S100B AS A POTENTIAL BIOMARKER FOR CONCUSSION IN MALE AND FEMALE COLLEGIATE RUGBY ATHLETES

By

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A Thesis Presented to

The Faculty of Humboldt State University

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Kinesiology: Exercise Science

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May 2018

Abstract

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Monica Ortiz

Sport related concussions are among the top ten most common sport injuries. Substantial and mounting evidence points to extensive short and long term negative consequences from mismanaged sport related concussions. Consequently, researchers have called for better diagnostic concussion tests that are rapid, reliable, sensitive, specific and objective. The purpose of this research was to determine the ability of salivary S100B to detect concussions, predict concussion symptom resolution time, and correlate with changes in neurocognitive performance over the course of one athletic season. A repeated measures of salivary S100B and neurocognitive performance using the ImPACT test and a concussion symptom questionnaire among collegiate male and female rugby athletes was proposed. An insufficient number of concussions were reported (n=1) to perform the original statistical analyses proposed. Alternatively, community baseline values were compared to athlete baseline values to validate previous research, trends (baseline averages compared to post practice and post-game), and a case study of the sole concussion were performed. Efforts to collect further neurocognitive data were abandoned as they weren't able to provide any insight into trends etc. The results of the independent t-test support previous research that rugby athletes have

significantly higher baseline values of S100B than non-rugby population. An increase in averages from baseline to post practice and post-game were noted. There has a notable increase from baseline to post injury for the participant who reported a concussion, although no statistical significance could be determined.

Acknowledgements

This research would not have been possible without the many contributions from my committee, research assistants and personal support system.

Without the mentorship of Elizabeth Larson my life would be on a completely, and far less fulfilling path. Not only did you impart a wealth of knowledge unto me, you've changed my entire approach on life. I will always be grateful and indebted to the investment you made on me and hope that I exceed your expectations.

To Dr. Ortega, thank you for believing in my design and allowing me to take the risk of using ELISA before it was validated. You've provided me with so many opportunities, I am forever grateful for. Thank you for all the times you went above and beyond throughout the years. In addition, thank you for helping me secure the Research Scholarship and Creative Grant to conduct this research.

I would also like to thank Dr. David S. Baston for helping so much with the ELISA testing. Without his assistance, this research would not have been possible. Not only did you guide me through the ELISA, you were a pleasure to work with. Even though I am not a student of yours, or even in your department, you still spent several twelve hour days in the lab with me from start to finish. You are a treasure to the university.

Thank you to the CNRS Core Research Facility for allowing me to use your equipment! Thank you to both of my research assistants, Mariah and Angel. Your

assistance on and off the field was invaluable. You helped me stay organized, inspired and sane.

While not directly involved, I thank the dedicated professors of the kinesiology department, especially Dr. Braithwaite, for providing a solid foundation on which my research was built. Your tutelage during my undergraduate program provided me with the self efficacy to pursue a graduate degree. In addition, I thank all subjects for their time, participation, and saliva.

To my incredible family and friends, thank you for forgiving me for every birthday, wedding or holiday I missed during my pursuit of this degree. Your support and encouragement has kept me moving forward during the more challenging times. To my loving partner Brett, thank you for everything.

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Introduction

Statement of the Problem

Greater than 200,000 people visit the emergency room for sport related concussions (SRC) each year in the United States (Centers for Disease Control 2007). When accounting for the majority of athletes who do not seek emergency care in the event of SRC, and the low reporting of sport related concussions, the number is likely closer to 3.8 million (Langolis, Rutland-Brown, & Wald, 2006). Additionally, this figure excludes sport related concussions that do not result in loss of consciousness, which evidence suggests occurs in less than 6% in collegiate athletes (Wasserman, Kerr, Zuckerman & Covassin, 2016). Nearly half a million college students (National Collegiate Athletic Association) participated in sports in the academic year 2014-2015. Approximately two million collegiate students participate in non NCAA club sports (http://www.nytimes.com/2008/12/02/sports/02club.html?_r=0).

Traumatic brain injuries are evaluated on a spectrum ranging from mild to severe based upon presence or absence of loss of consciousness, lesions, intracranial bleeding etc. Severe traumatic brain injuries may lead to death or severe disability (Selassie et al., 2008). A concussion, on the mild side of the traumatic brain injury spectrum, occurs when biomechanical forces are applied to the body or head, causing jarring of the brain, and resulting in temporary disruption of neurons and brain function (McCrory et al.,

2013). Symptoms are categorized as physical, behavioral, sleep, and cognitive impairment (McCrory et al., 2013). Physical symptoms include headache, nausea, vomiting, difficulty balancing, light and noise sensitivity (McCrory et al., 2013). Behavioral symptoms include sadness, increased anxiety and irritability (McCrory et al, 2013). Sleep symptoms include increase, decrease, or disruptions in sleep (waking up more often than usual), and taking longer than usual to fall asleep (Conder & Conder, 2015). Cognitive symptoms include difficulty remembering, concentrating, or focusing (McCrory et al., 2013).

