concussion***	***Concussion***			
	November															
	December														***3 Month***	
	January											***3 Month***				***3 Month***
	February															
	March														***6 Month***	
	April															***6 Month***

In Season, Off Season, Concussion, 3 Month, 6 Month ; Data used in analysis

Chapter 3

3 Brain Metabolite Levels in Female Rugby Athletes differ from Non-Contact Athletes and Sedentary Women

**Submitted July 18th, 2019 to the Journal of Neurotrauma*

Amy Schranz, Gregory A. Dekaban, Lisa Fischer, Kevin Blackney, Christy Barreira, Timothy J. Doherty, Douglas D. Fraser, Arthur Brown, Jeff Holmes, Ravi S. Menon, and Robert Bartha

The objective of this study was to investigate *in vivo* brain metabolite level differences between female varsity athletes engaged in contact and non-contact sports as well as sedentary women. These groups were chosen to examine the effects of long-term exercise and cumulative sub-concussive impacts on brain metabolites measured by proton magnetic resonance spectroscopy (MRS).

Single voxel MRS was acquired from the prefrontal white matter at the beginning (In-Season) and end (Off-Season) of season in contact (N=54) and non-contact (N=23) athletes. Sedentary women (N=23) were scanned once at a time equivalent to the Off-Season time point.

Metabolite levels in non-contact athletes did not change over a season of play, or differ from age matched sedentary women except that non-contact athletes had a slightly lower *myo*-inositol level. The non-contact athletes had different levels of *myo*-inositol, glutamate, and glutamine compared to levels previously found in contact athletes. Importantly, glutamine levels were significantly higher in contact athletes compared to the sedentary ($p < 0.001$) and non-contact ($p < 0.0001$) groups. Taken together, the measures from non-contact athletes and sedentary women do not demonstrate long-term exercise-induced changes in MRS measured metabolite levels. The differences in myo-inositol levels may reflect altered glial profiles in athletes. Altered glutamine levels as well as glutamate and myo-inositol between non-contact and contact athletes suggests that repetitive sub concussive impacts due to physical contact in high impact sports can

alter brain metabolite levels. This result underscores the need to use proper control groups in studies of concussion in high-impact sports.

3.1 Introduction

Athletes participating in contact sports are at a high risk of sustaining not just a single concussion, but multiple concussions, which can lead to structural and metabolic changes in the brain.[1] There is also growing concern that such injuries put athletes at an increased risk of neurodegeneration later in life.[2] These concerns may not be relevant in cases with rapid symptom resolution (e.g. 7-10 days), but may be of greater importance in the subset of athletes that develop post-concussion syndrome.[2] Relevant to this point, studies have shown that structural and functional brain changes persist beyond the time of symptom resolution.[3,4] However, the lack of correlation between existing clinical test scores and quantitative brain changes underlies the complexity of interpreting imaging findings related to sport concussion. Adding to this complexity are questions regarding the role of sex differences, the influence of repeated sub-concussive impacts on brain function and metabolism, and the use of proper control groups when evaluating the effects of concussion.

Although many different non-invasive imaging modalities have been used to study changes in the brain following concussion, magnetic resonance spectroscopy (MRS) provides neurochemical information that can be directly related to the known neurometabolic cascade.[5] MRS can be used to quantify specific metabolites relevant to concussion injury in a defined brain region of interest, including *N*-acetyl aspartate (NAA; neuronal integrity), creatine (Cr; energy metabolism), choline (Cho; phospholipid turnover and myelin), glutamate (Glu; neurotransmission and metabolism), glutamine (Gln; involved in neurotransmission and metabolism), and *myo*-inositol (Myo; glial marker).[6,7]

Numerous metabolite level changes have been reported in athletes following concussion in several different brain regions, primarily examining males.[8,9,18–20,10–17] Changes have been found, including reduced NAA in ex-boxers,[8] reduced NAA/Cr and Glu/Cr in concussed athletes (e.g. football players) compared to non-concussed [9,10] as

well as elevated Myo/Cr,[10] exacerbated Glu declines in former concussed athletes,[11] and changes in Myo and Cho in hockey and football players.[12] There have also been several studies that examined male and female athletes together.[21,22,31,23–30] Among these studies, reported changes included mostly reductions in NAA/Cr and NAA/Cho in the white matter of the frontal lobes,[21–23] elevated GABA/Cr in the frontal lobes of males and females,[31] as well as reduced NAA/Cr and NAA/Cho in the genu of the corpus callosum.[26] However, relatively few studies on female athletes have been reported.[4,32,33] In these studies, reduced Myo/Cr [32] and elevated Glx/Cr [33] (Glx = glutamate + glutamine) were found. The previous study by our group in female varsity rugby players,[4] found reduced glutamine following concussion compared to non-concussed rugby players, and decreased glutamine in the non-concussed players at the end of their season. However, this study did not include a group to control for an exercise effect across seasons of play.

Several previous studies measured metabolite changes in athletes during their athletic season, to investigate the effect of cumulative sub-concussive impacts.[13,14,16,25,28–30,34] In contact athletes, elevated Myo[25] and reduced Glx[14,25,34] have been reported, as well as reduced Cho, and Cr.[14,34] Moreover, elevated Glx/Cr,[28] and reduced NAA[16,29] has been observed after a season of play.

The interpretation of changes detected in previous studies depends to some degree on the control groups that are included. Many studies compare to an age-matched control group that are not athletes[21–23,27,35] or do not specify if they are athletes that participated in contact or non-contact sports,[9,10,26,31–33] leaving sub-concussive impacts as a potential confound in their interpretations. Studies have also demonstrated that the brains of athletes differ from sedentary controls, likely due to neuroanatomical adaptations and plastic changes in response to long-term training.[36,37] For example, a global increase in brain nonoxidative metabolism of carbohydrate substrates, potentially leading to changes in proton MRS findings, has been reported.[38] Specifically, studies have found increased lactate and Glx, as well as acute modulation of glutamate and GABA after vigorous exercise.[38,39] However, these studies investigated the acute effects of physical activity. How these changes translate into long term metabolite changes, such as over the course of a sports season, is not known. Perhaps closest to this paradigm is a study by Gonzales

and colleagues[40] who examined the effect of endurance training and found elevated NAA/Cr and Cho/Cr in the endurance trained group compared to normal healthy controls. However, this study included only athletes >40 years of age and did not report the duration of endurance training. Therefore, the effects of long-term exercise on MRS measured metabolites remains to be determined.

The main objective of the current study was to determine whether metabolite levels differed in female varsity rugby players (contact athletes) compared to female varsity rowers and swimmers (non-contact athletes) as well as sedentary females. The comparison of these groups allowed us to examine the effect of exercise on metabolite levels, as well as the effect of repetitive sub-concussive hits over the course of a season. Based on the results of previous studies, we hypothesized that there would be differences in NAA and Cho[40] between sedentary individuals and varsity athletes, but no differences in metabolite levels between athletes involved in non-contact sports (rowing and swimming), and contact sports (rugby).

3.2 Material and Methods

3.2.1 Participants

Sedentary and athletic females were recruited from the same university across three varsity sports teams, and studied at the same time during the beginning (In-Season) and end of season (Off-Season) to minimize sample heterogeneity. This study was approved by the University of Western Ontario's Health Sciences Research Ethics Board. Informed consent was obtained from each participant prior to the start of data collection. All participants in this study were university level athletes or students (age 18-30 years old). Athletes and sedentary recruits were required to be concussion free for at least 6 months to be included in the study. Athletes were recruited from the women's varsity rugby team over the course of five seasons, and women's varsity rowing and swim teams over a single season (for more details on these participants see Chapter 2 and Manning *et al.*, 2019).[4,41]

Briefly, sports seasons began at the end of August and early September (including tryouts). Rugby players had weekly contact practices in addition to weekly games until

November, followed by non-contact training until off-season data collection. Rowers trained six days a week with regular regatta competitions until November, and swimmers trained six days a week with monthly swim meets until March. In-Season data were acquired from the end of August to early September for all athletes, and Off-Season data were acquired from the end of January through February for rugby players and rowers, and in March for swimmers. Sedentary participants were scanned at a single time point from the end of January to end of March, to match with the Off-Season timepoint in athletes.

3.2.2 Clinical Measures

The SCAT3 (Sport Concussion Assessment Tool) was administered by a sports medicine physician at the In- and Off-Season timepoints for all athletes, and results are reported elsewhere.[41]

For the sedentary group, participants were recruited using the IPAQ (International Physical Activity Questionnaire, October 2002), a self-administered questionnaire that incorporates (1) job-related physical activity, (2) transportation physical activity, (3) housework, house maintenance, and caring for family, and (4) recreation, sport, and leisure-time physical activity, where each section is comprised of walking, moderate, and vigorous activity levels. Participants were asked to fill out the questionnaire based on their average routine over the course of the school semester (September 2018-January 2019), to match with the duration of the varsity sports seasons. This required participants to record their average time spent per activity outlined in each of the four categories. The raw time spent per activity was then weighted by its energy requirements defined in METs (METabolic equivalent; ratio of work metabolic rate to a standard resting metabolic rate) to yield a score of MET-minutes. A MET-minute is computed by multiplying the MET score of an activity by the minutes performed. The MET score used for walking, moderate, and vigorous activities were 3.3, 4.0, and 8.0 respectively.[42] Additionally, for transportation by cycling a MET score of 6.0 was used.[42] A threshold of 3000[43] MET minutes were used as the inclusion criteria for the sedentary group (A typical week of training for contact athletes would yield a MET score of approximately 5000).

3.2.3 Magnetic resonance imaging acquisition

Magnetic resonance spectroscopy data acquisition was identical to that reported previously.[4] Briefly, a Siemens 3T Magnetom Tim Trio and Prisma Fit MRI Scanners (Erlangen, Germany), both using a 32-channel head coil, were used for data acquisition. A rapid T_2-weighted FLAIR image was acquired to guide the placement of a 6 cm^3 (2 x 2 x 1.5 cm) voxel in the prefrontal WM region of the brain (Fig. 1A) for the acquisition of the spectroscopy data (slices = 50, TR/TE = 15000/139 ms, slice thickness = 3 mm, turbo factor = 38, matrix size 256 x 256, FOV = 256 mm, inversion time = 2850 ms). Water suppressed (192 acquisitions) and unsuppressed (8 acquisitions) spectroscopy data were acquired using the PRESS (point resolved spectroscopy) pulse sequence (TR/TE = 2000/135 ms, dwell time = 833 μs, number of points = 1024). A long echo-time was chosen in the current study to reduce the metabolite measurement error associated with the macromolecule baseline at shorter echo times.[44] Anatomical images for the estimation of voxel gray matter (GM), white matter (WM), and CSF volume were acquired using a sagittal T_1-weighted magnetization-prepared rapid acquisition gradient echo sequence (TR/TE = 2300/2.94 ms, flip angle = 98, matrix size 256 x 256, FOV = 256 x 240 mm, number of slices = 160, slice thickness = 1.22 mm).

3.2.4 Magnetic resonance spectroscopy

Spectra were processed and analyzed as previously described,[4] with absolute metabolite levels quantified using the approach described in Gasparovic and colleagues.[45] Spectra with a signal to noise ratio (SNR) <50 or water line width >12 Hz were not included in the analysis. SNR was measured as the NAA peak height divided by the standard deviation of the noise. Briefly, spectra were lineshape corrected by combined QUALITY (Quantification improvement by converting lineshapes to the lorentzian type) deconvolution and eddy current correction[46] and fitted in the time domain using a Levenberg–Marquardt minimization routine[47] using prior knowledge of metabolite line shapes (Fig. 1B). Analysis software created in our laboratory in the IDL (version 5.4 Research Systems Inc., Boulder, CO) programming language was used to model the spectra using prior knowledge acquired from *in vitro* spectra obtained from aqueous solutions of metabolites at pH 7.0 prior to the study.[47] Absolute concentrations are

reported using unsuppressed water from the MRS voxel as an internal standard as previously described.[47,48] This incorporates corrections to account for tissue partial volume (GM, WM, CSF) using the segmented T_1-weighted anatomical image, as well as corrections for signal loss due to T_1 and T_2 relaxation. The relaxation time constants used in this study are provided in Table 1. To eliminate the uncertainty associated with partial volume correction, and to compare to the literature, metabolite ratios relative to creatine (X/Cr) were also calculated.

3.2.5 Statistical Analysis

All statistical analyses were performed using GraphPad Prism Version 7.0 for Mac OS X (GraphPad Software, San Diego, CA). For each metabolite, the ROUT method was performed to remove outliers and the D'Agostino & Pearson normality test was used to test for normality. A two-way ANOVA, followed by two-tailed Sidak's multiple comparisons tests, was used to test for differences between rowers and swimmers at both measurement times, and to test for differences between contact (rugby) and non-contact (rowers and swimmers) athletes. A one-way ANOVA, followed by two-tailed Tukey's multiple comparisons test, was used to test for differences across contact, non-contact, and sedentary groups at the end of season. A *p*-value of 0.05 was used for all statistical tests.

Table 3.1 3 T Relaxation Constants used in Absolute Quantification*

Metabolite		Grey Matter		White Matter		Cerebrospinal Fluid	
	# of Protons	T_1(s)	T_2(ms)	T_1(s)	T_2(ms)	T_1(s)	T_2(ms)
N-acetyl aspartate	3	1.34	318	1.35	343	-	-
Choline	9	1.21	246	1.26	209	-	-
Creatine	3	1.34	158	1.36	159	-	-
Glutamine	5	1.17	134	0.98	134	-	-
Glutamate	5	1.27	167	1.17	143	-	-
Myo-inositol	6	1.17	221	0.98	195	-	-
Glucose	6	1.17	117	0.98	122	-	-
Water	2	1.46	95	0.94	75	4.3	503

**Values used were aeraged from values reported in the literature:*

Ethofer et al. (2003) *MRM*
Ganji et al. (2012) *NMR Biomed*
Harris et al. (2015) *J Magn Reson. Imaging*
Lu et al. (2005) *J Magn Reson. Imaging*
Mlynarik et al. (2001) *NMR Biomed*
Piechnik et al. (2009) *MRM*
Stanisz et al. (2005) *MRM*
Traber et al. (2004) *J MR Imaging*
Wansapura et al. (1999) *J Magn Reson. Imaging*
Wyss et al. (2018) *MRM*
Zhang e al. (2016) *MRM*

3.3 Results

3.3.1 Participants, Clinical Data, and Concussion History

Twenty-five participants were recruited to the sedentary group. Two participants were eliminated because one suffered a head injury prior to their scan, and another had a total MET-minutes >3000, leaving a total of N = 23 participants in the sedentary group. On average, participants had mean (± standard deviation) MET-minutes of 1252 ± 952. Of the 23 included participants, six reported having a previous concussion, 9.3 ±5.7 years prior to the start of the study.

A total of 31 athletes were recruited into the non-contact group from the Western varsity rowing and swim teams, with a total of 23 completing both scans at the In- and Off-Season time points. Of these 23 athletes, three reported having a previous concussion prior to the beginning of the study. For details on the 54 rugby athletes included in comparisons and associated concussion histories, please see Schranz *et al.*, 2018.[4] Additionally, the results of the SCAT3 scores for the contact and non-contact teams are reported in Manning *et al.*, 2019[41] and Schranz *et al.*, 2018.[4]

3.3.2 Quality assurance measures

All spectroscopy quality assurance measures calculated for the sedentary and non-contact groups did not differ significantly from the measures previously reported in the contact athletes.[4] The spectroscopy voxel was placed in the prefrontal region with mean (± standard deviation) tissue content in the sedentary group: GM 23±6%, WM 73±8%, CSF 3.5±2%, and non-contact group: GM 23±7%, WM 74±8%, CSF 2.7±2%. For all spectra from the sedentary and non-contact groups, the average full-width at half maximum of the water peak was 6.2 Hz and the average SNR was 92. All spectra and residuals were visually inspected prior to statistical analyses for artefacts and no spectra were eliminated from the analysis due to artefacts or insufficient quality (SNR <50 or water linewidth >12 Hz). Cramer-Rao Lower Bounds (CRLB) were calculated for all metabolites but not used to eliminate spectra to avoid bias selection.[49] For all spectra (n=184), the average CRLBs were 0.62% for *N*-acetyl aspartate, 1.5% for choline, 1.3% for creatine, 56% for glutamine, 4.2% for glutamate, and 6.9% for *myo*-inositol.

