ACUTE SUBCONCUSSIVE EFFECT ON PLASMA NEUROFILAMENT LIGHT POLYPEPTIDE IN COLLEGIATE SOCCER PLAYERS

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ABSTRACT

Research continues to allude to the efficacy of using blood biomarkers as tools to detect brain parenchymal damage in the periphery of the body. While emerging evidence supports the use of neurofilament light polypeptide (NF-L) as a specific blood biomarker of cerebral trauma, the cogency of its use with respect to repetitive subconcussive head impacts remains elusive. Therefore, the purpose of this study was to investigate the acute effects of subconcussive head impacts on plasma NF-L levels in the collegiate soccer cohort. To be considered for inclusion, participants were required to have at least three years of soccer heading experience and be between the ages of 18 and 26. Conversely, participants were excluded if they had a history of head injury during one year prior to the study or a history of neurological disorders.

In effort to test our central hypothesis that plasma levels of NF-L will increase in proportion to the amount of subconcussive head impact experienced, thirty-four participants were distributed between kicking-control and heading groups and assessed at four time points (pre-intervention, 0h-post, 2h-post, and 24h-post intervention). At each time point, a blood sample was collected from each participant. Between the pre- intervention and the 0h-post time point, each participant completed an intervention based on random group assignment. Participants in the heading group performed 10 soccer headers, while the kicking-control group performed 10 kicks. The ball was released by a JUGS machine approximately 40 feet from the participant at a speed of 25mph, inducing an average force of about 30g, similar to that of a long-throw during a soccer game, in one-minute intervals.

Our results support previous findings that NF-L expression markedly increases in proportion to subconcussive head impacts cumulatively, with a significant increase at 24h post-heading. Subconcussive head impacts gradually increased plasma NF-L expression, as illustrated

by a significant time by group interaction, $F(1, 31) = 9.17$, $p = 0.0049$, while the kicking-control group remain consistent throughout the study time points. A significant difference was revealed at 24h post-heading (3.68 ± 0.30 pg/mL) compared to pre-heading (3.12 ± 0.29 pg/mL, $p = 0.0013$; Cohen's $d = 1.898$). The heading group (3.68 ± 0.30 pg/mL) showed a significantly higher level of plasma NF-L than the kicking-control group (2.97 ± 0.24 pg/mL: $p = 0.038$: Fig 1). There are two chief findings from this study: 1) There was no significant increase in NF-L expression until the 24h post-heading assessment in the heading group and 2) NF-L expression remained consistent across all time points in the kicking-control group. These findings suggest potential for cumulative effects of subconcussive head impacts.

As research continues to reflect the negative neurocognitive effects of subconcussive head impacts, it is imperative that a gold standard for determining diagnosis, treatment, and prognosis is established. Our study further supports the efficacy of using biomarkers, specifically NF-L, in the triage and management of brain injuries.

INTRODUCTION

Subconcussion is a related, often overlooked condition defined as cerebral trauma that does not result in presentation of signs or symptoms and occurs secondary to repetitive lower-load impacts.[1] Subconcussion is ubiquitous in contact sports such as football, soccer, boxing, wrestling, rugby, hockey, or lacrosse.[2] Because of the invisible nature of the injury, athletes in contact sports endure a few hundreds to 1000 subconcussive head impacts in a single season. Long-term, repetitive subconcussive hits increase the risk of triggering concussion and are strongly linked to a trauma-induced neurodegenerative disease, referred to as chronic traumatic encephalopathy (CTE).[3]

While current knowledge of brain injuries is ever-evolving, a valid and objective measure of damage has yet to be identified and implemented as a gold standard for concussion diagnosis, treatment, or prognosis.[4-6] Given the current subjective nature of concussion assessment and management, the need for such a measure has become increasingly apparent.[7-11] Biological markers, hereafter referred to as "biomarkers," are measurable changes to cellular, molecular, or biochemical structures of the body. Blood biomarkers are free of subjective fluctuations and are able to detect subtle structural and metabolic changes after neural damage; therefore, it is inarguable that the blood biomarker has a cogent potential for a paradigm shift in triage and management of brain injuries.[12]

Biomarkers for brain injury each have specific derivations in the brain; for example, the marker S100B is expressed with astrological injury.[13] This aids clinicians in determining which biomarkers to prioritize in research as indicators of certain conditions. Biomarkers at the forefront of research on cerebral trauma include S100B, glial fibrillary acidic protein (GFAP), neurofilament light polypeptide (NF-L), and amyloid beta.[5, 11, 13, 14] Neurofilaments proteins

abundantly interspersed in and unique to intermediate axonal fibers comprise the bulk of the axonal skeleton.[15, 16] Of the five subunits of neurofilaments – neurofilament light (NF-L), neurofilament medium (NF-M), neurofilament heavy (NF-H), peripherin, and alpha-internexin, - NF-L is the most integral to large, myelinated axons deep in the brain.[6] In addition to providing protection from mechanical stress, NF-L is the primary cytoskeletal protein necessary for neuronal organization and allows for radial growth, myelination, and maintenance of the axon. [16-18]

NF-L has recently gleaned attention from the research community, as it may be the most sensitive and specific blood biomarker for axonal injury.[6, 12, 19] Recent studies have shown that plasma levels of NF-L are strongly associated with severe-moderate traumatic brain injury and neurodegenerative conditions such as Alzheimer's, multiple sclerosis, amyotrophic lateral sclerosis, frontotemporal dementia, Chronic Traumatic Encephalopathy, and Charco-Marie Tooth.[6, 9, 12, 18, 20-24] Most notably, preliminary reports indicate that plasma NF-L levels significantly increase after a concussion. Moreover, season-long exposure to subconcussive head impacts may induce a brazen increase in plasma NF-L levels.[12]