Barriers to Concussion Diagnosis. Acute diagnosis of a sport related concussion typically relies on commonly observed signs and reported symptoms which can be more subjective than typically preferred for clinical diagnosis. This requirement of honest reporting, coupled with the current lack of rapid diagnostic tools (Bazarian et al., 2006, Makdissi, Davis & McCrory, 2014), leads many athletes to play the remainder of a game or practice (often for days) with a concussion. Symptoms may not appear immediately following a concussion, potentially taking hours (McCrory et al., 2013) or days to present. This frequent gap in presentation of symptoms immediately following injury means athletes are often not removed from play, providing an opportunity for additional injury or even death. Metabolic dysfunction following SRC begins within minutes of injury and, if not removed from sport participation, leaves the athlete vulnerable to compounding injury and the rare but deadly second impact syndrome (Bowen, 2003).

Health effects of repeated and undiagnosed concussion. Recently, discoveries have been made regarding the possibility of long term negative consequences of mild traumatic brain injury, (Michetti, Bruschettini, et al., 2010, Strain et al., 2013) including SRC (Multani et al., 2016, Tremblay et al., 2012). Advanced neuroimaging has found abnormalities in lateral ventricle size, cortical thickness, neurometabolic function, and memory in former athletes with a history of concussions when compared to controls three decades after last concussion (Tremblay et al., 2012). Neurodegenerative disorders such as Chronic Traumatic Encephalopathy (CTE) have been linked to repetitive mild traumatic brain injury (Baugh et al. 2012, Omalu et al., 2011, Tagge et al., 2018). CTE is currently only able to be diagnosed post mortem (Baugh et al., 2012), and has been found in athletes as young as eighteen years old (Boston University CTE Center). No treatment currently exists for this neurodegenerative disorder that has a mean age of death of 59.3 years (Thor, Alvarez & McKee, 2015). An increase of research is being conducted to identify how to better detect or treat concussions, with dozens of articles published monthly. Many sport related medical professionals that specialize in traumatic brain injury agree that an easily obtained biomarker is needed to objectively evaluate the presence of a concussion (Bazarian et al., 2006), and determine long term cognitive dysfunction (Sun & Feng, 2014).

Sport ethics and concussion reporting. Aside from the difficulty of a medical diagnosis, many social issues arise surrounding accurate identification and diagnosis of concussions (Bazarian et al., 2006, Carman et al., 2015). In most settings, it is the

responsibility of the athlete to report that they may have received a concussion. For decades, sport ethics have encouraged playing through injuries, regardless of the nature, as a sign of toughness. Greater than 33% of former collegiate athletes admit to not reporting at least 1 SRC (Kerr, Register-Mihalik, Kroshus, Baugh & Marshall, 2016). Fear of removal from sports participation is cited as reason for lack of self-reporting in nearly 80% of collegiate athletes (Kerr et al., 2016). Athletes frequently (71.8%) report not wanting to let their team down as a motivation for lack of self-reporting (Kerr et al., 2016). Around 1 in 4 collegiate athletes have reported feeling pressure from a coach, teammate, parent, or fan to continue playing after experiencing a head impact (Kroshus et al., 2015). Unfortunately, this pressure leads to lower intention of reporting symptoms of a future suspected concussion (Kroshus et al., 2015). Additionally, research has shown that athletes will often under report concussion symptoms (Meier et al., 2015). Many athletes report not thinking the concussion was "serious enough" to report (Kerr et al., 2016). Many coaches (Faure & Pemberton, 2011) or athletes (Kerr et al., 2016) may not have proper education on what a concussion is, or the associated symptoms. Even after receiving the most commonly used tutorial for SRC education provided through the Centers for Disease Control, Heads Up, collegiate rugby players were not significantly more knowledgeable in basic concussion information (Whyte, 2016). While the Heads Up program did significantly improve collegiate rugby athletes attitudes towards concussions (Whyte, 2016), it remains unknown if the attitude improvement would produce an increase in self reporting concussions.

Issues with current concussion diagnostic tools. Common diagnostic tests for concussions include Immediate post-Concussion Assessment and Cognitive Testing (ImPACT), Standardized Concussion Assessment Tool, Standardized Assessment of Concussion, and Balance Error Scoring System (Rigby, Vela, Housman, 2013). ImPACT is considered the most reliable test, with literature confirming its specificity and sensitivity. Currently, ImPACT is the only Food and Drug Administration approved concussion diagnostic tool. However, ImPACT is not recommended to be used until a minimum of twenty four hours after suspected concussion. The reliability of BESS has ranged from moderate to below clinically acceptable (Bell, Guskiewicz, Clark, Padua, 2011). These commonly used tests require concentration and focus, which in turn may increase symptom severity. Researchers have questioned the necessity and benefits of tests such as ImPACT to confirm a clinically obvious SRC (Mayers and Redick, 2012).