3.3.3 Magnetic Resonance Spectroscopy: Absolute Metabolite Concentrations

3.3.3.1 Non-contact Compared to Contact Athletes over a Season of Play

No significant metabolite level differences were found between the rowers and swimmers at either time point, so these athletes were combined into a single non-contact athlete group. No metabolite changes were detected over a season of play in this non-contact group (Fig. 2). There were no significant differences between contact and non-contact groups in levels of NAA (Fig. 2A; Two-way ANOVA, $F_{(1,75)} = 3.63$, $p = 0.06$), choline (Two-way ANOVA, $F_{(1,75)} = 0.99$, $p = 0.32$, data not shown) or creatine (Two-way ANOVA, $F_{(1,74)} = 1.9$, $p = 0.17$, data not shown).

Mean glutamate concentrations were found to be significantly different between contact and non-contact groups (Fig. 2B; Two-way ANOVA, $F_{(1,73)} = 11.67$, $p = 0.001$). Glutamate was lower in the contact group at the In-Season (Fig. 2B; Sidak's, $t_{146} = 3.36$, $p = 0.002$) and Off-Season (Fig. 2B; Sidak's, $t_{146} = 2.53$ $p = 0.025$) time points, although glutamate levels did not change between time points within either group. Additionally, mean glutamine concentrations were also found to be significantly different between contact and non-contact groups (Fig. 2C; Two-way ANOVA, $F_{(1,75)} = 33.06$, $p < 0.0001$), with higher concentrations in the contact group at the In- Season (Fig. 2C; Sidak's, $t_{150} = 5.6$, $p < 0.0001$) and Off-Season (Fig. 2C; Sidak's, $t_{150} = 4.1$, $p = 0.0001$) time points.

Mean *myo*-inositol concentrations were found to be significantly different between contact and non-contact groups (Fig. 2D; Two-way ANOVA, $F_{(1,69)} = 36.78$, $p < 0.0001$), with lower concentrations in the contact group at the In-Season (Fig. 2D; Sidak's, $t_{138} = 6.14$, $p < 0.0001$) and Off-Season (Fig. 2D; Sidak's, $t_{138} = 3.36$, $p = 0.002$) time points. Additionally, a significant interaction was found between groups (Fig. 3, Two-way ANOVA, $F_{(1,69)} = 5.01$, $p = 0.028$), where non-contact Myo levels tended to decrease over a season, while contact Myo levels tended to increase.

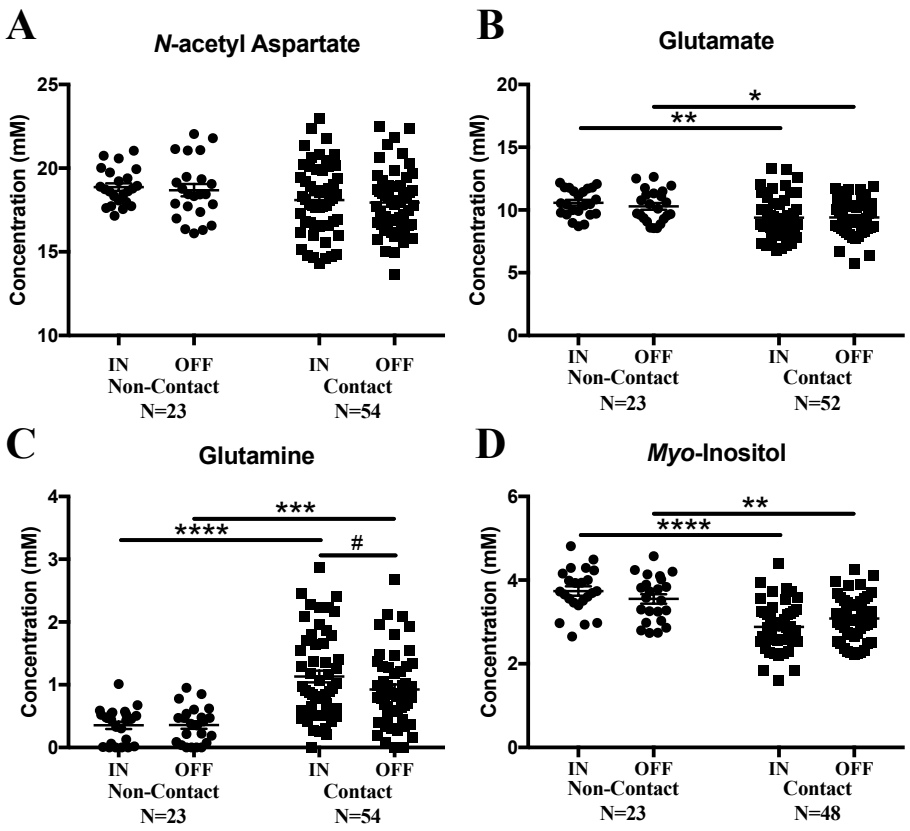


Figure 3.1: Measured concentration for (A)NAA, (B) *glutamate*, (C) glutamine, and (D) *myo*-inositol in the non-contact and contact group. Error bars represent the standard error of the mean. # represents the drop in glutamine levels previously reported in Chapter 2. *(p<0.0001****; p<0.001***; p<0.01**; p<0.05*)*

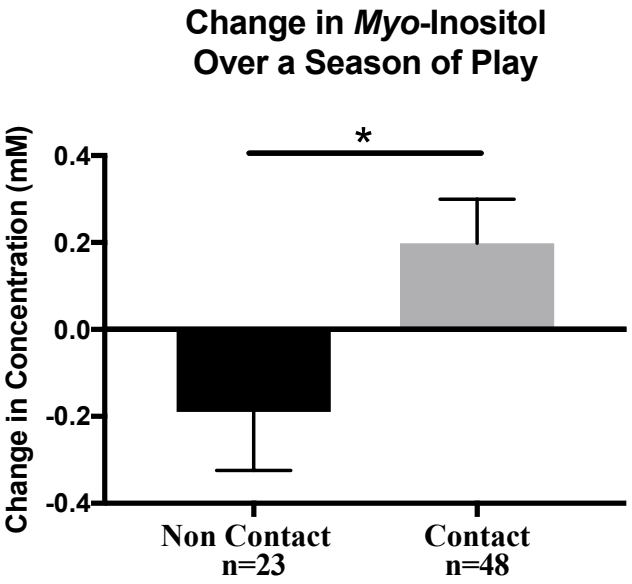


Figure 3.2: Change in *myo*-inositol over a season of play in non-contact and contact female athletes *(p<0.05*)*. Error bars represent standard error of the mean

3.3.3.2 Off-Season Athletes compared to a Sedentary Group

A one-way ANOVA was used to compare the contact Off-Season, non-contact Off-Season, and Sedentary females. The ANOVA revealed no significant differences among the three groups in NAA (Fig. 4A; $F_{(2,96)} = 2.91$, $p = 0.059$) or choline (Fig. 4B; $F_{(2,96)} = 1.43$, $p = 0.24$). However, significant differences were found in glutamate (Fig. 4C; ANOVA, $F_{(2,94)} = 9.39$, $p = 0.0002$), glutamine (Fig. 4D; ANOVA, $F_{(2,96)} = 15.5$, $p < 0.0001$), creatine (Fig. 4E; ANOVA, $F_{(2,95)} = 6.92$, $p = 0.0016$), and *myo*-inositol (Fig. 4F; ANOVA, $F_{(2,90)} = 21.02$, $p < 0.0001$) levels.

The contact group had significantly lower glutamate levels at the Off-Season compared to non-contact (Tukey's, $q_{94} = 3.82$, $p = 0.022$) and sedentary groups (Tukey's, $q_{94} = 5.72$, $p = 0.0003$), while glutamine levels were significantly elevated in the contact group compared to non-contact (Tukey's, $q_{96} = 6.68$, $p < 0.0001$) and sedentary groups (Tukey's, $q_{96} = 5.95$, $p = 0.0002$).

Creatine levels were significantly lower in the contact group compared to the sedentary group only (Tukey's, $q_{95} = 5.23$, $p = 0.001$), while *myo*-inositol levels were significantly lower in the contact group compared to the non-contact (Tukey's, $q_{90} = 4.75$, $p = 0.0033$) and sedentary groups (Tukey's, $q_{90} = 8.95$, $p < 0.0001$). No differences were found between the sedentary group and non-contact group, except for lower *myo*-inositol levels (Fig. 4F; Tukey's, $q_{90} = 3.69$, $p = 0.028$) in the contact group.

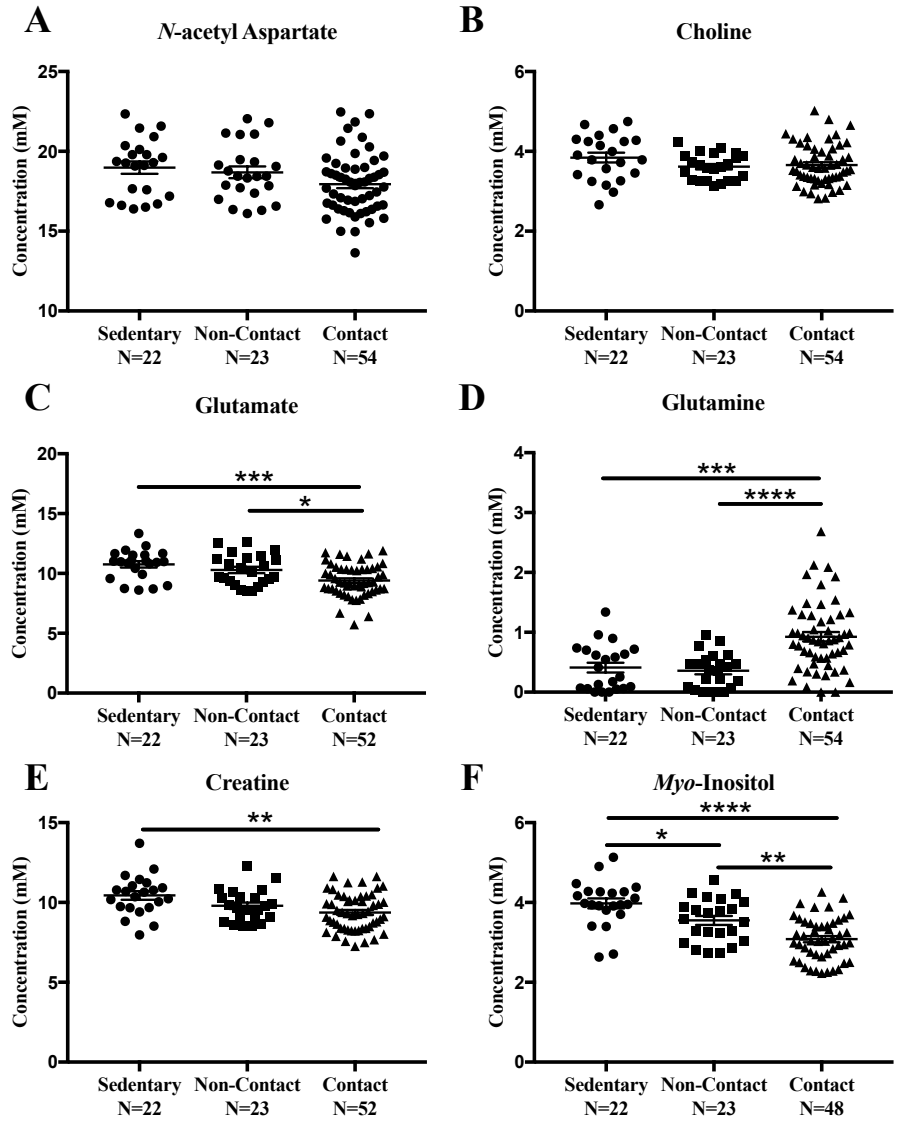


Figure 3.3: Measured concentration for (A) NAA, (B) choline, (C) glutamate, (D) glutamine, (E) creatine and (F) *myo*-inositol across groups. Error bars represent the standard error of the mean *(p<0.0001****; p<0.001***; p<0.01**; p<0.05*)*

3.3.3.3 Glutamine levels post-concussion: Group Comparisons

Due to the large differences in glutamine levels observed between contact and non-contact athletes in the current study, we chose to compare glutamine levels in the subset of contact athletes that obtained a concussion. Specifically, N = 13 contact athletes obtained a concussion, and seven of them had baseline scans (e.g. an In-Season scan prior to their concussion). Glutamine levels from 24-72 hours after concussion in our contact group and baseline levels (previously reported in Schranz *et al.*, 2018[4] as significantly reduced glutamine levels) were compared to the non-contact and sedentary groups (Fig. 5). A one-way ANOVA revealed significant differences across the 4 groups ($F_{(3,61)} = 15.22$, $p < 0.0001$). First, concussed contact group glutamine baselines measures were significantly higher than non-contact (Tukey's, $q_{61} = 8.87$, $p < 0.0001$) and sedentary groups (Tukey's, $q_{61} = 8.42$, $p < 0.0001$). Second, 24-72 hours post-concussion glutamine levels were significantly *higher* than non-contact levels (Tukey's, $q_{61} = 3.9$, $p = 0.037$), but significantly *lower* than contact baseline measures (Tukey's, $q_{61} = 5.28$, $p = 0.0023$, as previously reported). No significant differences were found between sedentary and post-concussion glutamine levels (Tukey's, $q_{61} = 3.36$, $p = 0.092$).

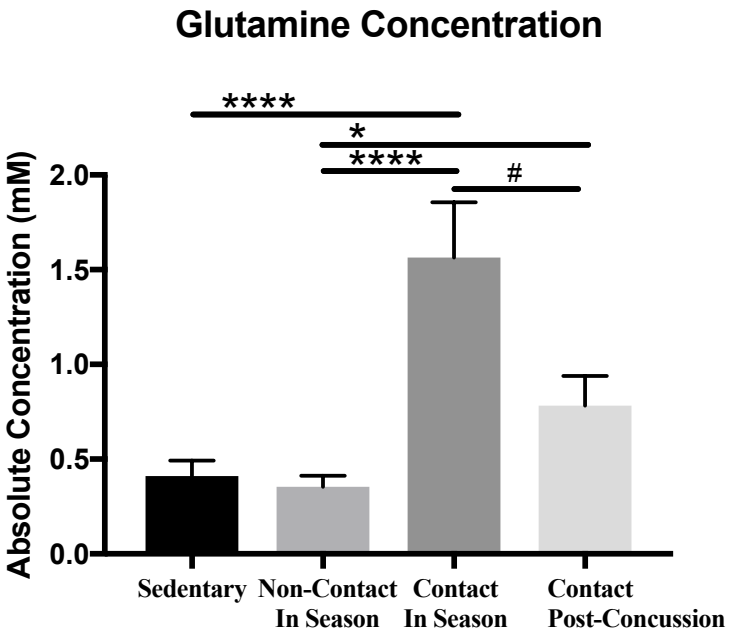


Figure 3.4: Measured glutamine concentration across groups. Error bars represent the standard error of the mean. # represents the decrease in glutamine levels previously reported between baseline and post-concussion in rugby athletes in Chapter 2. *($p<0.0001$****; $p<0.05$**)*

3.3.4 Magnetic Resonance Spectroscopy: Amplitude Ratios

3.3.4.1 Non-contact versus Contact Athletes over a Season of Play

No metabolite ratio changes were found over a season of play in the non-contact group (Supplementary Fig. 1). A two-way ANOVA found no significant differences between contact and non-contact groups in NAA/Cr (Supplementary Fig. 1A; $F_{(1,74)} = 0.059$, $p = 0.8$), while mean Cho/Cr was significantly different between athlete groups (Supplementary Fig. 1B; $F_{(1,73)} = 10.05$, $p = 0.0022$), with significantly higher Cho/Cr in the contact group at the In-Season time point (Sidak's, $t_{146} = 3.38$, $p = 0.0019$). Mean Myo/Cr were found to be significantly different between contact and non-contact groups (Supplementary Fig. 1C; $F_{(1,71)} = 14.19$, $p = 0.0003$), with lower concentrations in the contact group at the In-Season (Sidak's, $t_{142} = 3.82$, $p = 0.0004$). Mean Glu/Cr was found to be significantly different between contact and non-contact groups (Supplementary Fig.