Emerging evidence supports the efficacy of using blood biomarkers to detect brain parenchymal damage in the peripheral system. Although NF-L has been shown to reflect axonal damage secondary to traumatic force, evidence of this effect in the subconcussive population remains elusive. To fill the current knowledge gap, we conducted a randomized control trial using our established soccer heading model to control frequency and consistency in magnitude of head impacts. We examined changes in plasma NF-L levels at 0h, 2h, and 24h after 10 soccer headers compared to a pre-heading baseline. Kicking-control subjects underwent the same plasma sampling and accounted for potential exercise and musculoskeletal damage. We

hypothesized that there will be a significant elevation in plasma levels of NF-L after repetitive subconcussive head impacts, while there will be no change in those who performed soccer kicking. Therefore, the purpose of this study was to examine the effects of subconcussive head impacts on plasma NF-L levels in three acute phases compared to a baseline between groups in the collegiate soccer cohort.

METHODS

Study design

A laboratory study with a 2 x 4 repeated measure design was conducted to examine the acute effect of repetitive subconcussive head impacts on plasma NF-L expression. Independent variables included time (pre-intervention, 0h-post, 2h-post, and 24h-post interventions) and grouping (kicking control and heading). The dependent variable was plasma NF-L expression.

Participants

Thirty-four healthy adult soccer players volunteered to participate in the study. They were randomly assigned into either soccer heading or kicking-control group. Demographic information is provided in Table 1. For inclusion, participants were required to have at least three years of soccer heading experience and be between the ages of 18 and 26. Participants were excluded for a history of head injury during one year prior to the study or for history of neurological disorders. The study was conducted during the participants' off-season. Participants were instructed to refrain from any activity that involved head impacts during the study period. The Indiana University Institutional Review Board approved the study and written informed consent was obtained.

Procedure

Soccer Heading Protocol

A controlled soccer heading paradigm was employed to induce safe, repeatable, and mild subconcussive head impacts mimicking a 40-foot-long throw-in, a common drill used in soccer practices and games. This soccer heading protocol has been validated. A triaxial accelerometer (SIM-G, Triax Technologies, Inc., Norwalk, CT) was secured inside the headband and positioned directly below the external occipital protuberance (inion) to measure linear and rotational head acceleration (Table 1). Given that head impact variability between-header and between-participant is known to be small, head impact kinematics data were not incorporated in analysis.

Participants in all groups stood 40 feet across from the JUGS machine. The ball traveling speed was set at 25 mph for all groups. Participants in the heading group performed 10 standing headers, whereas participants in the kicking-control group performed 10 kicks. Participants in the kicking-control and heading groups were instructed to return the ball (by kicking or heading, respectively) to a researcher standing 20 feet in front of the participant (10° left: ensuring clear capture of the ball throughout its trajectory) at one-minute interval between each ball. The aim of the kicking group was to aid in distinguishing between the effects of subconcussive impact from exercise and simply a daily variation of the biomarker levels. A baseline was established via a pre-intervention biomarker assessment of both groups. The first phase of acute subconcussive effects was measured at 0h-post heading, indicating immediate cellular damage, followed by measurement of the second phase at 2h-post heading. Lastly, the 24h-post heading assessed the third phase of subconcussive effects. This time point is important because it provides the information about potential cumulative effects.

Biomarker Assessment

At each time point, the participants were asked to lay in the supine position with the arm relaxed at 10-degrees of flexion. Antecubital vein blood draws were performed each test session to help determine serum biomarker concentrations. A certified phlebotomist thoroughly cleaned the antecubital fossa with an alcohol swab and drew four mL of whole blood into serum vacutainer tubes with a 21G butterfly needle. With four time point measures of four mL, a total of 16 mL of blood per participant was necessary for the study. After the blood draw, the participant used gauze to maintain direct pressure for three minutes, then a Band-Aid was applied. The entire sample was centrifuged at 3,000 revolutions per minute for 20 minutes at 4°C after 40 mins of coagulation. The serum was then divided and transferred into one mL cryovials, flash frozen, and stored at -80°C in the exercise biochemistry lab.

Neurofilament light polypeptide measurements require an ultra-sensitive method to quantify changes in the blood. We utilized the Simoa digital-ELISA technology developed by Quanterix Inc. (Lexington, MA) to detect femtomolar changes in the neuron-derived biomarker (NF-L) after subconcussive head impacts compared with baseline values. A magnetic bead-based digital ELISA allows detection of proteins at subfemtomolar concentrations. The limit of detection (LOD) for the NF-L assay was 0.045 pg/mL, which is over 1000-fold more sensitive than conventional immunoassays (generally 30-60 pg/mL). Average intra-assay duplicate coefficient of variation for the samples was 6.5%. The assay is based on digital array technology and uses the neurofilament light polypeptide monoclonal antibody for NF-L capture (Covance, Princeton, NJ). The mouse monoclonal antibodies were used for detection of NF-L (Thermo Fisher Scientific Inc., Waltham, MA). Based on the standard sample curve, protein concentrations were calculated.

