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ORIGINAL ARTICLE

FLUID BIOMARKERS

Association Between Serum Neurofilament Light and Glial Fibrillary Acidic Protein Levels and Head Impact Burden in Women's Collegiate Water Polo

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Abstract

Recent investigations have identified water polo athletes as at risk for concussions and repetitive subconcussive head impacts. Head impact exposure in collegiate varsity women's water polo, however, has not yet been longitudinally quantified. We aimed to determine the relationship between cumulative and acute head impact exposure across pre-season training and changes in serum biomarkers of brain injury. Twenty-two Division I collegiate women's water polo players were included in this prospective observational study. They wore sensor-installed mouthguards during all practices and scrimmages during eight weeks of pre-season training. Serum samples were collected at six time points (at baseline, before and after scrimmages during weeks 4 and 7, and after the eight-week pre-season training period) and assayed for neurofilament light (NfL) and glial fibrillary acidic protein (GFAP) using Simoa[®] Human Neurology 2-Plex B assay kits. Serum GFAP increased over time (e.g., an increase of 0.6559 pg/mL per week; $p = 0.0087$). Neither longitudinal nor acute pre-post scrimmage changes in GFAP, however, were associated with head impact exposure. Contrarily, an increase in serum NfL across the study period was associated with cumulative head impact magnitude (sum of peak linear acceleration: $B = 0.015$, $SE = 0.006$, $p = 0.016$; sum of peak rotational acceleration: $B = 0.148$, $SE = 0.048$, $p = 0.006$). Acute changes in serum NfL were not associated with head impacts recorded during the two selected scrimmages. Hormonal contraceptive use was associated with lower serum NfL and GFAP levels over time, and elevated salivary levels of progesterone were also associated with lower serum NfL levels. These results suggest that detecting increases in serum NfL may be a useful way to monitor cumulative head impact burden in women's contact sports and that female-specific factors, such as hormonal contraceptive use and circulating progesterone levels, may be neuroprotective, warranting further investigations.

Keywords: acute brain injuries; serum markers; sports medicine; subconcussive head impacts; water polo

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Introduction

Mild traumatic brain injury (mTBI) affects both men and women, yet neurological sequelae of mTBI is thought to be influenced by sex.^{1,2} Women with a diagnosis of mTBI experience longer recovery times³ and worse symptoms up to 12 months post-injury compared with men.⁴ The neurobiological and neuropsychological mechanisms underlying this sex difference are not well understood, however.

In a similar context, women may also be more vulnerable to subconcussive head impacts or hits to the head that do not result in the clinical signs and symptoms of mTBI, which are thought to lie on the lower end of a severity spectrum with TBI.⁵ For example, female ice hockey players exhibited significant alterations in white matter integrity across one collegiate season whereas no changes in microstructural integrity were detected in the male athletes,⁶ despite a known greater cumulative head impact burden in men's ice hockey.⁷ A similar difference in head impact burden has been reported in club water polo games, where players on the men's team sustain head impacts of greater magnitudes than the women's team (mean peak linear acceleration [PLA]: 39.7 ± 16.3 g vs. 33.7 ± 12.6 g).⁸

Collapsed across men's and women's teams, collegiate (i.e., varsity) teams sustain more head impacts and greater cumulative impact magnitude than club teams during a single tournament.⁹ Head impact exposure and its putative effects, however, have not yet been longitudinally tracked in women's collegiate water polo athletes, a population identified as at increased risk for concussion.¹⁰

Subconcussive head impacts have the potential to transiently perturb neuro-ophthalmologic function,¹¹ disrupt white matter integrity,^{12–14} and alter functional connectivity,^{15,16} suggestive of overlapping pathophysiology with mTBI. Brain-derived blood biomarkers are minimally invasive and provide objective insight into the neuropathological processes after brain injury,^{17,18} including the subclinical neuronal and glial stress from subconcussive head impacts.^{19–22}

In particular, elevated circulating levels of neurofilament light (NfL), a structural protein abundantly expressed in myelinated axons,²³ have been associated with protracted recovery after concussion and persistent post-concussion symptoms in professional ice hockey players,²⁴ the number of recent sparring rounds in active boxers or mixed martial arts fighters,²⁵ and a greater number of punches to the head during a recent amateur boxing bout.²⁶

Glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed in astrocytes, is upregulated during astrocytic hypertrophy, and its presence in the bloodstream can indicate neuroinflammation and glial activation after injury.²⁷ The GFAP emerged as a potential biomarker for subconcussive head impacts when Papa and associates²² reported that serum GFAP levels

were higher in patients presenting to the emergency department with blunt head trauma but no signs or symptoms of concussion compared with a control group of patients with peripheral/body trauma.

To our knowledge, blood biomarkers for concussion or subconcussive head impact exposure have never been specifically examined in an all-female cohort. In addition, only two studies have examined directly sex differences in blood biomarkers after concussion,^{28,29} and only one has examined sex differences in blood biomarkers after subconcussive impact exposure, albeit retrospectively.³⁰ This limited effort is partly because the majority of investigations focusing on subconcussive head impacts in athletes have been conducted in men's sports with known high rates of head impacts, such as American football and ice hockey.³¹ Therefore, relatively little is known about the neurobiological effects of repetitive subconcussive head impact exposure in women.

Previous work suggests that underlying sex differences in axonal microstructure and female-specific factors may modulate the effects of subconcussive head impacts. First, female axons are smaller in diameter and contain fewer microtubules than male axons, making them more vulnerable to strain injury.³² Second, hormonal fluctuations across the menstrual cycle and hormonal contraceptive use appear to affect outcomes after mTBI such that women taking hormonal contraceptives reported better quality of life and general health compared with women who were in the luteal phase of the menstrual cycle, when progesterone is peaking, at the time of injury.³³ Altogether this suggests that to understand the effects of subconcussive head impacts in female brains, one must additionally examine the potential role of female-specific variables, such as hormonal fluctuations during the menstrual cycle and hormonal contraceptive use.

In this prospective longitudinal investigation of Division I collegiate women's water polo players, we aimed to investigate the associations between serum levels of biomarkers for axonal damage (NfL) and glial activation (GFAP) and subconcussive head impact exposure in collegiate women's water polo. First, we hypothesized that serum NfL and GFAP would increase over pre-season training, with potential neuroprotective effects of hormonal contraceptive use and salivary progesterone and estradiol levels (i.e., lower NfL and GFAP levels), and that this change from baseline to follow-up would be correlated with cumulative head impact exposure. Second, we hypothesized that serum NfL and GFAP would acutely increase after scrimmaging in an exposure-dependent manner.