Current best practices emphasize the need for baseline measures of aforementioned tests prior to the season. Ideally, a combination of these measures is conducted annually. Among NCAA athletic trainers (AT), up to 24% report not collecting any baseline measures (Rigby, Vela, & Housman 2013). In addition, most of the ATs report only conducting baseline measures once when the student athlete enters the athletic program, not annually as recommended (Kelly, Jordan, Joyner, Burdette & Buckley, 2014).

Issues with obtaining accurate neurocognitive data. Even in settings where baselines are conducted annually, there are multiple factors that may interfere with the

accuracy of a baseline measurement including “sandbagging”, effect of sleep, testing environment, and effects of learning disorders. Research has found that athletes will “sandbag” (intentionally trying to underperform in hopes that in the case of a concussion, a decrease in scores will not be detected) these tests to prevent possible future removal from competition. Szabo and colleagues (2013) found greater than 17% of football players at a Division 1 university intentionally engaged in “sandbagging” of ImPACT baselines, leading to invalid scores. Amount of sleep prior to testing has been found to effect 3 of 4 composite scores of ImPACT and increases symptom endorsement (McClure, Zuckerman, Kutscher, Gregory & Solomon, 2014). Group versus individualized neurocognitive testing significantly negatively interferes with all composite scores on ImPACT, even when excluding those with histories of learning disorders, attention deficit or concussions (Scolaro, Schatz, Neidzwski, & Ott, 2010). Considering that baseline testing often occurs in a group environment, and post injury testing is typically administered in an more quiet, distraction free, individual setting, testing environment may be a cause for concern. Athletes with a history of learning disorders, attention deficit, or both, performed significantly worse than their peers at baseline measures of ImPACT (Zuckerman, Lee, Odom, Solomon & Sillis, 2013).

Imaging techniques for diagnosing concussions such as computerized tomography (CT) are common practice, yet CT scans rarely produce remarkable results after a concussion (Bazarian, Blyth & Cimpello, 2006, McCrory et al., 2013). This imaging is unable to detect the subtle, often microscopic changes common of

concussions. Imaging equipment is not readily available at sporting events and taking an athlete to a hospital for tests that will likely not produce results is costly. The cost of an imaging test such as a CT scan ranges from three hundred dollars to greater than three thousand dollars (http://www.comparecatscancost.com/). Furthermore, imaging requires the athlete to be exposed to radiation, and a significant amount of stimulus in the form of questions asked of patients and lights and noises associated with an emergency room. A long history of contact sports could potentially expose an athlete's brain to radiation repeatedly. Additional concerns regarding the safety of using scanning techniques for patients with mild brain injury/concussion that experienced loss of consciousness have been raised (Jeter et al., 2013). This lack of reliable imaging leaves a gap for needed research. Such research may include the use of biomarkers that could be measured in near real-time.

Concussion monitoring and return to play. While the consensus states that most SRC will resolve spontaneously within 7-10 days (McCrory et al., 2013), in a study using only collegiate rugby players, up to 60% required 21 days or more to recover (Chermann et al., 2014). Meier and colleagues (2015) suggested that relying on ImPACT, self-reported symptoms, and clinical presentation may not accurately identify all symptomatic athletes. Up to 60% of the athletes studied were returned to play before all symptoms had resolved at 9 days' post injury (Meier et al., 2015). This is particularly concerning as concussion experts do not recommend initiating the graded return to play protocol until full symptom resolution for 24 hours.

S100B introduction

S100B is a calcium binding protein (Michetti et al., 2012). It is the most widely studied and promising biomarker in regard to traumatic brain injury (Di Pietro et al., 2014, Schulte et al., 2014, Wolf et al., 2013). It can be detected in cerebrospinal fluid (CSF), blood, urine and saliva (Michetti et al., 2012). The protein is most heavily concentrated in the central nervous system (CNS) within glial cells, but can also be found in low concentration in peripheral tissues (Michetti et al., 2012). Following traumatic brain injury, it is possible that S100B passes through the disrupted blood brain barrier after being leaked by CNS cells (Schulte et al., 2014). The concentration increase observed post traumatic brain injury in CSF and blood post traumatic brain injury typically resolves within 24 hours (Sandler, Figaji & Adelson, 2010). The cellular signaling mechanism responsible for the increase in concentration of S100B is still unknown (Schulte et al., 2014). At nanomolar concentrations, the protein is considered to have a neuroprotective or neurotropic effect (Michetti et al., 2012), however, when micromolar concentration is reached, it is indicative of neurotoxic conditions and brain injury (Michetti et al., 2012), and may amplify apoptosis (Marenholz et al., 2004).

Previous research of S100B. S100B has been shown to predict negative outcome following traumatic brain injury (Thelin, Johannesson, Nelson, & Bellander, 2013). Serum S100B has been found to accurately predict injuries (e.g., lesions) as shown by CT scans (Cervellin et al., 2012, Abbasi, Sajjadi, Fathi & Maghsoudi, 2014, Wolf et al.,

2013). Furthermore, serum S100B increases significantly after a mild traumatic brain injury and remains elevated above normal levels 1 to 14 days following injury in animal models (Rostami et al., 2012). At baseline conditions (not during state of brain injury), no significant differences have been found in athletes with and without concussion histories for blood levels of S100B, supporting its potential use as a concussion diagnostic tool (Di Battista et al., 2016).

