CONCUSSION

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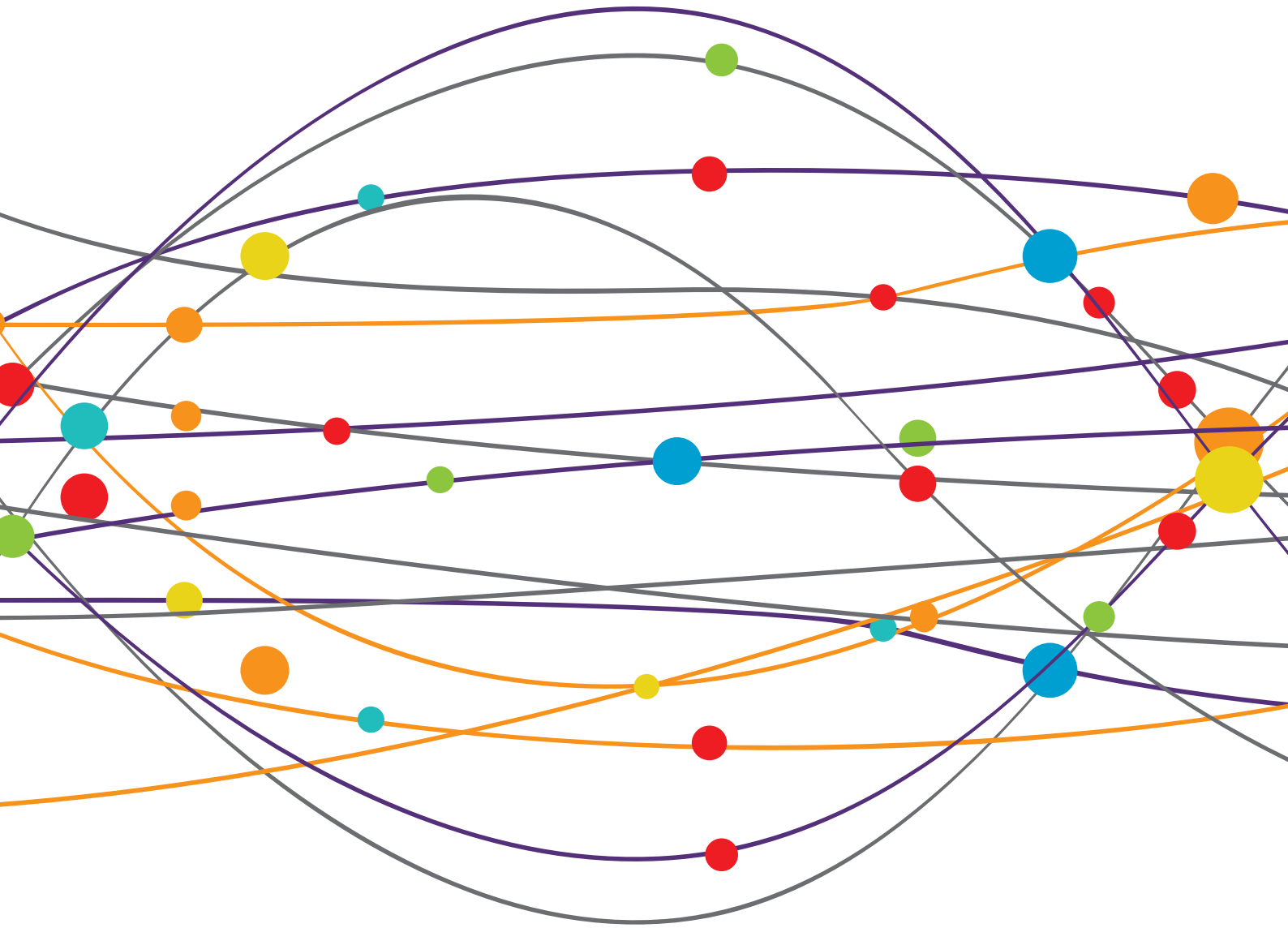

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CONCUSSION

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Concussion Subtype Identification With the Rivermead Post-concussion Symptoms Questionnaire

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Classifying concussion in key subtypes according to presenting symptomatology at an early post-injury stage is an emerging approach that may allow prediction of clinical trajectories and delivery of targeted treatments. The Rivermead Post-concussion Symptoms Questionnaire (RPQ) is a simple, freely available, and widely used tool for assessment of the presence and severity of various post-concussion symptoms. We aimed to probe the prevalence among athletes of symptom classes associated with identified concussion phenotypes using the RPQ at baseline and acutely after a concussion. Participants of organized sports aged 12–30 years were baseline-assessed with the expectation that some would experience a concussion during the study period. Concussed athletes were re-assessed within 2 weeks of their injuries. The RPQ was supplemented with three specific questions and reworded for baseline assessment. A binomial test was used to contrast the prevalence of an attribute in the concussed cohort against the probability established by the baseline observation. Three thousand and eighty-eight athletes were baseline-assessed and eighty-nine were re-assessed post-concussion. All concussed athletes endorsed having some elevated symptoms in the RPQ, and such endorsements were more prevalent than those among normal athletes. Moderate-to-severe post-concussion symptoms of specific classes tended to be endorsed with few additional symptoms of other classes of similar intensities. Elevated symptoms detected with the RPQ within as short as 2 weeks after a concussion may help delineate patients' clinical subtypes and guide their treatment. Further refinement of symptom questionnaires and use of objective measures will be needed to properly populate the concussion subtype classification.

Keywords: mild traumatic brain injury (mTBI), epidemiology, cognitive, fatigue, vestibular, migraine, sleep

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INTRODUCTION

Concussions are heterogeneous—there is a broad consensus among experts that a one-size-fits-all approach to post-concussion management is ineffective (1–4). Subtype classification is an emerging effort within the concussion-related field. Based on clinical and anecdotal evidence, it has been suggested that the six clinical phenotypes of concussion described below may be profiled according to symptoms observed within about 1 week post-concussion (5, 6). Although this particular

approach does not currently directly address the root-cause pathophysiology of symptoms as attempted in other approaches (7–9), such early-stage clinical profiling of concussion permits a targeted treatment by prioritizing management of most problematic issues while maximizing the initial impact on the patient's symptoms and impairment (6).

The six suggested profiles with partially overlapping symptoms are described as: (1) *cognitive-fatigue*, with symptoms of fatigue, decreased energy, non-specific headache, sleep disruption, or difficulty concentrating; (2) *vestibular*, with symptoms of dizziness, fogginess, nausea, feeling of being detached, or overstimulation in complex environments; (3) *oculomotor*, with symptoms of fatigue, distractibility, difficulties with visually based classes, pressure behind the eyes, or blurred or double vision; (4) *anxiety/mood*, with symptoms of anxiety, hypervigilance, feeling of being overwhelmed, sadness, or hopelessness; (5) *post-traumatic migraine*, with symptoms of headache with a pulsating quality associated with nausea, photosensitivity, or phonosensitivity; and (6) *cervical*, with symptoms of headache, neck pain, or numbness/tingling of the extremities.

We note that, among the six profiles, cervical attributes refer to extracranial origins and could be better conceptualized as an associated condition that affects recovery rather than a subtype of concussion. We also recognize that sleep disturbance is a condition that has emerged in the literature as affecting recovery from concussion (4, 10–12) and that it is considered to be a modifier in the recent update to this clinical profiling approach (6). We will thus distinguish sleep disturbance as a symptom class associated with concussion although not as characterizing a subtype of concussion.

The Rivermead Post-concussion Symptoms Questionnaire (RPQ) is a simple, freely available, and widely used tool for severity assessment of various post-concussion symptoms (13). The questionnaire was first of its kind when published in 1995, and has since been cited in hundreds of academic publications with an increasing trend. In its original form, the RPQ is based on a subjective five-scale rating of 0–4, relative to the premorbid levels, of 16 most commonly reported problems in the literature, with 0 indicating not experienced, and 4 severe. Respondents are also asked, if other difficulties exist, to rate on these additional symptoms. Here, we utilized slightly modified versions of the RPQ in a sports concussion study, in which more than 3,000 athletes were baseline-assessed and, in case of a subsequent concussion, re-assessed within 2 weeks post-injury.

Symptoms associated with concussion are not unique to concussion and can independently appear in normal settings (14, 15). The goal of this report is to probe the prevalence of symptom classes matching the current concussion profiling approach in a naturalistic athletic setting and during the acute post-concussion period using the RPQ. In such exploration, we also evaluate the ability of the RPQ to distinguish concussion clinical profiles.

METHODS

Data were collected as part of a larger research project on concussion with a particular focus on a normative characterization of eye movement performance in a variety of military and civilian groups. Collected data included demographic information, eye movements during visual tracking tasks, neurocognitive parameters, symptoms, and a history of previous head injuries. Some of the results from this project, not overlapping with the present report, have been published elsewhere (16, 17).

Subjects

The subject enrollment and assessment protocols were approved by the institutional review boards of Weill Cornell Medical College (WCMC) in New York, and Stanford University in California. In collaboration with school, university, and community athletic organizations in respective local areas, middle-school through adult athletes were enrolled and baseline-assessed. For equity's purpose, athletes were enrolled independently of the level of contact involved in their participating sports. The inclusion criteria were participation in organized competitive athletic activity, being of age 12–30 years, normal or corrected-to-normal vision, and for athletes over the age 18, a high school diploma or equivalent, or expected timely high school graduation. Prior to data collection, written informed consent by adult subjects, or legal guardians of minor subjects with the minors' assent, was obtained in accordance with the Declaration of Helsinki.

During the baseline consent process, the athletes were given the option of allowing their athletic director, trainer, coach, or school nurse to contact the researchers if they sustained a concussion, as well as the research staff to contact the athletic staff to check on injury status. Acute post-concussion enrollment was based on inclusion criteria consisting of having experienced a concussion within 2 weeks that resulted in loss of consciousness, post-traumatic amnesia, dizziness, nausea, headaches, balance problems, blurred or double vision, or daze, and confusion, and on an exclusion criterion of intoxication at the time of injury. A diagnosis by a physician was not required. For the purpose of this report, we will identify athletes who were under 18 years of age when they were baseline-assessed as pediatric subjects.

Data were collected inside a parked recreational vehicle outfitted as a mobile testing site, or at the Citigroup Biomedical Imaging Center at WCMC. Data collection spanned from September of 2012 through September of 2016.

Modified RPQ

The original form of the RPQ instructs the respondent to rate problems over the last 24 h relative to the premorbid levels (13). To assess the presence and severity of symptoms typically associated with concussion but instead in an everyday athletic setting, the instruction for baseline assessments was rephrased with the following sentence—"We would like to know if you have experienced any of the symptoms given below more than usual today or in the past week." The original instruction was retained for post-concussion assessments. Further, for both implementations the "Additional Symptoms" section was replaced with three specific items: balance problems; feeling mentally "foggy;" and drowsiness. To sum up, there were a total of 19 items in the modified RPQ, with which we associated the

following five subtypes of post-concussion clinical trajectories to determine the presence and severity of these symptom classes.

Cognitive-Fatigue

We identified the four items from the RPQ, "fatigue, tiring more easily," "forgetfulness, poor memory," "poor concentration," and "taking longer to think," as being associated with the cognitive-fatigue profile. The presence of a severe, at-least-moderate, or at-least-mild symptom was identified if any of the four items was rated as 4, $\geq$3, or $>$0, respectively.

Vestibular

We identified the two items, "feeling of dizziness" and "balance problems," as being associated with the vestibular profile. The presence of a severe, at-least-moderate, or at-least-mild symptom was identified if either of the two items was rated as 4, $\geq$3, or $>$0, respectively.

Oculomotor

We identified the two items, "blurred vision" and "double vision," as being associated with the oculomotor profile. The presence of a severe, at-least-moderate, or at-least-mild symptom was identified if either of the two items was rated as 4, $\geq$3, or $>$0, respectively.

Anxiety/Mood

We identified the four items, "being irritable, easily angered," "feeling depressed or tearful," "feeling frustrated or impatient," and "restlessness," as being associated with the anxiety/mood profile. The presence of a severe, at-least-moderate, or at-least-mild symptom was identified if any of the four items was rated as 4, $\geq$3, or $>$0, respectively.

Migraine

We identified the four items, "headaches," "noise sensitivity, easily upset by loud noise," "nausea and/or vomiting," and "light sensitivity, easily upset by bright light," as being associated with the migraine profile. The presence of a severe, at-least-moderate, or at-least-mild-symptom was identified if the headache item was rated as 4, $\geq$3, or $>$0 *and* in addition at least one of the other three items was rated as 4, $\geq$3, or $>$0, respectively.

No item from the RPQ was associated with the *cervical* profile/condition. In addition to the above five profiles, the presence of a severe, at-least-moderate, or at-least-mild symptom associated with *sleep disturbance* was identified if either "sleep disturbance" or "drowsiness" was rated as 4, $\geq$3, or $>$0, respectively. Thereby we specified a total of six symptom classes (five subtypes and one subtype-associated condition).

Statistical Analysis

A binomial test was used to contrast the prevalence of a specified attribute in the concussed cohort against the probability established by the baseline observation. A significant deviation from this probability was detected at the alpha level of 0.05. In addition, as described in the Results section below, being in the pediatric group and a history of a previous head injury (of unspecified severity) were both associated with a higher likelihood of getting concussed. To examine the association between these two attributes, we determined the odds ratio and its confidence interval.

RESULTS

Baseline assessments were conducted in a total of 3,091 qualified athletes. Three subjects had incomplete RPQ records. Of the remaining 3,088 subjects, 1,178 (38.2%) were female and 1,910 (61.9%) were male. There were 857 pediatric subjects (mean age: 15.3 years, SD: 1.4), of whom 317 (37.0%) were female and 540 were male (63.0%). A previous head injury was reported by 180 subjects of the pediatric group (21.0%) and 722 subjects of the adult group (32.4%), totaling 902 (29.2%).

After a baseline assessment, 89 subjects experienced a concussion and were re-assessed within 2 weeks of the injury, on average at 5.8 days post-injury (SD: 3.5). Consistent with the mostly random nature of concussive accidents, the distribution of timing of injuries since the baseline assessment closely followed a negative exponential pattern, with a time constant of $\sim$3 months (mean: 3.1, SD: 2.9, range: 0–15). All 89 concussed subjects had valid RPQ records at both baseline and post-concussion time points. Of the 89, 38 subjects were in the pediatric group, within which 26 were male (68.4%) and 12 were female (31.6%), and 51 subjects were in the adult group, within which 22 were male (43.1%) and 29 were female (56.9%). Four of the concussed subjects in the pediatric group were over 18 years of age at the time of post-concussion assessment, but only by at most 5 months. A previous head injury was reported by 12 of the 38 concussed pediatric subjects (31.6%) and 27 of the 51 concussed adult subjects (52.9%), totaling 39 (29.2%).

The presence and severity of six classes of symptoms identified through combinations of RPQ items are summarized in **Figure 1**. In the plots, the data are expressed cumulatively; thus, the percentages associated with the labels *+Mod* and *+Mild* indicate those of at-least-moderate and at-least-mild symptom presentations, respectively. In all symptom classes, problems that were present at an at-least-moderate level were more prevalent after a concussion than at baseline (**Table 1**). One or more of the four RPQ items related to the cognitive-fatigue profile were most prevalently endorsed by the concussed subjects, with as much as 47.2% reporting at-least-moderate symptoms (57.9% in the pediatric subgroup and 39.2% in the adult subgroup). The two RPQ items related to the oculomotor profile were least commonly endorsed by the concussed subjects. We also noted that the prevalences of vestibular- and migraine-related symptoms at the at-least-mild level were more than twice elevated after a concussion.

The identified post-concussion prevalence of at-least-moderate oculomotor symptoms was only marginally different from the probability derived from the baseline assessment (**Table 1**), and in the pediatric subgroup specifically, the prevalence at 2.6% was not statistically greater than the corresponding probability. In the pediatric subgroup, the identified prevalence of at-least-moderate anxiety/mood symptoms at 18.4% was also not statistically greater than the probability derived from the corresponding baseline assessment

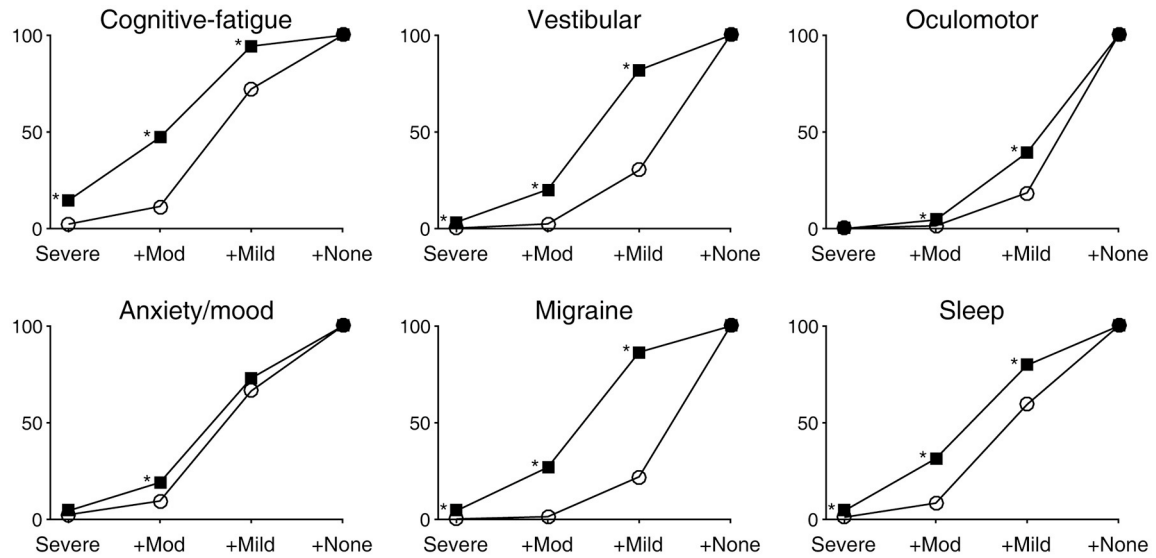

FIGURE 1 | Prevalences of six classes of symptoms expressed as cumulative distributions. Open circles and filled squares indicate baseline and post-concussion findings, respectively. An asterisk indicates a statistically significant ($p < 0.05$) deviation of a post-concussion prevalence from the baseline-derived probability as determined by a binomial test. Also see **Table 1**.

TABLE 1 | Prevalence of moderate-to-severe symptom identification.

	Prevalence (%)		*p**
	Concussed (*N* = 89)	**Baseline (*N* = 3088)**	
Cognitive-fatigue	47.2	11.5	<0.001
Vestibular	20.2	2.4	<0.001
Oculomotor	4.5	1.5	0.042
Anxiety/mood	19.1	9.5	0.006
Migraine	27.0	1.4	<0.001
Sleep	31.5	8.4	<0.001

**as determined by a binomial test.*

at 12.8%, which veered from the 9.5% overall and 8.2% adult probabilities. No substantial alteration was found between pediatric and adult subgroups in the prevalences of vestibular-, migraine-, or sleep-related symptoms.

We noted some sex-based symptom prevalence differences. All four subjects who reported severe migraine-related symptoms after a concussion were female (prevalence of 9.8 in female vs. 0% in male). Three of these subjects reported mild migraine-related symptoms at baseline while one was initially free of such symptoms. Similarly, all three subjects who reported severe vestibular-related symptoms after a concussion were female (prevalence of 7.3 in female vs. 0% in male). All these subjects reported mild vestibular-related symptoms at baseline. There was also a higher prevalence of at-least-mild sleep-related symptoms at baseline among female subjects (67.8 vs. 54.3% in male), which was further elevated after a concussion (92.7 vs. 68.8% in male). The trends were similar between pediatric and adult subgroups.

We next examined whether the prevalences of particular individual baseline attributes were different in those who experienced a concussion during the data collection period compared to the overall prevalences (**Table 2**). The sexes were not apportioned differently among the concussed subjects (53.9 male in the concussed vs. 61.9% male overall), but ages and previous histories of head injury were. In terms of baseline symptomatology, concussed subjects were more likely than typical to have endorsed cognitive-fatigue-, vestibular-, migraine-, and sleep-related items of the RPQ at varying levels.

As stated above, ages and previous histories of head injury were apportioned differently among the concussed subjects. We examined the possible association between age (pediatric vs. adult) and a previous head injury within the concussed group. The odds ratio was 0.41 with a confidence interval between 0.17 and 0.99, indicating that a concussed pediatric subject was less likely to have had a previous head injury than an adult counterpart.

To evaluate the ability of the RPQ to delineate among the five profiles presumed to predict post-concussion trajectories, we examined similarities and differences with which the subjects endorsed RPQ items that we associated with these profiles. We constructed 31 logical relationships regarding the presence or absence of endorsement of each symptom class (excluding the absence of all) and sorted the subjects correspondingly (**Figures 2–4**). Given the size of compartmentalization, we did not subdivide the 89 concussed subjects by their baseline characteristics in this analysis. A total of 18 subjects reported severe post-concussion symptoms in the RPQ, of whom 13 (72.2%) reported those associated with the cognitive-fatigue profile, and 11 (61.1%) reported only such symptoms (**Figure 2**). The subjects reported few overlaps in severe symptoms, with all but one of the 18 subjects (94.4%) reporting at most two classes of symptoms with a severe rating. A total of 58 subjects reported at-leased-moderate post-concussion symptoms, of whom 42

TABLE 2 | Baseline characteristics of those who became concussed during the study period compared against those of the entire sample.

	Prevalence (%)		*p**
	Concussed (*N* = 89)	**Overall (*N* = 3088)**	
Pediatric age	42.7	27.8	0.003
Previous head injury	43.8	29.2	0.003
COGNITIVE-FATIGUE			
At least moderate	22.5	11.5	0.004
At least mild	83.1	72.2	0.024
VESTIBULAR			
At least mild	44.9	30.2	0.004
MIGRAINE			
At least moderate	4.5	1.4	0.039
At least mild	37.1	22.1	0.001
SLEEP			
Severe	4.5	1.1	0.019
At least moderate	15.7	8.4	0.020

**as determined by a binomial test.*

(72.4%) reported those associated with the cognitive-fatigue profile (**Figure 3**). At this severity level also, the subjects reported relatively few overlaps in symptoms in general, with 47 of 58 subjects (81.0%) reporting at most two classes of symptoms with an at-least-moderate rating. All 89 concussed subjects reported at-least-mild post-concussion symptoms, of whom 59 (66.3%) reported symptoms with overlapping associations with at least four post-concussion profiles (**Figure 4**). Only 12 subjects (13.5%) reported symptoms with overlapping associations with at most two post-concussion profiles. Therefore, the RPQ may best identify patients' post-concussion profiles with moderate-to-severe increases in symptom severities.

DISCUSSION

The sample, represented by over 3,000 subjects at baseline, was a prospective cohort from a natural setting of competitive sports. Only 2.9% of the baseline-assessed athletes subsequently became concussed during the study period, likely reflecting the fact that the athletes were enrolled independently of the level of contact involved in their participating sports.

All concussed athletes in this study endorsed having some elevated symptoms in the RPQ. Although symptoms covered by the RPQ can appear in a normal setting as we also found, endorsements of these symptoms were measurably higher acutely following concussion. Among the six post-concussion symptom classes described by clinical and anecdotal evidence (5, 6, 11) and identifiable with the current implementation of the RPQ, cognitive-fatigue-related symptoms were most prevalently endorsed by concussed athletes at all levels of severity. Post-concussion increases in the prevalences of mild vestibular- and migraine-related symptoms were likewise substantial.

Symptoms related to the oculomotor profile were least prevalently endorsed through the RPQ by concussed athletes.

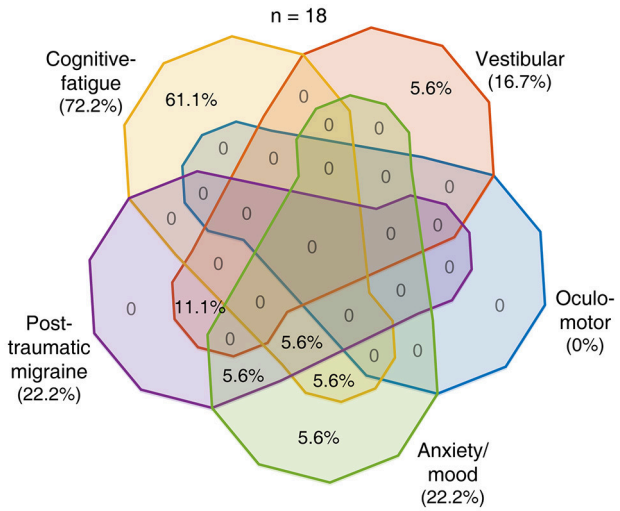

FIGURE 2 | Overlaps in post-concussion profile characteristics identified with severe symptoms**.** Eighteen of 89 concussed subjects reported having a severe post-concussion symptom. The distribution of these 18 subjects among the various combinations of presences and absences of endorsement of the five symptom classes is shown as percentages in the Venn diagram with areas of overlap among the five pear-shaped regions.

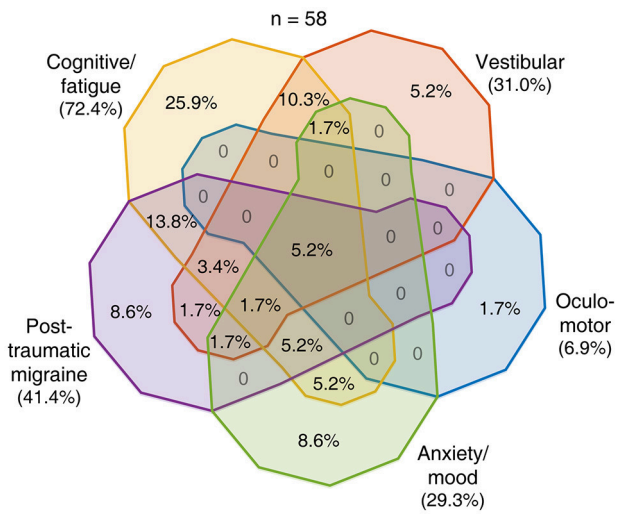

FIGURE 3 | Overlaps in post-concussion profile characteristics identified with at-least-moderate symptoms. Fifty-seven of 89 concussed subjects reported having an at-least moderate post-concussion symptom. The distribution of these 57 subjects among the various combinations of presences and absences of endorsement of the five symptom classes is shown as percentages in the Venn diagram.

This finding diverges from those derived from objective measures (18–21). It is possible that the low endorsement rate reflected a low representation of this profile in the study cohort. However, since the oculomotor profile were identified with only "blurred vision" and "double vision," the low endorsement rate may be better explained by the limitation of the scope of the questionnaire. Therefore, the RPQ may be supplemented with

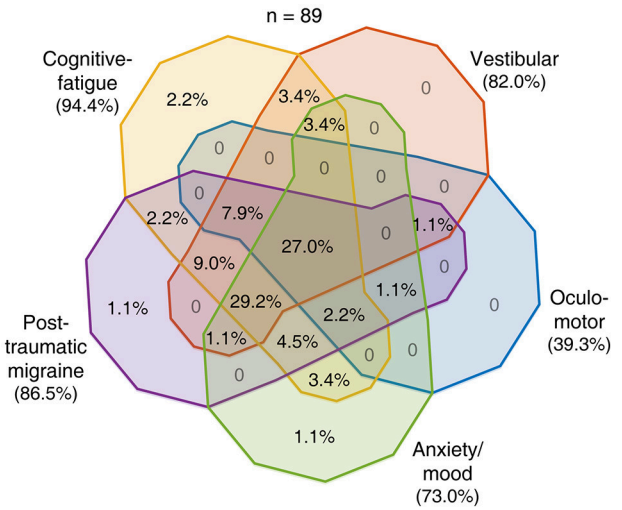

FIGURE 4 | Overlaps in post-concussion profile characteristics identified with at-least mild symptoms**.** All 89 concussed subjects reported having an at-least mild post-concussion symptom. The distribution of these 89 subjects among the various combinations of presences and absences of endorsement of the five symptom classes is shown as percentages in the Venn diagram.

additional items such as difficulty in reading/near work and in judging distances. Similarly, the RPQ contained no item related to the cervical profile, and it may be beneficial to supplement it with additional items such as neck pain and neck/upper extremity weakness.

Adult and pediatric athletes demonstrated generally similar symptomatology both at baseline and post-concussion. The pediatric athletes had comparatively elevated anxiety/mood problems at baseline, but such symptoms at a normal or subclinical level are common in adolescence (22, 23). Our pediatric athletes had a higher rate of concussion than adult athletes. Although age may play a role in concussion risk, there are contrasting findings in the literature (24). We also found some sex-based differences in symptomatology. Since our sample size and scope of collected data do not warrant a further analysis, the basis for the tendency we found is unclear. Age and a history of head injury were associated with a subsequent concussion. These two factors were related such that concussed adult athletes were more likely to have had a previous head injury than concussed pediatric athletes. This relation may be explained by a cumulative effect of concussion—adults are more likely to have had a previous head injury than youths, and a previous head injury can increase the chance of another concussion (24, 25).

Despite being a subjective measure and possibly limited in scope, the RPQ, in addition to its original purpose, may have the ability to delineate concussion profiles. In particular, among the five prescribed subtypes, there was a "sweet spot" in at-least-moderate symptom reports, which were endorsed by the majority of the concussed athletes and at the same time were observed within individuals with relatively few overlaps among different symptom classes. On the other hand, most concussed athletes reported at-least-mild problems of many different symptom classes. Therefore, moderate-to-severe increases in symptom severities as detected by the RPQ, when combined with objective measures, may well project patients' individual clinical trajectories and guide appropriate treatment approaches that maximize the treatment impact.

We also found that, in characterizing baseline RPQ responses, pre-existing symptoms that increase the chance of concussion may be identified, namely cognitive-fatigue-, vestibular-, migraine-, and sleep-related problems. Thus, in addition to a history of previous head injury, a well-known risk factor for concussion (24, 25), consideration of these symptoms may be integrated into concussion prevention strategies, such as promoting risk awareness and guiding the choice of activities. The female sex is also a recognized risk factor for sports concussion when comparisons between the sexes are made within specialties (24, 26, 27). However, this study did not identify a sex-based difference in concussion risk, possibly because we analyzed all sports collectively, the approach imposed by the protocol for subject selection.

CONCLUSION

Classifying concussion in key subtypes according to presenting symptomatology at an early post-injury stage may allow prediction of clinical trajectories and delivery of targeted treatments. We characterized with a modified RPQ prevalences of concussion-related symptoms in baseline naturalistic and acute post-concussion contexts within an athletic setting. The sample size for those who became concussed was reduced to <3% of the baseline. Although the RPQ is composed of simple self-report measures, symptom prevalences identified with it still rendered usefulness. That is, elevated symptoms detected with the RPQ within a week or so after a concussion may help identify patients' predicted clinical trajectories and guide care prioritization. The RPQ was still found to be limited in scope for such use, and thus supplementary question items may improve its ability. Finally, any interpretation must take place within the context of multiple measures, both subjective, and objective, applied to the evaluation of patients with concussion.

AUTHOR CONTRIBUTIONS

JM and JG designed experiments and oversaw data collection. JM conducted the statistical analyses and drafted the manuscript. All authors contributed to the interpretation of data and to revising the work.

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frontiers in Neurology

BRIEF RESEARCH REPORT
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Baseline Neurocognitive Performance and Symptoms in Those With Attention Deficit Hyperactivity Disorders and History of Concussion With Previous Loss of Consciousness

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Previous consensus statements on sports concussion have highlighted the importance of Attention Deficit Hyperactivity Disorder (ADHD) and loss of consciousness (LOC) as risk factors related to concussion management. The present study investigated how self-reported history of either ADHD diagnosis or history of previous concussion resulting in LOC influence baseline neurocognitive performance and self-reported symptoms. This analysis was performed retrospectively on data collected primarily from student-athletes, both Division 1 and club sports athletes. The dataset ($n = 1460$) is comprised of college students (age $= 19.1 \pm 1.4$ years). Significant differences were found for composite scores on the ImPACT for both history of concussion ($p = 0.016$) and ADHD ($p = 0.014$). For concussion history, those with a previous concussion, non-LOC, performed better on the visual motor speed ($p = 0.004$). Those with diagnosis of ADHD performed worse on verbal memory ($p = 0.001$) and visual motor speed ($p = 0.033$). For total symptoms, concussion history ($p < 0.001$) and ADHD ($p = 0.001$) had an influence on total symptoms. Those with ADHD reported more symptoms for concussion history; those with previous LOC concussion reported more symptoms than those with non-LOC concussion ($p = 0.003$) and no history ($p < 0.001$). These results highlight the importance of baseline measures of neurocognitive function and symptoms in concussion management in order to account for pre-existing conditions such as ADHD and LOC from previous concussion that could influence these measures.

Keywords: mild traumatic brain injury, concussion management, pre-existing conditions, ADHD, LOC

INTRODUCTION

In recent years, concussion research has quickly made its way to the forefront of the medias' attention and has been deemed a public health issue (1) and a silent, global epidemic (2). Each year it is estimated that there are between 1.6 and 3.8 million sports-related concussions (3) and they account for ∼5–9% of all sports-related injuries (4, 5). The use of neurocognitive measures has previously been deemed a cornerstone of concussion management (6, 7). In a recent consensus statement, the implementation of baseline measures of neurocognitive performance was believed to be helpful and useful for concussion management, but not mandatory (8).

Among the factors that have been hypothesized to influence recovery from concussion and may warrant additional research include diagnosis of Attention Deficit Hyperactivity Disorder (ADHD) and having a previous concussion that resulted in loss of consciousness (LOC) (6, 7). Recent studies have found that previous diagnosis of ADHD influences neurocognitive performance (9–13) and total symptom scores at baseline assessment (9–12). The influence of concussion history with LOC on recovery to subsequent concussions has been examined less than ADHD despite concussions at one time being graded with LOC being a main component of concussion diagnosis (14). Currently, however, it is estimated that LOC only occurs in 5–10% of sports-related concussions (7, 15, 16). Though concussions are no longer classified solely on the presence of LOC, evidence indicates that LOC is a potential modifier of concussion recovery (7, 17, 18). However, only one known study has examined whether LOC influences performance on neurocognitive measures (19). That study examined neurocognitive performance following a concussion and did not find any significant differences between groups (concussions with LOC and without LOC). This previous research suggests that those who are involved in concussion management should take into consideration factors such as ADHD and previous history of LOC when treating patients (7, 8, 20).

The current study examined the influence of self-reported diagnosis of ADHD and history of LOC at the time of concussion on neurocognitive performance and reporting of symptoms at a baseline pre-season assessment.

MATERIALS AND METHODS

Participants

Data was initially collected from 1514 college students, but 54 participants were taken out because of having an invalid baseline. The final sample include 1460 college students (665 females, 795 males; mean age = 19.1 ± 1.4 years). Most were student-athletes (Division I varsity = 565, club, $n = 821$) as well as dance majors ($n = 33$) who were tested as a result of university's concussion management protocol, plus an additional 41 students who were tested as controls for other projects. All participants were 18 years of age or older. The study received Institutional Review Board approval before testing began and all participants completed informed consent forms to participate in this study.

Measures

ImPACT (version 2.1) is a commonly used tool for concussion management which assesses neurocognitive performance and symptoms (21).

The self-reported demographic data was used to determine history of ADHD diagnosis, number of total concussions, and number of concussions with LOC. ImPACT contains six modules to measure neurocognitive performance; these modules include: word discrimination (measures attention and verbal memory), design memory (measures attention and visual memory), X's and O's (measures working memory, visual memory and processing speed), symbol matching (measures visual processing speed, learning and memory), color match (measures choice reaction time, impulse control, and inhibition), and three letter memory (measures working memory and visual motor response speed). From these six modules, ImPACT calculates four composite scores: verbal memory, visual memory, visual motor speed, and reaction time. Total symptom score was based on responses to the 22 symptom list which uses a 7-point likert scale (ranging from 0 to 6 on severity); therefore, the symptom score that is reported is based on number and severity of symptoms Previous research has suggested that the scores from the ImPACT may have somewhat poor reliability (21), but most studies have demonstrated good test-retest reliability for this measure (13, 15).

Procedures

The study was performed with a retrospective analysis of baseline pre-season ImPACT tests from spring 2011 until spring 2016. The Division I student-athletes, club athletes, and dancers all completed the ImPACT assessment as part of the university's concussion management protocols, which are required for athletes to complete prior to participation in their respective athletic endeavor. The "other" group were college students that participated in previous research as control subjects and had completed the same baseline ImPACT assessment. For most of the Division I student-athletes, the ImPACT was administered in a quiet computer laboratory with no more than two student-athletes at a time. For the rest of the sample the ImPACT was administered in a larger computer lab and often in a group setting. Testing was administered at various times during the day depending on availability of the participants. Generally, the ImPACT was completed in 20–30 min.

Statistical Analysis

All data were analyzed using SPSS 23 (IBM) with an alpha level of < 0.05. A Multivariate Analyses of Variance (MANOVA) was conducted with self-reported previous diagnosis of ADHD and history of a concussion (no history, history with LOC, history without LOC) as independent variables, with dependent variables derived from the ImPACT composite scores (e.g., verbal memory, visual memory, visual motor speed, and reaction time). Similarly, an ANOVA was conducted with self-reported previous diagnosis of ADHD and a history of concussion (no history, history with LOC, history without LOC) as independent variables, and total symptoms score derived from the ImPACT assessment as the dependent variable. LSD *post-hoc* tests were performed to determine where significant differences occurred.

RESULTS

Of the 1,460 participants, 168 (11.5%) self-reported a previous diagnosis of ADHD and 408 (27.9%) of the participants self-reported having a previous concussion. For the ADHD group, 48.2% (81 of 168) self-reported taking at least one medication typically prescribed for treatment of ADHD. Of the 408 participants with a concussion, 112 (27.4% of those with concussion and 7.7% of total sample) reported having history of concussion with LOC.

The MANOVA for the ImPACT composite scores found a statistically significant main effect for those diagnosed with ADHD [$F_{(4, 1451)} = 3.16$, *Wilks'* $\lambda = 0.99$, $p = 0.014$] as well as for concussion history [$F_{(8, 2902)} = 2.34$, *Wilks'* $\lambda = 0.99$, $p = 0.016$], but not a significant interaction for concussion history and ADHD (see **Table 1**). For those with a previous diagnosis of ADHD, there was a significant difference for performance on verbal memory [$F_{(1, 1454)} = 10.41$, $p = 0.001$] and visual motor speed [$F_{(1, 1454)} = 4.54$, $p = 0.033$] as those who reported a previous diagnosis of ADHD performed worse on those measures.

For concussion history, univariate analyses found significant differences for and visual motor speed [$F_{(2, 1454)} = 5.52$, $p = 0.004$], but not for the other scales. Those with history of non-LOC concussion performed better on visual motor speed than both those who reported history of concussion with LOC ($p = 0.023$) and those with no history of concussion ($p = 0.001$). While not significant, there were some trends for verbal memory [$F_{(2, 1454)} = 2.84$, $p = 0.059$] and reaction time [$F_{(2, 1454)} = 2.39$, $p = 0.092$] related to concussion history.

An ANOVA for total symptoms score revealed significant main effects for ADHD [$F_{(1, 1454)} = 11.72$, $p = 0.001$] and LOC [$F_{(2, 1454)} = 8.69$, $p < 0.001$], but not a significant interaction between the two (see **Table 1**). Those with a previous diagnosis of ADHD reported more symptoms than those without ADHD. Total symptom scores were higher for those with a history of concussion resulting in LOC than those with a previous concussion absent of LOC ($p = 0.003$) as well as those with no history of any concussion ($p < 0.001$).

DISCUSSION

Previous research has explored the extent to which ADHD influences neurocognitive performance and the self-reporting of symptoms at baseline (9–12), but there has been relatively little investigation into how history of a concussion with LOC influences these measures (19). This research study sought to further the research that has been previously performed to better understand how history of ADHD and LOC may influence baseline measures of neurocognitive performance and self-reported symptoms when student-athletes are assessed prior their participation in collegiate sports. This enables the examination of the potential residual effects of LOC and ADHD history on neurocognitive and symptom measures at baseline, which may ultimately influence concussion management strategies.

Our sample had 11.5% of our population report a previous diagnosis with ADHD, which is higher than the 4.2–8.1% reported in a recent review of literature on ADHD in athletes (20). While our percentage is higher than that with athletes, it is more consistent with previous research which shows the percentage to be around 11% in adolescents (22, 23). This could be due to the inclusion of non-varsity athletes (e.g., club athletes and dancers). In agreement with previous research, those with ADHD also reported significantly higher symptoms at baseline than participants without ADHD (9–11). The symptoms for ADHD closely resemble those that are commonly associated acutely with concussions, which may make the two difficult to distinguish. One possible explanation for this finding is a self-report bias as the population of participants with ADHD may be more accustomed to neuropsychological exams and reporting symptoms in their interactions with medical professionals. The neurocognitive test results showed that significant differences between those with ADHD and those without ADHD were that those without ADHD had significantly higher verbal memory and visual motor scores. This finding is congruent with other previous research (9–13, 24). However, it is important to note that these findings are based on self-reported diagnosis of ADHD and was not verified by a physician and could have possibly influenced our results.

Results from the neurocognitive assessments demonstrate that those reporting a history of concussions without LOC had significantly higher visual motor scores than both the LOC and no history of concussion group. However, concussion symptoms were significantly higher for those who had been concussed with LOC compared to the other two groups. These findings for the influence of LOC on neurocognitive performance is opposite of previous work which

TABLE 1 | Means (±SE) for ImPACT composite scores and total symptoms for concussion history and ADHD.

Group	Verbal memory	Visual memory	Visual motor speed	Reaction time	Total symptoms
No concussion	84.5 ± 0.5	73.6 ± 0.7	40.3 ± 0.3[a]	0.583 ± 0.004	4.7 ± 0.4[a]
Concussed without LOC	86.1 ± 0.8	76.0 ± 1.1	42.3 ± 0.5[a,b]	0.568 ± 0.006	5.7 ± 0.6[b]
Concussed with LOC	82.2 ± 1.5	75.3 ± 1.9	40.0 ± 0.9[b]	0.589 ± 0.011	9.4 ± 1.1[a,b]
No ADHD	86.2 ± 0.4[a]	75.8 ± 0.5	41.6 ± 0.3[a]	0.574 ± 0.003	5.1 ± 0.3[a]
ADHD	82.4 ± 1.1[a]	74.2 ± 1.4	40.1 ± 0.7[a]	0.587 ± 0.008	8.1 ± 0.8[a]

Those with similar letters designate statistically significant differences between groups, $p < 0.05$.

has found history of LOC to not influence neuropsychological performance (19, 25).

These results suggest that LOC at time of concussion may lead to residual deficits and symptoms, and, further, that chronic effects from previous concussions with LOC should be investigated further. This is consistent with current recommendations that LOC should be considered in concussion management (7, 8). One future direction could be investigating which, if any, specific brain structures and/or networks are involved in LOC to help elucidate the influence of LOC on neurocognitive performance and symptom reporting (26).

This research also raises a question about the combination of ADHD and a history of concussions that resulted in LOC. ADHD is known for having an influence on axonal integrity, white matter and function of certain areas of the brain (27–29), so it is possible that people with ADHD are more likely to experience concussions (30) or those with history of concussions to also report symptoms of ADHD (31). It might also be that these alterations in function and structure of the brain could predispose them to pathophysiology associated with LOC (26).

In conclusion, this research suggests that ADHD and LOC should be considered in concussion management as both of these pathologies may influence measures from baseline concussion testing, symptoms and neurocognitive performance, that are commonly used in concussion management.

ETHICS STATEMENT

Elon University IRB 13-017; 17-008; 17-043. This study was carried out in accordance with the recommendations of name of guidelines, name of committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the name of committee.

AUTHOR CONTRIBUTIONS

SK, EH, and CK were involved in development of research idea. SK, MS, EH, and CK were involved in writing of the manuscript. All authors reviewed the manuscript and assisted in data collection.

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Neuroendocrine Whiplash: Slamming the Breaks on Anabolic-Androgenic Steroids Following Repetitive Mild Traumatic Brain Injury in Rats May Worsen Outcomes

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Tabor J, Collins R, Debert CT, Shultz SR and Mychasiuk R (2019) Neuroendocrine Whiplash: Slamming the Breaks on Anabolic-Androgenic Steroids Following Repetitive Mild Traumatic Brain Injury in Rats May Worsen Outcomes. Front. Neurol. 10:481. doi: 10.3389/fneur.2019.00481

Sport-related concussion is an increasingly common injury among adolescents, with repetitive mild traumatic brain injuries (RmTBI) being a significant risk factor for long-term neurobiological and psychological consequences. It is not uncommon for younger professional athletes to consume anabolic-androgenic steroids (AAS) in an attempt to enhance their performance, subjecting their hormonally sensitive brains to potential impairment during neurodevelopment. Furthermore, RmTBI produces acute neuroendocrine dysfunction, specifically in the anterior pituitary, disrupting the hypothalamic-pituitary adrenal axis, lowering cortisol secretion that is needed to appropriately respond to injury. Some AAS users exhibit worse symptoms post-RmTBI if they quit their steroid regime. We sought to examine the pathophysiological outcomes associated with the abrupt cessation of the commonly abused AAS, Metandienone (Met) on RmTBI outcomes in rats. Prior to injury, adolescent male rats received either Met or placebo, and exercise. Rats were then administered RmTBIs or sham injuries, followed by steroid and exercise cessation (SEC) or continued treatment. A behavioral battery was conducted to measure outcomes consistent with clinical representations of post-concussion syndrome and chronic AAS exposure, followed by analysis of serum hormone levels, and qRT-PCR for mRNA expression and telomere length. RmTBI increased loss of consciousness and anxiety-like behavior, while also impairing balance and short-term working memory. SEC induced hyperactivity while Met treatment alone increased depressive-like behavior. There were cumulative effects whereby RmTBI and SEC exacerbated anxiety and short-term memory outcomes. mRNA expression in the prefrontal cortex, amygdala, hippocampus, and pituitary were modified in response to Met and SEC. Analysis of telomere length revealed the negative impact of SEC while Met and SEC produced changes in serum levels of testosterone and corticosterone.

We identified robust changes in mRNA to serotonergic circuitry, neuroinflammation, and an enhanced stress response. Interestingly, Met treatment promoted glucocorticoid secretion after injury, suggesting that maintained AAS may be more beneficial than abstaining after mTBI.

Keywords: concussion, adolescent, prefrontal cortex, pituitary, amygdala, HPA axis

INTRODUCTION

Mild traumatic brain injury (mTBI), or concussion, is a common form of head injury occurring mostly via falls, vehicular accidents, or by sports-related impacts to the head (1). mTBI imposes accelerative and rotational forces on the brain inside the skull that can result in structural, cellular, and mRNA expression changes (2), which are particularly disruptive during childhood and adolescence when the brain is still developing (3). While most people fully recover, a significant portion of those subjected to repetitive mTBIs (RmTBI), go on to exhibit clinical symptoms of post-concussive syndrome (PCS) (4). These symptoms include deficits in motor function, changes in mood and anxiety levels, as well as problems with complex mental processes and detriments to cognitive abilities (5). These symptoms may be brought about by the neurometabolic cascade that happens post-concussion (6). Following mTBI, the damaged cell membranes release excitatory neurotransmitters and cause ionic imbalances, contributing to a spread of depolarization and ionic flux in the brain (6, 7). Membrane ion pumps work exhaustively in attempts to restore ionic balance, requiring increased cellular ATP (6). The hyperglycolysis, and increased mitochondrial activity used to meet these energy demands is followed by a state of impaired metabolism, possibly contributing to behavioral impairments (7). mTBI has been shown to induce mitochondrial metabolic and genetic changes (8), in addition to mitochondrial dysfunction (9) associated with detrimental downstream effects such as axonal damage, neuron death, and a compromised blood-brain barrier (10). Children and adolescents are at highest risk for RmTBI (11), with adolescent athletes being a particularly vulnerable population (12). Given the long-term neurobiological and psychological consequences of RmTBI, and the increasing incidence of sport-related concussions in youth, understanding the pathology of RmTBI during this developmentally sensitive stage of brain maturation is crucial to predicting outcomes.

Further complicating this problem, it is not uncommon for adolescent athletes to consume anabolic-androgenic steroids (AAS) (13). AAS are a group of hormones including naturally produced testosterone and lab-made derivatives (14). Clinically they are used to treat various medical conditions, however they are also illicitly used to boost athletic performance (13). Global data indicate the prevalence of AAS use to be ∼3.3%, with adolescents having higher rates of AAS use than adults (13). AAS exposure during puberty has a wide range of neurological consequences that have the potential to affect brain development. Puberty is a hormonally sensitive period where the addition of exogenous hormones can have detrimental behavioral and neurohormonal effects (15, 16). Symptoms of chronic AAS use include varying levels of anxiety (17) and depression (18), as well as increased aggression, which may persist even after individuals stop taking the drug (19).

What's more, acute neuroendocrine dysfunction has been identified after mTBI, specifically in the anterior pituitary, disrupting normal function of the hypothalamic-pituitary-adrenal (HPA) axis (20). Elevated cortisol levels after injury are beneficial and help the body protect itself by promoting healing mechanisms (21). However, following RmTBI there is often a decrease in cortisol due to this HPA axis disruption which likely contributes to worse symptomology and increased recovery time (22). Interestingly, athletes who abuse AAS, and experience RmTBI often opt to abstain from their drug and exercise regimes in an effort to expedite recovery. Like those experiencing withdrawal due to their AAS dependence, some athletes have reported worse outcomes after abstaining from AAS (23, 24) suggesting that AAS maybe providing neuroprotective, or possibly therapeutic effects on the injured brain. Despite the prevalence of AAS, relatively few studies have looked at the interaction between AAS and RmTBI in adolescents, and to our knowledge, none have investigated whether quitting AAS following an mTBI is the best choice.

Given that adolescent athletes are at particularly high risk for RmTBI and have a high rate of AAS use, we sought to investigate the pathophysiological outcomes associated with the abrupt cessation of both exercise and the commonly abused AAS, Metandienone (25) (Met) on RmTBI outcomes using a clinically relevant rodent model of concussion. Adolescent males are consistently reported with higher AAS usage than females (26–28), in addition to higher lifetime prevalence rates of AAS use (6.4%) over females (1.6%) (13), which is why they were the selected for this study. Male rats received either Met or placebo and were then randomly assigned to the RmTBI or sham injury group. Following the RmTBIs or sham injuries, half the rats were returned to normal drinking water and were deprived of exercise wheels, while the other half continued to exercise and receive Met. A behavioral test battery was conducted to measure outcomes consistent with clinical representations of PCS and chronic AAS exposure. Neurobiological outcomes were further examined via changes in mRNA expression in the prefrontal cortex (PFC), the amygdala (AMYG), the hippocampus (HPC), and the pituitary gland (PIT). The PFC, HPC, and AMYG were selected because both concussion and AAS affect executive function, short-term working memory, mood regulation, and impulsivity, which all involve neural circuitry in these 3 brain regions. The PIT was also selected for investigation as it has a role in normal hormonal function, which may be disrupted by AAS and RmTBI.

METHODS

Subjects

All reported experiments were carried out in accordance with the Canadian Council of Animal Care and received approval from the University of Calgary Conjoint Faculties Research Ethics Approval Board. Forty-seven male Sprague Dawley rats (Charles Rivers Laboratories) were weaned at postnatal day 21 (P21), caged in groups of 4, and housed in an animal husbandry room at 21°C with a 12:12 hr light:dark cycle (lights on at 7 a.m.). The animals had *ad libitum* access to food and water.

Exercise Protocol

At postnatal day (P) 34, rats were randomly assigned to one of the following conditions: (a) *Placebo* + *Exercise* ($n = 8$), (b) *Steroid* + *Exercise* ($n = 8$), (c) *Placebo* + *Exercise* + *Steroid and Exercise Cessation (SEC)* ($n = 15$), (d) *Steroid* + *Exercise* + *SEC* ($n = 16$). The *Exercise* groups were housed in Lafayette Activity Living Chambers (model + 80859; Lafayette, IN, USA). *SEC* groups were returned to normal cages after the third mTBI or sham injury (P46) and were deprived of Met and running wheels for the remainder of the experiment. The Activity Living Chambers were maintained in the same husbandry room as the control cages and all animals had *ad libitum* access to food and water. Activity wheel counters were used to measure the distance the rats had run, and were recorded each day.

AAS Administration Protocol

Metandienone (Met) purchased from TripleBond (Guelph, ON, Canada) was orally administered to the rats in their drinking water by dissolving the drug at a concentration of 1.5 mg/kg, body weight/day, starting at P21. This dosage was selected as it closely mimics the dosage commonly used by humans (29) and was orally administered as this is the ingestion route most commonly observed in clinical populations. Met or placebo was administered daily to all *Steroid* and *Placebo* groups for 7 weeks up until sacrifice with the amount of water consumed was measured on a daily basis. The *SEC* groups were switched to placebo water after their third mTBI or sham injury for the remainder of the experiment (P47).

RmTBI Procedure

At P41, rats in each group were randomly assigned to receive 3 mTBIs with the Lateral Impact (LI) device or 3 sham injuries. The LI technique employed a protocol described by Mychasiuk et al. (2). Briefly, animals were lightly anesthetized with isofluorane until a toe pinch drew no response and were then placed in a prone position on a low friction Teflon board. A 50 g weight was pneumatically fired toward the rat's head at an average speed of 8.95 ± 0.12 m/s, resulting in TBIs at 81.66 Gs. The weight impacted a small "helmet" that protected the skull from structural damage but propelled the rat into a horizontal 180° rotation. This LI technique subjects the brain to acceleration/deceleration and rotational forces that mimic a sports-related concussion (2, 30). mTBIs or sham injuries were performed on P41, P44, and P47. *Time-to-right* measured the time each rat took to wake and move from a supine position to a prone or standing position following the injury, and was used as a surrogate measure for loss-of-consciousness.

Behavioral Testing

Rats underwent a behavioral test battery consisting of 6 behavioral tasks designed to measure post-concussive symptomology (31, 32). *Beam walking* is a test for balance and motor coordination used to measure hindleg foot slips on a tapered beam described in detail by Schallert et al. (33). This test was carried out at post-injury day 1 (PID1) and PID3.

On P49 or PID2 rats were tested in an *Open Field* paradigm as a measure of general locomotor activity (34). Animals were placed in the center of a circular arena with a diameter of 135 cm and allowed to explore their surroundings for 10 min. An overhead camera equipped with Noldus Ethovision XT 10.0 software tracked and analyzed the distance traveled and the time spent in the center of the arena for each rat. The arena was cleaned with Virkon between each testing session.

At PID3 (P50), rats were tested for general anxiety in the *Elevated Plus Maze* (EPM) (34). The EPM is constructed from black Plexiglas and was elevated 55 cm above ground. It contained two closed and two open arms, and each rat was permitted to explore the EPM for 5 min in a videotaped session. A research analyst blinded to the experimental conditions recorded how much time the rat spent in the center, open, and closed arms.

Novel Context Mismatch (NCM) was used to measure short term working memory. Training for the NCM occurred on P54–P56, where rats were placed in both Context A and Context B (5 min each) per training day, where Context B placement immediately followed Context A. Context A was a transparent rectangular box (70 × 40 × 33 cm) with 2 identical plastic cylinders; Context B was an opaque blue circular bin (36 cm high with a diameter of 47 cm), with two identical glass bottles. The probe trial for the test occured at PID10 (P57). Rats went from Context A (5 min) to Context B (5 min), into their home cage (5 min), then to the Novel Context (5 min). The novel context was a modified Context A, where the same rectangular box was used, and one object from Context A, as well as one object from Context B. The rats were videotaped exploring the novel context and a research assistant (blinded to the experimental conditions) scored the amount of time the animals investigated the novel object and the old object. All containers and objects were sanitized with Virkon between each testing session. The protocol employed was similar to that described be Spanswick & Sutherland (35).

Aggression levels were measured using the *Dominance Tube* test (36), which was administered on PID4. Rats were released into opposing ends of a clear tube, narrow enough to impede the animal's ability to turn around. The rats met in the middle of the tube, and the dominant animal would exhibit more aggression by forcing their opponent to back out of the tube. The rat was declared the winner when its opponent had all four paws out of the tube. The match ups at consisted of *RmTBI* vs. *RmTBI* & *Sham* vs. *Sham* rats, and were always *Steroid* vs. *Placebo* rats. There was a total of 3 trials per match up with trail wins, win percentage, and time spent in the tube recorded for each animal.

Finally, on PID14 rats were tested for 7 min in a modified *Forced Swim* paradigm similar to that employed by Yadid et al. (37) to examine depressive- or anxiety-like behaviors. A cylindrical tank (diameter of 30 cm, 60 cm high) filled with water (∼25°C) high enough that the rat's tail was unable to contact the bottom of the tank. After each session, the rats were dried with warm towels and returned to their home cages with the water being replaced before the next session. The amount of time spent immobile and number of escape attempts was scored for each rat.

Experimental duration and timepoints (P41–P61) were chosen as they are reflective of adolescence in rats (38, 39).

mRNA Analysis

Rats were euthanized at PID15 after completion of all behavioral testing. All rats were anesthetized via isoflurane inhalation, were quickly weighed and measured, and were then decapitated. Using the Zilles atlas (40) tissue from the PFC, HPC, AMYG, and PIT was removed, flash frozen on dry ice, and stored at −80°C. Total RNA was extracted from each brain region for molecular analysis with the Allprep RNA/DNA Mini Kit according to manufacturer protocols (Qiagen, Germany). Samples were tested for purity and concentration with a NanoDrop 2000 (Thermo Fisher Scientific, USA). Purified RNA (2 μg) was reverse transcribed into cDNA using the oligo(dT)20Superscript III First-Strand Synthesis Supermix Kit (Invitrogen, USA) according to manufacturer instructions.

A total of 6 genes were selected for analysis, which provided key information regarding the effects of AAS, RmTBI, and exercise on neuroinflammation, repair processes, and neurodevelopment. The 4 genes selected for PFC, AMYG, and HPC were: *Bdnf,* Brain-derived neurotrophic factor; *GR,* Glucocoritcoid receptor; *Iba1,* Ionized calcium-binding adaptor molecule 1; and *Maoa,* Monoamine oxidase A. A total of 6 genes were selected for analysis in PIT: *Bdnf*, *GR*, *Iba1*, *Maoa,* cAMP response-element binding protein (*Creb*), and Estrogen receptor (*ER*). *Bdnf* is involved in supporting the developing nervous system, with key roles in neurogenesis, neural plasticity, learning and memory (41), and is susceptible to changes in expression from mTBI (42). *GR* is a transcription factor activated by the stress hormone, cortisol (43). Under periods of injury or stress, sustained *GR* activation is toxic to neurons through increased excitotoxicity and oxidative stress (43, 44). *Iba1* is a marker of microglial activation as part of the neuroinflammatory response (45), involved in proliferation, migration, and immune responses at the site of injury (46). *Maoa* is an enzyme responsible for breaking down monoamine neurotransmitters (47) and has been implicated in the modulation of aggressive behavior (47) and stress responses via its effects on serotonergic circuitry (48, 49). *Creb* is a transcription factor that mediates complex learning and memory processes (50, 51) which can be disrupted through brain injury (52). *ER* is present throughout multiple brain regions, allowing estrogens to induce many effects on neuroprotection, synaptogenesis, and cognitive function (53).

