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Association of Acute Increase in Plasma Neurofilament Light with Repetitive Subconcussive Head Impacts: A Pilot Randomized Control Trial

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Abstract

The purpose of the study was to examine an association of repetitive subconcussive head impacts with changes in plasma neurofilament light (NF-L) levels following 10 bouts of controlled soccer heading. In this randomized control trial, 37 healthy adult soccer players were randomly assigned into either a heading ($n = 19$) or kicking-control group ($n = 18$). The heading group executed 10 headers with soccer balls projected at a velocity of 25 mph over 10 min. Plasma samples were obtained at pre-heading baseline, 0 h, 2 h, and 24 h post-heading. The kicking-control group followed the same protocol with 10 kicks. Plasma NF-L was measured using ultrasensitive single-molecule array technology. Data from 34 subjects were eligible for analysis (heading $n = 18$ and kicking $n = 16$). Ten subconcussive head impacts induced a gradual increase in plasma NF-L expression for the heading group ($\beta = 0.0297$, standard error [SE] = 0.01, $p = 0.0049$), whereas there was no significant time effect for the kicking-control group. A follow-up analysis revealed that a significant difference appeared at 24 h post-heading (3.68 ± 0.30 pg/mL) compared with pre-heading (3.12 ± 0.29 pg/mL, $p = 0.0013$; Cohen's $d = 1.898$). At the 24 h post-heading time-point, the plasma NF-L level for the heading group was significantly higher than that of the kicking-control group with an estimated mean difference of 0.66 pg/mL (SE = 0.22, $p = 0.0025$). The data suggest that the increased level of plasma NF-L was driven by repetitive subconcussive head impacts and required longer than 2 h after the head impacts for the increase to be detected. Plasma NF-L levels may serve as an objective marker to monitor acute axonal burden from subconcussive head impacts.

Keywords: blood biomarker; concussion; neurofilament; soccer; subconcussion

Introduction

Subconcussive head impact is defined as an impact to the head that does not trigger clinical symptoms of concussion. Athletes engaged in contact sports can sustain several hundred up to over one thousand subconcussive head impacts annually from soccer heading and American football tackling,[1–3] which have been shown to trigger degenerative responses in the brain, characterized by axonal growth cone collapse, tau protein disruption, abnormal cytoskeletal rearrangement,[4–6] and cerebral endothelial dysfunction.[7,8] The post-subconcussive cellular damage from these head impacts are thought to trigger lingering ocular-motor impairment,[9,10] transient vestibular dysfunction,[11,12] elevation in neural injury blood biomarkers,[13,14] and an alteration in neuronal connectivity.[15,16] If unabated, long-term exposure to these impacts likely leads to increased risk for developing a neurodegenerative disease linked to a history of head impact exposure, chronic traumatic encephalopathy (CTE).[17–19]

The use of brain-derived serological factors has been proposed as one potential avenue to gauge the severity of brain trauma, as they enable the detection of subtle cellular, structural, and metabolic changes.[20] In particular, neurofilament light polypeptide (NF-L), which is preferentially expressed to maintain structural integrity in subcortical axons, has emerged as a blood biomarker for traumatic brain injury (TBI). For instance, an increased level of serum NF-L was able to distinguish moderate to severe TBI patients from healthy controls with an area under the curve (AUC) of 0.99,[21] with a positive association between increased NF-L levels and the severity of axonal diffusion, as revealed by diffusion tensor imaging (DTI).[22] Moreover, professional boxers sustaining a higher frequency of subconcussive head impacts (>15 hits) expressed 200% higher serum NF-L concentrations than their counterparts who sustained fewer than than 15 hits.[23] In addition, NF-L levels have significantly risen in American football athletes in a dose-dependent manner, as athletes who have had more exposure to live competition display greater expression

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of serum NF-L than those who did not play in live competition.[14] However, because these studies lack appropriate control of confounding variables (e.g., head impact frequency/magnitude, exercise effects, musculoskeletal damage), it remains speculative whether the changes in NF-L expression were truly driven by subconcussive head impacts.

To fill the current knowledge gap, we conducted a randomized control trial using our established soccer heading model to control frequency and magnitude of head impacts. We examined changes in plasma NF-L levels at 0 h, 2 h, and 24 h after 10 soccer headers compared with a pre-heading baseline. Kicking-control subjects underwent the same plasma sampling and accounted for potential effect of exercise and musculoskeletal damage. Our hypothesis was that there would be a significant increase in plasma NF-L levels after subconcussive head impacts compared with the baseline, whereas NF-L levels in the kicking-control group would remain consistent across all time-points.