S100B and neurocognitive performance. Impaired neurocognitive performance has been found to be impaired in certain populations with elevated serum S100B levels unrelated to traumatic brain injury in both human and animal models. Impairment of memory in patients with chronic schizophrenia was correlated with increase S100B serum levels (Pedersen et al., 2008). In transgenic mice with Down Syndrome, a significant decrease in spatial learning was found (Gahtan, Auerbach, Groner, & Segal, 1998). Bjursten and colleagues observed a trend of decreased neurocognitive performance with an increase in serum S100B levels when studying the effect of exposure to high altitude. To the best of my knowledge, no research has monitored salivary S100B levels in relation to neurocognitive performance in an athletic population.

S100B and athletes. In the athletic population, age, body mass index (BMI) and concussion history have been found to be independent of S100B levels (Marchi et al., 2013). Research using salivary S100B has historically been used in hospital setting with preterm infants to detect neurological injury due to complicated births. Gazzolo et al., found elevated levels salivary of S100B at multiple time points predicted negative

outcome in infants who experienced asphyxia during birth. To date only one study has used saliva to obtain concentration levels of S100B related to sports (Michetti et al., 2011). Michetti and colleagues found rugby athletes have higher levels of S100B at baseline and post vigorous activity, and compared to non-athletes post vigorous activity. This analysis by Michetti et al., did not investigate any changes in neurocognitive function, nor did it include any subjects with traumatic brain injury.

S100B and exercise. Salivary S100B levels were found to increase after vigorous physical activity (Michetti, Bruschettini, Bruschettini, Frigiola, & Abella, 2011), and in serum post soccer games (Stålnacke, Tegner, & Sojka, 2004). Despite these changes post physical activity, S100B is still able to detect differences from sport related exertion and sport related concussion (Kiechle et al., 2013). Michetti et al., did not provide an operational definition of vigorous physical activity, nor did it provide the amount of time between the completion of the vigorous physical activity and the saliva collection which may have influenced the concentration.

S100B and brain trauma. S100B levels were found to increase after traumatic brain injury (Kiechle et al., 2014, Thelin et al., 2013). Many of the articles stated that concentrations normalized within 24 hours of injury (Marchi et al., 2013, Kiechle et al., 2014). Kellerman and colleagues found serum S100B levels greater than 0.7μg/l one day post admission to the hospital predicted patient mortality with 100% accuracy. A similar study found the best cutoff for predicting mortality was only 0.491 μg/l for serum and 0.025 μg/l for urine at 24 hours post injury (Rodriguez-Rodriguez et al., 2012). S100B

has also been significantly correlated to the number and severity of subconcussive blows athletes experience (Stålnacke, Tegner, & Sojka, 2004, Marchi et al., 2013). In amateur boxers, those who received multiple punches to the head had a significant increase in serum S100B (Graham et al., 2011). Schultz et al. (2016), found that serum S100B remained elevated in football collegiate athletes who had sustained a SRC and were deemed medically disqualified through the remainder of the season.

ELISA Test Principle. The microtiter plate is coated with an antibody specific to S100B. Standards and samples are added to wells with biotin conjugated antibody specific to S100B. Advidin conjugated to horseradish peroxidase is added and incubated. TMB substrate solution is then added, only wells with S100B, biotin-conjugated antibody and enzyme-conjugated advidin will exhibit change in color. The enzyme-substrate reaction is then terminated by the addition of sulfuric acid solution. The color change is then measured at 450 nm+/-2 nm using a Molecular Devices SpectraMax i3 spectrophotometer. The concentration is determined by comparing the optical density of samples to the standard curve.

Hypothesis and exploratory questions

The following hypothesis were proposed for this study:

S100B Laboratory Analysis

1. S100B will increase post sport related concussion.

Neurocognitive Performance/Change

1. A decrease in neurocognitive performance post sport related concussion will be observed with an increase of S100B.
2. As S100B returns to baseline value, neurocognitive performance will return to baseline values.

The following exploratory questions were also examined:

1. Does saliva have a high enough concentration to be detected using an Enzyme Linked Immunosorbent Assay (ELISA)?
2. Is saliva from an athletic population significantly higher than a community population?
3. Can saliva S100B significantly increase post practice?
4. Can a significant increase in S100B be detected post game?
5. Is it possible to identify a range in baseline S100B values among athletes?

Assumptions

The following assumptions were made for this study:

1. Participants were able to identify if they sustain/sustained a concussion.
2. Participants reported suspected concussions immediately.
3. Participants did not provide a baseline if they currently have a concussion.