Primers for the qRT-PCR were designed by an in-house research technician using Primer3 (http://bioinfo.ut.ee/primer3), and purchased from Integrated DNA Technologies (Coralville, USA). Duplicate samples were run on a 96-well-plate and each target gene was processed. qRT-PCR was performed and analyzed with the Applied Biosystems™ StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA) with 10 ng of cDNA, 10 μM of the forward and reverse primers for each target gene, and 1X SYBR Green FastMix with Rox (Quanta Biosciences, USA). Two housekeeping genes, CycA and Ywhaz (54) were used to determine relative target gene expression through the $2^{\Delta\Delta Ct}$ method as previously described by Pfaffl (55).

Telomere Length Analysis

Immediately after each rat was euthanized a sample of ear notch tissue was taken from each rat and stored at −80°C. Extraction of genomic DNA from ear notch samples using the Sigma REDExtract N-AMP Tissue PCR kit was performed according to manufacturer's protocol. Concentration and quality were measured using the NanoDrop 2000 (Thermo Fisher Scientific, USA). Samples were diluted to concentrations of 20 ng/μL to perform telomere analysis. A research technician designed primers for telomeres as well as the single copy 36B4 gene in-house with information previously described by Cawthorn (56). PCR reactions were run in duplicates on a 96 well-plate. Each PCR reaction was comprised of 1 μL of gDNA within a total volume of 20 μL using 1X SYBR Green FastMix with Rox (Quanta Biosciences, USA) for qRT-PCR on a CFX Connect Real Time PCR Detection System (Bio-Rad, Hercules, USA). The concentrations for primers were 20 μM for the forward and reverse primers of both Tel and 36B4. The telomere repeat number to single copy ratio (T/S) was calculated to determine telomere length with the single copy corresponding to the 36B4 gene. When T/S ratios = 1, the unknown DNA is equivalent to the reference DNA. If T/S > 1 there is an increase in telomere repeat number, whereas when T/S < 1, there is a decrease in telomere repeat number. T/S ratio was determined to be $[2^{Ct(telomere)}/2^{Ct(36B4)}]- 1 = -2^{\Delta Ct}$. Relative telomere length was determined using a linear regression equation, $y = 1910.5x + 4157$ (where y = telomere length and $x = -2^{\Delta Ct}$), described by Cawthorn (56).

Serum Biomarker Analysis

Rats were euthanized via rapid decapitation on PID15, and trunk blood was collected in serum separator tubes. Samples were clotted for 30 min at room temperature then centrifuged at 1,000 g for 15 min. The serum was stored at −80°C. ELISA kits were purchased for Testosterone, Corticosterone, and Alanine Transaminase (Abcam Inc, Canada). ELISAs for each biomarker were completed according to manufacturer's instructions. Standards, positive and negative controls, and samples were all run in duplicate on a 96-well plate and measured using the BioTek Synergy H.T. plate reader and Gen5 2.00.18 software with a path length correction algorithm. Samples were all in normal range of the standard curve.

Statistical Analysis

A research analyst blinded to all experimental conditions scored each of the behavioral tests and two analysts performed the qRT-PCR analysis for each gene, carried out in duplicate. A research analyst also carried out telomere and serum biomarker analysis.

TABLE 1 | Statisitcal results of the three-way anovas for the behavioral assessment of rmtbi, aas treatment, and SEC in adolescent rats.

Behavioral test	Effect of RmTBI F (*p*)	Effect of Met Treatment F (*p*)	Effect of SEC F (*p*)	Met Treatment × RmTBI F (*p*)	Met Treatment × SEC F (*p*)	RmTBI × SEC F (*p*)	Met Treatment × RmTBI × SEC F (*p*)
Time-to-right	40.56 (<0.01)	0.93 (0.34)	6.82 (0.01)	0.85 (0.36)	0.46 (0.50)	7.42 (<0.01)	0.98 (0.33)
Beam walk	10.52 (<0.01)	0.03 (0.86)	0.12 (0.74)	0.24 (0.63)	2.59 (0.11)	1.62 (0.21)	0.27 (0.61)
Open field: distance	0.22 (0.64)	0.38 (0.54)	15.65 (<0.01)	0.68 (0.41)	1.58 (0.21)	0.60 (0.44)	0.00 (0.95)
Open field: center	0.11 (0.75)	0.68 (0.41)	0.16 (0.69)	0.28 (0.60)	0.01 (0.91)	0.00 (0.97)	1.03 (0.31)
EPM	9.73 (<0.01)	0.02 (0.88)	0.29 (0.60)	0.12 (0.73)	0.00 (0.95)	5.08 (0.03)	0.01 (0.93)
NCM	10.11 (<0.01)	0.07 (0.80)	0.08 (0.78)	0.25 (0.62)	0.73 (0.40)	5.48 (0.02)	2.31 (0.13)
Dominance tube	0.01 (0.93)	0.13 (0.72)	0.12 (0.73)	5.88 (0.02)	0.12 (0.73)	0.02 (0.88)	4.41 (0.04)
Forced swim	0.45 (0.51)	12.98 (<0.01)	0.11 (0.74)	0.03 (0.87)	0.04 (0.84)	0.06 (0.81)	0.19 (0.67)

All analyses were performed with SPSS 23.0 for Mac, and $p \leq 0.05$ was considered statistically significant. Three-way ANOVAs with Injury (RmTBI vs. Sham), Treatment (Met vs. Placebo), and SEC (cessation vs. maintenance) as factors were run for each of the behavioral and molecular outcomes. *Post-hoc* pairwise comparisons (LSD), were carried out when appropriate. All error bars on graphs represent ± SEM.

RESULTS

Animal Characteristics

Weight gain from the initial mTBI to the end of the study (P41–P61), in addition to brain weight at time of sacrifice was recorded. The three-way ANOVA of body weight gain demonstrated a main effect of SEC [$F_{(1,78)} = 13.18$, $p < 0.01$], but not of RmTBI or treatment. Interestingly, steroid treatment alone had no effect on change in body weight, but cessation of treatment and exercise significantly increased the amount of weight gained between TBI and sacrifice (Steroid + SEC cessation; 125.9 g ± 4.9; Steroid + SEC Maintenance; 112.3 g ± 3.8; Placebo + SEC cessation; 123.3 g ± 4.9; Placebo + SEC Maintenance; 108.3 g ± 3.8). This result suggests that the weight gain was associated with a loss of running wheel access, rather than cessation of steroids. There were also no significant interactions between RmTBI, treatment, or SEC. Conversely, there were no significant effects of RmTBI, treatment, or SEC for brain weight, nor were there any significant interactions. There were also no significant differences in the average distance run per day for animals in the AAS and placebo groups.

Behavioral Testing

All statistical results from the three-way ANOVAs for the behavioral tests can be found in **Table 1** and graphical representation of these findings are in **Figure 1**. In summary, SEC affected behavioral performance on 2 of 7 measures (increased time-to-right and resulted in hyperactivity in the open-field) for both sham and RmTBI animals. The RmTBIs impaired performance on 4 of 7 tasks (increased time-to-right, increased hindleg foot slips in the beam walk task, and increased anxiety-like behavior in the elevated plus maze, while decreasing short-term working memory on the novel context mismatch task). AAS treatment was found to produce dysfunction in the forced swim task for both sham and RmTBI animals, whereby AAS animals displayed increased depressive-like behavior. In addition, there were multiple SEC by Injury interactions; (1) for time-to-right there was a two-way interaction between SEC and RmTBI. *Post-hoc* analysis demonstrated that the Injury × SEC interaction was driven by the animals in the RmTBI group, whereby injured animals that experienced cessation of AAS and exercise exhibited increased loss of consciousness, $p < 0.01$. (2) In the EPM there was also a two-way interaction between SEC and RmTBI. *Post-hoc* analysis of time in open arms of the EPM demonstrated that the Injury × SEC interaction was driven by the sham group, where animals that were exposed to sham injuries and experienced cessation of AAS and exercise displayed reduced anxiety-like behavior and increased time in the open arms of the EPM, $p = 0.05$. (3) In the NCM task, *post-hoc* analysis showed that the Injury × SEC interaction was driven by the RmTBI animals, whereby injured animals that experienced cessation of AAS and exercise exhibited worse performance on the NCM task than injured animals that maintained AAS and exercise treatment, $p < 0.01$. Finally, in the dominance tube task of aggression we identified a Met treatment by SEC interaction and a three-way interaction. *Post-hoc* analysis demonstrated that the Met Treatment by Injury interaction was driven by the RmTBI group. Animals that experienced RmTBI and placebo were more aggressive than RmTBI animals consuming AAS, $p = 0.05$. In addition, *post-hoc* analyses of the 3-way interaction between Met Treatment, Injury, and SEC found this interaction to be driven by animals in the S+E cessation group, whereby dominance/aggression increased in sham animals who experienced cessation of AAS and exercise ($p = 0.05$), but decreased in RmTBI animals that experienced cessation of AAS and exercise ($p = 0.03$).

mRNA Expression

mRNA was examined for 4 different genes (*Bdnf, GR, Iba1,* and *Maoa*) in the PFC, AMYG, and HPC, and 6 different genes (*Bdnf*, *GR*, *Iba1*, *Maoa*, *ER*, and *Creb*) in the PIT.

PFC

In the PFC expression, 2 of 4 genes were influenced by Met treatment (*GR* and *Maoa*), 2 were influenced by SEC (*Bdnf,*

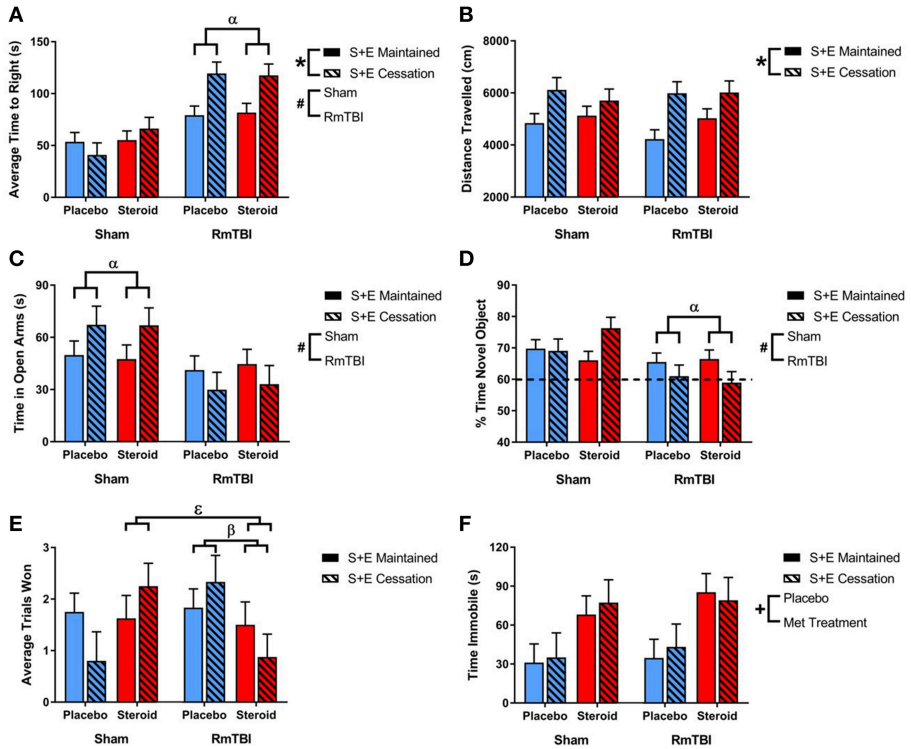

FIGURE 1 | Bar graphs displaying outcomes from behavioral test battery for all groups. Means ± standard error are displayed where *main effect for SEC, #main effect of RmTBI, +main effect of Met treatment, αsignificant RmTBI by SEC interaction, βsignificant Met treatment by RmTBI interaction, and εsignificant Met treatment by RmTBI by SEC interaction $p \leq 0.05$. **(A)** Displays the average time-to-right after sham injury or RmTBI. *Post hoc* analysis demonstrated that the Injury × SEC interaction was driven by the animals in the RmTBI group, whereby injured animals that experienced cessation of AAS and exercise exhibited increased loss of consciousness, $p < 0.01$. **(B)** Displays the mean distance traveled in the open field test. **(C)** Displays the average time spent in the open arms of the elevated plus maze. *Post hoc* analysis of time in open arms of the EPM demonstrated that the Injury × SEC interaction was driven by the sham group, where animals that were exposed to sham injuries and experienced cessation of AAS and exercise displayed reduced anxiety-like behavior and increased time in the open arms of the EPM, $p = 0.05$. **(D)** Displays the % of time spent with a novel object in the NCM task. *Post-hoc* analysis showed that the Injury × SEC interaction was driven by the RmTBI animals, whereby injured animals that experienced cessation of AAS and exercise exhibited worse performance on the NCM task than injured animals that maintained AAS and exercise treatment, $p < 0.01$. The hatched line indicates the % of expected time the rat will spend investigating the novel object. **(E)** Displays the average trials won out of 3 possible trials in the dominance tube test. *Post hoc* analysis demonstrated that the Met Treatment by Injury interaction was driven by the RmTBI group. Animals that experienced RmTBI and placebo were more aggressive than RmTBI animals consuming AAS, $p = 0.05$. In addition, *Post hoc* analyses of the 3-way interaction between Met Treatment, Injury, and SEC found this interaction to be driven by animals in the S+E cessation group, whereby dominance/aggression increased in sham animals who experienced cessation of AAS and exercise ($p = 0.05$), but decreased in RmTBI animals that experienced cessation of AAS and exercise ($p = 0.03$). **(F)** Displays the mean time spent immobile in the forced swim test. RmTBI, repetitive mild traumatic brain injury; S+E, steroid and exercise; NCM, novel context mismatch.

and *Maoa*), and 1 gene (*Iba1*) exhibited a Met treatment by SEC interaction. For *Iba1*, *post-hoc* analysis demonstrated that the Met Treatment by SEC interaction was driven by animals exposed to AAS. Rats that were exposed to AAS and exercise and then subsequently quit, exhibited increased expression of *Iba1*, when compared to rats that were exposed to AAS and exercise throughout the experiment, $p = 0.04$ (see **Figure 2**).

AMYG

SEC had more influence on gene expression in the AMYG, affecting 3 of 4 genes (*GR, Iba1,* and *Maoa*). The AMYG also exhibited significant interactions; (1) *GR* demonstrated a significant RmTBI by SEC interaction. *Post-hoc* analysis showed that the Injury by SEC interaction was driven by the sham animals, whereby expression of *GR* was significantly increased in sham animals that experienced cessation of AAS and exercise, compared to sham animals that maintained AAS and exercise throughout the experiment, $p < 0.01$. (2) *Bdnf* displayed a significant three-way interaction. *Post-hoc* analysis of the three-way interaction between Injury, Met Treatment and SEC demonstrated that the effect was driven by Sham animals that received AAS, whereby expression of *Bdnf* was significantly elevated in animals that experienced cessation of AAS and exercise as compared to those that maintained treatment throughout the experiment, $p < 0.05$ (see **Figure 3**).

HPC

Gene expression in the HPC was the least affected of the four brain regions, with only 1 gene (*GR*) altered by Met treatment, and 1 gene (*Iba1*) influenced by SEC (see **Figure 4**).

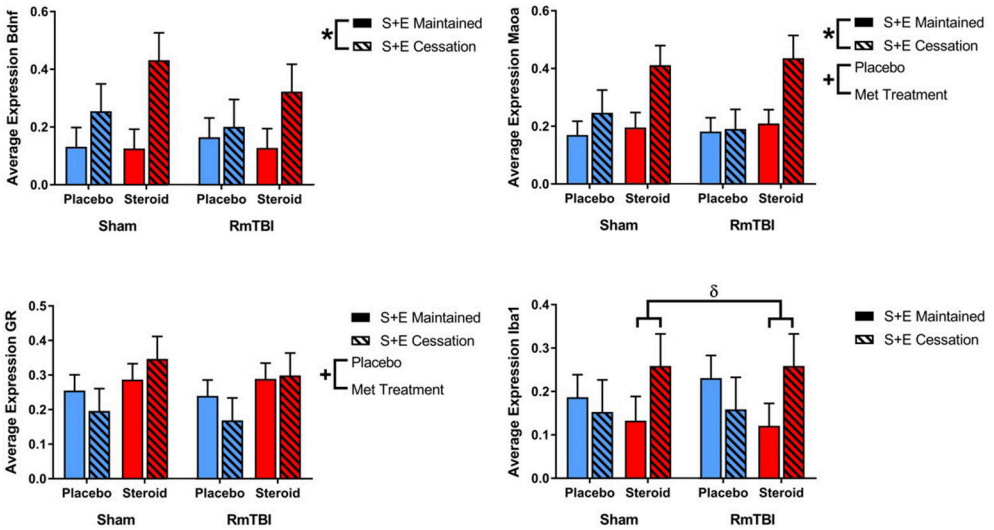

FIGURE 2 | Bar graphs displaying average mRNA expression levels in the PFC. Means ± standard error are displayed where *main effect for SEC, $^{+}$main effect of Met treatment, and $^{\delta}$significant Met treatment by SEC interaction $p \leq 0.05$. For *Iba1*, *post-hoc* analysis demonstrated that the Met Treatment by SEC interaction was driven by animals exposed to AAS. Rats that were exposed to AAS and exercise and then subsequently quit, exhibited increased expression of *Iba1*, when compared to rats that were exposed to AAS and exercise throughout the experiment, $p = 0.04$. mRNA, messenger RNA; PFC, pre-frontal cortex; RmTBI, repetitive mild traumatic brain injury; S+E, steroid and exercise.

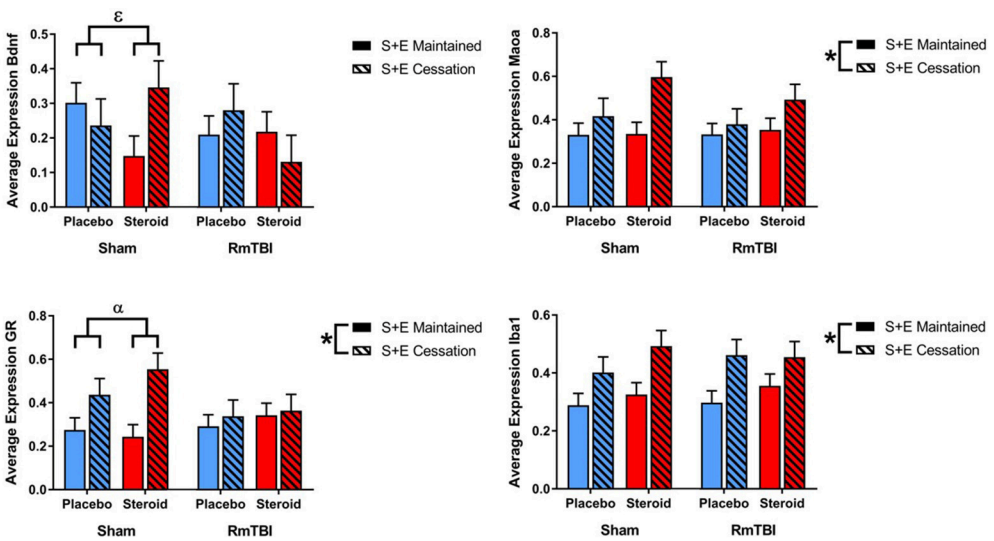

FIGURE 3 | Bar graphs displaying average mRNA expression in the AMYG. Means ± standard error are displayed where *main effect for SEC, $^{\alpha}$significant injury by SEC interaction, and $^{\varepsilon}$significant Met treatment by RmTBI by SEC interaction $p \leq 0.05$. *Post-hoc* analysis showed that the Injury by SEC interaction of *GR* was driven by the sham animals, whereby expression of *GR* was significantly increased in sham animals that experienced cessation of AAS and exercise, compared to sham animals that maintained AAS and exercise throughout the experiment, $p < 0.01$. *Post-hoc* analysis of the three-way interaction between Injury, Met Treatment and SEC of *Bdnf* demonstrated that the effect was driven by Sham animals that received AAS, whereby expression of *Bdnf* was significantly elevated in animals that experienced cessation of AAS and exercise as compared to those that maintained treatment throughout the experiment, $p < 0.05$. mRNA, messenger RNA; AMYG, amygdala; RmTBI, repetitive mild traumatic brain injury; S+E, steroid and exercise.

PIT

Lastly, in the PIT, 2 out of 6 genes (*ER* and *GR*) were altered by treatment and 4 of 6 genes (*ER, Iba1, Maoa,* and *Creb*) were affected by SEC. Expression of 1 gene (Creb) exhibited a Met treatment by Injury interaction, as well as a Met treatment by SEC interaction. *Post-hoc* analysis demonstrated that the Met Treatment by Injury interaction was driven by animals in the Steroid group. Rats with sham injuries in the steroid group

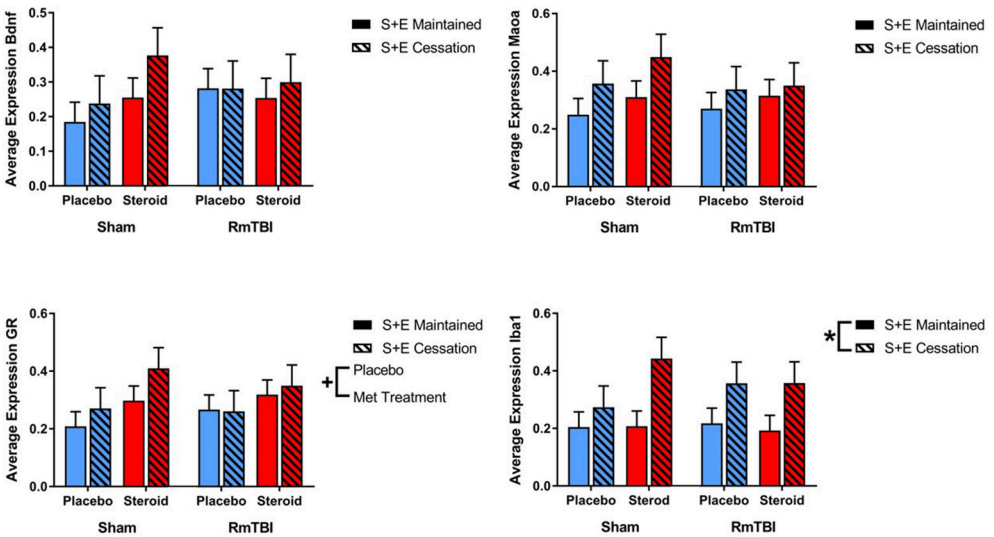

FIGURE 4 | Bar graphs displaying average mRNA expression in the HPC. Means ± standard error are displayed where *main effect for SEC and $^{+}$main effect of Met treatment $p \leq 0.05$. mRNA, messenger RNA; HPC, hippocampus; RmTBI, repetitive mild traumatic brain injury; S+E, steroid and exercise.

exhibited greater expression of *Creb* than rats with RmTBI in the steroid group, $p < 0.01$. The second *post-hoc* analysis found that the Met Treatment by SEC interaction was driven by the placebo group, whereby cessation of AAS and exercise significantly increased expression of *Creb* in animals that did not receive AAS, $p < 0.01$ (see **Figure 5**).

See **Table 2** for summary of statistical results of genes in all four brain regions.

Telomere Length

Ear notch skin samples were examined at PID15 for changes in telomere length. There was a significant RmTBI by SEC interaction [$F_{(1,62)} = 4.67$, $p = 0.04$]. *Post-hoc* analysis demonstrated that the Injury by SEC interaction was driven by the sham animals, whereby telomere length was significantly reduced in response to cessation of AAS and exercise if the rat did not receive repetitive injuries, but was not reduced in animals that experienced cessation of AAS and exercise, and RmTBI, $p = 0.02$. There were no other significant main effects or interactions. See **Figure 6**.

Serum Biomarkers

Serum levels of testosterone, corticosterone, and alanine aminotransferase (ALT) were examined at PID15. The three-way ANOVA for testosterone demonstrated a main effect of Met treatment, [$F_{(1,62)} = 12.53$, $p < 0.01$], but not of RmTBI, and SEC, nor were there any significant interactios between RmTBI, Met treatment, or SEC.

The three-way ANOVA for corticosterone failed to show any significant main effects of RmTBI, Met treatment, or SEC, however there was a significant Met treatment by Injury interaction, [$F_{(1,55)} = 7.92$, $p < 0.01$]. *Post-hoc* analysis showed that the Met Treatment by Injury interaction was driven by the placebo group, where sham animals demonstrated elevated corticosterone and animals in the placebo group that received RmTBIs exhibited reductions in corticosterone, $p = 0.02$.

The three-way ANOVA for ALT revealed a main effect of SEC [$F_{(1,31)} = 26.86$, $p < 0.01$], but not of RmTBI or treatment. However, there was a significant Injury by SEC interaction, [$F_{(1,31)} = 0.60$, $p = 0.03$]. *Post-hoc* analysis demonstrated that the Injury by SEC interaction was driven by animals in the RmTBI group, whereby those that experienced cessation of AAS and exercise exhibited significant decreases in ALT levels as compared to animals with RmTBI that maintained treatment throughout the experiment, $p < 0.01$. See **Figure 6**.

DISCUSSION

During adolescence, RmTBI, AAS use, and AAS withdrawal are all associated with changes in anxiety, depression, mood irritability, aggression, and cognitive function (5, 17, 23). As AAS-withdrawal symptoms overlap with that of PCS, we hypothesized that abrupt steroid and exercise cessation after RmTBI would potentiate behavioral impairments associated with PCS. In support of our hypothesis, SEC prolonged post-injury loss of consciousness and exacerbated, anxiety-like behaviors and short-term working memory. SEC also increased general activity in the open field and weight gain in both sham and RmTBI rats. However, these changes may have resulted from the absence of the running wheels inaccessibility, producing a need to exercise in addition to a calorie surplus induced weight gain (57, 58). Consistent with previous studies (59, 60) RmTBI alone produced loss of consciousness, motor and balance deficits, increases in anxiety-like behavior, and working memory deficits. Also, in line with previous findings, Met treatment produced increases in depressive-like behavior (61, 62). Contrary to prior studies however, we failed to identify an increase in aggression in our

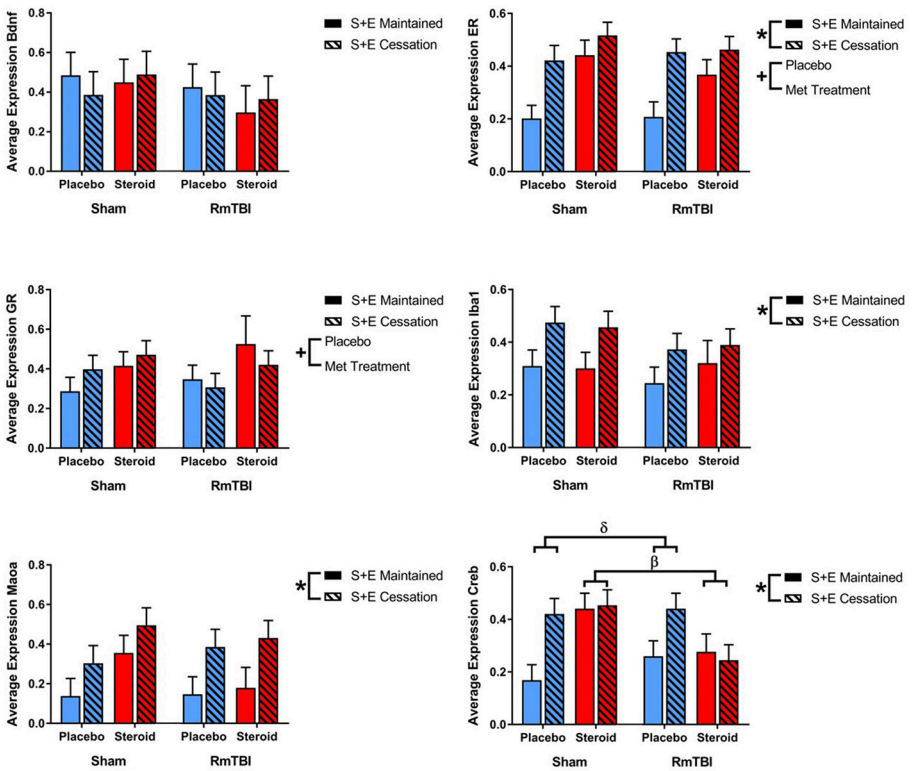

FIGURE 5 | Bar graphs displaying average mRNA expression levels in the PIT. Means ± standard error are displayed where *main effect for SEC, $^{+}$main effect of Met treatment, $^{\beta}$significant Met treatment by RmTBI interaction, and $^{\delta}$significant Met treatment by SEC interaction $p \leq 0.05$. *Post-hoc* analysis of *Creb* demonstrated that the Met Treatment by Injury interaction was driven by animals in the Steroid group. Rats with sham injuries in the steroid group exhibited greater expression of *Creb* than rats with RmTBI in the steroid group, $p < 0.01$. The second *post-hoc* analysis found that the Met Treatment by SEC interaction was driven by the placebo group, whereby cessation of AAS and exercise significantly increased expression of *Creb* in animals that did not receive AAS, $p < 0.01$. mRNA, messenger RNA; PIT, pituitary; RmTBI, repetitive mild traumatic brain injury; S+E, steroid and exercise.

Met treated rats (19, 63–65). Interestingly, when Met treatment was combined with RmTBI, we actually found a reduction in win percentage in the dominance tube, suggesting that that Met may affect the neural circuitry involved in aggression in a different manner than other commonly used AAS.

In addition to their effects on behavior, RmTBI and SEC interacted to induce changes in telomere length (TL). Previous findings in our laboratory have demonstrated mTBI induced reductions in TL (66), and shortened TL has been implicated in neurodegenerative diseases and age-related cognitive decline (67, 68). Telomeres are tandemly repeating, non-coding sequences of DNA capping the ends of eukaryotic chromosomes which are vulnerable to shortening, especially in neurons, as telomerase and other neuroprotective peptides are affected by oxidative stress (69, 70). The findings from the present study are therefore quite interesting. The SEC-sham animals had relatively similar TL to the RmTBI, suggesting that abruptly quitting Met in adolescence may have detrimental effects on TL, independent of injury. This reduction in TL may be in response to the sudden drop in androgen levels, as androgens have been shown to increase telomerase function (71), or could be associated with the absence of exercise, as exercise has also been shown to increase TL (59). Given that TL has been used as a gauge of neuronal aging and health (72), the SEC-induced shortening in TL further contributes to growing literature that AAS can potentiate neuronal impairments (17).

Typically, chronic AAS users experience a suppression of the hypothalamic-pituitary-gonadal (HPG) axis as a result of negative feedback inhibition, reducing the amount of naturally produced testosterone in circulation (73, 74). Met treatment was found to lower serum testosterone levels, verifying that in our study Met treatment did suppress the HPG axis. In addition, we also examined circulating corticosterone levels as previous literature suggested that AAS can alter the innate stress response, increasing circulation of glucocorticoids (75), while AAS withdrawal symptoms may be associated with changes in glucocorticoid action (23). Surprisingly, there were no effects of SEC on circulating levels of corticosterone, however there was an interaction between RmTBI and Met treatment, whereby RmTBI rats exposed to Met had increased corticosterone compared to the Met-exposed sham groups. This increase in corticosterone following RmTBI and Met treatment is likely due to injury- and AAS-induced dysfunction of the HPA axis; the HPA axis is vulnerable to increased androgen circulation (76), and

TABLE 2 | Changes in gene expression in the pfc, amyg, hpc, and pit after rmtbi, metandienone treatment, and SEC.

Brain region	Gene	Effect of RmTBI F (*p*)	Effect of met treatment F (*p*)	Effect of SEC F (*p*)	Met treatment × RmTBI F (*p*)	Met Treatment × SEC F (*p*)	RmTBI × SEC F (*p*)	Met Treatment × RmTBI × SEC F (*p*)
PFC	*Bdnf*	0.30 (0.59)	1.22 (0.28)	8.02 (<0.01)	0.13 (0.72)	2.15 (0.15)	0.72 (0.40)	0.01 (0.92)
	GR	0.30 (0.59)	5.16 (0.03)	0.14 (0.71)	0.00 (0.99)	1.59 (0.21)	0.15 (0.70)	0.06 (0.81)
	Iba1	0.04 (0.84)	0.05 (0.82)	0.74 (0.39)	0.11 (0.74)	4.10 (0.05)	0.02 (0.89)	0.08 (0.79)
	Maoa	0.00 (0.98)	6.89 (0.01)	8.90 (<0.01)	0.21 (0.65)	4.02 (0.05)	0.10 (0.75)	0.19 (0.66)
AMYG	*Bdnf*	1.01 (0.32)	0.94 (0.34)	0.35 (0.56)	0.26 (0.62)	0.32 (0.58)	0.61 (0.44)	4.87 (0.03)
	GR	0.88 (0.35)	0.77 (0.39)	8.43 (<0.01)	0.00 (0.95)	0.44 (0.51)	4.70 (0.04)	0.84 (0.37)
	Iba1	0.20 (0.66)	1.75 (0.20)	15.94 (<0.01)	0.32 (0.57)	0.01 (0.93)	0.02 (0.90)	0.76 (0.40)
	Maoa	0.44 (0.51)	3.04 (0.09)	8.53 (<0.01)	0.07 (0.79)	2.14 (0.15)	0.78 (0.38)	0.21 (0.65)
HPC	*Bdnf*	0.10 (0.75)	1.04 (0.32)	1.25 (0.27)	1.24 (0.27)	0.36 (0.56)	0.43 (0.52)	0.01 (0.92)
	GR	0.00 (0.96)	4.34 (0.04)	1.26 (0.27)	0.24 (0.63)	0.23 (0.63)	0.72 (0.40)	0.01 (0.94)
	Iba1	0.00 (0.98)	0.65 (0.43)	11.18 (<0.01)	1.17 (0.29)	1.12 (0.30)	0.00 (0.99)	0.59 (0.45)
	Maoa	0.22 (0.64)	1.19 (0.28)	3.20 (0.08)	0.24 (0.63)	0.00 (0.99)	0.56 (0.46)	0.10 (0.75)
PIT	*Bdnf*	1.01 (0.33)	0.06 (0.81)	0.01 (0.93)	0.41 (0.53)	0.54 (0.47)	0.06 (0.81)	0.01 (0.92)
	ER	0.35 (0.56)	11.02 (<0.01)	17.58 (<0.01)	1.21 (0.29)	3.77 (0.07)	0.09 (0.77)	0.00 (0.97)
	GR	0.02 (0.90)	4.44 (0.05)	0.01 (0.93)	0.15 (0.71)	0.262 (0.61)	1.77 (0.20)	0.00 (0.97)
	Iba1	1.37 (0.26)	0.13 (0.73)	7.93 (0.01)	0.42 (0.52)	0.14 (0.72)	0.46 (0.51)	0.07 (0.79)
	Maoa	0.34 (0.57)	3.62 (0.07)	9.61 (<0.01)	1.67 (0.21)	0.00 (0.96)	0.52 (0.48)	0.02 (0.88)
	Creb	2.38 (0.14)	0.56 (0.46)	5.93 (0.02)	8.16 (0.01)	7.08 (0.01)	0.47 (0.50)	0.02 (0.88)

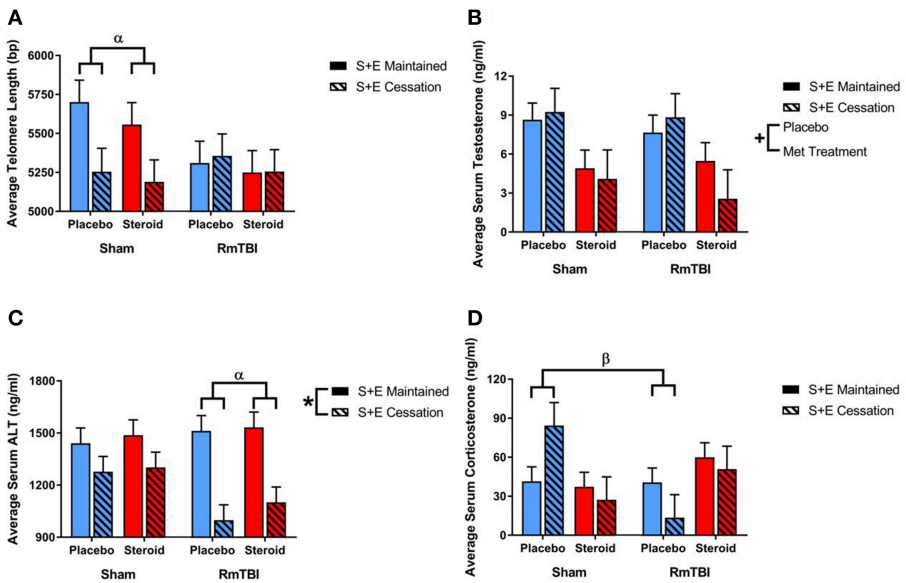

FIGURE 6 | Bar graphs displaying the results from telomere length analysis and serum ELISA results. Means ± standard error are displayed where *main effect for SEC, $^{+}$main effect of Met treatment, $^{\alpha}$significant RmTBI by SEC interaction, and $^{\beta}$significant Met treatment by RmTBI interaction $p \leq 0.05$. **(A)** Displays average telomere length for each of the groups at time of sacrifice. *Post-hoc* analysis demonstrated that the Injury by SEC interaction was driven by the sham animals, whereby telomere length was significantly reduced in response to cessation of AAS and exercise if the rat did not receive repetitive injuries, but was not reduced in animals that experienced cessation of AAS and exercise, and RmTBI, $p = 0.02$. **(B)** Displays the mean serum levels of testosterone at time of sacrifice. **(C)** Displays mean serum levels of ALT at time of sacrifice. *Post-hoc* analysis demonstrated that the Injury by SEC interaction was driven by animals in the RmTBI group, whereby those that experienced cessation of AAS and exercise exhibited significant decreases in ALT levels as compared to animals with RmTBI that maintained treatment throughout the experiment, $p < 0.01$. **(D)** Displays mean serum levels of corticosterone at time of sacrifice. *Post-hoc* analysis showed that the Met Treatment by Injury interaction was driven by the placebo group, where sham animals demonstrated elevated corticosterone and animals in the placebo group that received RmTBIs exhibited reductions in corticosterone, $p = 0.02$. RmTBI, repetitive mild traumatic brain injury; S+E, steroid and exercise; ELISA, enzyme-linked immunosorbent assay; ALT, alanine aminotransferase.

RmTBI (43, 77). However, given that pediatric mTBI may lower cortisol levels (22), Met may be compensating for this loss by escalating the glucocorticoid response in adolescence and ironically providing neuroprotection to the injured brain. Finally, with Met being an orally consumed AAS, which has been shown to induce hepatotoxic effects through first pass metabolism (14, 18), we sought to examine the effects of Met treatment, SEC, and RmTBI on liver function by measuring ALT levels, a known biomarker of liver damage (78). SEC was found to decrease circulating levels of ALT in both RmTBI and sham animals, but the SEC-induced reduction of ALT was significantly greater in the RmTBI group. Given that animals in the SEC group had abstained from Met consumption for ∼14 days prior to sacrifice, it is not surprising that they had lower ALT levels as the hepatotoxic compounds would have been removed from their systems. However, as the SEC also lowered ALT in the placebo groups, it is possible that ALT levels were more representative of exercise exposure than Met treatment, as wheel running in rodents has been shown to increase ALT (79). In addition, the intensified reductions in ALT levels we identified in the RmTBI + SEC groups, suggests that RmTBI influenced general liver health, and warrants future investigations. Overall, our results of circulating corticosterone and ALT demonstrate that Met exposure in adolescence can exacerbate the systemic response to injury and can significantly impact an individual's health.

As the PFC plays an important role in executive function, cognition (80), personality, and social behavior (81), it is particularly vulnerable to the negative consequences associated with AAS (61) and RmTBI (59). Given that the PFC matures much slower than other brain regions, undergoing significant development in adolescence, alterations to mRNA expression and protein translation may consequentially affect healthy neurodevelopment (82). In the present study mRNA expression in the PFC was examined in the context of Met treatment, SEC, and RmTBI. *Bdnf* plays a role in the developing PFC and is involved in brain plasticity related processes. Although not found in this study, AAS use has been shown to decrease *Bdnf* expression (61), which has been linked to abnormal social behavior (83). Therefore, SEC may have had positive effects in the PFC, as it increased levels of *Bdnf* expression. *Maoa* is involved in serotonin metabolism, and alterations in expression are associated with maladaptive behavioral responses to stress, such as depressive-like behaviors (47, 48). This increase in Maoa expression due to Met treatment and SEC would be consistent with our finding that Met promotes depressive-like behavior, in-line with existing literature on the depressive effects of AAS (62, 84). Complimentary to this finding, we also showed that *Iba1*, a known marker of microglial activation (45), exhibited increased expression in Met + SEC animals. Augmented inflammation in the PFC may contribute to the cognitive and withdrawal symptoms typically identified in AAS-users (85). *GR* expression was also increased due to Met treatment; possibly because androgens inhibit glucocorticoid binding to GRs (86, 87), or because Met treatment increased circulation of corticosterone. Nonetheless, increased GR activation is associated with alterations to the stress response, which could be responsible for increased neurotoxicity and further exacerbation of cognitive impairments (43, 44). We were able to show that Met treatment and SEC produced mRNA changes in the PFC that potentially contribute to stress, and in turn affect depressive-like behavior.

The AMYG is a brain region that is particularly vulnerable to both RmTBI and AAS action as it is heavily implicated in the stress response and emotional regulation (88, 89). Changes in gene expression in the AMYG can influence neuronal excitability and produce anxiogenic and depressive effects that may contribute to anxiety and mood-related disorders (90). SEC had substantial effects on gene expression in the AMYG. *Maoa* levels were affected, suggesting that serotonin levels are also altered in the AMYG, coinciding with previous literature demonstrating that AAS use may produce persistent changes to serotonergic circuitry after drug cessation in adolescence (64). In addition, increased expression of *Iba1* suggests a state of oxidative stress and neuroinflammation in the AMYG (45, 91). *GR* expression was also increased, but those with RmTBIs exhibited less of an increase, possibly because Met treatment upregulated *GR* expression before treatment stopped. Interestingly *Bdnf* expression in the AMYG was influenced by an interaction between RmTBI, Met, and SEC, which warrants further investigating as altered *Bdnf* levels in the AMYG have been implicated in depression (92). The mRNA expression changes suggest that SEC lead to increased neuroinflammatory and stress responses in the AMYG, which could interact with many brain regions to produce anxiogenic and depressive effects, while feeding into a persistent cycle of systemic stress.

Conversely, the HPC was the least affected by SEC and Met treatment. The HPC, like the AMYG, is part of the limbic system, and is in part responsible for emotional regulation (93). However, it also plays a role in cognitive function, memory consolidation, and a key site for adult neurogenesis (94). SEC increased *Iba1* expression in the HPC which as a marker of microglial activity (45), is associated with the suppression of neurogenesis in the HPC (95), contributing to symptoms of depression (96) which are prevalent in AAS-withdrawal (18). Conversely, increased GR activation has been associated with reduced neurogenesis and HPC damage (97). *GR* expression was increased by Met treatment, likely due to the altered stress response that AAS can enact on the brain (61) as well as an attempt by the HPC to lower HPA function through negative feedback mechanisms.

Lastly, the PIT is a neuroendocrine structure, implicated in the regulation of many physiological processes including growth, reproduction, and stress (98). Notably, the PIT plays a large role in the HPA axis, receiving input from the hypothalamus and subsequently leading to the downstream activation of glucocorticoid synthesis and secretion (99). Because of its involvement in the brain's response to stress and hormonal function, it is susceptible to permutation from RmTBI and the effects of AAS-withdrawal. Given that we identified changes in anxiety and depressive-like behaviors, in addition to serum markers of stress, we examined mRNA expression in the PIT. Like the PFC and AMYG, SEC had profound effects on gene expression. Firstly, *Maoa* expression was increased, potentially affecting the HPA axis, and downstream ACTH secretion (100). Previous findings have shown that treatment with selective

serotonin reuptake inhibitors (SSRIs) have increased HPA activation (101) and that general stimulation of serotonergic circuitry surrounding the HPA axis has led to increased HPA activation (102), therefore *Maoa* upregulation could be a function of increased serotonergic activity in the PIT. Given that *ER* is involved in the transcriptional control of endocrine functions such as the synthesis and release of growth hormone, prolacatin, and gonadotropin hormones (103, 104), alteration in expression levels of this gene due to SEC and Met treatment could have significant consequences during the neurodevelopmentally sensitive period of adolescence. *ER* can be greatly affected by AAS too since the excess androgens can aromatize in the brain to estradiols which bind to estrogen receptors, producing both genomic and non-genomic effects (14, 53). *Creb* was affected by SEC in the PIT, however there were also interactions between Met and SEC, and Met and RmTBI. These peculiar results likely reflect the integral role that *Creb* plays for healthy development of the PIT (105). Alterations to *Creb* levels through Met, SEC, or RmTBI could prove detrimental given the PIT is very sensitive to *Creb* levels, as seen in pituitary tumors or pituitary hypoplasia (105). Like the other 3 brain regions examined, SEC increased *Iba1* mRNA expression. The HPA axis has bidirectional communication with the neuroimmune system (76), and the release of pro-inflammatory cytokines due to microglial reactivity (106) affects PIT development and enhances HPA axis function through increased ACTH production (106). Finally, we saw increased *GR* expression due to Met exposure. Typically, acute HPA activation results in the release of glucocorticoids as a protective response (107) which also play a regulatory role by way of negative feedback inhibition on the PIT (108). However, AAS have been shown to alter behavior through increased glucocorticoid signaling (109) manifesting as depression and anxiety-like behaviors consistent with our behavioral findings. These effects can be further exacerbated in adolescence as previous findings have shown adolescent animals to have longer HPA responses than adults (97).

In summary, this study identified negative and alarming outcomes associated with RmTBI and SEC in adolescence. Behaviourally, we identified cumulative effects of RmTBI and SEC to loss of consciousness, anxiety-like behavior, and short-term working memory, in addition to changes in aggression and depressive-like behavior due to Met treatment. Interestingly, we saw even more robust effects of Met treatment and SEC in mRNA expression changes across 4 different brain regions, most of which demonstrated alterations to serotonergic circuitry, neuroinflammation, and an enhanced stress response. Interestingly, although serum corticosterone levels did not reflect any change due to SEC, Met was able to increase the glucocorticoid response to RmTBI. Opposite to our findings of lower corticosterone after RmTBI, studies of severe TBI show acute increases in cortisol after injury in both children (110) and adult patients (111, 112). As cortisol levels can fluctuate depending on TBI severity, there is a need for more research on the beneficial effects of AAS on glucocorticoid secretion since previous clinical trials have found corticosterone treatments for TBI to increase mortality rates (113). To date, only 2 studies have tried to investigate the interactions between AAS abuse and mTBI with varying results (114, 115), leaving a gap in the literature on a growing problem among both adolescent and adult athletes.

Although there are numerous strengths to this article, it is not without it's limitations. For example, future studies should examine the effects of SEC on female rats; while the usage of AAS in female adolescents is well-below that of males, their lifetime prevalence is still significant (13, 26, 27). In addition, mRNA was extracted from tissue in the specified brain regions, but this was not cell specific and we were unable to attribute mRNA changes to neurons or surrounding glial cells. While an ideal study would have examined cell specific gene expression changes, protein levels, and immunohistochemistry, this would have required a substantially larger sample size, but leaves ample opportunity for future discoveries in the field. However, to our knowledge, this is also the first study to examine the affects of sudden withdrawal of AAS has on the neurological function of athletes who have sustained multiple concussion. Given the findings from this study, we believe that future research examining the affects of SEC post-injury are warranted and suggest that clinical studies be done to confirm if athletes should taper the cessation of their AAS regime rather than quit cold turkey in an effort to minimize negative outcomes. Additionally, Met's ability to promote glucocorticoid secretion after injury opens potential therapeutic avenues of exogenous hormone administration for clinical treatment of mTBI.

ETHICS STATEMENT

All reported experiments were carried out in accordance with the Canadian Council of Animal Care and received approval from the University of Calgary Conjoint Faculties Research Ethics Approval Board.

AUTHOR CONTRIBUTIONS

JT was involved in the experimental design, data collection, and writing of the manuscript. RC was involved in data collection and writing of the manuscript. CD was involved in experimental design and manuscript writing. SS was involved in experimental design and manuscript writing. RM was involved with experimental design, data collection, data analysis, and writing of the manuscript.

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Disrupted White Matter Microstructure of the Cerebellar Peduncles in Scholastic Athletes After Concussion

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Concussion, or mild traumatic brain injury (mTBI), is a major public health concern, linked with persistent post-concussive syndrome, and chronic traumatic encephalopathy. At present, standard clinical imaging fails to reliably detect traumatic axonal injury associated with concussion and post-concussive symptoms. Diffusion tensor imaging (DTI) is an MR imaging technique that is sensitive to changes in white matter microstructure. Prior studies using DTI did not jointly investigate white matter microstructure in athletes, a population at high risk for concussive and subconcussive head traumas, with those in typical emergency room (ER) patients. In this study, we determine DTI scalar metrics in both ER patients and scholastic athletes who suffered concussions and compared them to those in age-matched healthy controls. In the early subacute post-concussion period, athletes demonstrated an elevated rate of regional decreases in axial diffusivity (AD) compared to controls. These regional decreases of AD were especially pronounced in the cerebellar peduncles, and were more frequent in athletes compared to the ER patient sample. The group differences may indicate differences in the mechanisms of the concussive impacts as well as possible compound effects of cumulative subconcussive impacts in athletes. The prevalence of white matter abnormality in cerebellar tracts lends credence to the hypothesis that post-concussive symptoms are caused by shearing of axons within an attention network mediated by the cerebellum, and warrant further study of the correlation between cerebellar DTI findings and clinical, neurocognitive, oculomotor, and vestibular outcomes in mTBI patients.

Keywords: magnetic resonance, diffusion weighted imaging, acquired brain injury, fractional anisotropy, tract-based spatial statistics

INTRODUCTION

Over 1.7 million Americans suffer a traumatic brain injury (TBI) each year (1). Moderate to severe TBI can often be diagnosed early through computed tomography (CT) and conventional magnetic resonance (MR) imaging. Concussion, or mild TBI (mTBI), however, makes up the great majority of TBI, but cannot be reliably detected by CT or conventional MR imaging techniques (2), which remain the standard of care.

The severity of a concussive injury is assessed by clinical evaluation of symptoms (3). Many concussed patients have symptoms including headaches, fatigue, insomnia, depression, attention problems, and memory problems (3), and while the majority recover within a few weeks, nearly a third continue to have persistent post-concussive symptoms (4–6). Current assessment does not reliably predict which mTBI patients will go on to suffer post-concussive symptoms (7); therefore, objective quantification of concussive injuries is needed.

Diffusion weighted imaging methods, including diffusion tensor imaging (DTI), provide useful tools for probing microstructural white matter changes in mTBI (2, 8). Traumatic axonal injury (TAI) may be inferred from CT or conventional MRI due to its association with small hemorrhages that those modalities can detect, but DTI can more directly detect changes in white matter microarchitecture (2). Past studies have found changes in DTI scalar metrics such as fractional anisotropy (FA), radial diffusivity (RD), and axial diffusivity (AD) in both acute and chronic mTBI patients, indicating microstructural alterations to white matter even in cases where CT and MRI scans were negative (7, 10). In the acute phase, FA has been seen to increase overall, while RD and AD have been seen to decrease. At chronic time points, the opposite effects are seen, with decreases in FA and increases in RD and AD (2, 9). Abnormal DTI scalar parameter values have been associated with cognitive functioning in mTBI patients (2, 8, 10, 11).

Prior DTI investigations of white matter microstructure have generally not studied different populations with concussions. However, athletes make up a population at high risk for concussive and subconcussive head traumas whose physical manifestations of injuries may differ from those due to mechanisms typically seen in the emergency room, such as motor vehicle collisions, assaults, and falls. The present study characterizes the early subacute abnormalities in DTI metrics in both athletes and ER patients after concussion, and compares the findings. In addition to the voxel-wise and tract-wise group comparisons of the conventional DTI literature, we also apply individual-patient tract-level analysis for greater clinical relevance and better robustness to the spatial heterogeneity of the white matter abnormalities associated with concussion (2, 7).

METHODS

Subject Recruitment

This study was carried out in accordance with the recommendations of the institutional review board of the Weill Cornell Medical College (WCMC). All subjects gave written informed consent in accordance with the Declaration of Helsinki except, in the case of minors, legal guardians gave written informed consent with the assent of the subjects. The protocol was approved by the WCMC institutional review board, and data were collected from September of 2012 through September of 2016.

A concussion was defined as an event of blunt impact on the head with loss of consciousness (LOC), post-traumatic amnesia (PTA), or at least one of the following symptoms: dizziness, nausea, headaches, balance problems, blurred or double vision, or feeling dazed/confused. Although for the purpose of this research we did not rely on formal medical diagnosis of concussion necessary for clinical management of the injury (12), this definition is consistent with the guidance of the American Academy of Neurology (13). Eighteen scholastic athletes between ages 13 and 22 years were recruited for testing within 2 weeks of a concussion, as were 42 ER patients with concussion aged 7 years and older, of whom 18 were selected to match the age range of the athlete subjects. For the purpose of equity, athletes were enrolled independently of the level of contact involved in their participating sports. The recruited athlete and ER subjects were scanned for MR imaging as soon as possible. In addition, 38 control subjects aged 7 years and older with no prior history of head injury were recruited and received imaging, of whom 10 were selected to match the age range of the athlete subjects.

Subjects over 18 were required to have a high school diploma or GED; 18-year olds set to graduate high school on time were also included. Exclusion criteria for subjects were a prior history of eye disease, neurological/psychiatric conditions, or substance abuse (**Table 1**). Subjects with contraindications for an MRI were also excluded. For athletes and ER patients, additional exclusion criteria were acute intoxication at the time of the concussion and LOC or PTA for more than 24 h. Subjects' symptoms and cognitive performance were assessed with an extensive battery of tests as reported elsewhere (14, 15).

Magnetic Resonance Imaging

MR imaging was performed at WCMC on a 3T Siemens Trio scanner. In each imaging study, whole-brain diffusion imaging was performed using an echo-planar imaging sequence (TE = 85 ms, TR = 7,500 ms) with one $b = 0$ s/mm^2 scan and $b =$ 1,000 s/mm^2 in 64 diffusion directions. Imaging was performed with 128 × 128 × 60 cubic voxels of 2 mm dimensions.

Image preprocessing was performed using tools within the Functional MRI of the Brain (FMRIB, Oxford University, Oxford, UK) Software Library (16–18). Correction for eddy currents and subject motion was performed and registered to the $b = 0$ s/mm^2 volume using the FMRIB Linear Image Registration tool (19). Image volumes were checked for excessive subject movement between diffusion weighted images and were accepted if mean and median movement were <2 mm.

Non-brain voxels were then excluded using the FMRIB Brain Extraction tool (20). Using the diffusion-weighted data, a diffusion tensor model was generated using the FMRIB DTIFit algorithm, from which fractional anisotropy (FA), mean diffusivity (MD), radial diffusivity (RD), and axial diffusivity (AD) were

TABLE 1 | Exclusion criteria.

Basis	Details
Neurological diagnosis	Prior Diagnosis of one or more of the following: Stroke, multiple sclerosis, epilepsy, brain tumor/cancer, encephalitis, dementia, movement disorder, spontaneous nystagmus
Eye-sight abnormalities	Amblyopia, uncorrected myopia, uncorrected presbyopia, uncorrected farsightedness, astigmatism, color blindness, macular degeneration
Eye diseases	Cataracts, glaucoma, retinal disorder
Psychiatric history	Any of the following: history of psychiatric hospitalization, history of legal trouble for violence, use of psychotropic medication other than a stable dose of SSRI
Psychiatric diagnoses	Prior Diagnosis of one or more of the following: Bipolar disorder, eating disorder, substance abuse disorder, personality disorder, sleep disorder, depressive disorder, anxiety disorder, ADHD
Questionnaires	Pediatric Subjects: *T*-Score $\geq$ 70 on Conners 3 Inattention Index or Hyperactivity Index, or *T*-Score $\geq$ 65 on BAI-Y or BDI-Y. 18+ Subjects: *T*-Score $\geq$ 70 on CAARS ADHD Index, $\geq$27 on CES-D, $\geq$26 on BAI.
Alcohol/drug abuse	Any of the following: • Score $\geq$ 6 on alcohol consumption survey • Answering 3 of 7 yes on MINI for alcohol dependence • History of daily/almost-daily use of illicit or prescription drugs • Use of any illicit or prescription drugs in past week • Past hospitalization/rehab for drugs • Past loss of job or suspension/expulsion from school for drugs • Multiple alcohol- or drug-related citation or arrest
MRI contraindications	Metal in body, claustrophobia, possibility of pregnancy

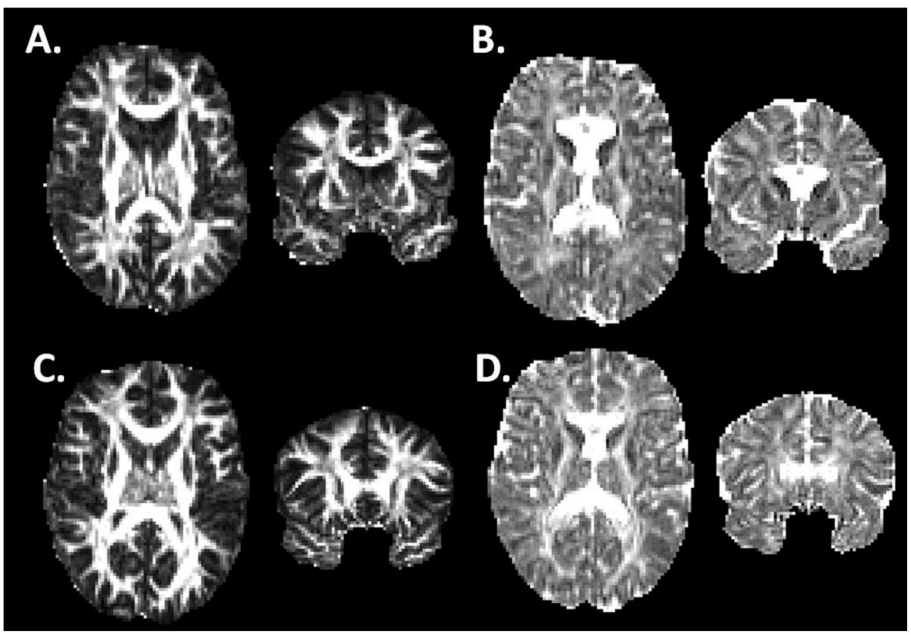

FIGURE 1 | Fractional anisotropy and Axial Diffusivity in Athlete and Control Brains. **(A,B)** Fractional Anisotropy **(A)** and Axial Diffusivity **(B)** maps for one of the athlete subjects in horizontal and coronal views. **(C,D)** FA and AD maps for one of the control subjects in horizontal and coronal views. The imaging data has undergone image preprocessing, but has not yet undergone non-linear coregistration to a common template.

determined at each voxel. Typical image quality is shown in **Figure 1**.