Methods

Trial design and randomization

This single-blind randomized clinical trial was a single-center prospective evaluation of changes in plasma NF-L levels of healthy participants in response to 10 acute bouts of mild repetitive head impacts. Using a simple, dice-based randomization method, participants were randomly assigned to either soccer heading or soccer kicking-control. After randomization, participants were unblinded as they needed to physically perform either heading or kicking, whereas all assessors (e.g., biomarker experimenter, statistician) remained blinded. Participants were assessed at four time-points (pre-intervention, and 0 h, 2 h, and 24 h post-intervention). At each time-point, venous blood draw was conducted. Between pre- and 0 h post-intervention, the heading group performed 10 soccer headers (see Soccer intervention section), whereas the kicking-control group performed 10 kicks. Subjects in both groups remained in the lab until 2 h post-intervention without engaging in strenuous cognitive or physical activities and returned to the lab approximately 24 h later for the final data collection. The primary outcome measure of the study was the slope of increased levels of NF-L over time relative to the within-group baseline and the slope of the kicking-control group. The Indiana University Institutional Review Board approved the study (protocol registered under ClinicalTrials.gov: NCT03488381), and written informed consent was obtained.

Participants

From August 2017 through March 2018, we recruited potential subjects who were enrolled at Indiana University-Bloomington, met the following inclusion criteria, and were free of exclusion criteria. For inclusion, subjects were required to have at least 5 years of soccer heading experience and be between the ages of 18 and 26. Subjects were excluded for a history of head injury during one year prior to the study; a history of vestibular, ocular, or vision dysfunction; or a history of neurological disorders. Our sample size calculation, based on previous subconcussion studies,[10,12,14] yielded 18 subjects per group being estimated to yield a statistical power of at least 0.80 with a significance level of $\alpha = 0.05$. As a result, 37 healthy adult soccer players were included in the study and were randomly assigned into either a heading ($n = 20$) or kicking-control group ($n = 20$; Fig. 1). Demographic information is provided in Table 1. 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 (see Supplementary Table S1, which is available online at www.liebertpub.com/neu).

Soccer intervention

A standardized and reliable soccer heading protocol was used to induce subconcussive head impacts.[24,25] A triaxial accelerometer (SIM-G; Triax Technologies, Inc., Norwalk, CT) was secured inside a headband and positioned directly below the external occipital protuberance (inion) to measure linear and rotational head acceleration (Table 1). A JUGS soccer machine (JPS Sports, Tualatin, OR) was used to simulate a soccer kick with standardized ball speed across subjects. A size 5 soccer ball was launched from the JUGS machine at a speed of 25 mph (11.2 m/sec), which is similar to a centering pass made from the sideline to midfield during regular play. Subjects in both groups stood approximately 40 ft away from the machine to perform the headers or kicks. Along with a tester's demonstration, each heading subject were instructed to head a ball in the air and aim for a tester standing approximately 16 ft in front of the subject. Kicking subjects were given an identical set of instructions of kicking the ball, rather than heading. Subjects performed 10 headers or kicks with a 1-min interval between each launch.

Blood sampling and NF-L measurement

Four-milliliter samples of venous blood were collected into EDTA vacutainer sterile tubes (BD Bioscience). Plasma was separated by centrifugation ($1500 \times g$, 15 min, 4°C) and stored at −80°C until analysis. Plasma NF-L concentrations were measured using the Simoa™ Platform (Quanterix, Lexington, MA), a magnetic bead-based, digital, enzyme-linked immunosorbent assay (ELISA) that allows detection of proteins at subfemtomolar concentrations, and an analytical protocol as previously described in detail.[26,27] Limit of detection (LOD) for the NF-L assay was 0.045 pg/mL. The analyses were performed by a board-certified laboratory technician blinded to the study design and subject characteristics. Serial samples from subjects were plated in a same-assay plate, to eliminate an inter-place effect on the within-subject NF-L data. The average intra-assay coefficient of variation for the samples was 6.5% (standard deviation [SD] 5.6).