Delimitations

The study was delimited by the following factors:

1. The participants were provided verbal and written concussion education.
2. An athletic trainer was present at games, and a safety officer was present during practices to identify SRC and immediately remove players with suspected concussions.
3. During baseline saliva and neurocognitive tests, participants were given the definition of concussion and asked if they suspect they had one.

Methodology

Design

This study employed a prospective cohort, *in vitro* quantitative design.

Athletic Subjects

Athletic subjects consisted of approximately 44 healthy adult male and female collegiate rugby club sport players (ages 18-25 years) at a small Pacific Northwest California State University. Participants were recruited during participation in the 2016-2017 rugby season. Subjects that experienced a concussion during the 2016-2017 rugby season either in practice or game were assigned to the "concussed" group. Based on data from the last 4-5 seasons, we anticipated that approximately 25-30 individuals would be included in the concussed group. The remaining participants who did not sustain a concussion during the season were assigned to the "control" group. The experiment was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and was approved by the Humboldt State University Institutional Review Board. All subjects gave written informed consent prior to participation in the study.

Community Subjects for ELISA validation

Prior to performing the ELISA on the athletic subject samples, validation/range finding experiments were performed to determine the efficacy of the ELISA using saliva. To perform this validation, 35 non NCAA male and female, age 18-35 'community members' were recruited to provide salivary samples. Recruitment was obtained via flyer and word of mouth. All subjects consented to participation prior to saliva collection.

All subjects completed a baseline assessment. During the baseline assessment session (Pre 1), subjects provided basic demographic information (age, gender, sport, position, concussion history, neurological history), completed an assessment of neurocognitive function using the ImPACT test (Pittsburgh, PA). At a later date, subjects provided two saliva samples (Baseline) for S100B quantification. All subjects diagnosed with a concussion by an athletic trainer underwent an acute post-injury assessment (Post Injury) within 1 day of the concussion injury. In this session subjects were to provide two saliva samples to be used to quantify S100B level (Post Injury). Following the post 1 session, all concussed subjects were to complete a "2-5 days post injury" (Post 2) assessment of neurocognitive function using the ImPACT test. The concussed subjects were to undergo neurocognitive assessment weekly until their ImPACT scores have returned to baseline/normal levels and they received clearance from a qualified health care professional to return to activity.

ELISA protocol. A sandwich ELISA kit (Cloud-Clone, item no. ABIN415069) was used to detect S100B concentration. The kit is an enzyme immunoassay for *in vitro* quantitative measurement of S100B designed for use of serum, cell culture supernatant, tissue homogenate, plasma, and bodily fluids. The detection range for this kit is 0.15-10ng/mL with a sensitivity of 0.059 ng/mL. The ELISA kits were performed per manufacturer's instructions. At the time of baseline assessment (Pre 1) and immediately after sustaining a concussion (Post 1) participant was given a saliva collection kit and instructed on proper use. Saliva was to be collected at S100B peak value of 60 minutes post injury, twenty-four hours post injury (Post 2), and one month post injury (Post 3). Baseline saliva collections were performed in laboratories. As it became clear that enough concussions would not be reported, saliva was collected after practice (Post Practice), and after games (Post Game). Post injury, post practice and post-game collections were performed on site at the fields. Samples collected on sidelines were immediately stored in a foam ice box containing dry ice, and then transferred to storage in a freezer at -80°C. Samples collected in laboratories were immediately stored at -80°C. Sample age ranged from 1 to 197 days.

Saliva was collected using the passive drool technique. Participants were instructed to allow saliva to pool into their mouths. Next, they tilted their heads downward and gently forced the saliva into the Saliva Collection Aid (Salimetrics Item No. 5016.02), where it was deposited into a 2 milliliter, polypropylene, cryovial (Salimetrics Item No. 5002.01-06). Participants provided water immediately before

providing sample. A minimum of 2 milliliters was collected. Cryovials were stored in cardboard cryostorage boxes (9x9 grids, 81 tubes per box) per manufacturers' instructions.

During the ELISA test, two changes were made to the manufacturers instructions. First, rather than being centrifuged as recommended, samples were vortexed after defrosting due to incompatibility with the available centrifuge, and tube in which the samples were stored. The second variation was due to a shortage of diluent A (approximately 6mls short of needed volume). In result, the amount of diluent A was decreased to allow for completion of the procedure.

Due to the limited number (96) of wells per plate, a total of 15 athlete baseline samples were used for the ELISA, each being run in duplicate as recommended by manufacturer. Although participants were directed to fill the entire saliva collection aid, the consistency of some saliva was frothy or bubbly, and after being frozen, defrosted and vortexed, sample volume decreased. Samples to be used were chosen by amount of saliva present after thawing, as a minimum of 0.2 milliliters was needed to run the analysis. All of the post practice (n=5), post game (n=4), and post concussion samples (n=1) were used for analysis and run in duplicates.

All samples and reagents were brought to room temperature prior to assay. Concentrations were predicted prior to analysis. At room temperature, reagents were prepared. 10mL of wash solution was diluted using 990mL of distilled water to prepare a total of 1000mL. This solution was used within 3 days.