Methods

Participants

This single-site, prospective observational study included active members of a National Collegiate Athletic Association Division I women's water polo team. Exclusion

criteria included a history of neurological conditions (e.g., seizures/epilepsy, stroke, closed head injuries with loss of consciousness greater than 15 min, brain tumor, multiple sclerosis), a history of vestibular, ocular, or vision dysfunction (e.g., macular degeneration), any head, neck, or face injury in the three months before the study (e.g., concussion, eye injury), and self-reported pregnancy or possibility of being pregnant.

One participant left the team during the second week of the eight-week head impact monitoring period and was excluded thereafter. One participant received a diagnosis of a concussion during the sixth week of the eight-week head impact monitoring period and was excluded thereafter. See Figure 1 for the study flow. All data collection activities were approved by the Institutional Review Board at Indiana University, and all participants gave written informed consent before participation.

Head impact monitoring

All participants were fitted with Impact Monitoring Mouthguards (firmware v2.0.17, Prevent Biometrics Inc., Minneapolis, MN). Embedded within the mouthguard, a triaxial accelerometer (ADXL372, Analog Devices, Boston, MA) and gyroscope (BMG250, Bosch, Gerlingen, Germany) captures six-degrees-of-freedom spatial and temporal estimates of linear and rotational head acceleration during impacts. When one axis of acceleration exceeds a threshold of 5 g, impact data are collected for 50 msec at a sampling rate of 3.2 kHz.

The impact data are processed using firmware within the mouthguard and are stored until a Bluetooth low-energy connection is established with the Prevent iOS application (v1.1.11). A proximity sensor within the mouthguard ensures data are only captured when the mouthguard is in contact with the upper dentition. In accordance with manufacturer recommendations, research personnel synced the mouthguards with the Prevent iOS application before and after every training session. Participants could opt to wear their mouthguards for a one-week familiarization period immediately before the head impact monitoring period. Impact data were collected across the eight-week head impact monitoring

period during fall pre-season training, for a total of 38 training sessions, one intrateam scrimmage, one friendly scrimmage against team alumni, and two intercollegiate scrimmages.

For all training sessions and scrimmages during the head impact monitoring period, mouthguards were distributed at the end of the swimming warm-up period. Participants wore their mouthguards through the conclusion of the training session/scrimmage. Research personnel collected the mouthguards, placed them in the solo charging cases, and then placed the solo charging cases in the team case for charging until the next session. During all training sessions, research personnel visually monitored the athletes and noted session start and end times. Impacts with PLA ≥10 g were retained for analysis. The outcome measures for head impact exposure as detected by the Impact Monitoring Mouthguards included frequency of impacts, PLA (g), and peak rotational acceleration (PRA, krad/s²).

Video validation of head impact measurements. Ten training sessions (eight practices and the two scrimmages) were recorded. Three research personnel independently categorized the impacts sustained during the recorded sessions as either true positives (defined as an observable impact to the head) or false positives (no impact observed in the recording). The positive predictive value (PPV) was calculated using the following formula.

PPV = True Positives / (True positives + False Positives)

Daily menstrual cycle and hormonal contraceptive use reporting

Participants completed daily surveys (Qualtrics, Provo, UT) to confirm they were still taking/using their prescribed hormonal contraceptive, if applicable, and to record any menstrual bleeding.

Serum sample collection and analysis

At each of the six time points involving a blood sample collection (Fig. 1), a trained phlebotomist collected up

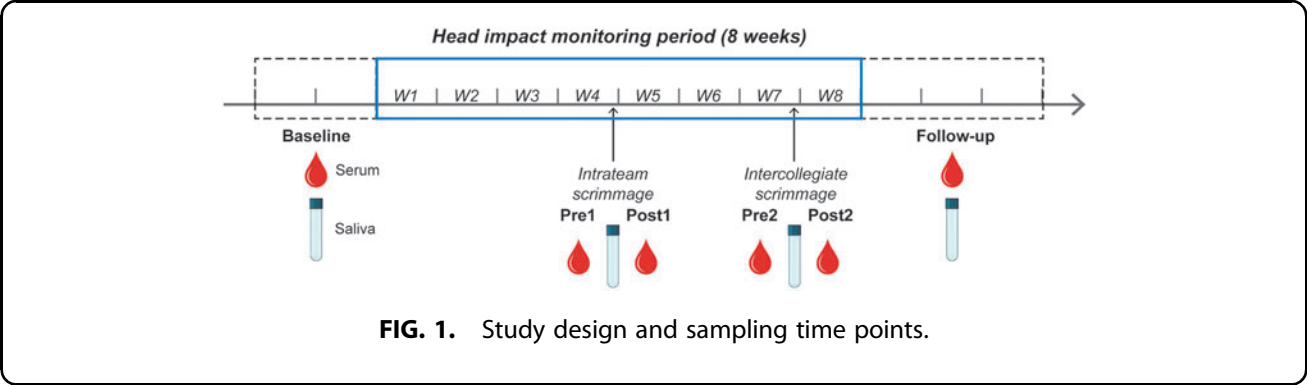


FIG. 1. Study design and sampling time points.

to 6 mL of venous blood from the antecubital region into a sterile serum vacutainer tube (BD Bioscience, Franklin Lakes, NJ). One participant reported a severe fear of needles during enrollment; therefore, all six of her samples were collected using Tasso-SST devices (Tasso, Inc., Seattle, WA). Both venous and capillary samples were allowed to clot at room temperature for a minimum of 30 min and then were centrifuged at 1500 g for 15 min at 4°C. Serum was aliquoted into 1.5 mL Eppendorf tubes and stored at -80°C until analysis. One serum sample from the post2 time point was missing because of phlebotomy difficulty; however, it should be noted that this participant did not play in the intercollegiate scrimmage because of an injury.

Serum NfL and GFAP concentrations were measured using Simoa® Human Neurology 2-Plex B assay (N2PB) kits and the Simoa® SR-X Biomarker Detection System (Quanterix™, Lexington, MA). The limits of detection (LOD) for the SR-X N2PB kit are 0.071 pg/mL and 0.410 pg/mL for NfL and GFAP, respectively. The functional lower limits of quantification (LLOQ) are 1.60 pg/mL and 16.6 pg/mL for NfL and GFAP, respectively. The concentration of NfL and GFAP in the samples were interpolated from a calibration curve fit with a four-parameter logistic equation ($1/y^2$ weighting). Twenty percent of the serum samples were assayed in duplicate. All samples from each participant were assayed on the same plate to minimize intrasubject variation. To minimize interplate variation, the same researcher performed all assays.