2D; $F_{(1,74)} = 10.7$, $p = 0.0016$), with a lower ratio in the contact group at the In-Season (Sidak's, $t_{148} = 3.43$, $p = 0.0016$). Additionally, mean Gln/Cr were found to be significantly different between contact and non-contact groups (Supplementary Fig. 2E; $F_{(1,74)} = 41.87$, $p < 0.0001$), with a higher ratio in the contact group at the In-Season (Sidak's, $t_{148} = 6.18$, $p < 0.0001$) and Off-Season (Sidak's, $t_{148} = 4.45$, $p < 0.0001$).

3.3.4.2 Off-Season changes compared to a Sedentary Group

A one-way ANOVA revealed no significant differences among the three groups in NAA/Cr (Supplementary Fig. 2A; $F_{(2, 95)} = 2.82$, $p = 0.065$), Cho/Cr (Supplementary Fig. 2B; $F_{(2, 95)} = 2.94$, $p = 0.058$), or Glu/Cr (Supplementary Fig. 2D; $F_{(2, 95)} = 2.099$, $p = 0.13$). However, significant differences were found among Myo/Cr (Supplementary Fig. 2C; $F_{(2, 94)} = 10.29$, $p < 0.0001$) and Gln/Cr (Supplementary Fig. 2E; $F_{(2, 95)} = 19.06$, $p < 0.0001$).

The contact group had significantly elevated Gln/Cr compared to non-contact (Tukey's, $q_{95} = 7.23$, $p < 0.0001$) and sedentary groups (Tukey's, $q_{95} = 6.77$, $p < 0.0001$), while Myo/Cr was significantly lower in the contact group compared to the non-contact (Tukey's, $q_{94} = 3.84$, $p = 0.021$) and sedentary groups (Tukey's, $q_{94} = 6.04$, $p = 0.0001$). No differences were found between the sedentary group and non-contact group.

3.3.4.3 Glutamine/creatine changes post-concussion: Group comparisons

Supplementary Fig. 3 shows the results of the one-way ANOVA across the 4 groups ($F_{(3,60)} = 20.57$, $p < 0.0001$). First, concussed contact Gln/Cr baselines measures were significantly higher than non-contact (Tukey's, $q_{60} = 10.23$, $p < 0.0001$) and sedentary groups (Tukey's, $q_{60} = 9.95$, $p < 0.0001$). Second, post-concussion Gln/Cr levels were significantly higher than non-contact levels (Tukey's, $q_{60} = 4.33$, $p = 0.017$) and sedentary groups (Tukey's, $q_{60} = 3.98$, $p = 0.033$), but significantly lower than baseline measures (Tukey's, $q_{60} = 6.24$, $p = 0.0002$).

3.4 Discussion

The objective of this study was to investigate brain metabolite level differences between female varsity athletes engaged in contact and non-contact sports as well as sedentary individuals, to examine the long-term effects of exercise and sub-concussive impacts. We found that *myo*-inositol, glutamate, and glutamine levels differ at both In-Season and Off-Season time points between contact and non-contact female varsity athletes. Furthermore, metabolite levels did not differ between non-contact athletes and age matched sedentary women, except that non-contact athletes had lower levels of *myo*-inositol. The current study also demonstrates that contact athletes had *higher* baseline glutamine levels compared to non-contact athletes and sedentary women. This unexpected finding suggests that the interpretation of glutamine changes previously reported post-concussion must be made carefully considering the control group used. For example, the glutamine levels previously reported post-concussion[4] were *higher* than that observed in non-contact athletes, but lower than baseline measures in the same athletes. This emphasizes the need to include proper control groups in studies of concussion.

3.4.1 Non-contact versus Contact Teams over a Season of Play

No changes were measured from In- to Off-Season in non-contact athletes. This suggests that previously reported reductions in glutamine and Gln/Cr observed in contact athletes from In- to Off-Season[4] is not the result of an exercise effect. Instead, it is more likely that the previously reported reduction in glutamine and Gln/Cr is caused by the repetitive impacts experienced by athletes throughout the season. To confirm that the contact athletes were indeed receiving high impacts, a head impact sensor that measured linear acceleration and rotational velocity was worn by a subset of players during a preseason rugby game.[41] During this single rugby game, a total of 151 impacts exceeding 15g were recorded across 26 players. Therefore, on average, a single player received six impacts >15g per practice, and two impacts >15g per game. For the detailed results on the impact data, please see Manning *et al.*, 2019.[41] It is important to recognize that previous studies reporting higher impact rates used a threshold of >10g,[50,51] rather than the >15g used in this cohort of athletes. Additionally, it has been shown that 45% of head impacts land between 10-15g,[52] meaning that there is a large portion of impacts not included in the

current analysis. Taken together, the female rugby athletes experience sub-concussive impacts throughout their season, and these impacts likely contribute to the reduction in glutamine and Gln/Cr, as well as the other major metabolite level differences observed in comparison to the non-contact and sedentary groups, further discussed below.

A significant interaction in *myo*-inositol levels was observed between contact and non-contact athletes. Even though *myo*-inositol levels are significantly lower in contact athletes at the In- and Off-Season timepoints, it appears that *myo*-inositol tends to increase over the season in contact athletes but decrease over the season in non-contact athletes (although, not significantly). A recent study by Lefebvre et al (2018)[25] examining male and female athletes found increased *myo*-inositol in contact athletes (rugby and soccer), compared to non-contact (swimming) and non-athletes in the motor cortex. This is opposite to our finding of lower *myo*-inositol levels at In- and Off-Season on contact athletes, but in line with our observed *myo*-inositol interaction between the groups. Furthermore, studies of retired contact athletes, found higher *myo*-inositol levels in the medial temporal lobe[12] and in the posterior cingulate gyrus.[18] This suggests that over time, *myo*-inositol levels may continue to rise in athletes playing contact sports.

3.4.2 End of Season Changes Compared to a Sedentary Group: Exercise versus Impacts

We found no metabolite differences in the prefrontal white matter between sedentary and non-contact athletes, asides from a marginal reduction in *myo*-inositol in non-contact athletes. However, there were many differences between the contact group and the sedentary and non-contact groups. This further suggests that the contact nature of the sport is having a cumulative effect on the brain, as described by others.[16,25,53] Although we saw no significant changes in NAA in the current study, trends towards reduced NAA in the contact athletes were observed ($p < 0.1$). Other studies have reported reduced NAA in male mixed martial arts fighters,[16] and in female and male athletes without concussion.[24,29,33] Moreover, Chamard and colleagues[24] found reduced NAA/Cr in female athletes only, but with subsequent absolute quantification, found NAA to be reduced in males and females.[29] However, these NAA changes were found in the corpus callosum, not in the prefrontal white matter. Another study that examined high school football

players found metabolite differences at the beginning of season, with lower choline and creatine levels during the season in a similar region of interest. These results are consistent with the lower creatine levels observed at the off-season compared to our sedentary group. Moreover, similar to the elevated glutamine levels in contact athletes observed in the current study, a study by Bari and colleagues[28] examining male football and female soccer players found elevated Glx (glutamate + glutamine) levels in the motor cortex in females. Furthermore, high school football players were found with elevated Glx in a similar region of interest at the beginning of a sports season, and found those Glx levels to drop during the season. Interestingly, this change in Glx follows the same pattern as glutamine in our contact athletes.

3.4.3 Elevated Glutamine in Contact Athletes

In our previous study on concussion in female varsity rugby athletes, we measured reduced glutamine levels after concussion compared to baseline measures.[4] Moreover, we demonstrated that the significance of this reduction is lost when (1) baseline measures are not used and (2) when glutamate and glutamine are fitted together as Glx. Now, perhaps of even greater interest, we demonstrate that if non-contact athletes or sedentary individuals were used as a control group, the directionality of the glutamine change would appear to be reversed. For example, by only having non-contact athletes as a control group, it would seem that glutamine levels were elevated immediately after concussion, while we know from comparison to same-subject baseline measures that glutamine is actually decreasing. This highlights the need for proper baseline measures, to correctly interpret metabolite changes following concussion.

We speculate that the increase in glutamine levels observed in contact athletes and the decrease previously reported after concussion, are driven by glutamine synthetase (GS) activity and expression. GS is the enzyme that converts glutamate to glutamine, and is present in glial cells, including astrocytes and microglia.[54] During neurotransmission, glutamate is released into the synaptic cleft and is then taken up by astrocytes to be converted to glutamine by GS.[55] This process is part of the glutamate-glutamine cycle. According to Giza and Hovda,[5] the neurometabolic cascade of events that occur immediately after a concussive injury includes altered ion flux and glutamate release.

Moreover, elevated levels of extracellular glutamate can lead to excitotoxicity and tissue injury.[56] If we assume that glutamate release also occurs in association with sub-concussive hits, but to a lesser extent than when associated with concussion, it is conceivable that over time epigenetic changes could lead to increased GS expression and activity to compensate for the elevated glutamate release. This compensation could manifest as elevated glutamine levels *as observed in our contact athletes*. In more severe head injuries, one of the most significant pathophysiological processes is the number of free radicals, oxygen (ROS) and nitrogen species (NOS), that are created as a consequence of glutamate efflux and calcium influx.[56] Furthermore, studies have shown reduced GS activity to be due to increased oxidative stress, and that this inhibition in GS leads to reduced glutamine.[54,57] Therefore, it is feasible that *following a concussive injury, if there is significant production of free radicals, GS inhibition would manifest as reduced glutamine levels*, as we previously reported post-concussion.[4]

3.4.4 Limitations, Strengths, and Future Work

There are several limitations to consider for this study. The MRS voxel was manually placed by the MRI technician, which limits the reproducibility of the voxel placement within subjects at follow up scans. However, given the diffuse nature of a concussive injury, and the heterogeneity of sub-concussive hits, it is likely that the effected brain tissue extends beyond our voxel. Moreover, having impact data from the contact athletes would have allowed us to investigate associations between the number of hits and glutamine levels. Such data were limited however, because the league did not allow impact recording devices to be worn during games.

There are several important strengths to the current study. Our contact and non-contact athletes were recruited from three sports teams, ensuring similar training schedules and matched activity levels, and we focused on only female athletes, as sex differences have been observed.[10,32] Additionally, we chose a longer echo time (TE = 135 ms) to remove any macromolecule signal, allowing more reproducible quantification of the lower amplitude metabolites.

3.4.5 Conclusions

The objective of this study was to compare metabolite levels in sedentary and athletic females from the same university and varsity sports teams during the In- and Off-Season at the same time of year. Metabolite levels in non-contact athletes did not differ from age matched sedentary women except for a slightly lower myo-inositol level in the non-contact athletes, suggestive of no long-term exercise-induced change in MRS measured metabolite levels. Comparing metabolite levels to a group of female contact athletes previously reported, the non-contact athletes had different levels of *myo*-inositol, glutamate, and glutamine compared to the contact athletes. Importantly, glutamine levels were significantly higher in contact athletes compared to the sedentary and non-contact groups, demonstrating that repetitive sub-concussive impacts due to physical contact in high impact sports can alter metabolite levels. These data also demonstrate that the use of a sedentary or non-contact control group, instead of a contact control group or same subject baseline scans, can change the interpretation of the directionality of metabolite changes, specifically glutamine, after concussion.

3.5 References

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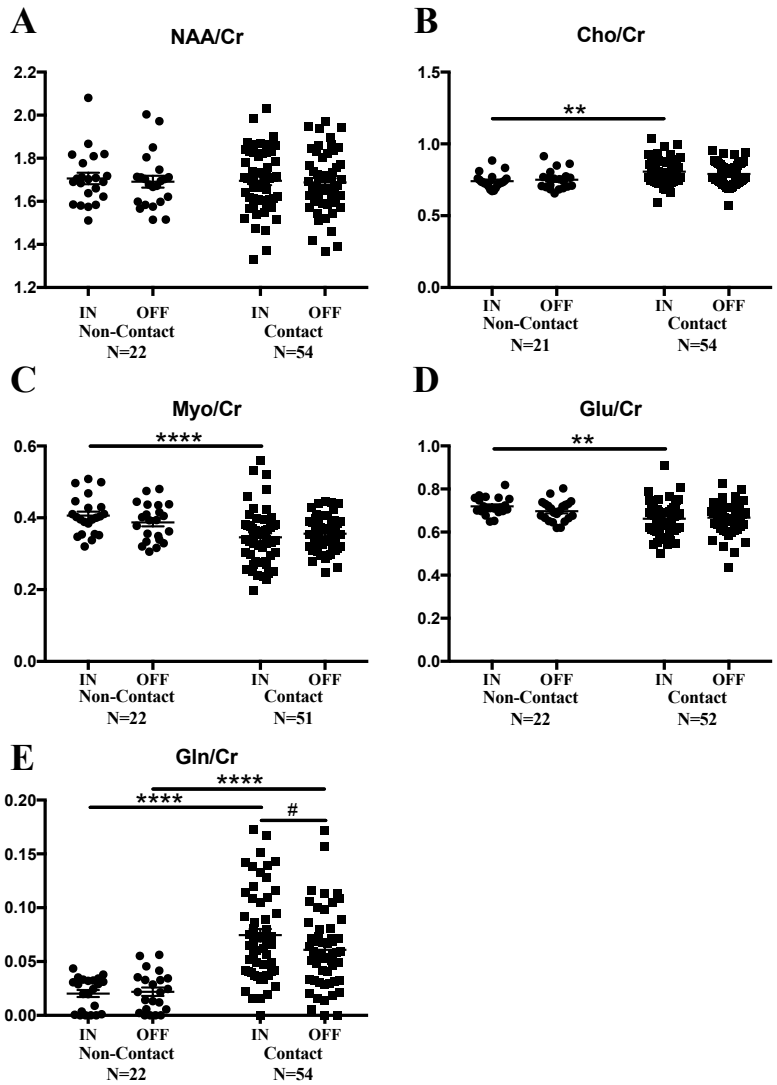
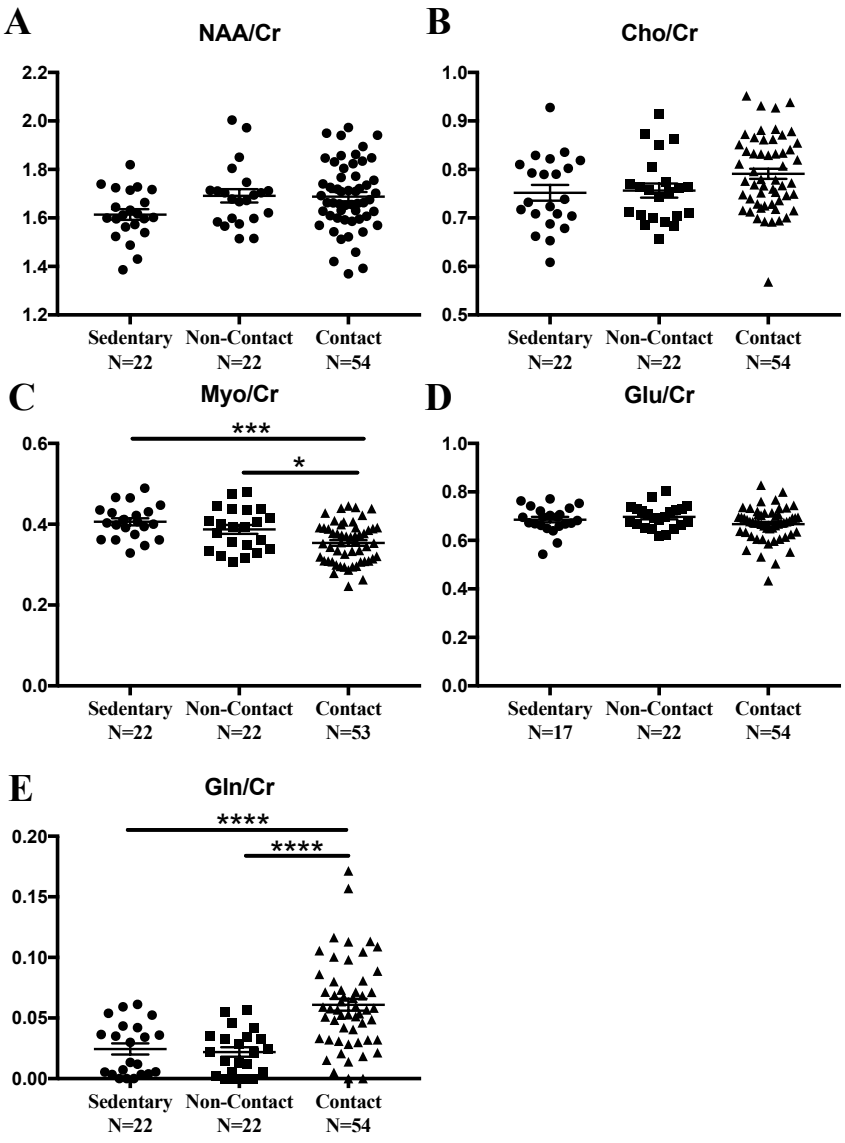
3.6 Supplementary Material