Data Analysis

Our primary interest was to identify the changes in NF-L expressions after subconcussive head impacts in the heading group; hence, NF-L expressions at post-heading time points (0h, 2h, and 24h) was compared with pre-heading baseline. Each time point of the control groups (standing and kicking) assisted in interpretation of whether or not changes in the heading group were due to subconcussive impact, exercise, bodily injury, or simply a daily variation.

A two-way repeated measures analysis of variance (RMANOVA) was used to compare the outcome measurement (biomarker levels) with group (heading and kicking control) and time (pre-intervention, 0h-post, 2h-post, and 24h-post), as well as a time by group interaction. RMANOVA was used to assess both within-group and between-group differences. A parametric test was chosen over a nonparametric test based on the explicit assumptions of an ANOVA test. This study meets the assumption that there was one dependent variable (biomarker expression) and the participants experienced the same conditions within groups. Each group was assessed for biomarker expression at the same four time points. Finally, remaining assumptions include normal distribution of data, sphericity, no significant outliers, and homogeneity of variance. Cohen's d was used to calculate effect size of the NF-L change at specific time points compared to the pre-intervention baseline. If a significant interaction is present, a Bonferonni post-hoc correction was used to determine where effects of subconcussive head impact occur. A priori alpha level was set at 0.05.

RESULTS

Demographic Characteristics

There was no significant difference in any demographic characteristics between groups. In the heading group, median peak linear head acceleration was 31.8 g (interquartile range: 31.1

– 34.5 g) and median peak rotational head acceleration was 3.56 krad/s^2 (2.93 – 4.04 krad/s^2). Demographic information is detailed in Table 1.

Changes in NF-L levels

Subconcussive head impacts gradually increased plasma NF-L expression, as illustrated by a significant time by group interaction, $F(3, 90)=5.284$, $p=0.002$, while the kicking-control group remained consistent throughout the study time points. Post hoc analysis for the heading group with Bonferroni correction revealed that a significant difference appeared at 24h post-heading (3.68±0.30pg/mL) compared to pre-heading (3.12±0.29pg/mL, $p=0.0013$; Cohen $d=1.898$). The heading group (3.68±0.30pg/mL) showed a significantly higher level of plasma NF-L than the kicking-control group (2.97±0.24pg/mL: $p=0.038$: Fig 1).

DISCUSSION

In this study, we showed a marked increase in plasma NF-L at 24h after 10 bouts of subconcussive head impacts, inducing approximately 30 *g* and 35 krad/s^2. These magnitudes of subconcussive impacts are comparable to the hits observed in football and ice hockey practices and games. There are two chief findings from this study: 1) There was no significant increase in NF-L expression until the 24h post-heading assessment in the heading group, and 2) NF-L expression remained consistent across all time points in the kicking-control group. These findings suggest potential for cumulative effects of subconcussive head impacts. The clinical significance of the modest NF-L elevation (0.56pg/mL) at 24h post-heading compared to the baseline is difficult to delineate, however, we were able to detect the subtle change resulting from relatively mild head impacts, supporting the highly sensitive nature of NF-L assessment.

Traumatic brain injury (TBI) continues to be at the forefront of research, although the focus to date has been on mild to severe TBI. Subconcussion, however, has become increasingly

relevant as recent research reveals its role in neurocognitive deficits and neurodegeneration. Cumulative impacts of subconcussion can be comparable to bouts of mild to severe TBI. Bailes et al. found that neurofilament dephosphorylation occurs in days one to three, even after subconcussive trauma, and that football players at various levels of participation may experience between 100 and 1000 head impacts during a season.[3] Broglio et al. reported an average of 16,746.lg of force in high school football players over the course of a season.[7] Our study demonstrated a traumatic subconcussive effect after only 10 head impacts with an average of only 20-30g of force.

Our results support previous findings that NF-L expression markedly increases in proportion to subconcussive head impacts cumulatively. Oliver et al. examined the changes in NF-L expression over the course of a football season in starters versus non-starters; NF-L gradually increased and peaked at the end of the season in the starter group, illustrating the cumulative neuronal burden of subconcussive head impacts, while there was no change in the non-starter group.[12] Shahim et al. examined NF-L fluctuations across time in boxers and hockey players compared to non-competitive individuals; NF-L increased in both boxing and hockey groups and decreased with rest. Boxers who presented with a greater number of hits or post-hit symptoms showed a more significant elevation of NF-L expression.[25] Similarly, Neselius et al. examined NF-L levels in Olympic boxers experiencing a minimum of 45 bouts of boxing compared to non-boxing controls and revealed a significant difference in the boxing group, comparatively.[26] Cumulative NF-L CSF levels were increased up to 4.1-fold even after a three-month follow up in a study by Zetterberg et al. comparing amateur boxers with a high severity of bouts versus low severity to healthy controls. [27] In our study, we examined, quite similarly to these studies, the inverse relationship between subconcussive head impacts and NF-L expression

versus no impact and the absence of expression, although we controlled for the exact number and magnitude of hits within an exact time frame.