Saliva sample collection and hormone assays

Saliva samples were collected at four time points: (1) at baseline, before the start of the eight-week head impact monitoring period, (2) the day of Scrimmage I, (3) the day of Scrimmage II, and (4) at the final, follow-up time point (Fig. 1). For all sample collections, participants were instructed to passively drool into the provided, labeled conical tube immediately on waking and before eating, drinking, or brushing teeth. Participants collected at least 2 mL of saliva and brought the samples with them to the pool in the morning. Samples were separated into 500–1000 μ L aliquots and stored at -80 °C until analysis.

On the day of analysis, samples were thawed in a warm water bath for 60 min and centrifuged at 1500 g for 15 min. Salivary progesterone was measured using Progesterone Saliva ELISA kits (PRG32-K01, Eagle Biosciences, Inc., Nashua, NH). Salivary estradiol was quantified using Salivary 17 β -Estradiol EIA kits (1-3702, Salimetrics, State College, PA). The assay range for the progesterone assay was 20–5000 pg/mL with a lower limit of sensitivity of 20 pg/mL; the dynamic assay range for the estradiol assay was 1–32 pg/mL with a lower limit of sensitivity of 0.1 pg/mL. Eighty-eight (88) percent and 81% of samples were assayed in duplicate with mean coefficients

of variations (CV) of 1.8% and 3.1% for the progesterone and estradiol assays, respectively.

For both assays, absorbance was measured by a microplate reader (BioTek PowerWave XS, Winooski, VT) with Gen5™ Reader Control Software. Salivary hormone concentrations were interpolated from concentrations on a standard curve. To minimize intraparticipant variance, all samples from each participant were tested on the same plate. To minimize interplate variance, one researcher performed all hormone assays. Three samples from the follow-up time point were unusable because of high viscosity.

Statistical analysis

To test the first hypothesis, we fit linear mixed effects models with blood biomarker concentrations (NfL or GFAP) at baseline, pre1, pre2, and follow-up time points as the outcome variable with fixed effects for time (a continuous variable, defined as the number of days from the first day of the head impact monitoring period), hormonal contraceptive use (a binary variable), salivary estradiol concentration, and salivary progesterone concentration. A random intercept was set for each participant to account for baseline differences in NfL and GFAP concentrations. Models were estimated using the Restricted Maximum Likelihood method. Variance inflation factors (VIF) were calculated and examined to check for multi-collinearity.

Biomarker concentrations from the two post-scrimmage time points (post1 and post2) were not included in the mixed effects regression models because of the potential acute effect of participating in the scrimmages, as suggested by previous reports that NfL and GFAP peak 20–24 h after subconcussive head impact exposure.^{19,22} Next, the relationship between change in biomarker concentration from baseline to follow-up and head impact kinematics was examined by fitting three linear regression models per biomarker with either total number of impacts, cumulative PLA, or cumulative PRA as the predictor and hormonal contraceptive use included as a covariate. The level of significance was adjusted by Bonferroni correction to account for the three comparisons per biomarker (i.e., adjusted $p = 0.05/3 = 0.017$).

Next, we examined the relationship between acute exposure and acute changes in serum NfL and GFAP using two sets of linear regression models. The change in serum biomarker concentration from pre- to post-scrimmage was the outcome variable, and acute head impact exposure (number of hits, cumulative PLA, or cumulative PRA) was the predictor. The model was adjusted for cumulative head impact exposure before the scrimmage (number of hits, cumulative PLA, or cumulative PRA). For each set of three linear regression models per biomarker, the level of significance was adjusted by Bonferroni correction (i.e., $p = 0.05/3 = 0.017$) to account for multiple comparisons.

All analyses were performed using R (version 4.1.2). Mixed effects models were fit using “lme4” and “lmerTest” packages.^{34,35} All tests were two-sided, and the level of significance was set to $p=0.05$ unless otherwise specified.

Results

Participant characteristics and head impact exposure

Demographic and other participant characteristics are described in Table 1. While the majority of participants used hormonal contraceptives (14 of 22, or 63.6%), hormonal contraceptive types were split between either an oral contraceptive pill ($n=8$) or an intrauterine device ($n=6$).

Across the study period, a total of 124 head impacts (≥ 10 g) were captured by the Impact Monitoring Mouthguards. The total number of head impacts per participant across the study period ranged from 0 to 15 impacts. The average PLA of the impacts was 14.6 ± 5.5 g, and the mean PRA was 1.6 ± 0.7 krad/s² (Fig. 2A,2B). The average number of impacts sustained during the intrateam scrimmage at the end of week 4 was 0.43 ± 0.75 impacts. During the intercollegiate scrimmage at the end of week 7, participants sustained an average of 0.65 ± 0.93 impacts. Of note, cumulative head impact exposure did not significantly differ between participants using hormonal contraceptive and non-users, as suggested by a non-significant unpaired t test ($p=0.649$; Fig. 2C).

Table 1. Participant Characteristics and Cumulative Head Impact Exposure

Demographic variable	N = 22
Age, y	19.3 ± 1.4^a
Water polo playing experience, y	9.0 ± 3.0^a
Year in school, n (%)	
Freshman	7 (31.8)
Sophomore	5 (22.7)
Junior	6 (27.2)
Senior	5 (22.7)
Primary water polo position, n (%)	
Goalie	3 (13.6)
Attacker	9 (40.9)
Center or center defender	5 (22.7)
Utility	5 (22.7)
Lifetime concussion history, n (%)	
No history	13 (59.1)
1 prior concussion	7 (31.8)
2 prior concussions	2 (9.1)
Hormonal contraceptive use, n (%)	
User	14 (63.6)
Non-user	8 (36.4)
Head impact exposure per participant	
Number of head impacts	5.6 ± 4.3^a
Cumulative PLA, g	82.2 ± 61.9^a
Cumulative PRA, krad/s ²	9.2 ± 7.1^a

PLA, peak linear acceleration; PRA, peak rotational acceleration. ^aSummary statistics are presented as mean $\pm$ standard deviation.

Salivary estradiol and progesterone varied widely between and within individuals at the four time points with saliva collection across pre-season training (Fig. 3). Notably, salivary hormone levels were generally lower in participants using hormonal contraceptives than non-users.

Video validation of head impact exposure

A total of 16 head impacts were detected by the mouthguards during the 10 recordings. Fifteen head impacts were observed during the review of the recordings, yielding a PPV of 93.8%. The interrater agreement was excellent (95.8%).

Serum GFAP increased while serum NfL decreased over time

Serum GFAP concentrations significantly increased over time, as supported by a significant and positive association between serum GFAP and time ($B=0.138$ [SE=0.054], $p=0.013$; Fig. 4A and Table 2). Serum NfL concentrations significantly decreased over time (Fig. 4B), as supported by a significant and negative association with time, in days ($B=-0.010$ [SE=0.004], $p=0.027$; Table 2).