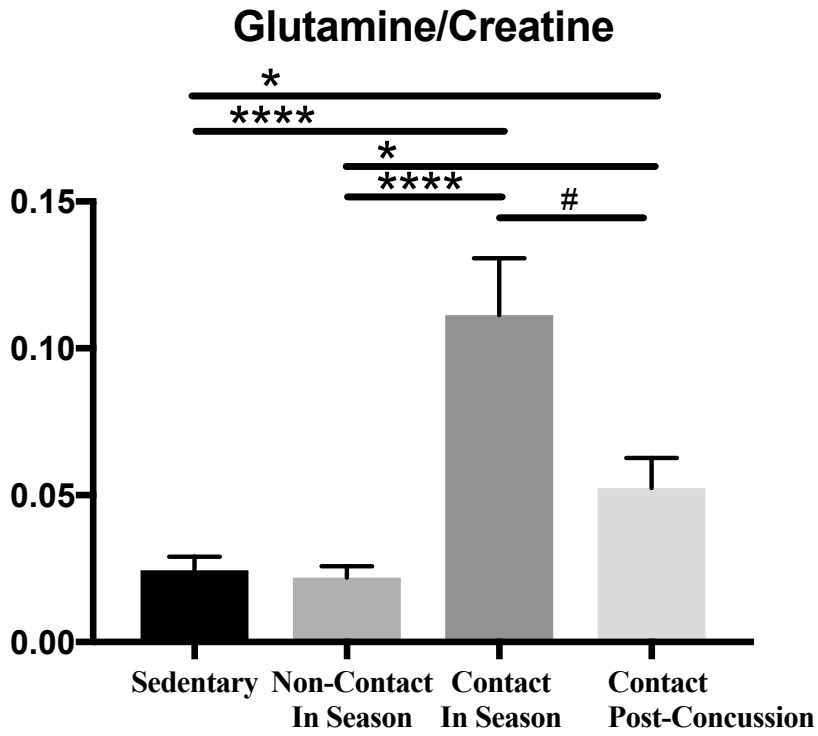


Supplementary Figure 3.1: Amplitude ratio of (A)NAA/Cr, (B) Cho/Cr, (C) *myo*-inositol/Cr, (D) glutamate/Cr, and (E) glutamine/Cr in the non-contact and contact group. Error bars

represent the standard error of the mean. # represents the drop in glutamine levels previously reported in Chapter 2. *(p<0.0001****; p<0.01**)*



Supplementary Figure 3.2: Amplitude ratio of (A) NAA/Cr, (B) Cho/Cr, (C) Myo/Cr, (D) Glu/Cr, and (E) Gln/Cr across groups. Error bars represent the standard error of the mean *(p<0.0001****; p<0.001***; p<0.05*)*



Supplementary Figure 3.3: Measured glutamine/creatine amplitudes across groups. Error bars represent the standard error of the mean. # represents the drop in glutamine/creatine previously reported between baseline and post-concussion in rugby athletes in Chapter 2. *(p<0.0001****; p<0.05*)*

Chapter 4

4 Longitudinal brain metabolite changes in a murine model of mild repetitive closed head injury

**In preparation for publication*

Amy Schranz, BMSc, Patrick McCunn*, BSc, Kathy Xu, Arthur Brown, PhD, Robert Bartha, PhD

**Patrick McCunn provided the data analysis, interpretation, and written sections on the DTI portion of this study*

The number and severity of impacts to the head triggers an unpredictable cascade of neurometabolic events. The final outcome of a head injury depends in part on a complex network of biochemical pathways, involving both oxidative metabolism and anaerobic pathways. These immediate changes in brain neurochemistry following an impact may precede microstructural changes. Previous studies in animal models of concussion have normally examined brain metabolism after a single insult. However, this approach does not replicate the environment of competitive contact sports, where athletes receive repetitive sub-concussive impacts often daily for months prior to a concussion. The purpose of this study was to investigate the changes and potential associations between brain metabolite levels measured by magnetic resonance spectroscopy (MRS) and brain microstructure measured by diffusion tensor imaging (DTI) in the mouse brain after a repeated mild closed head injury paradigm.

Male mice were divided into five groups, control (n=12), 48 hours post-injury (n=12), 1-week (n=12), 4 weeks (n=12), and 10 weeks post-injury (n=12). Mice received a closed-head mild controlled cortical impact, with one impact per day for five days, followed by *in vivo* MRS and DTI data acquisition.

In this study, we observed changes in DTI and NAA early on (48 hours and 1 week), that resolved by 4 and 10-weeks, indicating a mild injury that recovered with time. However, small changes at 10-weeks in creatine, lactate, taurine, Glu/Cr and

Gln/Cr, point towards a subtle shift in these biochemical pathways in order to re-establish homeostasis. Moreover, the change in Gln/Cr is consistent with that observed in a previous human study of female varsity rugby athletes in comparison to non-contact athletes. We speculate that the five hits in five days paradigm used in the current study may simulate the impact conditions that contact athletes experience over a season of play, eventually causing elevated glutamine levels.

4.1 Introduction

Despite recent research efforts to understand the long-term effects of concussion, identifying those at risk of post-concussion syndrome, and the development of effective treatment methods remains elusive. Failure to make significant progress in this area is largely due to the complexity of the underlying pathophysiology of concussion and the inability of conventional CT and MRI contrasts to detect brain changes.[1] As a result, over the last two decades, there have been many studies dedicated to investigating the utility of more specialized MRI techniques to detect subtle brain changes following concussion, including magnetic resonance spectroscopy (MRS) and diffusion tensor imaging (DTI).[1]

DTI is a complementary MRI modality that provides sensitive measurements related to tissue microstructure and structural connectivity, that can be associated with pathological changes within the brain.[2] Previous work applying DTI in humans after sport concussion has shown changes in fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD),[3] and it has been suggested that altered diffusion persists well after clinical assessment scores return to normal.[4] For a more comprehensive review on DTI in sports concussion see Chamard et al., 2018[3] and Asken et al., 2017.[5] Unfortunately, the measured changes in these metrics have been inconsistent, and it is important to study them further in pre-clinical models to verify their accuracy and suitability for diagnostic medicine. In line with the human literature, increases and decreases in DTI metrics have been found in animal models. For example, a study performed by Bennett et *al.* (2012)[6] observed decreased FA in the corpus callosum of mice after repeated closed-skull TBI, while Robinson et *al.* (2017)[7] found reduced AD in mice but no changes in FA within the corpus callosum. It is important to note, that even though animal models have the benefit of reducing the heterogeneity in

the injury, the model used and chosen parameters still vary from study to study making comparisons challenging.

After an insult to the head, there is a cascade of neurometabolic events that depends on factors including the number and severity of impacts.[8–10] The final outcome of a head injury is determined by the ability of the brain to modulate a complex network of biochemical processes, including the TCA cycle and oxidative metabolism, as well as anaerobic pathways. These immediate changes in brain neurochemistry as well as altered neurotransmission from the secondary chemical cascade of ion flux that occurs after receiving a hit to the head[11] precede structural changes,[12] and produce metabolite level changes that can be detected and quantified by ^{1}H MRS.

The effect of concussion on the brain has been largely studied by MRS in athletes participating in various contact sports. These studies have observed changes in the levels of several different metabolites. For example, altered *N*-acetyl aspartate (NAA),[13,14] choline,[15–17] *myo*-inositol,[16,18,19] glutamate[20] and glutamine[21] have been reported in WM[4,16,22,23] and GM[18,19,24] regions in the brain. The large variability in metabolite findings across the literature is likely due to the heterogeneous nature of concussion (e.g. impact site, force of impact, timing of imaging post impact, concussion history, sex, diet, etc.).[25] Animal models can be utilized to control many of these variables, allowing exploration of the mechanisms and relevance of specific metabolite changes after concussion.[1] Numerous animal models exist, and studies to date have reported changes including reduced NAA/Cr,[26,27] Tau/Cr,[8] creatine and glutamine,[28] and *myo*-inositol,[29] as well as increased glutamine,[10,30] lactate, *myo*-inositol, and choline[31] after injury. However, the majority of these studies used rather severe models, with craniotomies, that are more representative of moderate to severe traumatic brain injury, not concussion.[10,27,29–31] Therefore, investigating concussion or the effects of repetitive impacts with MRS in animal models, remains relatively unexplored. A synopsis of the relevant literature in animal models is summarized in Table 1.4.

Few studies have examined the association of MRS and DTI metrics in concussion. A recent study in rats by Li et *al.*, (2017)[9] did find a positive correlation

between FA and *myo*-inositol, which could indicate glial proliferation as the main cause of increased FA post TBI. Additionally, we previously reported that changes in FA and RD correlate with changes in the MRS measured metabolite, glutamine[21] over a season of play in female varsity rugby players. Moreover, we further found that these same female contact athletes have elevated Gln/Cr and glutamine levels compared to non-contact athletes and nonathletes,[32] suggesting that repetitive sub-concussive impacts can influence brain metabolism.

The force and direction of impact can vary greatly in different animal models of concussion. The majority of studies utilizing MRS have examined the effects of moderate and severe traumatic injuries using a fluid percussion injury,[33–35] controlled cortical impact,[10,36–38] or a blast injury,[28,29] rather than concussion (e.g. closed head impact model, see Table 1.4). Additionally, for closed head impact (CHI) studies, MRS has mainly been used to examine the effects of a single impact, rather than repetitive head injury (See Table 1.4). The majority of single CHI have used a weight drop model of 450g[8,31,39,40] with drop heights from 25-100 cm characterized as mild injuries, and >100 cm characterized as moderate to severe, with high mortality rates. Closed-head controlled cortical impact models have also been used with 5mm diameter tips at 5-4 m/s at a depth of 1 mm being characterized as mild.[26,41,42]

Investigating the effect of mild repetitive head impacts is critical to interpreting the changes observed following sport induced concussion. We propose that the metabolic response of the brain after a single concussive injury will be different to the response observed after a similar impact delivered within the context of multiple sub-concussive hits akin to what occurs in high impact sports. Understanding the metabolic changes that occur following multiple light impacts is the first step in answering this important question, which has immediate parallels to the effects of sub-concussive impacts in contact sports. Therefore, the objective of this study was to investigate the changes and potential associations between brain metabolite levels measured by MRS and brain microstructure measured by DTI in the mouse brain after a repeated closed head injury paradigm. Based on the results of previous studies,[42,43] as well as our own recent findings[21,32] in humans, we hypothesized reduced NAA levels, as well as increases

in glutamine, would be associated with altered tissue microstructure.

4.2 Methods

4.2.1 Study Design

Sixty C57BL/6J male mice (weight 22.5 ± 2.5 g) were divided into five groups, control (n=12), 48 hours post-injury (n=12), 1-week (n=12), 4 weeks (n=12), and 10 weeks post-injury (n=12). In preparation for injury, mice were anaesthetized by induction with 4% isoflurane followed by maintenance with 2% isoflurane. The animal was positioned under a traumatic brain injury device (TBI 0310, Precision Systems and Instrumentation, LLC). Following a 10 mm midline incision, the skin and fascia were reflected, and the animals received a mild controlled cortical impact, centered at the sagittal suture, with a 5 mm-diameter, pliant, silicone tip. The device was programmed to impact at a depth 1.0 mm at a 3.5 m/s velocity with a 500 ms dwell time. Animals received one impact per day for five days. Imaging followed at 48-hours, 1 week, 4 weeks, or 10 weeks after the final impact (different mice were studied at each time point). Control mice received no surgery or impacts.

4.2.2 Data Acquisition

All imaging and spectroscopy were performed on an Agilent 9.4 Tesla small-bore MRI scanner (Santa Clara, CA, U.S.A.) at the Robarts Research Institute using a Millipede MP30, or MP40 radio frequency coil. The MP40 coil was used at the last timepoint due to failure of the MP30 coil. Diffusion Tensor Imaging (DTI) was acquired using a spin-echo acquisition sequence (TE=36 ms, TR=1s, max b-value=1085 s/mm^2, 12 gradient directions, FOV=18.75 mm, 128x128 matrix, 31 – 0.5 mm thick slices). A 2D fast spin-echo anatomical image (TR/TE=4000/10ms; FOV=19.2x19.2 mm^2; matrix=128x128; slice thickness=0.5 mm) was acquired for tissue/CSF segmentation. Magnetic resonance spectra were acquired from a 2 x 6 x 3 mm^3 voxel encompassing both hippocampi (Figure 4.1) using VAPOR water suppression[44] and the localization by adiabatic selective refocusing (LASER) pulse sequence (TR/TE=3250/18.9 ms; averages=128/8; HS2 R15 adiabatic full passage pulses; bandwidth=6000 Hz).[45] Acquisition of a macromolecule only spectra was interleaved with the acquisition of the full spectrum using a single-

inversion recovery technique as previously described[46] to remove the contribution of macromolecules to the spectrum in post-processing.