Statistical analysis

The demographic differences between the heading and kicking-control groups were assessed by independent samples t tests. To examine group differences (heading vs. kicking-control) in NF-L levels over time (pre-, 0 h, 2 h, and 24 h post-intervention), a mixed effects regression model with repeated measurements was used on the plasma NF-L level as the primary outcome with baseline NF-L, time, group, and time × group interaction as independent variables. Covariates such as age, gender, and years of soccer heading experience were adjusted if they appeared significant. If a significant time effect was present, paired t tests (two-tailed) were used to determine significant differences within group across time-points compared with the baseline. The level of statistical significance for the paired t tests was set to $p < 0.017$ after adjusting for Bonferroni corrections. We calculated the Cohen's d to identify effect size for the observed significant change. A linear regression, with the baseline NF-L level as a covariate, was used to determine the group differences at the certain time-point(s) where the significant elevation of NF-L in the heading group occurred. All the data were analyzed with SAS™ software, version 9.4 (SAS Institute Inc., Cary, NC).

Results

Demographics and head impact kinematics

Forty-two individuals were assessed for eligibility, and 37 individuals who met inclusion criteria and were free of exclusion criteria proceeded to the study. There were three voluntary withdrawals at

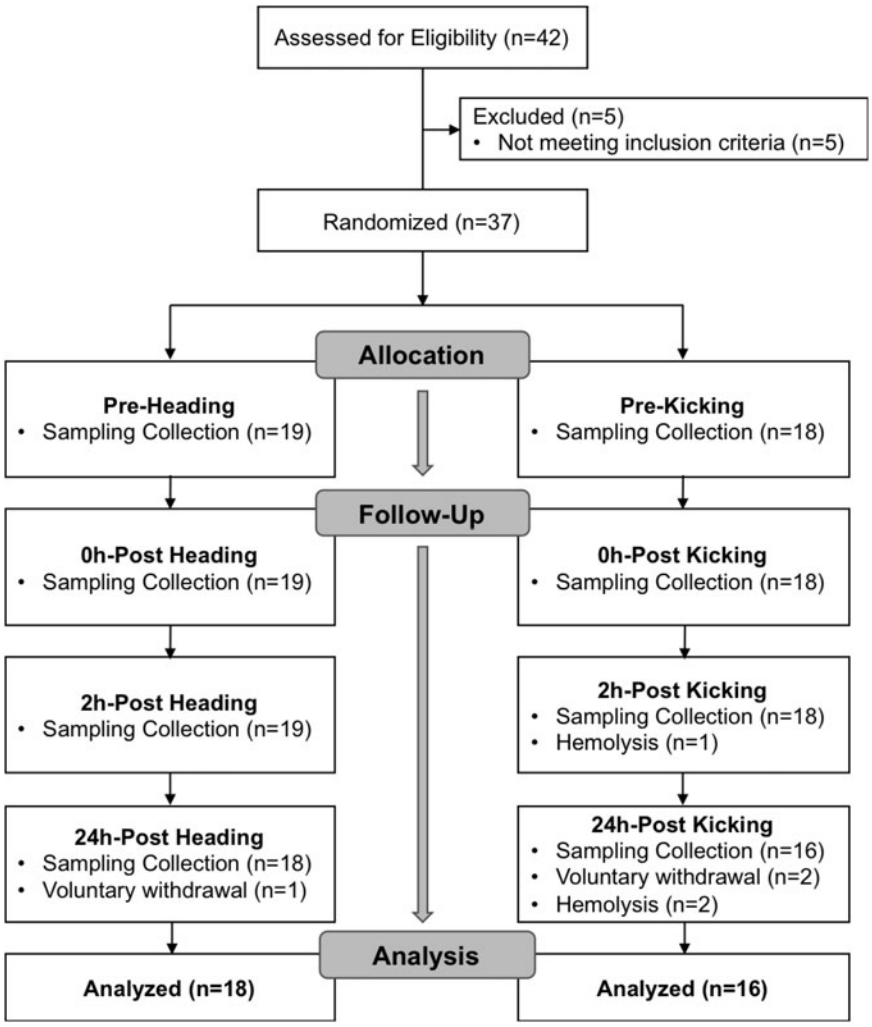

FIG. 1. Study flow chart.

24 h follow-up (heading $n=1$, kicking $n=2$). Data from 34 subjects were eligible for analysis (heading $n=18$ and kicking $n=16$; see flow chart, Fig. 1). There were no significant difference in any demographic characteristic between groups. Demographics and head impact kinematics are detailed in Table 1. The heading group experienced a median linear head acceleration of 31.8*g* per head impact (interquartile range: 31.1–34.5*g*) and a median rotational head acceleration of 3.56 krad/sec^2 per head impact (interquartile range: 2.93–4.04 krad/sec^2). Conversely, the result for the kicking-control group did not show a detectable level of head acceleration (Table 1).