Because subconcussive head impacts are often painless and asymptomatic, clinical significance of the elevation in plasma NF-L after repetitive head hits remains difficult to interpret. However, circulating NF-L has been proposed as one of the most sensitive surrogate markers for neuronal damage and degeneration. Furthermore, the diagnostic and prognostic value of NF-L has been established in individuals with TBI and various neurodegenerative pathologies. For example, Shahim et al. concluded that NF-L is predictive of long-term sequelae from severe TBI of varying etiology, with the peak levels measured 12 days post-injury.[25] Over a three-fold elevation of serum NF-L was detected in frontotemporal dementia patients compared to healthy controls.[22] In Alzheimer's disease patients, there were significant associations of increased serum NF-L with estimated years from symptom onset, reduced brain volume, and whole-brain atrophy rate.[28] Meta-analysis by Xu et al. demonstrated that amyotrophic lateral sclerosis patients exhibited a significantly higher level of serum NF-L (with a large effect size of 1.448) than that of healthy controls.[29] Findings from clinical studies were corroborated in a recent mechanistic paper published in *Neuron*, whereby an extent of axonal degeneration was associated with increased levels of plasma NF-L in various neurodegenerative mouse modes (i.e. Parkinson's disease, dementia with Lewy bodies, corticobasal syndrome, Alzheimer's disease),[30] indicating the sensitive and specific nature of circulating NF-L in distinguishing a pathological brain from a healthy one.

Limitations

Due to possible shifting of the Triax headband, complete accuracy of the impact sensor cannot be assumed. Palpation skills for exact placement of the headband cannot be validated.

Additionally, since sleep quality and quantity, diet, hormone (particularly menstrual hormone), and exercise assessments were not included in this study, and participants were not monitored continuously between time points, potential fluctuations in NF-L expression influenced by these factors could not be evaluated. Abnormal metabolic processes have been identified consistently in patients with TBI, which can affect the nutritional status of the patient; thus, nutritional support has been suggested as a necessary treatment in patients with TBI.[31] Metabolic endocrinopathies and hormone dysregulation (particularly in women) may be an effect of TBI.[32] Furthermore, sex hormones (namely, estrogen and progesterone) have reported neuroprotective effects secondary to TBI.[33] Light exercise may be considered in the treatment of patients with TBI, given the role proteins have in synaptic function and metabolic function, which may have a neuroplastic enhancing effect.[34, 35] Sleep disorders are an established result following TBI, [36] while rest is a necessary treatment in patients with TBI,[37] indicating the intimate relationship between sleep and TBI.[35] Given the potential of these factors to alter brain function and healing processes, future studies should aim to rule out the influential possibilities they could have in the subconcussive population.

CONCLUSION

As research continues to reflect the negative neurocognitive effects of subconcussive head impacts, it is imperative that a gold standard for determining diagnosis, treatment, and prognosis is established. Our results support previous findings that NF-L may be particularly effective in identifying neural trauma in this population. Further studies must examine the efficacy of long-term blood biomarker assessment and the potential for use in neurodegenerative cases.

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Table 1. Demographics and impact kinematics by group.

Variables	Heading	Kicking Control	*P*-value
n	18	16	-
Gender	7M 11F	6M 10F	-
Age, y	20.3 ± 1.5	21.2 ± 1.4	0.089
BMI	23.2 ± 2.4	24.4 ± 3.2	0.236
No. of previous concussion	0.78 ± 1.0	0.63 ± 1.7	0.753
Years of soccer heading experience	9.5 ± 3.6	10.0 ± 4.5	0.725
Head impact kinematics, median (IQR)[a]			
PLA, *g*	31.8 (31.1 – 34.5)	- [b]	-
PRA, krad/s^2	3.56 (2.93 – 4.04)	- [b]	-

Note: BMI, body mass index. IQR, interquartile range. PLA, peak linear acceleration. PRA, peak rotational acceleration. krad, kiloradian. [a]Based on the sum of 10 soccer headers. [b]Soccer kicking did not cause a detectable level of head acceleration.

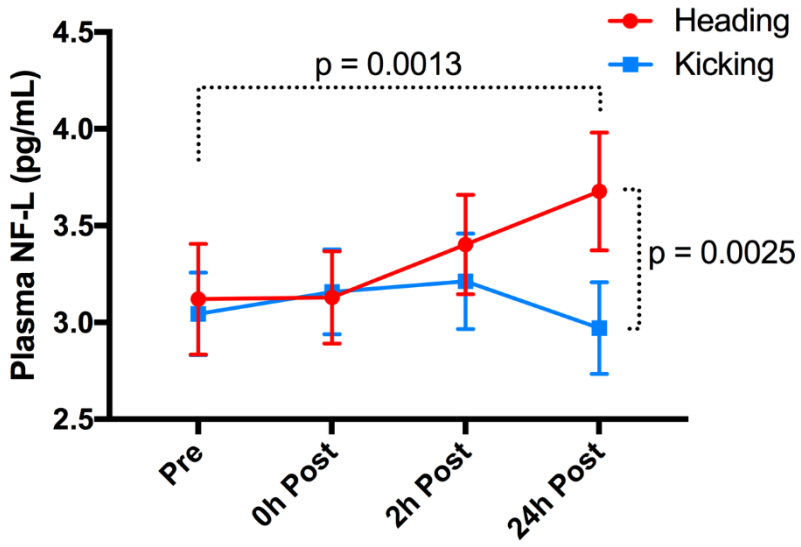

FIGURE LEGEND

Figure 1. Changes in plasma NF-L levels before and after subconcussive impacts. In the heading group, NF-L was elevated at 24h post-heading compared to pre-heading and 0h post-heading time points, but the kicking control group remained static across all time points. The heading group's NF-L levels at 24h post-heading were higher than that of the kicking-control group. Data are presented as means ± SEM.