Tract-based Spatial Statistics (TBSS) were used to perform non-linear registration on the FA volumes to the FMRIB58_FA standard-space image, constructed from an average of 58 FA images taken of healthy subjects aged 20–50. After brain volumes were registered into a common space, a mean FA skeleton was generated using a threshold of FA $\geq$ 0.2 to limit the analysis to white matter voxels. TBSS alignment and white-matter skeleton generation was performed separately for each of the comparison groups (see *Statistical Analyses*) (21).

In addition to voxel-based analysis, masks were applied corresponding to the 27 white matter tracts previously labeled

TABLE 2 | Subject demographics.

	Athletes (18 subjects)	ER Patients (18 subjects)	Controls (10 subjects)
Age	13–22 years (17.7 ± 3.0)	13–25 years (17.1 ± 3.7)	13–25 yrs (21.2 ± 4.0)
Gender	9 Female/9 Male	11 Female/7 Male	5 Female/5 Male
Time after injury	9.4 ± 5.2 days (3–23 days)	10.4 ± 2.7 days (4–14 days)	N/A
Inclusion criteria	All athlete subjects	Age-matched to athletes	Age-matched to athletes/ER patients

in the Johns Hopkins University (JHU) white-matter atlas (16), with bilateral tracts collapsed. Within each of these regions of interest (ROI), mean values for the four DTI scalar parameters were calculated for each subject.

Statistical Analyses

Using the FMRIB Software Library randomize tool, permutation tests ($n = 5{,}000$) were performed to evaluate significant differences between groups on a voxel-wise basis, using Threshold-Free Cluster Enhancement, with correction for family-wise error. For each comparison group, permutation tests were performed for FA, MD, RD, and AD to control for false-positive voxels (22).

Mean FA, MD, RD, and AD within each of the 27 white-matter ROIs were compared between groups with two-sided t-tests, with false-detection rate (FDR) correction for the multiple comparisons. Comparisons were also made between the groups in terms of the number of individuals with extremely high or low parameter values for each of the four DTI metrics in each white matter tract. For this purpose, an abnormal parameter value was defined as >2.2 control group standard deviations above or below the control mean, which is the threshold used by Yuh et al. (7) in their DTI study of acute mTBI in ER patients. Significance of these group differences were determined with Pearson's χ^2 test.

RESULTS

Subject Demographics

Table 2 summarizes characteristics of the athlete and ER subjects. In our dataset, patient, and control ages were imperfectly matched, while differences in gender were not significant. Despite the best effort to have the recruited athlete and ER subjects scanned for MR imaging quickly, the timing of imaging since concussion ranged from 3 to 23 days, with an overall mean (SD) of 9.9 (4.1) days. There was no statistical difference in the timing of imaging between the athlete and ER groups.

Voxel-Wise Group Comparisons

No significant differences between athlete and ER subjects were found in the voxel-wise comparison. For athletes, significant regional decreases were observed compared to controls in AD (**Figure 2**). These differences were seen in the uncinate fasciculus, external capsule, posterior limb of the internal capsule, and regions of the thalamus. All three of the cerebellar peduncles also saw significant decreases in AD.

White Matter Tract Group Comparisons

Comparisons of white matter ROI means of DTI metrics demonstrated no significant group differences between athletes and controls or between ER patients and controls when adjusting for FDR ($q = 0.2$).

Outlier/Abnormal Tract Analysis Using Normative Control Subjects

For each athlete and control subject, the number of high outlier and low outlier regions were counted for each DTI parameter (**Table 3**). Athletes had more abnormally low FA and AD, and high RD ROIs. The finding of abnormal regional low AD was the only one which remained significant after applying a Bonferroni correction. In athletes, the most common tracts with abnormally low AD were the middle cerebellar peduncle (11 athletes), inferior cerebellar peduncle (8 athletes), superior cerebellar peduncle (8 athletes), uncinate fasciculus (7 athletes), and the superior fronto-occipital fasciculus (5 athletes).

The comparison of white matter ROIs in ER patients and controls showed similar trends, with significantly more ER patients demonstrating abnormally low FA and low AD in one or more ROIs compared to controls (**Table 4**). In ER patients, the most common tracts with abnormally low FA were the medial lemniscus (6 patients), posterior thalamic radiation (4 patients), and retrolenticular part of the internal capsule (4 patients). In ER patients, the most common tracts with abnormally low AD were the inferior cerebellar peduncle (6 patients), middle cerebellar peduncle (5 patients), and the uncinate fasciculus (4 patients).

Athletes vs. ER Patients

Given the above results, we also compared the 18 athletes against the 18 ER patients. For the outlier analysis, we treated the ER patients as "controls" for determining means and standard deviations in ROI.

No significant differences were seen between the patient groups in voxel-wise or tract-wise group comparisons. However, the outlier analysis at the individual-subject level showed a significant number of athletes with one or more tracts exhibiting extremely low AD compared to the group of ER patients (**Table 5**). The athletes had the most low-AD counts in the fornix/stria terminalis (4 athletes), middle cerebellar peduncle (4 athletes), and the superior cerebellar peduncle (3 athletes).

DISCUSSION

The present study shows early subacute post-concussion abnormalities in regional white matter microstructures in both scholastic athletes and ER patients. The abnormalities were observed as a greater frequency of extreme deviations in regionally defined DTI parameters, especially in the cerebellar peduncles of scholastic athletes, with our ROI-based outlier analysis. This method of analysis was modeled on that of (11) for chronic mTBI and Yuh et al. (7) for acute mTBI, in which high and low outliers in FA were compared

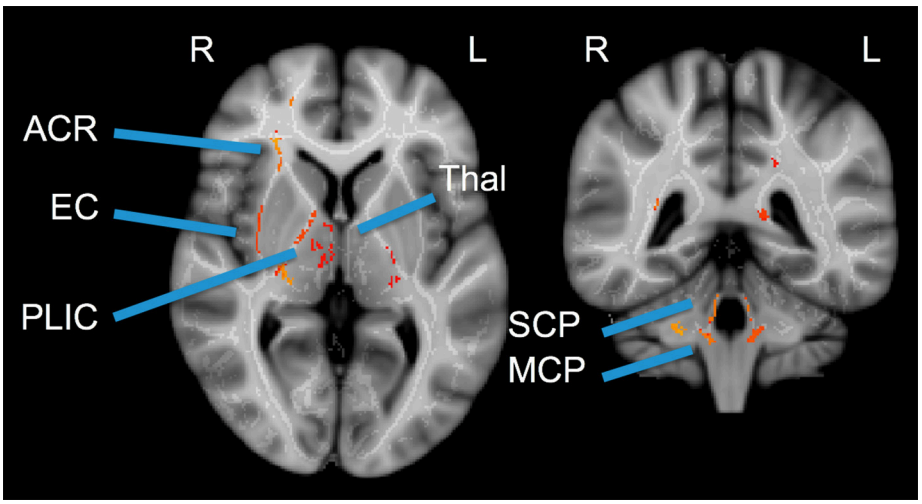

FIGURE 2 | Cross-sectional voxel-wise comparison: Control > Patient Axial Diffusivity. Two cross-sections illustrating regions of significant decrease in athlete AD (acute post-injury time point) compared to controls. TBSS Analysis with Threshold-Free Cluster Enhancement FWE corrected at $p < 0.025$. ACR, Anterior Corona Radiata; EC, External Capsule; PLIC, Posterior Limb of Internal Capsule; Thal, Thalamus; SCP, Superior Cerebellar Peduncle; MCP, Medial Cerebellar Peduncle.

TABLE 3 | Proportion of 18 athletes vs. 10 controls with one or more abnormally high/low DTI parameter value in a JHU atlas white matter tract.

	Contains ≥ 1 JHU tract more than 2.2 SD above control mean	**Contains ≥ 1 JHU tract more than 2.2 SD below control mean**
Fractional anisotropy	Control: 2 (20%) Athlete: 6 (33%) ($p = 0.47$)	**Control: 1 (10%)** **Athlete: 11 (61%) ($p = 0.007$)**
Mean diffusivity	Control: 1 (10%) Athlete: 6 (33%) ($p = 0.19$)	Control: 2 (20%) Athlete: 10 (56%) ($p = 0.07$)
Radial diffusivity	**Control: 0 (0%)** **Athlete: 8 (44%) ($p = 0.011$)**	Control: 1 (10%) Athlete: 7 (39%) ($p = 0.11$)
Axial diffusivity	Control: 1 (10%) Athlete: 5 (28%) ($p = 0.29$)	**Control: 2 (20%)** **Athlete: 16 (89%) ($p < 0.001$)**

P-values determined by Pearson's χ^2 test. Significant values in bold.

TABLE 4 | Proportion of 18 ER patients vs. 10 controls with one or more abnormally high/low DTI parameter value in a JHU atlas white matter tract.

	Contains ≥ 1 JHU tract more than 2.2 SD above control mean	**Contains ≥ 1 JHU tract more than 2.2 SD below control mean**
Fractional anisotropy	Control: 2 (20%) ER patient: 8 (44%) ($p = 0.21$)	**Control: 0 (0%)** **ER patient: 11 (61%) ($p < 0.001$)**
Mean diffusivity	**Control: 0 (0%)** **ER patient: 6 (33%) ($p = 0.04$)**	Control: 2 (20%) ER patient: 7 (39%) ($p = 0.32$)
Radial diffusivity	**Control: 0 (0%)** **ER patient: 9 (50%) ($p = 0.005$)**	Control: 1 (10%) ER patient: 5 (28%) ($p = 0.29$)
Axial diffusivity	Control: 1 (10%) ER patient: 8 (44%) ($p = 0.065$)	**Control: 2 (20%)** **ER patient: 11 (61%) ($p = 0.038$)**

P-values determined by Pearson's χ^2 test. Significant values in bold.

between controls and patient subsets (7, 11). This method of classifying individual patients as having or lacking abnormal tracts can account for the spatial heterogeneity of mTBI, which often confounds group comparisons at both the voxel and tract spatial scales. This patient-specific analysis is also much more clinically relevant than findings at the group level.

Of particular note are our results demonstrating abnormally low AD both in the athlete and ER cohorts. The large majority of athletes had at least one white matter tract with a markedly

TABLE 5 | Proportion of 18 athletes vs. 18 ER patients with one or more abnormally high/low DTI parameter value in a JHU atlas white matter tract.

	Contains ≥ 1 JHU tract more than 2.2 SD above ER mean	**Contains ≥ 1 JHU tract more than 2.2 SD below ER mean**
Fractional anisotropy	Athletes: 7 (39%) ER patients: 4 (22%) ($p = 0.29$)	Athletes: 7 (39%) ER patients: 3 (17%) ($p = 0.15$)
Mean diffusivity	Athletes: 4 (22%) ER patients: 4 (22%) ($p = 1$)	Athletes: 5 (28%) ER patients: 2 (11%) ($p = 0.22$)
Radial diffusivity	Athletes: 5 (28%) ER patients: 3 (17%) ($p = 0.44$)	Athletes: 6 (33%) ER patients: 3 (17%) ($p = 0.26$)
Axial diffusivity	Athletes: 3 (17%) ER patients: 4 (22%) ($p = 0.68$)	**Athletes: 9 (50%)** **ER patients: 3 (17%) ($p = 0.034$)**

P-values determined by Pearson's χ^2 test. Significant values in bold.

low AD value, and while these low values were spatially heterogeneous, there was a striking consistency of having them in at least one of the three cerebellar peduncles. Some ER patients showed abnormally low AD values as well. Our direct comparison of ER patients and athletes suggests that those with sports concussions may be more prone to abnormalities in the cerebellar peduncles than typical ER concussion patients. The group differences may indicate differences in the mechanisms of the concussive impacts as well as possible compound effects of cumulative subconcussive impacts in athletes.

Our results are consistent with a prior DTI study showing decreases of white matter AD in male scholastic American football players at both 24 h and 8 days after concussion (23); however, that investigation focused on cerebral tracts and did not specifically investigate the cerebellum. A recent meta-analysis has shown variable findings from various DTI studies of sports concussions that may relate to differences in techniques and in the timing of imaging relative to the injury (24). Despite such differences, our study substantiates previous reports that suggest selective effects of TBI on the cerebellar peduncles—cerebellar white matter volume was found be reduced in children years after a TBI, implicating lasting cognitive and behavioral consequences (25), and FA alteration in cerebellum-related white matter tracts and associated cognitive deficits were found in acute-phase adult mTBI patients (26) and chronic phase combat veterans exposed to mTBI (27).

Neuropathological studies have shown cerebellar Purkinje cell loss in boxers (28) as well as in mice, non-human primates and combat veterans exposed to blast injuries (29, 30). The anatomic arrangement of the cerebellar peduncles as relatively unsupported bridges of white matter between the bulk of the cerebellum and the brainstem renders them uniquely vulnerable to shear stress from acceleration/deceleration effects, as shown in biomechanical studies (31–35).

These findings in the cerebellar peduncles provide some support to the hypothesis of predictive brain state disruption in TBI (36). By this hypothesis, the collection of clinically observed post-concussive symptoms could be explained by timing-related disruptions in an attention network mediated by the cerebellum (36), which was supported by a study that combined DTI, neurocognitive tests, and eye movement measurements (37). It should be noted that our analysis was data-driven across all of the major white matter tracts of the cerebrum and cerebellum; therefore, it was not influenced by any hypothesis regarding the location of injury such as the cerebellar peduncles. To further test the predictive brain state hypothesis, future studies can and should attempt to correlate cerebellar DTI findings to clinical, neurocognitive, oculomotor, and vestibular outcomes relevant to cerebellar function. This can pave the way toward targeted rehabilitation strategies aimed at improving the affected domains, especially attentional function which is one of the most disabling impairments in activities of daily living and can be ameliorated using goal-oriented mindfulness training regimens (38, 39).

A concern specific to our dataset is the wide window of "early subacute" post-concussion scan latencies. The differences between DTI parameters 3 days and 23 days after a concussion could be significant (9). The ROI-based outlier analysis proved to be a useful workaround for the range and variability in the timing of MR scan. Another limitation of the present study is that age matching between patients and controls was imperfect. On the whole, the control cohort was older than the concussion cohorts, as the total pool of control subjects was small. Since there are non-linear age-dependent changes in DTI measures that are also region-dependent, controlling for age by statistical regression is difficult (40). A linear regression, even done on a tract-by-tract basis, would not add any clarity to the results. Therefore, we did not attempt to use regression to adjust for age differences. However, the average age difference between the controls and mTBI patients is not expected to produce meaningful differences in the DTI measures, especially for most of the white matter tracts shown here to be significantly different between mTBI subjects and controls. Of special note, cerebellar white matter consists of early-maturing tracts (41) that reach their asymptotic DTI metric values during the first decade of life, with no significant changes in FA or MD during the rest of childhood or adolescence (42, 43). In particular, DTI metrics of all three cerebellar peduncles bilaterally plateau at 70 months of age (44). Therefore, no age-related changes in the cerebellar peduncles would be expected in the range of 13–25 years of the subject groups that we studied. Instead, any age-related differences between groups would have been observed in slower-maturing cerebral white matter, such as that of the frontal lobes. Finally, overall sample size was small. A larger statistical power may reveal more subtle group differences.

We did not exclude patients based on lesions that would be visible on routine clinical MRI. However, these otherwise healthy young athletes suffered concussions that rarely produce visible

brain lesions, especially not in the cerebellum. Also, as our results are not explained by one or two outliers, we believe our results cannot be explained by lesions that could be detected by routine clinical MRI.

In designing future studies of athlete populations, it would perhaps be best to design image acquisition protocols that allow for more advanced diffusion models than DTI. For instance, neurite orientation dispersion and density imaging (NODDI) uses high angular resolution diffusion data with multiple *b*-values, and applies a three-compartment model that takes into account the neural microstructure in describing the diffusion pattern within a voxel (45). The NODDI model rests on certain assumptions regarding normal brain physiology, so in studying pathology such as mTBI, it is possible that the resulting NODDI parameters do not accurately describe the underlying brain architecture. However, Palacios et al have recently shown greater sensitivity in mTBI when studying changes in NODDI parameters compared to the DTI parameters analyzed in this study (46).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the institutional review board of the Weill Cornell Medical College (WCMC). All subjects gave written informed consent in accordance with the Declaration of Helsinki except, in the case of minors, legal guardians gave written informed consent with the assent of the subjects. The protocol was approved by the WCMC institutional review board.

AUTHOR CONTRIBUTIONS

JM, JG, and PM: conception and design. JM and JG: acquisition. JMM, EP, and PM: analysis. JMM, EP, JM, JG, and PM: interpretation.

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Longitudinal Assessment of Cortical Excitability in Children and Adolescents With Mild Traumatic Brain Injury and Persistent Post-concussive Symptoms

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Introduction: Symptoms following a mild traumatic brain injury (mTBI) usually resolve quickly but may persist past 3 months in up to 15% of children. Mechanisms of mTBI recovery are poorly understood, but may involve alterations in cortical neurophysiology. Transcranial Magnetic Stimulation (TMS) can non-invasively investigate such mechanisms, but the time course of neurophysiological changes in mTBI are unknown.

Objective/Hypothesis: To determine the relationship between persistent post-concussive symptoms (PPCS) and altered motor cortex neurophysiology over time.

Methods: This was a prospective, longitudinal, controlled cohort study comparing children (8–18 years) with mTBI (symptomatic vs. asymptomatic) groups to controls. Cortical excitability was measured using TMS paradigms at 1 and 2 months post injury. The primary outcome was the cortical silent period (cSP). Secondary outcomes included short interval intracortical inhibition (SICI) and facilitation (SICF), and long-interval cortical inhibition (LICI). Generalized linear mixed model analyses were used to evaluate the effect of group and time on neurophysiological parameters.

Results: One hundred seven participants (median age 15.1, 57% female) including 78 (73%) with symptomatic PPCS and 29 with asymptomatic mTBI, were compared to 26 controls. Cortical inhibition (cSP and SICI) was reduced in the symptomatic group compared to asymptomatic group and tended to increase over time. Measures of cortical facilitation (SICF and ICF) were increased in the asymptomatic group and decreased over time. TMS was well tolerated with no serious adverse events.

Conclusions: TMS-assessed cortical excitability is altered in children following mild TBI and is dependent on recovery trajectory. Our findings support delayed return to contact sports in children even where clinical symptoms have resolved.

Keywords: transcranial magnetic stimulation, mild traumatic brain injury, pediatrics, persistent post-concussive symptoms, cortical silent period

INTRODUCTION

Mild traumatic brain injury (mTBI) is a significant health concern due to its frequency and the possibility that it can contribute to long-term morbidities (1–4). The pediatric population is at the highest risk of incurring a mTBI (5, 6) and ∼30% of children will go on to have prolonged symptoms lasting 4 weeks or longer (7), referred to as Persistent Post-Concussive Symptoms (PPCS) (8–10). Children with PPCS often have difficulty returning to school and sport, and experience a significant negative impact on quality of life of both the child and family (11, 12).

Disturbances of cortical excitability and neurophysiology may be involved in the pathogenesis of ongoing post-concussive symptoms. Current understanding of the pathophysiology of mTBI includes a cascade of cellular damage that may result in excitotoxicity, neuronal death, and cellular energy crisis (13–15). Underlying many of these consequences are changes to ion concentration (16, 17) and thus, resting membrane potential (17). As resting membrane potential is altered, so too is the conductivity of the surrounding neurons. Alterations to membrane potential in broader cortical regions may have a global effect on cortical excitability.

Transcranial magnetic stimulation (TMS) is a useful modality for measuring neurophysiological changes after various forms of brain injury increasing our understanding of how the brain responds and changes after injury (18–20). TMS studies in adults with mTBI and PPCS have demonstrated acute alterations in primary motor cortex excitability (21–26). Findings have varied across studies but potential changes include alterations in cortical inhibitory systems such as the prolongation of the cortical silent period (cSP) (21, 22) or reduction of long interval intracortical inhibition (LICI) (23, 24, 27). Longitudinal studies examining how these alterations change over time however have been limited and none include children.

We conducted a prospective, longitudinal, controlled cohort study to determine the relationship between motor cortex excitability, and post-concussive symptoms over time in children with mTBI. We hypothesized that measurements of cortical excitability would be associated with severity of post-concussive symptoms, and that these would change over time as symptoms decreased.

METHODS

Population

Participants were recruited in the setting of a randomized controlled trial of melatonin for the treatment of PPCS following pediatric mTBI performed in the Complex Concussion Rehabilitation Program at the Alberta Children's Hospital (28) (clinicaltrials.gov/NCT01874847). Children (aged 8–18 years) were included if they presented to the Emergency Department or Concussion Clinic with a mTBI or concussion (diagnosed by a physician *and* a history of a mechanically-induced alteration of consciousness, or change in neurological function satisfying the ACRM criteria for mTBI (7, 12, 29) and persistent PPCS at 1 month post injury. Persistent PPCS was defined as an increase in post-concussive symptoms by at least 10 points compared to baseline using the Post-Concussion Symptom Inventory (PCSI). Children were not eligible if they had a Glasgow Coma Score of <13, loss of consciousness >30 min, a previous head injury in the last 3 months, the injury was due to an assault, or if there was alcohol or illicit substance use at the time of injury. Children with a significant past medical history (e.g., epilepsy, moderate/severe developmental delay, inflammatory bowel disease) or psychiatric history (e.g., hospital admission, regular follow-up by a psychiatrist, or requiring the use of psychiatric medications) or any contraindications to TMS (30).

Eligible families were contacted by telephone at 4 weeks post injury (**Figure 1**). Typically developing controls were recruited from friends and siblings of mTBI participants who satisfied all exclusion criteria including no history of TBI. Controls were recruited to maintain equal proportions of age and sex to the case population. Participants in the TBI groups were seen at 1 and 2 months post-injury and were not receiving any study medications. Each session included symptom evaluation and TMS neurophysiology. Control participants were seen at 1 month post-injury. Written informed consent was obtained from the parents of each participant, as well as verbal assent from the participant themselves. This study was approved by the University of Calgary Conjoint Health Research Ethics Board (REB13-0372).

Clinical Outcome and Classification

The Post-Concussion Symptom Inventory (PCSI) is a validated questionnaire of 26 symptoms using a Likert scale (0–6), that provides an overall score of PPCS symptoms (range 0 to 156) (8, 31). It has four clinical domains; somatic, cognitive, affective and sleep. As PCSI symptoms are common in healthy populations, an assessment of the pre-injury PCSI score was obtained at enrollment (4 weeks post injury). Participants were designated as "symptomatic" if the PCSI score was increased by 10 or more points compared to the pre-injury PCSI score. A smaller cohort of children with clinical recovery by 4 weeks post injury was also recruited. This "asymptomatic" group had PCSI scores at or below the pre-injury score. Participants completed the PCSI at the initial TMS session (1 month post-injury), and at the follow-up TMS session at 2–3 months post injury. At follow-up, the symptomatic group was classified as "recovered" if their PCSI returned to pre-injury levels or below.

TMS Neurophysiology Measures

Participants attended the Alberta Children's Hospital Pediatric Non-invasive Brain Stimulation Laboratory and were oriented to the TMS procedures. Participants could watch a movie of their choice while seated comfortably. Ag-AgCl electrodes (Kendall; Chicopee, MA, USA, 1.5-cm radius) were placed on first dorsal interosseous (FDI) muscles bilaterally to record surface electromyograms (EMG). Grounds were attached to a wrist band. EMG signals were amplified 1,000x and band-pass filtered from 20 to 2,000 Hz and then digitized at a rate of 5,000 Hz using CED1401 hardware and Signal 6.0 software (Cambridge Electronic Design, Cambridge, UK).

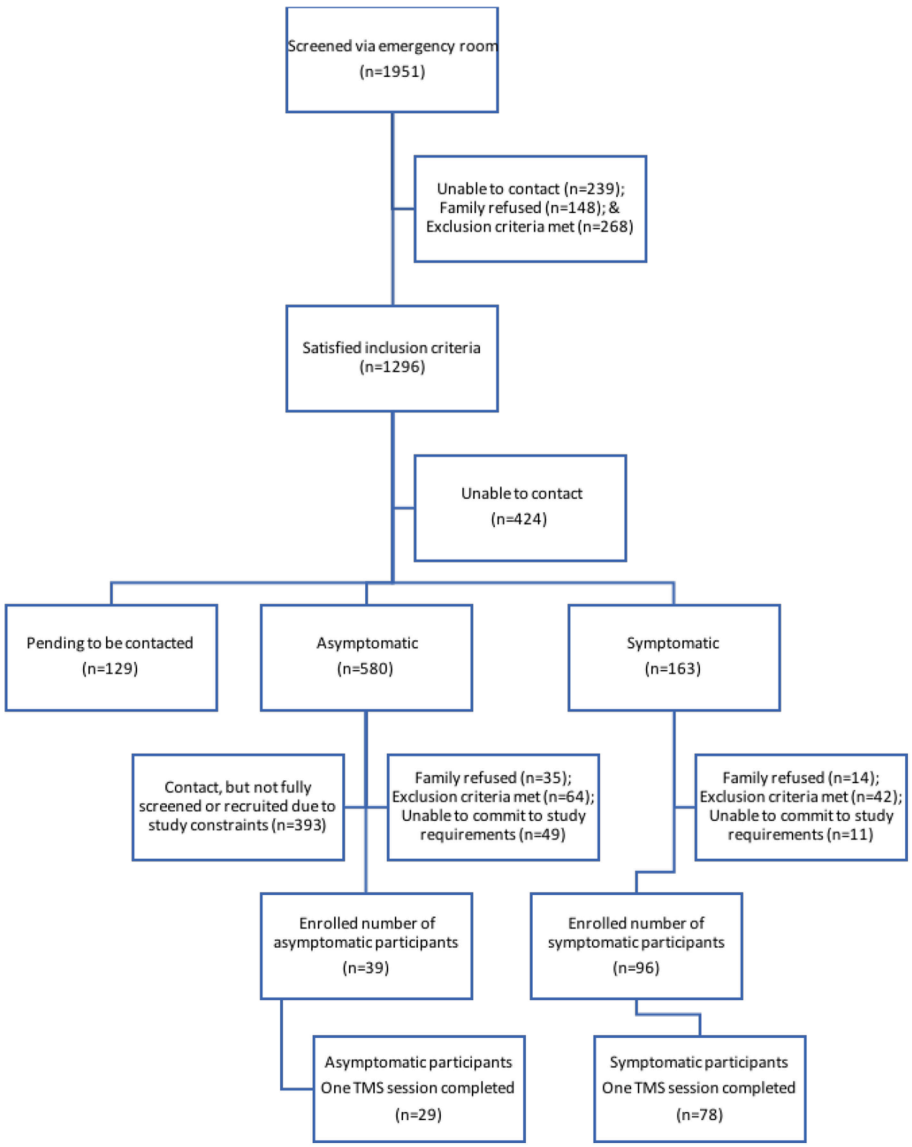

FIGURE 1 | Participant recruitment flow. Schematic of individuals at each stage of the recruitment process. Final boxes show participants from each TBI group who completed the first TMS session.

Single-Pulse TMS

Assessment of motor cortex neurophysiology was performed by eliciting motor evoked potentials (MEPs) in the dominant FDI muscle using single pulse TMS of contralateral primary motor cortex (M1). TMS was performed with a figure-of-eight coil (70 mm) connected to a Magstim Bistim^2 stimulator (Magstim; Dyfed, UK) which induced posterior-anterior currents in the dominant motor cortex while recording bimanual FDI. The optimal location or 'hotspot' that produced the largest and most consistent MEP was determined using suprathreshold intensities, which were then reduced. The hotspot was marked on a standard T1 MRI using neuronavigation (Brainsight 2, Rogue Research, Montreal, CA) and used for all further testing. For active TMS trials, FDI contraction was held at 20 or 50% maximum voluntary contraction with continuous visual feedback via an oscilloscope (GDS-1022, GwINSTEK, Taiwan) with the full-wave EMG rectified and smoothed (100 ms time constant, Neurolog NL703EMG Integrator, Digitimer UK).

Following localization of the motor hotspot, the resting motor threshold (RMT) was determined per published standards (32) as the minimum stimulator intensity (percentage of maximum stimulator output, MSO) required to elicit >50 μV FDI motor-evoked potentials (MEP) in 5/10 consecutive trials. MEP were recorded by Signal software and imported into MATLAB R2014b (Mathworks, Inc., Natick MA) for scripted analysis. The EMG from each paradigm was manually inspected for artifacts and pre-stimulation muscle activation, with poor quality frames removed. EMG traces were blinding prior to manual analysis to eliminate

experimenter bias. All MEP analyses were performed using Matlab scripts developed by our team.

Single Pulse TMS Outcomes

Stimulus Response Curve (SRC)

Ten single pulse TMS stimulations at each of six intensities (100–150% RMT, in steps of 10%) were delivered in random order to produce recruitment curves. MEP were recorded bilaterally from FDI muscles. Recruitment curves were analyzed by measuring peak to peak amplitude of 10 MEP from six intensities (100–150% RMT, in steps of 10%) then averaged to produce a sigmoidal input/output curve.

Cortical Silent Period (cSP)

Fifteen suprathreshold stimuli were applied with a three second separation while the dominant hand was contracted at 20% maximum voluntary contraction. The silent period was defined as a continuous disrupted EMG waveform beginning after the end of MEP waveform, continuing until the return of the background EMG waveform.

Ipsilateral Silent Period (iSP)

Ten suprathreshold (120% RMT) TMS stimuli were applied during 50% maximal voluntary contraction in the non-dominant hand (ipsilateral to the stimulated M1). Silent periods were measured synonymously to cSPs. The start of the silent period was manually determined as the point at which the EMG trace dropped below 25% of the background. The end of the silent period was determined when the EMG trace returned above 25% of the normal background. Silent periods were averaged to produce a mean silent period duration.

Paired Pulse TMS Outcomes

Paired-pulse TMS used two stimulators (Bistim^2 and 200^2 Magstim, UK) connected by an adaptor. Paired pulses were separated by an interstimulus interval (ISI) between an initial conditioning stimulus (CS, 80% RMT), followed by a test stimulus (TS, 120% RMT). Paired pulse outputs were expressed as a ratio of CS:TS, with >1 indicating facilitation and <1 indicating inhibition.

Short Interval Intracortical Inhibition/Intracortical Facilitation (SICI/ICF)

Ten unconditioned TS were randomly intermixed with 10 paired stimulation each at 10 ms ISI for ICF and 2 ms ISI for SICI (total of 30 stimulations). *Long Interval Intracortical Inhibition (LICI):* Here, the CS and TS were separated by a 100 ms ISI. Ten CS-TS pairs were randomized with ten TS alone. *Short Interval Intracortical Facilitation (SICF)*: Ten unconditioned and 10 conditioned stimuli at each of three separate ISIs (1.5, 2.6, 4.3 ms) were delivered in random order (40 stimulations in total).

Safety and Tolerability

After each session, participants completed a previously developed pediatric TMS tolerability questionnaire (33) that documented the presence and severity of five common potential side effects (headache, unpleasant tingling, neck pain, nausea, and light-headedness) (34). Participants were also asked to rank their TMS experience against 7 other common childhood experiences.

Statistical Analyses

The sample size was estimated as 24 per group using the cSP data from Miller et al. (23). Demographic characteristics were compared between experimental and control groups using student *t*-tests and Chi-square tests. Between group contrasts of SRC data indicated no significant changes in activation across sessions (using a two-way ANOVA). One sample *t*-tests were performed to determine the presence of inhibition or facilitation, as per our stipulated paired pulse ratio criterion. Partial correlations were used to analyse the relationship between change in cortical excitability and PCSS symptoms controlling for the PCSI pre-injury scores. We used generalized linear mixed models (GLMM) were used to analyse the relationships between Group and Time characteristics in a linear mode (cSP and iSP) and log-normal mode (SICI, LICI, SICF, and ICF) in our neurophysiological data. Time and group were fitted as fixed factors and participant as a random factor. Where there was an effect of time, GLMM in logistic mode (with Satterthwaite approximation) was used to analyze the relationship between recovery and cortical excitability change in the symptomatic group. Analyses were performed using SigmaPlot (version 13.0) and SPSS (version 24) software.

RESULTS

Population

Details of participant recruitment are shown in **Figure 1**. Population demographics are summarized in **Table 1**. One hundred and sixty-one participants were enrolled, and 133 had measurable and complete stimulus response curves allowing them to complete the entire study protocol. The final experimental sample consisted of 107 participants (78 symptomatic and 29 asymptomatic) with a median age of 15.1 (range: 9.0–18.0 years, 57% female). A smaller experimental population completed a second session of TMS (see **Table 1**). The healthy control population included 26 participants (median age = 14.6 years, range: 9.9–18.0 years, 54% female). The median age of the experimental groups was comparable, as was the ratio of males to females.

Single Pulse TMS

Stimulus Response Curves

Resting SRCs were obtained from all participants across each experimental group. Sigmoidal curves are shown in **Figure 2**. Responsiveness to increased TMS intensity were similar across groups at the initial measurement ($p = 0.5$), and at follow-up ($p = 0.9$).

Cortical Silent Periods

CSP was measured in a total of 124 participants (29 asymptomatic, 69 symptomatic, 26 control). cSP did not differ between groups overall, [$F_{(2, 121)} = 0.281$, $p = 0.281$]. Mean MEP amplitude, which may effect cSP, did not differ

TABLE 1 | Population demographics. Demographic characteristics and recovery status[a].

	Control	Asymptomatic	Symptomatic
N at Session 1	26	29	78
N at Session 2	-	9	54
Age ± SD, Range	14.6 ± 3, 9–18	14.2 ± 2, 9–17	15.2 ± 2, 9–17
Gender, F%	54	48	60
Handedness, R%	88	86	90
Cause of Injury %			
• Sport		82	71
• Fall		12	7
• MVA		3	10
• Other		3	12
PCSI Δ 1mo (median)	-	1	28
PCSI Δ 2mo (median)	-	4	3
Cognitive Domain Score	0.55 (1.2)	0.93 (1.6)	11.8 (8.1)
Physical Domain Score	1.6 (2.4)	2.3 (3.4)	16.2 (10.4)
Emotional Domain Score	0.75 (1.3)	0.65 (1.5)	7.2 (6.2)
Fatigue Domain Score	0.85 (1.0)	0.9 (1.8)	5.4 (4.1)

[a] *TBI participants were divided into 2 groups; symptomatic and asymptomatic. Mean age was expressed in years, ± standard deviation. Dominant handedness was reported as % right hand dominant. PCSI scores were expressed as the median change from baseline. Asterisk indicates significantly different from controls.*

between groups. There was no effect of age or sex on cSP change over time. Generalized linear mixed-effects model demonstrated shorter cSP durations (18.84 (SD 2.82) ms) in the symptomatic group compared to asymptomatic group at the initial timepoint [$F_{(1,\ 167)} = 4.838$, $p = 0.029$], with a trend for cSP duration to increase over time (11.36 (SD 2.60) ms; [$F_{(1,\ 84.51)} = 3.27$, $p = 0.074$]. There was significant subject heterogeneity (random intercept) accounting for 22% of the variance (95% CIs: 12.14, 36.18). Recovery in the symptomatic group at 2–3 months post injury could be predicted by cSP duration at 1 month (OR 1.029, $p = 0.049$) and change in cSP over time (OR.984, $p = 0.006$). Although the overall model was significant (LR Chi (2) = 9.78, $p < 0.001$), the effect size was small.

Ipsilateral Silent Periods

ISP was measured in a total of 123 participants (28 asymptomatic, 69 symptomatic, 26 healthy controls). Significant differences in iSP across all groups were observed [$F_{(2,\ 119)} = 3.828$, $p = 0.024$]: Symptomatic EM = 18.044 (95% CI: 16.092, 19.996), Asymptomatic EM = 14.150 (95% CIs: 10.823, 17.478), Control EM = 16.263, (95% CIs 12.433, 20.093). Generalized linear mixed-effects modeling demonstrated longer iSP durations in the symptomatic group [$F_{(1,\ 112)} = 4.052$, $p = 0.046$] but no effect of time [$F_{(1,\ 76)} = 0.977$, $p = 0.324$] (**Figures 3C, D**).

Paired Pulse TMS

Short Interval Intracortical Inhibition

SICI was evaluated in 131 participants (30 asymptomatic, 76 symptomatic, 25 control). An average inhibitory effect consistent with SICI was present in all groups [$t_{(25)} = 4.372$, $p < 0.001$]. Generalized linear mixed-effect modeling revealed significant differences in SICI at 1 month post injury between groups, [$F_{(2,\ 128)} = 3.752$, $p = 0.026$]: Symptomatic EM = 0.164 (95% CI:0.132, 0.202), Asymptomatic EM = 0.277 (95% CIs:0.198, 0.388), Control EM = 0.161, (95% CIs.112, 0.233) (**Figure 4A**). Change over time was analyzed in the TBI groups. Here, the effect of Group was not significant [$F_{(1,\ 179)} = 1.005$, $p = 0.317$] but there was a significant effect of Time post injury [$F_{(1,179)} = 18.746$, $p < 0.001$] and a significant Group × Time interaction [$F_{(1,\ 179)} = 18.746$, $p < 0.007$] (**Figure 4B**). Logistic regression, however, failed to demonstrate a relationship between recovery and change in SICI over time in the symptomatic group (LR chi(2) = 0.59, $p = 0.744$).

Long Interval Intracortical Inhibition

LICI was evaluated in 128 participants (30 asymptomatic, 73 symptomatic, 25 control). A significant inhibitory effect consistent with LICI was present in each group [$t_{(25)} = 4.372$, $p < 0.001$]. There were no significant difference in LICI between groups, [$F_{(2,\ 125)} = 0.678$, $p = 0.509$]. Over time, GLMM demonstrated no effect of mTBI group [$F_{(1,\ 174)} = 1.453$, $p = 0.230$] or Time post-injury [$F_{(1,\ 174)} = 0.623$, $p = 0.431$].

Intracortical Facilitation

SICF was measured in 120 participants (28 asymptomatic, 69 symptomatic, 23 control). The SICF effect was present at both ISI in the control group at 1 month (ISI 1.5 ms, $p = 0.05$; 4.3ms, $p = 0.02$) (**Figure 5**). There was a significant effect of Group [$F_{(2,\ 117)} = 5.46$, $p = 0.005$] on the SICF effect: Asymptomatic estimated mean (EM) = 0.348 (95%CI:0.262, 0.463), symptomatic EM = 0.206 (95% CI:0.172, 0.247), and control EM = 0.297 (95% CI:0.217, 0.406), see **Figure 5**. Change was analyzed using a generalized linear mixed effects model, there was no effect of mTBI Group [$F_{(1,\ 168)} = 0.021$, $p = 0.885$] on the SICF at 1.5 ms ISI effect but a significant effect of Time [$F_{(1,\ 168)} = 9.064$, $p = 0.003$]. There was a significant Group × Time interaction effect, [$F_{(1,\ 169)} = 10.719$, $p = 0.001$]. There were similar effects of Group and Group × Time on SICF at 2.6ms and 4.3 ms (data not shown). Change in SICF at 1.5 ms ISI however was not related to recovery in the symptomatic group ($B = -0.959$, $p = 0.423$).

A typical range of facilitation was observed in all groups using the 2 ms ICF paradigm ($n = 131$). There was a significant effect of Group at 1 month post injury, [$F_{(2,\ 128)} = 4.076$, $p = 0.019$]: Symptomatic EM = 0.328 (95% CI:0.273, 0.395), Asymptomatic estimated mean (EM) = 0.492 (95%CI:0.367, 0.660), and control EM = 0.271 (95% CI:0.196, 0.374), see **Figure 6**. When change was analyzed with GLMM, there was no longer an effect of Group [$F_{(1,\ 179)} = 0.785$, $p = 0.377$], but ICF decreased significantly over time [$F_{(1,\ 179)} = 11.826$, $p = 0.001$] with a Time × Group interaction effect [$F_{(1,\ 179)} = 6.347$, $p = 0.013$]. (**Figure 6**). Change in ICF in the symptomatic group however was not related to recovery in the symptomatic group ($B = 1.226$, $p = 0.072$).

Tolerability

No serious adverse events were reported. TMS was well-tolerated with sessional tolerability measures summarized in **Table 2**. Mild headache was reported in 13% of participants with a higher proportion in the symptomatic group (27%). This

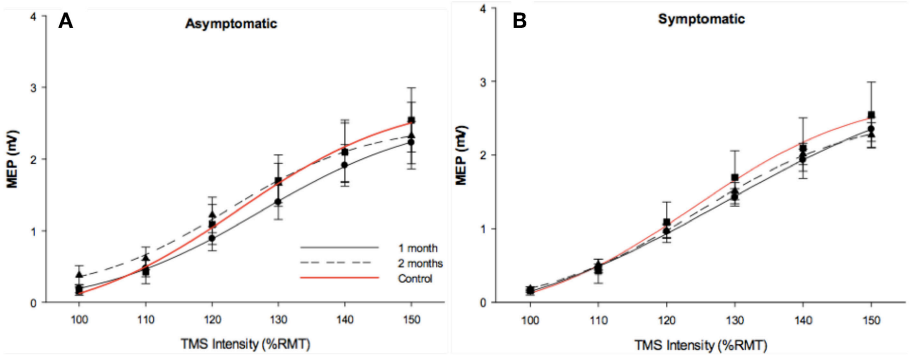

FIGURE 2 | Stimulus response curves**.** Stimulus response curves from dominant FDI muscle in 1 month post injury (solid black line), 2 months post injury (dotted black line). Control data (red line) was included in each figure as a reference. Longitudinal asymptomatic data shown in plot A ($n = 29$), symptomatic data in plot B ($n = 78$). Group means were comparable across session.

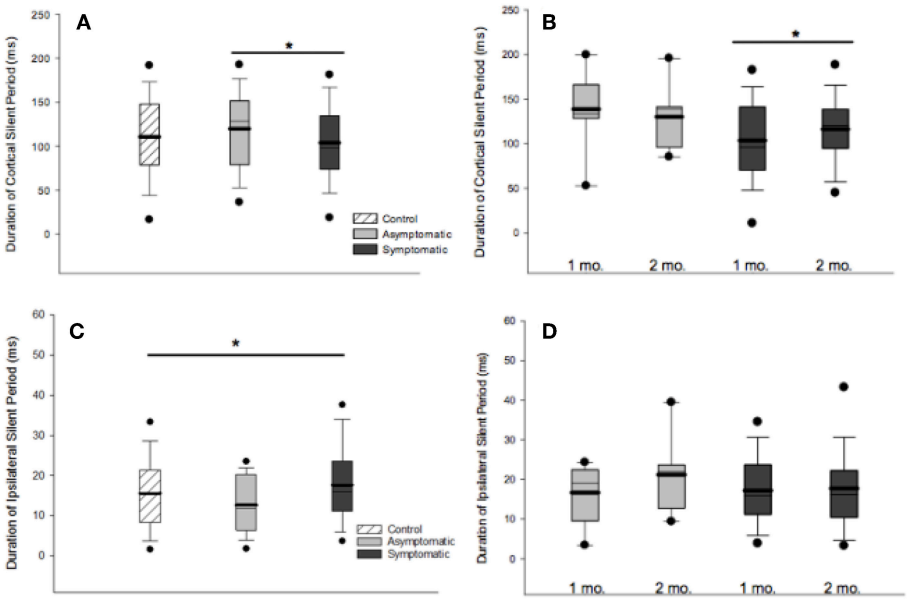

FIGURE 3 | Cortical and ipsilateral silent periods. **(A)** Comparison of cSP duration across groups at 1 month post injury found shorter cSP duration in the symptomatic compared to the asymptomatic group. **(B)** cSP durations increased over time in the symptomatic group at 1 month than 2 months post injury [$F_{(1, 167)} = 4.838$, $p = 0.029$]. **(C)** iSP durations differed across groups at 1 month post injury, [$F_{(2, 119)} = 3.828$, $p = 0.0240$]. **(D)** Generalized linear model mixed-effects modeling demonstrated longer iSP durations in the symptomatic group [$F_{(1, 112)} = 4.052$, $p = 0.046$] but no effect of time [$F_{(1, 76)} = 0.977$, $p = 0.324$]. For all boxplots, thick line is mean, thin line is median, edges are quartiles, and whiskers are 5 and 95th percentiles. *$p < 0.05$.

difference at 1 month did not persist to the session completed at 2-months post-injury.

DISCUSSION

In this study, we have characterized multiple aspects of motor cortex neurophysiological change in children recovering from mTBI and PPCS. The cSP is shortened at 1 month in the symptomatic children and increases over time. Overall, parameters associated with cortical inhibition (cSP and SICI) were more likely to be relatively increased in asymptomatic children. Conversely, parameters associated with cortical excitation/facilitation (SICF and ICF) were decreased in symptomatic children. Taken together, these findings suggest that longitudinal neurophysiological measurements, via TMS, over the motor cortex suggest a demonstrable change in cortical excitability between symptomatic and asymptomatic PPCS groups. Our findings substantially add to current understanding of neurophysiological alterations that occur following a pediatric mTBI, and support the safety and tolerability of TMS in this population.

There is a paucity of research regarding the neurophysiological changes occurring in PPCS in adults and especially children. Increased cSP duration has been

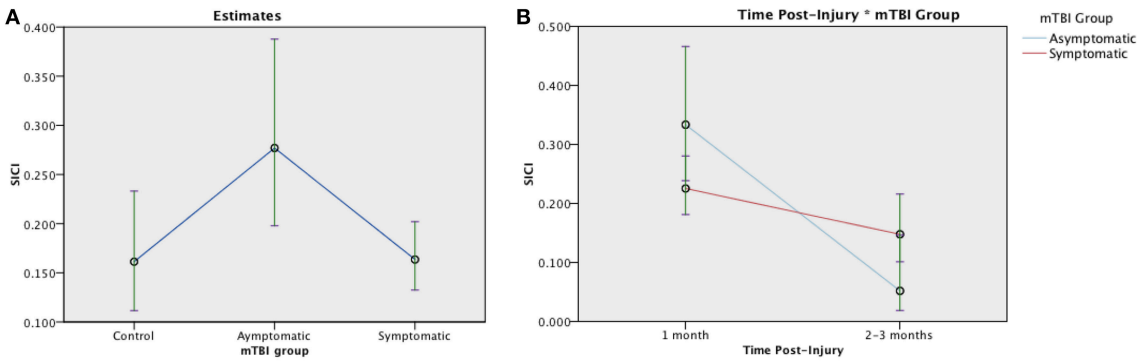

FIGURE 4 | Estimated means charts for SICI. SICI is expressed as a ratio of conditioning stimulus (CS, 80% RMT) to test stimulus (TS, 120% RMT) at interstimulus interval of 2 ms. Values <1 indicate inhibition. **(A)** Significant differences were found in SICI between groups at 1 month post injury, [$F_{(2,\ 128)} = 3.752$, $p = 0.026$]. **(B)** Generalized linear mixed model analysis demonstrated a significant effect of Time and Time × Group interaction on SICI in mTBI groups corrected model [$F_{(3,\ 179)} = 8.135$, $p < 0.001$]. Although both the symptomatic (red) and asymptomatic (blue) groups show decreasing SICI over time, the trajectory is steeper in the asymptomatic group. The error bars represent 95% confidence intervals of the estimated means.

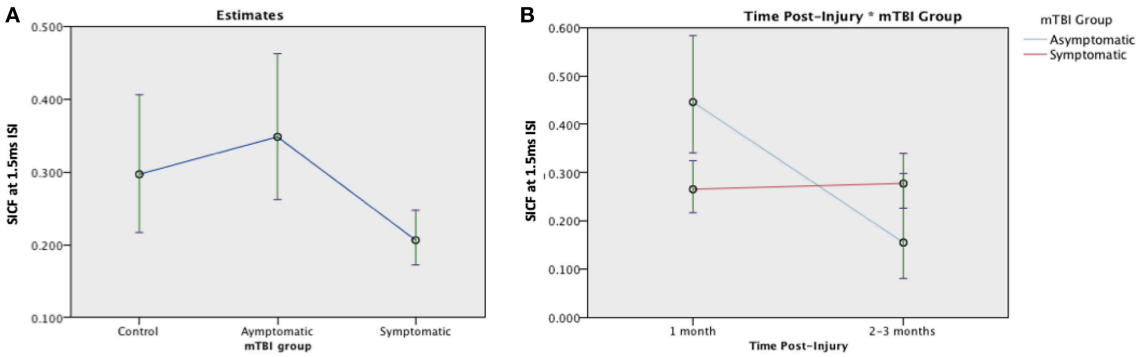

FIGURE 5 | Estimated means charts for SICF effect at 1.5 ms ISI. **(A)** The SICF effect differed significantly across groups [$F_{(2,117)} = 5.46$, $p = 0.005$]. **(B)** There was a significant effect of Time and a Group × Time interaction effect, corrected model [$F_{(3,168)} = 6.058$, $p = 0.001$]. The error bars represent 95% confidence intervals of the estimated means.

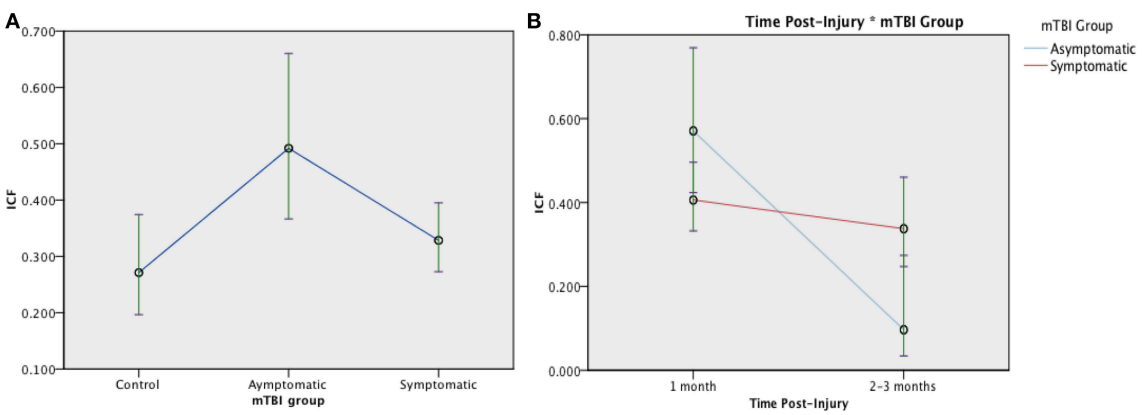

FIGURE 6 | Estimated means charts for ICF. ICF is expressed as a ratio of conditioning stimulus (CS, 80% RMT) to test stimulus (TS, 120% RMT) at interstimulus interval of 10 ms. **(A)** There was a significant effect of Group at 1 month post injury, [$F_{(2,\ 128)} = 4.076$, $p = 0.019$]; **(B)** There was a significant effect of Time and Time × Group interaction on ICF [corrected model $F_{(3,\ 179)} = 9.154$, $p < 0.001$]. Although both the symptomatic (red) and asymptomatic (blue) groups show decreasing ICF over time, the trajectory is steeper in the asymptomatic group. The error bars represent 95% confidence intervals of the estimated means.

TABLE 2 | Tolerability. Longitudinal tolerability data[b].

	Healthy	Asymptomatic	Symptomatic	X^2	*P* (2)
Headache					
1mo	1 (4)	4 (12)	21 (27)	8.1	0.02
2mo	–	4 (30)	9 (12)	0.6	0.4
Neck Pain					
1mo	1 (4)	5 (15)	11 (14)	2.2	0.3
2mo	–	1 (8)	17 (24)	0.9	0.3
Tingling					
1mo	0 (0)	5 (15)	11 (14)	4.3	0.1
2mo	–	1 (8)	10 (14)	0.03	0.9
Nausea					
1mo	0 (0)	2 (6)	4 (5)	1.5	0.5
2mo	–	2 (15)	3 (4)	0.9	0.3
Lightheaded					
1mo	2 (8)	1 (3)	8 (10)	1.6	0.4
2mo	–	2 (15)	3 (4)	0.9	0.3

[b] *Tolerability values were expressed as the total number of participants in each group that reported each side effect. The number in parentheses represented the corresponding percentage of the group.*

TABLE 3 | Summary of results.

	Control	Asymptomatic	Symptomatic
N at Session 1	20	32	72
N at Session 2	-	12	71
cSP			
Mean (SD) T1	110.52 (8.9)	120.38 (8.4)+	103.24 (5.4)+
Mean (SD) T2	-	134.16 (11.87)	113.55 (5.4)
iSP			
Mean (SD) T1	15.62 (1.9)	14.15 (1.7)*+	18.04 (0.9)*+
Mean (SD) T2	-	19.29 (3.3)	18.12 (1.3)
LICI			
Mean (SD) T1	0.06 (0.01)	0.08 (0.008)	0.06 (0.008)
Mean (SD) T2	-	0.05 (0.06)	0.15 (0.03)
SICI			
Mean (SD) T1	0.16 (0.03)	0.28 (0.05)*+	0.16 (0.02)*+
Mean (SD) T2	-	0.0.5 (0.03)	0.15 (0.03)
SICF			
Mean (SD) T1	0.30 (0.05)	0.35 (0.05)	0.21 (0.02)
Mean (SD) T2	-	0.16 (0.05)	0.28 (0.03)
ICF			
Mean (SD) T1	0.27 (0.04)	0.49 (0.07)*+	0.33 (0.03)*+
Mean (SD) T2	-	0.1 (0.05)	0.34 (0.05)

* *indicates significantly different from control group.* + *indicates significantly different between symptomatic and asymptomatic groups.*

reported previously in adults with mTBI (21, 22, 24, 35). The only other longitudinal TMS study following 15 adults with acute mTBI reported increased cSP durations at 72 h post-injury and persisted beyond 8 weeks post injury (23). Although not reported specifically, these adults seem to have experienced normal recovery trajectories with symptom scores similar to controls by 1-month post-injury i.e., similar to our asymptomatic group (21, 27, 36). Tremblay et al. (25) Other research in asymptomatic adults with a history of mTBI found increased cSP durations from 9 months to 30 years post-injury (19, 21, 23, 25). Interestingly, the cSP duration increased over time in our symptomatic group i.e., becoming similar to the asymptomatic group. However, this was not significantly related to clinical PPCS scores or recovery. Further longitudinal studies are required to elucidate the trajectory of cSP duration over time.

Short-interval intracortical inhibition was increased in the asymptomatic group and changed significantly over time. This finding is somewhat in keeping with the changes in cSP reported above and support the cortical origin of the neurophysiological changes. Indeed, inhibitory interneurons have been shown to be particularly vulnerable to injury in TBI (37). In contrast, long-interval intracortical inhibition was not significantly different between groups in our study. Previous research in smaller samples of asymptomatic athletes with concussion has reported both enhanced LICI (25) and no significant differences to controls (25). LICI and cSP have often been thought to operate by similar cellular and neurotransmitter mechanisms including alterations in $GABA_b$ receptor-mediated processes (38, 39). However, studies in other populations of acquired brain injury and neurodegeneration have suggested a dissociation between the neuronal systems reflected in these two paradigms (38, 39). Although Tremblay et al. report possible metabolic imbalances between GABA and glutamate concentrations in previously concussed athletes using MR spectroscopy (25), further well-powered research is required using multimodal imaging to tease out whether this occurs in mild TBI.

Our previous pilot study examining cortical excitability at 1 month post injury found reduced LICI in children with persistent symptoms following mTBI (40). The current study follows on from this. It is likely that the main reason for the discrepant finding is likely due to sample size, especially as logarithmic transformation can underestimate the variability in the sample. This is particular important as children with mTBI have greater variability in motor cortex neurophysiology (33). This has been addressed in our current larger study by employing generalized linear mixed effect modeling in order to take into account the between and within subject variability and maximize sample size.

In keeping with changes in cortical inhibition, we also found significant differences in intracortical facilitation (ICF and SICF). This is the first study to examine SICF in pediatric mTBI. Increased ICF was present in the asymptomatic group, and decreased SICF in the symptomatic group with both groups changing toward "normal" over time. Similar SICF results were found across the different ISI intervals which supports the legitimacy of this finding. SICF is also suggested to be mediated by GABAergic systems, with increased GABA activity resulting in reduced SICF (41). Loss of facilitation in the asymptomatic group, combined with prolonged cSP in the same population, might suggest underlying differences between the normal behavior of GABA in the motor cortex compared

with those of controls or symptomatic children. Interestingly, the rate of change was greatest in the asymptomatic group. This finding supports other recent literature suggesting that there is ongoing cerebral recovery despite resolution of clinical symptoms (42, 43). The change in ICF and SICF in the symptomatic group did not predict recovery of symptoms. This may be due to the slow rate of change in ICF and SICF in the symptomatic group during this time period. The next step to better elucidate these underlying mechanisms might be to quantify GABA levels using neuroimaging methodology such as MR spectroscopy. This technique would allow quantification of GABA release patterns over time, which may be involved in mediating changes in cortical neurophysiology (44).

Transcallosal inhibition was measured using iSP. Transcallosal tracts have been shown to be particularly sensitive to damage in TBI (45, 46). iSP values were significantly different between groups with the asymptomatic group having decreased transcallosal inhibition. Measurement of iSP in children has been infrequent and somewhat inconsistent, in part due to differences in methodology. We used 120% RMT, based on methods of 40, who found iSP to be associated with paired-pulse TMS measures of interhemispheric inhibition and motor performance in a pediatric population (47). Transcallosal injury provides a complex research target due to the elaborate circuitry involved. Future research into transcallosal injury using iSP, IHI and imaging measures of both structural and functional connectivity might better elucidate any alteration of interhemispheric interactions in children with mTBI.

Significant limitations of our study should be considered. Groups were comparable in age- and gender but a significant proportion lacked repeated measures, this decreased the power in the longitudinal component of our study (especially in the asymptomatic group) although this was somewhat limited by our statistical approach. Nevertheless, the intersession variability of TMS neurophysiology remains a significant challenge in this type of research (48). Pre-injury neurophysiological data was not available in our participants. Although it is it is possible that the TMS parameter changes could have been present pre-injury, the rate of change observed over time would suggest otherwise. No differences between the SRC measures of groups, or of sessions, were observed. Our population did not include children younger than 8 years of age as younger children often have thresholds too high to stimulate at 150% RMT. Our findings suggest that the cortical excitability properties reflected by SRC are similar across participants and perhaps not altered by mTBI. This finding suggests that SRC may not be sensitive to such changes and is perhaps a paradigm that is less likely to yield interesting information in future studies.

Despite these limitations, the power of our study was much higher than the previous work in this field. Additional strengths of our study included well-standardized TMS methodologies designed to deliver stimulus intensity based on each individual participants RMT, as well as a participant group displaying a broad range of PPCS phenotypes. TMS has been safe and well tolerated all participants, with no adverse events (35).

CONCLUSIONS

In summary, children with different recovery trajectories after mTBI show significant and complex alterations in TMS measures of cortical excitability which change during recovery. Cortical inhibition is increased in children who have early recovery whereas cortical excitation is decreased in children with persistent symptoms. Motor cortex neurophysiology changed significantly over time. These finding suggests that there is ongoing cerebral recovery at 1-month post-injury even where there is resolution of clinical symptoms. Further well-powered longitudinal studies of pediatric TBI can help to inform our knowledge and monitor neurophysiological recovery following pediatric mTBI.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical clearance was granted by the University of Calgary Conjoint Health Research Ethics Board (REB13-0372).

AUTHOR CONTRIBUTIONS

KB and AK conceived study design. RK and KB performed participant recruitment. RK, EZ, PC, and TS performed data collection. RK, KB, EZ, PC, and TS performed data analysis and drafting the manuscript. RK, AK, EZ, PC, TS, and KB revised the manuscript. KB and AK obtained funding.