Table 1. Demographics and Impact Kinematics by Group

Variables	*Heading*	*Kicking control*	P-*value*
N	18	16	-
Sex	7M 11F	6M 10F	-
Age, years	20.3 ± 1.5	21.2 ± 1.4	0.089
BMI, kg/m^2	23.2 ± 2.4	24.4 ± 3.2	0.236
No. of previous concussions	0.78 ± 1.0	0.63 ± 1.7	0.753
Soccer heading experience, y	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/sec^2	3.56 (2.93–4.04)	- [b]	-

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

Subconcussive effects on plasma NF-L levels

Ten acute bouts of subconcussive head impacts gradually increased plasma NF-L expression, as illustrated by a statistically significant time effect for the head impact group, $F(1, 31)=9.17$, $p=0.0049$. For example, 0.03pg/mL of NF-L is estimated to increase every hour after 10 headers (SE = 0.001). On the other hand, there was no significant time effect for the kicking-control group, $F(1,31)=1.20$, $p=0.28$ (Table 2). Follow-up paired *t* tests after adjusting for Bonferroni correction within the heading group revealed that a significant difference appeared at 24 h post-heading (3.68 ± 0.30 pg/mL) compared with pre-heading (3.12 ± 0.29pg/mL, $p=0.0013$; Cohen's d = 1.898; Fig. 2). A linear regression, adjusting for the baseline NF-L level, was used to assess the between-group difference at the 24 h post-intervention time-point and distinguished that the heading group was significantly higher than the kicking-control group with an estimated mean difference of 0.66 pg/mL (SE = 0.22, $p=0.0025$; Fig 2).

Table 2. Changes in Plasma NF-L Levels over Time

Variables	*Group*	*Coefficient*	*SE*	P
Time	Heading	0.02969	0.00980	0.0049
	Kicking	-0.00947	0.00732	0.2054
Group	Heading	-0.02492	0.09909	0.8031
	Kicking (reference)	0	0	-
Baseline NF-L	-	0.8494	0.04285	0.0001

Data results are from mixed effects regression model. NF-L, neurofilament light; SE, standard error of the estimate.

Discussion

In this short report, we showed a marked increase in plasma NF-L at 24 h after 10 bouts of subconcussive head impacts. There are three chief findings from this study: (1) plasma NF-L was sensitive enough to indicate a subtle axonal damage caused by repetitive subconcussive head impacts, (2) plasma NF-L gradually increased and peaked at 24 h post-impacts indicating relatively longer plasma half-life than existing biomarkers ($\sim$6 h),[20] and (3) plasma NF-L levels were consistent across time-points in the kicking-control group, suggesting unremarkable effects of daily fluctuation, exercise, and bodily damage. These findings support previous clinical studies in professional boxers and football players in such a way that axonal injury caused by subconcussive head impacts can be peripherally inspected by serological levels of NF-L expression.

An earlier report in 2007 examined whether 10 to 20 soccer headers increase NF-L levels in cerebrospinal fluid in amateur soccer players, and found that the assessment method they employed was not sensitive enough to detect NF-L levels (the limit of detection: 125 pg/mL).[28] The same group developed a novel immunoassay using a magnetic bead-based single-molecule array (Simoa) in 2013 that enabled detecting proteins at subfemtomolar concentrations.[26,27] The breakthrough in the assay improvement exponentially advanced biomarker science, and NF-L has demonstrated one of the most consistent results in distinguishing neuronal pathology from a healthy state. For instance, over a three-fold elevation of serum, NF-L was detected in patients with frontotemporal dementia and Alzheimer's disease compared with healthy controls[29] and a significant inverse association between the increased level of serum NF-L, reduced brain volume, and whole-brain atrophy rate.[30] These findings were further corroborated by a recent mechanistic article, showing that an extent of axonal degeneration was associated with increased levels of plasma NF-L in various neurodegenerative mouse models (e.g., Parkinson's disease, dementia with Lewy bodies, corticobasal syndrome, Alzheimer's disease).[31] Additionally, NF-L has shown its excellent diagnostic and prognostic values in TBI. An increased level of serum NF-L was able to distinguish moderate to severe TBI subjects from healthy controls with an AUC of 0.99.[21] With magnetic resonance DTI (MR-DTI), the higher concentrations of peripheral NF-L were significantly associated with higher trace ($R^2 = 0.79$) and lower fractional anisotropy (FA) ($R^2 = 0.83$), indicating blood concentration of NF-L reflects the severity of diffuse axonal injury.[22] Similarly, serum NF-L levels completely discriminated between patients with sport-related concussion and healthy controls.[23]