APPENDIX A

PURPOSE STATEMENT, SPECIFIC AIMS AND HYPOTHESES, OPERATIONAL DEFINITIONS, ASSUMPTIONS, LIMITIATIONS, AND DELEMITATIONS

STATEMENT OF THE PROBLEM

Emerging evidence indicates the efficacy of using blood biomarkers as a means to detect brain parenchymal damage in the peripheral system. Preeminently, neurofilament light polypeptide (NF-L) has been shown to reflect axonal damage in response to traumatic force to the brain. Although plasma NF-L levels after a concussion are reported to significantly increase, the NF-L profile following repetitive subconcussive head impacts remains elusive. Therefore, the purpose of the current investigation is to examine the effects of subconcussive head impacts on plasma NF-L levels in three acute phases compared to a baseline between groups. Positively connecting the accuracy of blood biomarkers as indicators of cerebral trauma can supplement and guide future diagnosis, treatment, and prognosis of brain injuries.

SPECIFIC AIMS AND HYPOTHESES

Specific Aim 1. To evaluate acute subconcussive head impact effects on plasma expression of neurofilament light polypeptide (NF-L).

Hypothesis 1-1. Within group

H_A: There will be a significant increase in plasma NF-L expression at post-heading time points compared with baseline in the heading group.

H_o: There will be no significant increase in plasma NF-L expression at post-heading time points compared with baseline in the heading group.

Hypothesis 1-2. Between group

H_A: There will be a significant increase in plasma NF-L expression at post-heading time points in the heading group compared to the control groups.

H_o: There will be no significant increase in plasma NF-L expression at post-heading time points in the heading group compared to the control groups.

OPERATIONAL DEFINITIONS

Blood Biomarker – A biological maker, or biomarker, is a measurable cellular, biochemical, or molecular change in the body that has the potential to aid in diagnosis, treatment, and prognosis of medical conditions. In this study, we examine a protein biomarker that presents in the blood secondary to cerebral trauma, thus, a blood biomarker.[19, 38]

Blood Brain Barrier – The blood brain barrier (BBB) is composed of endothelial cells lining the walls of cerebral capillaries and serves as the principal exchange site between blood and the central nervous system (i.e. the brain and spinal cord). It is hypothesized that BBB compromise may allow for biomarker release from the brain to the periphery to be detected in bodily fluids such as the blood.[39, 40]

Tight Junction – Tight junctions (TJs) are located between the endothelial cells of the BBB and act as a selective diffusion barrier against substances crossing between the blood and the brain cells. BBB damage affects TJs such that they become less selective in what is allowed to cross the barrier, which may explain how cerebral proteins escape from the brain.[41, 42]

Concussion – Concussion is a type of mild traumatic brain injury that occurs when bodily impacts cause linear or rotational acceleration of the brain and, consequently, cerebral damage.[9, 43]

Subconcussion – Subconcussion is caused by head impacts at a lower magnitude than a concussion that may cause damage to the brain without presenting itself clinically as a concussion does. Without objective assessments such as biomarkers, subconcussion could go overlooked.[2]

Neurofilament light polypeptide – Also known as NF-L, this protein is a crucial component of the axonal skeleton. As such, it is an indicator of brain injury, specifically to the neural axon when present in the blood.[12, 18, 19, 44]

ASSUMPTIONS

The following assumptions apply to this study:

1. The soccer players have a proficient, similar soccer heading technique and can, therefore, minimize the variation of head impact experienced between each participant in the subconcussive head impact group.
2. Participants will give an accurate and truthful account of their history of brain injuries.
3. Data collected from the Triax accelerometer and the Quanterix Simoa HD-1 analyzer are accurate and reliable.
4. Each participant has been exposed to a similar socioeconomic status, and, therefore, experienced similar dietary habits.
5. Participants will complete the procedure exactly as intended and instructed.
6. Participants in the soccer heading group are representative of other similar-experienced soccer players.

DELIMITATIONS

The following delimitations apply to this study:

1. Participants in the subconcussive head impact group will be experienced soccer players.
2. Participants will only be those that have indicated no history of cerebral damage within one year of the study.
3. Blood biomarkers will be examined as opposed to cerebrospinal fluid, saliva, urine, or tears.

4. Neurofilament Light Polypeptide (NF-L) will be the only measure examined.
5. NF-L expression will be measured in ng/ml.
6. Data collection will be obtained and analyzed by the PI and graduate assistants.
7. Data collection will begin with baseline measures.
8. Data collection will continue with three subsequent collection time points.
9. A triaxial accelerometer will be used as a headband to monitor linear and rotational accelerations of head in the experimental group.
10. A Quanterix Simoa HD-1 analyzer will be used to assess expression of the NF-L biomarker in the blood.

LIMITATIONS

The following limitations apply to this study:

1. Participants' behaviors throughout the study such as diet, exercise, and sleeping habits cannot be controlled.
2. Menstrual hormones cannot be regulated in the female population.
3. Each head impact participant may head the ball differently regardless of optimal control measures.
4. Expression of biomarkers of brain injury will only be examined in the blood and not in other potentially indicative biological media such as saliva, urine, tears, or cerebrospinal fluid.
5. Palpation skills for exact placement of the headband cannot be validated.