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Chronic Neurobehavioral Sex Differences in a Murine Model of Repetitive Concussive Brain Injury

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Traumatic brain injury (TBI) resulting from repeated head trauma is frequently characterized by diffuse axonal injury and long-term motor, cognitive and neuropsychiatric symptoms. Given the delay, often decades, between repeated head traumas and the presentation of symptoms in TBI patients, animal models of repeated injuries should be studied longitudinally to properly assess the longer-term effects of multiple concussive injuries on functional outcomes. In this study, male and cycling female C57BL/6J mice underwent repeated (three) concussive brain injuries (rCBI) delivered via a Leica ImpactOne cortical impact device and were assessed chronically on motor (open field and rotarod), cognitive (y-maze and active place avoidance), and neuropsychiatric (marble-burying, elevated zero maze and tail suspension) tests. Motor deficits were significant on the rotarod on the day following the injuries, and slight impairment remained for up to 6 months. All mice that sustained rCBI had significant cognitive deficits on the active place avoidance test and showed greater agitation (less immobility) in the tail suspension test. Only injured male mice were significantly hyperactive in the open field, and had increased time spent in the open quadrants of the elevated zero maze. One year after the injuries, mice of both sexes exhibited persistent pathological changes by the presence of Prussian blue staining (indication of prior microbleeds), primarily in the cortex at the site of the injury, and increased GFAP staining in the perilesional cortex and axonal tracts (corpus callosum and optic tracts). These data demonstrate that a pathological phenotype with motor, cognitive, and neuropsychiatric symptoms can be observed in an animal model of rCBI for at least one year post-injury, providing a pre-clinical setting for the study of the link between multiple brain injuries and neurodegenerative disorders. Furthermore, this is the first study to include both sexes in a pre-clinical long-term rCBI model, and female mice are less impaired functionally than males.

Keywords: traumatic brain injury, mouse, concussion, repetitive brain injury, behavior, active place avoidance, microbleeds, sex differences

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INTRODUCTION

The effects of traumatic brain injury (TBI) are far-reaching, with a global incidence estimated in 2007 to be ∼10 million (1). In 2013, there were ∼2.5 million TBI-related emergency room visits in the United States alone (2). Although the majority of sustained TBIs are mild and classified as concussions (concussive brain injury; CBI), many of the millions of the survivors suffer persistent

post-injury complications that include cognitive, motor, and neuropsychiatric symptoms, and in some cases these issues can persist for over 10 years (3).

In recent years, more attention has been directed to the effects of repeated TBI, particularly in the context of military operations and contact sports (e.g., American football, soccer, boxing). Both military personnel and contact sports participants are at greater risk for exposure to multiple concussive and sub-concussive TBIs (repeated concussive brain injuries; rCBI). A recent study estimated the incidence of TBI in Iraq and Afghanistan veterans to be ∼17%, with half sustaining multiple head injuries (4). In the context of sports, head injuries are associated with multiple contact sports in both men and women (5), and in the National Football League up to 30% of players sustaining a concussion go on to receive repeat TBIs (6). Multiple TBIs are associated with delayed neurodegenerative conditions including chronic traumatic encephalopathy (7), a condition characterized by perivascular accumulation of hyperphosphorylated tau (8) and behavioral symptoms including (but not limited to) cognitive dysfunction, violence, depression, and suicidality (9, 10).

As there are no fully effective therapies to treat TBI, or rCBI specifically, translational studies remain of importance. Animal models that replicate at least a subset of the pathological and behavioral symptoms that are observed following clinical rCBI create a context in which therapies can be tested. Many functional symptoms (i.e., cognitive and neuropsychiatric problems) may not appear in human TBI patients for months or years following sustained injuries, and there is a critical need to develop chronic animal models with persistent functional and neuropathological symptoms to provide a context in which delayed treatments can be assessed. Many rodent models of rCBI have been developed and described, and many have clinical relevance with behavioral symptoms similar to rCBI patients, including cognitive deficits and/or neuropsychiatric changes and axonal damage [e.g., (11–16)]. However, most of the observations have been limited to more acute time periods (days, or up to a few months) following the injuries.

There are a small number of rCBI studies in mice that have extended observations to a year or longer following injuries and continue to report functional and neuropathological effects of rCBI (17–20). These studies, however, have been limited to male mice. Clinically, female athletes are at equal or greater risk of sustaining head injuries than males during participation in sports (21–26), and although many studies have demonstrated a poorer clinical outcome in women following CBI [e.g., (27, 28)], data remain inconsistent (29). Thus, there is a continued need to be inclusive of both sexes in pre-clinical rCBI research.

The goal of this study was to study sex differences in behavioral symptoms over a chronic period in a murine model of rCBI that results in axonal damage. Although prior studies included both male and female rodents in TBI behavioral studies [e.g., (15, 30–33)], most have employed the controlled cortical impact (CCI) brain injury model and evaluated outcomes at more acute time points. The injury model employed in this study has an advantage of being less invasive than traditional CCI and fluid percussion injury models that require craniectomy. Although this injury method does not include rotational acceleration, an important component in a majority of clinical head trauma cases, CBI allows for relatively consistent control of the mechanical effects of impact injury, which can be a disadvantage in at least some rotational injuries where there is no precise control of rotational effects due to inherent differences in head response.

METHODS

Animals and Housing

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences (Bethesda, MD). Male and female mice, 8 weeks old, were obtained from Jackson Laboratories (C57BL/6J, 000664; Bar Harbor, Maine) and group-housed in Association for Assessment and Accreditation of Laboratory Animal Care-Accredited facilities with a standard 12-h light-dark cycle, with food and water available *ad libitum*. Animals acclimated to facilities for at least 1 week prior to baseline behavioral testing.

Repetitive Concussive Brain Injury (rCBI) Procedures

Mice were randomly assigned to sham or injury groups. Mice in the injured group received three injuries at 24-h intervals. Concussive brain injury methods were performed as described by Velosky et al. (15). Briefly, mice were anesthetized with isoflurane and moved to a stereotaxic frame with anesthesia maintained via a flow-through nose cone. All fur was removed from the scalp with depilatory cream, Bregma was visualized under the skin with bright illumination and marked by a small dot with permanent marker. Anesthesia was discontinued with continuation of 100% oxygen, and a Leica ImpactOne impactor was immediately employed to deliver the injury to the scalp over parietal cortex (2.5 mm posterior to bregma and 2.5 mm left of bregma), with a 5-mm impact tip, 1.5 mm depth, 5.0 m/s velocity. Sham-treated mice underwent all procedures including the same duration of anesthesia, but the impactor was not activated.

Apnea and Righting Reflexes

Immediately following concussive impact (rCBI mice) or discontinuation of anesthesia (sham controls) each day, any occurrence of cessation of breathing was measured and recorded as duration of apnea. Following injury or sham procedures, each mouse was placed into an individual clean cage over a warming pad in a supine position, and the time to return to a prone position was recorded as the righting reflex. Each day after all injury and sham procedures were complete mice were returned to group-housing.

Body Weight Measurements

Baseline body weights were taken on the first day of injury, and mice were weighed at each behavioral testing time and on the day of euthanasia. Post-injury body weights are expressed as a percent of baseline measurements.

Behavioral Testing

The number of mice in each sex and injury group at each behavioral testing time point are listed in **Table 1**. Two male and

TABLE 1 | Number of animals (*n*) in each group and mean body weights (expressed as a percent of baseline weight, measured on the first injury day) at each behavioral testing time point.

	Post-injury Day 1		Post-injury Day 30		Post-injury Day 90		Post-injury Day 180		Post-injury Day 360	
	n	Weight (%)	*n*	Weight (%)	*n*	Weight (%)	*n*	Weight (%)	*n*	Weight (%)
Male—Sham	19	98.51	19	113.49	19	132.76	19	152.92	19	187.66
Male—rCBI	19	91.05**	19	106.33**	19	124.81**	19	139.69**	18	169.60**
Female—Sham	17	101.36	17	117.17	17	125.16	17	144.50	17	182.14
Female—rCBI	21	93.74	21	111.25	20	130.52	20	152.63	17	186.67

**Significant difference in weights between injured male and sham-treated male mice across all post-injury days ($p = 0.0064$).*

two female mice died due to rCBI procedures. Some mice were lost throughout the year of the study prior to the final behavioral test (tail suspension test), but the deaths were spontaneous (unexplained), or veterinarian-recommended euthanasias due to skin lesions. Thus, injury could not be determined to increase mortality rate during the 1-year study period. Open field (OF) and rotarod testing were performed multiple times during the experimental period of 1 year (see below); elevated zero maze (EZM), marble burying test (MBT), y-maze, active place avoidance (APA), and tail suspension test (TST) were only performed once at the end of the experiment.

The procedures for OF and rotarod testing are found in more detail in a previous publication (32). For 3 days prior to injury, mice were trained to perform on an accelerating rotarod (4–60 rotations/min over 3 min, three trials/day); average latency to fall from the rotating rod on the final day was recorded as baseline performance. Prior to the third day of rotarod baseline testing, mice were placed in a 40 × 40 cm OF box (∼5 Lux) with opaque walls connected to Any-Maze software (Stoelting Co., Wood Dale, IL) that tracked the position of the animal for 20 min. The software reported the total distance traveled in the arena, as well as the distance traveled in the center zone (20 × 20 cm), expressed as a percentage of the total distance traveled. Baseline OF testing was performed prior to rCBI; mice were re-tested in the OF and on the rotarod (in that order) on Days 1, 30, 90, 180, and ∼1 year following the final CBI.

The EZM and MBT were performed as previously described 1 year after the injury on the first and second days following rotarod and OF testing, respectively (33). The EZM (Stoelting) is an annular platform raised 50 cm above the floor, divided into two opposing quadrants that are darkened and enclosed (∼200 Lux) and the remaining two quadrants exposed and with greater amounts of light (∼1,600 Lux). Movements of the mice were tracked with Any-Maze software for 5 min. For the MBT, mice were placed in a clear Plexiglas box (45 cm long × 24 cm wide × 22 cm high) filled with wood shavings to a depth of 5 cm. Twelve glass marbles were placed in a rectangular shape 6.5–9.5 cm apart; mice were placed in the boxes for 30 min. At the end of the test session, an experimenter blinded to the injury status of the mice counted the number of marbles buried to at least 2/3 depth.

On the fifth day following rotarod and OF testing, the y-maze novel arm test of spatial episodic memory, based on rodents' preference for novelty (34), was performed. The y-maze (Stoelting) has three arms at a 120° angle to each other, with a triangular central zone. For the first 5-min trial, one of the arms is blocked and the mice can explore the remaining two arms. After a 2-h inter-trial interval, the mice are placed back into the apparatus and all three arms are available. A camera above the maze recorded the movements of the mouse and Any-Maze software calculated the percent of time the mouse spent in the novel (previously blocked) arm. A mouse will normally spend a greater percentage of time in the novel arm, given it remembers the apparatus from the first trial in which that arm was blocked.

Three days following the y-maze test, spatial learning was tested in the active place avoidance test (APA; BioSignal Group, Brooklyn, NY) using methods similar to those described by Sangobowale et al. (35). The mice were first habituated to the circular arena (40 cm diameter) for 10 min, after which they underwent four testing trials with approximately a 40-min inter-trial interval. During each 10-min trial, the arena rotated slowly (∼1 rotation/min); a fixed 60° segment of the apparatus was software-defined such that if the mouse entered that zone for longer than 500 ms an electric shock was applied (0.2 mA, 500 ms, every 1.5 s until the mouse left the zone). Thus, the mouse had to keep moving with the arena to avoid being shocked. Visual cues placed on the room walls around the arena facilitated spatial learning. On the following day, the 60° shock zone was rotated 180° from its location the previous day, and the mice had to learn to avoid the new location in four, 10-min trials.

The final test performed approximately a year following the injuries, 4 days following APA, was the TST. This test employed the procedures described by Can et al. (36). Mice were suspended by their tails from laboratory benches with tape (12 mm wide, 24 cm long) attached approximately 1 cm from the tip of the tail. To prevent tail-climbing, a 4-cm length of hollow polycarbonate tubing (1.3 cm inner diameter; McMaster-Carr, Santa Fe Springs, CA; #8585K41) was placed around the base of the tail prior to the test. Mice remained suspended for 6 min; sessions were videotaped, and the amount of time spent immobile was later scored by an investigator blinded to the injury condition of the animals.

Histology and Immunohistochemistry

Following all behavioral testing, mice were transcardially perfused with 4% paraformaldehyde and tissue was prepared for immunohistochemistry as previously described (15). Brains from 14 injured male and 15 injured female mice (and five sham mice from each sex) were processed for Prussian blue staining

combined with pararosaniline nuclear stain to detect regions of previous brain hemorrhages (manufacturer's instructions were followed for staining of mounted sections; Sigma-Aldrich, St. Louis, MO; HT20). Six mice from each injury/sex group were randomly selected for glial fibrillary acidic protein (GFAP) staining of free-floating sections as previously described (15), using a mouse monoclonal antibody (1:500, Thermo Fisher Scientific, MS-280-P) and a biotinylated secondary antibody (1:500; AffiniPure Goat Anti-Mouse IgG [H + L], Jackson ImmunoResearch Laboratories, 115-065-003) with DAB staining solution (Vector Labs, SK-4100). Sections were mounted onto slides, allowed to air-dry overnight and coverslipped.

GFAP-stained slides were scanned with a Zeiss Axio Scan.Z1 with Zen 2.5 blue edition software (Zeiss) and images of the corpus callosum (CC) and right and left optic tracts (OPT) and hippocampi (HP) were imported into ImageJ software. The measurement feature was employed to determine the mean gray density of areas of the CC, OPT and HP traced via freehand selection (**Figure 1**). An area with absence of immunostaining was selected from each section to remove background. Three sections per animal for each region were averaged to calculate average density values of GFAP staining (density = mean gray density – background). Assessments were performed by a single investigator blinded to the sex and injury condition of the images.

Cortical depth near the impact site was measured using methods described previously (15). The line tool in the Zen software was employed to draw a 1.75 mm horizontal line lateral from the superior sagittal fissure. A vertical line was then placed and measured at this location, tangential to the surface of the cortex and extending to the dorsal boundary of the corpus callosum (**Figure 1**, red dashed line, "d"). A 500 μm-wide region surrounding the vertical line, extending the depth of the cortex (CTX), was captured and imported into ImageJ for analysis of GFAP staining density as described.

Statistical Analyses

Statistical analyses were performed with SPSS 21.0 (IBM Corp., Armonk, NY, USA) or SAS Studio 3.71 (SAS Institute Inc., Cary, NC, USA). Righting reflex and cortical depth data did not pass the homogeneity of variance test (Levene's test of the equality of variances) and were analyzed with Kruskal–Wallis tests and adjusted pairwise comparisons (SPSS). Apnea data were analyzed with a mixed model ANOVA (PROC MIXED; SAS) with sex as a fixed factor and day as a repeated measure. For body weight data and behavioral tests performed at multiple time points, mixed model ANOVAs (PROC MIXED) were performed with sex and injury as fixed factors and time as a repeated measures factor. Distance traveled data from open field testing were converted to natural log values to meet homogeneity of variance requirements. Tests performed at one time point (y-maze, EZM, MBT, TST) were analyzed with two-way ANOVAs (PROC GLM; SAS), with sex and injury as fixed factors. GFAP staining density in the CC was also analyzed with a two-way ANOVA (sex x injury); OPT, CTX, and HP staining density were analyzed with three-way ANOVAs, with sex and injury as fixed factors and side (left vs. right) as a repeated measure. CTX GFAP data were converted to inverse values to meet homogeneity of variance requirements. Where *post-hoc* testing was necessary, Bonferroni-corrected planned contrasts were performed. Following statistically significant main effects or planned contrasts, effect size (Cohen's *d*) was calculated as $\left|\frac{\mu_1-\mu_2}{s_{pooled}}\right|$, where $s_{pooled} = \sqrt{\frac{s_1^2+s_2^2}{2}}$. Figures were made using Microsoft Excel 2016 and Daniel's XL Toolbox 7.2.13, and data shown in all figures represent the means ± standard error of the means unless otherwise specified.

RESULTS

Apnea and Righting Reflexes

Apnea was not observed in any mice following sham procedures; apnea in mice following CBI each day is shown in **Figure 2A**. Durations of apnea were equivalent between male and female mice and across injury days ($p > 0.1406$). Righting reflexes following sham and CBI procedures on the three injury days are shown in **Figure 2B**. Righting reflexes were longer in injured mice than in sham mice of the same sex on all three injury days [$H(3) = 56.069, 50.479, 49.442$, $p < 0.0001$ for injury days 1, 2, and 3, respectively]. There were no sex differences in righting reflexes in either the sham-treated mice or the injured mice on any injury days (adjusted $p = 1.0$).

Body Weights

There was a significant sex by injury interaction effect on post-injury body weights [$F_{(1, 73.2)} = 7.38$, $p = 0.0082$]. Bonferroni-adjusted planned contrasts revealed that post-injury, the body weights of injured male mice remained lower than the weights of sham-treated male mice ($p = 0.0064$, $d = 0.30$), but injury did not affect body weights of female mice ($p = 1.0$; **Table 1**).

Histological Findings

The brains from 29 injured mice and 10 sham mice underwent Prussian blue staining to detect microbleeds. Examples of Prussian blue staining in injured mice are shown in **Figure 3**. (No positive staining was seen in sham mice). Of the 29 injured mice, 11 of the mice were negative for Prussian blue staining (data not shown). Eleven animals (six females and five males) showed evidence of microbleeding only on the very surface of the brain near the impact site (**Figure 3A**), primarily observed in the glia limitans. An additional five mice (three males and two females) had surface bleeding with extension into cortical layers (**Figure 3B**), and two mice (one of each sex; **Figure 3C**) had more extensive injury, with evidence of cortex compression and bleeding that extended into the corpus callosum. Three more of the 29 mice, all female, had lateral/temporal bleeding near the rhinal sulcus as seen in **Figure 3D**. (Two mice had both mild surface bleeds and lateral bleeding). The more extensive injuries as assessed by Prussian blue staining were not associated with increased apnea or longer righting reflex times (data not shown).

Figure 4 is a summary of cortical atrophy, as measured by cortical thickness, and astrogliosis after rCBI. Analysis of cortical depth on the left side of the brain near the impact site showed

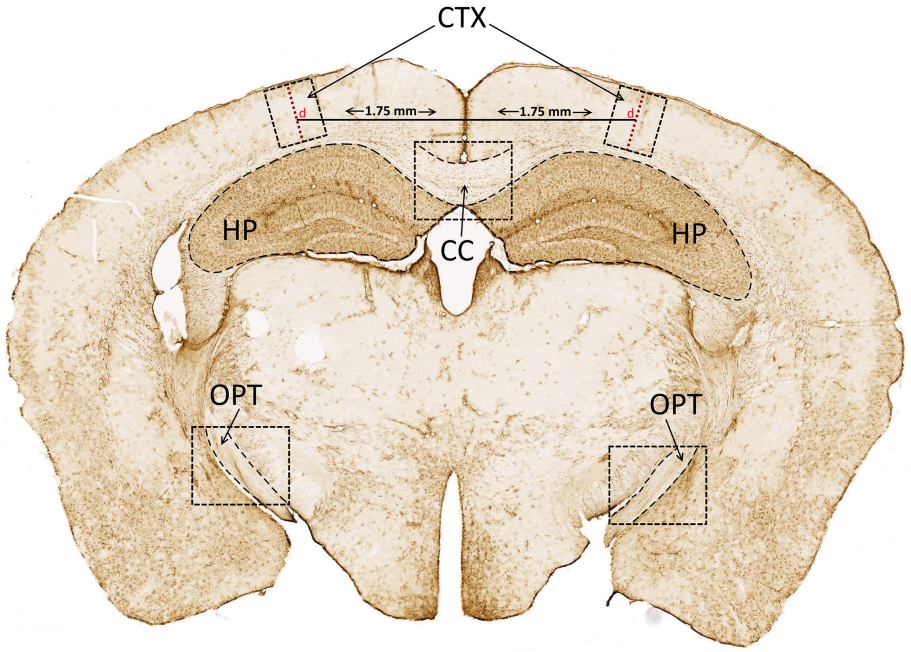

FIGURE 1 | Cortical depth (d) and GFAP analysis. Cortical depth (d; red dashed line extending from surface of cortex to dorsal boundary of corpus callosum) was measured 1.75 mm from the superior sagittal fissure. GFAP staining density was analyzed in the CC, and bilaterally in the CTX, HP, and OPT. GFAP, glial fibrillary acidic protein; CC, corpus callosum; CTX, cortex; HP, hippocampus; OPT, optic tracts.

a significant effect of group [H(3) = 16.786, $p = 0.001$]; both male and female injured mice had significantly thinner CTX at the injury site than respective sham controls ($p = 0.021$ and 0.026, respectively; **Figure 4A**). There was also increased GFAP staining density in the CTX beneath the impact site on the injured side of the brain [injury × side interaction effect: $F_{(1, 19)} = 15.36$, $p = 0.0009$; **Figure 4B**]. Astrogliosis was greater in the left CTX of injured mice than on the right side of injured mice ($p = 0.0012$, $d = 1.71$) and staining density in the injured left CTX was also greater than that in the left CTX of sham-control mice ($p < 0.0001$, $d = 2.01$). There was also a main effect of sex [$F_{(1, 19)} = 12.10$, $p = 0.0025$, $d = 0.41$], with female mice having greater levels of GFAP staining in the CTX than male mice (data not shown). Although there was an injury × sex interaction effect on GFAP staining in the HP [$F_{(1, 19)} = 5.01$, $p = 0.0373$], *post-hoc* tests did not reveal significant comparisons (male sham vs. male rCBI: $p = 0.2248$; female sham vs. female rCBI: $p = 1.0$; male rCBI vs. female rCBI: $p = 1.0$; male sham vs. female sham: $p = 0.0896$; data not shown).

However, rCBI increased astrogliosis in white matter tracts. Representative samples from each sex and injury group of GFAP staining in white matter tracts, the CC and OPT, are shown in **Figure 4C**. There was a significant main effect of injury on GFAP-immunostaining in the CC [$F_{(1, 20)} = 25.23$, $p < 0.0001$, $d = 1.89$]; mice that had sustained rCBI had greater GFAP-immunostaining in the CC than sham-treated mice. In the OPT, a three-way mixed-models ANOVA showed a significant main effect of injury on GFAP staining density [$F_{(1, 20.2)} = 21.54$, $p = 0.0002$, $d = 1.79$]. Injured mice had significantly greater amounts of GFAP staining in the OPT than sham-treated animals.

Open Field

In the OF, there was a significant injury × sex × day interaction effect for the distance traveled in the arena [$F_{(5, 354)} = 7.55$, $p < 0.0001$] (**Figure 5A**). Separate two-way ANOVAs (injury × day) were performed for each sex. In males, there was a significant injury × day interaction effect [$F_{(5, 179)} = 22.98$, $p < 0.0001$]; sham-treated and injured male mice were equally active in the OF during baseline testing and on the day following the final injury ($p = 1.0$), but injured male mice ambulated greater distances on all subsequent testing days compared to sham-treated male mice (days 30, 90, 180, and 360; $p < 0.0001$; $d = 1.28$, 1.47, 2.06, 2.02, respectively). In females, there was only a main effect of day [$F_{(5, 175)} = 28.29$, $p < 0.0001$]; the female mice were less active in the arena on all subsequent days compared to baseline testing ($p < 0.0001$, $d = 1.71$, 1.52, 1.58, 1.56, 2.46 for days 1, 30, 90, 180, and 360, respectively), and at the 1-year time point the female mice were less active when compared to day 1 ($p = 0.0078$, $d = 0.62$), day 30 ($p = 0.0001$, $d = 0.87$), and day 90 ($p = 0.0054$, $d = 0.71$).

There was an injury × day interaction effect on the distance traveled in the center zone of the OF (expressed as a percentage of the total distance traveled) [$F_{(5, 355)} = 7.03$, $p < 0.0001$; **Figure 5B**]. On the day following the final injury, injured mice were less active in the center of the arena than sham controls ($p = 0.0018$, $d = 0.98$). The effect on day 360 neared significance ($p = 0.0990$, $d = 0.44$), where injured mice were more active in the center of the arena.

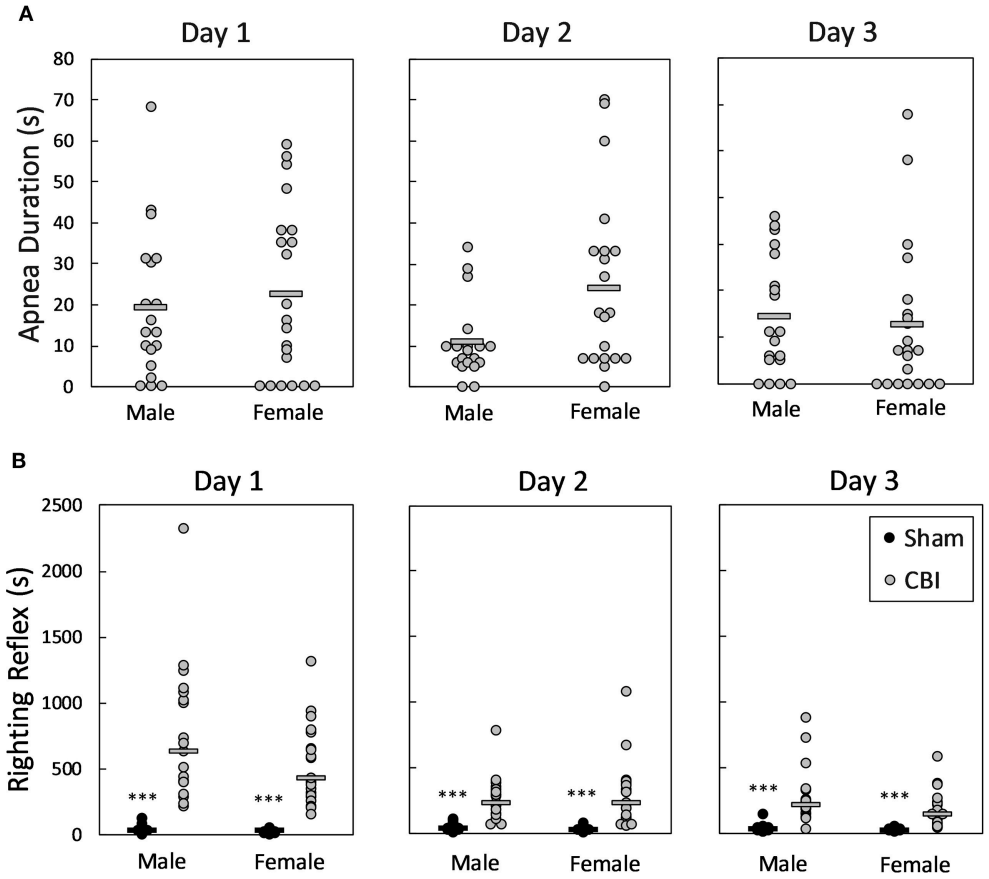

FIGURE 2 | Apnea **(A)** and righting reflexes **(B)** following CBI on each injury day. Shown are individual data points; bars in **(A)** represent group means, in **(B)** represent group medians. The legend in lower right panel applies to all plots. There were no significant differences between male and female mice in the duration of apnea following CBI on any of the injury days **(A)**. On each of the injury days, mice sustaining injury had significantly longer righting reflexes than sham-treated mice of the same sex **(B)**. The asterisk (***) in **(B)** represents a significant effect of injury in the given sex, where CBI > Sham, $p < 0.0001$. There were no significant differences in righting reflex times between male and female mice, either injured or sham-treated. CBI, concussive brain injury.

Rotarod

For rotarod testing (**Figure 5C**), there was a significant injury by day interaction effect [$F_{(5, 355)} = 11.96$, $p < 0.0001$] and a significant sex by day interaction effect [$F_{(5, 355)} = 3.93$, $p = 0.0017$]. Planned Bonferroni-corrected comparisons showed that there was a main effect of rCBI on rotarod performance on days 1 ($p < 0.0001$, $d = 1.65$), 90 ($p = 0.0138$, $d = 0.73$), and 180 ($p = 0.0402$, $d = 0.57$) following injury, with mice sustaining rCBI falling from the rotarod at shorter latencies than sham controls. Also, planned contrasts revealed that overall, female mice stayed on the rotarod longer than male mice at the 1-year time point (day 360; $p = 0.0096$, $d = 0.78$).

Elevated Zero Maze, Marble Burying Test, y-Maze

There was an injury by sex interaction effect on anxiety-like behaviors tested in the EZM 1 year following rCBI [$F_{(1, 68)} = 6.31$, $p = 0.0144$; **Figure 5D**]. Bonferroni-corrected planned contrasts showed that sham-treated females and injured females spent equivalent amounts of time in the open quadrants of the maze ($p = 1.0$), but injured male mice spent significantly more time in the open quadrants than the male sham-treated mice ($p < 0.0001$, $d = 1.48$). There were no effects of injury or sex on the number of marbles buried in the MBT ($p > 0.2966$; data not shown). Likewise, there were no effects of injury or sex, or interaction between the two factors on the percent of time spent in the novel arm in the y-maze test [$F_{(1, 67)} < 0.942$, $p > 0.335$; data not shown].

Active Place Avoidance

There was a main effect of sex on distance traveled during the 10-min acclimation trial of the APA test [$F_{(1, 66)} = 9.11$, $p = 0.0036$, $d = 0.73$], with females ambulating greater distances in the arena (data not shown). A mixed-models analysis (injury × sex × trial) showed a significant main effect of injury on spatial learning in the APA test on the first day of testing [$F_{(1, 49.4)} = 31.23$, $p < 0.0001$, $d = 1.34$; **Figure 5E**], and an injury x trial interaction effect on the second day when the shock zone was relocated [$F_{(3, 133)} = 9.89$, $p < 0.0001$]. Sham-treated mice had fewer entrances to the shock zone of the apparatus on all trials

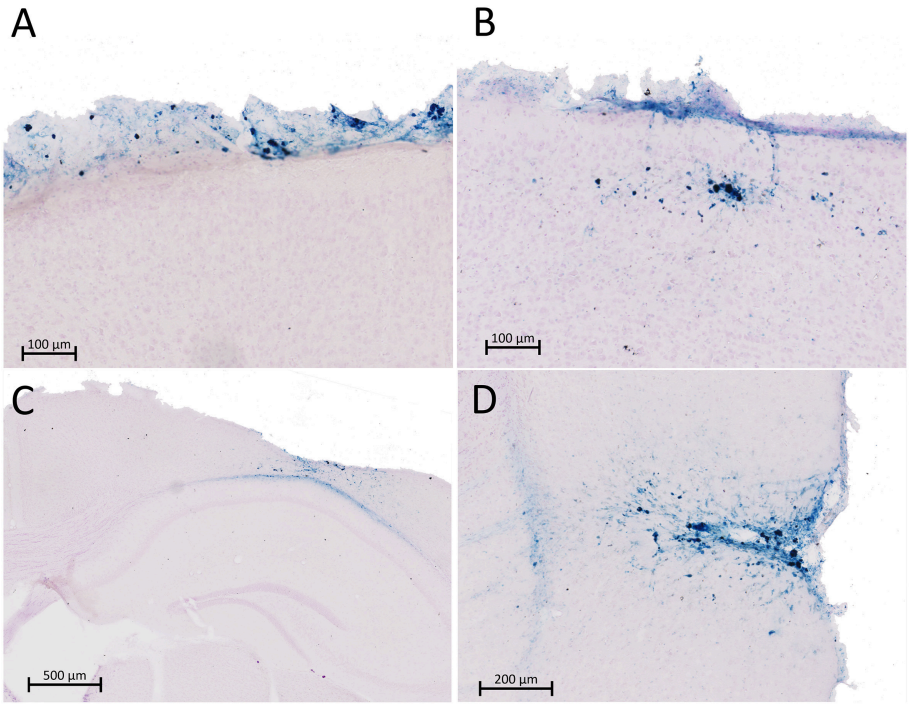

FIGURE 3 | Prussian blue (PB) staining was performed in 29 mice for microbleeds after rCBI. Sections shown in **(A–D)** represent four different animals that stained positive for microbleeds. Eleven injured mice were negative for PB staining (not shown). Most mice positive for PB staining presented with microbleeds limited to the surface of the brain in the glia limitans ($n = 11$; **A**) or extending into the most superficial layers of the cortex ($n = 5$; **B**). A very limited number of animals had more severe injury involving the corpus callosum ($n = 2$; **C**) or temporal regions of cortex ($n = 3$; **D**). Two mice had both mild surface bleeds **(A)** and temporal bleeding **(C)**.

on the first day of testing demonstrating quicker learning of the location of the zone; on the second day, injured mice had equal performance to sham controls on the first trial, but shams had fewer entries to the shock zone on the remaining three trials ($p < 0.0001$; $d = 1.33, 1.53, 1.62$ for trials 2, 3, and 4, respectively).

Tail Suspension Test

There was a significant effect of injury on immobility in the TST for depressive-like behaviors [$F_{(1, 67)} = 10.62$, $p = 0.0018$, $d = 0.78$; **Figure 5F**]; sham-control mice spent greater time in an immobile state than injured mice.

DISCUSSION

Summary of Pathological and Behavioral Findings Following rCBI

This study employed a mouse model to study the effects of repetitive concussive brain injuries on behavior and brain pathology in both male and female animals up to 1 year following injuries. A summary of significant findings is found in **Table 2**. Cortical atrophy was found at the lesion site (**Figure 4A**); our previous study in which mice were euthanized at a more acute time point (32 days) found equal cortical thickness (15), suggesting this is a chronic, degenerative process rather than an immediate effect of the impacts. GFAP staining was unaffected by injury in the hippocampus but increased in the perilesional cortex and axonal tracts (corpus callosum and optic tracts) in both male and female mice (**Figure 4B**). In addition, Prussian blue staining (**Figure 3**) showed that the injuries led to cerebral microbleeds primarily limited to the site of the injury and the cortex beneath, consistent with clinical findings following mild TBI [e.g., (37, 38)] and with prior rCBI studies in mice (39, 40). A subset of the mice also had positive Prussian blue staining distal from the primary injury site near the rhinal sulcus; Sauerbeck and colleagues recently reported pathology in similar locations following concussive-rotational injury (CHIMERA) (41).

Behaviorally, both male and female mice had motor impairments on the rotarod for up to 6 months following the concussions (**Figure 5C**), and both sexes had significant cognitive deficits at the 1-year time point on the APA task (**Figure 5E**). Injured male mice showed some behavioral differences compared to sham controls that were not seen in female mice: in the OF the injured male mice were hyperactive on post-injury days 30 and beyond (**Figure 5A**), and in the EZM 1 year following injury they spent greater amounts of time in the open/exposed quadrants (**Figure 3D**). Additionally, male mice sustained weight loss as a result of the injuries (**Table 1**), and their weights remained lower compared to uninjured mice for the duration of the study.

Axonal Injury and CBI

Axonal injury is a prominent feature of CBI, both clinically and in animal models (42, 43), and the CC may be particularly vulnerable to injury. Activated microglia, together with axonal

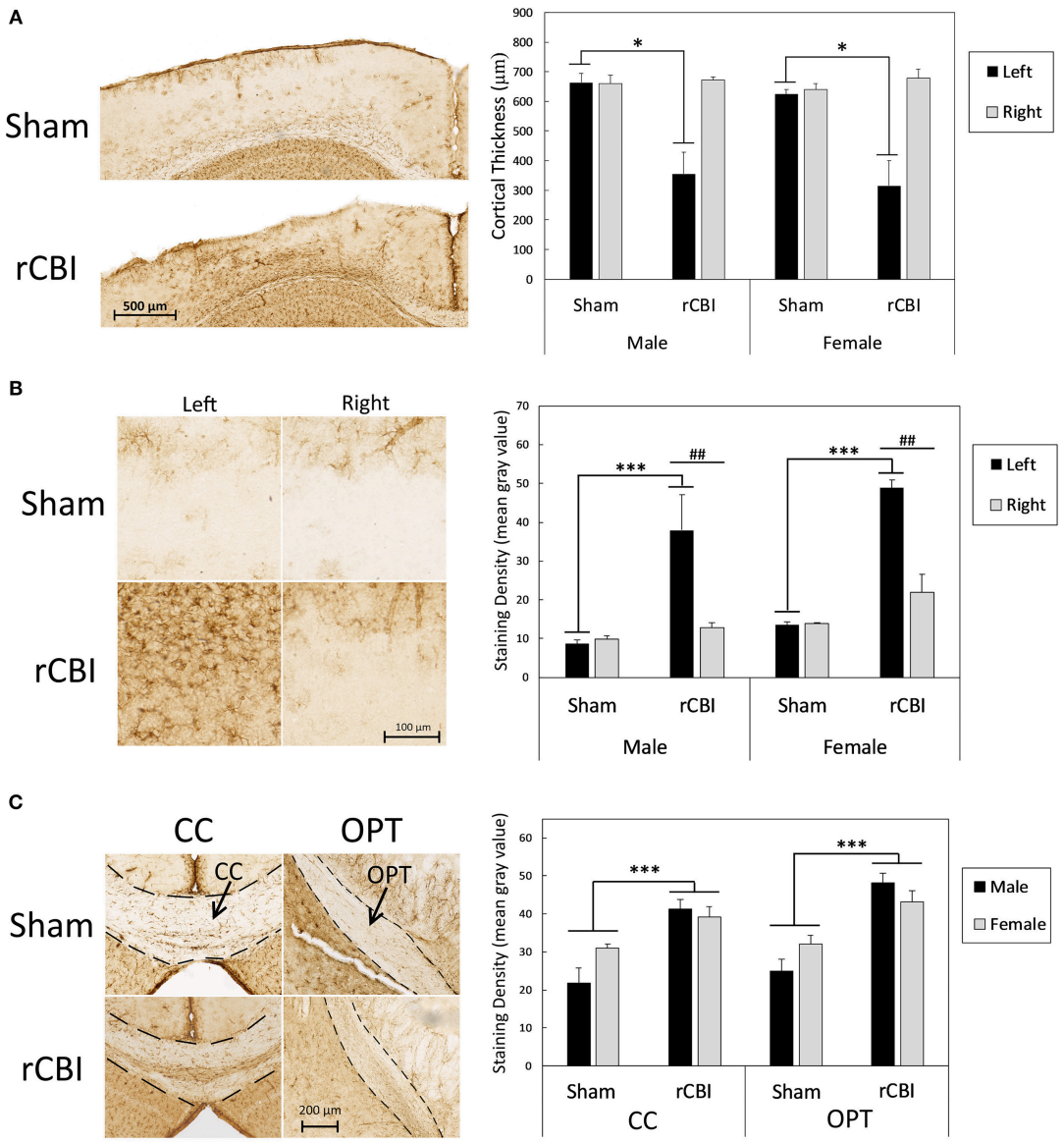

FIGURE 4 | Cortical atrophy as measured by cortical thickness and astrogliosis following rCBI as evidenced by GFAP staining. Representative photomicrographs represent approximate median values. One year following injuries, injured mice had significantly thinner CTX at the injury site than sham-treated mice **(A)**. Asterisks (*) in **(A)** represent a significant main effect of injury in the represented sex (Sham > rCBI; $p < 0.05$). There was also significantly increased astrogliosis in the perilesional CTX on the injured (left) side of the brain compared to the right side and compared to the left side of sham mice **(B)**. Asterisks (***) in **(B)** represent a significant effect of injury on the left (injured) side of the brain (rCBI > Sham; $p < 0.0001$); pound signs (##) represent significant differences in staining density between the right (uninjured) and left (injured) sides of the brain in the represented sex (Left > Right; $p < 0.01$). GFAP staining density was significantly increased following injuries in white matter tracts, specifically the CC and OPT **(C)**. Asterisks (***) in **(C)** represent a significant main effect of injury (rCBI > sham) in the represented brain region ($p < 0.001$). rCBI, repetitive concussive brain injury; GFAP, glial fibrillary acidic protein; CTX, cortex; CC, corpus callosum; OPT, optic tracts.

degeneration and atrophy in the CC have been described in chronic (up to 18 years post-injury), but not acute, TBI patients (44). In a mouse model of single CBI, Marion and colleagues recently described axonal damage in the CC, including degenerating and demyelinated axons, disruption of paranodes where myelin attaches to axons, and overall CC atrophy at 8 weeks post-injury (45). Wild-type mouse models of rCBI describing axonal damage and/or neuroinflammation in the CC and other axonal tracts at more acute time periods are numerous (11–15, 40, 46–50). Gold and colleagues recently reported a decrease in CC volume 6 months following 10 CBIs (16), and there are a smaller number of studies that have extended observations to more chronic time points: CC atrophy as measured by thickness has been reported in mouse rCBI models 1 (17) and 2 (19) years following the injuries. These changes were paralleled by increases in the CC in markers for astrocytes (GFAP) and microglia (Iba1) (16, 17, 19), indicative of neuroinflammation, consistent with the current data.

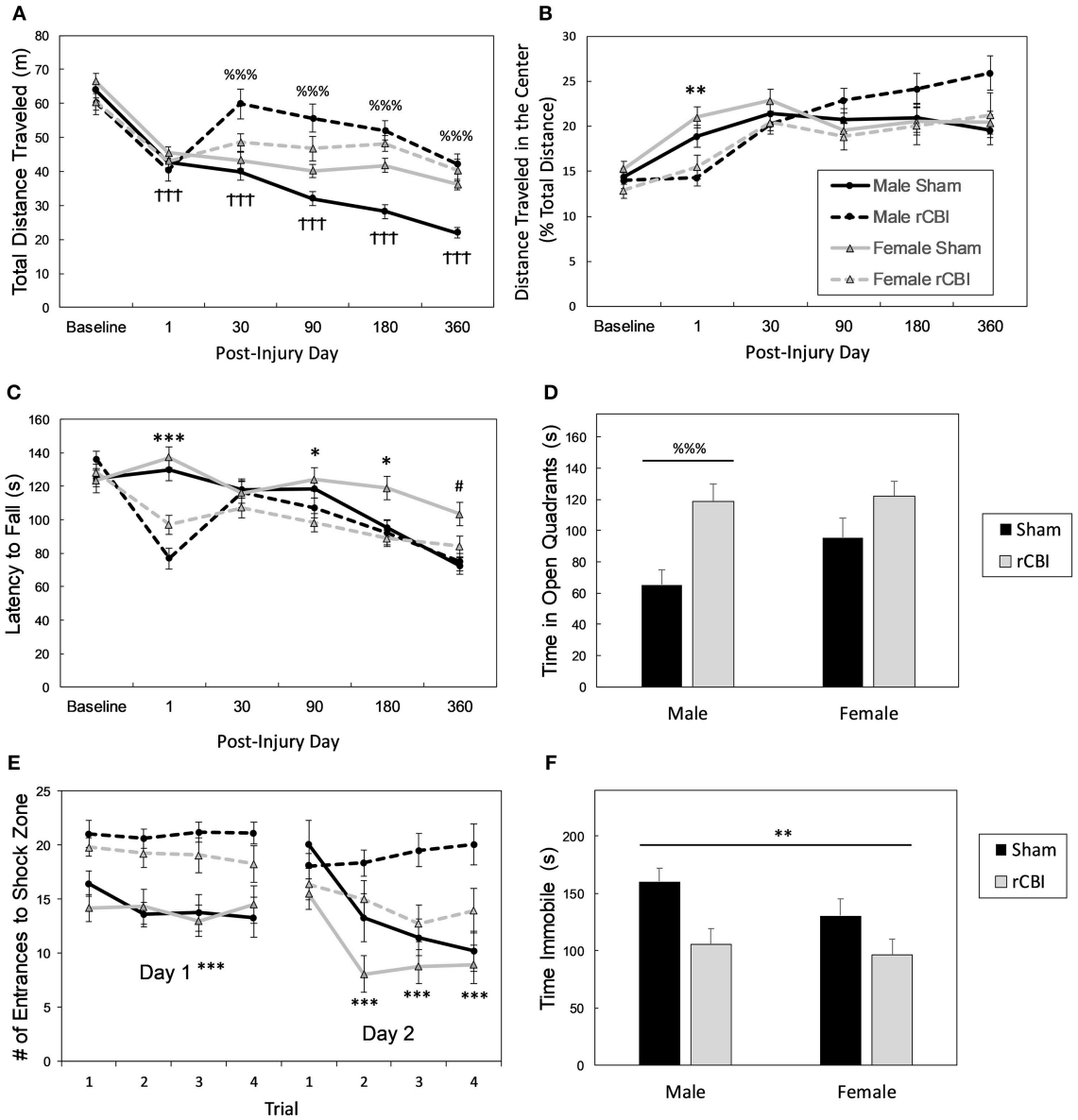

FIGURE 5 | Chronic behavioral effects of rCBI. Legend in **(B)** applies to **(A,B,C,E)**. Behavioral testing following rCBI revealed functional deficits in the OF **(A,B)**, rotarod **(C)**, EZM **(D)**, APA **(E)**, and TST **(F)** tests. Injured male mice began exhibiting a hyperactive phenotype in the OF about a month following injuries **(A)**. All injured mice were significantly less active in the center zone of the OF the day following the final injury (expressed as a percentage of total activity), suggesting anxiety **(B)**. On the rotarod **(C)**, there were significant motor deficits the day following the injuries; slight impairment remained for up to 6 months. Only injured male mice had significantly different behavioral performance in the EZM (1 year; **D**); they spent increased time in the bright and exposed quadrants compared to sham-treated mice. One year post-injuries, all mice had significant cognitive deficits as assessed on the APA test **(E)**, and showed significantly greater agitation (less immobility) in the TST for depressive behaviors **(F)**. The cross signs († † †; $p < 0.0001$) in **(A)** indicate a significant effect of testing day in female mice; female mice became less active in the OF arena following baseline testing. The percent signs (%%% $p < 0.0001$) in **(A,D)** represent an injury effect in male mice only where Injured > Sham on the measured behavior. Asterisks (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.0001$ in **B,C,E,F**) represent a main effect of injury on the given behavior (C: rCBI < Sham; E: rCBI > Sham); The pound sign (# $p < 0.05$) in **(B,C,F)** represents a main effect of sex (female > male). rCBI, repetitive concussive brain injury; OF, open field; EZM, elevated zero maze, APA, active place avoidance; TST, tail suspension test.

Changes in Spontaneous Activity and Motor Behaviors Following rCBI

During the year of study, the mice had significant motor deficits on the rotarod the day following the final injury but also remained slightly impaired up to 6 months following injuries, and male mice developed a hyperactive phenotype in the OF after 1 month. Rotarod deficits have been reported up to 1 year following 30 closed head injuries in mice (18). Motor deficits following experimental TBI are often acute and relatively transient compared to cognitive deficits [e.g., (14,

TABLE 2 | Summary of significant findings between injured and sham mice, or main effects of sex.

Measure	Significant effect	*p*-value	Effect size (Cohen's *d*)
Post-injury weights (% baseline)	Male Sham > Male rCBI	$p = 0.0064$	$d = 0.30$
Righting reflex, Injury Days 1-3	rCBI > Sham	$p < 0.0001$	N/A
Cortical thickness	rCBI < Sham	$p < 0.05$	N/A
GFAP staining density			
Cortex (male vs. female)	Female > Male	$p = 0.0025$	$d = 0.41$
Cortex (injured (left) side of brain)	rCBI > Sham	$p < 0.0001$	$d = 2.01$
Corpus callosum	rCBI > Sham	$p < 0.0001$	$d = 1.89$
Optic tracts	rCBI > Sham	$p = 0.0002$	$d = 1.79$
Rotarod, Latency to Fall			
Day 1	Sham > rCBI	$p < 0.0001$	$d = 1.65$
Day 90	Sham > rCBI	$p = 0.0138$	$d = 0.73$
Day 180	Sham > rCBI	$p = 0.0402$	$d = 0.57$
Day 360	Female > Male	$p = 0.0096$	$d = 0.78$
OF, Distance traveled; Males			
Day 30	Male rCBI > Male Sham	$p < 0.0001$	$d = 1.28$
Day 90		$p < 0.0001$	$d = 1.47$
Day 180		$p < 0.0001$	$d = 2.06$
Day 360		$p < 0.0001$	$d = 2.02$
OF, Center distance traveled (% Total); Day 1	Sham > rCBI	$p < 0.0018$	$d = 0.98$
EZM, Time in open quadrants (1 year)	Male rCBI > Male Sham	$p < 0.0001$	$d = 1.48$
APA, Acclimation, Distance traveled (1 year)	Female > Male	$p = 0.0036$	$d = 0.73$
APA, # of entries to shock zone; Day 1 (1 year)	rCBI > Sham	$p < 0.0001$	$d = 1.34$
APA, # of entries to shock zone; Day 2 (1 year)			
Trial 2	rCBI > Sham	$p < 0.0001$	$d = 1.33$
Trial 3		$p < 0.0001$	$d = 1.53$
Trial 4		$p < 0.0001$	$d = 1.62$
TST, Time immobile (1 year)	Sham > rCBI	$p = 0.0018$	$d = 0.78$

Shown are the dependent measures in the current experiment for which significant effects of injury or sex were found. P-values from ANOVAs (or Bonferroni-corrected post hoc comparisons) or Kruskal–Wallis tests, and calculated Effect sizes (Cohen's d) are provided. Cohen's $d = \left| \frac{\mu_1 - \mu_2}{s_{pooled}} \right|$.

16, 51)] and here we show that motor deficits are resolved while cognitive deficits are observed 1 year following injuries. Hyperactivity in male mice has been previously reported in rCBI models (52, 53), as well as in more severe injury models such as controlled cortical impact (CCI) that result in overt damage to the hippocampus (32, 54–59). Ultimately, there are a variety of experimental manipulations that can result in increased locomotion, and it has been suggested that any lesion or damage involving a complex control system that modulates and suppresses activity, located along the axis between the entorhinal cortex and olfactory bulb, may result in hyperactive behavior (60).

Effects of rCBI on Cognition

Consistent with prior long-term rCBI studies that employed the Barnes maze as a spatial cognitive test 1 year or longer post-injuries (17, 19), all injured mice in this study had significant cognitive deficits as assessed in the APA test (a test of spatial learning and memory), despite showing no deficits on the y-maze test of novel arm recognition (a test of spatial episodic memory). Deficits in spatial learning and memory are most often attributed to hippocampal damage and dysfunction, but in the current study parietal association cortical atrophy and astrogliosis may have been a factor as the parietal cortex is involved in linking motion and visual information during the early steps of map formation in spatial tasks (61). The APA test has been shown to detect cognitive deficits following single and rCBI in mice at a more acute time period (12) and for up to 12 months following repetitive mild TBI in a human tau-expressing transgenic mouse model (62); it is a particularly difficult task as it requires the animal to attend only to stationary visual room cues, ignoring the olfactory cues on the rotating apparatus and to continue to move with the arena in order to avoid the shock zone (63). In addition, two intact hippocampi and the fimbria containing the hippocampal commissural fibers (a white matter axonal tract) are required for successful performance of the task (64). Thus, our disparate findings on the y-maze and APA are likely due to test difficulty but could also relate to test modality. Sangobowale and colleagues recently demonstrated significant deficits on the APA task 8 days following a single CBI in mice, an injury that was characterized by reduced myelin in the CC (and other white matter regions) as assessed by Luxol fast blue. Both the cognitive deficits and axonal damage were reduced by treatment administered 12 h following injury with a combination of minocycline, an agent that inhibits microglial activation and prevents myelin loss, and N-acetylcysteine, an antioxidant drug with anti-inflammatory actions (35). These results suggest that cognitive deficits associated with damage in white matter tracts

may serve as useful functional and neuropathological endpoints for assessing delayed effects of potential therapeutic agents.

Chronic "Emotional Dysregulation" in Rodent Models of rCBI

Finally, we employed the EZM and TST, respectively, to assess chronic neuropsychiatric symptoms following rCBI, as anxiety and depression are among the most common long-term complaints in clinical TBI populations. Injured male, but not female, mice spent increased amounts of time in the bright and open regions of the maze compared to sham controls, suggesting reduced anxiety, consistent with other studies 1 year following rCBI in mice (17, 19), but see (18), or at more acute time periods (16, 53, 65). A curious finding of this study is increased amount of agitation (less immobility) in the TST of the injured mice compared to the sham controls. Greater amounts of immobility in this test [and in a similar test, the forced swim test (FST)], are interpreted as a state of despair or "depression" in animal models (66, 67). Although they found no differences in the TST, Gold and colleagues reported greater amounts of "highly mobile" time in the FST in a murine rCBI model with CC atrophy, microgliosis, and astrogliosis 6 months following injury (16). This finding was interpreted as a symptom of "emotional dysregulation," and in our model, together with hyperactivity in the OF and the increased amount of time in the open quadrants of the EZM, could suggest a pathological phenotype of agitation and risk-taking. However, these findings do not model the more common clinical symptoms of depression and anxiety that are diagnosed following TBI. Overall it has been noted that compared to motor and cognitive deficits that are employed in pre-clinical studies, findings regarding neuropsychiatric symptoms have been much more inconsistent and that further study, as well as the inclusion of different functional testing models, is needed (33, 67, 68).

Sex Differences in Functional Deficits Following TBI

Injured female mice in this study fared better than males. Unlike injured male mice their weights remained at the same level as sham controls, they did not exhibit the hyperactive phenotype in the OF, and they had normal behavior in the EZM. Chronic injury-induced astrogliosis cannot explain the long-term sex-differences in behavior in this study, as males and females showed the same neuropathological profile at the 1-year time point. However, we previously showed that male mice have increased astrogliosis compared to females at a more acute time point (∼1 month) following rCBI (15). Translational TBI studies are becoming more inclusive of both sexes, and there is mounting evidence that female rodents may fare better acutely (within days following injury) in measures of behavior and neuroinflammation than males (15, 30, 69–75). As noted in a recent review (76), there are also many reports showing no sex differences or that males have better outcomes than females [e.g., (32, 76–78)], and definitive conclusions from rodent studies are difficult at this point as investigators are using different species at different ages, different functional testing paradigms at varying times following TBI, and different injury models. However, although phase III clinical trials of progesterone to date have failed, the neuroprotective effects of estrogen and progesterone are well-established in animal models of neurotrauma (79–81), and it is possible that these hormones are exerting long-term protective/therapeutic effects in this model via biological mechanisms that were unexplored in this study. In addition to developmentally programmed sex differences, the on-going effects of sex hormones in the brain are far-reaching. Sex hormone receptors are located in glial cells and throughout neurons, where they have many actions, including regulation of signaling pathways and direct and indirect effects on gene expression, leading to alteration of many physiological and behavioral functions (82). Although the initial focus of studies on sex hormones and behavior focused on the hypothalamus and sexual behaviors, it is now realized that steroids have more global effects on the brain, including regions such as the hippocampus, prefrontal cortex, cerebellum, and periacquadectal gray, resulting in sex differences in addiction, responses to stress, mood regulation, and pain sensitivity, among others (82). Ultimately, the potential effects of steroid hormones following injury, both neural and glial, genomic and non-genomic, are complex and will require dedicated, targeted study.

CONCLUSIONS

In summary, this study employed a moderately severe chronic rCBI model in wild-type mice. Consistent with reports of other investigators (17–19), there were significant behavioral deficits concurrent with ongoing neuroinflammation in axonal tracts 1 year following the injuries, Furthermore, this is the first long-term rCBI study in rodents that has been inclusive of both sexes, and we have demonstrated that male mice fare worse on two behavioral tasks, despite showing a similar neuropathological profile to injured female mice. There is a need for continued development of translational models of chronic rTBI with measurable functional and pathological features that lend themselves to therapeutic intervention.

ETHICS STATEMENT

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences (Bethesda, MD) and the mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care-Accredited facilities.

AUTHOR CONTRIBUTIONS

LT and JM designed and planned the study. TBI procedures were performed by AF. Behavioral testing was performed by LT and AV. Immunohistochemical processing and analyses were performed by LT, AF, and AV. Data analyses were performed by LT. Final editing of the manuscript by LT and JM.

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REVIEW
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Neuroimaging and Neuropsychological Studies in Sports-Related Concussions in Adolescents: Current State and Future Directions

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Sports-related concussion, is a serious neurological concern that many adolescent athletes will face during their athletic careers. In some instances, the effects of sports-related head injury are long-lasting. Due to their still-developing brains, adolescents appear to be more vulnerable to long-term repercussions of these injuries. As all sports-related concussions are mild traumatic brain injuries (mTBI), this review we will examine the pathophysiology of mTBI, its acute effects and long-term risks from sustaining injury, and current and needed advancements in the areas of neuropsychological testing, accelerometer telemetry, and neuroimaging. Current methods do not adequately measure the extent of an injury that an athlete may sustain, potentially putting these athletes at a much greater risk for long-term effects. To better understand mTBI, neuropsychological testing best practices need to be developed, standardized, and implemented based on sound scientific evidence in order to be propagated as clinical guidelines. Wearable accelerometers can be used to assess thresholds for mTBI and cumulative effects of concussive and subconcussive injuries. Novel neuroimaging methods that can detect anatomical abnormalities and functional deficits with more specificity and sensitivity should be developed. Young athletes are particularly a vulnerable population warranting immediate and significant research aimed at protecting them against sports related injury and mitigating their long-term deficits.

Keywords: mild traumatic brain injury, neuroimaging, neuropsychological testing, accelerometers, fMRI, DTI, MEG, TMS

INTRODUCTION

Sports-related concussion (a type of mTBI) is a serious neurological concern that many adolescent athletes will face during their athletic careers. Sports-related concussion is the second most common cause of head injury in children, second only to motor vehicle accidents (1). It has been estimated that 1.6 to 3.8 million concussions occur in sports and recreational activities each year in the United States (1). The highest incidence for sports-related concussion in young male athletes is reported in high school football, whereas soccer and basketball show the highest prevalence in female athletes (2). Emerging research suggests that younger athletes take longer to recover and may be more vulnerable than adults to the effects of an mTBI (3). Thus, the problem of mTBI in youth or adolescents needs urgent and extensive investigation, especially regarding diagnosis, management, and return-to-play guidelines. Mismanagement of mTBI puts athletes at risk for the short-term risk of second impact syndrome as well as long-term neurological sequelae such as chronic traumatic encephalopathy and chronic neurocognitive impairment (4). In this article, we will review the current understanding of the pathophysiology of mTBI, and highlight neuropsychological testing and neuroimaging methods that can be used to detect and monitor recovery of a youth sustaining an mTBI.

Brain Development

Prior to discussing the potential effects of mTBI on adolescents, it is important to understand normal brain development in this population. Although a child's total brain volume reaches ∼90% of its adult size by 5 years of age, the brain continues to undergo significant changes throughout childhood and adolescence (5). Studies have demonstrated an increasing volume of white matter and reorganization of synaptic connections throughout the adolescent period (6–8). Gray matter in frontal, parietal, and temporal lobes reach their maximum volume around 12–16 years of age, while the gray matter in the occipital lobe continues to increase in size and density until 21 years of age (9). While gray and white matter changes in association cortices continue through the second decade of life, the greatest delay in maturation occurs in the prefrontal cortex, where myelination and synaptic pruning continues well into adulthood (10, 11). Similarly, the posterior part of corpus callosum continues to increase during adolescence as a result of increased myelination in the interhemispheric white matter tracts (12). These processes can be altered by environmental factors, including head injury.