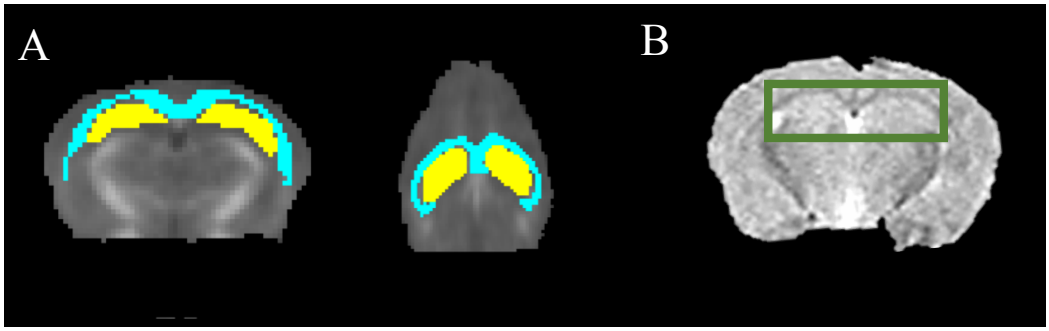


Figure 4.1: Masks utilized for diffusion tensor imaging analysis of brain microstructure. (A) Regions of interest were defined for white matter including Corpus Callosum (teal binary mask), and hippocampus (yellow binary mask). (B) A typical voxel placement (green outline) for ^{1}H MRS in the mouse brain including both hippocampi (2 x 6 x 3 mm^{3}).

4.2.3 Post-Processing and Statistical Analysis

DTI images were pre-processed using fMRI Software Library (FSL, v.5.0.10, Oxford, UK). Images were first registered to a C57BL/6J atlas using a linear transformation (FLIRT)[47] followed by a non-linear registration (FNIRT)[48] in FSL. The resulting transformation matrices were then inverted and used to bring masks of the corpus callosum and hippocampus back into the diffusion space of each subject, where mean diffusion parameter values for each region were then extracted. Mean region of interest (ROI) analysis was performed and focused on two relevant regions of interest: the corpus callosum and the hippocampus (Figure 4.1). Within each ROI the mean fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD) values were extracted.

All spectra were lineshape and eddy current corrected using combined QUALITY deconvolution (400 points) and eddy current correction.[49] Macromolecules were then fitted using a Hankel singular value decomposition (HSVD)[50] and subtracted from the metabolite spectrum. Using in-house analysis software, suppressed (post macromolecule

subtraction) and unsuppressed spectra were fitted in the time domain using prior knowledge produced by density matrix simulations[51] incorporated into a Levenberg-Marquardt minimization routine[52] (Figure 4.2). Metabolites included in the prior knowledge template included *N*-acetyl aspartate (NAA), alanine, γ-aminobutyric acid (GABA), aspartate, choline, glycerophosphorylcholine (GPC), phosphorylcholine (PCho), creatine (Cr), glucose, glutamate (Glu), glutamine (Gln), glutathione, glycine, *myo*-inositol (Myo), lactate, and taurine (See Table 4.1 and Figure 4.2).

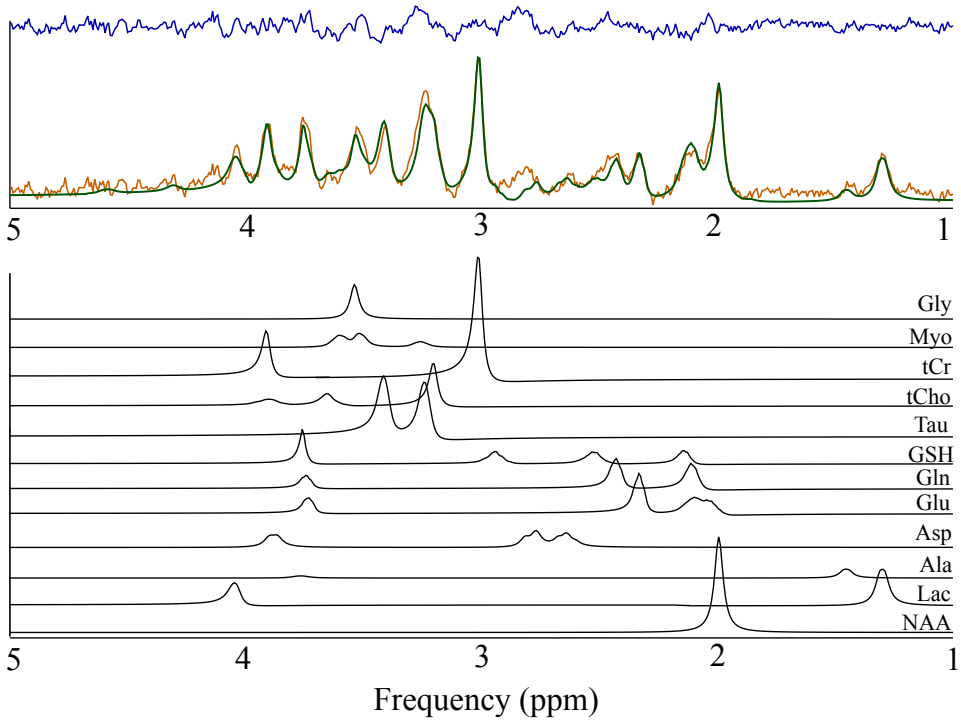


Figure 4.2: A typical ^{1}H spectrum after post-processing and macromolecule subtraction (orange), with the fitted spectrum (green) superimposed. The residual after fitting (blue) is shown above, and the individual prior knowledge components of the spectrum are shown below in black. Gly, Glycine; Myo, *Myo*-inositol; tCr, total Creatine; tCho; total Choline; Tau, Taurine; GSH; Glutathione; Gln, Glutamine; Glu, Glutamate; Asp, Aspartate; Ala, Alanine; Lac, Lactate; NAA, *N*-acetyl asparate; ppm, parts per million. (Note that GABA and glucose are not shown, as they fit to zero in this dataset)

Metabolite absolute concentrations and ratios relative to Cr were calculated in this study. Absolute concentrations incorporated a correction to account for tissue partial volume (Tissue and CSF) obtained by segmenting the 2D fast spin-echo anatomical image. Absolute concentrations also account for T1 and T2 relaxation related signal loss using the constants listed in Table 4.1. For spectra acquired from the MP40 coil, the unsuppressed spectra were scaled to match the mean water signal obtained from MP30 coil. Metabolite ratios relative to Cr were also calculated, to eliminate the uncertainty associated with partial volume and relaxation corrections from influencing the results.

Signal-to-noise ratio (SNR) was measured as the *N*-acetyl aspartate peak height divided by the standard deviation of the noise. Spectra with an SNR <10 or water linewidth >30 Hz were not included in the analysis. Metabolites with a coefficient of variation >40% were excluded from the statistical analysis, except for glutamine (CV>40%), which was left in due to the overall study hypothesis.

All statistical analyses were performed using GraphPad Prism Version 7.0 for Mac OS X (GraphPad Software, San Diego, CA). The ROUT method and D'Agostino & Pearson normality test were performed on each metabolite in each group to remove outliers and test for normality, respectively. A one-way ANOVA (or Kruskal-Wallis) was used to assess statistical significance (α=0.05) across groups, using Tukey's multiple comparisons correction (or Dunn's Test). The associations between metabolic changes and metrics of tissue microstructural integrity were examined using two-tailed Spearman correlations (α=0.05).

Table 4.1: Relaxation Constants used to correct for T_1 and T_2 related signal loss

		Grey Matter		White Matter		CSF	
Metabolite	Protons	T_1 (s)	T_2 (ms)	T_1 (s)	T_2 (ms)	T_1 (s)	T_2 (ms)
NAA	3	1.67	294	1.67	294	-	-
NAAG	3	1.67	294	1.67	294	-	-
Alanine	4	1.37	148	1.37	148	-	-
GABA	6	1.37	105	1.37	105	-	-
Aspartate	3	1.37	148	1.37	148	-	-
Choline	9	1.35	441	1.35	441	-	-
GPC	9	1.35	441	1.35	441	-	-
PCho	9	1.35	441	1.35	441	-	-
Creatine	3	1.04	128	1.04	128	-	-
Glucose	6	1.37	104	1.37	104	-	-
Glutamate	5	1.50	89	1.50	89	-	-
Glutamine	5	1.50	116	1.50	116	-	-
Glutathione	7	1.37	106	1.37	106	-	-
Glycine	2	1.37	148	1.37	148	-	-
Myo	5	1.37	148	1.37	148	-	-
Scyllo	6	1.37	148	1.37	148	-	-
Lactate	3	1.37	148	1.37	148	-	-
Taurine	4	2.33	93	2.33	93	-	-
Water	2	2.16	44	2.16	44	4.29	111

4.3 Results

4.3.1 Quality Assurance Measures

The spectroscopy voxel placed over the hippocampus was composed of (mean $\pm$ standard deviation) 94 $\pm$ 3 % tissue and 6 $\pm$ 3 % CSF. For all data sets the average full-width at half maximum of the water peak was 20 $\pm$ 3 Hz and the average SNR was 14.7 $\pm$ 4. All spectra were visually inspected prior to statistical analyses for artefacts. Four spectra were eliminated due to insufficient data quality and SNR (<10), and outliers were removed for each data set according to the ROUT test (including DTI data sets). Cramér-Rao Lower Bounds (CRLB) were not used to eliminate metabolite measurements to avoid bias selection,[53] however, average CRLBs were 3% for NAA, 13% for lactate, 5% for glutamate, 56% for glutamine, 9% for glutathione, 2% for creatine, 2% for taurine, and 21% for choline.

4.3.2 Diffusion Tensor Imaging

DTI results of the hippocampus and corpus callosum are shown in Figure 4.3. Significant differences were observed in FA ($p < 0.0001$) and RD ($p = 0.0004$) in the hippocampus,

as well as in FA ($p = 0.003$) and RD ($p = 0.0048$) in the white matter (corpus callosum). There was a statistically significant increase in FA in the hippocampus (Figure 4.3A) at 48 hours in comparison to the control group ($p = 0.019$), that persisted out to 1-week ($p = 0.015$). A similar increase in FA was observed in the white matter (Figure 4.3B; $p = 0.037$) but it did not persist at later time points ($p > 0.05$). This increase in FA was driven by a significant reduction in RD. In the hippocampus (Figure 4.3C) RD was reduced at 48 hours in comparison to the 10-week group ($p = 0.0027$), and the decrease remained at 1-week ($p = 0.0014$) and 4-weeks ($p = 0.043$). In the white matter (Figure 4.3D) RD was reduced at 48 hours in comparison to the control group ($p = 0.0026$), but by 4-weeks had significantly increased ($p = 0.036$) back toward baseline levels. There were no statistically significant changes in mean diffusivity (MD) or axial diffusivity (AD) at any timepoints in either region.

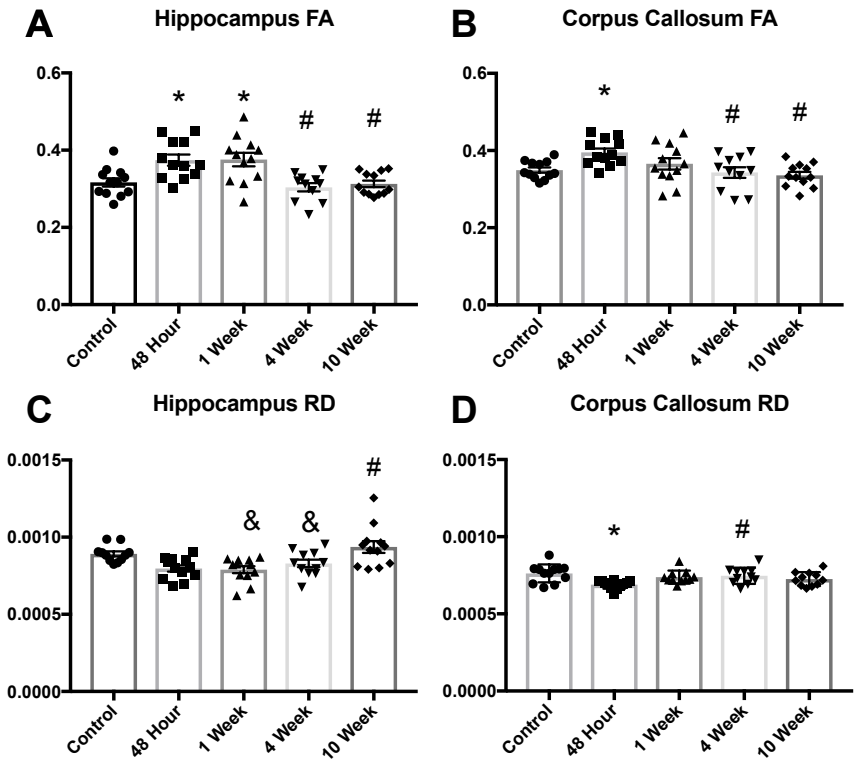


Figure 4.3: Bar graphs indication the individual mouse and mean DTI metric, with standard error bars, for the Hippocampus and Corpus Callosum. An asterisk (*) indicates significant difference from the control group, the pound (#) indicates significant

differences from 48 hours, and the symbol (&) indicates significant differences from the 10-week group

4.3.3 Magnetic Resonance Spectroscopy and Correlations

Gln/Cr was significantly different (Figure 4.4A; $p = 0.033$), with increased levels at 10 weeks compared to 48 hours ($p = 0.045$). Furthermore, examining absolute glutamine levels, the ANOVA showed a significant difference (Figure 4.4B; $p = 0.025$) among groups, but individual comparisons between groups were did not reach the level of significance ($p = 0.07$). Additionally, negative correlations between glutamine and MD (Figure 4.4C; $p = 0.037$, $r^2 = 0.4$), and RD (Figure 4.4D; $p = 0.046$, $r^2 = 0.37$) were observed at 10 weeks in the white matter.

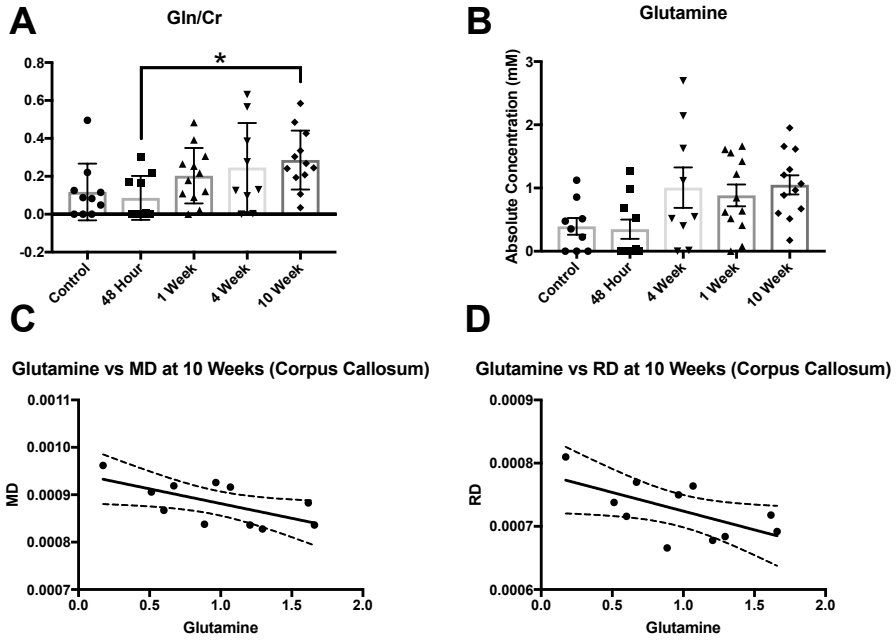


Figure 4.4: (A-B) Bar graphs indicating the individual mouse and mean values for Gln/Cr and Glutamine absolute concentration, with standard error of the mean. (A) Gln/Cr was found to be significant (ANOVA, $p = 0.033$) with elevated levels at 10 weeks compared to 48 Hours ($p = 0.045$). (B) Glutamine was found to be significant (ANOVA, $p = 0.025$) but significance was lost after post hoc testing ($p = 0.07$). Correlations between Glutamine and (C) MD ($p = 0.037$, $r^2 = 0.4$) and (D) RD ($p = 0.046$, $r^2 = 0.37$) in the corpus callosum are plotted with 95% confidence band

NAA/Cr was also found to be different across groups (Kruskal-Wallis $p = 0.04$, Figure not shown), but individual comparisons between groups did not reach significance (Dunn's test, *p>0.05)*. Measurement of absolute metabolite levels revealed decreased NAA (Figure 4.5A; $p = 0.018$) from 48 hours to the 1-week group ($p = 0.018$). Additionally, creatine concentrations were significantly different (Figure 4.5B; $p = 0.013$), with reduced levels at 10 weeks in comparison to the control group ($p = 0.011$). Furthermore, NAA concentrations were positively correlated with FA in the white matter (Figure 4.5C; $p = 0.011$, $r^2 = 0.11$). Examining individual time points, this correlation was only significant at 48 hours (Supplementary Figure 4.1A; $p = 0.0094$, $r^2 = 0.54$). A similar positive correlation was observed between creatine and FA in the white matter ($p = 0.015$, $r^2 = 0.11$, data not shown) and at 48 hours (Supplementary Figure 4.1B; $p = 0.013$, $r^2 = 0.51$).