APPENDIX B

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Cerebral Physiology

The human body is comprised of trillions of cells, and those that are specific to the nervous system are referred to as neurons. Neurons are the brain's mode of transportation of electrical signals, i.e. action potentials, to control all brain, and consequently, bodily functions. Action potentials are generated at the axon hillock and propagated down the axon, which is the long, cylindrical projection of the neuron that spans the distance between the cell body, or soma, and the dendrites. The mechanical strength of the axon comes from the cytoskeleton matrix consisting of microtubules, neurofilaments, actin microfilaments, and proteins.[1]

Dendrites are small tree-like projections of the neuron that are responsible for releasing the signals to the next neuron. In this fashion, the brain cells communicate with each other to send signals throughout the body.[1] In order to maintain healthy neurons, glial support cells must accompany each neuron. Glial cells (derived from the Greek language meaning "glue") include oligodendrocytes, astrocytes, and microglia. [2]

Since the brain controls so many processes of the body, neurons must fire at a rapid rate to sustain function. Insulation from the myelin of neuronal axons remits the action potentials expeditiously down to the next cell. In the central nervous system (CNS), oligodendrocytes are responsible for myelination of axons and forming a supportive network around neurons.[3] Oligodendrocytes are spherical with small processes that branch out to wrap around the adjacent axon.[3]

Star-shaped glial cells named astrocytes are responsible for supporting the metabolic requirements of neurons.[4] Astrocytes are the most diverse and numerous glial cell in the CNS

and maintain copious amounts of projections (end-feet) that wrap around cerebral blood vessels, which allows for adequate maintenance of brain homeostasis and defense. [2, 5]

While the aforementioned glial cells promote normal function of neurons, small microglia exist to protect the CNS. Microglia are the primary immune response cells in the brain that respond to foreign substances and remove them from the CNS to restore homeostasis. [5, 6]

Blood Brain Barrier

There are two proposed routes of escape for biomarkers into the blood. First, Blood Brain Barrier (BBB) compromise has traditionally been believed to be the sole site of cerebral by-product exchange.[7] Neural homeostasis and interaction with the vascular system is maintained by the BBB, which is the principal site of blood and CNS exchange in the body.[5, 8-10] It is composed of endothelial cells lining the walls of cerebral capillaries.[4, 8, 9, 11, 12] Between the endothelial cells are tight junctions (TJs) that serve as a diffusion barrier by selectively permitting or excluding substances across the BBB.[2, 5, 8-10, 13, 14] Astrocytic end-feet surround the vessels creating a physical connection and tight barrier between cerebral vasculature and neurons.[4, 15, 16] Further anchoring the endothelial cells, the basal lamina plays an important role in BBB structure and tightness, though the mechanisms for this are poorly understood as of yet.[5] Receptors expressed in the basal lamina mediate cell signaling for differentiation, proliferation, migration, and adhesion – all critical in maintenance of the BBB. [5, 17, 18]

Since the primary function of the BBB is to maintain brain homeostasis, disruption of the BBB triggers neuronal damage and dysfunction. In fact, BBB compromise has been cited in numerous studies of cerebral pathophysiology- namely, traumatic brain injury, epilepsy, stroke, and neurodegenerative diseases.[8, 10] A compromised barrier not only allows potentially harmful substances into the brain, but it can produce an efflux of substances from the brain into the blood

that can indicate damage. These substances when detected in the blood are referred to as biomarkers.[17]

Tight Junctions

Maintenance of molecular selectivity in the BBB is the role of TJs, which act as seals between the barrier epithelium.[4, 10] These junctions are composed of the proteins occludin, claudin, and junction adhesion molecules (JAMs), which the expression of may be induced by astrocytes.[4, 5, 10, 17]

Occludins selectively protect the barrier permeability via regulation of electrical resistance and solute flow.[5, 8, 9, 19] Disruption of occludin has proved to cause a drop in transepithelial electrical resistance across the BBB.[18] While both possess adhesive properties, claudin, derived from the Latin word meaning, "to close," is the primary protein that seals the TJ.[11, 13] Claudins also incite fibril formation crucial to barrier structure, which is why they are considered the primary sealant even though they are smaller and shorter proteins than occludin.[5, 8, 9, 14] Disruption of claudin has demonstrated an increase in barrier permeability secondary to compromised fibril organization.[5, 17, 18] JAMs abet cell-to-cell adhesion in the BBB, providing stability to the barrier.[5, 9, 13, 20] JAMs also play an important role in maintenance of BBB polarity that allows the barrier to be selectively permeable to solutes.[19]

Glymphatic System

Alternatively, a more novel explanation of biomarker seepage into the periphery is through the glymphatic system. The glymphatic system is the brain-wide paravascular pathway for fluid transport and waste elimination.[2, 21-23] Three mechanisms allow efficiency of the pathway: para-arterial cerebrospinal fluid (CSF) influx, para-venous interstitial fluid (ISF) clearance, and a water channel for transport.[7, 24] CSF, ISF, and para-arterial spaces interact to

exchange soluble proteins, metabolic waste, and excess extracellular fluid and, ultimately, eliminate byproducts via the blood stream. Of particular importance in bulk flow of solutes from the brain to the blood in this system are the aquaporin-4 water channels secured near the perivascular astrocytic end feet. [21, 22, 25-27] Aquaporin-4 channel expression increases with traumatic brain injury, [28, 29] which may contribute to the solute efflux observed in biomarker expression.[21]

When neural damage occurs, proteins are released into the cerebrospinal fluid, which further mixes with the interstitial fluid and can be propelled into the periphery as cerebral arterial pulse pressure increases and pushes the mixture through the astrocytic aquaporin-4 channels into the paravenous space.[21, 23, 24] Subsequently, the proteins (biomarkers) are expressed in the blood, indicating neural damage.[2, 25, 30] Dissolution of this system has revealed decreases in biomarker expression,[30] thus supporting the notion that biomarkers reach peripheral blood via this mechanism. While this system poses an intriguing explanation for peripheral biomarker expression, current literature lacks support of this mode in human populations and adequate investigation of the role of glymphatic impairment preceding protein expression. [23]