Influences of hormonal contraceptive use and hormone levels on blood biomarker levels

The VIFs for all fixed effects in both mixed effects regression models ranged from 1.008 to 1.398, indicating that multi-collinearity was not a concern and that including all three female-specific covariates was statistically justifiable. Hormonal contraceptive use appears to be associated with lower levels of serum GFAP and NfL while holding time point and salivary hormone levels constant, as demonstrated by negative, significant associations between use of hormonal contraceptives and serum levels of GFAP and NfL (GFAP: $B=-21.119$ [SE=7.616], $p=0.012$, Fig. 5A; NfL: $B=-2.657$ [SE=0.966], $p=0.012$, Fig. 5B; Table 2).

In addition, while salivary progesterone levels were negatively associated with NfL levels ($B=-0.004$ [SE=0.002], $p=0.029$, Fig. 6B), progesterone levels were positively associated with GFAP levels ($B=0.061$ [SE=0.022], $p=0.007$, Fig. 6A). In other words, higher salivary levels of progesterone were associated with lower serum NfL levels and higher serum GFAP levels, irrespective of hormonal contraceptive use or time point during pre-season training as these other fixed effects are held constant. Salivary estradiol levels were not associated with serum NfL or GFAP.

Greater head impact burden was associated with an overall increase in NfL

The overall change in serum NfL from baseline to follow-up was positively and significantly associated with

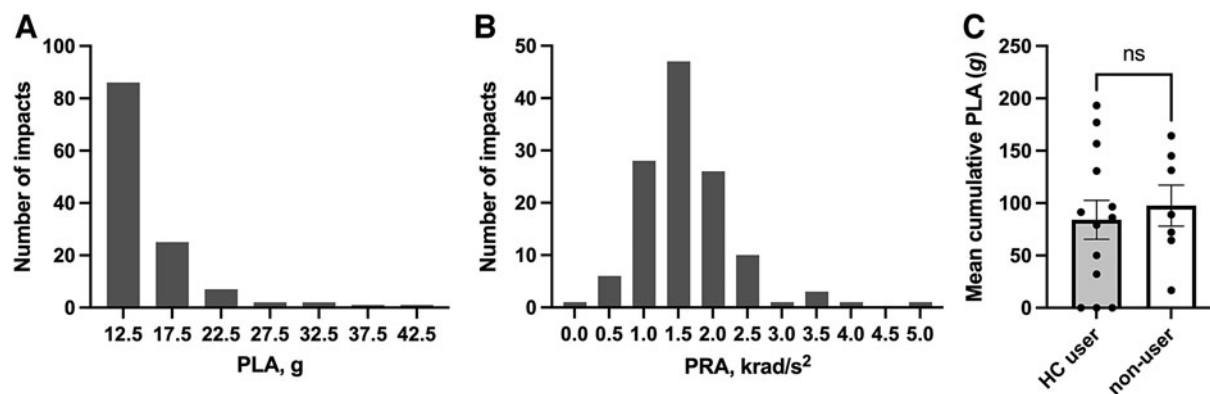


FIG. 2. Head impact magnitude distributions. (A) Histogram of impact magnitude in terms of peak linear acceleration (PLA) and (B) peak rotational acceleration (PRA). (C) Cumulative head impact exposure, in terms of sum PLA, did not differ between hormonal contraceptive (HC) users and non-users. Bars and error bars represent mean $\pm$ standard error of the mean.

cumulative head impact burden across the head impact monitoring period (Table 3). After adjustment for multiple comparisons, cumulative PRA was significantly associated with increased serum NfL (PRA: $B=0.148$ [$SE=0.049$], $p=0.008$; Fig. 7C). The total number of impacts and cumulative PLA were associated with an increase in serum NfL (Fig. 7A,7B), but these relationships did not survive correction for multiple comparisons. The overall change in GFAP from baseline to follow-up was not associated with any of the head impact kinematic variables (Table 3).

Acute changes in GFAP and NfL were not associated with head impact exposure during scrimmages

Acute changes in serum GFAP and NfL from pre- to post-scrimmages were not associated with head impacts, in terms of the number of impacts, cumulative PLA, or

cumulative PRA, sustained during the scrimmages, as demonstrated by non-significant associations between change in GFAP or NfL and each of the three head impact kinematic variables (Fig. 8, Table 4). After adjustment for cumulative head impact exposure before each scrimmage and hormonal contraceptive use, the relationships between change in biomarker concentrations and head impact exposure during the scrimmages remain non-significant (Table 4).

Discussion

To our knowledge, this is the first study to prospectively examine brain-derived blood biomarker expressions, head impact exposure, and potential neuroprotective effects of hormonal levels and contraceptive use in female collegiate athletes. The primary findings from the present study can be summarized as follows: (1) there was an overall linear increase in serum GFAP and a decrease

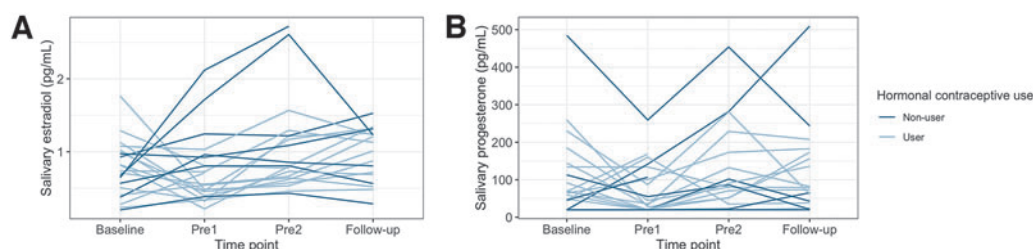


FIG. 3. Fluctuations in (A) salivary estradiol and (B) salivary progesterone across the head impact monitoring period. When comparing salivary hormone levels to self-reported menstrual cycle patterns derived from the daily reports, it became apparent that any examinations of phase-dependent effects would not be possible given the small sample size, as a large proportion of the participants reported abnormal menstrual cycle patterns (e.g., amenorrhea, anovulatory cycles, irregular intermenstrual durations), typical of hormonal contraceptive users and/or elite athletes.^{66,67}