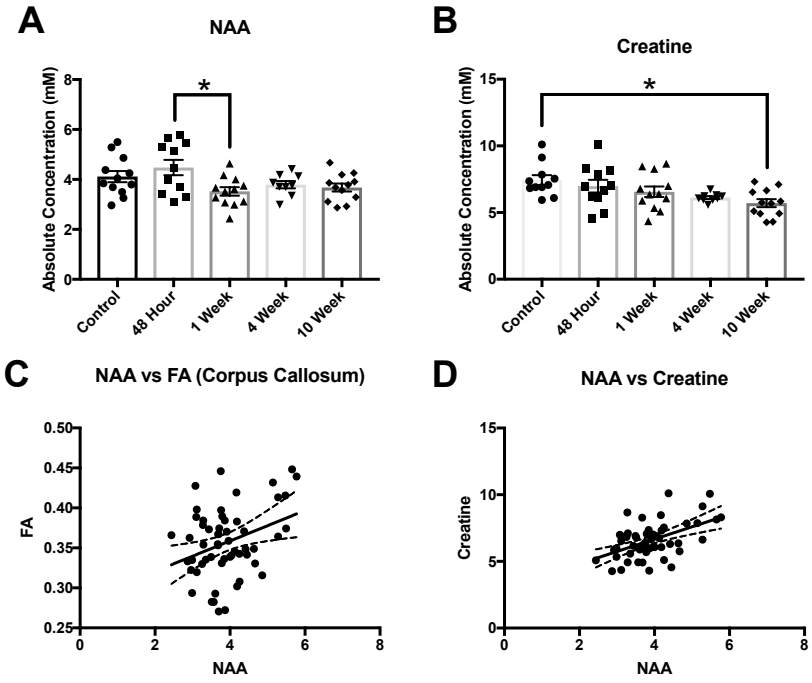


Figure 4.5: (A-B) Bar graphs indicating the individual mouse and mean absolute concentration for NAA and Creatine, with standard error of the mean. (A) NAA was found to be significant (ANOVA, $p = 0.018$) with reduced concentration between 48 hours and 1 week ($p = 0.018$). (B) Creatine was found to be significant (ANOVA, $p = 0.013$) with reduced concentration at 10 weeks ($p = 0.011$). Significant Pearson Correlation between NAA levels

and (C) FA in the corpus callosum ($p = 0.011$, $r^2 = 0.11$), and (D) Creatine levels ($p<0.0001$, $r^2 = 0.27$) are plotted with 95% confidence bands

Glu/Cr was also found to be different across groups (Figure 4.6A; $p = 0.026$), with significantly higher levels at 10 weeks in comparison to control levels ($p = 0.035$) and 1-week levels ($p = 0.049$). Measurement of absolute metabolite levels revealed differences in glutamate (Figure 4.6B; $p = 0.044$), but the increase did not survive post-hoc testing ($p = 0.056$). Additionally, a negative correlation was observed between glutamate and FA in the hippocampus (Figure 4.6C; $p = 0.0014$, $r^2 = 0.18$), which was driven by an increase in RD (Figure 4.6D; $p = 0.003$, $r^2 = 0.16$). A similar correlation with FA (Figure 4.6E; $p = 0.005$, $r^2 = 0.15$) and RD (Figure 4.6F; $p = 0.036$, $r^2 = 0.09$) was found in the white matter.

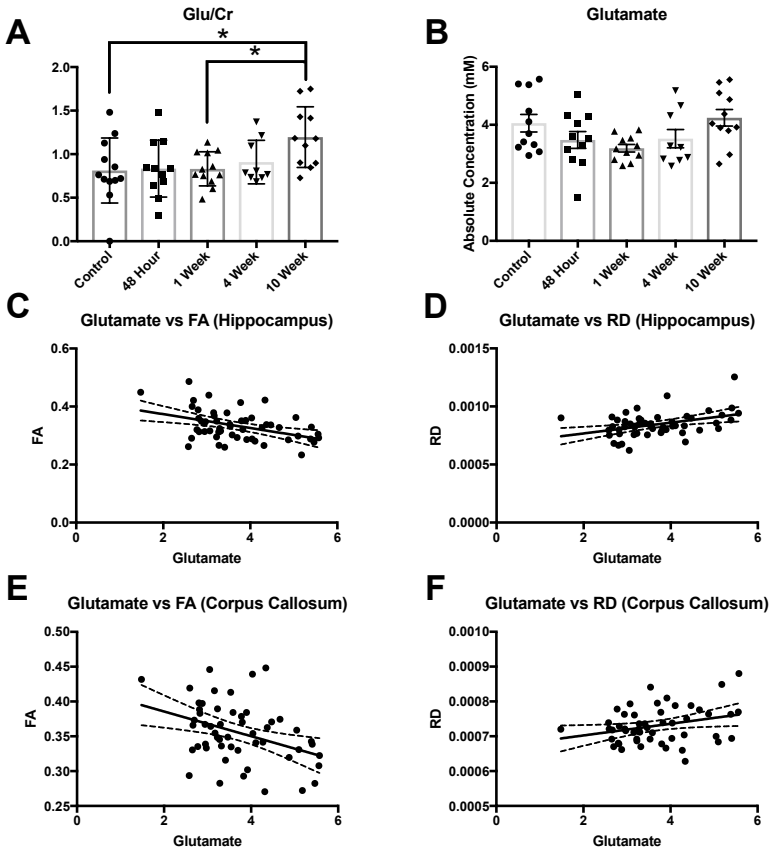


Figure 4.6: (A-B) Bar graphs indicating the individual mouse and mean values for Glu/Cr and Glutamate, with standard error of the mean. (A) Glu/Cr was found to be significant

(ANOVA, $p = 0.026$) with increased levels at 10weeks in comparison to controls ($p = 0.035$) and 1week ($p = 0.049$). (B) Glutamate concentration was found to be significant (ANOVA, $p = 0.044$), no significance held past post-hoc testing ($p = 0.056$). Correlations between Glutamate and (C) FA ($p = 0.0014$, $r^2 = 0.18$) and (D) RD ($p = 0.003$, $r^2 = 0.16$) in the hippocampus, and between (E) FA ($p = 0.005$, $r^2 = 0.15$), and (F) RD ($p = 0.036$, $r^2 = 0.09$) in the corpus callosum are plotted with 95% confidence bands

No significant changes were observed in Tau/Cr, but absolute taurine levels significantly changed (Figure 4.7A; $p = 0.0011$), with reduced levels compared to controls observed at 48 hours ($p = 0.0007$), 1-week ($p = 0.013$), and 10 weeks ($p = 0.027$). No correlations were found between taurine and DTI metrics. Although Lac/Cr did not significantly change, absolute lactate levels did (Figure 4.7B; $p = 0.016$), showing increased levels at 48 hours in comparison to 10 weeks ($p = 0.01$). Additionally, a positive correlation was observed between lactate and FA in the white matter (Figure 4.7C; $p = 0.005$, $r^2 = 0.14$), which was driven by an increase in AD (Figure 4.7D; $p = 0.001$, $r^2 = 0.19$). Examining individual time points, this FA correlation was only significant at 48 hours (Figure 4.7E; $p = 0.0019$, $r^2 = 0.47$). The same was true for AD (Figure 4.7F; $p = 0.015$, $r^2 = 0.5$). No other significant changes or correlations were observed.

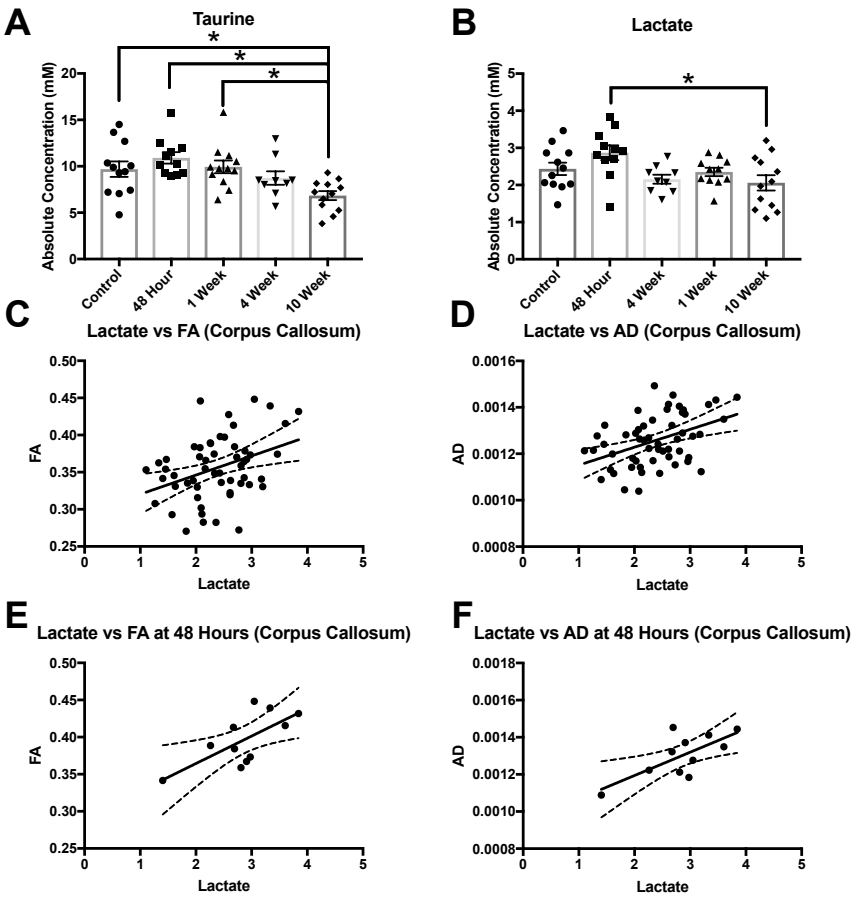


Figure 4.7: (A-B) Bar graphs indicating the individual mouse and mean absolute concentrations for Lactate and Taurine, with standard error of the mean. (A) Taurine was found to be significant (ANOVA, $p = 0.0011$) with reduced concentration at 10 weeks compared to Control ($p = 0.027$), 48 hours ($p = 0.0007$), and 1 week ($p = 0.013$). (B) Lactate was found to be significant (ANOVA, $p = 0.016$) with reduced levels at 10 weeks compared to 48 hours ($p = 0.01$). Correlations between Lactate and (C) FA ($p = 0.005$, $r^2 = 0.14$) and (D) AD ($p = 0.001$, $r^2 = 0.19$) in the corpus callosum, and between (E) FA ($p = 0.0019$, $r^2 = 0.47$), and (F) AD ($p = 0.015$, $r^2 = 0.5$) at 48 hours in the corpus callosum are plotted with 95% confidence bands

4.4 Discussion and Conclusion

The purpose of this study was to investigate the changes and potential associations between brain metabolite levels measured by MRS and brain microstructure measured by DTI in the mouse brain after a repeated mild closed head injury paradigm. We

hypothesized reduced NAA levels, as well as increases in glutamine, would be associated with altered tissue microstructure. Acutely, at 48 hours, we found elevated FA driven by reduced RD in the hippocampus and white matter within the MRS voxel (corpus callosum). We additionally observed elevated lactate at 48 hours, followed by reduced NAA at 1 week. By 10 weeks we observed reduced Taurine levels and elevated Gln/Cr and Glu/Cr which were driven by reduced creatine and elevated Gln and Glu (trends). Moreover, several correlations were observed at various timepoints after injury. Most importantly, glutamine was found inversely correlated with MD and RD at 10 weeks, and NAA was positively correlated with FA.

4.4.1 Diffusion Tensor Imaging

In the current study, ROI analyses of the hippocampus and white matter revealed transient increases in FA and decreases in RD. These results are consistent with several previous studies of concussion. For example, Kikinis et al. (2017)[54] found increased FA in the corpus callosum, the fimbria of the hippocampus and in other regions of the injured rat brain, using a weight drop model. Moreover, increases in FA in the corpus callosum has been reported in mTBI studies in humans.[55–58] These changes could be associated with neuroinflammation as well as remyelination.[59,60] Additionally, Li et al., (2017)[9] speculated that astrocyte proliferation is the main cause of increased FA after TBI. However, two previous studies of repeated head injury in mice found somewhat different results. Bennett and colleagues (2012)[6] reported no changes in DTI metrics at 24 hours post-injury but found reduced AD and MD at 1-week post-injury in the corpus callosum. The timeline of changes was similar to the current study, however the DTI metrics observed to change were different. Bennett and colleagues (2012)[6] used a 2-hit paradigm, rather than the 5-hit paradigm used in the current study, as well as different impact parameters including a larger impactor tip (9 versus 5 mm), greater depth (3.3 versus 1 mm) and velocity (5 versus 3.5 m/s). Another study by Yu and colleagues (2016)[61] found reduced AD at 3, 6, and 42 days post injury in the cerebral cortex, and reduced FA at 42 days in the corpus callosum. Interestingly, Yu et al., (2016)[61] also used a 5-hit paradigm and similar impact parameters (3 mm tip, 4 m/s, 1 mm depth), but followed a longitudinal study design, rather than a cross-sectional design as implemented in the current study.

The increased FA and reduced RD in the current study are also consistent with our own previous findings in *non-concussed* contact sport athletes.[21] Specifically, we observed increased FA and reduced RD within the white matter of the MRS voxel in female rugby players after a season of contact play. Interestingly, a correlation was observed between the change in Gln/Cr and the change in FA and RD over the sports season. We observed a similar correlation in the current study between glutamine and RD at 10 weeks. The similarities observed here with non-concussed rugby players suggest that the mild 5- hit paradigm may be a suitable model of repetitive sub-concussive impacts.