Subconcussion

Concussion is a brain injury that occurs when impacts cause linear or rotational acceleration of the brain and, consequently, cerebral damage.[4, 31, 32] Since many concussions go unreported (50-70%), it is difficult to be exact, but it is estimated that 1.6-3.8 million sport or recreation-related concussions occur each year in the United States alone. [33-35]

While there is increased awareness of the incidence, mechanism, and treatments of concussions, a related condition referred to as “subconcussion” is often overlooked. Subconcussion is defined as a head impact that causes damage to the brain at a lower magnitude

than a concussion, such that the neurological symptoms exhibited in concussed patients may not readily present in the subconcussed patients. [36] Nevertheless, diagnostic images have shown neurological changes in both populations.

There is no single mechanism for subconcussed patients as there is with concussions; rather, subconcussion is the result of repetitive, lower-load impacts to the brain that accumulate over time. Accelerations of the brain can cause compromise to the natural viscoelasticity, causing subsequent shearing of the axon and accumulation of by-products in the periphery.[37] Subconcussion is most prevalent in contact sports such as football, soccer, boxing, wrestling, rugby, hockey, or lacrosse.[38]

Biomarkers

Research on the mechanisms and consequences of brain injury has been extensively studied to date. Although current knowledge of brain injury triage and management expands continuously, a valid and objective measure of damage has yet to be identified and implemented as a gold standard for diagnosis, treatment, or prognosis. [4, 7, 36, 39-41] Biological markers, or biomarkers are cellular, biochemical, or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids.[42] Biomarkers for brain injury each have specific derivations in the brain, which allows clinicians to prioritize and direct research for each condition. For example, Tau is expressed after axonal shearing, which indicates neural damage.[43]

Biomarkers can be assessed via CSF, blood, tears, urine, and saliva. Despite the accuracy of CSF use in detection of cerebral damage, the invasive and difficult nature of the lumbar puncture procedure precludes many researchers from using this method.[6, 44] Alternatively, the blood biomarker has risen as an appropriate and specific means for biomarker measurement.[7] The exact structures, functions, and mechanisms of fluid biomarkers are still at the forefront of

current research throughout the health field; however, recent studies have shown positive correlations of blood biomarkers as indicators of axonal, neuronal, and astrological injury associated with concussive and subconcussive brain injuries.[44, 45] Unlike subjective measurements, blood biomarkers are a powerful objective approach to detect subtle structural and metabolic changes after neural damage.

While researchers surmise that presentation of neurological media in the blood is secondary to axonal shearing of the cytoskeleton from torsional forces, the exact mechanism has yet to be isolated.[37] Identification and validation of appropriate biomarkers of concussion and subconcussion could change the management of cases to provide more efficient and precise diagnosis and determination of severity across all settings.[36] If research determines the efficacy of using biomarkers to monitor progression of brain injuries, return to activity guidelines could be standardized according to levels of expression in the blood. Linear documentation of injury progression may further be used to determine prognosis.[36, 41]

Neurofilament Light Polypeptide

Neurofilaments are phosphorylated neuronal-specific proteins abundantly interspersed in intermediate axonal fibers between the actin and microtubules, thus comprising the bulk of the axonal skeleton.[36, 46] Each filament contains light (NF-L), medium (NF-M), and heavy (NF-H)[1, 6, 47] subfilament chains, which is determined by the molecular weight. Sidearm emanations of the neurofilaments possess both repulsive and attractive properties critical to network formation of the cytoskeleton. Phosphorylation and viscoelasticity of neurofilaments foments the radial growth and cellular organization of the axon.[46] Viscoelastic properties are derived from the stretching ability of the NF-H and NF-M subunit composition, which aids in prevention of repetitive mechanical stress. NF-L, however, plays a more critical role in organization of the cell

as it supports the positioning of neural mitochondria and endoplasmic reticulum.[46] NF-L is expressed in the large myelinated axons deeper in the CNS and presented in the blood in studies following mTBI in boxers. [41]

Dephosphorization of the neurofilaments occurs secondary to axonal injury.[48] Importantly, alterations of neurofilaments interferes with nerve conduction velocity. Initial injury is a result of direct head impact that shifts the brain, causing linear or rotational acceleration. This initiates a cascade of neural inflammation and decrements by causing stretching and, subsequently, local swelling of the axons as neurofilaments begin to disassemble. Axotomy, or disconnection and failure of that portion of the neuron, and/or nerve conduction interference will be elicited. [31, 34, 38, 44, 48, 49]

Neurofilament disruption has been correlated with numerous neurodegenerative diseases. Studies of NF-L as a marker of axonal damage has been linked to conditions such as Alzheimer's, frontotemporal dementia, multiple sclerosis, amyotrophic lateral sclerosis, Charco-Marie Tooth, and concussions.[16, 36, 37, 41, 44, 46, 50-56] Recent studies have further indicated that neurofilament light polypeptide (NF-L) could be the most sensitive and specific blood biomarker for axonal injury.[41, 44, 53] Mild repetitive traumatic brain injury (mTBI) causes axonal or cytoskeletal alterations, which can present in the blood upon BBB compromise. This ultimately leads to cognitive impairment of varying degrees, and use of these biomarkers may be beneficial in determining severity and guiding future treatment.