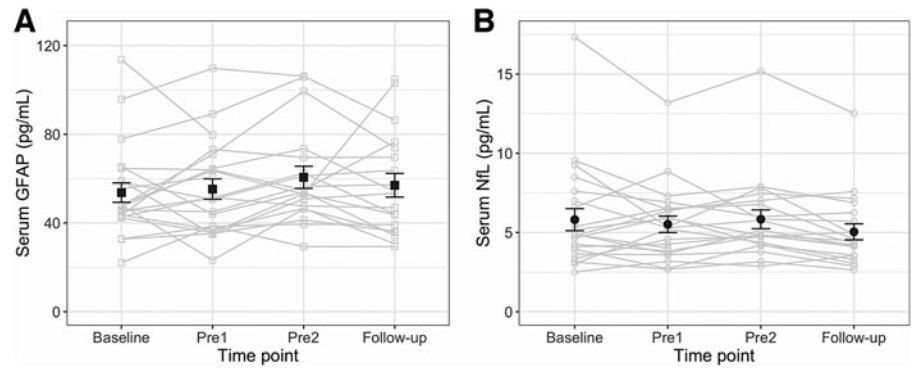


FIG. 4. Longitudinal pattern of changes in blood biomarker concentrations were assessed using linear mixed effects regression models. **(A)** Serum glial fibrillary acidic protein (GFAP) increased over time ($B=0.138$, $SE=0.054$, $p=0.013$), adjusted for hormonal contraceptive use and salivary levels of estradiol and progesterone. **(B)** Serum neurofilament light (NFL) slightly decreased over time ($B=-0.010$, $SE=0.004$, $p=0.027$), adjusted for hormonal contraceptive use and salivary levels of estradiol and progesterone. Solid points and error bars represent means $\pm$ standard error of the mean.

in serum NfL concentrations over pre-season training; (2) hormonal contraceptive use was associated with lower serum NfL and GFAP levels over time, while elevated salivary progesterone levels were associated with lower serum NfL but higher serum GFAP; (3) NfL increased from baseline to the conclusion of pre-season training in association with cumulative head impact magnitude; (4) acute changes in NfL and GFAP between pre- and post-scrimmages were not associated with head impact exposure during scrimmages.

Collectively, these results suggest that increases in serum NfL levels may reflect greater cumulative head impact exposure and that hormonal contraceptive use and progesterone levels may influence neural response to head impacts.

The most novel aspect of this study was the inclusion of hormonal contraceptive use and salivary progesterone

and estradiol levels in the examination of serum NfL and GFAP levels over time. The neuroprotective effects of reproductive hormones have been documented for decades across numerous pre-clinical TBI studies.^{36–38} Treating ovariectomized female rodents or male rodents with estradiol results in reduced post-injury hypoperfusion³⁹ and an improved bioenergetic state,⁴⁰ while treatment with progesterone is associated with reduced edema,⁴¹ lesion size, and post-injury cognitive deficits,⁴² and attenuates white matter disruption in the corpus callosum.⁴³

Table 2. Serum Neurofilament Light and Glial Fibrillary Acidic Protein Levels Modulated Over Time by Contraceptive Use and Salivary Hormone Levels

Effect	B	SE	t	p value
GFAP				
Time	0.138	0.054	2.567	0.013*
Hormonal contraceptive use	-21.119	7.616	-2.773	0.012*
Progesterone	0.061	0.022	2.793	0.007**
Estradiol	-6.800	4.881	-1.393	0.168
NfL				
Time	-0.010	0.004	-2.278	0.027*
Hormonal contraceptive use	-2.657	0.966	-2.752	0.012*
Progesterone	-0.004	0.002	-2.239	0.029*
Estradiol	0.697	0.407	1.712	0.092

GFAP, glial fibrillary acidic protein; NfL, neurofilament light. Time was quantified as the number of days from the first day of the head impact monitoring period. The p values were estimated via t tests using the Satterthwaite method (“lmerTest” R package). Asterisk (*) denotes significance; $p < 0.05$.

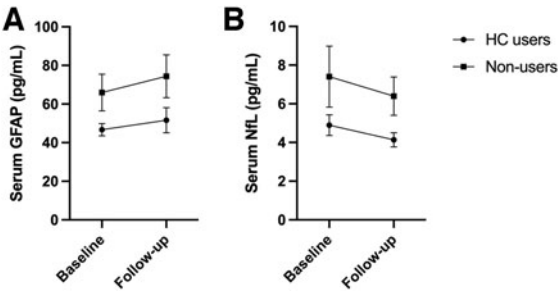


FIG. 5. Trajectories of blood biomarkers across preseason training by hormonal contraceptive use. **(A)** Overall change in serum glial fibrillary acidic protein (GFAP) between hormonal contraceptive (HC) users and non-users. **(B)** Overall change in serum neurofilament light (NfL) between hormonal contraceptive users and non-users. Biomarker levels are depicted as means, and error bars represent standard error of the mean.