Coated wells were placed in the holder followed by the addition of 50μL of the samples to the appropriate well of the pre-coated antibody microtiter plate. 100μL of conjugate was added to each well and thoroughly mixed. The wells were covered and incubated at 37°C for 1 hour. Following incubation, the mixture was removed by aspirating contents from the wells. This procedure was repeated for a total of five washes. After the final wash the plate was inverted, and blotted onto absorbent sheets until dry. 50μL of substrate A and B were added to each well. Wells were covered and incubated for 15 minutes at 20-25°C using a Forma Scientific Incubator (Model 310). 50μL of the stop solution was added to each well and thoroughly mixed. Optical density was then determined at 450nm using a microplate reader.

The duplicate readings were averaged for each standard and sample. All optical density values were subtracted by the mean value of the blank. A standard curve was developed by plotting the average optical density reading for each standard on the vertical axis, against concentration on the horizontal axis. A line of best fit was produced using Microsoft Excel to generate a linear regression. The concentration was then determined by calculating to the corresponding mean absorbance from equation for the trendline developed from the standard curve.

Tables

Table 1 Independent t-test comparing baseline athletes to baseline community S100B (ng/ml)

Group	Mean	SD	Range
Athletes	1.41*	1.12	4.48
Community	0.41	0.37	1.17

Note: *$p < 0.05$

Table 2 Baseline to Post Game S100B (ng/ml)

Participant	Baseline	Post Game	Percent Change
Subject 1	0.99	0.99	.7
Subject 22	.835	1.796	115.08
Subject 23	.614	1.336	117.58
Subject 32	1.67	1.382	-17.36

Table 3 Comparing Baseline Concentrations to Previous Research

Author	Technique	Source	Baseline Median
Schulte et al., 2016	ELISA	Serum	89 ng/L
Michetti et al., 2011	ILMA	Saliva	.75 μg/L
Present Study	ELISA	Saliva	.9867 ng/ml
Stalnacke et al., 2004	ILMA	Serum	.066 μg/ml

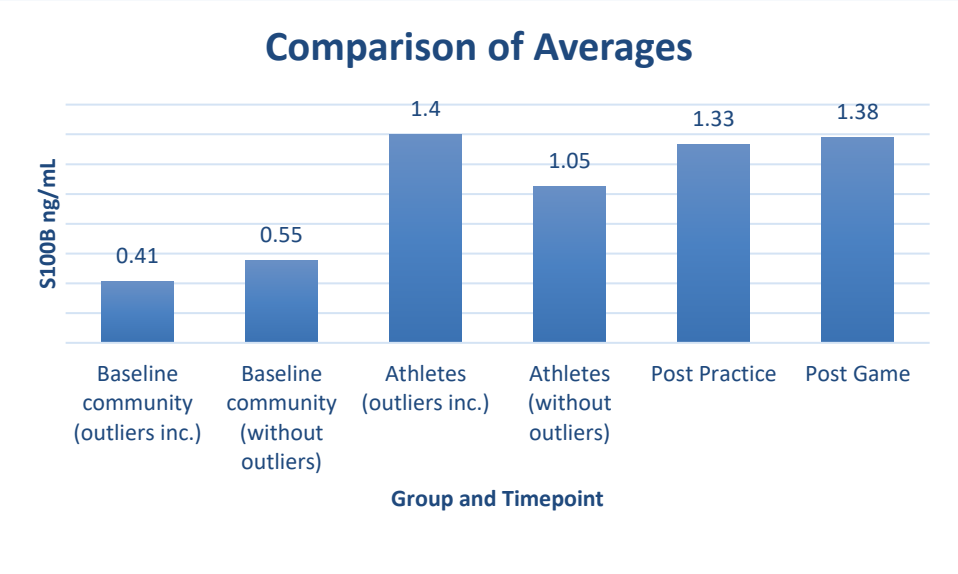

Figure 1 Averages for Each Collection Type

Results

An insufficient number of concussions were reported (n=1), making it unrealistic to test the initial hypothesis. The low number of reported concussions is likely explained by several factors including cancelled games, increase in player safety, and fear of being removed from play if a concussion was reported. In lieu of the original hypothesis being tested (repeated measures, baseline to post injury), it was decided that exploratory questions be more deeply analyzed to fill gaps in S100B salivary data, replicating the lone previous research using athletes and salivary S100B (Michetti et al., 2011), and the sole concussion reported be presented as a case study. Post-concussion ImPACT data collection was abandoned as it would provide no statistical or exploratory function.

Assumptions relevant to an independent t-test were tested. Two outliers were detected through boxplots, 1 belonging to each group (community and athletes). Levine's test for equality of variances was violated ($p=0.31$) leading to interpret the data as equal variances not to be assumed. Tests for normality revealed the assumption was met (Kolmogorov-Smirnov) for community $p = 0.51$, athletes $p = 0.12$). The assumption for independence was met. Observed power for this analysis was .880. Effect size, as measured by Cohen's *d* was large, 1.18.