Thus, it is reasonable to hypothesize that young children involved in contact sports could have a more prolonged course of recovery following mTBI due to the physiologic immaturity of their brains as compared to adults. In fact, recovery times in children average approximately 1 month while symptoms in adults generally resolve within 10 days (3). Additionally, studies of adolescents have shown impaired working memory up to 1 year from injury and impaired attention up to 2 years from injury (13). These findings suggest that children are at risk for permanent impairments following mTBI (13). In addition, children with a prior history of migraines, learning disabilities, and attention deficit disorder may experience an even further prolonged course of recovery as well as more severe symptoms (4). Furthermore, injuries sustained during childhood and adolescence has the potential to change the trajectory of brain development, resulting in long lasting effects well into adulthood.

Definition of mTBI and Its Effects

An mTBI can be defined as a temporary alteration in brain function and mental status resulting from blunt trauma to the head or body that rapidly displaces the brain within the skull (14). It is important to note two items surrounding this definition (1) all concussions are mTBI, but not all mTBI are concussions; and (2) In any blunt trauma to the head or body, load of that force is also transmitted and causes fracture, contusion, hemorrhages, which are not addressed in this review of milder injuries. A loss of consciousness can occur, but is not required for a head injury to be classified as an mTBI (14). Typically, mTBI is characterized by rapid onset of symptoms that last for a short time and resolve spontaneously. Symptoms may develop more slowly in some individuals, so athletes suspected of suffering an mTBI should be assessed multiple times following an impact (15). Headache and dizziness are the most common physical symptoms of mTBI (16). Other physical symptoms include fatigue, photosensitivity, and nausea (15). Patients with mTBI also report cognitive symptoms such as an inability to concentrate ("feeling foggy"), as well as various other problems with memory and confusion (15). Emotional symptoms can include anxiety, irritability, emotional lability, and depression (4). Patients with mTBI may also experience difficulty falling asleep, excessive drowsiness, and/or other changes in sleep habits (4).

While one instance of concussion is not ideal, those young players who sustain multiple concussions over the course of their career may have even bigger cause for concern. A history of a previous concussion is associated with a greater risk of sustaining another concussion and puts athletes at an increased risk for longer-term deficits (16). Young athletes who sustain 3 or more concussions are more likely to exhibit a greater symptom burden including loss of consciousness, post-traumatic amnesia, and confusion (17). Furthermore, on neuropsychological testing, cognitive symptoms are found to persist longer for those with a history of 2 or more concussions (18). In addition, the grade point averages of student athletes with 2 or more concussions have been shown to be significantly lower (18).

Athletes with an history of multiple concussions are more likely to suffer from long-term neurological sequelae including deficits of working memory and visuospatial processing, epilepsy, early-onset Alzheimer's disease, chronic depression, and chronic

Abbreviations: AAN, American Academy of Neurology; AMS, American Medical Society of Sports Medicine; ANAM, Automated Neurocognitive Assessment Metrics; BOLD, Blood oxygen level-dependent; CNT, Computerized neuropsychological tests; CISG, Concussion in Sports Group; CRI, Concussion Resolution Index; CT, Computed tomography; DTI, Diffusion tensor imaging; EEG, Electroencephalogram; fMRI, Functional MRI; ImPACT, Immediate Post-Concussion Assessment and Cognitive Test; MEG, Magnetoencephalography; MRI, Magnetic resonance imaging; MRS, Magnetic resonance spectroscopy; mTBI, Mild traumatic brain injury; TMS, Transcranial Magnetic Stimulation.

traumatic encephalopathy (19). Second-impact syndrome can cause severe cerebral vascular congestion and result in death (2, 20). There are currently no long-term prospective studies that examine the effects of concussions in young athletes who participated in contact sports before the college and professional levels. Future studies should be directed at understanding the effects of concussions, and the cumulative effects of repeat concussions throughout the entire career of young athletes.

PATHOPHYSIOLOGY OF mTBI

An mTBI can be caused by direct trauma to the head or the rotational acceleration of the brain within the skull. These changes result in cellular damage to neurons, axons, and the vasculature of the brain which disrupts neurotransmission and neurometabolism (21, see 22 for a review). Animal models have provided the majority of information regarding the pathophysiology of the damage done by mTBI (2). These studies have demonstrated that a concussive injury damages the neuronal cell membrane, beginning a cascade of abnormal neuronal events. Disruption of the membrane causes an efflux of potassium to flow back into the extracellular space coupled with influx of sodium and calcium and thereby prompting the release of glutamate, an excitatory amino acid from the neuron (2, 21). Glutamate triggers the release of potassium that in turn causes further depolarization of the cell (2). As the sodium-potassium ion pumps attempt to compensate for this ion imbalance, adenosine triphosphate and glucose stores are depleted. The accompanying decrease in cerebral blood flow then leads to an energy crisis and lactate accumulation (2, 21). In addition to the potassium imbalance, calcium builds up in cell at the same time, thereby impairing mitochondrial function and exacerbating the energy crisis (22). All of these events can also release free radicals and lead to long lasting injury to neurons making them vulnerable to repeat injury (2).

Apart from these acute changes, more protracted impairments in metabolism lasting several days are also noted. Several animal studies have investigated other molecular impacts of mTBI. Across studies, changes in brain amino acids including N-acetylaspartate (NAA), arginine and γ-aminobutyric acid (GABA) and ATP/ADP ratio were noted, illustrating the energy crisis resulting from mTBI (23–26). In addition the degree and duration of the changes in these amino acids correlated with the severity of mTBI (23). These results highlight the role of the initial impact has on the cascade of events that follow, including the changes to metabolism. Further, in animals sustaining multiple mTBIs, the severity of the metabolic alterations increased and, did not demonstrate complete recovery despite prolonged follow up (24). Alterations in glycolytic gene expression and enzymatic activities have been reported following mTBI, indicating to genetic factors mediating brain responses to injury (27). The results of these studies warrant further investigation, particularly to investigate how these results may be applied to the discovery of drugs that could target specific mitochondrial functions and prevent the catastrophic effects of mTBI (26).

In addition to the energy crisis, the biomechanical impact has been shown to damage the cytoskeleton in the form of collapse of neurofilaments and disruption of microtubules. Such injury to axons can lead to impairments in axonal transport, neurotransmission and even axonal disconnection (21). Emerging data also indicate that proteins like integrins that form cytoskeletal anchors in the cell membrane in both neurons and vascular endothelium likely are the main molecular target of mTBI (21). These axonal changes have been shown to correlate with impaired cognition in both animal models and human studies (21). Additional sequalae of mTBI includes disruption of excitatory and inhibitory neurotransmitter balance, activation of inflammatory cytokines, and in extreme cases cell death.

It is important to note that these pathophysiological changes can persist beyond the resolution of clinical symptoms. Since the cellular functions can take several days to weeks to return to baseline, the patients remain physiologically vulnerable during this period (4). Thus, a second injury during this phase may result in a worsening of the original effects and create increased risk for additional long-term sequelae (4). It has also been shown that younger individuals are more susceptible to the cumulative effects of repeat concussions, most likely due to developmental differences such as brain size, myelination levels, and differences in cerebral blood flow (4). Further studies that examine the pathophysiology of such brain trauma in adolescents are needed to better understand the underlying mechanisms of such injury and recovery. This work is critically needed for better diagnosis and for information on when it is safe to return to normal activities.

CURRENT STATE OF DIAGNOSTIC METHODS IN mTBI

Guidelines for mTBI diagnosis, management, and return-to-play decisions have been released by the American Medical Society (AMS) of Sports Medicine, the American Academy of Neurology (AAN), and the Concussion in Sport Group (CISG). The AMS and AAN guidelines were last updated in 2013, while the CISG more recently updated its consensus statement in 2016. Despite these three highly regarded guidelines, there is no one agreed-upon protocol for when a child should return-to-play. However, they all recommend that athletes exhibiting symptoms of concussion should be seen by a licensed health care professional or neuropsychologist and that return-to-play should be gradual once symptoms both at rest and with exertion are resolved (28).

Neuropsychological Testing in mTBI

There is a longstanding history of neuropsychological testing in the objective assessment of cognitive and behavioral changes in individuals with mild traumatic brain injury. It is well-established that individuals with mild traumatic brain injury can suffer neurocognitive deficits, most notably in the areas of processing speed/reaction time, attention, working memory, executive function, and memory (retrieval); these domains of function are readily assessed with formal neuropsychological testing. Several case-controlled and meta-analytic reviews are

available on this topic, establishing the reliability and validity of testing in this population (29–34). A detailed discussion of these meta-analytic studies is beyond the scope of the current review; however, we would note the general trend of these meta-analyses, which have suggested that neurocognitive effect sizes tend to diminish spontaneously as the post-injury window broadens. In adult samples, a majority of the variance in post-concussive cognitive complaints has been found to be primarily related to psychological distress factors and litigation.

In the context of sports-related concussion management, the seminal work of Dr. Jeffery Barth and the sports laboratory assessment model at the University of Virginia provided a model of baseline and post-injury assessment that has become a model assessment strategy in many contemporary systems (35, 36). This strategy involves measuring cognition in the asymptomatic athlete, then re-assessing cognition (with the same testing battery) in the injured patient. When baseline testing is available, one is able to not only compare scores of the injured patient to the general population (norm-referenced measurement), but also a more sophisticated intra-individual assessment of change (baseline vs. post-injury). Over time, the use of neuropsychological testing has expanded in the assessment and management of concussion, especially in the field of sports medicine. An inter-organizational neuropsychology group (American Academy of Clinical Neuropsychology, American Board of Neuropsychology, Division 40 (Neuropsychology) of the American Psychological Association, and the National Academy of Neuropsychology) produced a consensus statement suggesting neuropsychologists can provide value-added data in the assessment and management of concussed athletes (37).

The Concussion in Sports Group (CISG) is an international, multi-disciplinary clinical and research group which have published sports concussion consensus guidelines since 2001, with the most recent iteration coming from the group's 5th meeting in 2017 (38). The CISG has addressed neuropsychological/cognitive assessment in their statements. Initially, there was a recommendation that formal cognitive assessment should be an essentially universal component of concussion assessment and management. Over time, this recommendation has been tempered. Specifically, in CISG-5, the group asserted that neuropsychological testing still contributes significant information in sports concussion; however, they found limited evidence suggesting neuropsychological testing prevented concussions or altered concussion outcomes. They go on to state that the current literature fails to support mandatory global neuropsychological testing for all athletes, including both baseline and post-injury assessment. Return-to-school planning was considered to be an area where neuropsychological testing can be especially helpful. More recently, the Centers for Disease Control reviewed literature pertaining to the diagnosis and management of concussion in children. Cognitive testing was discussed, with the overall summary suggesting moderate evidence supporting the use of graded symptom checklists in distinguishing injured patients. In contrast, brief cognitive screening (e.g., Standardized Assessment of Concussion), computerized measures (e.g., CNS Vital Signs), and specific measures of motor reaction time and vestibular-ocular motor screening were found to be of low to very low confidence. We would note that the review did not include studies with more detailed or robust neurocognitive batteries that would commonly be use in a typical clinical neuropsychological battery.

TABLE 1 | Advantages and disadvantages specific to CNT.

Advantages	Disadvantages
Easily administered to large groups (37)	Group administration could potentially alter individual results (38)
Increased availability of multiple assessments (37)	Computers introduce the potential for software and hardware failure; many tools require internet access (39)
Portability allows rapid assessment and testing in more remote environments (37)	Most CNT still require scoring and interpretation by trained neuropsychologists (40)
Electronic medium facilitates compilation of a centralized database for normative data and research (37)	Difficulties remain for interpretation in the absence of baseline data or until complete normative data has been established (42)
Improved accuracy of time-based response measurements (37)	
Test forms can be modified to adjust for individualized presentation (37)	

Of course, traditional neuropsychological testing in the context of mTBI/concussion evaluation and management is not without limitations. As noted above, the ability to detect neurocognitive impairments drops off within a few days to weeks following injury, and in some cases, performance-based deficits will resolve before subjective symptoms. Thus, if a clinician is reliant on neurocognitive testing data as the primary marker of recovery, they may risk false negative errors, which further risks prolonged recovery or re-injury in a patient who may still be in the midst of neurologic recovery. Another limitation are interpretations of psychometric change scores. For example, confident interpretation relies on adequate test-retest reliability, which for some tests may be poor, include significant practice effects, or simply not be available (39). Although statistical methodologies are available to control for reliability and practice effects (i.e., reliable change indices, regression-based change scores), the statistical knowledge required to calculate these changes scores often requires formal training, which a vast majority of clinicians may not be exposed to. Other limitations are more pragmatic in nature. For example, large-scale baseline testing with traditional neuropsychological tests can be challenging as they require one-on-on administration, with scoring and interpretation of results requiring trained neuropsychologists (40). Of course, this has been shown to be feasible in some settings. For example, the National Hockey League has employed a dual-approach, employing general screening instruments, computerized assessment, and traditional neuropsychological (paper and pencil) testing (41). Of course, this protocol does not easily generalize to a majority of concussion clinics or general medical practices due to the availability of specialists, time, space, and financing.

Given the changing landscape of concussion, including increased awareness of injury and market pressures for rapid diagnosis and assessment, recent trends have seen the

development of computer-based neuropsychological testing. Overall, computerized neuropsychological tests (CNTs) is somewhat of a mixed bag with regard to advantages over traditional measures (see **Table 1**). In a joint position paper by the American Academy of Clinical Neuropsychology and the National Academy of Neuropsychology, the panel agreed that CNTs can be administered by a wide range of professionals and distributed to a wide clinical population; however, caution was encouraged for users potentially viewing these instruments as a "plug and play" strategy that provides a diagnosis or interpretive statement at the exclusion of meaningful factors when considering cognition (e.g., similarities between normative data setting and clinical testing environment; cultural factors; malingering and effort problems; test theory and psychometric development; degree of computer naivete) (43). The authors of the paper also encourage a clear understanding of the difference between *neuropsychological testing* vs. *neuropsychological assessment*, with the latter implying clinical activities (i.e., clinical history integration, behavioral observations, physical findings, and interpretive and diagnostic conceptualization) beyond the relatively simple task of test administration. Considering the advantages and disadvantages of CNTs, the selection of which CNT instrument to use presents its own problem. Limited studies have considered head-to-head comparisons between CNTs, limiting one's ability to optimally select an instrument with the operational characteristics for their population at hand. Numerous studies have considered psychometric properties of common CNTs, utilized in concussion populations, with results suggesting quite a bit of variability across measures and across test-retest intervals (44, 45). Select studies have attempted to address this gap in the extant literature. Resch et al. (45) examined the psychometric properties of four of the most widely employed CNTs, including the Immediate Post-Concussion Assessment and Cognitive Test (ImPACT), Axon/CogSport, Automated Neurocognitive Assessment Metrics (ANAM), and the Concussion Resolution Index (CRI). Resch and colleagues failed to identify superiority with any specific test and found inconsistent psychometrics across instruments, including varying validity, test-retest reliability, sensitivity, and specificity. Even with ImPACT, a measure that has become ubiquitous with sports concussion assessment, demonstrated mixed validity results ($r =$ 0.20–0.88), mixed to unacceptable reliability ($r =$ 0.23–0.88), and a broad range of sensitivity (79.2–94.6%) and specificity (89.4–97.3%) (44–46). In another similar study, Nelson et al. (47), compared reliability and validity between the ImPACT, ANAM, and Axon Sports/CogState over 24 h and 8, 15, and 45 day intervals. Sensitivities tended to be strongest at short intervals (47.6–67.8%); however, test-retest reliabilities dropped off for all instruments over longer intervals.

The extant literature has not identified a "perfect" neuropsychological test for individuals who have suffered concussion. As such, many have advocated for a multidimensional test to manage mTBI (such as the SCAT5) that considers observable symptoms as well as cognitive and neurological screening (4, 48, 49). Within these trends, some have recommended that performance-based testing be geared toward and focused on vulnerable populations, such as children and adolescents, when feasible; however, this data in isolation should not be used as a litmus test to determine an athlete's readiness to play (49). We would note that return-to-play decision making has received a vast majority of the attention with regard to clinical decision making. In youth athletics, we would argue that return-to-learn would be a more significant outcome. Unfortunately, there have been limited studies with respect to the value-added nature of neuropsychological testing in school decision making, which we would expect to be much greater. In order to advance the science, future efforts must work toward establishing a best practice guideline for the use, administration, and interpretation of neuropsychological tests, regardless of the modality. Although some have suggested neuropsychological testing can be helpful even in the absence of an asymptomatic baseline exam, further studies are needed to understand what populations may or may not need baseline examinations (50). Moreover, studies should also be geared toward addressing questions regarding standardization of administration practices and hardware/technology used for CNTs, as well as ongoing studies to establish psychometric and operational characteristics of various instruments, across diverse age, sex, sport, intellectual and educational levels, in order to optimize measure selection (40, 51). The establishment of these guidelines would also be best served with a clearer understanding of the relationship between variations in an individual's scores and the recovery curve for each individual assessment (45).

Accelerometer Telemetry

The biomechanics of head injury can be assessed using wearable accelerometers that precisely measure the force of impact and provide real-time assessments of linear and angular acceleration as well as g-force. Being unobtrusive, these accelerometers can be worn discretely behind the ear or within headbands and caps and allows monitoring of both helmeted and non-helmeted athletes. Since these devices transmit data in real-time, qualified professionals can be alerted in case of high impact events. While one study using accelerometers embedded in helmets in collegiate football players found no correlation between acute symptoms of mTBI, postural stability, and neuropsychological deficits and the impact magnitude or impact location (52), a more recent study found that the athletes suffering concussion who demonstrated persistent neurophysiological deficits after despite complete symptom resolution had received significantly higher number of side impacts (53). These findings indicate that number and type of impact may be just as important as the force of impact in determining the immediate and long-term sequelae following mTBI.

Neuroimaging in mTBI

Current standards of care for examining an mTBI include neuroimaging methods such as computed tomography (CT) scans and magnetic resonance imaging (MRI). A CT scan is usually performed first to rule out intracranial hemorrhage and skull fracture (2). While useful in this context, artifacts caused by beam hardening and partial volume effects limits the visualization of posterior fossa, frontal, and temporal regions. Further, CT cannot effectively resolve the gray-white matter

interface for mTBI, resulting in poor visualization of deep white matter tracts, hippocampus and brainstem-structures that can be better visualized by MRI (54). However, structural MRI is still not sensitive enough to identify changes in the microstructure of the white matter. Therefore, neither CT nor MRI has the sensitivity or specificity of identifying mTBI related brain injury. Furthermore, it is important not only to determine the specific structural changes, but also functional consequences that occur as a result of an mTBI. We will discuss the current state of neuroimaging methods in turn.

Structural Imaging-Diffusion Tensor Imaging (DTI)

Diffusion tensor imaging (DTI) is superior to structural MRI and CT scans in detecting white matter alterations, localized lesions, and diffuse axonal damage, especially in younger children where such alterations are typically more difficult to detect (2, 55). DTI provides information regarding white matter microstructure and fiber tract integrity by measuring the motion of the water molecules within the brain. Changes in fractional anisotropy (a measure of the direction of water movement within axons) and mean diffusivity (a measure of the overall diffusion in a tissue) are able to indicate alterations in the structure of white matter after mTBI (3, 56–58). White matter abnormalities have been found in the anterior corona radiata, the uncinate fasciculus, corpus callosum, inferior longitudinal fasciculus, and the cingulum bundle of patients that exhibit persistent cognitive impairment after mTBI (55). Changes in attention and memory have also been associated with altered fractional anisotropy levels within the left anterior corona radiata and uncinate fasciculus (59).

The global changes in fractional anisotropy and radial diffusivity have been seen even months after the initial concussive injury (56). Many of these fractional anisotropy changes are localized to the temporo-occipital white matter (60), but deep white matter changes may show an even longer time course of recovery, especially for female athletes. In a study of female contact sport athletes, microstructural changes could be persistently identified, even as long as 7 months after the initial injury occurred, and despite these athletes denying the presence of post-concussive symptoms (61). These data highlight the importance of improving our current diagnostic methods to better understand structural changes related to mTBI.

Magnetic Resonance Spectroscopy (MRS)

Magnetic resonance spectroscopy measures the level of metabolites in the brain non-invasively by quantifying the changes in the proton spectrum induced by the metabolites. Individual metabolites and their concentrations can be quantified by their characteristic pattern of resonance frequencies. While MRS can detect signals from many metabolites, it is limited primarily by the concentrations of these metabolites in the tissue being examined and the magnetic field strength of the MRI scanner. Most common metabolites studied using MRS include N-acetylaspartate (NAA) and creatine (indices of neuronal viability), choline (measure of cell membrane integrity), glutamate and glutamine (assess excitatory neurotransmission), and γ-aminobutyric acid (GABA, an inhibitory neurotransmitter). The main advantage of MRS is its ability to monitor alterations in metabolic function over time even in the absence of structural abnormalities.

Researchers are beginning to use MRS to examine the association between mTBI and mitochondrial metabolism. Acutely decreased levels of NAA were noted in athletes who had suffered a concussion (< 3days), similar to that observed in rodent studies. The levels of NAA returned to non-concussive levels by 30 days (62). Additional alterations in concentrations of Creatine and Choline in the frontal lobe white matter have been observed that progressively return to normal by 30–45 days post injury (63). These findings highlight the possible clinical application of MRS as a non-invasive method of assessing changes caused by mTBI that are not evident by routine clinical, neuropsychological, or imaging evaluations (62).

However, MRS does not have the spatial resolution of an anatomical MRI and provides information on metabolites in small areas of the brain. Usually, in each scanning session, one or two areas of interest as well as control regions can be studied, making a priori identification of the critical brain areas a necessity. For example, only few brain areas including the frontal white matter, primary motor cortex, cingulate cortex and thalamus have been studied using MRS (64). But, due to differences in types of sports and locations of impact, there is no clear consensus on which brain areas should be examined. This problem is further compounded by on-going developmental changes in brain metabolites in adolescent athletes as well as normal variations of metabolite concentrations across different brain areas and individuals. Recent advances in whole brain MRS imaging will be helpful in studying metabolic consequences of sports related concussion particularly in children.

Functional Imaging-Functional Magnetic Resonance Imaging (fMRI)

Functional MRI (fMRI) measures brain activity by detecting changes in blood flow in real time as an individual is presented with a task (2, 65). Through such functional imaging assessments it is possible to associate any alterations in this blood flow with neurocognitive dysfunction post injury. The tasks that are presented during fMRI can be personalized for detection of deficits for each individual concussive case, and thus provide the most specific and accurate information for each patient (65). Another advantage of fMRIs is the capability to run multiple cognitive tests and trials within a short period of time (65). Such multicomponent studies could potentially elucidate the effect of concussive events on the brain, and enable medical professionals to detect even very small changes in neuronal function-changes that may go undetected otherwise and which will put the individual at a greater risk for secondary complications (65).

Results from fMRI assessments are obtained by measuring changes in local blood oxygen level-dependent (BOLD) contrast levels (65). A prospective fMRI study conducted on eight college football players found that those athletes who experienced an mTBI during the season showed increased BOLD responses while completing memory, sequencing, and sensorimotor tasks. These responses were increased when compared to their own baseline

scan at the beginning of the season, but were also increased when compared to the other players with no reports of an mTBI (65). Results from this study imply that the athletes who have sustained an mTBI require more input from neighboring neuronal networks in order to complete the same tasks as controls, probably due to axonal damage near the area of impact (65).

Another study using fMRI and the n-back paradigm (a visual working memory test) demonstrated BOLD signal changes between a preseason baseline assessment and a post-injury or an end of the season assessment. These changes were proportional to the number of head collision events and are possibly linked to alterations in glucose (66) and oxygen (67) metabolism after head impact (65). With these findings, fMRI shows promise as a valuable diagnostic and research tool in the detection, assessment, and tracking of mTBI in athletes.

Functional Imaging-Magnetoencephalography

Magnetoencephalography (MEG) is another functional neuroimaging modality, one that can identify brain areas involved in language perception, language production, somatosensory perception, and motor performance. Like fMRI, brain activity changes are measured during the performance of a task, and so MEG can be used in mapping the functional organization of the brain. Unlike conventional electroencephalogram (EEG), the surface distributions of the magnetic signals arise mainly from the primary or source currents and are not distorted as they spread to the surface of the head. This allows MEG to provide more accurate spatial localization than EEG (68, 69). Resting state MEG data can assist in establishing normal functional connectivity patterns between different areas of the brain (68). Studies have shown that the neurological symptoms experienced following an mTBI may be a result of alterations in these functional connectivity patterns (68, 69). Healthy neuronal tissues produce resting-state MEG data with frequencies above 8 Hz, while injured tissues produce lower frequencies (70). This low-frequency imaging has shown strong potential as a diagnostic imaging biomarker for mTBI. In one study measuring frequencies of 1–4 Hz, a control group was used to generate a diagnostic threshold that allowed 87% positive detection of mTBI (70). In another study, 55 control subjects and 31 mTBI subjects participated in a MEG assessment that analyzed the proportions of long-range vs. short-range connections within functional connectivity patterns. The study showed that the long-range connections accounted for 60% of all connections in mTBI patients vs. 20% in the control group, and this characteristic could distinguish the mTBI subjects from the control group subjects with 100% accuracy (68). With further research MEG has the potential to diagnosis mTBI, as well as the potential for monitoring recovery.

Transcranial Magnetic Stimulation

Transcranial Magnetic Stimulation (TMS) is a non-invasive brain stimulation technique that is being increasingly used in studying mTBI. TMS introduces an external, focal magnetic field to brain areas to elicit or disrupt brain activity. Often, TMS is used in conjunction with other neuroimaging methods, such as MRI, and is quickly emerging as an important diagnostic tool in investigating motor and speech and language networks in several neurological disorders. In addition to being non-invasive, TMS can be safely used in adolescents and in the patients with head injury. Using TMS, a study found that in athletes who suffered a concussion, the severity of concussion correlated with degree of inhibition in the motor cortex. This pattern was further exaggerated in athletes who sustained a repeat concussion, indicating to the cumulative effects of repeated injuries (71). In addition, studies of motor excitatory and inhibitory networks using TMS demonstrate persistent functional abnormalities long after symptom recovery and neuropsychological tests return to baseline (72–75). These findings further highlight the need for accurate diagnosis and long term follow up well-beyond symptom recovery in patients with mTBI.

DISCUSSION

In this review, we examined the pathophysiological cascade and the neuropsychological tests and advanced neuroimaging techniques employed in studying sport related concussion or mTBI in children. A similar examination of traumatic brain injuries resulting from more direct collision was not included in this review since the injury sustained has different biomechanical mechanisms and load distribution. Such injuries are often associated with different clinical symptoms including fracture, hemorrhage, and brain contusions and likely have different metabolic and pathophysiological sequalae. However, this review is confined to sports related concussion as the research on diagnosis, treatment, and the effects of mTBI is increasing, possibly due to greater awareness of concussion in professional sports. There are many areas of progress, yet there is still much to be done. One area that is markedly lacking is the longitudinal follow-up of youth that play sports, and the incidence of long-term post-concussion sequelae. It is unclear whether, and to what extent, concussions sustained in early life increase the risk for other neurological disease processes in later life.

Diagnosis and management of concussion, while much improved over the past 20 years, still is an area that needs further research in the areas of neuropsychological testing and neuroimaging. There remains a lack of evidence speaking to the superiority within and across neuropsychological tests and modalities (e.g., paper and pencil vs. computerized; baseline model vs. norm-referenced). Current recommendations for inclusion of neuropsychological testing in vulnerable populations and in return-to-school decision making is notable; however, general assessment and management should be a multimodal strategy. Wearable accelerometers can be used to assess the threshold of impact needed to initiate an injury and further explore the cumulative effects of subconcussive impacts to the head, particularly in young children and adolescents. Additionally, neuroimaging techniques are crucial to fully understanding the extent of an injury, but advanced techniques such as DTI, fMRI, MEG, and TMS need further development before they can be used clinically to diagnose concussion or

determine return-to-play. Extensive research is needed in these areas across all age groups in order to develop more sensitive diagnosis methods, improve clinical guidelines, and prevent avoidable long-term deficits.

FUTURE DIRECTIONS

A Recent study reported increased blood levels of T-tau in ice hockey players with sports-related concussion. The concentrations of T-tau were highest immediately after the injury, and the T-tau concentrations at 1 h after concussion predicted the time to recovery (76). Future studies should be directed toward replicating this promising finding in order to significantly improve the way clinicians diagnose and manage sports related concussions and make return-to-play decisions. Toward this end, the NCAA-U.S. Department of Defense Concussion Assessment, Research and Education (CARE) Consortium (http://www.ncaa.org/sport-science-institute/topics/ncaa-dod-care-consortium) was launched in 2014 as the largest study on concussion and repeated head trauma in athletes. Thirty campuses across the country are participating in this study to evaluate both acute and long term effects of head injury in athletes. In particular, the study hopes to define the clinical evolution of concussion and identify the neurobiological underpinning of concussion by using accelerometer telemetry, advanced neuroimaging and biological markers including genetic testing. The long term follow-up phase of the study started in the winter of 2018 and will follow the athletes for 4 years after their athletic career has ended. It is expected that the findings from this study will provide crucial information to better understand the pathophysiology of concussion as well as toward improved diagnosis and management of acute and long term effects of concussion from sport participation. It is also hoped that the neuroimaging, blood and genetic testing can help determine return-to-play. Despite being the largest such study, women athletes continue to be under represented in this consortium. Even as we await the results from this study, large scale studies must also to be initiated in children of both genders playing in a variety of organized sports from a very young age. The advances in neuroimaging hardware and computational capabilities will continue to improve the spatial and temporal resolutions of these techniques and the accuracy of these methods in the diagnosis of sports related concussion will continue to improve in the coming years.

AUTHOR CONTRIBUTIONS

All authors take responsibility for the integrity and the accuracy of the review. SN and BB: study concept and design. CC, KC, AS, SN, and BB: literature review. CC, KC, AS, JT, SN, and BB: data analysis and interpretation, drafting and editing of the manuscript, contributing important intellectual content in manuscript review.

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BRIEF RESEARCH REPORT
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Longitudinal Changes in Magnetic Resonance Spectroscopy in Pediatric Concussion: A Pilot Study

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Background: Nearly 20% of US adolescents report at least one lifetime concussion. Pathophysiologic models suggest that traumatic biomechanical forces caused by rotational deceleration lead to shear stress, which triggers a neurometabolic cascade beginning with excitotoxicity and leading to significant energy demands and a period of metabolic crisis for the injured brain. Proton magnetic resonance spectroscopy (^{1}H MRS) offers a means for non-invasive measurement of neurometabolic changes after concussion.

Objective: Describe longitudinal changes in metabolites measured *in vivo* in the brains of adolescent patients with concussion.

Methods: We prospectively recruited 9 patients ages 11 to 20 who presented to a pediatric Emergency Department within 24 h of concussion. Patients underwent MRI scanning within 72 h (acute, $n = 8$), 2 weeks (subacute, $n = 7$), and at approximately 1 year (chronic, $n = 7$). Healthy, age and sex-matched controls were recruited and scanned once ($n = 9$). ^{1}H MRS was used to measure N-acetyl-aspartate, choline, creatine, glutamate + glutamine, and myo-inositol concentrations in six regions of interest: left and right frontal white matter, posterior white matter and thalamus.

Results: There was a significant increase in total thalamus glutamate+glutamine/choline at the subacute ($p = 0.010$) and chronic ($p = 0.010$) time points, and a significant decrease in total white matter myo-inositol/choline ($p = 0.030$) at the chronic time point as compared to controls.

Conclusion: There are no differences in ^{1}H MRS measurements in the acute concussive period; however, changes in glutamate+glutamine and myo-inositol concentrations detectable by ^{1}H MRS may develop beyond the acute period.

Keywords: pediatric, adolescent, mild traumatic brain injury, concussion, magnetic resonance spectroscopy

INTRODUCTION

Nearly 20% of US adolescents report experiencing at least one lifetime concussion, also referred to as mild traumatic brain injury (TBI), and concussion accounts for approximately 200,000 visits annually to pediatric emergency rooms (1, 2). Current guidelines for assessing and diagnosing pediatric concussion rely on clinical judgment without the use of routine laboratory or imaging tests (3, 4). While most children recover within 1 month, 20–30% will experience persistent symptoms beyond 1 month (5, 6). It remains unknown why some patients experience prolonged symptoms while others do not. Investigations into the pathophysiologic underpinnings of concussion are crucial to guide development of tools that will allow researchers and clinicians to more accurately diagnose, prognose, and guide future interventions.

Animal models suggest concussion results from shear stress caused by rotational deceleration from traumatic biomechanical forces (7). This shear stress triggers a neurometabolic cascade that begins with excitotoxicity and leads to significant energy demands and a period of metabolic crisis for the injured brain (8, 9). Non-invasive neuroimaging-based biomarkers have provided important insights into structural and functional brain abnormalities in concussion. For example, studies using advanced neuroimaging techniques in adults have shown that concussion can be associated with alterations in white matter (WM) microstructure (diffusion tensor imaging, DTI), functional connectivity (fMRI), and cerebral blood flow (MR angiography) (10, 11).

Proton magnetic resonance spectroscopy (^{1}H MRS) allows direct investigation of metabolic shifts that occur during the neurometabolic cascade of concussion. ^{1}H MRS enables non-invasive measurement of metabolite and neurotransmitter concentrations in brain tissue. Metabolites with distinctive prominent spectra in ^{1}H MRS are N-acetyl aspartate (NAA), choline-containing compounds (Cho), and creatine-containing compounds (Cr). NAA is an amino acid highly concentrated in neurons, often interpreted as a marker of neuronal integrity and density, though some have shown dynamic changes in neuronal concentrations, suggesting a role as an indirect marker of the neuronal energy state (12, 13). Cho is a marker for cellular membrane turnover. Cr is a marker for cellular energetic systems and has also been proposed to have trophic and neuroprotective roles (14, 15). ^{1}H MRS can also be used to measure neurotransmitter concentrations (e.g., γ-aminobutyric acid [GABA], glutamate), lactate, and myo-inositol (Ins), among many other molecules with subtler, more difficult to resolve spectra. One meta-analysis of ^{1}H MRS data in adult TBI show decreased NAA, NAA/Cr and increased Cho/Cr at the subacute and chronic time points in moderate to severe cases, but did not reveal significant differences in mild TBI (16). One notable study, excluded from this meta-analysis, demonstrated decreased NAA/Cr at the acute post-concussive time point that normalized by 1 month (17).

Fewer neuroimaging studies have focused on pediatric concussion (18). Systematic reviews of neuroimaging in pediatric concussion have shown that DTI, fMRI and angiography findings do not always align with changes detected in adults (18–20). The adolescent brain may be uniquely vulnerable to the neurometabolic cascade, and this population requires special attention. Only a few studies, to our knowledge, have used in ^{1}H MRS in the adolescent population, and the methodology and reported results are highly variable (21–25).

The purpose of our study was to use magnetic resonance spectroscopy imaging (MRSI), an advanced type of ^{1}H MRS that allows multi-voxel measurements as compared to single-voxel spectroscopy, to explore possible differences in an extensive array of brain metabolites in pediatric patients with concussion as compared to controls. We further explored longitudinal trends in metabolites within concussed patients. Given the variable findings in preceding studies, this study was exploratory in nature. Nonetheless, we hypothesized that findings would be relatively consistent with observations in adult concussion as described above.

METHODS

Subjects

This study was approved by the Institutional Review Board at Boston Children's Hospital and written informed consent was obtained from parent proxies for all subjects. We prospectively recruited patients who presented to a pediatric Emergency Department within 24 h of concussion. Each patient underwent MRI scanning within 72 h (acute), 2 weeks (subacute), and approximately 1 year (chronic) after the concussion. Concussion was defined as a blunt, sports-related injury to the head resulting in either (1) alteration in mental status (including loss of consciousness, disorientation, or amnesia) or (2) any of the following symptoms that started within 4 h of injury and were not present before the injury: headache, nausea, vomiting, dizziness/balance problems, fatigue, drowsiness, blurred vision, memory difficulty or difficulty concentrating. Patients were excluded from the study if they presented to the Emergency Department with Glasgow Coma Scale < 14, focal symptoms or other indications for head imaging or intracranial hemorrhage seen when imaging was obtained, orthopedic fracture, co-existing intra-abdominal or intra-thoracic trauma, or spinal-cord injury, or an underlying neurologic disorder or psychiatric illness requiring medications. Healthy, age and sex-matched controls were recruited and scanned once. Controls were recruited without current neurological complaints or recent head trauma at least a year prior to scanning.

MRI Image Acquisition

Structural T1-weighted and MRSI volumes were collected at each time point. Imaging data were acquired on a 3T Siemens Tim Trio system (Erlangen, Germany), equipped with 32-channel receive coil array and maximum gradient strength and slew rate of 40 mT/m and 180 mT/m/ms, respectively. T1-weighted motion mitigated multi-echo MPRAGE (26) parameters were: TR = 2520 ms; TE = 1.74, 3.54, 5.34, and 7.14 ms; inversion time = 1,350 ms; field-of-view (FOV) = 240 mm^2; voxel size = 1 mm^3. For MRSI, we used an accelerated 3D MRSI sequence with spiral k-space trajectories (27), which has been fully implemented on Siemens MR platforms (28). This sequence provides for the first time in clinical settings volumetric CSI coverage of

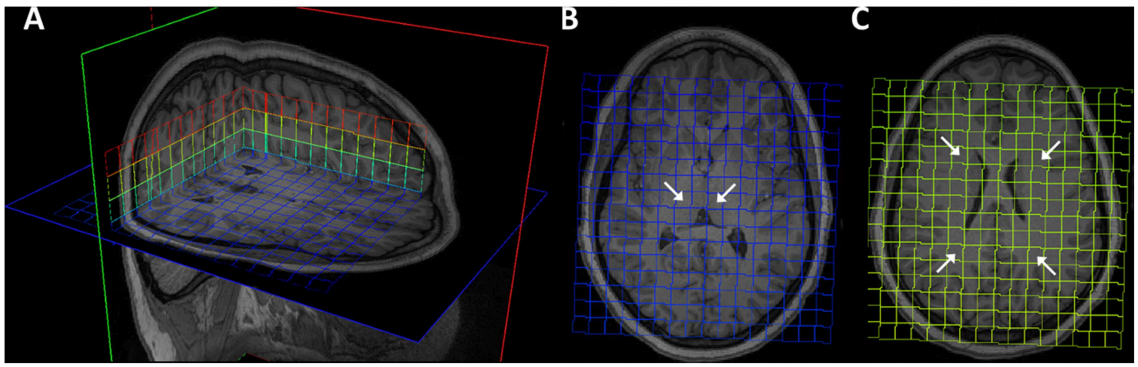

FIGURE 1 | Magnetic resonance spectroscopy excitation grid overlaying T1 MPRAGE images of representative brain. **(A)** 3D visualization. **(B)** Bilateral thalamus ROIs indicated by arrows on transverse slice. **(C)** Bilateral frontal and parietal ROIs.

isotropic voxel size of 2 cc in 1 min of acquisition time. Specific imaging parameters were: Matrix size (x, y, z, f) = (13, 13, 8, 512), zero-padded to (16, 16, 8, 512) and encoded over FOV_{XY} = 16 cm^2, FOV_Z = 10 cm, FOV_f = 1.2 KHz for an overall isotropic voxel size of 2 cc; excitation box was prescribed entirely within the brain to avoid lipid contamination from the skull, such that the center of the excitation box is centered around basal ganglia; TE = 30 ms, TR = 1.8 s, number of averages = 8 (for signal-to-noise-ratio [SNR] purposes), for an overall acquisition time of 7:48 min. An additional spectroscopy scan with no water suppression and with one average and TR = 1 s (overall acquisition time of 36 s) was acquired for the purposes of obtaining absolute quantitative estimates of brain metabolites.

MRI Image Processing

MRSI was used to measure NAA, Cho, Cr, glutamate + glutamine (Glx), Ins concentrations. MRSI data was analyzed using TARQUIN and custom Matlab (Natick, MA, USA) routines (29). We obtained a 3D MRSI acquisition with a single short TE (30 ms) and without any spectral editing, which meant that GABA and lactate signals were not analyzed/fitted due to their inherent strong coupling with other metabolites in the spectra. Spectra from individual voxels were fit using default TARQUIN parameters, except the chosen reference signals were NAA, Cr, Cho, and Lipids, and the excitation scheme was LASER(30). Absolute concentrations were calculated assuming a water attenuation factor of 0.7 (metabolite T_2 = 200 ms, water T_2 = 80 ms, TE = 30 ms) and water concentration of 35,880 mM (WM water concentration). Concentration ratios were also determined for each metabolite concentration relative to Cho. Data quality was evaluated on a voxel-wise basis by first visually inspecting a selection of spectra to determine SNR and Q thresholds so that low quality spectra are rejected from the remaining dataset. SNR was determined by TARQUIN by taking the ratio between the maximum in the spectrum minus baseline divided by two times the root mean squre of the residual between 0.5–4 ppm. Q is a measure of how well the spectrum was fit by TARQUIN determined by the ratio of the fit residual to the noise level of the spectrum, specifically the standard deviation of the fit residual (between around 0.5–4 ppm) divided by the standard deviation of the noise region.

For each patient and time point, the 3D MRSI excitation grid was overlaid onto the T1-weighted structural volume to allow visualization and manual selection of region of interest (ROI). Six MRSI voxels were selected as each regions of interest: left and right frontal WM, posterior WM and thalamus (**Figure 1**). Given the large voxel size in MRSI, voxels were selected to include as much of the desired ROI as possible. For each subject, measurements from individual thalamus and WM voxels were averaged to create total thalamus and total WM meta-ROIs, respectively. T1-weighted images were pre-processed with the FreeSurfer "recon-all" pipeline (https://surfer.nmr.mgh.harvard.edu) and output were visually inspected, yielding four whole brain segmentation masks of the WM, cerebrospinal fluid (CSF), cortical gray matter (cGM), and subcortical gray matter (sGM) in native space. sGM comprised bilaterally the thalamus, caudate, putamen, pallidum, hippocampus, amygdala, and insula. For each MRSI ROI, the percentages of WM, CSF, cGM, and sGM within the ROI were computed.

Statistical Analyses

All statistical analyses were performed using STATA statistical software package (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC). To exclude low-quality data, voxels were removed if SNR was <5 and the Q-statistic was >2.5. Fisher's exact test was used to analyze for sex differences between concussed and control patients. ANOVAs were used to analyze differences in age and partial volumes. Differences in metabolite concentrations at each time point were compared between patients and controls using Mann-Whitney U-tests. We first looked for differences in the meta-ROIs, and if a trend ($p < 0.1$) was detected, we further investigated for differences in the individual ROIs and for longitudinal trends. To assess longitudinal trends within the concussed patients, we used linear mixed effects models fit by maximum likelihood with metabolite concentration as the outcome and fixed and random effects of time point. Separate models were performed for each metabolite and ROI combination of interest. A threshold of p <0.05 was used to determine statistical significance. Due to the

TABLE 1 | Demographics and ROI partial volumes.

	Control ($n = 9$)	Concussion ($n = 9$)	*p*-value
Age, mean years (SD)	13.52 (1.26)	14.23 (2.81)	0.50
Female sex, *n* (%)	1 (11%)	1 (11%)	> 0.99
L thalamus, % sGM, mean (SD)	80.43 (14.90)	80.43 (12.14)	> 0.99
R thalamus, % sGM, mean (SD)	82.97 (17.19)	78.22 (11.23)	0.52
L frontal WM, % WM, mean (SD)	93.44 (6.17)	91.70 (10.97)	0.69
R frontal WM, % WM, mean (SD)	98.25 (1.97)	98.11 (1.60)	0.88
L parietal WM, % WM, mean (SD)	96.72 (2.54)	91.42 (10.73)	0.17
R parietal WM, % WM, mean (SD)	95.93 (3.01)	93.81 (5.50)	0.33

Partial volumes refer to the first (baseline) collection time point. ROI, region of interest; SD, standard deviation; L, left; R, right; sGM, subcortical gray matter; WM, white matter.

exploratory nature of our pilot study, *p*-values were not corrected for multiple comparisons.

RESULTS

Demographics

Baseline group demographics and ROI partial volume data are presented in **Table 1**. In total, spectroscopy data was collected on nine concussed subjects and nine controls. Due to inability to collect MRSI data on every subject, it must be noted that these cohorts were not perfectly age-matched. After exclusion of corrupted data and accounting for loss to follow up, six concussion subjects had full longitudinal data at all three time points, one had data at the acute and chronic time points, and two subjects had only a single time point (one acute, and one subacute). Among all voxels from all participants, only five were excluded from analysis due to poor quality spectra (SNR < 5 and Q > 2.5). There were no significant differences in sex or age between concussion and control groups. There were no significant partial volumes differences between groups.

MRSI Analyses

Spectroscopic measurements are presented in **Table 2**. On examination of the meta-ROIs, there was a significant increase in total thalamus Glx/Cho at the subacute ($p = 0.010$) and chronic ($p = 0.010$) time points as compared to controls (**Figure 2**). For total WM, there was a significant decrease in Ins/Cho ($p = 0.030$) and a trending decrease in Ins ($p = 0.050$) at the chronic time point (**Figure 3**).

Further targeted investigation into the individual ROIs revealed a significant increase in left thalamus Glx/Cho at subacute ($p = 0.017$) and chronic ($p = 0.039$) time points. There was a significant decrease in right frontal WM Ins/Cho ($p = 0.011$) at the chronic time point, and a trending decrease in left frontal WM Ins/Cho ($p = 0.064$) at the chronic time point. There were no other significant differences in metabolite absolute concentrations or ratios for any of the individual or meta-ROIs at any time point when comparing concussed patients to controls.

Within the concussed groups, there was a significant linear increase over time in total thalamus Glx/Cho ($b = 0.37$, CI: 0.03–0.71, $p = 0.034$) and in total thalamus Ins/Cho ($b = 0.46$, CI: 0.11–0.81, $p = 0.010$). There were no significant linear trends over time in total WM Glx, Glx/Cho, Ins, or Ins/Cho.

DISCUSSION

This study used MRSI to broadly explore longitudinal changes in the neurometabolic state of the brain after pediatric concussion. Our study demonstrates the feasibility of using ^{1}H MRS in the pediatric population to measure a range of neurometabolites. Our analyses suggest that Glx/Cho in the thalamus may be increased at the subacute time point following concussion, and this elevation may persist chronically up to 1 year. Our study also suggests that concussion may be associated with a delayed decrease in Ins that is not seen in the acute or subacute time period. These results could inform future studies investigating potential therapeutic targets or prognostic biomarkers, which are critically needed for concussion generally, but also specifically in pediatric patients who may be particularly vulnerable to these neurological insults.

Our study adds to a small but growing body of literature using ^{1}H MRS in pediatric concussion research. In 2012, Maugans et al. were the first group to report MRS findings in the adolescent population. Using single-voxel spectroscopy, they found no longitudinal changes in thalamus, frontal GM, or frontal WM NAA, NAA/Cr, or lactate in mild TBI, and no differences compared to controls (21). Their study looked at three time points, and final imaging for all subjects occurred < 3 months from the initial injury. While our findings align with their observations of NAA, we included imaging done approximately 1 year from injury, which offers further insight into the neurometabolic processes when symptoms have resolved for most patients.

Other pediatric studies have shown variable results. Our Ins findings are congruent with work by Poole et al. who followed football athletes longitudinally over the course of a season and found decreasing Cr and Ins in the dorsolateral prefrontal cortex (22). Although Poole et al. did not assess a control group, which limits comparison to the present study, we also demonstrated decreased Ins over time in our sample. Ins has been proposed to be a glial marker, among other functions, and decreased concentrations may represent glial dysfunction (15). We did not find significant differences in NAA/Cho nor the absolute concentrations of NAA, Cho, and Cr. Other studies have found increased GABA/Cr in the frontal lobe at approximately 1 month following concussion compared to controls (23), decreased Cho in the prefrontal region 3 months following concussion as compared to controls (24), decreased NAA/Cr and NAA/Cho in the corpus callosum and parietal WM at various time points ranging from 3 to 12 months post-concussion as compared to controls (25). It is important to note that the methodology and findings of these five studies are highly variable, and further studies are needed to continue to reproduce results and more deeply evaluate the neurometabolic consequences of pediatric concussion.

We also report a novel finding of increased thalamic Glx/Cho at the subacute and chronic time points. The spectra for glutamate and glutamine overlap highly, and concentrations are generally correlated, so their combined concentrations (i.e., Glx) are reported in this study. On further inspection of the quality of the spectral fitting for Glx spectra, the average Cramér-Rao-lower-bound value for all Glx peaks

TABLE 2 | Median metabolite concentrations (mM), or concentrations ratios, and interquartile range by group and time point.

		Control	Concussion			CRLB
			Acute ($n = 8$)	Subacute ($n = 7$)	Chronic ($n = 7$)	
Days since injury, mean (SD)		7.78 (4.29)	1.13 (0.64)	16.14 (2.73)	417.86 (19.14)	
Cho	Total WM	22.21 (2.87)	22.43 (3.80)	24.04 (5.68)	24.72 (4.60)	6.67%
	Total thalamus	25.19 (6.54)	26.01 (13.52)	21.66 (8.74)	21.80 (11.38)	
Cr	Total WM	71.87 (11.15)	61.88 (40.68)	69.69 (10.20)	66.31 (6.16)	7.07%
	Total thalamus	71.20 (17.80)	69.49 (11.56)	61.72 (11.49)	63.37 (15.47)	
NAA	Total WM	104.26 (16.22)	101.12 (9.22)	109.33 (8.11)	102.96 (4.02)	6.72%
	Total thalamus	92.54 (19.74)	90.28 (34.58)	96.50 (42.43)	82.55 (27.36)	
NAA/Cho	Total WM	4.87 (0.92)	4.72 (0.47)	4.86 (0.84)	4.43 (0.69)	–
	Total thalamus	3.30 (1.11)	3.53 (0.90)	3.50 (1.44)	3.79 (1.45)	
Glx	Total WM	72.75 (9.23)	65.07 (31.19)	63.87 (21.36)	70.34 (12.84)	16.20%
	Total thalamus	68.80 (29.00)	78.29 (53.18)	78.40 (16.82)	81.89 (7.73)	
Glx/Cho	Total WM	3.57 (1.01)	2.83 (0.55)	2.98 (0.49)	2.87 (0.70)	–
	Total thalamus	2.87 (0.30)	3.36 (1.49)	**3.75 (1.16)	**3.72 (1.30)	
Ins	Total WM	54.75 (7.50)	47.71 (12.26)	50.17 (16.25)	*49.71 (5.64)	12.91%
	Total thalamus	44.15 (16.90)	41.45 (22.16)	48.37 (20.92)	42.51 (11.62)	
Ins/Cho	Total WM	2.35 (0.89)	2.28 (1.06)	2.33 (0.69)	**1.87 (0.35)	–
	Total thalamus	1.82 (0.29)	1.59 (0.41)	2.06 (0.45)	1.88 (1.20)	

*Total WM is the average of the 4 individual WM ROIs and total thalamus is the average of the 2 individual thalamic ROIs. SD, standard deviation; WM, white matter; Cho, choline compounds; Cr, creatine compounds; NAA, N-acetyl-aspartate; Glx, glutamate + glutamine; Ins, myo-inositol; CRLB, Cramér-Rao-lower-bound. *P < 0.1, **p < 0.05 by Mann-Whitney U-test.*

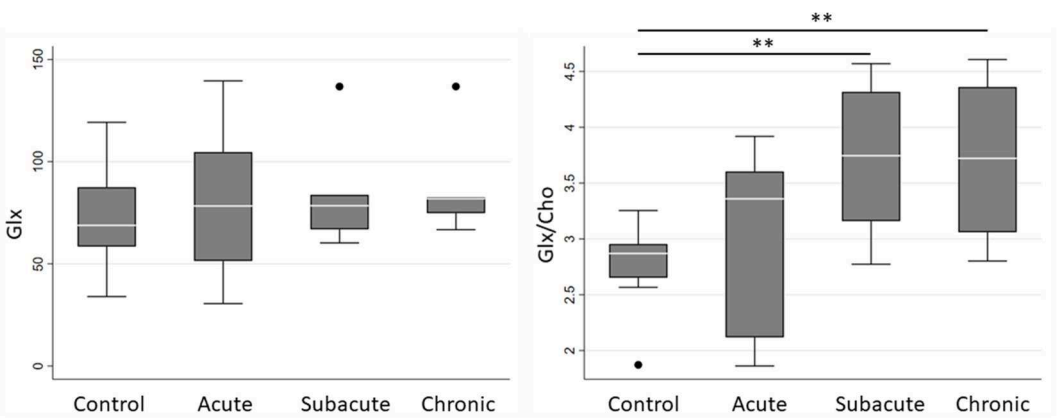

FIGURE 2 | Boxplot representations comparing median and interquartile range for total thalamus Glx and Glx/Cho between controls and concussed patients at acute, subacute and chronic time points. Individual horizontal bars are presented for each significant group comparison. Glx = glutamate + glutamine, Cho = choline compounds. $^{**}P < 0.05$ by Mann-Whitney U-test.

was approximately 16%, which indicates a fair degree of certainty in the Glx concentrations. Current pathophysiologic models suggest an acute and transient period of neuronal excitotoxicity and glutamate release at the beginning of the neurometabolic cascade of concussion (7). It is possible that our acute time point did not occur soon enough after injury to detect this cascade. Additionally, our findings indicate a delayed increase in glutamate concentrations, which suggests that altered neurotransmission may persist chronically following concussion. It should be noted that the differences in absolute Glx concentrations were not significant. In fact, further inspection of the data in **Table 2** shows increasing absolute Glx values and decreasing absolute Cho values, suggesting that the trend observed in Glx/Cho could be a product of these two insignificant, opposing trends.

Our study has several limitations. First, this study was underpowered to detect small changes in metabolite concentrations, and it remains possible that additional meaningful neurometabolic changes occur during the longitudinal course of pediatric concussion. Due to the small sample size, we did not include covariates in our statistical model, which limits the generalizability of our findings and allows for the possibility of confounders that may have impacted these results. For instance, while there is evidence that brain metabolites are relatively stable above 4 years of age, there may still be age-related effects on metabolites concentrations, and this

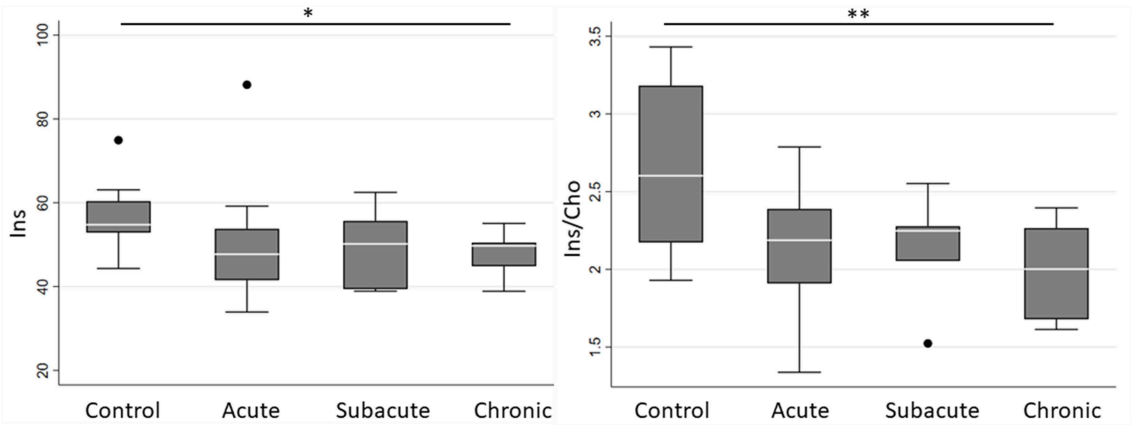

FIGURE 3 | Boxplot representations comparing median and interquartile range for total white matter Ins and Ins/Cho between controls and concussed patients at acute, subacute and chronic time points. Individual horizontal bars are presented for each significant group comparison. Ins = myo-inositol, Cho = choline compounds. *$P < 0.1$, **$p < 0.05$ by Mann-Whitney U-test.

remains an important area for future research (30, 31). Given the limited sample size, we did not correct for multiple comparisons, and as a result our findings are preliminary and require confirmation with better-powered studies. Second, normal ranges for absolute concentrations are not well established in either pediatric or adult populations, making it difficult to interpret effect sizes. Though we believe our methodology for absolute concentration of metabolites was suitable for the longitudinal analysis we undertook, concentrations we report for GM would need to be refined for comparison to other studies that make different assumptions about metabolite T_2 values or tissue water concentrations. Finally, obtaining estimates of the GABA and lactate concentrations will also be explored in future studies, as we expect that their values might change in the event of concussion. To obtain reliable estimates of these metabolites though, we need to employ special spectral editing MRSI acquisition techniques [e.g., MEGA-PRESS 2DJ (32) or 2D COSY MRSI (33)] in order to be able to uncouple the GABA and lactate signals from the other metabolites that are spectrally overlapping with them.

The current results align with the adult literature, which has shown minimal differences in ^{1}H MRS measurements in the acute concussive period. Our study suggests that there may be significant longitudinal neurometabolic changes in the pediatric population leading to differences detectable beyond the acute time point, and we add to previous ^{1}H MRS studies of pediatric concussion by examining the chronic time point at 1 year post-concussion. It remains to be seen how these results will fit with hypothesized biological pathways implicated in pediatric concussion, and larger sample sizes are needed to further evaluate smaller effects and to determine if the observed neurometabolic changes are reproducible.

DATA AVAILABILITY

The datasets generated and analyzed for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Review Board at Boston Children's Hospital with written informed consent obtained from all subjects. For minors, consent was obtained from parent proxies. All subjects or parent proxies gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board at Boston Children's Hospital.

AUTHOR CONTRIBUTIONS

PG, RM, and BG designed and supervised data collection. EM, JS, AC, and RM were responsible for imaging analysis design. EM, JS, and AC conducted the experiments. EM analyzed the results. RM and AC provided statistical analysis guidance. EM, JS, and AC wrote the manuscript, and all authors reviewed the manuscript.

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Sex-Based Differences in Transcranial Doppler Ultrasound and Self-Reported Symptoms After Mild Traumatic Brain Injury

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The possibility of sex-related differences in mild traumatic brain injury (mTBI) severity and recovery remains a controversial subject. With some studies showing that female subjects suffer a longer period of symptom recovery, while others have failed to demonstrate differences. In this study, we explored the sex-related effects of mTBI on self-reported symptoms and transcranial Doppler ultrasound (TCD) measured features in an adolescent population. Fifty-eight subjects were assessed—at different points post-injury—after suffering an mTBI. Subjects answered a series of symptom questions before the velocity from the middle cerebral artery was measured. Subjects participated in breath-holding challenges to evaluate cerebrovascular reactivity. The Pulsatility Index (PI), the ratio of the first peaks (P2R), and the Breath-Hold Index (BHI), were computed. Linear mixed effects models were developed to explore the interactions between measured features, sex, and time since injury while accounting for within subject variation. Over the first 10 days post-injury, the female group had significant interactions between sex and time since injury that was not present in the TCD features. This is the first study to compare sex-related differences in self-reported symptoms and TCD measurements in adolescents suffering an mTBI. It illustrates the pitfalls clinicians face when relying on subjective measures alone during diagnosis and tracking of mTBI patients. In addition, it highlights the need for more focused research on sex-related differences in concussion pathophysiology.