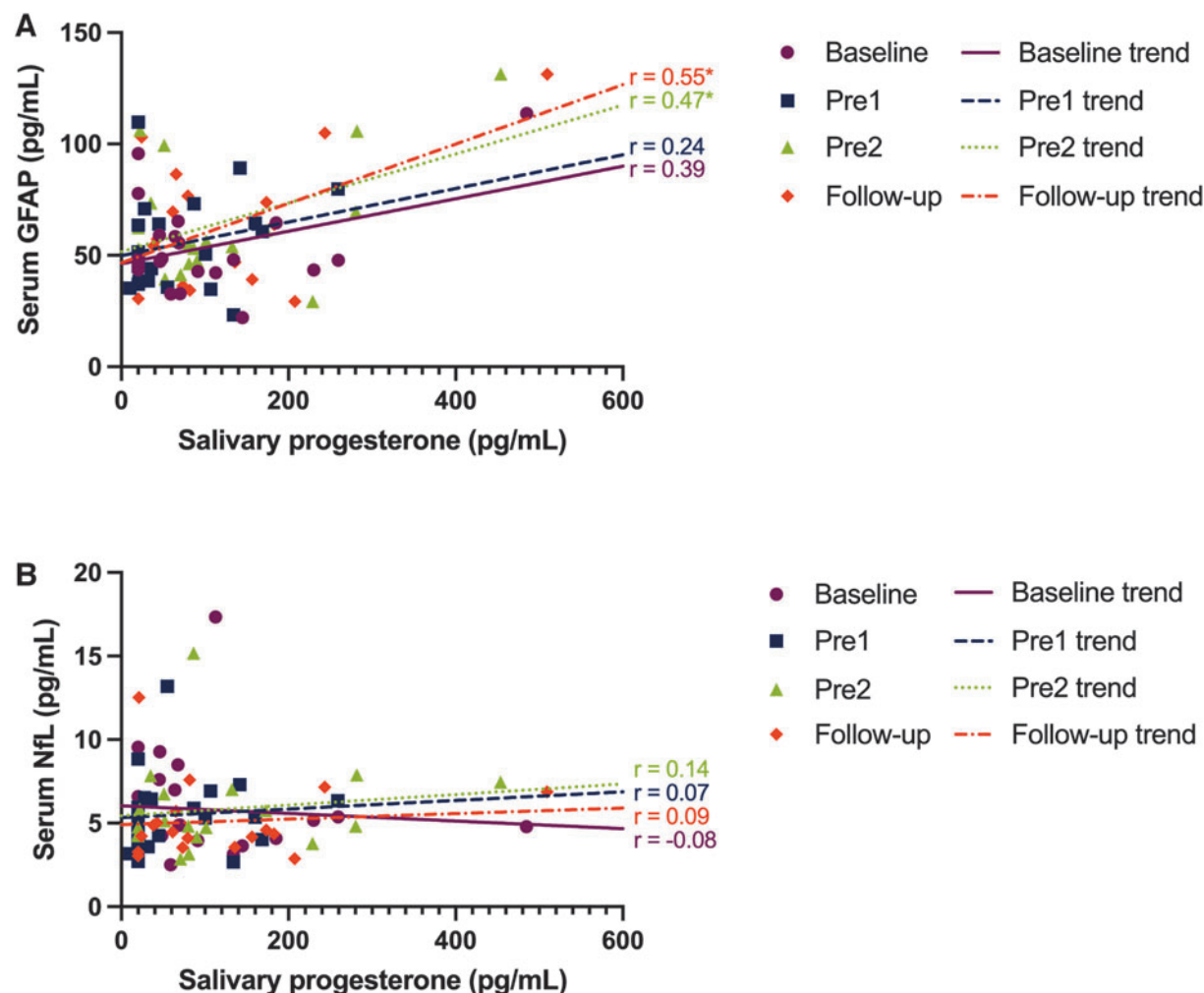


FIG. 6. Association between hormonal contraceptive use and blood biomarker levels across pre-season training. **(A)** Serum glial fibrillary acidic protein (GFAP) and salivary progesterone levels were positively associated across pre-season training. *Post hoc* exploratory analysis examined the relationship between progesterone and GFAP levels using Pearson correlations. Correlations between GFAP and progesterone were significant at pre2 ($r = 0.47$, $p = 0.04$) and at follow-up ($r = 0.55$, $p = 0.02$). Asterisk denotes $p < 0.05$. **(B)** Despite a significant, negative association between salivary progesterone levels and serum neurofilament light (NfL) levels in the linear mixed effects model, exploratory analysis did not reveal any significant Pearson correlation coefficients for any of the four time points.

This neuroprotection, however, does not appear to translate to clinical neurotrauma: progesterone as a post-TBI therapeutic failed Phase III clinical trials because six-month functional outcomes were not improved in the treatment group, as assessed by the Glasgow Outcome Scale.^{44,45} Also contrary to pre-clinical findings, women who sustain a mTBI during the luteal phase of the menstrual cycle, when endogenous progesterone levels are at their highest, were more likely to report worse somatic symptoms and quality of life at one-month post-injury.³³

Hormonal contraceptive use, however, has been reported to be protective, in terms of reduced peak symptom

severity³ and better self-reported quality of life and general health at one-month post-injury.³³ Hormonal contraceptive use downregulates endogenous production of progesterone and estradiol, possibly exerting a neuroprotective effect by reducing the influence of any functional or structural disruption of the hypothalamic-pituitary-ovarian axis observed after TBI.^{46–48}

A post-traumatic reduction in the normally elevated levels of endogenous progesterone could precipitate worse outcomes, either directly or mediated by hormones synthesized from progesterone: estradiol, cortisol, and testosterone.^{33,47} Further, the pre-clinical findings of neuroprotective effects

Table 3. Associations Between Head Impact Exposure Across the Eight-Week Monitoring Period and Change in Serum Biomarker Concentrations from Baseline to Follow-Up

Effect	B	SE	t	p value
NfL				
Number of impacts	0.215	0.086	2.510	0.022
Cumulative PLA	0.015	0.006	2.574	0.020
Cumulative PRA	0.148	0.049	2.985	0.008*
GFAP				
Number of impacts	-1.721	1.111	-1.550	0.140
Cumulative PLA	-0.062	0.081	-0.761	0.457
Cumulative PRA	-0.907	0.687	-1.321	0.204

NfL, neurofilament light; PLA, peak linear acceleration; PRA, peak rotational acceleration; GFAP, glial fibrillary acidic protein.

Models were adjusted for hormonal contraceptive use. Asterisk (*) denotes a *p* value less than the adjusted level of significance, correcting for multiple comparisons.

of progesterone and estradiol generally examined one hormone in isolation, yet these two hormones can have antagonizing or opposing effects in the brain. For example, Woolley and McEwen (1993)⁴⁹ observed that administration of estradiol in ovariectomized rats resulted in increased dendritic spine density and that while administration of estradiol followed by progesterone initially had the same effect, dendritic spine density rapidly decreased.

In terms of protection against N-methyl-D-aspartate (NMDA) excitotoxicity, progesterone treatment in conjunction with estradiol, blocked estradiol-mediated neuroprotection.⁵⁰ The underlying molecular mechanism for this phenomenon was examined in a follow-up investigation: the estradiol-dependent increases in protein levels of estrogen receptors and brain-derived neurotrophic factor (BDNF), a neurotrophin with potent neuroprotective effects, were reversed by co-administration with progesterone.⁵¹ Estradiol and progesterone have also been shown to have, at times, opposing effects on astrogliosis, depending on the region and injury model.⁵²

In the present study, we observed a diverging association between progesterone and levels of NfL and GFAP and the absence of an association between estradiol and blood biomarker levels, which could potentially be attributed to the complexity of the interaction between estradiol and progesterone. A larger sample size with more dense sampling of hormones and blood biomarkers may allow for a more in-depth examination of hormone levels, their interactions, hormonal contraceptive use, and biomarkers for brain injury during repetitive head impact exposure.

While the scope of the analyses in the present study was limited by the relatively small sample size, the results indicate that hormonal contraceptive use may be protective against increases in biomarkers of brain injury. As such, we encourage future investigations of the effects subconcussive head impact exposure in women to account for both hormonal contraceptive use and hormonal levels, because focus on these female-specific factors in subconcussive head impact research is severely lacking.