SPSS version 25 was used to conduct an independent t-test to compare baseline samples between the athletic group ($n = 15$) and the community group ($n = 15$). Level of significance was set to $p < 0.05$. Community members included in the analysis were

chosen using an internet based randomizer program designed to eliminate bias. In line with previous research, results support the hypothesis that significantly higher S100B concentrations would be observed in the athletic population than the community non athletes ($p = 0.006$).

Evaluation of Hypotheses and Exploratory Questions

1. Does saliva have a high enough S100B concentration to be detected using an ELISA?
2. Is saliva from an athletic population significantly higher than a community population?
3. Can S100B increase post practice?
4. Can a significant increase in S100B be detected post game?
5. Will there be a large range in baseline values among athletes?

The ELISA kit was sensitive enough to detect S100B levels in both the athletic and community participants, at each time point, within all samples. Sample size was not required to be reduced (serial dilutions), nor is the use of protease inhibitor required (data indicated the protein had not been degraded). The data produced was also intended to be used for a technical paper on the validation of saliva using this kit.

Case study. One concussion was reported during the last game of the season. A saliva collection was obtained approximately 60 minutes post injury. An increase of from

.614ng/ml to 1.33ng/ml (114.7% increase) was observed. While limited due to the small number, this notable increase does provide promise for future use as a biomarker, but is diminished by a similar increase (see Table 2) observed post game in non-concussed athletes (n=2). In addition, it's possible that the increase was due to subconcussive blows (Stålnacke, Tegner, & Sojka, 2004, Marchi et al., 2013) most contact sport athletes will sustain during a competitive game.

Comparison of Baseline, Post Game, and Post Practice Averages. Among the athletes, baseline values ranged from 0.278ng/ml to 4.754ng/ml, and had an average of 1.426 ng/ml. As aforementioned, within the athletic and community participants, an outlier was identified in each group during the statistical analysis. For exploratory purposes, an additional average was created with the removal of the sole outlier, which decreased the average to 1.05ng/ml. The post-practice and post-game averages were 1.332ng/ml, and 1.377ng/ml, respectively (see Figure 1). The increase between baseline and post practice and game may be explained by adipolysis from sustained physical activity. There is however, the possibility that the small increase (0.0045ng/ml) between post practice and post game is due to subconcussive blows sustained during the game. Theoretically, if due solely to adipolysis, the post practice average should be higher than the post game values, as practice was 120 minutes in duration, and games were 80 minutes in duration, and the athletes likely played at a higher intensity during the game than during practice. Interestingly, one participant exhibited a decrease (-17.36%) from baseline (1.67ng/ml) to post game (1.38ng/ml).

Discussion

Notably, when comparing the results of this study to that of similar studies, there is a clear difference in the values obtained at each time point, potentially explained by the different method of S100B collection and techniques used to quantify S100B and source of collection S100B (see Table 3). In addition to athletes exhibiting a higher average baseline value than non athlete participants, a greater range of baseline values was present among the athletic participants than the non athlete participants (4.48ng/ml, 1.17ng/ml respectively). There are two possible explanations for the greater range among the athletes: an athletic participant(s) may have provided the baseline sample during a state of concussion (wittingly or unwittingly), as symptoms may not have been present prior or during to baseline collection, or the athletic population may have included participants who were in a state of adipolysis or skeletal muscle repair.

Variants observed in the present study of baseline S100B values may be better disentangled by a more thorough series of sampling. Collecting serial baseline sample after a mandatory three day period of abstinence from all exercise, e.g., three days off then baseline once daily for three subsequent days, may decrease the wide range of variation seen in rugby athletes and establish a more reliable frame of reference for baseline values. Furthermore, asking future participants to report on their recent and normal exercise habits utilizing well substantiated definitions of level of activity (i.e. sedentary, lightly active, moderately active, very active) from an organization such as

the Centers for Disease Control, could provide additional clarity when deciphering baseline variants. This reporting should be separate from reporting on both historical contact sport exposure and current contact level in practice and game settings.

A controlled, methodical monitoring of S100B during exercise without subconcussive blows should be performed with saliva collected immediately prior, during and post exercise. Post exercise and post game (non vs. contact) serial S100B should be taken at one hour, three hours, six hours, twelve hours, twenty four hours, forty eight hours and one week post exercise or game for comparison. A controlled monitoring of S100B levels along with subconcussive blows (frequency (number) and intensity (force) recorded) in both a practice and game settings should be conducted. Using videography and head gear that detects and records the magnitude of external forces applied may help identify expected changes in saliva S100B levels in response to subconcussive blows. Further examination using neurocognitive tests would potentially identify any relationships between changes in salivary S100B levels under conditions of subconcussive blows and neurocognitive performance.

Collecting a more thorough medical history may too help explain S100B salivary concentration variation. Medical history should include past or current history of melanoma, mood disorders, other neurological disorders, etc. Recent

exposure to high altitude or any other external or internal conditions that may affect the permeability of the blood brain barrier should be accounted for.