Keywords: sex differences, traumatic brain injury, vascular reactivity, CBF autoregulation, blood flow

INTRODUCTION

The presence of sex-related differences in mild traumatic brain injuries or concussions (mTBI), is a contentious subject. Several studies have found increased symptoms in females (1–3), as well as increased length of recovery (4–6). In the case of Preiss-Farzanegan et al. (7), a difference existed in adults (18 years-old or older), but not in minors (17 years-old and younger). Conversely, others have failed to demonstrate increases in female symptoms at all (8–12). In general, the current state of the literature suggests that the existence of gender dependence in concussion recovery and severity is still an open question (13, 14).

One commonality between these studies however, is that they all rely on neurocognitive evaluations, patient symptoms, or physical performance testing. Although these have been shown to provide insight into concussion severity and prognosis, they do not objectively measure physiological changes resulting from concussive injury. Here, we present a study comparing features of the cerebral blood flow velocity (CBFV) as measured with Transcranial Doppler (TCD) ultrasound in addition to self-reported symptoms to investigate sex-related differences in concussion.

With recent research demonstrating abnormalities in cerebral blood flow after a mTBI, it is clear that the microvasculature is affected (15–24). In Thibeault et al. (23), the cerebral hemodynamic changes in adolescents between 14 and 19 years old after suffering a clinically diagnosed mTBI were assessed using TCD. In that study two distinct phases of hemodynamic alterations after a concussive injury were identified. In the initial phase, beginning within an hour of injury and lasting through the first 48 h, Pulsatility Index (PI), and peak ratio (P2R), showed a significant difference from controls. After 48 h however, these differences in pulsatile features were no longer observable. At this point in their recovery the breath-holding index (BHI), a measure of the cerebral vascular reactivity (CVR), was significantly increased when compared to controls. This lasted through day seven. After which, the population level increase was no longer significant.

Although Thibeault et al. (23) was the first study to suggest the presence of multiple phases of hemodynamic dysfunction, there have been others demonstrating measurable alterations in mTBI subjects using TCD. Utilizing a hypercapnia challenge, Len et al. (15) found significant changes in a population of concussed subjects. A subsequent study found significant differences during hypocapnia (24). Similarly, the study from Albalawi et al. (25), found vasoreactivity was linearly related to both severe headaches and cognitive symptoms. Baily et al. (18), found lowered CVR in a population of subjects suffering from chronic symptoms. The present study, however, appears to be the first to explore sex specific abnormalities in mTBI subjects with both self-reported symptoms and an objective physiological measure.

METHODS

Patient Population

Participants in this study consisted of adolescents between 14 and 19 years old from the Los Angeles, California metropolitan area. Subjects classified with an mTBI were diagnosed by independent physicians and were scanned at different times post-injury. For this analysis these longitudinal measurements were restricted to 13 days post-jury from 58 unique subjects. The population was comprised of 37 male and 21 female participants, with 81 and 57 total exams for each group, respectively. Within the male group, 17 subjects had more than one scan during the course of recovery and a median number of scans of 1.0 with an IQR of 2.0. In the female group, 13 subjects had more than one scan and there as an overall median 2.0 scans with an IQR 3.0. The control group consisted of 109 age-matched subjects, 89 male and 12 female, who had no reported head-injuries in the preceding 12-months. The control group was only scanned a once. The study was approved by Western Institutional Review Board (IRB #20141111). This data was previously used in Thibeault et al. (23).

Data Collection

The TCD signals were acquired from the middle cerebral arteries (MCA) transtemporally by ultrasonographers utilizing 2 MHz probes held by an adjustable headset. End-tidal CO_2 was collected concurrently through a nasal cannula. The exam protocol, illustrated in **Figure 1**, began with a 5-min baseline period of normal breathing. This was followed by a series of 4 breath-holding challenges as an estimate of CVR. Each of these consisted of a 25-s period where the subject was instructed to hold their breath, followed by 35-s of normal breathing.

Symptom Reporting

Before each of the data collection session, subjects were asked to answer a number of questions similar to the graded symptom scale checklist. **Table 1** presents the list of questions where subjects were asked to numerically rate their current symptom state. The ratings were used both individually and summed together as an estimate of severity.

Analysis

The TCD features found to correlate with mTBI in Thibeault et al. (23), were used to compare with the self-reported symptoms. The first pulse level feature, extracted from the baseline section, was the PI. This is generally believed to be related to distal resistance however, it appears to be more modulated by a number of physiological processes (26), PI is found by

$$PI = (P_1 - D) / \overline{V}_B.$$

Where P_1, D, and V_B are defined in **Figure 1**.

The second, P2R, is the ratio of P_2, and P_1, as illustrated in **Figure 1**. This has been hypothesized to be related to distal bed compliance (27). This is found by

$$P2R = P_2/P_1.$$

These features were individually averaged across all the extracted pulses from the baseline section.

The CVR was estimated using the BHI. This was found by first finding the highest peak of the low-pass filtered CBFV waveform between the four breath-hold sections as illustrated in **Figure 1**. This is then related to the baseline mean velocity by

$$BHI = \frac{P_{BH} - V_{BL}}{V_{BL}}.$$

Statistical Modeling

Linear mixed-effect models were developed to explore the interactions between effects of time and sex on the measured variables while compensating for the unbalanced groupings and the potential individual subject variation. The models were developed in R using the lme4 package (28). Summary statistics and significance values—using the satterthwaite method of

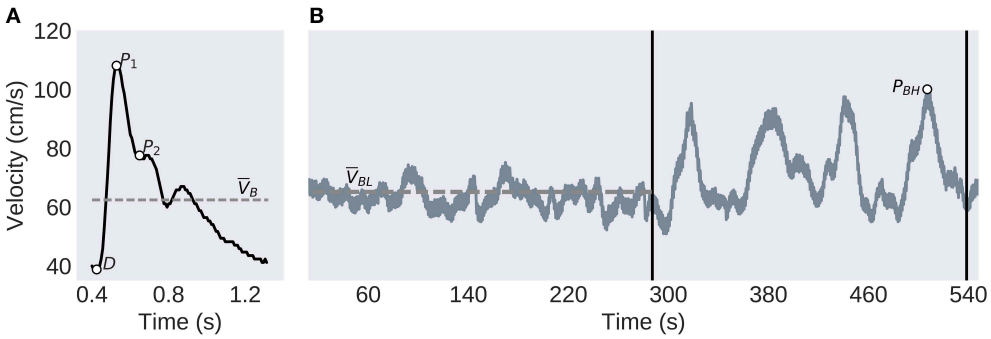

FIGURE 1 | Experimental Protocol and TCD Analysis. **(A)** The individual pulses are extracted before the systolic peak (P1), diastolic trough (D), second peak (P2), and the mean velocity (V_B) are identified. **(B)** The CVR protocol consists of a 5-min baseline, left of the first vertical bar, followed by the four breath-holding challenges, between the vertical bars. The low-frequency component of the global signal (solid gray line), is used to compute the baseline mean velocity (V_{BL}, dashed line) and the largest peak velocity (P_{BH}) used to calculate the BHI.

degrees of freedom and *t*-test—were computed with the lmertest package (29). Additional model analysis was completed with the Psycho library (30). Effects were considered significant if $p < 0.05$, and the reported beta was at least twice the standard error (SE).

The models were developed for each of the three TCD features as well as the summed symptom scores, as dependent variables. For symptoms and BHI, the random-effects were explored by fitting different models with the maximum likelihood method and comparing with the likelihood ratio test—the models with significant improvement were selected. The fixed-effects and interactions were similarly compared, and the final models were then fit with the restricted maximum likelihood method. The models for PI and P2R failed to converge with the maximum likelihood method, however, the restricted method did reach convergence. Because of this, the resulting models both used a similar structure, with days-post injury, sex, and their interactions as fixed effects, and subject specific intercepts as random effects. For the sex category, a contrast encoding of [0.5, −0.5] with males as the reference was employed. Similarly, a dummy encoding with the controls as the reference group was used for the days-post category. These were grouped similar to Thibeault et al. (23). Correlations between features were evaluated using the Pearson correlation coefficient and the sex dependent interactions of the resulting regression lines were explored using the ANCOVA method with a set of linear models fit with the ordinary least squares method from lme4 (28). A one-way ANOVA was conducted to compare the effect of sex and condition (case or control), on age using the StatsModels package (31) in Python.

TABLE 1 | Subjects were asked score themselves on the following symptoms based on how they feel now–None (0), Mild (1,2), Moderate (3,4), Severe (5,6).

Symptoms
Headache
Pressure in head
Neck pain
Nausea or vomiting
Dizziness
Blurred vision
Balance problems
Sensitivity to light
Sensitivity to noise
Vision problems
Feeling like in a "fog"
"Don't feel right"
Difficulty concentrating
Difficulty remembering
Fatigue or low energy
Confusion
Drowsiness
Trouble falling asleep
More emotional
Irritability
Sadness
Nervous or anxious

RESULTS

Population

There was no significant interaction between the effects of sex and condition on age [$F_{(1,163)} = 0.08$, $p = 0.78$], or main effects of either sex [$F_{(1,163)} = 1.86$, $p = 0.17$] or condition [$F_{(1,163)} = 0.004$, $p = 0.96$]. All subjects identified as athletes, with the majority of males, 26 (74%) subjects from the mTBI group and 77 (87%) from the control group, playing football as their primary sport. The other subjects were split between rugby, soccer, basketball, baseball, lacrosse, ice hockey, and quidditch. Although within the female population soccer was the most popular, 10 (45%) from the mTBI group and 7 (75%) subjects from the control group, the overall spread was more diverse and included volleyball, dance, track, swimming, basketball, softball and cheer. Within the mTBI population there was a slight difference in the reported mechanism of injury. For the male population 33 (89%)

TABLE 2 | Mixed effect model results for symptoms.

Variable	β (SE)	95% CI	*t*(DF) *p*
(Intercept)	2.7 (1.97)	[−1.03, 6.52]	1.37(215) $p > 0.1$
Sex	2.91 (3.94)	[−4.58, 10.51]	0.74(215) $p > 0.1$
0–1 Days-Post	**27.54 (3.99)**	**[19.89, 35.06]**	**6.9(214) $p < 0.001$**
2–3 Days-Post	**24.4 (3.5)**	**[17.65, 30.99]**	**6.97(222) $p < 0.001$**
4–5 Days-Post	**18.84 (3.15)**	**[12.78, 24.78]**	**5.97(223) $p < 0.001$**
6–7 Days-Post	**21.98 (3.01)**	**[16.01, 27.64]**	**7.29(230) $p < 0.001$**
8–9 Days-Post	**13.34 (3.15)**	**[7.23, 19.28]**	**4.23(230) $p < 0.001$**
10–11 Days-Post	**9.42 (3.25)**	**[3.21, 15.56]**	**2.9(228) $p < 0.01$**
12–13 Days-Post	**8.56 (3.48)**	**[1.95, 15.14]**	**2.46(222) $p < 0.05$**
Sex:0–1 Days-Post	**30.85 (7.98)**	**[15.43, 45.85]**	**3.87(214) $p < 0.001$**
Sex:2–3 Days-Post	**20.4 (7.01)**	**[−1.08, 23.6]**	**2.91(222) $p < 0.01$**
Sex:4–5 Days-Post	**16.88 (6.31)**	**[−2.29, 24.09]**	**2.67(223) $p < 0.01$**
Sex:6–7 Days-Post	**27.98 (6.03)**	**[6.93, 33.6]**	**4.64(230) $p < 0.001$**
Sex:8–9 Days-Post	**23.46 (6.31)**	**[4.82, 28.79]**	**3.72(230) $p < 0.001$**
Sex:10–11 Days-Post	11.22 (6.49)	[−1.08, 23.6]	1.73(228) $p > 0.05$
Sex:12–13 Days-Post	10.92 (6.95)	[−2.29, 24.09]	1.57(222) $p > 0.1$

The bold rows indicate significant effects or interactions.

TABLE 3 | Mixed effect model results for BHI.

Variable	β (SE)	95% CI	*t*(DF) *p*
(Intercept)	0.41 (0.02)	[0.37, 0.44]	23.35(215) $p < 0.001$
Sex	−0.03 (0.03)	[−0.09, 0.04]	−0.73(215) $p > 0.1$
0–1 Days-Post	−0.02 (0.04)	[−0.09, 0.05]	−0.58(215) $p > 0.1$
2–3 Days-Post	**0.14 (0.03)**	**[0.08, 0.21]**	**4.44(224) $p < 0.001$**
4–5 Days-Post	**0.09 (0.03)**	**[0.04, 0.15]**	**3.17(222) $p < 0.01$**
6–7 Days-Post	**0.06 (0.03)**	**[0.01, 0.12]**	**2.32(231) $p < 0.05$**
8–9 Days-Post	0.05 (0.03)	[0, 0.11]	1.81(231) $p > 0.05$
10–11 Days-Post	−0.01 (0.03)	[−0.07, 0.05]	−0.32(228) $p > 0.1$
12–13 Days-Post	0.01 (0.03)	[−0.05, 0.07]	0.24(223) $p > 0.1$
Sex:0–1 Days-Post	0.15 (0.07)	[0.01, 0.29]	1.98(215) $p < 0.05$
Sex:2–3 Days-Post	0.05 (0.07)	[−0.1, 0.12]	0.71(224) $p > 0.1$
Sex:4–5 Days-Post	0.05 (0.06)	[−0.05, 0.2]	0.82(222) $p > 0.1$
Sex:6–7 Days-Post	0 (0.06)	[−0.08, 0.17]	0.09(231) $p > 0.1$
Sex:8–9 Days-Post	−0.01 (0.06)	[−0.06, 0.16]	−0.23(231) $p > 0.1$
Sex:10–11 Days-Post	0.01 (0.06)	[−0.1, 0.12]	0.17(228) $p > 0.1$
Sex:12–13 Days-Post	0.08 (0.06)	[−0.05, 0.2]	1.18(223) $p > 0.1$

The bold rows indicate significant effects or interactions.

subjects reported being injured playing a sport, while 4 (11%) did not provide a mechanism. Within the female population 16 (76%) identified their cause of injury from a sport, whereas 5 (24%) reported another mechanism or did not provide a cause.

Symptoms

The model for symptoms had an explanatory power (conditional R^2) of 76.74%, in which the fixed effects explain 49.95% of the variance (marginal R^2). Though there was no overall main effect of sex in the model (β = 2.91, SE = 3.94, 95% CI [−4.58, 10.51], $t_{(215)} = 0.74$, $p > 0.1$), there were significant interactions between sex and days-post groups for the first 11 days, see **Table 2**. This is illustrated by the increased self-reported summed symptoms scores in the female population in **Figure 2A**. In addition, the individual symptom averages in **Figure 3** illustrate that it was not a small subset of symptoms dominating the summed score for the female population. Additionally, there were large main effects for all days-post groupings, see **Table 2**.

TCD Features

BHI

The BHI model had a total a total explanatory power (conditional R^2) of 45.16%, in which the fixed effects explain 13.83% of the variance (marginal R^2). There was no main effect of sex found in the model, (β = −0.03, SE = 0.03, 95% CI [−0.09, 0.04], $t_{(215)} = -0.73$, $p > 0.1$), but there was one large interaction between sex and days-post at the 0–1 days grouping (β = 0.15, SE = 0.07, 95% CI [0.01, 0.29], $t_{(215)} = 1.98$, $p < 0.05$), **Table 3**. However, within this grouping, all of the male subjects were collected on the day of their injury, while the female subjects were all collected the day after their injury occurred. In this instance, it seems more feasible that the interaction is a product of the female subjects being collected closer to the period of hyperreactivity found in Thibeault et al. (23), as opposed to a sex-related disparity.

The overall longitudinal profile found in Thibeault et al. (23) was also predicted here by main effects for days-post 2–3 (β = 0.14, SE = 0.03, 95% CI [0.08, 0.21], $t_{(224)} = 4.44$, $p < 0.001$), 4–5 (β = 0.09, SE = 0.03, 95% CI [0.04, 0.15], $t_{(222)} = 3.17$, $p < 0.01$), and 6–7 (β = 0.06, SE = 0.03, 95% CI [0.01, 0.12], $t_{(231)} = 2.32$, $p < 0.05$), see **Figure 1B**.

PI

The model for PI had total explanatory power (conditional R^2) of 72.02%, in which the fixed effects explain 8.35% of the variance (marginal R^2). Here, a main effect of sex was predicted (β = −0.13, SE = 0.04, 95% CI [−0.21, −0.04], $t_{(195)} = -2.96$, $p < 0.01$). Exploring the population results in **Figure 2C** suggest that this effect may be a product of an inherent difference between males and females in the control population, as opposed to a sex-related difference. This is supported by the lack of significant interactions between sex and days-post injury grouping, **Table 4**. There was a significant main effect found at days-post 8–9 (β = 0.07, SE = 0.03, 95% CI [0.01, 0.13], $t_{(227)} = 2.14$, $p < 0.05$), that cannot be fully explained.

P2R

The model predicting P2R had a total explanatory power (conditional R^2) of 74.42%, in which the fixed effects explain 10.32% of the variance (marginal R^2). A main effect of sex was present (β = 0.05, SE = 0.02, 95% CI [0.01, 0.10], $t_{(196)} = 2.21$, $p < 0.05$). However, similar to PI, **Figure 2D** illustrates a difference between control groups. There were no significant interactions between sex and days-post found, see **Table 5**.

Correlations

The correlations between features provides additional information about the sex-related differences in this population, **Figures 4A,B**. Both sexes had significant negative correlations

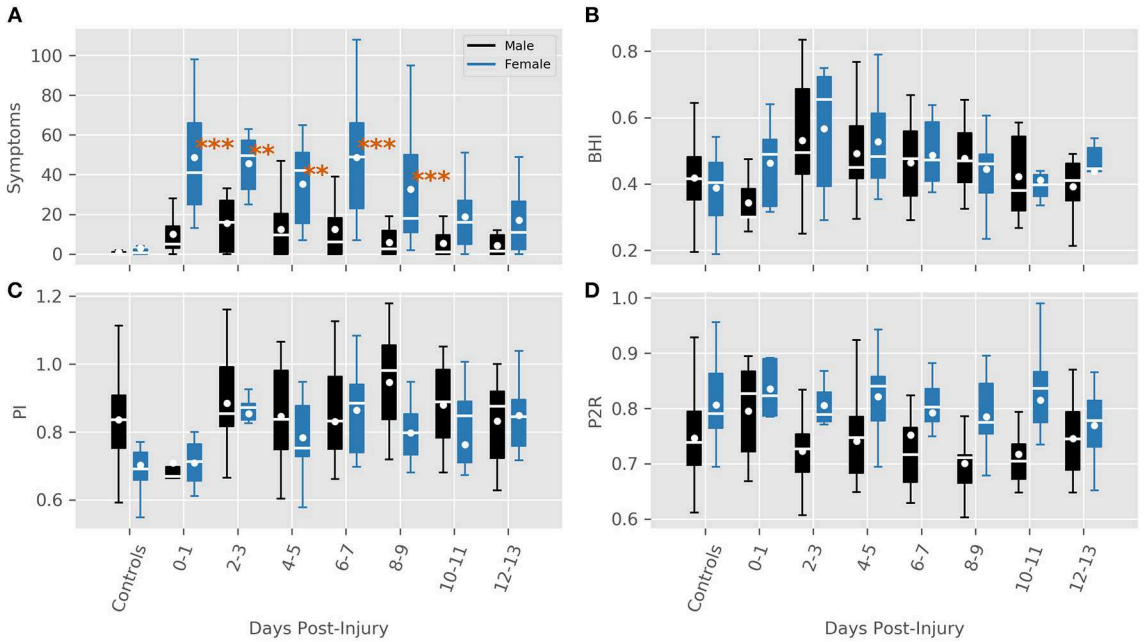

FIGURE 2 | Features for each sex grouped by days post-injury. **(A)** Summed symptoms. **(B)** BHI. **(C)** PI **(D)** P2R. Significance values are indicated where a large interaction between sex and days-post was found (**$P < 0.01$, ***$P < 0.001$).

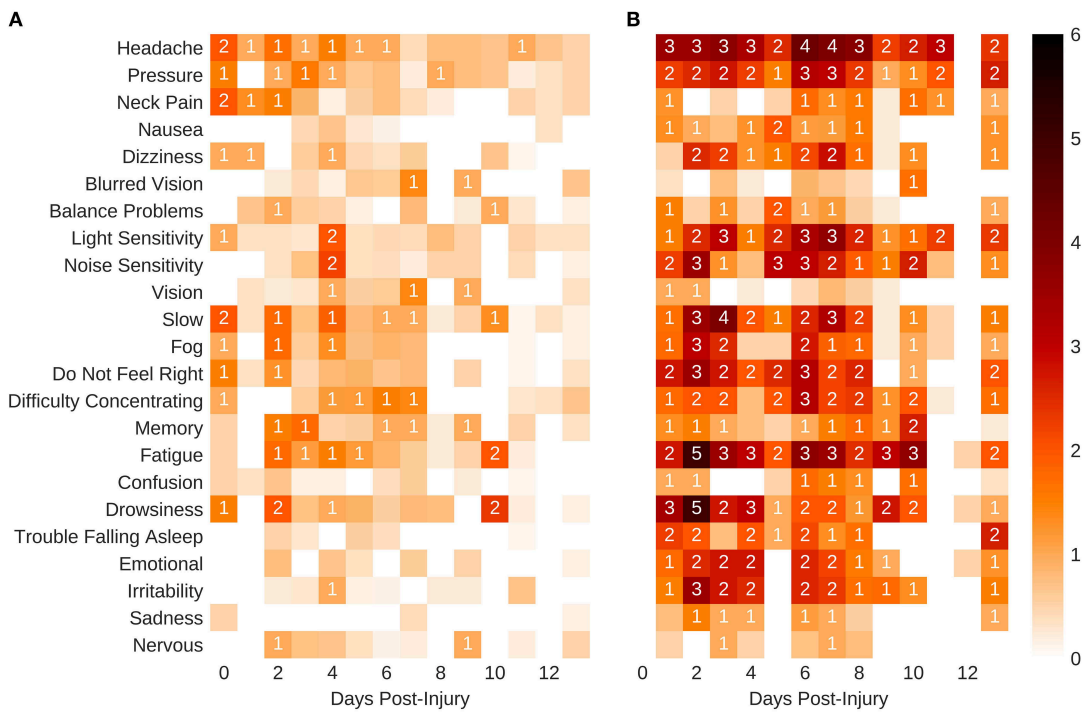

FIGURE 3 | Self-reported mean symptom scores. **(A)** Males. **(B)** Females. The shaded squares without a value are below 1.

between PI and P2R ($r_{male} = -0.8$, $p < 0.001$; $r_{female} = -0.67$, $p < 0.001$). For the male population there were significant correlations between BHI and PI ($r = 0.27$, $p < 0.001$), as well as BHI and P2R (-0.18, $p = 0.02$), that were no present in the female population, **Table 6**. Conversely, the female population had significant correlations between symptoms and BHI ($r =$

TABLE 4 | Mixed effect model results for PI.

Variable	β (SE)	95% CI	t(DF) p
(Intercept)	0.77 (0.02)	[0.73, 0.81]	35.95(195) $p < 0.001$
Sex	**−0.13 (0.04)**	**[−0.21,−0.04]**	**−2.96(195) $p < 0.01$**
0–1 Days-Post	−0.05 (0.04)	[−0.13, 0.02]	−1.33(186) $p > 0.1$
2–3 Days-Post	0.03 (0.04)	[−0.04, 0.09]	0.8(205) $p > 0.1$
4–5 Days-Post	0.02 (0.03)	[−0.04, 0.08]	0.62(211) $p > 0.1$
6–7 Days-Post	0.06 (0.03)	[0, 0.12]	1.99(228) $p = 0.05$
8–9 Days-Post	**0.07 (0.03)**	**[0.01, 0.13]**	**2.14(227) $p < 0.05$**
10–11 Days-Post	0.03 (0.03)	[-0.04, 0.09]	0.8(220) $p > 0.1$
12–13 Days-Post	0.05 (0.04)	[-0.01, 0.12]	1.55(205) $p > 0.1$
Sex:0–1 Days-Post	0.05 (0.08)	[−0.1, 0.2]	0.68(186) $p > 0.1$
Sex:2–3 Days-Post	0.11 (0.07)	[−0.09, 0.16]	1.54(205) $p > 0.1$
Sex:4–5 Days-Post	0.05 (0.06)	[0, 0.27]	0.72(211) $p > 0.1$
Sex:6–7 Days-Post	0.08 (0.06)	[−0.02, 0.24]	1.23(228) $p > 0.1$
Sex:8–9 Days-Post	0.01 (0.07)	[−0.08, 0.17]	0.21(227) $p > 0.1$
Sex:10–11 Days-Post	0.04 (0.07)	[−0.09, 0.16]	0.56(220) $p > 0.1$
Sex:12–13 Days-Post	0.14 (0.07)	[0, 0.27]	1.96(205) $p > 0.05$

The bold rows indicate significant effects or interactions.

TABLE 5 | Mixed effect model results for P2R.

Variable	β (SE)	95% CI	*t*(DF) *p*
(Intercept)	0.78 (0.01)	[0.75, 0.8]	62.97(196) $p < 0.001$
Sex	**0.05 (0.02)**	**[0.01, 0.1]**	**2.21(196) $p < 0.05$**
0–1 Days-Post	0.04 (0.02)	[0, 0.08]	1.69(185) $p > 0.05$
2–3 Days-Post	0.01 (0.02)	[−0.02, 0.05]	0.72(205) $p > 0.1$
4–5 Days-Post	0 (0.02)	[−0.03, 0.04]	0.17(210) $p > 0.1$
6–7 Days-Post	0 (0.02)	[−0.03, 0.04]	0.28(227) $p > 0.1$
8–9 Days-Post	−0.01 (0.02)	[−0.05, 0.02]	−0.57(226) $p > 0.1$
10–11 Days-Post	0 (0.02)	[−0.03, 0.04]	0.16(220) $p > 0.1$
12–13 Days-Post	0 (0.02)	[−0.03, 0.04]	0.14(204) $p > 0.1$
Sex:0–1 Days-Post	0.02 (0.04)	[−0.06, 0.11]	0.42(185) $p > 0.1$
Sex:2–3 Days-Post	0.01 (0.04)	[−0.06, 0.09]	0.17(205) $p > 0.1$
Sex:4–5 Days-Post	0 (0.04)	[−0.1, 0.05]	0.09(210) $p > 0.1$
Sex:6–7 Days-Post	0 (0.04)	[-0.07, 0.08]	0.08(227) $p > 0.1$
Sex:8–9 Days-Post	0.01 (0.04)	[−0.07, 0.07]	0.31(226) $p > 0.1$
Sex:10–11 Days-Post	0.01 (0.04)	[−0.06, 0.09]	0.39(220) $p > 0.1$
Sex:12–13 Days-Post	−0.03 (0.04)	[−0.1, 0.05]	−0.65(204) $p > 0.1$

The bold rows indicate significant effects or interactions.

0.28, $p < 0.01$), as well as symptoms and PI ($r = 0.28$, $p < 0.01$), that were not found in the male population, **Table 6**.

Further exploring the sex-related correlation structure by an ANCOVA analysis reveals a significant difference in the slopes of the regression lines for symptoms and BHI between sexes ($\beta = 58.55$, SE = 19.07, 95% CI [20.98, 96.12], $t = 3.07$, $p < 0.01$). A difference in slopes was also found comparing symptoms and PI ($\beta = 69.08$, SE = 17.58, 95% CI [34.45, 103.70], $t = 3.93$, $p < 0.001$). For symptoms and P2R however, there was no significant difference in slope ($\beta = -56.21$, SE = 31.79, 95% CI [−118.82, 6.40], $t = -1.77$, $p = 0.08$), instead a difference in y-intercepts was found ($\beta = 69.45$, SE = 25.11, 95% CI [20.00, 118.91], $t = 2.77$, $p < 0.01$).

DISCUSSION

Symptoms

Several studies have found a similar increase in self-reported symptoms for female subjects (1, 2). In the study from Baker et al. (4), the increased symptoms in the acute stage may have influenced recovery time—explaining the prolonged recovery for females. Although the difference between sexes here does appear more pronounced, comparing that difference to those other studies is not possible given the heterogeneity of the symptom collection.

The mechanism of injury presents a potentially confounding factor. In this study the majority of male subjects played helmeted sports (68%). The protection afforded by these helmets could have contributed to the overall lowered symptoms. However, in the study from Broshek et al. (1), female subjects were more than twice as likely to experience cognitive impairments than males in unhelmeted sports—illustrating that a difference existed even when accounting for helmets. The sex differences in reported symptoms observed in the current study were not accompanied by evidence for corresponding concussion-related differences in the TCD features. Moreover, the main effects of time observed for BHI suggest the progression of vascular injury to be similar for both sexes. A more compelling explanation would be an inherent reporting bias in the female group. Other studies have shown that female athletes tend to report more symptoms than males (7, 32). In addition, females are generally more focused on, and aware of, their health (33), suggesting that there is more of a motivation to ensure a complete recovery. Conversely, male athletes have a number of societal and cultural motivations to perceptually diminish the magnitude of their injury and return to sport as soon as possible (34). Similarly, it was shown in Kerr et al. (35), that male athletes were significantly more likely to hide a concussive injury.

There have been several studies exploring the physiological differences between sexes that contribute to the susceptibility and recovery from concussion, many of which center on the possible role of estrogen. In more severe traumatic brain injuries, estrogen has been shown to have a neuroprotective effect in male rats, but a deleterious one in females (36). In humans, the study from Gallagher et al. (5), found that female subjects suffering from a sport-related concussion who used hormonal contraceptives reported lower symptom severity than those who did not, suggesting that hormonal contraceptives may play a role in modulating the collapsing neurometabolic cascade that is a hallmark of concussive injuries (16). Another consistent theme in mTBI gender differences is decreased neck strength in women (37, 38), which has been shown to be inversely related to concussion susceptibility. A similar confound of this study is the role physical maturity plays in how someone responds to an mTBI. The study from Krix et al. (39) found that male subjects in early stages of puberty had increased odds of a prolonged recovery from a concussive injury. Although puberty clearly

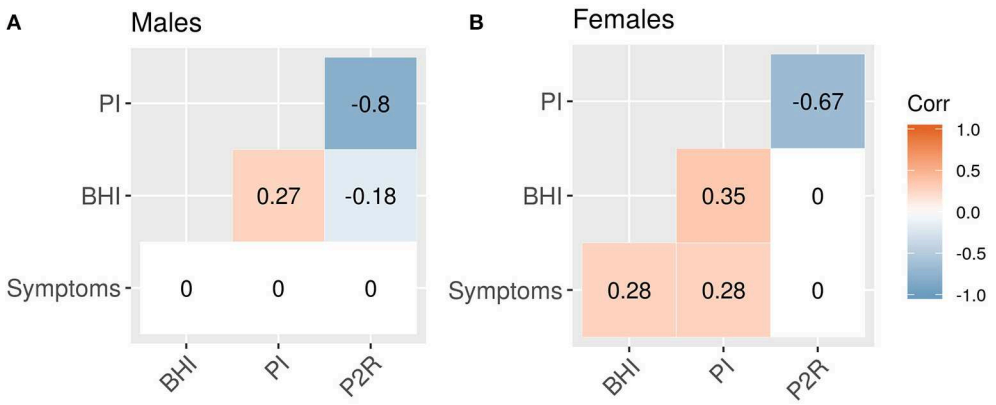

FIGURE 4 | Feature correlations. **(A)** Male correlations. **(B)** Female correlations. Correlations that are not significant at the 0.05 level are set to zero.

TABLE 6 | Feature Correlations for the male and female groups.

	BHI	PI	P2R
MALE GROUP			
Symptoms	0.05 ($p = 0.5$)	−0.12 ($p = 0.12$)	0.14 ($p = 0.07$)
BHI		0.27 ($p < 0.001$)	−0.18 ($p < 0.05$)
PI			−0.8 ($p < 0.001$)
FEMALE GROUP			
Symptoms	0.28 ($p < 0.01$)	0.28 ($p < 0.01$)	−0.11 ($p < 0.05$)
BHI		0.35 ($p < 0.01$)	−0.01 ($p > 0.05$)
PI			−0.67 ($p < 0.001$)

affects the adolescent brain (40), it is still unclear how that would contribute to the results of this work.

TCD Features

It is important to note that in this context BHI is not meant an exact measure of reactivity. Breath-holding can introduce other autonomic and sympathetic responses that can confound its use for directly quantifying reactivity. However, as illustrated in Thibeault et al. (23), and confirmed by the main effects of days-post here, BHI as measured in this population, is a robust biomarker of mTBI. The difference in slopes of the regression lines between symptoms and BHI illustrate the vulnerability of relying on subjective measures alone.

The overall sex-related main effect for both PI and P2R is most surprising aspect of this analysis. For both features that effect did not appear to be based on the injury, but rather an inherent sex-related difference in this population. Previously, when sex was ignored both were altered immediately following an mTBI (23). The alterations of these features here, as illustrated in **Figure 2**, appear to only be present in the male population. PI is a complex metric that is influenced by the combinations of cerebral perfusion pressure, cerebrovascular resistance, arterial bed compliance, heart rate, and the pulse amplitude (26). Similarly, It has proposed that P2R is associated with distal bed compliance dynamics (41), however there is no established physiological correlation. Why either of these features would have a sex-related dependence is unclear and will need to be explored further in the future. Regardless, these results illustrate that that dependence is not due to the injury.

The study from Esposito et al. (42) showed that women had higher Cerebral Blood Flow (CBF) compared to males. Although, in the population here there was no significant trend in mean velocity during injury recovery, that may be because TCD cannot measure CBF directly, only the velocity. In addition, a post-concussive change in mean velocity has not been demonstrated with TCD (23).

CONCLUSIONS

This is the first study to compare sex-related differences between clinical symptoms and TCD measurements in adolescent mTBI subjects. The objective measures highlight the need to mitigate patient heterogeneity when assessing concussion recovery and the discrepancy in clinical symptoms illustrates how difficult this can be for clinicians. In the case of males the possibility of under-reporting may need to be considered. A physiological measurement such as TCD may eventually help remove ambiguity and provide clinicians with an objective physiological measure of mTBI recovery.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Western Institutional Review Board (IRB #20141111), with written informed consent from all subjects. All subjects gave written informed consent in accordance with

the Declaration of Helsinki. The protocol was approved by the Western Institutional Review Board.

AUTHOR CONTRIBUTIONS

CT had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. RH: study concept and design. CT and ST: analysis. CT, ST, and RH: interpretation of data. CT and ST: drafting of the manuscript. RH, SW, and ST: critical revision of the manuscript for important intellectual content. CT and ST: statistical analysis. NC and SW: technical or material support. RH and CT: study supervision.

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The NIH had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Incidence of Mild Traumatic Brain Injury: A Prospective Hospital, Emergency Room and General Practitioner-Based Study

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Skandsen T, Nilsen TL, Einarsen C, Normann I, McDonagh D, Haberg AK and Vik A (2019) Incidence of Mild Traumatic Brain Injury: A Prospective Hospital, Emergency Room and General Practitioner-Based Study. Front. Neurol. 10:638. doi: 10.3389/fneur.2019.00638

Background: There are no recent estimates of incidence rates of mild traumatic brain injury (MTBI) from Norway. Moreover, reported incidence rates rarely comprise cases of MTBI evaluated in the primary care setting. In this study, we utilized existing data collected as part of the recruitment to a large, follow-up study of patients with MTBI. We estimated the incidence rate of MTBI, including patients who visited outpatient clinics, in the age group 16–59 years in a Norwegian region.

Methods: During 81 weeks in 2014 and 2015, all persons aged 16–59 years, presenting with possible MTBI to the emergency department (ED) at St. Olavs Hospital, Trondheim University Hospital or to the general practitioner (GP)-run Trondheim municipal outpatient ED, were evaluated for a diagnosis of MTBI. Patients were identified by computerized tomography (CT) referrals and patient lists. Patients referred to acute CT from their primary GP with suspicion of MTBI were also recorded. This approach identified 732 patients with MTBI. Age- and sex-specific incidence rates of MTBI were calculated using population figures from the regional catchment area.

Results: Overall incidence of MTBI in people between 16 and 59 years was 302 per 100,000 person-years (95% confidence interval 281–324). The incidence rate was highest in the age group 16–20 years, where rates were 835 per 100,000 person-years in males and 726 in females.

Conclusion: The overall incidence rate of MTBI was lower than expected from existing estimates. Like other reports, the incidence was highest in the late teens.

Keywords: incidence, concussion, mild traumatic brain injury, epidemiological, glasgow coma scale, Norway, emergency room, primary care

INTRODUCTION

Traumatic brain injury (TBI) is a complex injury comprising a spectrum from mild TBI (MTBI) with low risk of persistent disability, to the most severe TBI with devastating brain damage. However, MTBI constitutes 80–90 % of all TBI. It can therefore be appropriate to study characteristics of MTBI, such as incidence, separately. Moreover, a review recently highlighted that the monitoring of the epidemiology of MTBI was incomplete (1). Internationally, the reported incidence rates of mild traumatic brain injury (MTBI) vary extremely, from 100 to 749 cases per 100,000 person-years (2–7). This may reflect a real variation in the burden of traumatic brain injury (TBI), especially in a global context, since injuries are more common in low-income countries (8). However, the variation also results from heterogeneity in study design and data sources (5). Importantly, patients with MTBI who are evaluated outside hospitals, are often not included in incidence estimates (9).

The Nordic countries have been considered safe communities with a decreasing number of TBI (10, 11), but reported rates vary (9, 12). Most of the existing Nordic epidemiological studies on MTBI, however, were conducted decades ago and mostly report incidence rates of hospitalized TBI, either TBI of all severities (9, 11–15) or only MTBI (16). Only a few studies included both hospitalized and non-hospitalized patients (17–19), but none of these dealt exclusively with MTBI.

In the present study, the aim was to estimate the incidence of MTBI in an adult population within a regional Norwegian catchment area. We utilized existing data collected as part of the recruitment to a large, prospective cohort study of patients with MTBI aged 16–59 years (20). Cases were persons who presented with a possible MTBI to the general practitioner (GP) run municipal emergency department (ED) or the ED at a level 1 trauma center, as well as cases referred to computerized tomography (CT) by the patient's primary GP.

METHODS

Study Period and Setting

Patients aged 16–59 years with MTBI were identified during 81 weeks between April 1st 2014 and December 5th 2015 in two emergency departments (ED): St. Olav's Hospital, Trondheim University Hospital, a Norwegian regional level 1 trauma center and the Trondheim Municipal Emergency clinic (only out-patients), run by the GPs in the area, working shifts. This ED is co-located at the hospital. Their catchment area for MTBI is mostly urban: the city of Trondheim and four neighboring municipal entities with 229,000 residents. The EDs are state run, like most health care in Norway. During normal weekday work hours, patients can contact either their primary GP at one of the 41 GP centers in the catchment area, or one of the EDs. After 3 p.m. on workdays and the whole weekend, patients present to one of the Eds, since the GP centers are closed Mostly, however, patients with mild TBI present directly to the municipal ED at all times of the day. During the study period, indication for CT was assessed with the Scandinavian Guidelines for Head Injury Management from 2000 (21). In these, CT was recommended in patients with either amnesia/ suspected loss of consciousness (LOC) or certain risk factors. Hence, more patients have been referred to CT in Norway than in countries adhering to other, stricter, CT rules.

MTBI Criteria, and Case Ascertainment

TBI was defined as "an alteration in brain function, or other evidence of brain pathology, caused by an external force" (22), and cases identified with *TBI* were further categorized as *mild* (MTBI) according to the WHO criteria: GCS score 13–15 at presentation, LOC <30 min, and posttraumatic amnesia (PTA) <24 h (23). Patients were identified by daily manual screening of both the patient lists at the EDs and the referrals to head CT, and by daily contact with neurosurgical residents. Notably, also lists of the patients at the municipal ED, who had *not* been referred to CT were prospectively screened. This screening was performed 1–2 times per day by a research assistant who read the notes on all patients who had presented with injuries in the head and neck area or when location was poorly described. Our goal was to identify *all* patients with possible TBI and approach them for case ascertainment. If attempt to contact failed, we used information from the medical records regarding GCS score, amnesia or LOC for MTBI criteria evaluation. Patients who had been referred to head CT from their primary GPs in the catchment area, and met the diagnostic criteria for MTBI were registered, but not approached. For details regarding case ascertainment, see our previous publication (20).

During the study period, 1,095 patients were examined with head CT due to trauma, 624 of these were evaluated to have MTBI. Furthermore, 79 patients who had not been examined with head CT, but who had been clinically evaluated to meet the MTBI criteria and enrolled in the original follow-up study were included in the incidence analysis. In addition, 29 patients were retrospectively identified as being directly referred to CT from their primary GP center. Hence, 732 patients were identified with MTBI (**Figure 1**). As recently published, 517 patients (71%) were treated without hospital admittance and more than 70% had a GCS score of 15. CT showed intracranial lesions in 6%. For details on the clinical and demographic characteristics see (20).

Incidence Rate Estimation

We estimated age- and sex specific incidence per 100,000 person-years by dividing cases of MTBI during the study period by the population at risk, i.e., total population of catchment area. Cases of MTBI were identified from; (1) all patients who had head CT at the study hospital and who met the diagnostic criteria for MTBI; (2) patients meeting MTBI criteria who eventually consented to participation in the longitudinal study, but who had not been examined with head CT; and (3) patients who had been referred directly to head CT by their primary GPs in the catchment area, and were considered to meet the diagnostic criteria.

The population at risk was obtained from the Norwegian National Registry and Norwegian State Educational Loan Fund, and the total number of people, as well as number of men and women between 16.0 and 59.9 years residing in the catchment area was extracted. This figure included students attending school/college/university in the area, but who had

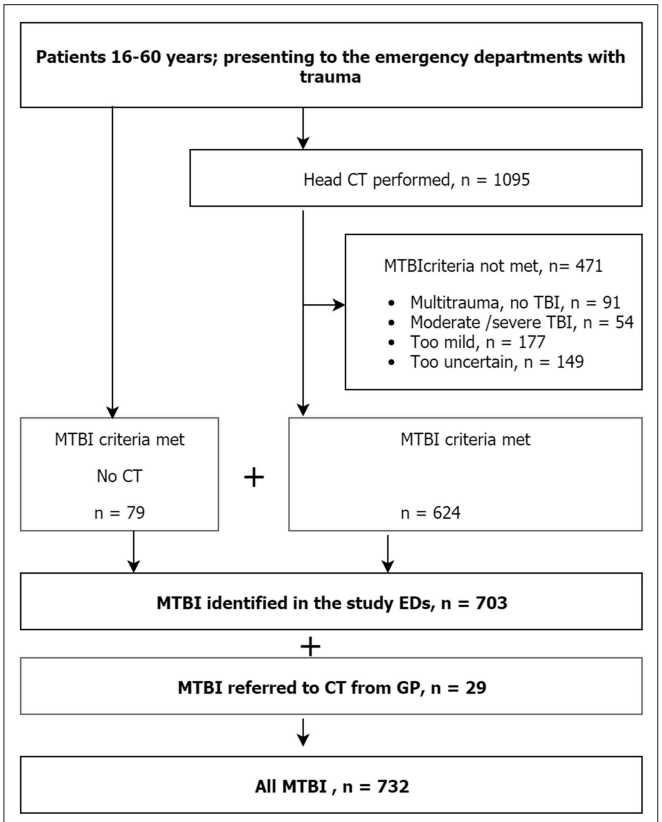

FIGURE 1 | Flow chart of the identification of patients with MTBI. The flow chart demonstrates which figures from the screening procedure that were used for the computation of incidence rates.

TABLE 1 | Mild traumatic brain injury (MTBI) incidence by age and sex.

	Total population in the study area (*n*)	MTBI cases (*n*)	Incidence per 100,000 person years (95% CI)
Males			
16–20	7,226	94	835 (682–1022)
21–25	15,536	96	396 (324–484)
26–30	12,358	54	280 (214–366)
31–35	8,801	44	320 (238–431)
36–40	8,219	34	265 (189–371)
41–45	8,435	26	197 (134–290)
46–50	8,122	48	379 (285–503)
51–55	6,886	32	298 (210–421)
56–59	5,198	21	259 (169–397)
Total	80,781	449	357 (325–391)
Females			
16–20	6,981	79	726 (582–905)
21–25	14,888	57	245 (189–318)
26–30	10,219	33	207 (147–291)
31–35	8,087	25	198 (134–293)
36–40	7,314	13	114 (66–196)
41–45	7,867	16	130 (79–213)
46–50	7,847	24	196 (131–292)
51–55	6,773	22	208 (137–316)
56–59	5,001	14	179 (106–303)
Total	74,977	283	242 (216–272)

home address elsewhere, and conversely, excluded students with a home address in the catchment area, but who attended school/college/university elsewhere. For the computation of incidence rates, we used the Stata Statistical Software: Release 12. College Station, TX: Stata Corp LP (2011).

RESULTS

Applying the total of 732 patients who had been identified with MTBI in the catchment area, the overall incidence rate of MTBI in persons aged 16–59 years was 302 per 100,000 person-years (95% CI, 281–324). The incidence rate was 357 (95% CI, 325–391) in males and 242 (95% CI, 216–272) in females. The rate was highest in the age group 16–20 years, where males had a rate of 835 per 100,000 person-years and females a rate of 726 per 100,000 person-years. **Table 1** shows the population size, number of MTBI cases, and incidence rates by age groups and sex.

DISCUSSION

The estimated incidence rates in this study were derived from visits at all medical services available for patients with acute MTBI; the hospital, the GP-run municipal out-patient ED, and the primary GPs in the catchment area. Importantly, 71% were treated without hospital admission. Few other epidemiological studies report such complete coverage. A population-based study from a region in New Zealand also applied case ascertainment at the community level as well as the WHO criteria for MTBI (3). They found an overall incidence rate for MTBI, of 749 per 100,000 person-years across all age groups. Based on their reported data, the incidence of MTBI in the 15–64 year age group can be calculated to 710 per 100,000 person-years, which is substantially higher than in the present study. However, in the New Zealand study, multiple overlapping sources of information about possible cases was used, such as schools and sports organizations, and not only health care providers. Still, the cases identified outside hospitals and GPs in their study constituted only 28%, which cannot explain the large discrepancy in MTBI incidence in the New Zealand study compared to the present study. In line with the estimates from New Zealand, a systematic review on MTBI concluded that the population-based MTBI rate probably is above 600 per 100,000 person-years if accounting for cases not treated at hospitals (2). Hence, in comparison with these figures, the incidence found in the current study using Norwegian data was low.

In contrast, the incidence rate found in the present study corresponds with an overall incidence rate of 354 per 100,000 person-years found in a population based Swedish study of all TBI, where the proportion of MTBI was 97.5% (18). The Swedish study also covered both the catchment hospital and a GP-run ED and assumed to capture all cases of TBI seeking medical

evaluation. In that study, only 36% had a CT, while 88% were treated as in-patients, possibly reflecting that the Scandinavian guidelines for TBI from 2000 had not been fully implemented (24). The only Norwegian study covering all medically evaluated head injuries in a defined area, is a retrospective study from Northern Norway in 1993. They found an incidence rate of TBI of all severities of 229 per 100,000 person-years and a rate of hospital admittance of 74% (19) Possibly, the lower estimated incidence in that study, compared to the incidence in the present study, reflects that also in Norway, awareness and recognition of mild TBI has been increasing during the last years, like in the US (25).

In line with most previous studies (18, 19, 26), we found that adolescents in the age group of 16–20 years had much higher incidence rate of MTBI than other ages. It may well be that they have a more active, and possibly more careless, lifestyle involving a higher risk of trauma. Moreover, they typically live with their parents, and may be brought to medical evaluation for a head injury more often.

We assume, however, that the incidence of MTBI was underestimated in the present study since there will always be missed cases of MTBI in a screening process. First, our study procedures did not capture patients seen only by their primary GP unless they were referred to CT, and results must be interpreted with some caution. While it is most common that patients with acute MTBI present directly to the municipal ED, patients who present *more than 24 h* after injury, are more likely to present to their GP, and may not be examined with CT is the risk of intracranial bleeding is over.

Second, we did not register patients not examined with CT, yet meeting criteria for MTBI, unless they were enrolled in the follow-up study. In the recruitment to the follow-up study, we experienced that around 50% of eligible patients eventually got enrolled (20). Hence, the group of 79 consenting patients without CT, should at least be twice as large. If we had calculated with that, the total incidence would, however, only increase to around 330 per 100,000. Seemingly, a large proportion of the patients with MTBI were examined with CT according to our results. We believe that this result reflects the low threshold for CT in the EDs, also shown in a previous publication from our setting (27). Actually, the Nordic countries, and Norway in particular, have been recognized for an increasing use of CT (28). Moreover, only 6% had intracranial findings on CT, and many were examined with CT despite not meeting criteria for MTBI, but rather for minimal head injury, as shown in **Figure 1**. Third, some patients could not be reached for case assignment, and could be missed for inclusion if clinical signs of MTBI were not clearly described in the record, a recognized source of error in epidemiological studies (29). Fourth, some of the patients evaluated to have "uncertain MTBI" (**Figure 1**) should possibly have been included. Finally, it should also be mentioned that patients presenting to the EDs with MTBI, but who resided outside the catchment area, were not excluded, likely counterbalanced by the residents in the area who may have sustained MTBI outside the catchment area.

Since there is a large university in the catchment area, there were many students in the population at risk, and the average level of education in the population may be somewhat higher than in Norway as a whole. The incidence of MTBI might therefore not be the same in more rural parts of the country.

Taken together, MTBI was estimated to be less frequent in this Norwegian area than in many other high-income countries, especially among persons older than 20 years. A similar trend has also been shown in previous Norwegian epidemiological studies of all hospitalized (14) and all severe TBI (30). Reasons for the lower incidence might be that people tend to follow security/safety regulations, at home, at work and in the traffic. The true incidence of MTBI, however, will remain unknown, since many patients with MTBI consider it unnecessary to seek medical treatment (31, 32).

In summary, our data indicate that the incidence of medically evaluated MTBI in Norway is lower than 600 per 100,000 person-years anticipated in a recent review, but likely higher than 302 per 100,000 person-years as computed here due to inherent difficulties identifying all MTBI cases as discussed above.

ETHICS STATEMENT

The Regional committee for research ethics approved the study (approval number 2013/754). According to this approval, patients in the follow-up study gave written consent, while consent was not required for use of the information obtained via the screening and case ascertainment procedures.

AUTHOR CONTRIBUTIONS

TS, IN, DM, AH, and AV designed the study. TS and TN performed the analyses. TS, IN, and CE collected the data. TS drafted the manuscript. All authors critically reviewed the manuscript. All authors read and approved the last version of this manuscript.

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Relationships Between Sleepiness, Mood, and Neurocognitive Performance in Military Personnel

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Neurocognitive computerized assessment tools (NCATs) were developed to assist military clinicians with the tracking of recovery from injury and return to full duty decisions with a recent focus on the setting of post-concussion evaluations. However, there is limited data on the impact of deployment on neurocognitive functioning, sleepiness, and mood in healthy, non-concussed Service members. Automated Neuropsychological Assessment Metrics version 4 TBI Military (ANAM) data was obtained for a sample of active duty deployed personnel ($n = 72$) without recent history of mild traumatic brain injury (mTBI). A linear regression was conducted to examine the effects of sleepiness and mood state on neurocognitive performance. The overall multivariate regression was statistically significant. Negative mood states were the most salient predictors of neurocognitive performance with higher levels of endorsement associated with lower scores. The findings support measures of negative mood state, but not sleepiness, as relevant predictors of neurocognitive performance as measured by the ANAM. These results indicate that mood needs to be considered when reviewing neurocognitive data to ensure that appropriate clinical decisions are made; in particular for return-to-duty decisions in deployed settings after concussion recovery.

Keywords: assessment, depression, statistical methods, sleep disorders, military

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INTRODUCTION

Traumatic brain injury (TBI), specifically mild TBI (mTBI), was considered to be the signature battlefield injury of Operations Iraqi Freedom and Enduring Freedom (1). TBI is defined by the United States Department of Defense (DoD) as "a structural or physiological disturbance of the brain, caused by an outside force that is followed by clinical symptomology" (e.g., loss of memory, slowed thinking, and confusion). It is recognized that some military Service Members (SMs) diagnosed with mTBI, especially those experiencing multiple injuries, may also experience changes in personality, sleep problems, and cognitive impairment and can increase the risk for suicide, post-traumatic stress disorder, depression, and anxiety (2, 3).

Due to these immediate and long-term impacts of mTBI it has been of importance to DoD clinical personnel to properly screen for injury and to monitor recovery from injury. Neurocognitive assessment tools (NCATs), also known as computerized neuropsychological

assessment devices (CNADs) and computerized neurocognitive test (CNT) batteries, are often used to assess athletes and US military service members following an mTBI. In 2008, following the lead of the sports medicine community, Congress mandated that all SMs receive a pre-deployment neurocognitive assessment using an NCAT, the Automated Neuropsychological Assessment Metrics 4 TBI-MIL (hereafter referred to as the ANAM), prior to any deployment to a combat zone (4) for the purposes of establishing a baseline for post-injury comparative purposes. Such comparisons can be used to evaluate neurocognitive functioning to screen for deficits in cognition following an mTBI event in both the acute and post-acute phases of injury, to track recovery, and provide data to assist with return-to-duty decisions. However, it should be noted that any comparison to pre-deployment data is only valid if the assessment is an actual representation of an individual's typical neurocognitive functioning. Researchers have recently identified numerous threats to the validity of baseline assessments, including sleepiness, and mood (5).

Military personnel are especially vulnerable to any adverse effect sleepiness may have on NCAT performance as chronic insufficient sleep is prevalent among personnel deployed to combat environments (nightly average of 6.25 h) (6). Chronic insufficient sleep or sleep insufficiency occurs when sleep is insufficient to support adequate alertness, performance, and health, either because of reduced total sleep time (decreased quantity), or fragmentation of sleep by brief arousals (decreased quality). A recent meta-analysis reported sleep restriction significantly impairs cognitive functioning across a numerous cognitive domains (7). Insufficient sleep has been specifically linked to impairments in attention, reaction time, learning and memory, and decision-making (8–17). These impairments are associated with altered functioning of the dorsolateral prefrontal cortex and parietal regions of the brain (18). Research has shown that even one night of insufficient sleep can alter the connectivity of neural networks to the detriment of cognitive processes (19). Numerous studies have reported that when sleep is reduced to <7 h cognitive performance is lower in tests for vigilance, alertness, reaction time, memory, and decision-making (11, 12, 20, 21).

Abnormal mood may also challenge the validity of NCATs. The ANAM contains a self-report mood scale designed to assess several dimensions of mood (e.g., anger, anxiety, and depression) (22). Post-injury assessments may be useful for detecting changes in mood resulting from mTBI-related depression and heightened anxiety. Research has established that both depression and anxiety adversely affect cognitive processes (23, 24). Depression has been linked to decreases in both short and long-term memory, executive function, attention, and simple reaction time (23, 25). Similarly, anxiety has been linked to decreases in executive functioning and inhibition (24). Additional research focusing on SMs who served in Operations Iraqi Freedom and Enduring Freedom has indicated that combat exposure is a risk factor for both anxiety and depression (26). All the studies suggest that mood should be examined as part of any post-injury assessment.

To best of our knowledge, no study has examined the degree insufficient sleep affects performance on the ANAM4 TBI-MIL; however, research involving previous iterations of the ANAM have reported that both sleep restriction and deprivation were associated with lower scores. None of these studies reported on the relationship between insufficient sleep and the ANAM mood-scale, but Acheson et al. (27) reported that insufficient sleep resulted in increased fatigue and decreased positive mood states as indicated by the Profile of Mood States (POMS). Other studies focusing on military populations using NCATs other than the ANAM have indicated that insufficient sleep associated with high stress environments, such as high-stakes training, survival (28), and/or simulated operational environments, results in decreases in mood, and neurocognitive performance (29–32).

The aim of this study was to elucidate and compare the relationships among self-reported sleepiness, mood state, and neurocognitive performance in deployed service members as measured by the ANAM.

MATERIALS AND METHODS

Records

The study used a retrospective cross-sectional design. A subset of healthy non-concussed SMs who received medical care for minor deployment-related orthopedic injuries at a concussion care center were extracted from an archival database containing demographic and neurocognitive assessment data from deployed Marine Corps units. Inclusion criteria was based on no history of any severity of TBI in the preceding 12 months based on the DoD and Veterans Affairs consensus criteria. History of concussion was based on the results of a self-report TBI questionnaire that is administered as part of the ANAM and determined by an endorsement of at least one of the following symptoms immediately following the injury event: feeling dazed or confused, experiencing loss of consciousness, or experiencing loss of memory for the injury. After inclusion criteria was applied, there were 72 SMs for analyses.

The original protocol was approved by the Naval Air Warfare Center Aircraft Division institutional review board, Patuxent River, MD (protocol NAWCAD.2011.0003-CR01-EMC) (33). Data from that protocol were de-identified and archived for further use. Subsequent analyses on the de-identified archival database were reviewed by the Navy Experimental Diving Unit Institutional Review Board and determined to be exempt human subject research in compliance with all applicable Federal regulations governing the protection of human subjects.

Neurocognitive Testing

The ANAM is an automated, CNT battery that includes a sleepiness scale, mood scales, a questionnaire for self-reporting TBI, and the following subtests: Code Substitution (CDS), Matching-to-Sample (M2S), Mathematical Processing

Abbreviations: ANAM, Automated Neuropsychological Assessment Metrics version 4 TBI Military; DoD, Department of Defense; ImPACT, Immediate Post-Concussion Assessment and Cognitive Test; NCAT, Neurocognitive Computerized Assessment Tools; NCS, Neurocognitive Composite Score; SMs, Service Members; TBI, Traumatic Brain Injury; mTBI, Mild Traumatic Brain Injury.

(MTH), Procedural Reaction Time (PRO), Simple Reaction Time (SRT), Code Substitution Delayed (CDD), and Simple Reaction Time Repeated (SR2) (34). Detailed descriptions of the TBI questionnaire and subtests can be found elsewhere (35). Throughput scores (mean correct responses per 60 s) from each subtest were used in all analyses. The ANAM has been shown to be a reliable and valid tool that has clinical utility as a population screening tool for the detection of neurocognitive dysfunction following a single, uncomplicated concussion within a 72-h window (36).

The sleep scale (i.e., sleepiness) is a self-reported measure of sleepiness rated on a 7-point Likert scale, with values closer to 7 indicating increased sleepiness and fatigue. The mood scale is a self-reported measure assessing seven mood dimensions, including happiness, vigor, restlessness, depression, anxiety, fatigue, and anger. Each dimension consists of six adjectives, rated on a 7-point Likert scale, with higher values representing greater degrees of endorsement of each mood state. Confirmatory factor analysis supports a 7-factor model of the mood scale, although there is evidence to suggest an alternative 2-factor model encompassing positive and negative mood states (22).

Statistical Analyses

All analyses were performed with MATLAB 2013b (Mathworks, Natick, MA) and SPSS Version 22 (IBM, Armonk, NY).

Normative Data

The use of normative data is a cornerstone of neuropsychological assessment (37). An extensive normative database for the ANAM exists with over a hundred thousand data points, which stratifies the performance of healthy, non-concussed SMs according to age and gender; these data were collected as part of SMs pre-deployment baseline assessments (35).