Biomarkers in Subconcussion

Given the successful identification of biomarkers that show a correlation between concussion and neurodegenerative diseases,[22, 35] it is not only indicated, but necessary to continue investigation of similar correlations in the subconcussive population.[31] In fact, athletes

have been reported to receive up to or surpassing 10,000 subconcussive impacts throughout an athletic career,[49] which can place them at a higher risk of neurodegenerative diseases, neuronal loss, and decreased cognitive or motor activity.[22, 31, 57]

These individuals express neural damage similar to the formerly cited neuropathies, and, therefore, also have the potential to exhibit a relationship between this damage and expression of this damage in the blood. Neural degradation has been noted to present within merely an hour after damage has occurred, peak between twelve and thirty-six hours, and potentially remain detectable in the periphery for several weeks.[23, 37] Biomarkers at the forefront of research on cerebral trauma include S100B, glial fibrillary acidic protein (GFAP), neurofilament light polypeptide (NF-L), and amyloid beta.[6, 23, 40, 43]

Future studies will further need to continue to differentiate between what specific biomarkers correlate with each neuropathy, what is indicated by the amount of expression in the blood, and what this suggests about the extent of damage. In turn, this will influence treatment regimens and, subsequently, prognosis of the subconcussive population.[4, 38, 41, 58]

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APPENDIX C

STUDY PROCEDURES/ IRB Form

Procedure Outline		
1. Email will be distributed		
2. Interested participants will contact PI (KK) to discuss project		
3. Participants meet with the PI to discuss the project and ask further questions		
4. Participants take informed consent form with them and return with signature if they are willing to participate in the study		
a. Pre-test	1. Symptom Assessment (5 mins)	
(~40 mins if TMS)	2. Ocular-motor assessment (10 mins)	
(~1h if EEG)	3. Blood Draw (Exercise Biochemistry Lab; 5 mins)	
	4. TMS Assessment (Neuromotor Lab; 15-20 mins)	4. EEG Assessment (EEG lab; 30-40 mins)
Intervention: Soccer heading, kicking, or standing (10 mins)		
b. 0h-Post-test	1. Symptom Assessment (5 mins)	
(~40 mins if TMS)	2. Ocular-motor assessment (10 mins)	
(~1h if EEG)	3. Blood Draw (Exercise Biochemistry Lab; 5 mins)	
	4. TMS Assessment (Neuromotor Lab; 15-20 mins)	4. EEG Assessment (EEG lab; 30-40 mins)
c. 3h-Post-test	1. Symptom Assessment (5 mins)	
(~40 mins if TMS)	2. Ocular-motor assessment (10 mins)	
(~1h if EEG)	3. Blood Draw (Exercise Biochemistry Lab; 5 mins)	
	4. TMS Assessment (Neuromotor Lab; 15-20 mins)	4. EEG Assessment (EEG lab; 30-40 mins)
1. 24h-Post-test	1. Symptom Assessment (5 mins)	
(~40 mins if TMS)	2. Ocular-motor assessment (10 mins)	
(~1h if EEG)	3. Blood Draw (Exercise Biochemistry Lab; 5 mins)	
	4. TMS Assessment (Neuromotor Lab; 15-20 mins)	4. EEG Assessment (EEG lab; 30-40 mins)

APPENDIX D

DATA COLLECTION FORM AND SURVEYS

Health and Concussion History Questionnaire

Subject Number_____ Date_____

Please answer the following questions honestly and to the best of your ability.

1. **Age ____**

2. Are you a current member of a soccer team? YES **___**NO**___**

2a. What level? Collegiate ___ Club ___ Recreation___ Professional___

3. How long have you a. played soccer? ____ yrs **b. been soccer heading? ____** yrs

4. Have you ever been diagnosed by a certified athletic trainer or physician with a concussion? YES **___** NO**___**

4b. For your concussion(s), approximately when did the concussion(s) occur, how long did signs and symptoms last, and how long did you miss athletic participation? Please list per concussion. Use back of paper if necessary.

Concussion	**Date (month-year)**	**Signs and Symptoms Duration (# of days)**	**Length of time until you returned to practice or game (# of days)**
1			
2			
3			
4			
5			

5. Please circle yes or no to the following, and explain as needed.

YES NO Have you had any head, neck, or face injury in the one year prior to the study?

If yes, then please explain.

YES NO Do you have a history of vestibular dysfunction (e.g. vertigo)?

If yes, then please explain.

YES NO Do you have a history of hearing dysfunction (e.g. deafness)?

If yes, then please explain.

YES NO Do you have a history of vision problems (e.g. macular degeneration)?

If yes, then please explain.

YES NO Do you need corrective eyewear? (e.g. glasses or contacts)?

YES NO If yes, would you be able to wear contacts during testing?

YES NO Are you *currently* taking any medications affecting balance (e.g. antibiotics)?
If yes, then please explain.

YES NO Have you had any lower extremity injuries *in the past six months*.

If yes, then please explain.

YES NO Are you currently pregnant?

YES NO Are you claustrophobic?

YES NO Do you have any metal implants (e.g. pacemaker, ferromagnetic aneurysm clip)?

If yes, then please explain.

YES NO Do you have any neurological disorders (e.g. seizure disorders, closed head injuries with loss of consciousness greater than 15 minutes, CNS neoplasm, or history of stroke)?

If yes, then please explain.

YES NO Do you have hypertension, cardiac arrhythmia, or pulmonary disease?

If yes, then please explain.