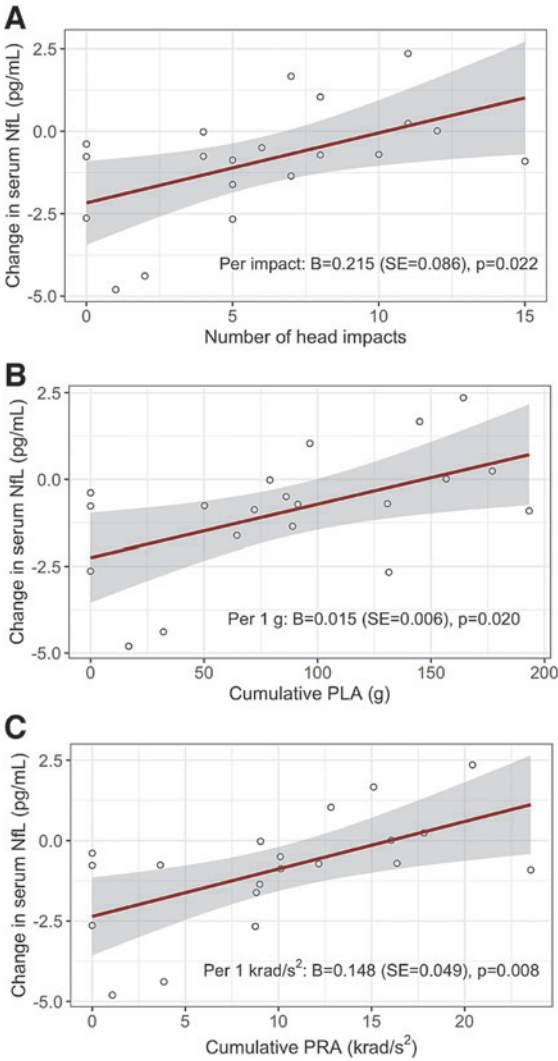


FIG. 7. Change in serum neurofilament light (NfL) from baseline to follow-up was associated with head impact exposure. The overall change in serum NfL is shown in relation to (A) the total number of head impacts, (B) cumulative peak linear acceleration (PLA), and (C) cumulative peak rotational acceleration (PRA). After correcting for multiple comparisons, the relationship between change in serum NfL and cumulative PRA remained significant.

There is a substantial body of knowledge about the relationship between circulating NfL levels after head trauma, from severe TBI to subconcussive head impact exposure. The NfL has been touted as an excellent blood biomarker to reflect the severity of brain injury and neurodegeneration, capable of distinguishing patients with TBI from uninjured controls through 180 days post-injury.²⁴ While the exact kinetics of human NfL in the

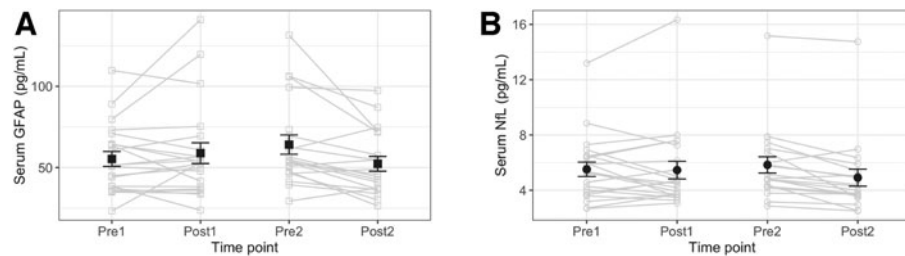


FIG. 8. Blood biomarker concentrations from pre- to post-scrimmage. The changes in serum concentrations across the two scrimmages are shown for **(A)** glial fibrillary acidic protein (GFAP) and **(B)** neurofilament light (NfL). No associations were observed between changes in serum levels and acute head impact exposure during scrimmages, with and without adjustment for cumulative head impact exposure before the scrimmages. Solid points and error bars represent means $\pm$ standard error of the mean.

bloodstream remain unknown, the half-life is most likely on the time scale of a few weeks,^{53,54} which lends a temporal profile conducive to the use of this biomarker to gauge axonal damage after traumatic brain injury or subconcussive head impact exposure.

Compared with previous investigations of circulating levels of NfL after TBI, the results of the present study, in which the mean serum NfL levels never exceeded 7 pg/mL across pre-season training, are quite reassuring: individuals with moderate or severe TBI exhibit median serum NfL levels in the range of 20–50 pg/mL, even months after injury.²⁴ The present study detected increases in serum NfL that were positively associated with head impact exposure across pre-season training in women's collegiate water polo players. A similar pattern of elevation has been observed in pre-season training in collegiate football players, such that starters exhibited a greater increase in serum NfL levels after the pre-season training camp relative to non-starters and non-contact control athletes (swimmers).⁵⁵ Further investigation is warranted to ascertain the clinical relevance of these minor increases in serum NfL.

To that end, the utility and clinical relevance of circulating NfL as a biomarker for axonal damage from acute

subconcussive head impact exposure is, at present, still being determined because conflicting findings have been reported in soccer heading models. For instance, plasma NfL levels in experienced young adult soccer players were significantly elevated at 24 h after performing 10 controlled soccer headers in 10 min.¹⁹ Another group employed a similar model and found that elevated serum NfL levels at 1 h post-heading (40 headers in 20 min) persisted at three weeks post-heading.⁵⁶ Austin and colleagues,⁵⁷ however, did not observe an increase in serum NfL over the 24 h period after 10, 20, or 40 controlled soccer headers.

These conflicting results from soccer heading models may stem from methodological differences (i.e., launching a soccer ball with a JUGS machine^{19,56} versus dropping the soccer ball from above the research participant⁵⁷) that resulted in fundamental differences in subconcussive head impact exposure. Regardless, the low doses of head impacts sustained by participants during water polo scrimmages may explain why we did not observe any significant changes in serum NfL from pre- to post-scrimmage: the average number of head impacts sustained in the intrateam scrimmage and the intercollegiate scrimmage were 0.43 ± 0.75 and 0.65 ± 0.93

Table 4. Associations Between Acute Head Impact Exposure and Change in Serum Biomarker Concentrations from Pre- to Post-Scrimmage

Effect	Unadjusted				Adjusted			
	B	SE	t	p value	B	SE	t	p value
NfL								
Number of impacts	0.312	0.229	1.365	0.180	0.406	0.237	1.711	0.096
Acute cumulative PLA	0.003	0.011	0.268	0.790	0.011	0.013	0.878	0.386
Acute cumulative PRA	0.102	0.121	0.839	0.407	0.196	0.131	1.500	0.142
GFAP								
Number of impacts	2.046	3.489	0.587	0.561	3.95	3.591	1.101	0.278
Acute cumulative PLA	-0.064	0.163	-0.393	0.696	0.104	0.186	0.558	0.580
Acute cumulative PRA	-0.028	1.834	-0.015	0.988	1.562	1.947	0.802	0.428

NfL, neurofilament light; PLA, peak linear acceleration; PRA, peak rotational acceleration; GFAP, glial fibrillary acidic protein.

Linear regression models were adjusted for cumulative head impact exposure prior to each scrimmage and hormonal contraceptive use; unstandardized estimates are reported for both the unadjusted and adjusted models.

impacts, respectively, substantially less than the minimum 10 impacts experienced by participants in the aforementioned soccer heading studies.