Descriptive Statistics

Examinations of histograms, normality plots, and Lilliefors statistics revealed that there were minor violations of univariate normality. Thus, non-parametric statistics were used for any comparisons of means. Outliers were assessed as three times the interquartile range above the third quartile or three times below the first quartile. All outliers were removed for the analysis.

Descriptive statistics were calculated for each subtest and it was determined if any mean was outside the normal range of functioning (i.e., normal limit; defined as within the 25th to 75th percentile ranks of previously published normative ANAM data) (35). Due to the normality violations, a series of one-sample Wilcoxon signed rank tests were used to evaluate differences in sleepiness, mood scale, and subtest data between the sample and an-age matched normative sample. Effect size was evaluated using rank biserial (r_{sb}) correlations and the results were interpreted using the following criteria for strength of association: small = 0.1, medium effect = 0.3, and large effect = 0.5 (38, 39). No adjustment was made for multiple comparisons. A p-value of ≤ 0.05 was considered significant.

Data Reduction

All data were converted to Z-scores using age-matched (21–25 years) normative data (35). The Z-score means for CDD, CDS, M2S, MTH, PRT, SRT, and SRT2 were averaged to create a neurocognitive composite score (NCS). Use of composite scores in neuropsychological testing has been reported to minimize floor and ceiling effects and reduce the risk of a Type I error (40).

Due to multicollinearity concerns within the mood scale, a principal component analysis was conducted on the seven mood subscales to reduce the dimensionality of mood data to a smaller number of latent components. As a general rule, mood dimensions that loaded below 0.4 on all extracted components were removed and the principal component analysis was repeated. The resulting uncorrelated principal components were used as predictors in regression analyses.

Regression Models

Multiple linear regression models with simultaneous predictor entry were run for each ANAM subtest and the ANAM composite score using sleepiness and each mood scale principal component as predictors. Collinearity diagnostics were run to ensure partial regression coefficients derived from regression analyses were estimated precisely and that the relative importance of each predictor for neurocognitive performance could be assessed reliably. Multicollinearity was measured using variable inflation factors (VIF) for each predictor. Significant multicollinearity was indicated if any VIF exceeded 4 (with values approaching 10 indicating serious multicollinearity) (41). Violations of multivariate normality were checked using histograms and QQ-plots of the standardized residuals.

RESULTS

Descriptive Statistics

Table 1 presents descriptive data. The available demographic data for the overall record sample consisted of all males (100%), who were enlisted (100%), with a mean age of 25.4 ($SD = 5.0$) years. The majority of means fell within the range of normal functioning with only PRT, SRT, and SRT2 falling below the 25th percentile and anger and depression above the 75th percentile compared to the age-matched comparative norm. There was an indication toward lower scores in the deployment sample for all of the subtests, with M2S having the most prominent difference (−19%), and for vigor and happiness (one-sample Wilcoxon signed-rank tests, all $p < 0.05$). There were also indications of higher scores for sleepiness, restlessness, anxiety, anger, and fatigue.

Table 2 presents correlations between NCS, sleepiness, and mood. There were significant associations between NCS and each of the predictor variables, with only vigor having lower than a medium effect ($r = 0.27$). There were negative correlations between neurocognitive performance and sleepiness ($r = -0.34$), restlessness ($r = -0.40$), anxiety ($r = -0.36$), depression ($r = -0.40$), anger ($r = -0.34$), and fatigue ($r = -0.39$), and positive correlations between neurocognitive performance and vigor ($r = 0.27$) and happiness ($r = 0.38$).

TABLE 1 | Descriptive Statistics of the ANAM Subtest, Sleepiness, and Mood Data.

Measures	Normative data		Sample			
	M	*SD*	*M*	*SD*	*p*	*ES*
NEUROCOGNITIVE SUBTESTS						
Code substitution delayed	48.20	15.80	42.25	17.76	0.008*	−0.37††
Code substitution	54.60	11.20	50.46	11.95	0.004*	−0.39††
Matching to sample	36.40	11.00	29.60	10.11	0.001*	−0.65‡‡
Mathematical processing	20.80	6.20	18.30	6.10	0.001*	−0.44††
Procedural reaction time	101.60	14.00	90.99†	21.37	0.001*	0.47††
Simple reaction time	237.80	28.40	210.69†	57.03	0.002*	0.42††
Simple reaction time repeated	237.40	30.60	204.27†	64.06	0.001*	−0.44††
Sleepiness	2.40	1.20	2.77	1.30	0.004*	−0.39††
MOOD SCALE						
Anger	16.20	20.00	26.16‡	24.41	0.010*	0.35††
Anxiety	14.80	15.00	20.46	19.92	0.049*	0.27**
Depression	12.20	17.20	17.45‡	19.85	0.143	0.20**
Fatigue	24.40	19.00	30.75	21.47	0.032*	0.29**
Happiness	64.60	21.60	54.07	25.45	0.002*	−0.42††
Restlessness	17.40	17.00	26.17	22.99	0.015*	0.33††
Vigor	57.60	19.60	48.16	20.83	0.001*	−0.46††

M, mean; SD, standard deviation; ES, rank biserial correlations.

**Significant differences compared with the normative data (according to the Wilcoxon test).*

† Below the 25th percentile rank of normative data.

‡Above the 75th percentile rank of normative data.

***Effect size exceeds the threshold for small effect ($r_{sb} > 0.10$).*

†† Effect size exceeds the threshold for small effect ($r_{sb} > 0.30$).

‡‡ Effect size exceeds the threshold for small effect ($r_{sb} > 0.50$).

Data Reduction

The Kaiser-Meyer-Olkin (KMO) Test of sampling adequacy (KMO = 0.829) indicated that enough cases were present in the dataset to support the PCA. The PCA converged on a two-factor solution, with components accounting for 65 and 22% of variance, respectively. As shown in **Table 3**, Component 1 included negative mood states (restlessness, anxiety, depression, anger, and fatigue) whereas Component 2 included positive mood states (vigor and happiness). Vigor and happiness negatively cross-loaded onto Component 1, although this was expected given negative associations between self-reported negative and positive mood states. For multiple linear regression analyses, the negative and positive mood components, along with sleepiness, were used as predictors of neurocognitive performance in lieu of individual mood subscales.

Regression Models

Results of multiple linear regression analyses are presented in **Table 4**. Only regression models for M2S, PRT, SRT, SRT2, and NCS reached statistical significance at $p < 0.05$. Adjusted R^2 values indicated that sleep and mood accounted for ∼10–20% of the variability in ANAM throughput scores for M2S (Adjusted $R^2 = 0.11$), PRT (Adjusted $R^2 = 0.12$), SRT (Adjusted $R^2 = 0.19$), SRT2 (Adjusted $R^2 = 0.22$), and NCS (Adjusted $R^2 = 0.18$). This relationship was driven by negative mood, which emerged as the only significant predictor of ANAM throughput scores in these models. Lower throughput was associated with higher negative mood state values. There were no indications of multicollinearity based on VIF and tolerance values for sleepiness (VIF = 2.10, tolerance = 0.48), negative mood (VIF = 1.82, tolerance = 0.55), and positive mood (VIF = 1.28, tolerance = 0.78), nor were there violations of multivariate normality.

DISCUSSION

This study was the first to examine the relationships between measures of sleepiness, mood, and neurocognitive performance in deployed SMs. Elucidating the relationships between these variables is imperative for the proper interpretation of neurocognitive assessment data as negative mood and sleepiness have been reported to adversely affect neurocognitive performance (42, 43). Thus, it is especially important for clinical personnel to consider such relationships and how mood may affect performance if the data are to be used to make post-concussive injury decisions, such as return-to-duty decisions. The primary findings of the study were that measures of mood, particularly negative mood states, within the ANAM predicted neurocognitive performance during deployment. These negative effects of mood on neurocognitive performance were most pronounced for ANAM subtests indexing sensorimotor speed (i.e., SRT, SRT2, PRT), as well as an overall ANAM composite score.

TABLE 2 | Correlations between the ANAM sleepiness, mood, and neurocognitive composite scores.

Measure	1	2	3	4	5	6	7	8	9
1. NCS	1								
2. Sleepiness	−0.365**	1							
3. Vigor	0.274*,†	−0.615**,‡	1						
4. Restlessness	−0.386**,†	0.503**,‡	−0.143,†	1					
5. Depression	−0.364**,†	0.534**,‡	−0.274*,†	0.827**,‡	1				
6. Anger	−0.341**,†	0.539**,‡	−0.236*,†	0.852**,‡	0.830**,‡	1			
7. Fatigue	−0.374**,†	0.684**,‡	−0.370**,†	0.765**,‡	0.784**,‡	0.714**,‡	1		
8. Anxiety	−0.329**,†	0.337**,†	0.005	0.841**,‡	0.804**,‡	0.788**,‡	0.637**,‡	1	
9. Happiness	0.375**,†	−0.592**,‡	0.834**,‡	−0.423**,†	−0.514**,‡	−0.494**,†	−0.524**,‡	−0.274*,†	1

NCS = neurocognitive composite score.
**Correlation is significant at the 0.05 level (2-tailed).*
***Correlation is significant at the 0.001 level (2-tailed).*
†Effect size exceeds the threshold for small effect (r > 0.10).
‡Effect size exceeds the threshold for a medium effect (r > 0.50).

TABLE 3 | Component matrix loadings of unrotated components extracted by principal component analysis.

Measure	Component Matrix	
	Component 1	Component 2
Restlessness	**0.91**	0.26
Depression	**0.92**	0.13
Anger	**0.90**	0.15
Fatigue	**0.87**	−0.02
Anxiety	**0.83**	0.42
Vigor	−0.41	**0.88**
Happiness	−0.66	**0.70**

Bold values indicate membership of mood scales to respective principal components.

This is a key finding that needs to be considered when examining post-injury data as changes in mood; specifically changes in negative mood (e.g., fatigue, depression, anxiety, irritability, and emotional lability) are commonly associated with mTBI (44–47). Most mood-related symptoms, regardless of injury severity, remain elevated for 2 weeks post-injury (48); however, depressive symptoms have been reported to remain elevated for a month or more post-injury (49). Mood-related symptomology is so common following TBI that a mood-related symptom profile is one of the suggested symptom-based profiles for combat-related mTBI (44) and one of the suggested clinical profiles for sports-related concussion (50–53). To complicate matters, mood-related symptoms are often comorbid with cognitive symptomology (e.g., problems with attention, multitasking, distractibility) (47) making it very difficult to parse out the specific effects of mood-related symptoms on neurocognitive functioning.

There were significant changes in our deployed sample compared to normative data with decreases in neurocognitive scoring and positive mood and increases in sleepiness and negative mood. The overall mood state of the sample compared to the normative data was expected due to the high levels of psychological and physical stress associated with deployment (54, 55). Recent research has linked deployment stressors to negative post-deployment psychological health outcomes including, but not limited to, increased risk of physical health problems, increased fatigue, mood swings, suicidality, irritability, anxiety disorders, major depression, and substance abuse (56). Deployment-related stressors can also be compounded by other stressors distinct to military service, including mission ambiguity, engagement ambiguity, leader climate, cultural, and situational ambiguity (56). To compound matters, the sampled Marine units are known to have high rates of repeat deployments, longer deployments, and less time between deployments, both of which are associated with decreased morale, changes in mood, decreased psychological health, stress-related work problems, and sleep dysfunction (57–59).

Studies have reported that 75% of SMs rated their sleep as worse during deployment compared to pre-deployment levels (6) and that as many as 27% of SMs returning from Operations Iraqi Freedom and Enduring Freedom have reported trouble sleeping while deployed (56). The literature is clear that service members are at risk for insufficient sleep; in terms of sleep quantity and poor sleep quality and that, these risks are aggravated by deployment, especially in redeployers (6, 59, 60). Sleep dysfunction historically has been viewed as a symptom of anxiety and/or depression with insomnia being common between the two disorders. Research has shown that there is bidirectional relationship where poorer sleep quality may be a mechanism through which work stress results in increased depression and that increased depressive symptoms may result in poorer sleep (61, 62).

Ultimately, deployment-related increases in sleepiness and negative mood states, particularly those associated with depression and anxiety, are of concern as these factors may confound the results of mTBI-related neurocognitive assessments and lead to invalid patient dispositions. It should be noted that there is currently no agreement on whether anxiety and/or depression adversely affect neurocognitive

TABLE 4 | Summary of multiple regression models predicting ANAM test performance.

ANAM subtest	*F*-test	Adjusted R^2	Sleep	Negative mood state	Positive mood state
CDD	$F_{(3,68)} = 1.55, p = 0.208$	0.02	$t = -0.22, p = 0.827$	$t = -1.74, p = 0.087$	$t = 0.01, p = 0.994$
CDS	$F_{(3,68)} = 1.47, p = 0.231$	0.02	$t = -0.18, p = 0.858$	$t = -1.35, p = 0.183$	$t = 0.07, p = 0.607$
M2S	$F_{(3,68)} = 3.98, p = 0.011^{*}$	0.11	$t = -0.65, p = 0.518$	$t = -2.07, p = 0.042^{*}$	$t = -0.08, p = 0.934$
MTH	$F_{(3,68)} = 0.29, p = 0.830$	0.00	$t = 0.11, p = 0.912$	$t = -0.12, p = 0.476$	$t = 0.36, p = 0.722$
PRT	$F_{(3,68)} = 4.09, p = 0.01^{**}$	0.12	$t = -0.30, p = 0.766$	$t = -2.24, p = 0.028^{*}$	$t = 0.87, p = 0.386$
SRT	$F_{(3,68)} = 6.45, p < 0.001^{***}$	0.19	$t = -0.33, p = 0.741$	$t = -2.93, p = 0.005^{**}$	$t = 0.81, p = 0.421$
SRT2	$F_{(3,68)} = 7.59, p < 0.0001^{***}$	0.22	$t = -0.37, p = 0.711$	$t = -3.22, p = 0.002^{**}$	$t = 0.59, p = 0.557$
NCS	$F_{(3,68)} = 6.16, p < 0.001^{***}$	0.18	$t = -0.30, p = 0.763$	$t = -2.90, p = 0.005^{**}$	$t = 0.66, p = 0.513$

CDD, Code Substitution Delayed; CDS, Code Substitution; M2S, Match to Sample; MTH, Mathematical Processing; PRT, Procedural Reaction Time; SRT, Serial Reaction Time; SRT2, Simple Reaction Time Repeated; NCS, neurocognitive composite score.

*Coefficient is significant at the 0.05 level (2-tailed).

**Coefficient is significant at the 0.01 level (2-tailed).

***Coefficient is significant at the 0.001 level (2-tailed).

performance, with research supporting both sides (63–66). However, there are numerous ways in which mood, depression, and anxiety can be related to decreased neurocognitive performance: changes in mood (i.e., depression and anxiety) and decreased neurocognitive performance can be direct symptoms of the same injury, the mood symptoms may be a response to decreased cognitive functioning, or that mood symptoms, in and of themselves, may adversely affect cognitive functioning (67–69).

Although it is well-accepted that there is an association between sleepiness and neurocognitive performance (9, 70), sleepiness was not predictive of NCS when controlling for the presence of negative and positive mood states in regression models. This could be suggestive of mediation by the positive mood factor for the effects of sleepiness. It also could be speculated that a single Likert scale may not accurately capture changes in sleepiness that occur during deployment and a more objective measure of sleep (i.e., quality or quantity) may have more predictive utility.

Limitations

This study has substantial limitations. First TBI history data were based on self-report rather than objective data; therefore, the results are subject to recall biases (i.e., under-reporting). Second, data were not screened for invalid test performance (i.e., poor effort) as we did not have access to all metrics produced by the ANAM. Third, there was limited demographic and service-related characteristics available in the dataset. This limited our ability to examine common confounding factors of NCS (i.e., age, education, rank, time in service, and number of deployments) in the current sample. Additionally, no clinical information regarding the presence of comorbid diagnoses, such as post-traumatic stress disorder, post-concussive syndrome, depression, anxiety, and/or adjustment disorders was available, which could also confound test results. Finally, a single Likert scale may not accurately capture changes in sleepiness that would have been hypothesized to occur during deployment because of poor sleep quality or quantity.

CONCLUSION

Measures of negative mood states were found to have significant negative relationships to several neurocognitive performance domains, whereas measures of positive mood states and sleepiness did not. There were also significant differences in neurocognitive performance, sleepiness, and mood between our deployment sample and normative data. These results taken together indicate that both changes in mood, particularly negative mood states, need to be considered when reviewing data from a neurocognitive assessment, especially if the SM is deployed, to ensure that the appropriate clinical decisions are made.

ETHICS STATEMENT

The original protocol was approved by the Naval Air Warfare Center Aircraft Division institutional review board, Patuxent River, MD (protocol NAWCAD.2011.0003-CR01-EMC) (33). Data from that protocol were de-identified and archived for further use. Subsequent analyses on the de-identified archival database were reviewed by the Navy Experimental Diving Unit Institutional Review Board and determined to be exempt human subject research in compliance with all applicable Federal regulations governing the protection of human subjects.

AUTHOR CONTRIBUTIONS

FH and JT contributed conception and design of the study. FH retrieved and managed the data. FH and JH performed the statistical analysis. FH, PS, and JH contributed to the initial drafting of the manuscript and wrote sections of the manuscript. All authors contributed to editing and revising of the manuscript, read and approved the submitted version.

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The Role of TDP-43 in Military-Relevant TBI and Chronic Neurodegeneration

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Due largely to the use of improvised explosive devices (IEDs) and other explosives in recent military conflicts, blast-related TBI has emerged as a prominent injury sustained by warfighters. In the recent wars in Iraq and Afghanistan, traumatic brain injury (TBI) has been one of the most common types of injury sustained by soldiers and military personnel; of the ∼380,000 TBIs reported in service members from 2000 to 2017, 82.3% were classified as mild (mTBI). While mTBI is associated with normal structural imaging, brief or no loss of consciousness, and rapid recovery of mental state, mTBI can nevertheless lead to persistent behavioral and cognitive effects. As in other cases of mTBI, exposure to low-level blast often does not cause immediate overt neurological effects, but may similarly lead to persistent behavioral and cognitive deficits. These effects are likely to be compounded when multiple exposures to blast and/or impact are sustained, since there is increasing evidence that multiple mTBIs can lead to chronic neurodegeneration. One common form of this deleterious outcome is frontotemporal lobar degeneration (FTLD), which is a progressive neurodegenerative process marked by atrophy of the frontal and temporal lobes, leading to frontotemporal dementia, a common form of dementia affecting behavior, cognition and language. About half of all cases of FTLD are marked by TAR-DNA binding protein (TDP-43)-positive protein inclusions. TDP-43, a DNA/RNA binding protein, controls the expression of thousands of genes and is associated with several neurodegenerative diseases including amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, and chronic traumatic encephalopathy. TDP-43 abnormalities have also been associated with traumatic brain injury in both pre-clinical and clinical studies. The role of TDP-43 in the manifestation of FTLD pathology in military TBI cases is currently unclear, and to date there has been only a limited number of pre-clinical studies addressing the effects of repeated blast-related mild TBI (rbTBI) in relation to FTLD and TDP-43. This review will summarize some of these findings and address the concerns and critical knowledge gaps associated with FTLD manifestation with military populations, as well as clinical findings on other forms of mTBI.

Keywords: blast-induced brain injuries, TDP43 proteinopathy, frontotemporal lobar degeneration, TBI, Frontotemporal dementia (FTD)

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INTRODUCTION

Traumatic brain injury (TBI) is one of the most common types of injuries sustained by military personnel. Between 2000 and 2017, TBI was reported in more than 380,000 service members, and more than 82% of these TBIs were classified as mild (mTBI) (1). mTBI is associated with brief or no loss of consciousness, normal structural imaging, and rapid recovery of memory and mental state (2), making it difficult to diagnose, and it is likely that the incidence of mTBI in military personnel is higher than reported. A specific problem for the military population is TBI caused by blast overpressure. Blast injury likely has a different primary injury mechanism than impact TBI due to different loading conditions, but there are similar downstream neurocognitive effects. However, identification of pathological processes and post-mortem studies are at a relatively nascent stage, as discussed in this review. Blast-related TBI has been a signature of Operation Iraqi Freedom and Operation Enduring Freedom, though DVBIC does not differentiate between mTBI caused by blast vs. impact, and currently this is based on retrospective analysis of a smaller cohort of warfighters (3). Concussive and sub-concussive symptoms resulting from blast exposure are often associated with unfavorable long-term clinical outcomes (4, 5). In addition to blast from improvised explosive devices (IEDs) in combat operations, repetitive mild blast overpressure exposure sustained during training and breaching exercises is also of concern. Military and law enforcement personnel who participate in training for breaching and heavy weapon systems are often exposed to multiple low-level blast exposures throughout training (6). In addition, instructors overseeing these exercises are exposed to even more low-level blasts over a longer period of time throughout their careers and report symptomatology similar to that seen following concussion (5, 6).

Though the acute symptoms of mTBI generally resolve quickly, mTBI can lead to persistent behavioral and cognitive deficits, which are thought to be compounded cumulatively following multiple mTBIs, with a "dose"-response relationship between number of injuries and symptoms (7–9). Increasingly, TBI is being causally linked to neurodegenerative diseases. The possible relationship between repetitive injury from blast overpressure exposure and chronic neurodegeneration will be discussed, with focus on clinical and preclinical findings as well as research gaps that remain to be addressed. This review is primarily focused on the emerging evidence implicating the protein marker transactive response DNA binding protein 43 kDa (TDP-43), and its role in impact and blast TBI and chronic neurodegeneration.

ASSOCIATION OF TDP-43 WITH TBI AND NEURODEGENERATION

Among the variety of protein mediators that are of interest with respect to chronic neurodegenerative diseases, the pathological accumulation of TDP-43 has emerged as a potentially pivotal contributor. TDP-43, a DNA/RNA binding protein encoded by the *TARDBP* gene, controls the expression of thousands of different genes. In its pathological form, TDP-43 is hyperphosphorylated, ubiquitinated, cleaved into 25 and 35 kDa fragments, and mislocalized to cytoplasmic protein inclusions (10). These inclusions are found in various neurodegenerative diseases, including chronic traumatic encephalopathy (CTE), and frontotemporal lobar degeneration (FTLD) (11, 12).

Preliminary research reports have shown that repetitive mTBI may cause a type of progressive neurodegeneration now known as CTE (13, 14). Neuropathological hallmarks of CTE include brain atrophy, white matter loss, and abnormal protein accumulation (14, 15). The main protein pathology and diagnostic criterion indicative of CTE is accumulation of neurofibrillary tangles (NFTs) of hyperphosphorylated tau, a microtubule-associated protein, in neurons and glia, particularly in perivascular regions and deep in the sulci (16). In his 1928 paper on "punch-drunk" syndrome in boxers, Martland described chronic physical deficits and, importantly, cognitive dysfunction and mental deterioration (13). More recently, clinical symptoms such as irritability, aggression, depression, memory deficits, and suicidality have been described (14, 15). Because diagnosis of CTE, which is most often observed in chronic impact cases, can only be confirmed by post-mortem pathological analysis, there is a lack of consensus in the research field as to how and if the clinical features of CTE relate to the neuropathological findings, TBI and symptomology (17). There is also debate about whether CTE can result from repeated blast exposure or whether blast leads to different chronic neurodegenerative conditions (18, 19). More epidemiology studies with comorbidities of TBI and post-mortem brain pathology could inform classification of disease pathology.

In 2010, McKee et al. found that in 12 cases of CTE-diagnosed athletes, a disease previously thought of as a tauopathy, 10 individuals showed widespread TDP-43 proteinopathy (12). They later found that TDP-43 pathology progresses with the stages of CTE, which are determined based on tau NFT pathology (20). In stage I CTE, TDP-43-positive neurites can be found in the subcortical white matter and fornix; in stage II, there are isolated TDP-43-positive neurites or inclusions in the subcortical white matter, brainstem, or medial temporal lobe; in stage III, most cases of CTE show TDP-43-positive neurites in the cortex, medial temporal lobe, or brainstem; and nearly all cases of stage IV CTE have TDP-43-positive neurites and inclusions in glia and neurons in the cortex, medial temporal lobe, diencephalon, basal ganglia, brainstem, and spinal cord (16). The strong association of CTE with repetitive impact-related TBI, along with the observation of TDP-43 pathology in many cases of CTE, may point to some association between subconcussive brain trauma and TDP-43 pathology (12), though this needs to be further supported and confirmed by more independent clinical studies.

FTLD is a neurodegenerative process characterized by selective, progressive degeneration of the frontal and temporal lobes (21). There are a variety of subtypes of FTLD, characterized by the type of intracellular protein accumulations found post-mortem. The most common subtypes have accumulation of either tau (FTLD-tau) or TPD-43 (FTLD-TDP), while the remaining 10–15% have accumulation of the protein fused in sarcoma (FUS) (22).

Frontotemporal dementia (FTD), the clinical manifestation of FTLD, has been reported in military Veterans. Various epidemiological studies have found that FTD is one of the most common forms of dementia in individuals under 65 years of age, after Alzheimer's disease and vascular dementia (23–25). It is a progressive neurodegenerative disease causing changes in cognition and behavior, with three clinical variants. Behavioral variant FTD (bvFTD) is the most common form of FTD and is characterized by disinhibition, apathy, and impulsivity (26). The other forms of FTD are non-fluent/agrammatic variant primary progressive aphasia (nfvPPA), characterized by difficulty in speech production, and semantic variant primary progressive aphasia (svPPA), characterized by impaired speech comprehension (27). About 40% of FTD cases are familial, with a known family history of the disease, and are caused by a variety of genetic mutations including in the *MAPT, GRN, C9ORF72,* and *TARDBP* genes (28). The remaining 60% of FTD is sporadic, with no known family history. There is overlap between the type of protein accumulation found in the brain and the FTD variant, such that, for example, FTD-tau and FTD-TDP can both cause bvFTD (29). Though cases of FTD caused by *TARDBP* mutation are very rare, FTLD-TDP is the most common form of FTLD and is seen in both familial and sporadic FTD (28).

Preclinical

Several preclinical studies have examined TDP-43 pathology, using various impact TBI models. These impact studies identified alterations in TDP-43 including proteolysis, phosphorylation, and formation of cytoplasmic TDP-43 granules even after only one impact. This has been shown in several studies using fluid percussion injury (FPI) or cortical contusion injury (CCI) in rodents, where TDP-43 was phosphorylated, mislocalized, and cleaved into TDP-25 and TDP-35 following injury, leading to neuronal loss (30–32). The cleavage products are also increased in astrocytes following a weight drop TBI (33). In general, these models are of moderate-to-severe impact injury rather than a mild or blast injury and involve open head surgery and a severe deformation of the brain tissue after injury. A model of repetitive mTBI, using 3 daily controlled cortical impacts, persistently increased TDP-43 expression and aggregation in the mouse cortex and hippocampus (34).

FTD-relevant behavioral and cognitive changes have also been observed in various preclinical TBI studies using single and repetitive models. Rats subjected to a single CCI display learning and memory deficits as measured by Morris water maze (30). TDP-43^{A315T} transgenic mice had significantly impaired cognition as measured by the Y maze following FPI (32). While a single mild impact causes learning impairments, repeated mTBI causes more generalized progressive impairments in cognition, learning, and behavior (35). Repetitive mild lateral FPI and weight drop models have been shown to cause learning and memory deficits, as measured by Morris water maze, novel object recognition tasks, Barnes maze, and Y maze (36, 37). Few studies of repetitive TBI and dementia have been conducted using non-rodent models, which have more similar head and brain anatomy to that of humans, leading to difficulty in scaling these studies to humans. One study using a swine model of acceleration injury, found that repeated injury causes more severe cognitive dysfunction than a single injury (38).

While the majority of studies on TBI and FTD-like symptoms have used impact injury models, there are some using blast overpressure-related TBI models. In one such study, mice exposed to low-level (2.5 psi) blast exhibited impaired memory, as measured by the novel object recognition task (39). Our research group has similarly demonstrated that blast exposure can lead to impaired learning and memory along with tau pathology (40, 41). There are few preclinical longitudinal studies investigating the relationship between blast-induced TBI, TDP-43, and FTLD. In one study, mice exposed to blast were found to develop CTE-like tau pathology and cognitive deficits (42), though this blast has high accelerative forces that are not characteristic of primary blast exposure. This study did not examine TDP-43 pathology, but it does provide a link between blast exposure and development of CTE-like symptoms. In another study, mice subjected to blast overpressure exhibited TDP-43 fragmentation and mislocalization, hallmarks of TDP-43 proteinopathy (43). A preliminary study using a rat model of blast TBI demonstrated significantly increased TDP-43 in the brain following 3 or 4 exposures to 19psi blast (44). This indicates that repetitive blast exposure may lead to disruption in TDP-43, which is likely an important aspect of neurodegenerative processes. Based upon these promising findings, further study is needed to define the relationships between brain injury sustained from blast overpressure exposure, TDP-43 proteinopathy, and the development of FTD-like symptoms. Long-term preclinical studies, particularly ones focusing on repetitive blast exposure relevant to training and breaching environment of Warfighters, are currently lacking in this field and remains an important area of research.

Clinical

There has been limited clinical research associating chronic TBI, TDP-43 and neurodegeneration, but there is some preliminary evidence that links these factors. The clinical behavioral symptoms associated with bvFTD, such as apathy and social dysfunction, are also frequently reported in cases of TBI and CTE (45) and there have been multiple studies correlating TBI with increased risk of developing FTD. Patients with a history of TBI had earlier ages of onset for symptoms and were 3.3 times more likely to develop FTD than patients with no head trauma (30, 46, 47) In a study of Veterans diagnosed with dementia, the prevalence of TBI history was significantly greater in those with FTD than in those with other types of dementia (48). A systematic review of 47 original research studies on concussion found that multiple concussions are a risk factor for development of cognitive impairment (49). A meta-analysis of 18 journal articles on the risk of neurodegeneration after TBI found that TBI is a risk factor for development of dementia (odds ratio (OR) of 1.93), TDP-43-associated diseases (OR of 4.44), and FTD (OR of 2.97), showing an association between TBI, TDP-43, and FTD (50). While there is a growing body of literature describing observations of FTD in military Veterans (48, 51), the cause, onset, and prognosis of disease is currently unclear in military populations. Similarly, although current research trends show that brain trauma could be associated with FTD

and possibly a risk factor toward sporadic FTD, a clearly defined cause-effect relationship between FTD and brain trauma is at present unclear.

A common link between repetitive impact TBI and FTD is mislocalization and aggregation of TDP-43 in neurons and glial cells. In a study of human subjects who sustained a single moderate/severe TBI, TDP-43 expression was increased in the cytoplasm, though no increase in TDP-43 phosphorylation was observed (52). A case study of a woman who sustained a single severe TBI found that she subsequently developed symptoms of dementia as well as intraneuronal TDP-43 inclusions in the frontal and temporal lobes (53). The existing reports on TDP-43 pathology following TBI are limited in both scope and number of patients, and further examination of TDP-43 in various TBI populations is necessary to establish a causal link between TBI and TDP-43 pathology.

In cases of CTE with severe TDP-43 pathology, the pattern of TDP-43 expression mimics the pattern of expression seen in FTLD-TDP, with TDP-43 proteinopathy found in all layers of the cortex, particularly in layer II, and in the dentate fascia of the hippocampus (12, 16). Additionally, among individuals with a history of repetitive mTBI who were diagnosed with CTE, 6% were diagnosed with CTE-FTLD. Of these, half had FTLD-TDP and half had FTLD-tau (20). This demonstrates that brain trauma may be a risk factor contributing to chronic neurodegeneration, potentially mediated by TDP-43 proteinopathy.

Longitudinal studies which comprehensively monitor blast overpressure exposure and resultant chronic debilitations are currently lacking in the clinical literature. In a study of individuals with a history of repetitive mTBI, 16 of 21 military Veterans were diagnosed with CTE, three of whom were exposed to blast from IEDs (20). Of those three, one was found to have TDP-43 pathology in the brain (20). Similarly, another study of military Veterans with a history of blast exposure and/or concussion found tau pathology in the brain (42). In such studies of blast-exposed Veterans, it is difficult to ensure that the subjects have only been exposed to blast and do not have a history of impact TBI. The individuals in the McKee et al. study with a CTE diagnosis and history of IED blast exposure also had a history of playing high school football and/or motor vehicle accidents (20), and therefore likely had sustained impact mTBI(s). Likewise, in the Goldstein et al., study, the military Veterans had a history of "blast exposure and/or concussive injury" (42) and therefore, it is impossible to attribute the findings to blast exposure itself. The current state of blast/neurodegeneration literature is greatly limited by a small number of subjects and many confounding factors. There have been studies showing a divergence in CTE diagnosis and blast exposure. One study reported a unique pathological finding in chronic blast-exposed Veterans: significant perivascular astrogliosis and glial scarring at boundaries between the brain and CSF and between the gray and white matter (18). This pathology was not found in individuals with chronic impact TBI, indicating that it might be a unique consequence of blast-related brain injury (18). Further, this glial scarring is in the absence of any tau pathology, which is the current diagnostic hallmark of CTE (19). This lack of consensus

TABLE 1 | Summary of previous research conducted on TBI, TDP-43, and FTD.

	Clinical TBI		Preclinical TBI	
Cause/evidence of disease progression	**Civilian TBI**	**Military TBI (blast exposure studies only)**	**Models of civilian TBI**	**Models of blast exposure**
FTD/FTD-like behavioral deficits	• History of TBI is linked to FTD (30, 47) TBI history leads to earlier-onset bv FTD (46) • Veterans with dementia: TBI prevalence higher in those with FTD than other dementias (48) • Meta-analysis: TBI is a risk factor for FTD with an odds ratio of 4.44 (50)	• Military personnel exposed to blast showed a dose-response relationship between number of exposures and symptoms (9) • **More clinical studies are needed on blast-specific injury**	• Repetitive impact TBI causes cognitive and behavioral deficits (34–38) • Repeated injury causes more severe cognitive dysfunction than single injury (38) • TDP-43^{A315T} transgenic mice have impaired cognition following TBI (30, 32)	• Mice exposed to low-level blast exhibited impaired memory (39) • **Preclinical studies on repetitive bTBI are lacking**
CTE/CTE-like symptoms	• Repetitive mild TBI was associated with CTE (12, 14, 20) • **More independent studies are lacking** • Multiple concussions are a risk factor for cognitive impairment (49)	• Veterans exposed to blast can develop CTE* (20, 42) *subjects also had history of impact TBI	• Repetitive impact injury in mice causes CTE-like neuropathologic changes (34)	• **Lack of preclinical studies linking repetitive blast and CTE neuropathology**
TDP-43 proteinopathy	• Repetitive mild TBI leads to TDP-43 proteinopathy, including protein inclusions, cytoplasmic mislocalization, phosphorylation, and cleavage (12, 20, 32) • A single TBI causes increased expression and intraneuronal inclusions of TDP-43 (52, 53) • TBI is a risk factor for TDP-43 associated diseases with an odds ratio of 1.93 (50)	• CTE is found in humans exposed to blast from IEDs* (20) *subjects also had history of impact TBI	• Repetitive impact TBI leads to TDP-43 aggregation (34) • A single impact injury causes TDP-43 pathology, including cleavage, phosphorylation, mislocalization, and cytoplasmic aggregation (30–33, 43, 54) • This proteinopathy is exacerbated in transgenic mice (32, 54)	• Single blast lead to TDP-43 cleavage (43) • **Lack of preclinical studies on repetitive blast and TDP-43 proteinopathy**

of whether blast exposure can lead to CTE is an important knowledge gap to be addressed.

SUMMARY

While there is ample evidence that multiple impact head traumas resulting in TBIs can lead to neurodegeneration, longitudinal studies addressing Warfighter needs in this domain are currently lacking, despite the emergence of evidence in Veteran populations that FTD is a primary concern. Studies targeting mechanisms underlying the pathogenesis of neurodegeneration using pre-clinical models addressing this Warfighter population are largely non-existent in the literature. **Table 1** summarizes the work that has been conducted in this field to date and illustrates pertinent research gaps to be addressed in the future, including a need for more clinical research on blast-related TBI and independent studies to demonstrate CTE pathology. Although a number of studies have focused on repetitive impact TBI experienced by civilians, they generally do not include military populations and tend to not differentiate between TBI resulting from impact and blast. Moreover, those that do address blast exposure generally rely on self-reported history of blast exposure, which creates shortcomings due to recall and diagnostic error. There is also an increasingly recognized need to study multiple repetitive exposures to low-level blast such as that experienced by breachers and heavy weapons systems users. While some studies have shown associations between TDP-43, TBI, and neurodegeneration, more studies are needed to establish this paradigm. In terms of preclinical work, there has been little research on repetitive blast exposure and its effects on TDP-43 and cognitive impairment. These research gaps are areas of future study in our laboratory, using appropriate animal models. Filling these gaps will help to understand how military personnel are particularly vulnerable to neurodegenerative processes and help identify areas of possible intervention.

AUTHOR CONTRIBUTIONS

LH wrote the manuscript. VS and LH structured the manuscript. VS and JL edited the manuscript.

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Racial and Ethnic Differences in Emergency Department Utilization and Diagnosis for Sports-Related Head Injuries

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Background: Prior studies have shown racial differences in concussion awareness and outcome.

Objective: To assess if racial or ethnic differences exist in Emergency Department (ED) utilization and diagnosis for children with sports-related head injuries.

Methods: We performed a retrospective, cross-sectional analysis of ED visits from 2008 to 2017 using National Electronic Injury Surveillance System (NEISS) data. Population-weighted ED visits for children age 7–18 years with a sport-related injury were included. We compared the probability of an ED visit being for an injury to the head or diagnosed as a concussion between children of different races/ethnicities. Analyses were adjusted for age, gender, sport, year, and location where the injury occurred.

Results: We identified 11,529,994 population-weighted ED visits for pediatric sports-related injuries, of which 1,497,717 (13.0%) were injuries to the head and 619,714 (5.4%) received a diagnosis of concussion. Black children were significantly less likely than non-Hispanic white children to have their ED visit be for an injury to the head [Odds Ratio (OR) 0.72, 95%CI 0.65–0.79] or concussion (OR 0.58, 95%CI 0.50–0.68). Black children presenting to the ED with an injury to their head were less likely than non-Hispanic white children to be diagnosed with a concussion (OR = 0.71, 95%CI 0.59–0.85).

Conclusions: Racial differences exist in both ED utilization for pediatric sports-related head injuries and in the diagnosis of concussion. Further work is needed to understand these differences to ensure all brain injured athletes receive optimal care, regardless of race.

Keywords: concussion, head injury, children, emergency department, race, ethnicity, disparities

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INTRODUCTION

Sports-related injuries are one of the most common causes of head injuries and concussions in children in the United States (1, 2). Recently, concerns have been raised about the long-term effects of concussions on neurocognitive function, including the risk of chronic traumatic encephalopathy (CTE) and other neurodegenerative disorders (3–6). As a result, there have been significant efforts to increase awareness and recognition of sports-related head injuries, as well as to define protocols for concussion management and return-to-play guidelines (7, 8). Without proper recognition and

diagnosis, athletes may prematurely return to play, potentially exacerbating their underlying head injury and putting themselves at increased risk of second-impact syndrome (7, 9, 10). Therefore, the identification of patients with sports-related head injuries and concussion has important implications for how young athletes, parents, and coaches approach return to play, rehabilitation, as well as the long-term risks/benefits of continued sports participation after a sports-related head injury. The Emergency Department (ED) represents a common location of initial evaluation of sports-related head injuries and concussions for children, with ED visits for young athletes increasing (1, 11).

Previous data demonstrate racial disparities in recognition of concussion symptoms and knowledge about the significance of concussion, with white athletes having higher knowledge about concussions then their African American counterparts (12). Furthermore, racial differences have been observed in the management of pediatric head injuries, with race being an important predictor of which children with head injuries undergo emergent neuroimaging (13, 14). However, it is not known whether these disparities translate into differences in patterns of ED utilization for sports-related head injuries. Nor is it known if race impacts the probability that a head-injured child will be diagnosed with a concussion. Therefore, our objectives were to: (1) assess if race/ethnicity affects the likelihood that a sports-related ED visit is for a head injury; (2) to determine if race/ethnicity impacts the likelihood that an athlete presenting to the ED with a head injury is diagnosed with a concussion.

MATERIALS AND METHODS

Study Design

We performed a retrospective, cross-sectional analysis of ED visits from the National Electronic Injury Surveillance System (NEISS) over 10 years between 2008 and 2017. The NEISS is a division of the United States Consumer Product Safety Commission (CPSC) and reports population-weighted estimates of injuries and consumer product related injuries. The NEISS collects data from approximately 100 hospitals located throughout all geographic regions of the United States (15, 16). Emergency Department records are reviewed at the end of each day by study research coordinators and coded according to the NEISS coding manual. Clinical information including diagnostic evaluations such as laboratory testing and diagnostic imaging results are not available in NEISS. The NEISS has been utilized for multiple published reports on the epidemiology of injury (17–20). Included in the NEISS are ED visits for sports-related injuries, coded by sport of participation. This study was approved by the Institutional Review Board of Boston Children's Hospital.

Study Population

The unit of analysis for our study was the population-weighted ED visit. We included all ED visits for children age 7–18 years with an ED visit that occurred while participating in the following sports: baseball, basketball, cheerleading, field hockey, football (American), gymnastics, ice hockey, lacrosse, rugby, soccer, softball, tennis, track and field, volleyball, and wrestling. Included in the NEISS are ED visits for injuries, as well as for medical problems such as heat-exhaustion. ED visits in our analysis include those that occurred in both organized as well as recreational sports participation, as these are not distinguished in the NEISS database.

Study Definitions and Outcomes

We abstracted the following data elements from the NEISS database: age, sex, race, ethnicity, year of presentation, sport of participation, location where the injury occurred, body part injured, and final diagnosis. The NEISS classifies each ED visit by both body part involved and final diagnosis. We categorized injuries by the involved body region into: head, neck, torso, upper extremity, lower extremity, and other. "Other body region injuries" included heat-stroke/exhaustion, cardiac events and injuries to the ear, eye, and face. ED visits were categorized into the following final diagnoses: concussions, fractures/dislocations, sprains/strains/contusions, internal injuries, and other. "Other diagnoses" included: accidental poisonings, amputation, anoxia, avulsion and crush injuries, dental injuries, dermatitis, foreign bodies, frostbite, hematoma, hemorrhage, lacerations, nerve damage, puncture wounds, heat-related injuries, and injuries not otherwise classified. Final diagnoses were assigned by the treating ED provider and classified based on the previously defined NEISS methodology. When multiple injuries or diagnoses are present, NEISS classifies the visit according to the most severe injury (15).

NEISS classified race as White, Black/African American, Asian, American Indian/Alaska Native, Native Hawaiian/Pacific Islander, and other. In NEISS "other" includes patients who indicate more than one race or for whom none of the above categories applies. A patient's ethnicity is separately coded in NEISS as Hispanic or non-Hispanic. For our analysis, we categorized race and ethnicity into the following categories: non-Hispanic white, Hispanic, black, Asian, and other (included all patients not categorized by our aforementioned categories) (21).

The primary outcomes for our analysis were an ED visit for an injury to the head region of the body, or an ED visit for an injury diagnosed as a concussion.

Analysis

We first compared patient characteristics between ED visits for all reasons to those for head injuries and injuries diagnosed as concussions. We describe continuous variables using medians and interquartile ranges and categorical variables using proportions and 95% confidence intervals (CI). For our primary outcome, we calculated the odds of an ED visit being for an injury to the head region or diagnosed as a concussion by race/ethnicity. For our secondary outcome, we calculated the odds of a patient with an injury to the head region being diagnosed as a concussion by race/ethnicity. We performed sub-analyses stratified by sport of participation to evaluate if sport of participation was an important confounder in the relationship between race and head injury/concussion diagnoses.

Our analyses were performed using survey-weighted logistic regression with statistical weighting provided by the NEISS (15) and results are reported as odds ratios and associated 95% CIs. Multivariate analyses evaluating the association between race/ethnicity and ED visits for injuries to the head region or

TABLE 1 | Characteristics of population-weighted Emergency Department (ED) visits for pediatric sports-related injuries.

	All ED visits ***N* = 11,529,994**	**Head injury ED visits** ***N* = 1,497,717**	**ED visits diagnosed as concussion** ***N* = 619,714**
Age, median (IQR)	13.5 (11.3-15.4)	13.5 (11.2-15.3)	13.9 (11.9-15.5)
Male sex, *n* (%)	8,218,044 (71.3)	1,080,753 (72.2)	452,299 (73.0)
Race/ethnicity, *n* (%)			
White, non-hispanic	5,676,695 (49.2)	780,448 (52.1)	342,904 (55.3)
Hispanic	754,286 (6.5)	93,408 (6.2)	37,005 (6.0)
Black	1,779,354 (15.4)	176,553 (11.8)	64,124 (10.3)
Asian	91,256 (0.8)	14,997 (1.0)	5,735 (0.9)
Other	163,154 (1.4)	21,065 (1.4)	9,048 (1.5)
Missing	3,065,248 (26.6)	411,245 (27.5)	160,879 (26.0)
Year, *n* (%)			
2008	1,140,558 (9.9)	98,252 (6.6)	39,866 (6.4)
2009	1,133,525 (9.8)	121,480 (8.1)	43,045 (6.9)
2010	1,221,117 (10.6)	145,356 (9.7)	51,198 (8.3)
2011	1,203,043 (10.4)	151,872 (10.1)	59,768 (9.6)
2012	1,260,242 (10.9)	174,270 (11.6)	71,299 (11.5)
2013	1,157,985 (10.0)	167,983 (11.2)	72,485 (11.7)
2014	1,144,094 (9.9)	164,812 (11.0)	72,556 (11.7)
2015	1,124,372 (9.8)	162,507 (10.9)	69,413 (11.2)
2016	1,084,923 (9.4)	158,513 (10.6)	69,283 (11.2)
2017	1,060,134 (9.2)	152,672 (10.2)	70,800 (11.4)
Location of injury, *n* (%)			
Place of recreation	5,857,505 (50.8)	784,291 (52.4)	347,746 (56.1)
School	2,668,799 (23.1)	387,834 (25.9)	174,694 (28.2)
Not recorded	2,379,814 (20.6)	259,186 (17.3)	80,766 (13.0)
Home	494,912 (4.3)	50,626 (3.4)	11,791 (1.9)
Other	128,694 (1.1)	15,780 (1.1)	4,717 (0.8)
Sport, *n* (%)			
Baseball	935,242 (8.1)	132,139 (8.8)	34,306 (5.5)
Basketball	3,173,919 (27.5)	312,136 (20.8)	108,840 (17.6)
Cheerleading	331,063 (2.9)	50,564 (3.4)	19,597 (3.2)
Field hockey	36,940 (0.3)	5,202 (0.3)	2,210 (0.4)
Football	3,442,602 (29.9)	522,011 (34.9)	259,025 (41.8)
Gymnastics	253,909 (2.2)	16,682 (1.1)	4,911 (0.8)
Ice hockey	114,487 (1.0)	32,475 (2.2)	15,436 (2.5)
Lacrosse	151,669 (1.3)	29,909 (2.0)	12,673 (2.0)
Rugby	48,566 (0.4)	11,944 (0.8)	5,235 (0.8)
Soccer	1,531,774 (13.3)	222,474 (14.9)	93,386 (15.1)
Softball	512,320 (4.4)	63,608 (4.2)	22,183 (3.6)
Tennis	58,056 (0.5)	5,824 (0.4)	1,245 (0.2)
Track and field	222,201 (1.9)	12,955 (0.9)	3,884 (0.6)
Volleyball	362,986 (3.1)	33,147 (2.2)	13,115 (2.1)
Wrestling	354,260 (3.1)	46,646 (3.1)	23,667 (3.9)

diagnosis of concussion were adjusted for age, sex, year of visit, location where the injury occurred, and sport (22–24). These were determined *a priori* to be important factors in determining the types of injuries that present to an ED. Sport-specific analyses were adjusted for age, sex, year of visit, and location of injury. Due to the small number of male participants in field hockey, sex was not included in models evaluating associations between field hockey and our outcomes of interest. Secondary to the small number of female and young participants in rugby, both sex and age were not included in models evaluating associations between rugby and our outcomes of interest. In our analysis of the relationship between race and having an ED visit be for a head injury or concussion, an odds ratio of <1 represents decreased odds of the ED visit being for a head injury/concussion

relative to non-Hispanic white athletes. In our analysis of the relationship between race and a diagnosis of concussion among head injured athletes, an odds ratio of <1 represents decreased odds of being diagnosed with a concussion relative to non-Hispanic white athletes.

All statistical analyses were performed using SAS software, copyright© 2012 SAS Institute Inc.

RESULTS

We identified 360,103 unweighted ED visits for pediatric sports-related injuries. These visits correspond to 11,529,994 population-weighted ED visits, including 1,497,717 (13.0%) visits for injuries to the head region and 619,714 (5.4%) visits for injuries diagnosed as concussions (**Table 1**). Data on race and ethnicity were available for 8,464,746 (73.4%) ED visits. We found no association between not reporting race/ethnicity and ED visits for injuries to the head region [Odds ratio (OR): 1.05, 95%CI 0.91–1.22] or injuries diagnosed as concussions (OR: 0.97, 95%CI 0.74–1.26).

After adjustment for age, gender, sport, year of ED presentation, and location of injury, black children were less likely to have their ED visit be for an injury to the head region or for an injury that was diagnosed as a concussion relative to non-Hispanic white children (**Table 2**). Similar findings were not observed for Hispanic children, Asian children or children who identified as other races. In sport-specific analyses, black children were significantly less likely than white children to have their ED visit be for a head injury if playing: basketball, football, cheerleading, soccer, or volleyball. Asian children injured playing baseball were more likely than white athletes to have their ED visits be for a head injury (**Table 3**).

Among the sub-set of children presenting to the ED with an injury to their head region, black children were less likely than non-Hispanic white children to be diagnosed with a concussion (**Table 4**). In contrast, we found no significant difference in rates

TABLE 2 | Associations between race and having an emergency department visit for a sports-related injury be for an injury to the head region or diagnosed as a concussion (compared to other body regions and diagnoses, respectively).

	Injuries to the head region		Concussions	
Race/ethnicity	**Unadjusted**	**Adjusted[a]**	**Unadjusted**	**Adjusted[a]**
White, non-hispanic	Ref	Ref	Ref	Ref
Hispanic	0.89 (0.60–1.30)	0.89 (0.64–1.24)	0.80 (0.46–1.36)	0.83 (0.54–1.28)
Black	0.69 (0.63–0.76)	0.72 (0.65–0.79)	0.58 (0.50–0.68)	0.58 (0.50–0.68)
Asian	1.23 (0.92–1.66)	1.25 (0.92–1.69)	1.05 (0.72–1.52)	1.08 (0.72–1.62)
Other	0.93 (0.78–1.11)	0.93 (0.78–1.11)	0.91 (0.72–1.16)	0.86 (0.68–1.10)

[a]*Adjusted for age, sex, location of injury, sport, and year of ED presentation.*

TABLE 3 | Sports-specific adjusted associations between race and having and emergency department visit for a sports-related injury be for an injury to the brian/head region.

Sport	**White**	**Hispanic**	**Black**	**Asian**	**Other**
Baseball[a]	Ref	1.21 (0.84–1.75)	0.91 (0.69–1.21)	1.44 (1.02–2.03)	0.73 (0.38–1.39)
Basketball[a]	Ref	0.88 (0.50–1.19)	0.71 (0.63–0.79)	1.42 (0.90–2.24)	0.86 (0.59–1.25)
Cheerleading[a]	Ref	1.31 (0.90–1.92)	0.61 (0.46–0.81)	1.77 (0.83–3.77)	0.91 (0.41–2.03)
Field hockey[b]	Ref	0.10 (0.01–1.13)	1.16 (0.38–3.53)	***	0.16 (0.03–1.01)
Football[a]	Ref	1.05 (0.74–1.48)	0.71 (0.63–0.80)	1.38 (0.92–2.06)	1.10 (0.90–1.35)
Gymnastics[a]	Ref	0.93 (0.47–1.81)	0.98 (0.58–1.64)	1.42 (0.70–2.88)	0.69 (0.28–1.69)
Ice hockey[a]	Ref	1.64 (0.21–12.72)	0.96 (0.32–2.87)	1.86 (0.50–6.89)	1.92 (0.72–5.11)
Lacrosse[a]	Ref	0.55 (0.30–1.02)	1.10 (0.65–1.86)	1.90 (0.73–4.91)	0.74 (0.33–1.66)
Rugby[c]	Ref	0.75 (0.27–2.07)	0.60 (0.28–1.30)	0.31 (0.07–1.44)	2.32 (0.89–6.0)
Softball[a]	Ref	1.44 (0.90–2.29)	0.79 (0.57–1.10)	0.89 (0.46–1.74)	1.10 (0.47–2.56)
Soccer[a]	Ref	0.67 (0.54–0.84)	0.72 (0.57–0.92)	0.92 (0.60–1.39)	0.70 (0.57–0.87)
Tennis[a]	Ref	0.54 (0.21–1.44)	1.33 (0.64–2.79)	1.39 (0.35–5.58)	0.90 (0.16–5.12)
Track and field[a]	Ref	0.56 (0.30–1.07)	0.81 (0.53–1.24)	0.84 (0.43–1.62)	1.14 (0.47–2.81)
Volleyball[a]	Ref	0.88 (0.51–1.53)	0.67 (0.45–0.98)	1.23 (0.63–2.41)	0.74 (0.36–1.51)
Wrestling[a]	Ref	1.09 (0.77–1.55)	0.79 (0.56–1.10)	0.79 (0.52–1.19)	0.94 (0.38–2.23)

[a]*Adjusted for age, sex, location of injury, and year of ED presentation.*
[b]*Adjusted for age, location of injury, and year of ED presentation.*
[c]*Adjusted for location of injury and year of ED presentation.*
[***]*Unable to calculate due to small number of weighted ED visits.*

TABLE 4 | Association between race and having an ED visit with an injury to the head region be diagnosed as a concussion.

Race/ethnicity	Unadjusted	Adjusted[a]
White, non-hispanic	Ref	Ref
Hispanic	0.84 (0.61–1.14)	0.84 (0.65–1.10)
Black	0.73 (0.62–0.86)	0.71 (0.59–0.85)
Asian	0.79 (0.62–1.02)	0.85 (0.65–1.12)
Other	0.96 (0.73–1.27)	0.86 (0.64–1.17)

[a]*Adjusted for age, sex, sport, location of injury, and year of ED presentation.*

of concussion diagnoses between head-injured, non-Hispanic white children and Hispanic children, Asian children or children who identified as other races. In adjusted analyses, other factors associated with receiving a diagnosis of concussion among pediatric patients presenting to the ED with an injury to the head region included: age (OR 1.08, 95%CI 1.07–1.10 for each year over age 7), female gender (OR 1.18, 95%CI 1.09–1.29), injuries that occurred at places of recreation (OR 2.06, 95%CI 1.58–2.67) or school (OR 2.08, 95%CI 1.58–2.67) compared to those injuries occurring at home, and year of presentation (OR 1.08, 95%CI 1.04–1.10 for each year after 2008). Among children presenting to the ED with a head injury, the greatest differences in the diagnosis of concussion were found in basketball, cheerleading, football, gymnastics, and soccer (**Table 5**).

Differences in the percent of children presenting with a head region injury and those diagnosed as a concussion are summarized in **Figure 1**. The difference in diagnoses was driven by a higher percentage of black children with head injuries receiving a diagnosis of contusion, internal injury, or "other injury." We found no significant differences in the rates of diagnosis of fracture among children with injuries to the head region.

DISCUSSION

Among ED visits for sports-related injuries, black children were nearly 30% less likely than non-Hispanic white children to have their ED visit be for an injury to the head region and over 40% less likely to have their ED visit be for the diagnosis of a concussion. Among children presenting to the ED with an injury to the head region, black children were more than 30% less likely to be diagnosed with a concussion. In addition, we found increasing rates of ED visits for sports-related head injuries and concussion over time. These findings are consistent with prior data and may represent increased awareness and diagnosis rather than an absolute increase in the actual number of concussions. The results of our analysis raise important questions about how race affects the decision to seek ED level care for a sports-related head injury, as well as how race impacts the likelihood that a head-injured athlete receives a diagnosis of concussion.

Prior research has demonstrated racial differences in concussion knowledge and recognition of concussion symptoms (12). Specifically, white athletes in a prior study demonstrated increased concussion knowledge and more frequently recognized the signs and symptoms of concussion. In addition, racial disparities in the use of neuroimaging in evaluation of head injuries in the ED have been reported (13, 14). In these two studies, white children were more likely to have emergent neuroimaging (MRI or CT) performed than black children. Finally, racial differences have been reported in the site of initial care for brain-injured children (25), with black children more likely to have their point of entry into the health care system for a concussion be in the ED. Our study is consistent with these prior data, demonstrating racial differences in the evaluation and management of head injured children. However, while prior data have shown the ED is a common site of initial care for brain-injured black children, our data demonstrate significant racial differences in care utilization and diagnosis in the ED setting, with black children less likely to have an ED visit be for a head injury or concussion. Taken together, these findings suggest the magnitude of underutilization and underdiagnosis may be even greater than we observed. Finally, our study is the first to our knowledge to demonstrate how race affected ED utilization and diagnosis for athletes with head injuries.

We found that among athletes with an ED visit for a sports-related injury, black children were less likely to have that visit be for a head injury or diagnosed as a concussion. While our study demonstrates racial differences in ED utilization, our findings could represent either overutilization of the ED for head-injured non-Hispanic white athletes or underutilization by black athletes. The observed racial differences in ED utilization could be due to a variety of factors including differences in the recognition of signs and symptoms of head injuries, differences in level of concern about head injuries, access to certified athletic trainers, differences in where patients seek initial care for head injuries, and differences in referral patterns to the ED for head injuries (12, 25, 26).

We found that black children were 30% less likely than their non-Hispanic white counterparts to be diagnosed with a concussion when presenting with an injury to the head region. Head injured black children were more likely than their white counterparts to receive a general diagnosis of head contusion or internal injury. It is possible that many of these children receiving these diagnoses did in fact have a concussion. These findings raise important questions about potential racial bias in the evaluation and diagnosis of head-injured athletes (27). It is not clear if our finding represents under-diagnosis of concussion in young, black athletes, or over- diagnosis in non-Hispanic white athletes. Importantly, the differences were most pronounced for football, soccer, basketball, and cheerleading. These results are significant as these sports have been associated with high rates of head trauma, including repetitive head trauma (24). Because most clinically important traumatic brain injuries can be ruled out without the need for emergent neuroimaging (28), most ED visits for children with sports-related concussions focus on education regarding cognitive rehabilitation and return to play guidelines (7). With increasing research and guidelines around concussion management and return to play, diagnostic precision is becoming increasingly important. Racial differences in the clinical diagnosis of concussion could result in disparities in the post-injury care of children with sports-related head injuries and

TABLE 5 | Sport-specific adjusted associations between race and the diagnosis of concussion in athletes presenting to the emergency department with an injury to the head region.