4.4.2 ^{1}H MRS: Glutamine

In a previous study by our group in female varsity rugby players, we observed a large reduction in glutamine levels after concussion, as well as a smaller reduction after a season of contact play in athletes with no diagnosed concussion.[21] More recently, we have shown that Gln/Cr and absolute glutamine levels are *increased* in these same female varsity rugby players compared to varsity athletes involved in non-contact sports and non-athletes.[32] These previous studies provided the motivation to investigate the effects of multiple light impacts over a short period of time in an animal model to determine whether glutamine levels would increase in comparison to controls and to explore potential associations with DTI metrics. Despite the large variability in the glutamine measurements, the current study did find increased Gln/Cr at 10 weeks post injury compared to the 48-hour time point in the hippocampus (and corpus callosum). There was also a trend ($p<0.1$) toward higher absolute glutamine levels at 10 weeks compared to 48 hours after injury. Therefore, the significant increase in Gln/Cr is likely due to a small increase in glutamine, and a concomitant reduction in creatine. Interestingly, this change in Gln/Cr level is consistent with that observed in the varsity rugby athletes in comparison the non-contact athletes.[32] We speculate that the five hits in five days paradigm used in the current study may simulate the impact conditions that contact athletes experience over a season of play, eventually causing elevated glutamine levels. Although the reason for this glutamine increase is currently unknown, there are several potential explanations that could be explored in future studies. For example, we speculate

that elevated glutamine levels may be the results of a primed microglia profile[62] in the brain after repetitive impacts. Glutamine's main role in the brain is its involvement in neurotransmission by shuttling glutamate back to the neuron through astrocytes.[63] The conversion of glutamate to glutamine is performed by the enzyme, glutamine synthetase.[63] Microglia have also been shown to express this enzyme, although they hardly convert glutamate into glutamine.[62,63] However, studies have shown persistent microglia activation after brain injury,[62] and have shown that in this state microglia will increase the metabolism of glutamate to glutamine, to prevent dangerous levels of extracellular glutamate.[63] It is possible that persistent microglia activation manifests as a shift in the glutamate-glutamine equilibrium, resulting in elevated glutamine levels.

We also previously found changes in Gln/Cr and glutamine to negatively correlate with changes in FA and positively correlate with changes in RD in female contact athletes after a season of play.[21] In the current study, we did observe negative correlations between glutamine and MD as well as RD at the 10-week time point. However, a direct comparison cannot be made as a cross sectional study design was used in the current study, while our previous study in rugby players used longitudinal measures.

4.4.3 ^{1}H MRS: *N*-acetyl Aspartate and Glutamate

Reduced NAA concentrations were observed in the current study as we hypothesized, but not until 1-week post-injury, compared to 48 hours. There was no change detected compared to the control group, which may be in part due to the lack of a sham surgery. For example, Lyons and *colleagues* (2018)[26] used a block experimental design in mice with a traditional sham set up (surgery without TBI/CHI), and found reductions in NAA 3 days post injury, *in a similar voxel to this current study*, in a closed head impact model (See Table 1.4). Moreover, a recent study by Fidan et al., (2018)[42] that incorporated a repeated measures paradigm in male rats, observed reduced NAA/Cr in the hippocampus compared to shams at 1-week post injury. Signoretti et *al*., (2010)[40] also found reduced NAA at 2 hours, 4 hours, 1 day and 2 days post injury, but by using high-performance liquid chromatography on deproteinized whole brain extracts rather than *in vivo* MRS. Furthermore, using a repeated measures paradigm might also have increased sensitivity to

changes in NAA levels after injury. For example, a study by Vagnozzi and colleagues (2005)[43] using a 2 hits paradigm found reduced NAA in whole brain extracts 2 days post-injury using a weight drop model.

The NAA correlation with FA, and lack of correlation with glutamate, may suggest altered oxidative metabolism that reflects impaired glutamatergic signaling. NAA is synthesized in the neuronal mitochondria and is constantly transported along axons into oligodendrocytes to be used in myelin synthesis and maintenance, and is also thought to be an osmolyte.[64] NAA and FA values have been found to correlate in other studies[65] suggesting that changes in NAA and tissue microstructure are linked. Interestingly, NAA levels were positively correlated with FA in the white matter in this study. Furthermore, a study looking at normal aging in mice, showed that NAA levels and glutamate levels correlated extremely well (r^2=0.859), because both metabolites mainly reside in neurons.[66] However, no such correlation was found in the current study, suggesting alteration from healthy aging. Additionally, in the same study of normal aging,[66] reductions in glutamate were observed in the hippocampal region at 12, 18, and 24 months, when compared to 3 months. The current study found increased Glu/Cr over the 10-week period post-injury, with a trend towards increased glutamate, reflective of altered oxidative metabolism or excitotoxicity. This is further supported by the elevated Gln/Cr, discussed earlier. Alternatively, other studies in the literature tended to observe decreases in glutamate,[38,67] however both of these studies used ^{13}C MRS *ex vivo*. Lama and colleagues (2014)[67] used a modified weight drop model of severe TBI, and observed lower glutamate labeling 60 mins post TBI, while Robertson and colleagues (2013)[38] used a controlled cortical impact model of TBI and found reduced glutamate labeling 24 hours post TBI.

4.4.4 ^{1}H MRS: Other Metabolite Changes

A small decrease in creatine at 10 weeks post-injury compared to control, and a positive correlation with FA was observed. Several other studies using mice, rats, and rabbits have also observed decreases in creatine after head injuries.[26,28,68] Although a small decrease in creatine was observed, the magnitude is larger than the expected reductions in creatine observed over 21 months in healthy mice.[66] The creatine signal is composed of creatine

and phosphocreatine, which are involved in tissue energy metabolism.[69] It has been suggested that phosphocreatine serves as an energy shuttle between mitochondria and other sites in the brain.[69] Interestingly, NAA and creatine levels were positively correlated. Given that NAA is synthesized in the mitochondria where phosphocreatine serves as an energy shuttle, mitochondrial energetics may explain why these metabolites were correlated.

Lactate was also increased at 48 hours compared to 10-weeks. Interpreting this is challenging without a significant difference from the control group, which again could be due to the lack of a traditional sham group. Lactate is the end product of anaerobic glycolysis,[69] and has been shown to increase in more severe cases of TBI,[31,67] due to the presence of hypoxic/ischemic conditions. This result suggests a reduction in oxidative metabolism in favour of anaerobic glycolysis 48 hours after the 5-hits paradigm, which resolves by 10-weeks.

In addition to the lactate change, lower taurine levels were observed 10-weeks post injury compared to all other time points. Singh et al., (2016)[8] found reduced Tau/Cr five days after a single mTBI, and suggested that the reduction may be due to taurine's role in cell volume regulation, where astrocytes re-establish normal volume after injury by extrusion of ions and amino acids such a taurine.[70] Additionally, it has been suggested that taurine release may be driven by activation of glutamate receptors,[71] however no correlations were observed between taurine and glutamate. The subsequent decrease in taurine and creatine likely explain why no change was observed in Tau/Cr, further demonstrating the importance of quantifying absolute measures in addition to using the ratio method.

4.4.5 Limitations and Future Work

There are several limitations to the current study. With regard to the animal model, we studied repeated impacts at 24-hour intervals in male mice. The first limitation of this work is that we did not examine differences between male and female animals. Since differences have been reported between male and female mice,[26] and our hypotheses were mainly derived from our previous studies of female athletes, future work must

include direct comparison of metabolite changes in male and female mice. Another limitation is that we did not explore the effect of the number of hits delivered, or the timing between hits, which was beyond the scope of the current work. A third limitation is that the mice used in the current study did not undergo cognitive and behavioural testing. A separate cohort of mice was used for this purpose limiting the examination of direct associations between imaging and behavioral measures (5-choice reaction time and paired discrimination), and will be reported elsewhere with the histology findings (GFAP, Iba-1, silver staining, NeuN/APP, and cleaved casp3). Finally, relating to the metabolite level measurements, the variance was high for many of the lower amplitude metabolites, including glutamine, making interpretations challenging. Lower variability may be achieved in the future with a larger sample size, the incorporation of a longitudinal study design, and the use of spectral editing techniques to improve measurement of glutamine specifically.[72,73]

4.4.6 Conclusions

The purpose of this study was to investigate the changes and potential associations between brain metabolite levels measured by MRS and brain microstructure measured by DTI in the mouse brain after a repeated mild closed head injury paradigm. In this study, we observed changes in hippocampal and white matter FA and RD, as well as NAA, and lactate at early time points (e.g 48 hours and 1 week) compared to baseline and later time points. Additional changes in creatine, taurine, Glu/Cr and Gln/Cr later following injury (e.g. 10 weeks), point towards a subtle shift in the biochemical pathways involving these metabolites to re-establish homeostasis.

4.5 References

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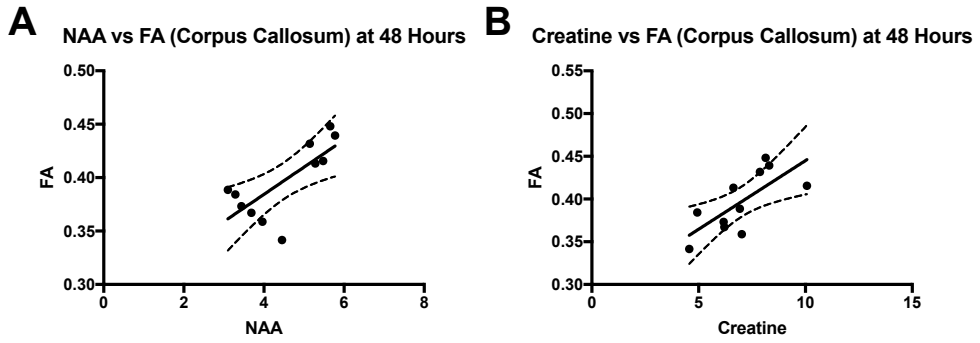
4.6 Supplementary Material



Supplementary Figure 4.1: Correlations between (A) NAA and FA ($p = 0.009$, $r^2 = 0.54$), and (B) Creatine and FA ($p = 0.013$, $r^2 = 0.51$), in the corpus callosum are plotted with 95% confidence band

Chapter 5

5 Summary and Future Work

5.1 Summary

Prior to the work contained within this thesis, previous research applying proton MRS to study concussion had demonstrated that sex differences exist post-concussion[1,2], and the most consistent finding was reduced NAA ratios[1,3–8], but the vast majority of studies focused on male athletes, or chose not to explicitly differentiate between sex. Moreover, studies across labs have examined different brain regions using different methods to quantify absolute metabolite concentrations, making it difficult to compare MRS results across sites. Furthermore, most studies have chosen a short echo time (TE<45ms), and have not separately measured glutamate and glutamine, which are difficult to distinguish due to signal overlap with each other and with less well-defined macromolecule signals[9]. Therefore, proton MRS studies tend to report the sum of glutamate and glutamine as GLX, or do not report these metabolites at all. However, it is important to quantify glutamate and glutamine separately due to their involvement in neurotransmission within the glutamate-glutamine cycle[10]. After a concussive injury, there is altered ion flux and neurotransmitter release[11], which could shift the glutamate-glutamine equilibrium.

The overall objective of this thesis was to first examine metabolite changes using MRS in the prefrontal white matter of female contact athletes before and after concussion, with the added goal of quantifying glutamate and glutamine separately. The second objective was to replicate metabolite level changes in an animal model of concussion, to position future studies to probe the reasons for these changes, and to explore whether these changes represent potential therapeutic targets.

5.1.1 Reduced brain glutamine in female varsity rugby athletes after concussion and in non-concussed athletes after a season of play

Chapter two applied proton MRS to monitor changes in prefrontal white matter metabolite levels in female rugby players with and without concussion, following athletes

over multiple seasons of play, as well as up to 6 months post-concussion. Reduced glutamine and Gln/Cr were found following concussion, and in the Off-Season in non-concussed athletes. Specifically, a 52% reduction in glutamine was observed immediately following concussion and persisted out to 3 months post-concussion, whereas a 21% reduction in glutamine was observed at Off-Season in non-concussed athletes. These changes could reflect a reduction in oxidative metabolism or a shift in the glutamate-glutamine cycle of neurotransmission. Although the magnitude of the glutamine reduction was larger after concussion, the directionality of the changes was similar in both groups. However, the cause of these changes may be different in the concussed and non-concussed athletes. A reduction in glutamine in non-concussed athletes may be due to an exercise effect. Alternatively, the reduction in glutamine in the concussed athletes may be the result of concussion, while the change in non-concussed athletes may be due to the cumulative effect of sub-concussive impacts over the course of the season. Examining the effect of exercise and metabolite levels in non-contact athletes was the main motivation for the thesis Chapter that follows.

Additionally, within the MRS voxel, increased FA and reduced RD were found, which could be interpreted as neuroinflammation or re-myelination. Furthermore, no clinical correlations were found with the reduction in glutamine, which demonstrates the insensitivity of current clinical measures to these persistent brain changes following concussion.

5.1.2 Brain Metabolite Levels in Female Rugby Athletes are different from Non-Contact athletes and Sedentary Women

Chapter three used proton MRS to determine whether metabolite levels were altered in female varsity rugby players (contact athletes) compared to female varsity rowers and swimmers (non-contact athletes) as well as sedentary females. The inclusion of these groups allowed us to examine the effect of exercise on metabolite levels, as well as the effect of repetitive sub-concussive hits over the course of a season. In this study we showed that metabolite levels in non-contact athletes do not differ from age matched sedentary women, except for a small difference in *myo*-inositol. We also demonstrated that *myo*-inositol, glutamate, and glutamine levels differed between contact and non-

contact athletes. Specifically, glutamine levels were significantly elevated in contact athletes compared to sedentary and non-contact groups. Most importantly, these data show that having a sedentary or non-contact control group, instead of baseline scans, can change the interpretation of the directionality of a metabolite change, specifically glutamine, after concussion.

We speculated that the changes in glutamine levels in contact athletes and after concussion, are driven by glutamine synthetase (GS) activity and expression, the enzyme that converts glutamate to glutamine[12]. During neurotransmission, glutamate is released into the synaptic cleft and is then taken up by astrocytes to be converted to glutamine by GS, as part of the glutamate-glutamine cycle. If we assume that glutamate release also occurs in association with sub-concussive hits[11,13], but to a lesser extent than when associated with concussion, it is conceivable that over time GS expression and activity would increase in order to compensate for the elevated glutamate release, manifesting as elevated glutamine levels *as observed in our contact athletes*. In more severe head injuries, one of the most significant pathophysiological processes is the creation of free radicals as a consequence of glutamate efflux and calcium influx[13]. Furthermore, studies have shown GS activity to be sensitive to oxidative stress, and that this inhibition in GS leads to reduced glutamine[12,14]. Therefore, it is feasible that *following a concussive injury, if there is significant production of free radicals, GS inhibition would manifest as reduced glutamine levels*, as we observed post-concussion in Chapter two.

5.1.3 ^{1}H MRS Metabolite changes in a mouse model of repetitive closed head injury

The purpose of Chapter four was to investigate metabolite level changes in the male mouse brain in a model of multiple sub-concussive hits. The main goal with this 5-hit paradigm was to replicate the elevated glutamine levels observed in the female rugby athletes compared to non-contact and sedentary women as we observed in Chapter three. Understanding the metabolic changes that occur following multiple light impacts in animal models is the first step towards understanding the effects of sub-concussive impacts in contact sports. In this study we observed changes in NAA early on (48 hours

and 1 week), that resolved by 4 weeks, indicating a mild injury that was able to recover. However, small changes at 10-weeks in creatine, lactate, taurine, Glu/Cr and Gln/Cr, suggested a subtle shift in the biochemical pathways involving these metabolites to re-establish homeostasis.