Due to the peripheral storage of S100B primarily in adipose tissue, body fat may partially account for variation in concentration, however previous research (Marchi et al., 2013) has relied on BMI. While BMI is commonly used as a rapid measure for health risk stratification, it is often inaccurate for classifying athletes (Mazic et al., 2009) as they tend to have higher amounts of muscle mass (compared to non athletes), which is much heavier than fat mass. Using more accurate ways to obtain body composition (e.g., hydrostatic tank, skin folds) may reveal a relationship between overall body fat and S100B at baseline.

Rugby is a collision sport played without protective gear, and therefore comes with inherent danger. While rare relative to overall number of rugby players worldwide and absent from existing scholarly literature, there have been several brain injury caused deaths among rugby players in recent years reported by the media. Given perceived stress has been found to have a relationship with S100B levels (Li et al., 2014), player position, experience and self efficacy may also play a part, and therefore should also be further investigated. At each of the aforementioned serial

collection points, a simple self-report of perceived stress should be obtained to help determine the effect perceived stress may have on S100B levels.

Limitations

Several factors limited the present research. While a statistically sufficient number of athlete baseline data was analyzed (n=15), it is possible that the athlete baselines that were not analyzed (due to a limited number of wells) may have produced a different mean. Although data for post-game and post practice was used simply for investigating trends, the numbers for both were small. In addition, none of the participants who provided post practice samples had other data points to compare them to. Thus, the comparisons of baseline, post practice and post-game averages must be taken with caution of this in mind as the data may be skewed. Another limiting factor present in this research is the saliva sample age range, as rate of decay during storage has not been established.

Conclusions

Salivary S100B shows promise as a biomarker for future research as a diagnostic tool for SRCs and is a good fit for use in ELISA. Salivary S100B was detectable without addition of protease inhibitor, does not require serial dilutions, and detected a wide range of concentrations among both groups. Diagnosing SRCs using an ELISA kit to test salivary S100B concentrations shows promise to providing results in quicker turnaround times, (for indicating both an SRC and a return to baseline). In addition, it only requires a one hour wait period between suspected injury and data collection, which is twenty three hours less than the required twenty four hour period between suspected injury and administration of the FDA approved ImPACT test. Importantly, it requires virtually no effort on the subjects' behalf, isn't able to be 'sandbagged,' and if used as a precautionary measure (i.e. all athletes submit a sample after each game) may detect SRCs before symptoms may appear.

Future Research Participants and Methods. Future researchers would benefit from using non club sport participants and club sport participants in the same studies. While it is true that most research has examined NCAA athletes and more club sport participants should be used, the less predictable nature (cancelled games, cancelled practices, high attrition rate, athletes who consistently miss practice) of club sports presents unique challenges to data collections and thus decreases the efficiency of research. It would serve future researchers who aim to use ELISA as a method of

diagnostics well to collect throughout the season rather than rely on self-reporting (i.e., after several games and several practices). Furthermore, this approach may allow a retrospective analysis for athletes who do not report immediately, continue to play or practice, and report after.

Future research S100B ELISA methods. Future research should focus on effects of exercise, optimal collection time, decreasing ELISA time, comparing concentrations of CSF, urine, saliva and serum, comparing sandwich based methods (i.e., ILMA versus ELISA), and hydration status. Future research should quantify the effects of exercise to changes in salivary S100B levels. Previous researchers were able to quantify peripheral sources of S100B by using a creatine kinase factor derived from serum. If replicated using saliva, peripheral sources may be deducted from total quantity and allow a more accurate level of neurological sources. More research is needed to determine peak S100B levels in saliva. Currently, only serum and CSF have research supported S100B peak levels and the debate is ongoing. More research should be invested in reducing time required to perform ELISA. There is currently an “instant” ELISA kit which may reduce analysis time by several hours and has yet to be validated using saliva. While only one operator conducted all ELISA tests in the present study, potential changes in concentration may occur via different operator conducting the ELISA. It seems that automation may be the optimal route to pursue as variances in operator performance may be eliminated and may decrease total time required to perform the analysis and allow for more accurate comparisons between research. Future research should determine the

necessity or possible interference with having participants drink water immediately prior to saliva collection. Hydration status appeared to influence thickness of saliva, which in turn may have affected the concentration of S100B in salivary samples.

What this research adds to the body of knowledge. S100B is detectable in saliva when using an ELISA technique, which is the least invasive means of collection relative to the procurement of other sources (e.g., CSF, serum). Individual baselines for athletes have a wide range, supporting previous researchers' claims that an individualized baseline is required rather than using a 'normal range cutoff' approach. In accordance with Mitchetti et al., 2011, rugby athletes exhibit higher salivary levels of S100B during a baseline state than non-rugby players. Serial dilutions are not required to perform the ELISA, nor is a protease inhibitor. The lack of needing dilutions or protease inhibitor decreases the cost and time required to perform the ELISA.

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