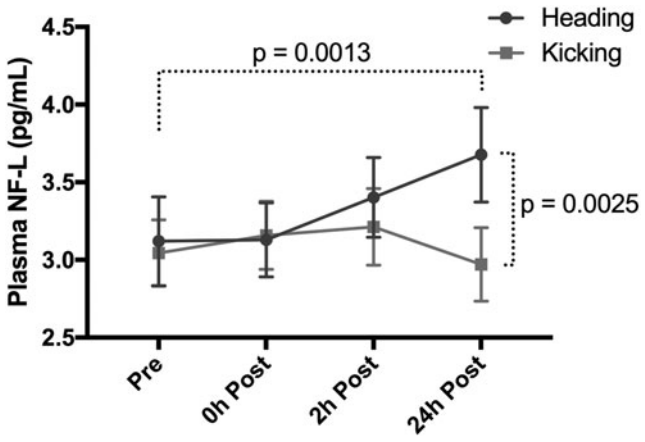

FIG. 2. Changes in plasma NF-L levels before and after subconcussive impacts. In the heading group, NF-L was elevated at 24 h post-heading compared with pre-heading and 0 h post-heading time-points, but the kicking-control group remained static across all time-points. The heading group's NF-L levels at 24 h post-heading were higher than that of the kicking-control group. Data are presented as means ± SEM. NF-L, neurofilament light; SEM, standard error of mean.

The current study along with a few other clinical studies have now begun to elucidate the subtle, but absolutely detectable changes caused by subconcussive head impacts. Soccer heading may account for the dominant share of subconcussive exposure in sports, given soccer is one of the most popular sports with approximately 265 million people playing around the globe.[32] Ten bouts of soccer headers in the current study induced approximately 300*g* and 35krad/sec^2, which were near identical to previous reports using a similar subconcussion model.[33,34] These impact kinematics were comparable to the hits observed in football, where average college football player incurs 7.0 to 9.4 hits during practice and 25 hits during a game with a mean peak linear acceleration per hit of 28.8 to 32.0*g*.[9,35–37] Therefore, the results from 10 soccer headers can be translated into other contact sports, indicating the vast clinical implication of the current finding. However, the clinical significance of the modest NF-L elevation (0.56 pg/mL) at 24 h post-heading compared with the baseline is difficult to delineate, but many athletes engaged in contact sports incur these subconcussive head impacts on a daily basis. When an individual constantly experiences subconcussive head impacts, axonal injury becomes more prominent [15,16,38] and reflects on a robust increase in peripheral expression of NF-L, as alluded to by Oliver and colleagues; that the starting football players increased NF-L levels after summer camp and the season by 1.52 pg/mL and 4.82 pg/mL compared with the pre-season baseline, respectively.[14]

There are several key limitations in the study. There are two possible pathways for NF-L to reach the peripheral bloodstream. Extracellular NF-L may either cross the disrupted blood–brain barrier or be drained into lymphatic vessels as part of the glymphatic system, then ultimately drained into the bloodstream.[20] Because both pathways cannot be visualized or tracked in a human model, an animal TBI model coupled with two-photon laser scanning microscopy[39,40] should be employed to determine the role of the blood–brain barrier and the glymphatic system in increased brain-derived blood biomarker levels. Because our design monitors the participants up to 24 h after headers, we were unable to study how long the elevation lasts before returning to baseline, which warrants future investigations. Other potential confounders such as sleep quality and quantity, diet, and menstrual cycle were not assessed, yet our repeated measures study design with randomization should account for potential contribution of these factors. Although

we observed a significant increase in plasma NF-L levels at 24 h post-heading, we did not monitor participants' behavior or activity between 2 h and 24 h post-heading. There is a possibility that factors about which we were unaware outside the study protocol may have contributed in the increase in plasma NF-L. However, the change observed in the heading group at 24 h post-heading was significantly higher than not only the within-group baseline, but also when compared with the kicking-control group. Therefore, we believe that the 24 h post-heading elevation in plasma NF-L was driven by subconcussive head impacts.

Conclusion

There is growing concern that even mild head impacts can cause significant insidious neurotrauma if sustained repetitively. Whereas subtle changes caused by subconcussive head impacts are difficult to detect and characterize, plasma NF-L concentrations have proven effective in reflecting repetitive subconcussive head impacts. Our data suggest that the increased level of plasma NF-L was driven by 10 acute bouts of subconcussive head impacts and required longer than 2 h after the impacts for the increase to be detected. Plasma NF-L levels may serve as an objective marker to monitor acute axonal burden from subconcussive head impacts.

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Author Disclosure Statement

No competing financial interests exist.

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