Although the elevation in Gln/Cr was successfully replicated 10 weeks after mild repetitive injury, quantification of glutamine levels in this mouse study was challenging due to the complexity of the spectrum at a short echo time (TE = 18.9 ms). Lower variability may be achieved in the future with a larger sample size, the incorporation of a longitudinal study design, and the use of spectral editing techniques to improve measurement of glutamine[15,16].

5.1.4 ^{1}H MRS and Imaging Concussion: Clinical Translation

Using MRS in the clinic as a diagnostic or prognostic tool for concussion is currently not feasible. However, there are several ways to further advance MRS, that can bridge this gap between concussion research and clinical translation, as discussed next, under future work. As outlined earlier, along with MRS research, studies have also investigated changes in blood flow (PET, ASL), neuroinflammation (PET), water diffusion and tractography (DTI), and connectivity (rs-fMRI) after concussion, and changes have been identified in all of these metrics, but clinical translation is still limited.

We are now at a point in concussion research where it is imperative that we start using results from these different imaging metrics, together, to interpret and understand what is happening after concussion, and why. With a better understanding of these mechanisms, therapies can be optimized. For example, MRI provides many different types of contrast fundamentally based on the density or diffusion of water throughout the brain. However, these metrics are insensitive to the biochemical mechanisms underlying pathophysiological changes, such as those present after concussion. This is where MRS fits in, by providing a way to measure the chemical content of MR-visible nuclei, allowing for evaluation of brain metabolism and the biochemical pathways involved. Therefore, MRS can aid in the interpretation of changes found with other MRI techniques. Moreover, as the medical field shifts towards

personalized medicine, there should be a parallel shift towards real-time patient specific data analysis. In regards to MRI, it is feasible that this real time analysis could allow the identification of abnormal brain regions, such as with DTI or rs-fMRI, to which the MRS voxel could be localized for signal acquisition.

5.1.5 Conclusions

The first aim of this thesis was to use proton (^{1}H) MRS to explore metabolite changes in the prefrontal white matter of female contact athletes before and after concussion. In the first ^{1}H MRS study done at 3 Tesla (Chapter 2), reductions in glutamine were observed after concussion at 24-72 hours and 3 months post-injury in female varsity rugby players. In a second ^{1}H MRS study at 3 Tesla (Chapter 3), it was realized that glutamine levels were significantly elevated in the same rugby players (contact athletes) compared to non-contact varsity athletes and sedentary women. These studies taken together demonstrate the importance of investigating metabolites that are implicated in neurotransmission (glutamate and glutamine). Moreover, these data show that having a sedentary or non-contact control group, instead of baseline scans, can change the interpretation of the directionality of a metabolite change, specifically glutamine, after concussion. To begin to understand why these changes occur in contact athletes, we must develop an animal model that can be used for intervention studies.

The second aim of this thesis was to replicate the human findings in an animal model of repetitive mild head injury, to position future studies to probe the reasons for these changes, and to investigate the effects of potential therapeutic targets. In the final study, we used ^{1}H MRS 9.4 Tesla in a mouse model of repeated head injury to investigate metabolite level changes post-injury, with the hypothesis that similar changes to the contact athletes (elevated glutamine) would be observed. Elevated Gln/Cr was observed 10-weeks post-injury, suggesting that the model may be an appropriate model of sub-concussive injury.

5.2 Future Work

5.2.1 Human ^{1}H MRS studies in Athletes and Sport Concussion

This thesis used single voxel ^{1}H MRS in the prefrontal white matter of female varsity athletes, and sedentary women. However, other brain regions are also of interest and have been studied including the hippocampus[17,18], motor cortex[6,19], prefrontal cortex[20], corpus callosum[8,21], and posterior cingulate[22,23], to name a few. It would be interesting to investigate a collection of these brain regions within the same cohort of athletes, to see how metabolites, such as glutamine, may vary across the brain. This is especially intriguing, considering that concussion is a diffuse injury, but how sub-concussive impacts change brain metabolite levels in different brain regions is less clear. Furthering the work from Chapter 3, it would also be useful to follow non-contact athletes and sedentary women over multiple years, as was done with the contact athlete group (female varsity rugby team), to observe how these metabolite differences between groups evolve over time.

Another important aspect to consider when designing future ^{1}H MRS human studies, is the choice of acquisition parameters, TE (echo time) in particular. The majority of sport concussion ^{1}H MRS studies to date, use a TE < 45 ms, which increases the overlap of glutamate and glutamine with macromolecule signals potentially decreasing quantification precision[9]. In this thesis a long-echo time of TE = 135 ms was chosen to minimize macromolecule contributions to the spectrum in an effort to increase quantification precision of glutamate and glutamine. This change in acquisition parameters speaks to the need for standardization of ^{1}H MRS methodology across sites. More importantly, reporting absolute quantification methods and constants, to enable the reproducibility of results across MRI centres, is lacking in the ^{1}H MRS literature. Public databases with metabolite T_1 and T_2 relaxation constants, for example, would allow for easy use in the quantification process and easy referencing for future studies. Moreover, a step towards improved inter-site data quality standards, such as choosing to use CRLBs to eliminate data, is needed to prevent data bias.

5.2.2 Using ^{1}H MRS in Animal Models of Concussion and Sub-Concussion

With the replication of elevated glutamine in a mouse model of repetitive sub-concussive injury (Chapter four), the next questions to investigate include (1) why is glutamine changing after repeated hits to the head, and (2) is this change protective or detrimental? As eluded to in Chapters 3 and 4, the changes in glutamine may be manifesting from changes in the activity level and expression of glutamine synthetase. Therefore, studies examining the activity of this enzyme and expression levels in conjunction with ^{1}H MRS would aid in the interpretation of these data.

In parallel with the animal studies outlined above, it is also pertinent that the acquisition of lower-level metabolites continues to be improved. Although the elevation in Gln/Cr was successfully replicated, quantification of glutamine levels in this study was challenging due to the complexity of the spectrum at a short echo time (TE = 18.9 ms). This includes not just glutamate and glutamine, but also glutathione and GABA, which are all tightly regulated together in the GABA-glutamate-glutamine cycle[10]. Spectral editing techniques with subtraction have become a popular choice, especially for quantification of GABA, however even with editing, separating glutamate and glutamine, as well as removal of macromolecules, remains challenging. Newer spectral editing techniques that do not require subtraction[16] should be considered in future studies. Another possibility for future studies, includes ^{13}C MRS, to explore glutamine changes through glutamine labeling. In fact, studies using this technique have found reductions in glutamine labeling after a fluid percussion injury[24].

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Appendices

Appendix A : Research Ethics

Western Research

Date: 13 March 2019

To: Dr. Arthur Brown

Project ID: 102857

Study Title: London Brain Injury Consortium: A Longitudinal Patient Centered Program to Uncover the Cellular and Molecular Determinants of Neurological Outcomes after Brain Injury.

Application Type: HSREB Amendment Form

Review Type: Delegated

Full Board Reporting Date: 26March2019

Date Approval Issued: 13/Mar/2019 14:41

REB Approval Expiry Date: 10/Aug/2019

Dear Dr. Arthur Brown ,

The Western University Health Sciences Research Ethics Board (HSREB) has reviewed and approved the WREM application form for the amendment, as of the date noted above.

Documents Approved:

Document Name	Document Type	Document Dat
Brown Protocol HSREB Delegated_SSMD_version_date_13 march 2019 changes accepted	Protocol	13/Mar/2019
Letter_of_Information Women's NonAthletes march 2019 changes accepted	Consent Form	13/Mar/2019

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Nicola Geoghegan-Morphet, Ethics Officer on behalf of Dr. Joseph Gilbert, HSREB Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Western

AUP Number: 2018-176
PI Name: Brown, Arthur
AUP Title: Investigations of CNS Injury and Regenerative Therapies
Approval Date: 03/01/2019

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2018-176:1: entitled " Investigations of CNS Injury and Regenerative Therapies "
has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15
   http://www.uwo.ca/univsec/policies_procedures/research.html

   b) University Council on Animal Care Policies and related Animal Care Committee procedures
   http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm
2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
   e) http://uwo.ca/research/services/animalethics/animal_use_protocols.html
3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training (training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.
4) As per MAPP 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
   c) UCAC policies and related ACC procedures will be followed, including but not limited to:
      i) Research Animal Procurement
      ii) Animal Care and Use Records
      iii) Sick Animal Response
      iv) Continuing Care Visits
5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to
hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

Appendix B: Permissions to reprint the published Human Brain Mapping article found in Chapter 2

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Aug 07, 2019

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Curriculum Vitae

University Educational Background

Current Position, PhD Candidate
Medical Biophysics, The University of Western Ontario
Supervised by Dr. Robert Bartha
Centre for Functional and Metabolic Mapping, Robarts Research Institute

2011 – 2015, Bachelors of Medical Science
Honors Specialization in Medical Biophysics (Medical Science Concentration), The University of Western Ontario

Publications

- Ryan K., Schranz A., Duggal N., Bartha R. Differential Effects of Transcranial Direct Current Stimulation on Antiphase and Inphase Motor Tasks: a Pilot Study. *Behav Brain Res.* 2019 March. Pii:S0166—4328(18)31413-X.

- Wong D., **Schranz A.L.**, Bartha R. Optimized in vivo brain glutamate measurement using long-echo-time semi-LASER at 7T. *NMR in Biomedicine.* 2018 August; e4002.

- **Schranz A.L.**, Manning K.Y., Dekaban G.A., Fischer L., Jevremovic T., Blackney K., Barreira C., Doherty T., Fraser D., Brown A., Holmes J., Menon R.S., Bartha R. Reduced Brain Glutamine in Female Varsity Rugby Athletes after Concussion and in Non-Concussed Athletes after a Season of Play. *Hum Brain Mapp.* 2018 April;39(4): 1489-1499.

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Abstracts and Presentations

- Poster at Imaging Network of Ontario (ImNO) Annual Conference. London, Canada (2019). Schranz A.L., *et al*. "Metabolite Levels Differ in Contact and Non-Contact Sport Female Varsity Athletes."
  Poster Presentation Award ($200)
  Represented as a digital poster at the International Society for Magnetic Resonance in Medicine (ISMRM) Montreal, Canada (2019)

- Oral Presentation at Robarts Research Retreat. London, Canada (2018). Schranz A.L., Stanley O. "Ultra-High Field Imaging of the Negative Blood Oxygen Level Dependent Responses in the Primary Motor Cortex using fMRI and fMRS."
  Collaborative Research Competition Award

- Oral Presentation at Imaging Network of Ontario (ImNO) Annual Conference. Toronto, Canada (2018). Schranz A.L., Xu K., *et al*. "Magnetic Resonance Spectroscopy in a Rodent Concussion Model."
  Represented as a poster at the Annual Rotman Research Institute Conference (2018) and Traumatic Brain Injury Conference (2018).

- Poster at Alzheimer's Association International Conference (AAIC). Chicago, USA (2018). Borrie M., Fogarty J., Best S, Haddad SMH., Schranz A., VanderPloeg K., Copeland G., Burhan AM., Montero-Odasso M., Naklweewa S., Bartha R. "Jazzercise as an Intervention for Subjective Cognitive Decline in Postmenopausal Women: Pilot Study Rationale and Feasibility."

- Oral Presentation at Imaging Network of Ontario (ImNO) Annual Conference. London, Canada (2017). Schranz A., Manning K.Y. *et al*. "Reduced Brain Choline in Adolescent Hockey Players after Concussion"
  Represented as a poster at London Health Research Day (2017) and Oral Pitch at Robarts Research Retreat (2017)

- Poster at the Annual Rotman Research Institute Conference: Neural Dynamics and Brain Health. Toronto, Canada (2017). Manning KY, Schranz A., *et al*. "Axonal damage and global hyperconnectivity persist 3-month after concussion in young hockey players."
  Represented as a poster at London Health Research Day (2017)

- Oral Presentation at Imaging Network of Ontario (ImNO) Annual Conference. London, Canada (2017). Manning KY., Schranz A., *et al*. "Axonal damage and global hyperconnectivity persist 3-month after concussion in young hockey players."

- Poster at Imaging Network of Ontario (ImNO) Annual Conference. London, Canada (2017). Wong D., Schranz A., *et al*. "Optimal Echo Time for In-Vivo Glutamate Detection at 7T Using semi-LASER 1H-MRS."
  Represented as an ePoster at International Society for Magnetic Resonance in Medicine (2017)

- Oral Presentation at Imaging Network of Ontario (ImNO) Annual Conference. Toronto, Canada (2016). Schranz A., Blackney K., *et al*. "Reduced Brain Glutamine in Female Varsity Rugby Athletes after Concussion."
  Magna cum Laude Award for the Scientific Presentation
  Represented as a poster at London Health Research Day (2016), Oral Pitch at Robarts Research Retreat (2016) and ePoster at International Society for Magnetic Resonance in Medicine (2017, Magna cum Laude Merit Award)

- Poster at London Health Research Day (LHRD). London, Canada (2016).
  Wong D., Schranz A., *et al*. "Enabling Quantification of ultra-high field 1H-MRS of the brain using simulated *a priori* knowledge."

- Poster at Robarts Research Retreat. London, Canada (2016). Ryan K., Schranz A., *et al*. "Enhancing Manual Dexterity in Healthy Older Adults with the use of Transcranial Direct Current Stimulation."
  Represented as a Poster at London Health Research Day (2016)

Invited Lectures and Teaching Experience

- 2017: Invited Guest Lecture at the Centre for Functional and Metabolic Mapping (CFMM) Winter School, Robarts Research Institute. London, Canada. Schranz A., Wong D. "MR Spectroscopy in Concussion and at Ultra-High Field."

- 2017: Invited Oral Presentation in the Department of Neuropsychology at Western University. London, Canada. Schranz A. "An Introduction to MR Spectroscopy."

- 2017-2018: Teaching Assistant for the Biomechanics Course.
  Help developed concepts during tutorials, hold office hours for student questions, and mark assignments and exams

- 2017: Pedagogy in Biophysics.
  Developed formal understanding of pedagogical concepts underlying high quality science curriculum design and delivery
  Created a new Medical Biophysics undergraduate course, including learning

outcomes, lecture slides, and assignments

- 2016-2019: Teaching Assistant for the General Biophysics Laboratory Course. Introduced and developed concepts including scientific writing skills and statistics, biomechanics, imaging and biophysical analysis; mark assignments and research projects

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Honors, Scholarships and Awards during University

Research Specific

2019/03 Poster Presentation Award ($200)
Imaging Network of Ontario (ImNO)

2018 Collaborative Research Competition Award ($600)
Robarts Research Retreat

2017/04 Magna cum Laude Merit Award
International Society for Magnetic Resonance in Medicine

2017/04 Trainee Educational Stipend ($600 value)
International Society for Magnetic Resonance in Medicine

2016 – 2017 Ontario Graduate Scholarship ($15,000)
Ontario Ministry of Training, Colleges and Universities

2016 – 2017 The Jonathan & Joshua Memorial Scholarship ($1,400)
The University of Western Ontario

2016/03 Magna cum Laude Award for the Scientific Presentation
Imaging Network of Ontario

2015 – 2019 Western Graduate Research Scholarship ($4,500 annually)
The University of Western Ontario

2015 Dean's Undergraduate Research Opportunities Program

Scholarship ($3000)
The University of Western Ontario

Non-Research specific

2018 – 2019 A.C. Groom Award ($1,000)
Awarded to the senior student who presented the most effective seminar from the 2017-2018 seminar series

2011 – 2012

2013 – 2015 Dean's Honor List
Awarded to students who achieve 80% average or higher

2011 – 2012 Western University Scholarship of Excellence ($2,000)
Entrance scholarship awarded to students with a 90-94.9% average

2011 – 2012 Western Scholar
Awarded to students with 90% or higher high school average