As recently reported by Monroe and colleagues⁹ in a novel investigation examining changes in salivary S100B and NfL, increased salivary NfL was associated with greater head impact exposure during a water polo tournament, consisting of three or four games over a one- or two-day period. This was considerably more playing time, and specifically playing time during competitive play against opposing teams, than our cohort experienced in any two-day period during their pre-season training.

In the present study, the primary objective was to examine change in serum NfL and GFAP over time and in relation to cumulative head impact exposure. Notably, salivary NfL may not be as representative of axonal damage as serum/plasma NfL⁵⁸ and requires validation in the context of TBI and subconcussive head impact exposure because the kinetics of salivary NfL as a biomarker for neuronal damage are not yet understood.

While we observed a significant increase in serum GFAP over the course of pre-season training, this increase was not associated with cumulative head impact frequency or magnitude. Reports of GFAP as a biomarker for subconcussive head impact exposure are presumably forthcoming but currently limited at both the clinical and pre-clinical level. Elevated serum GFAP levels were detected in a head trauma control group, or a group of patients presenting to the emergency department after similar traumatic mechanisms of injury as the primary concussion group but did not exhibit clinical signs and symptoms of concussion, compared with a body trauma control group (no head trauma).²² These elevated serum GFAP levels peaked at 20 h post-trauma.²²

Yet, in a pilot case-control study, Nowak and coworkers⁵⁹ did not observe an increase in plasma levels of GFAP at 2 h or 24 h after performing 10 controlled soccer headers in a group of healthy, young adult soccer players. Granted, a bout of 10 soccer headers is likely not equivalent to the mechanisms of injury experienced by the head trauma control group examined by Papa and colleagues (2019),²² over half of whom presented to the emergency department after a motor vehicle accident or a major fall.

In a pre-clinical model of subconcussive head impact exposure, rats in a high exposure group (600 impacts over four weeks, scaled from a study of head impact exposure in collegiate football players⁶⁰) exhibited significantly greater volume of GFAP+ surfaces in the basolateral and central amygdala relative to the sham injury group two weeks after the last injury.⁶¹ No differences in GFAP+ staining in these regions, however, were observed in the moderate exposure group (160 impacts over four weeks) or in the single injury group compared with the sham injury group,⁶¹ suggesting that the thresh-

old to trigger robust astrogliosis after subconcussive head impact exposure may be relatively high.

The timing of the pre- and post-scrimmage blood sample collections in the present study was designed with the half-life of GFAP (about 24–48 h in the bloodstream⁵⁴) in mind. The acute head impact exposure experienced by our participants during the scrimmages may not have been a large enough stimulus to trigger astrogliosis and the release and eventual elevation of GFAP in the blood.

Wearable head impact sensors have allowed researchers to determine associations between head impact exposure and various outcome measures. Skull cap and skin patch sensors, while convenient methods to quantify head impact exposure in non-helmeted sports, generally overestimate peak head impact kinematics because of non-rigid coupling between the skull and the sensor.⁶² Mouthguard sensors, however, can provide more reliable and accurate kinematic measurements because of the direct coupling with the upper dentition.⁶² The Prevent Biometrics boil-and-bite mouthguard has been demonstrated to be highly accurate in terms of measuring rotational acceleration, rotational velocity, and linear acceleration at the impact peak and across the kinematic trace for the entire measurement time window (50 msec).⁶³

In the present study, the mean PLA was 14.6 ± 5.5 g, and the mean PRA was 1632.5 ± 715.5 rad/s², considerably lower magnitudes than those previously reported in collegiate water polo players (e.g., 36.1 ± 12.3 g PLA and 5000 ± 2900 rad/s² PRA during men's collegiate water polo games⁶⁴; 33.7 ± 12.6 g PLA and 4000 ± 2800 rad/s² PRA during women's collegiate club water polo games⁸). The head impact kinematics reported by Cecchi and colleagues, however, were collected by SIM-G sensors (Triax Technologies, Norwalk, CT) held in place at the occipital protuberance within pockets sewn into the back of water polo caps. Further, all head impact kinematic data were collected during games, with an exception for several male goalies in the investigation reported by Cecchi and colleagues (2019),⁶⁴ who wore their cap sensors for a selection of practices in addition to games.

In summary, the head impacts sustained by the female water polo players in the present study were slightly lower in magnitude, which could be attributed to sensor type, setting (pre-season practices vs. competitive play), or a combination of these two factors, but appear to be comparable in terms of frequency relative to the exposure reported in previous studies in water polo.^{8,9}

The results and implications of the present study should be interpreted within the context of its limitations. First, while this is the first report of documented head impact exposure in women's collegiate water polo practices, the sample size was limited to a single collegiate team and was therefore relatively small. This small sample size unfortunately prevented us from examining the effect of hormonal contraceptive use by type or any

menstrual cycle phase-dependent effects on serum GFAP and NfL levels. Second, the monitoring period was limited to eight weeks of pre-season training, while the competitive season may involve greater head impact exposure for many players because of high frequency of intercollegiate competitions, a pattern previously observed in soccer players.⁶⁵

Third, we would have ideally preferred to video-verify all impacts that were detected by the mouthguards in the head impact monitoring period. While a member of the research team was physically present at all practices and scrimmages, however, these training sessions were “closed,” and therefore only eight practices, in addition to the two public scrimmages, were recorded. Thus, video verification was limited to these recordings.

Conclusions

The present study was the first study to date to examine blood biomarkers of brain injury in a women's contact sport, collegiate water polo. The NfL, a marker of axonal injury, increased over pre-season training in association with larger cumulative head impact magnitude, while changes in serum GFAP were not associated with cumulative head impact burden. Further investigation is merited to determine the clinical relevance of minor changes in NfL. Acute head impact exposure during scrimmages was not associated with changes in biomarker levels from pre- to post-scrimmage.

Preliminary evidence from this study suggests that hormonal contraceptive use may be neuroprotective, and future investigations of head impacts in women's sports should account for hormonal contraceptive use and circulating levels of progesterone and estradiol.

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Authors' Contributions

Megan E. Huibregtse: Conceptualization, Methodology, Formal Analysis, Investigation, Writing—Original Draft, Visualization, Project Administration, Funding Acquisition. Sage Sweeney: Investigation, Writing—Review and Editing. Mikayla Stephens: Investigation, Writing—Review and Editing. Hu Cheng: Methodology, Writing—Review and Editing, Supervision. Zhongxue Chen: Methodology, Writing—Review and Editing, Supervision. Hannah J. Block: Methodology, Writing—Review and Editing, Supervision. Sharlene D. Newman: Conceptualization, Methodology, Writing—Review and Editing, Supervision. Keisuke Kawata: Conceptualization, Methodology, Writing—Review & Editing, Supervision, Project Administration, Funding Acquisition.

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Author Disclosure Statement

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