	White	Hispanic	Black	Asian	Other
Baseball[a]	Ref	0.98 (0.60–1.61)	0.72 (0.50–1.04)	1.03 (0.55–1.93)	1.06 (0.44–2.56)
Basketball[a]	Ref	0.61 (0.40–0.94)	0.71 (0.57–0.90)	1.12 (0.63–2.01)	1.00 (0.61–1.63)
Cheerleading[a]	Ref	1.28 (0.70–2.35)	0.56 (0.32–0.99)	0.87 (0.32–2.35)	1.44 (0.47–4.47)
Field hockey[b]	Ref	***	***	***	***
Football[a]	Ref	0.88 (0.63–1.24)	0.68 (0.54–0.85)	0.65 (0.47–0.88)	0.77 (0.48–1.24)
Gymnastics[a]	Ref	2.39 (0.50–11.3)	0.22 (0.07–0.68)	0.80 (0.11–5.92)	21.57 (1.93–241.35)
Ice hockey[a]	Ref	1.07 (0.08–13.89)	0.52 (0.14–1.98)	0.56 (0.09–3.59)	18.50 (1.88–181.74)
Lacrosse[a]	Ref	0.60 (0.12–2.99)	0.71 (0.30–1.68)	0.07 (0.02–0.25)	0.44 (0.13–1.52)
Rugby[c]	Ref	0.56 (0.10–3.06)	0.80 (0.23–2.75)	1.57 (0.05–46.52)	2.71 (0.43–16.92)
Softball[a]	Ref	1.05 (0.60–1.84)	1.39 (0.77–2.51)	0.29 (0.05–1.81)	0.87 (0.12–6.61)
Soccer[a]	Ref	0.78 (0.63–0.97)	0.76 (0.47–1.20)	0.91 (0.64–1.30)	0.71 (0.36–1.38)
Tennis[a]	Ref	***	***	***	***
Track and field[a]	Ref	0.71 (0.05–9.78)	0.62 (0.26–1.47)	0.24 (0.02–2.95)	0.45 (0.12–1.62)
Volleyball[a]	Ref	1.20 (0.57–2.53)	0.57 (0.28–1.17)	0.81 (0.30–2.23)	1.47 (0.54–4.04)
Wrestling[a]	Ref	1.18 (0.66–2.09)	1.16 (0.51–2.66)	0.70 (0.23–2.13)	0.28 (0.05–1.73)

[a]*Adjusted for age, sex, location of injury, and year of ED presentation.*
[b]*Adjusted for age, location of injury, and year of ED presentation.*
[c]*Adjusted for location of injury and year of ED presentation.*
[***]*Unable to calculate due to small number of weighted ED visits.*

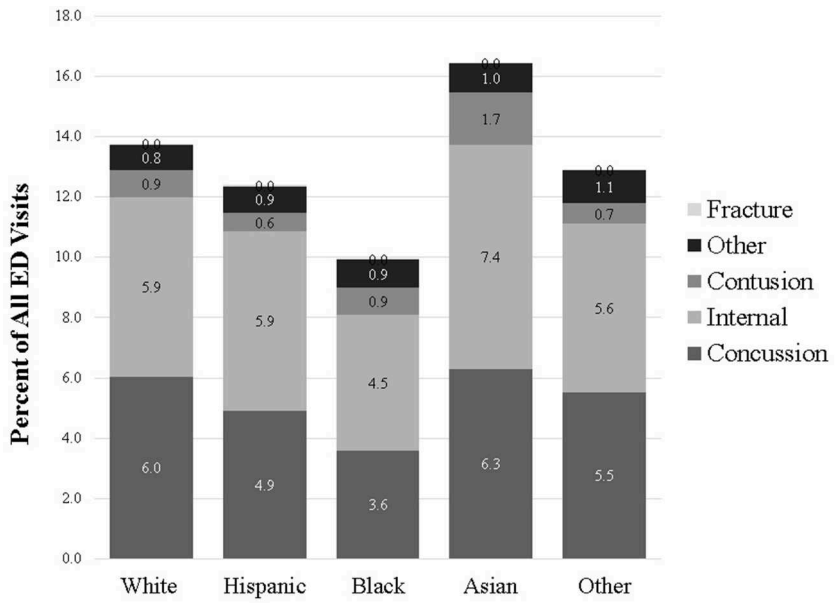

FIGURE 1 | Percent of ED visits for sports-related injuries attributed to head injuries and sub-divided by final diagnosis.

concussions. Specifically, broad diagnoses like head "contusion" or "internal injury" may not signal to athletes, their parents, trainers, and coaches that a concussion has occurred. In the absence of a diagnosis of concussion, these athletes may return to play prematurely, and risk repeat head injury before the brain has had adequate time to recover. Conversely, if over diagnosed, children with concussion could be inappropriately held out of play. Therefore, clinicians must ensure that all athletes receive the appropriate diagnosis and return to play instructions, regardless of race.

The findings of our study must be interpreted in the context of its limitations. First, a significant proportion of children in our study did not report race. However, we found no significant association between not reporting race and having an ED visit be for a head injury or concussion. Second, we only evaluated care in the ED, meaning our findings may not be generalizable to

other care settings. Prior work has suggested that only evaluating ED care for concussion may significantly underestimate the incidence of pediatric head trauma (25). Third, we had limited clinical data on children in our analysis and could not assess what clinical factors contributed to the diagnosis of concussion. However, it is unlikely that patterns of head injuries vary by race, after adjustment for age, gender, sport, year, and location where the injury occurred. Fourth, we are unable to assess if sports-related head injuries occurred in the context of organized or recreational sport participation. Last, some of the observed differences in the diagnosis of concussion among athletes with injury to the head region may reflect race-related differences in case-mix. However, the observed difference in our study was driven by an increased proportion of head-injured black children being diagnosed with "internal injuries," "contusion," or "other." While it is possible these differences may represent an increased incidence of intracranial hemorrhages in head-injured black children, these diagnoses in sports are rare and unlikely to vary to by race or ethnicity. Therefore, the observed disparities likely represent differences in how the diagnosis of concussion is given to head-injured athletes in the absence of other more significant clinical diagnoses.

In conclusion, we identified race-based differences in ED utilization for injuries to the head region and injuries diagnosed as concussions. We also found race-based differences in the diagnosis of concussion among young athletes who presented to the ED with injuries to their head region. Further work is needed to understand factors leading to these observed differences to help ensure that all head-injured athletes receive optimal care, regardless of race.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of our hospital with a waiver of informed consent, given the anonymous nature of the dataset.

AUTHOR CONTRIBUTIONS

TL conceptualized and deigned the study, performed the data analyses, drafted the initial manuscript, and reviewed and revised the manuscript. KM and AM aided in the conceptualization and design of the study, reviewed data analyses, and reviewed and revised the manuscript. RM conceptualization and designed the study, supervised data analyses, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Making Headway for Discussions About Concussions: Experiences of Former High School and Collegiate Student-Athletes

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In order to better understand how to identify and treat student-athletes who experience concussions, better insight into reporting behavior of athletes is needed. This study aims to identify information influencing athletes' attitudes toward reporting their concussions and the perceived trajectory of their recovery both athletically and academically. Twenty-five former high school and collegiate athletes who experienced concussions in a wide variety of sports, organizational levels, and time periods gave insight through structured, qualitative interviews. A number of factors regarding education about concussions, proper diagnosis of concussions, and motivational pressures within high school and collegiate athletics were assessed. Eight major themes were identified regarding the participants' experiences with sport-related concussion: optimism bias, invisibility of the injury, diagnostic barriers, desire to play, external support and pressures, uncertainty of long-term prognosis, generational factors, and protection of future athletes. The findings support that underreporting of concussions among those players interviewed is related to misperceived risk, lack of education, and a struggle between internal and external pressures to play through injury. However, those who did seek medical and academic support, often did receive the necessary aid.

Keywords: concussion, traumatic brain injury, athlete, sports, adolescent

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INTRODUCTION

Despite mandated concussion education programs and increasing media attention to the risks of playing while concussed, many high school and collegiate athletes continue to misrepresent and underreport concussion symptoms (1). Several factors lead to this underreporting behavior. Clinical diagnosis of concussion is highly dependent on self-reported symptoms, including subjective neurological signs such as headaches, confusion, and amnesia. In addition, athletes' current understanding of concussion often does not coincide with the accepted medical definition (2, 3). Motivational issues also contribute to the underreporting of symptoms and continuing to play through injuries (4).

Some have suggested that targeted concussion education may increase reporting, but there is also evidence to suggest that such efforts may not equate to increased reporting; possibly due to some of the reporting motivation issues as previously mentioned (4–8). High school and collegiate athletes continue to have some of the highest rates of unreported concussion (9–11).

But concussive injuries during adolescence and early adulthood can have serious medical repercussions for the developing brain, though most notably in the subacute phase of the injury (12, 13). Although removal from play decisions are of immediate importance post-injury, in the absence of clear guidelines for return to classes, some student athletes may suffer academically as well. Few studies have been done to establish more effective removal/return to play or return to school guidelines (14, 15).

The purpose of this work was to gain information about reasons that they did or did not report their injury and the perceived trajectory of recovery athletically and academically. The sample was not limited to those that were more recent athletes, allowing analysis of the effect of increasing media attention and concussion education efforts on recent experiences and comparison to older individuals. The goal of this work is to enhance the knowledge of these factors in order to design effective educational interventions that will increase reporting behavior for these athletes and to establish more effective guidelines for supporting a concussed student athlete during their recovery process.

METHODS

Design

A retrospective, survey study was performed and individual, face-to-face, structured interviews were conducted with former high school and college student athletes that sustained a sports-related concussion while playing organized sports. The interview responses were reviewed to identify key themes. These themes were further analyzed as they may have or have not related to the demographic background of the participants in order to reveal any trends, consistencies, or differences in the athletes' concussion experiences and reporting behaviors.

Participants

Participants were recruited by university email announcements to faculty, staff, and students, and by fliers placed on campus and in local coffee shops and gyms. Inclusion criteria consisted of English-speaking, over the age of 18, having participated in organized athletics during their high school and collegiate career and having sustained one or more sport-related concussions through this participation. Additionally, the former athletes must have access to an email account for initial contact and scheduling purposes. Exclusion criteria was a history of severe head trauma or neurologic disease (e.g., headache disorder/migraines, mood disorder, epilepsy/seizure disorder, etc.) prior to the experience of sports related concussion. The study staff explained the study procedures, risks, benefits, and alternatives to participation, and the participants signed an informed consent document. This protocol was approved by the Institutional Review Board (IRB#16-04937-XP).

Procedures

All participants were interviewed face to face with a single examiner in an office suite. Interview questions were read verbatim by the interviewer and are provided in **Table 1**. Individual interviews' content validity was established at the end of each session. Then, the interviewer verbally summarized the subjects' comments. Subjects were allowed to clarify any comments and to add any further thoughts on their concussion experience. Each interview was recorded by an audiotape recorder by the interviewer. All audiotapes were transcribed and verified by a second member of the study staff.

Analysis

The data analysis team followed the method that is described in the "Long Table Approach" (15). Briefly, 6 of the authors individually read the transcripts multiple times and summarized the main points and key quotes from each interview. From these items, individual team members identified themes in the data. Then the team met as a group, debated and provided evidence for each of the identified themes until a consensus was reached. From this iterative process, the research team developed major and minor overarching themes and recommendations for factors to be considered when designing future guidelines for student athletes experiencing a concussion.

RESULTS

Demographic Characteristics of the Study Participants

The study cohort included 25 former high school and college athletes. Demographics of these participants are described in **Table 2**.

Eight major themes were identified regarding the participants' experiences with sport-related concussion: optimism bias, invisibility of the injury, diagnostic barriers, desire to play, external support and pressures, uncertainty of long-term prognosis, generational factors, and protection of future athletes. A list of these themes can be found in **Table 3**, and each of these is described below.

Optimism Bias

Participants expressed varying levels of understanding regarding the chances of experiencing a sports-related concussion. However, those who were familiar with the risk of concussion displayed an ignorant awareness, with numerous athletes stating that they did not believe that they themselves would sustain a concussion: "It just happened out of nowhere… I never thought that it would happen to me."

Eighty-four percent (21/25) of subjects felt that concussions were not preventable, often explaining that their own experiences were out of their control: "My particular accident was a pretty random accident, and there wasn't anything necessarily that could have been done."

TABLE 1 | Structured interview script.

The first set of questions ask you to describe the circumstances of your concussion. If you have had multiple concussions, please answer the questions for each instance of injury.

1. Please tell me about the type of athletics you participated in, how it was organized by your school (such as club or varsity athletics) and the sport or sports you were playing when you experienced a concussion.
2. Please tell me general details about the size, location, and athletic division ranking of the school that sponsored these athletic activities, but do NOT tell me the name of the specific school.
3. About how old were you when you experienced your concussion or concussions? What year or years were you in school?
4. How long has it been since then?
5. How old were you when you first started playing this sport?
6. Prior to your concussion in high school or college, were you aware of the risk of head injury in playing your particular sport or sports?
7. Would you rate your particular sport or sports as being high risk, medium risk, or low risk for head injury?
8. What education have you received regarding concussions? Would you say that this education has come from your school or schools in general, your coaches, your parents, the media, or some other source?
9. Do you feel that there is anything that could have prevented your concussion (such as different safety equipment, more or less intense practices, etc.)?
10. What were the circumstances of your injury?
11. How was your injury discovered? Did you lose consciousness?
12. What other kinds of symptoms do you remember having? How long did these symptoms last, and how bothersome were they in your daily life?
13. How were you evaluated immediately after your injury?
14. Did you receive any medical care or other special evaluation in the days following the injury?
15. Did you receive any additional information about concussion in the days following the injury?
16. If you reported the event, did you feel comfortable going to your coach or trainer? Would you say that your teammates, or others in your sport felt the same way?

The next set of questions ask you to describe the recovery after your concussion.

17. When were you able to return to play after your injury? Were there any restrictions once you were allowed to return to practice or competition?
18. Did you feel that there were any repercussions from your coach or trainer because of your injury? If so, please describe.
19. Were you concerned about repercussions for athletic or academic scholarships because of your injury? In what way?
20. How long do you feel that full recovery took?
21. What changes did you, your professors or teachers, or school administrators have to make to ensure your academic success during the recovery period?
22. Were there any other changes you had to make in your daily life in the time right after your concussion?
23. What kind of social support network did you have during your recovery? Who were the members of that network? What did this network specifically help you with during the recovery period?
24. Did you have to withdraw or drop any courses related to your injury?
25. Did you feel that your academics were affected by your concussion? If so, how were they affected?
26. Do you have any lasting effects now that you feel are due to your concussion or concussions? Would you say that these are problems that you have daily, occasionally, or rarely?

(Continued)

TABLE 1 | Continued

The first set of questions ask you to describe the circumstances of your concussion. If you have had multiple concussions, please answer the questions for each instance of injury.

27. Have you ever been diagnosed with post-concussion syndrome or any other health issues related to your concussion?
28. Do you feel that the current media attention surrounding concussion (such as the Concussion movie featuring Will Smith, and litigation in NCAA and NFL athletes) has changed any of your perceptions or feelings about your past injury? In what way?
29. Do you have any other thoughts about sports-related concussion that you would like to share with us?

Before we leave today, I am going to briefly summarize what I feel are the most important themes of your comments. Please stop me or correct me if I have misunderstood anything that you have said. [Insert summary of the subject's comments and allow for subject agreement].

TABLE 2 | Characteristics of the sample.

Characteristic	Category	*n*
Gender	Male	21
	Female	4
Time since concussion	< 10 Years	13
	> 10 Years	12
Sport played	Football	8
	Basketball	5
	Soccer	4
	Softball/Baseball	4
	Rugby	3
	Cheerleading	1
	Polo	1
	Running	1
	Roller derby	1
	Wrestling	1
Type of school	High school	13
	College	14
	Other	2
Organizational level	High school	13
	Junior varsity	3
	Varsity	8
	Collegiate	17
	Intramural/Club	6
	Junior college	1
	Division I	1
	Division II	5
	Division III	4

N.B. Subtotals of individual categories may not add up to 25, as some participants may have reported concussions during involvement in multiple sports, at multiple ages, or in multiple organizational levels.

Invisibility of the Injury

Student athletes often reported that because of the lack of visible signs or symptoms, it was easy for the concussion to not be recognized not only in diagnosis, but also in the post-injury recovery setting: "It's because it is such an invisible injury. You know I didn't have a cast. I didn't have a broken leg."

TABLE 3 | Interview response themes and supporting quotations.

- **Optimism bias**
  - *"It just happened out of nowhere… I never thought that it would happen to me."*
  - *"My particular accident was a pretty random accident."*
- **Invisibility of the injury**
  - *"You know I didn't have a cast. I didn't have a broken leg."*
- **Desire to play**
  - *"I wanted to be out there every single play, regardless of how I felt."*
- **Diagnostic barriers**
  - *"My coaches knew to ask me repetitively, "Are you okay?" and I just didn't know the correct answer. I just didn't know to say "No." I just felt like I had to say "Yes.""*
  - *"You just got hit in the head, so you are not thinking real clearly. You need other people to watch out for you."*
- **External support and pressures**
  - *"I almost felt like it was held against me-that I had to miss a week because I had a concussion. It wasn't my fault."*
  - *"The teachers and professors were very helpful…they gave me a good program to get me back in."*
- **Uncertainty of long-term prognosis**
  - *"I don't think we thought much about head injuries or being turned into a vegetable or something from it."*
  - *"It would be useful to make it more clear like we do for smoking, like what is the risk…they give you a very vivid picture of the down side, and I don't feel like you get that at all…"*
- **Generational factors**
  - *"I'm kind of jealous of the medical care that players get now compared to what my era of players got. So, I am really jealous about…what kind of impact that might have had on my life."*
  - *"It was "Rub some dirt on it, get back out there, let's go!""*
  - *"I felt okay going to the trainer because that's what he's there for."*
- **Protection of future athletes**
  - *"Some of the things that happened to me, I don't want them to happen to other athletes."*
  - *"I think the more restrictions put on it, the better."*

This lack of a visual indicator for injury led many participants to describe circumstances where coaches and peers did not fully comprehend the severity of the concussion. These circumstances led to feelings of isolation. One participant said "My coaches didn't understand, my trainer didn't understand, my teammates certainly didn't understand. They don't know anything about head injuries…I felt like I was on my own."

Desire to Play

Some athletes utilized the invisibility of concussion to allow them to participate in sports while injured, as depicted by this remark: "I didn't really have any limitations because I was silent about [my concussion]."

Participants shared feelings that they were unaware of the seriousness of their injury or simply did not care about the consequences of continuing to play. One commented "I was asked if I was all right, and I just said I was, and did not say anything further."

Other justifications for not reporting concussion symptoms included wanting to keep their starting role and sensing an obligation as a team player, depicted by this response: "There is this internal pressure…because I didn't want to let my team down… I wanted to be out there every single play, regardless of how I felt."

Diagnostic Barriers

As previously mentioned, concussions pose unique barriers for diagnosis. If a head trauma is not witnessed or if no loss of consciousness takes place, recognition and diagnosis of a concussion is reliant only on an athlete's self-reported symptoms. Many athletes often did not recognize their symptoms and continued to play: "I could tell something was wrong because I couldn't remember the play…I didn't know what to do. I had to ask a teammate what I did on each play."

Many symptoms that participants reported (such as headaches, fatigue, or light sensitivity) can be difficult to recognize by coaches or medical staff. Several former student athletes stressed the importance of someone else recognizing their concussion, as described by the following response: "You just got hit in the head, so you are not thinking real clearly. You need other people to watch out for you."

External Support and Pressure

Participants gave a variety of responses in terms of describing external support and pressure in sharing their symptoms after experiencing a concussion. Some described a continued lack of understanding from coaches. One participant stated, "I almost felt like it was held against me-that I had to miss a week because I had a concussion. It wasn't my fault." Even with athletic staff who recognized a concussive injury, one participant shared their internal struggle to balance pressures from coaches: "My coaches knew to ask me repetitively, 'Are you okay?' and I just didn't know the correct answer. I just didn't know to say 'No.' I just felt like I had to say 'Yes'." Also, a sense of comradery among teammates led to athletes playing through injury both in support of their peers, as well as out of fear of judgment from their peers if they were to sit out.

The former student athletes did consistently report that they received many academic accommodations after reporting their concussion, whether it meant missing class, delaying tests, or even receiving one-on-one tutoring. One participant describes their academic experience after suffering a concussion: "The teachers and professors were very helpful…they gave me a good program to get me back in."

Uncertainty of Long-Term Effects

Former athletes shared their concern about the increased media attention for concussions, and the potential for more lasting effects post-concussion. The recent studies on NFL football players have often been highlighted (16), although there may be dangers in overstating some of these claims (17). One person said, "You know we got injured all the time in football, but I don't think we thought much about head injuries or being turned into a vegetable or something from it." Another participant alluded to the greater certainty of risk with other disease processes and behaviors "It would be useful to make it more clear like we do for smoking, like what is the risk…they give you a very vivid picture of the down side, and I don't feel like you get that at all, especially if you are doing it at my level." While many participants were very competitive in high school and collegiate athletics, it is currently unclear what the actual risk of long-term sequelae is for them.

Many interviewees voiced discontent with the knowledge of their coaches and medical staff when they were playing. One

participant stated the following: "When they say, 'You got a concussion,' you just shook it off. You just got up and you got going. The medical care was very poor and the knowledge of the long-term was nonexistent."

Generational Factors

Interviewees displayed a dichotomy in responses between those that experienced their concussion greater than 10 years ago, and those that experienced their sports-related concussion within the past 10 years. Twelve of the 25 interviewees sustained a concussion over 10 years ago, with 5 having sustained their concussions more than 20 years ago. Common trends for these older individuals suggested a general lack of education and awareness regarding concussion risks. One interviewee, who sustained a sports-related concussion over 20 years ago stated, "I'm kind of jealous of the medical care that players get now compared to what my era of players got. So, I am really jealous about…what kind of impact that might have had on my life." These injured athletes returned to play almost immediately, with very few mentioning any type of restrictions. One older participant explained, "It was 'Rub some dirt on it, get back out there, let's go'!"

In comparison, players who sustained a concussion within the last decade reported a more positive outlook regarding these same questions. Younger participants mentioned receiving education on concussions prior to injury, as well as feeling more comfortable reporting the injury to athletic staff members. One participant commented, "I felt okay going to the trainer because that's what he's there for." Athletes also described receiving formal evaluations from athletic trainers and/or physicians following the injury, and underwent a more progressive, delayed return to play.

Protection of Future Athletes

The majority of participants across all age groups expressed support for the strategies being implemented to address concussive injuries. Several individuals reflected on their own injuries when discussing the protection of future athletes, such as one interviewee who commented: "Some of the things that happened to me, I don't want them to happen to other athletes, and having the media attention… and the litigations that are happening I think have helped educate many parents, coaches, trainers, everybody that is involved within organized sports, to be more aware and attentive to what needs to be done."

Various participants shared feelings of concern for their own children playing certain sports that they judged to be at high risk for concussion, such as the parent who said, "I will probably never let my kids play football." Other former athletes advocated for more stringent concussion-prevention protocols and rules, with one person stating, "I think the more restrictions put on it, the better."

DISCUSSION

Prior qualitative studies have explored student-athletes' education regarding concussion, past or present personal experiences of concussion, and support of concussed individuals as they return to school. From this work, it becomes very apparent that healthcare providers, coaches, athletic trainers, teachers, and parents should learn more about concussions, understand that they can occur in all sports, and recognize signs or symptoms of injury (18). The current study is consistent with, but adds to, this prior literature. The themes identified reveal that multiple barriers still exist for athletes to recognize, report, and safely recover from concussions. Overall, participants described several ideas that are amenable to future intervention, leading to better concussion reporting behaviors.

Foremost, educational interventions must be done to help athletes recognize a concussion and its associated symptoms. Even though the participants were responding to a call for individuals that had experienced concussion, there was still confusion on what constituted such an injury. One participant asked "When you say concussion what's the definition? Are you talking about where I black out and had to be woken up? Or are you talking about bell-ringers? What is the definition of a concussion?" This lack of clarity occurs within the context of the diversity of concussion symptoms (including sleepiness, headache, photosensitivity, and short-term memory loss). Often, no two concussions are exactly alike, even when experienced by the same athlete. Until there is a truly objective and accurate assessment method (such as a concrete and measurable biomarker), medical personnel remain dependent on reported symptoms.

Concussion diagnosis remains subjective, and so there must be self-advocacy of the athlete to get the care they require. Beyond being able to recognize appropriate symptoms of concussion, athletic training staff and coaches must remain approachable and supportive for an athlete to report an injury that may have not been witnessed. Ultimately, the burden of reporting currently still falls to the athlete, not only during the acute period, but also for seeking out appropriate medical follow up. Nearly all participants stated that they did not receive additional medical care or education regarding concussion in the days following injury. The participants who did seek medical attention reported receiving a thorough work up, one which included radiological imaging and observational stays in the hospital. Of note, some younger athletes did refer to new protocols in place that included re-evaluation by medical professionals periodically after their initial assessment at the time of injury.

In the area of academics, student-athletes reported concussions to have little impact on their academics once certain accommodations were made. School professionals see other barriers. For instance, school nurses and athletic trainers report that legislative efforts supported their management of return-to-play and return-to-school, but barriers still included lack of educator knowledge and inconsistent care from physicians (19), as well as established social norms for recognition and underreporting of concussions (20). Still, none of the interviewees provided any indication of educational staff opposition to academic workload modifications. Only one athlete reported a significant negative impact on their academic performance even after modifications were made. Currently, there is no legislative enforcement for clearance in returning to classwork.

Overall, there still remains a clear barrier in that many athletes are simply not motivated to report symptoms of concussion.

When educational efforts about concussion safety are directed at the athletes themselves, one study reported an increased desire to avoid collisions, but behaviors for reporting concussions that occur may still be unchanged (21). Indeed, many athletes want to play despite any potential cost to their own health, an attitude which creates ethical conflicts between the needs of the player, the needs of the team, and the goals of the medical community (22). Kuhn has shown that participation in some sports, such as football, is a risk factor for not disclosing injury (23). In certain sports, there can be a cultural trend to "tough it out." Another factor driving this desire to play is the comradery felt among teammates, and continued play may happen to avoid being isolated from this social structure (24). Adolescence is commonly noted for increased peer influence, especially regarding risk-taking behaviors, however, other studies demonstrate peer influence on positive behaviors as well (25, 26). Future interventions would be wise to capitalize on this relationship in order to increase reporting behavior, perhaps by focusing on the safety of the team or peer-group as a whole. Healthcare providers could advocate for this developmental consideration to be made in educational efforts. Perhaps a push for "buddy-system" type efforts could increase reporting compliance. Such a peer-based approach could aid in targeting the motivational struggle in reporting concussions, and over time improve social customs that could lead to safer behaviors.

Regarding limitations of the study, despite efforts to recruit off-campus, many participants had university affiliations and higher educational backgrounds which limited the diversity for these demographics. Furthermore, the respondents were mostly male (21/25), which limits the generalizability of these findings for female athletes that sustain a concussion. Also, all former athletes experienced concussions during team athletics, so these responses may have limited applicability to individual sports. These features of the recruitment method and sample limit generalizability. One advantage of this method was that it allowed both athletes from club and intramural level sports to be included in this study, which often do not have formal coaches and/or training staff present during activities. These subgroups were included in hopes of creating a diverse array of athletic backgrounds, and they emphasize the barriers of recognizing and treating concussion in these various settings.

As the study is within the qualitative field of research, the themes identified are subject to the bias of those analyzing the interview responses. This bias is somewhat unavoidable. Still, the authors minimized potential bias by repeating analyses with each member of the team, each of whom is familiar with Long Table Approach, and then iteratively and collectively agreeing upon themes that were reflective of subject responses.

There are several avenues of future inquiry that are possible based upon these results, and which could influence effective interventions toward increasing reporting behavior. First, continued inquiries into the motivational aspects of reporting behavior remain relevant, and this study should ideally be repeated with a larger study population, as the current group only had 25 individuals. A larger group would be more representative of a collective concussed population, and, therefore, would be helpful in general education and demographic-specific education, alike. More specifically targeted recruitment (i.e., for a particular sport) would likewise enhance educational efforts. Further studies should be conducted involving: evaluations of the risk-taking behavior of those athletes who continue to play while concussed (despite adequate knowledge of the risks); the nature of coach-athlete relationships and their changing influences on reporting behavior; and the influence of team vs. individual sports on reporting behavior. In addition, there is little investigation regarding appropriate timing for a return to academics for student athletes, nor modifications to academic workload. Ultimately, the search for an objective and definitive biomarker of concussion is ongoing and should be continued (27).

Interventions recommended based on these current findings include: (1) continuing student-athlete education on recognizing concussion symptoms in themselves and their peers, (2) encouraging coaching staff and athletic trainers to create a team culture where athletes feel comfortable reporting their injuries and symptoms after injury, (3) stressing to all members of the athletic community, including athletes, family members, coaches, and training staff the value of player safety over player performance, (4) stressing the importance of requesting academic support after a concussion and (5) continuing research both in barriers and facilitators of reporting concussion symptoms as well as objective measures, such as biomarkers.

Many former athletes were aware of the potential for concussions in athletics, yet they felt that such injuries would still not happen to them. Once a concussion did occur, many athletes did not report their injury due to lack of education regarding concussions, an internal drive to play and/or external pressure to play by coaching staff. From the individuals captured, academic needs were met, however, this study included participants from a relatively well-educated background. Participants continued to be plagued by the lack of information regarding long term effects of concussions, and as more information is revealed, many of these former athletes support increased awareness and steps taken to protect future athletes.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of UTHSC Institutional Review Board with written informed consent from all subjects. The study staff explained the study procedures, risks, benefits, and alternatives to participation, and the participants signed an informed consent document in accordance with the Declaration of Helsinki. This protocol was approved by the Institutional Review Board (IRB#16-04937-XP).

AUTHOR CONTRIBUTIONS

JT and AS contributed conception and design of the study. EO performed the structured interviews. AO, EO, SS, MP, KG, and AS performed qualitative analysis. AO wrote the first draft of the manuscript. AO, EO, SS, MP, KG, and AS wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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BRIEF RESEARCH REPORT
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Preliminary Evidence for a Window of Increased Vulnerability to Sustain a Concussion in Females: A Brief Report

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A difference exists between sexes for the incidence of concussion injuries and severity of post-injury outcomes with females having a higher incidence rate (in comparable sports) and experience more robust symptoms than males. The basis for this disparity has remained largely unresolved. Recent findings point to a potential biological mechanism that may be related to the menstrual cycle as an arbiter of post-injury outcomes. What has not been addressed, is whether the phase of menstrual cycle (inferred fluctuations of ovarian hormones) contributes to an increased vulnerability to sustain a concussion injury. This prospective, observational study sought to determine if concussions occurred at different frequencies throughout the phase of the menstrual cycle. Female athletes who sustained a concussion injury were queried three times over the 7-day study (e.g., within 48 h of injury, and 4 and 7 days after injury) to recall the number of days that have elapsed since the beginning of their most recent menstruation. Twenty female athletes enrolled after sustaining a concussion; 18 were eumenorrheic and 2 amenorrheic. Among eumenorrheic participants at the time of injury, 2 were in the follicular phase, 4 were in the early luteal phase and 9 were in the late luteal phase. Two athletes were injured on the first and 1 was injured on the second day of menstruation. The greatest number of concussions were sustained during the late luteal phase and during the first 2 days of menstruation. This 9-day window accounted for 2/3rd of the sustained concussions in our study.

Keywords: menstrual cycle, luteal phase, concussion, brain injury-traumatic, womens health

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INTRODUCTION

A longstanding discussion in the field of concussion injury and management centers on whether a difference exists between sexes for the incidence of injury and severity of post-injury outcomes (1–6). In short, does the athlete's sex contribute to the risk for sustaining a concussion when participating in similar sports, or exaggerate symptoms and recovery following an injury?

From a pure incidence perspective, epidemiological studies report that females (e.g., high school and college-aged athletes) have a higher rate of concussion injuries than males in comparable sports (5, 7). In high school aged girls, the overall concussion rate per 10,000 athlete exposures, defined as one athlete participating in one practice or competition, was 2.64 (95% confidence interval: 2.37, 2.90) compared to 1.69 (1.51, 1.88) in boys competing in similar sports (7). In college-aged athletes, the respective concussion rate per 1,000 athlete exposures was consistently higher in females than males in basketball [e.g., 0.53 (0.43, 0.64) vs. 0.38 (0.29, 0.46)], lacrosse [e.g., 0.45 (0.32, 0.57) vs. 0.30 (0.22, 0.39)], and soccer [e.g., 0.54 (0.43, 0.64) vs. 0.26 (0.17, 0.35)], respectively, (5).

The presentation and range of symptomatic experiences after a concussion are well-documented and include potential impairment to affect (8–11), cognition (12–15), sleep (16–18), vesitbular-occulomotor (19–21), and cardiovascular autonomic function (22, 23). The challenge for clinicians has been a limited ability to predict the type, number, and severity of symptoms that may emerge, and equally important, how long they persist after a concussion. A snapshot of evidence suggests that females are more likely than males to present with reduced performance on visual memory tasks (3), experience more discomfort after injury, and are more likely to seek treatment for post-concussion headaches (24), while other studies have shown no differences in symptom burden between males and females (3, 25–27). But, the question remains as to why an applied mechanical force to the head or body is more likely to cause a concussion or lead to differing post-injury symptoms in females vs. males?

A few recent reports (28, 29) demonstrated that a biological basis for these observations may arise from the menstrual cycle as a potential arbiter for the post-injury symptomatic experience from concussion. In one study (29), women who sustained a mild traumatic brain injury (MTBI) during the luteal phase of their menstrual cycle had a significantly lower quality of life and indicators of health 1 month after discharge than those females who were in the follicular phase of the cycle or were taking hormone contraceptives. In a separate study (28), authors found that males tended to recover from injury more quickly than females and that the initial symptom severity after concussion in male athletes was correlated to the length of recovery. In females, however, the symptom burden after concussion was more closely associated to the use of hormone contraceptives. In other words, females, who were not taking hormone contraceptives at the time of injury experienced more severe symptoms compared to those taking hormone contraceptives. Thus, a differential in effective hormone concentrations at the time of injury partially contributed to the post-injury symptomatic experience in females who were taking hormone contraceptives vs. those who were not. But, what has not been addressed, is whether the inferred changes in circulating hormone concentrations throughout the menstrual cycle, and its respective phases, contribute to a difference in the frequency of concussion injuries that occur in female athletes. The purpose of this study was, therefore, to document the frequency of concussion injuries across the respective phases of the menstrual cycle (e.g., menstruation, follicular phase, early luteal phase, and late luteal phase) in college-age female athletes. Based on the available evidence, we hypothesized that the greatest number of concussions would be sustained during the late luteal phase when hormone concentrations that are speculated to impart neuroprotective effects are anticipated to be on the decline to their monthly nadir.

MATERIALS AND METHODS

A prospective, cross-sectional institutional review board (IRB) approved study, which is part of a larger on-going initiative with separate outcome measures, was performed in female collegiate athletes aged 18–22 years old. A player suspected of sustaining a head injury was evaluated using clinically accepted practices of concussion assessment for both immediate on-field and subsequent office-based follow-up evaluations. Prospective participants were alerted to the existence of the research study through IRB approved means including recruitment flyers and word-of-mouth referrals. Upon initial contact between the researchers and the prospective participant, the study aims and expectations of participating were described. If, at that time, there were any questions, they were addressed. If not, a study visit was scheduled and written informed consent was obtained from all participants prior to initiating the study procedures. To be eligible for study participation, the concussed athlete must have sustained a concussion within the previous 48 h, demonstrate capacity to provide written informed consent, not be taking medications or substances with direct or indirect actions on the cardiovascular or central nervous systems, and be free of acute illness or trauma that would otherwise preclude their participation. During the intake examination at each of 3 study visits occurring within the first post-injury week after sustaining a concussion (e.g., within 48 h of injury/symptom presentation, 4 and 7 days later), participants were asked to indicate the number of days since the beginning of their most recent menstruation. All participants provided the start date of the most recent menstruation after reviewing a calendar, by immediate recall, or in some cases, an athlete referred to an application on her personal smart phone specific to tracking menstrual function/ patterns. Each athlete provided the identical dates for the start of their most recent menstrual cycle. The participants were also asked to provide their age of menarche, use/non-use of hormone contraceptives and characterize their recent menstrual cycle history (e.g., prior 3 months) as being regular (eumenorrhea: ∼1 cycle per month), or irregular (amenorrhea: absence of menstruation; oligomenorrhea: >35 to <90 days between successive cycles). For the purposes of this report, a normal menstrual cycle was defined as 28 days and the phases were standardized: menstruation (0–4 days); follicular phase (5–14 days); early luteal phase (15–21 days); and late luteal phase (22–28 days) (**Figure 1**). As part of the research intake, participants were asked to recall their past concussion history and were evaluated at each visit by the same rater with the Sport Concussion Assessment Tool (SCAT) version 3 (SCAT 3) (30) to characterize their post-injury affect and symptom presentation. Due to the descriptive nature of this report and small sample sizes, statistical analyses were not performed.

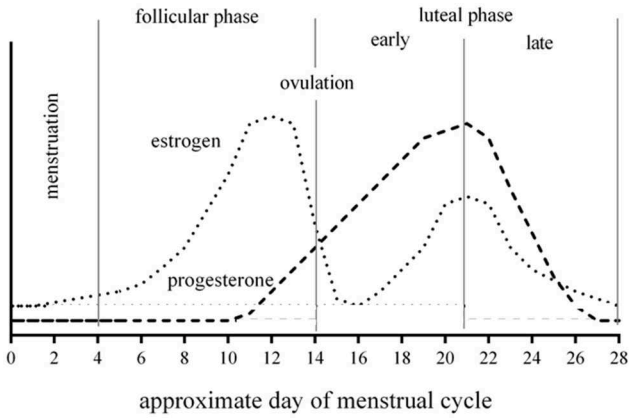

FIGURE 1 | Example 28-day menstrual cycle with changes of ovarian hormones during respective phases.

RESULTS

As of this report, 20 female athletes enrolled in our study after sustaining a concussion; 18 were eumenorrheic and 2 were amenorrheic. Of the eumenorrheic females, 12 participants reported that they did not take hormone contraceptives, while 6 reported that they took oral hormone contraceptives (**Table 1**). All eumenorrheic participants reported having a routine, predictable monthly cycle in the months preceding their enrollment in the study. Two athletes taking hormone contraceptives were found to be amenorrheic; upon further discussion it was revealed that this was a voluntary decision to continuously suppress ovulation. **Table 1** provides information about the participants including the phase of menstrual cycle, use/non-use of hormone contraceptives, number of past diagnosed concussions and the SCAT 3 symptom score for each visit. Specific demographic detail and sports played are not included to maintain the confidentiality of the athletes in this small cohort study.

Upon review of the data, it was revealed that 3 participants sustained their injury on the first or second day of menstruation (1 taking hormone contraceptives), 2 participants were in the follicular phase (1 taking hormone contraceptives), 4 participants were in the early luteal phase (2 taking hormone contraceptives), and 9 participants were in the late luteal phase (2 taking hormone contraceptives) at the time of injury. In total, 9 of 18 (50%) eumenorrheic participants sustained their concussion during a 7-day window characterized by the late luteal phase and no other phase exceeded a 22% injury rate. We hypothesized that the occurrence of concussion injuries would be the greatest when hormone concentrations were expected to be on the decline or were at their monthly nadir. In this context and in consideration for the timing of the concussion injuries early in the menstrual phase, it may be argued that when including these 3 injuries to the 9 from the late luteal phase, 66.7% (12/18 eumoenrrheic athletes) of the concussions occurred during a narrow 9-day window. There was a wide range of early symptom severity, but no emerging trends as to whether the symptom burden was attributable to the phase of menstrual in this small cohort. Similarly, all but 6 participants had a past history of at least 1 diagnosed concussion injury. Because the composition of injury occurrence varied during the menstrual phase in our cohort of concussed female athletes, the use of statistics to evaluate the potential role for past concussion history on our outcomes would be inappropriate. However, as presently constituted, the average number of past concussions for each cohort are provided: late luteal phase = 1.2; menstrual phase = 1.3; early luteal phase = 1.8; and, follicular phase = 2.0.

DISCUSSION

This preliminary observation demonstrates that 50% of concussions experienced in our cohort of college-aged female athletes occurred during a 7-day window characterized by the late luteal phase. If including cases where females sustained their concussion on the first or second day of menstruation, 66.7% of concussions happened during a 9-day window. The significance of this window of time, is that the hormones estrogen and progesterone are declining to, or are at their monthly nadir. For those taking oral hormone contraceptives, this window of time corresponds to when the placebo pill is often taken (or skipped), and as such, there is no exogenous support of circulating hormone concentrations.

The role of menstrual cycle phase at the time of MTBI was explored by Wunderle and colleagues who followed 144 females (16–60 years old) for 1 month after emergency department discharge (29). The investigators found that women who sustained a MTBI during the luteal phase of injury ($n = 37$) had a significantly lower quality of life and indicators of health 1 month after discharge than those females who were in the follicular phase of the cycle ($n = 72$) or were taking hormone contraceptives ($n = 35$). To describe their 1-month post-injury observation of more adverse outcomes in women who sustained a MTBI during the luteal phase despite a presumed increase in circulating progesterone concentrations at the time of injury, Wunderle et al. proposed the "withdrawal hypothesis" (29). Specifically, the hypothesis stated that a "…TBI occurring in the setting of high progesterone results in a sudden decrease of progesterone." The basis for the decline in progesterone among their luteal phase injury participants was not specified, nor was a blood concentration obtained to verify it. However, secondary hypopituitarism has been shown to emerge following MTBI across a wide range of clinical presentations (31–37). In their study cohort, nearly twice as many females who met the inclusion criterion sustained the MTBI during the follicular phase than in the luteal phase, and women taking hormone contraceptives were separated into their own group without consideration for the phase of the cycle that they were in (e.g., where they were in the pill pack), if they had a monthly menstruation or were suppressing menstruation altogether. This latter observation challenges our hypothesis for the presence of a narrow window of increased vulnerability to sustain a concussion during the menstrual phase. However, it should be noted that the

TABLE 1 | Characteristics of study participants.

Subject #	Phase of menstrual cycle upon injury	Use of hormone contraceptives	Age of menarche	# of past diagnosed concussions	SCAT 3 symptom score visit 1	SCAT 3 symptom score visit 2	SCAT 3 symptom score visit 3
1	ELP	No	12	1	48	51	34
2	FP	Yes	13	1	5	0	0
3	MP	Yes	13	2	30	26	13
4	LLP	No	10	3	19	17	16
5	LLP	No	11	1	51	12	0
6	LLP	No	14	0	55	29	10
7	MP	No	12	0	20	0	0
8	FP	No	12	3	18	0	0
9	LLP	No	13	2	13	3	7
10	LLP	No	13	0	36	10	8
11	Amenorrhea	Yes	13	1	41	18	3
12	Amenorrhea	Yes	11	0	29	32	11
13	ELP	No	12	1	23	12	6
14	LLP	Yes	15	0	32	16	23
15	ELP	Yes	13	5	6	4	0
16	MP	No	14	2	36	22	5
17	LLP	No	12	2	65	41	24
18	LLP	No	14	0	8	0	0
19	LLP	Yes	12	3	33	25	26
20	ELP	Yes	12	0	24	12	8

ELP, early luteal phases; FP, follicular phase; LLP, late luteal phase; MP, menstrual phase; SCAT, sport concussion assessment tool.

etiology for the MTBI in their participants was not disclosed (e.g., motor vehicle accident, fall, occupational, sports, violence, etc.). And, unlike organized sporting activities where any suspected head trauma is promptly evaluated by a qualified healthcare professional within the university setting, the experience of an injury occurring under free-living conditions in the community may result in individuals not seeking medical attention, or going to a provider/facility other than where the research study was performed. As a result, there are challenges in comparing the inclusive findings to that from the Wunderle study.

In a report by Gallagher et al. entitled, "The Effects of Sex Differences and Hormonal Contraception on Outcomes after Collegiate Sports-Related Concussion," the authors demonstrated that males tended to recover from injury more quickly than females and that the initial symptom severity after concussion in male athletes was correlated to the length of recovery (28). In females, however, the symptom burden after concussion was more closely associated to the use of hormone contraceptives such that females who were not taking hormone contraceptives at the time of injury experienced more severe symptoms compared to those taking hormone contraceptives. The post-injury consequences of concussion injuries on menstrual cycle dysregulation were recently highlighted in an article by Snook et al. (38) entitled "Association of Concussion with abnormal menstrual patterns in adolescent and young women." The authors demonstrated that ∼1 in 4 females (12–21 years old) with concussion experienced at least 2 abnormal menstrual patterns within the first 120 days after injury, compared to only 5% of participants with non-head sport-related orthopedic injury; this disparity contributed to a significantly elevated odds ratio of 5.85 (95% confidence interval: 1.61–21.22) for the occurrence of menstrual dysfunction after concussion injury. The collection of evidence highlights an interesting inter-related paradigm: hormones that regulate the menstrual cycle and their circulating concentrations at the time of head injury appear to influence the post-injury symptomatic experience; a head injury may influence the post-injury secretory patterns of hormones that regulate the menstrual cycle leading to an increased risk of dysfunction in subsequent months; and, with our findings, concussion injuries appear to occur more frequently during the phase of the menstrual cycle commonly associated with declining or low estrogen and progesterone concentrations.

In addition to having a very specific and potent effect on the uterus, ovarian hormones (e.g., estrogen and progesterone) are demonstrated to exert neuroprotective effects (39–42). Estrogen and progesterone-mediated neuroprotection is thought to be related to their effects on hormone receptors, direct antioxidant effects, effects on astrocytes and microglia, modulation of the inflammatory response to injury, and effects on mediating glutamate excitotoxicity, among others (40–42). Neuroprotection by testosterone has also been explored, and appears to be related to its action on androgen receptors (43–45). However, neuroprotection by testosterone has been suggested to be difficult to directly study because the hormone can aromatize to estrogen through the action of enzymes (46). Because testosterone concentrations in females are generally about 10% or less than

that of males, the focus of hormonally-mediated neuroprotection will focus on estrogens and progesterone. The luteal phase of the ovarian cycle is characterized by two distinct patterns of change in estrogen and progesterone concentrations; in the early phase after ovulation, these hormones ascend to their peak concentration (during the phase), and then steadily decline through the late phase until the initiation of menstruation where they stay low for the first few days (estrogen) or ∼10 days (progesterone).

If we appreciate the time-course of progesterone secretion as part of the normal menstrual cycle and our 66.7% incidence rate of concussion during the late luteal phase and early menstruation, it stands within reason to expect that progesterone concentrations would continue to fall or remain low for an ∼2–3-week window after injury. Under these circumstances, the ability to mobilize and exploit neuroprotection (40–42) or repair via increasing estrogen or progesterone concentrations may be constrained because of the intrinsic secretory patterns that regulate the menstrual cycle or the potential for hypothalamic-pituitary-gonadal axis impairment following concussion. For those taking oral hormone contraceptives, the initiation of the next cycle of pills would exogenously support hormone concentrations, and perhaps facilitate changes to ameliorate symptoms. As such, these circumstances and the potential role(s) of ovarian hormones in recovery from injury may have contributed to the protracted symptoms and reductions to quality of life and health described by Wunderle et al. (29). This position may also assist in explaining the past observation of females who were not taking hormone contraceptives at the time of injury experiencing more severe symptoms compared to those taking hormone contraceptives (28).

Caution should be exercised against presuming that increasing concentrations of estrogen and/or progesterone through exogenous supplementation would facilitate healing, or recovery or management of post-injury symptoms, as there is no evidence to suggest that such an intervention would be warranted, unless, of course, the presence of a clinically-significant hormone deficiency has been identified after screening by an endocrinologist. In addition, the synthesis of this collective evidence does not mean that higher ovarian hormones are prophylactic against sustaining a concussion or attenuating the post-injury symptomology because we know that injuries can occur at any time. At this time, one can only conclude that that lower hormone concentrations that may be associated with the late luteal phase or early menstruation appears to create a window of vulnerability where females sustain concussion at a greater rate than other intervals during the menstrual cycle.

There are several limitations from this preliminary analysis of an on-going study. First, we are reporting outcomes from a small cohort of female athletes who participated in varsity athletics and sustained an injury at a single site. Second, we are working under the assumption that each eumenorrheic female had a consistent 28-day cycle. Third, we did not obtain blood samples to support the narrative of the potential role for low hormone concentrations. Finally, we relied on the participant's self-reported accounting for the number of days since their last cycle; to minimize bias, we asked each participant to recall the date at each visit without prompting for the information previously provided. There was only 1 participant who sustained a concussion on a transitional day (e.g., last day of one cycle) for the phase of the cycle; in this case, she sustained the injury 14 days since the first day of her most recent menstruation and is described as being in the follicular phase in our dataset.

In conclusion, there appears to be a 9-day window of increased vulnerability to sustain a concussion during the late luteal phase of the menstrual cycle and the first few days of menstruation. This risk may be associated with declining or low concentrations of estrogen and progesterone that could be speculated to reduce neuroprotection, and perhaps, blunt the recovery time from symptoms. Because of the inferred reduction to neuroprotection as a result of the declining hormone concentrations during these phases of the menstrual cycle, we speculate that females participating in sporting activities during this epoch of time may be more vulnerable to sustain a concussion injury when exposed to a diverse magnitude of applied mechanical forces to the head and/or body compared to other phases where circulating hormone concentrations may be higher and impart greater neuroprotection. Whether or not these hormonal changes contribute to the differences in concussion between sexes previously described for the incidence and severity of outcomes remains to be seen. In order for the field to develop a substantive base of knowledge on this topic, a more comprehensive study is needed and should include comparisons of the frequency of injuries across the phases of menstrual cycle to other forms of traumatic sports injury (e.g., soft-tissue tear and/or rupture, fractures, etc.) and what role, if any, past concussion history, or use/type of hormone contraceptive (e.g., oral, implant, injection, etc.) has on the reported outcomes. To facilitate this narrative, stakeholders responsible for clinical surveillance or those engaging in prospective research should consider incorporating the following measurements to their prospective practices and observations:

1. Screen for health/behavior indicators that may negatively impact eumenorrhea,
2. Ask females to disclose their use/non-use of hormone contraceptives during the pre-participation physical examination and alert clinical staff to changes in their medication(s),
3. Ask females who sustain a concussion to disclose the first day of their last menstruation, and,
4. Follow-up with and document menstrual cycle patterns (e.g., time of arrival, length of cycle, changes to flow/discharge of fluids, associated symptoms) for several cycles after a concussion injury.

DATA AVAILABILITY

The data supporting the findings presented in this study are available within the article. Because the primary study is on-going, data will not be shared at this time.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of name of guidelines, name of committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the name of committee.

AUTHOR CONTRIBUTIONS

ML: conceptualization, funding acquisition, formal analysis, and writing original draft. ML, VH-L, AH, CL, and AT: investigation and writing, review and editing, formal approval of final draft. ML and AT: methodology. ML, VH-L, AH, and CL: project administration. ML, VH-L, and AT: supervision.

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A Moderate Blast Exposure Results in Dysregulated Gene Network Activity Related to Cell Death, Survival, Structure, and Metabolism

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Blast exposure is common in military personnel during training and combat operations, yet biological mechanisms related to cell survival and function that coordinate recovery remain poorly understood. This study explored how moderate blast exposure influences gene expression; specifically, gene-network changes following moderate blast exposure. On day 1 (baseline) of a 10-day military training program, blood samples were drawn, and health and demographic information collected. Helmets equipped with bilateral sensors worn throughout training measured overpressure in pounds per square inch (psi). On day 7, some participants experienced moderate blast exposure (peak pressure ≥5 psi). On day 10, 3 days post-exposure, blood was collected and compared to baseline with RNA-sequencing to establish gene expression changes. Based on dysregulation data from RNA-sequencing, followed by top gene networks identified with Ingenuity Pathway Analysis, a subset of genes was validated (NanoString). Five gene networks were dysregulated; specifically, two highly significant networks: (1) Cell Death and Survival (score: 42), including 70 genes, with 50 downregulated and (2) Cell Structure, Function, and Metabolism (score: 41), including 69 genes, with 41 downregulated. Genes related to ubiquitination, including neuronal development and repair: UPF1, RNA Helicase and ATPase (*UPF1*) was upregulated while UPF3 Regulator of Nonsense Transcripts Homolog B (*UPF3B*) was downregulated. Genes related to inflammation were upregulated, including AKT serine/threonine kinase 1 (*AKT1*), a gene coordinating cellular recovery following TBIs. Moderate blast exposure induced significant gene expression changes including gene networks involved in (1) cell death and survival and (2) cellular development and function. The present findings may have implications for understanding blast exposure pathology and subsequent recovery efforts.

Keywords: blast, overpressure, gene expression, RNA-sequencing, NanoString

INTRODUCTION

When an individual is in close proximity to a blast, the resulting overpressure (i.e., shock wave) can cause injury to the brain and/or body (1). The increased use of improvised explosives, sophisticated weaponry, and explosive entry techniques has led to increased risk of blast exposure. Specifically, in military personnel who deployed to recent conflicts of Operation Iraqi Freedom (3) and Operation Enduring Freedom (OEF), an estimated 300,000 service members were exposed to at least one blast from adversary attack (2), and blast overpressure from firing weapons is increasing commensurate with increases in weaponry power. High intensity blast exposure events can damage connective tissues, including the central nervous system, resulting in cerebrovascular damage and blood-brain barrier disruption. Significant overpressure can result in tearing of the long axons of neurons (diffuse axonal injury) leading to the associated deficits and comorbidities of a traumatic brain injury (TBI) (4, 5). Although there is evidence suggesting blast-induced TBI (biTBI) has distinct features from blunt-force or penetrating TBI (6), it is difficult to evaluate the consequences of blast in isolation using human subjects because there is often concomitant blunt force or penetrating TBI when objects are propelled and contact the skull (e.g., shrapnel) or the individual is thrown. These challenges contribute to the relatively poor understanding of the pathophysiologic responses to blast and lack of therapies to treat blast-exposed individuals. Moreover, the response and subsequent recovery from blast exposure represent an important line of research, which remains to be further explored and may elucidate the biological mechanisms associated with blast.

Differential gene expression is reported in a small number of clinical TBI studies (7–10), with few studies relevant to blast TBI (11–13). Gene expression regulation is imperative to appropriate cellular response to external mechanical, environmental, or biological stimuli, and the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) complex is a main transcription factor of these adaptive gene expression changes (14). More specifically, the NF-κB complex is a transcription factor central to numerous cellular pathways influencing cell survival and proliferation, including inflammatory and immune responses, gene activation, and ubiquitination (14). Animal models demonstrate that the NF-κB complex regulates the innate immune response through upregulation of proinflammatory cytokines including tumor necrosis factor (TNF) (15), interleukin 1 (IL-1) (16), and interleukin 6 (IL-6) (17). In addition, mutations and epigenetic changes within the NF-κB pathway have been linked to immune and inflammatory diseases (18). Cytokines are among a number of factors that may activate NF-κB. NF-κB becomes activated when ubiquitin degrades its inhibitory protein, IκK, freeing NF-κB to enter the nucleus and activate gene transcription (17, 19). Study of gene expression changes following blast exposure may elucidate some of these complexities surrounding the roles and relationships of ubiquitin and inflammatory cytokines following blast exposure.

Within clinical studies of TBI, changes in the NF-κB network are reported in a limited number of studies (7–10); however, they have not yet been examined in biTBI. Preclinical studies of blast exposures have demonstrated altered gene expression, including cognitive impairment (20, 21) and immune function (22). Recent work in military training that involves personnel exposure to blast has demonstrated that ubiquitin carboxy-terminal hydrolase-1 (UCH-L1) is weakly correlated with repeated exposure to low-level blast (12), which is consistent with previous work in TBI (23) and blast exposure (11, 24). In particular, Heinzelmann et al. (11) found protein ubiquitination genes (associated with neuronal recovery, central regulator in IPA) to be downregulated in military personnel with chronic symptoms following blast head injury. UCH-L1 is predominately expressed in the neurons and neuroendocrine cells within the brain (25, 26) and is an enzyme responsible for protein degradation, thus providing a role in ubiquitin stability within neurons and maintaining neuronal health (27). In animal models, a mutation in the UCH-L1 gene causing a truncated protein is associated with neurodegeneration, likely due to the buildup of ubiquitin and subsequent lack of protein clearance (28). Given this limited number of clinical studies, this study sought to further examine differential gene expression pathways in a blast-exposed population. The purpose of this study was to examine gene networks involving cell death and survival as well as cell structure, function, and metabolism to investigate the role of these networks specific to biTBI.

MATERIALS AND METHODS

To address the gaps in knowledge surrounding the consequences of exposure to isolated blast, a unique cohort of military personnel engaged in training on advanced techniques for breaching buildings with controlled explosives was utilized. The breaching activities were conducted under close supervision and with personal protective equipment and established safety procedures, eliminating the chance of concomitant blunt-force or penetrating TBI. Moreover, recruiting from a training environment (as opposed to real-world combat) facilitated accurate measurement of isolated blast exposures using helmets equipped with pressure sensors (see "Blast Measurement"). This novel sampling also facilitated a collection of baseline data, including pre-exposure blood draws to support assessment of gene expression changes after blast. During the 2 week training program, some participants ($n = 29$) experienced a moderate blast exposure with peak pressure exceeding 5 pounds per square inch (psi), which exceeded the training range limit of 4 psi and was more than 200% greater than typical exposures measured in such training [e.g., Carr et al. (12)]. The moderate blast exposure was an isolated event, and blast exposures remained $\leq$2 psi on all other training days. These 29 cases were studied for gene expression changes related to cell death and survival as well as cell structure, function, and metabolism from training day 1 to 10. Unbiased RNA-sequencing (RNA-seq) was used to detect dysregulated genes (13). Ingenuity pathway analysis (29) of dysregulated genes was used to identify gene networks, two

TABLE 1 | Demographic and previous explosive exposure of participants exposed to moderate blast.

	Moderate blast ($N = 29$)
Mean age in years (SD)	31.2 (4.4)
Mean Years of Service (SD)	11.2 (4.7)
Number of Prior Explosive Breaches and Artillery Fires, % (no.)	
0–9	20.7% (6)
10–39	34.8% (10)
40–99	17.2% (5)
100–199	20.6% (6)
200–399	6.9% (2)

of which were validated in the present study using NanoString's nCounter system.

Participants

All study protocols were reviewed and approved by the Institutional Review Boards (IRBs) at the Naval Medical Research Center and Walter Reed Army Institute of Research (NMRC#2011.0002; WRAIR#1796) as described in a past publication (12). Prior to study participation, each participant provided informed consent. The parent study from which the present study is drawn was comprised of ($N = 108$) male active-duty military service members who were engaged in 2 week blast training programs (as either a student or instructor). The goal of the course was to teach advanced techniques for explosive breaching, a tactic used to gain access into secured structures. All participants provided demographic and health history data at baseline, as well as blood samples. For the present study, participants ($n = 29$) examined were those who experienced a moderate blast exposure ($\geq$5 psi). These 29 individuals, who provided blood samples at the end of training (day 10), were used in the present study to examine gene expression changes from baseline to 3 days post-moderate blast exposure.

Self-reported data were provided by participants at baseline included demographic, health, and blast-history information. Demographic data included age, military rank, and educational status; health information collected included smoking status and history of TBI (**Table 1**). Previous blast exposure data were also obtained through self-reports on how many blast exposures had been experienced during breaching and artillery fires using the following ordinal scale: 0, 1–9, 10–39, 40–99, 100–199, 200–399, and 400+ blast exposures. Details regarding the surveys used to collect data have been previously described (12).

Blast Measurement

Objective blast data were collected using standard Army combat helmets equipped with bilateral sensors capable of measuring blast parameters greater than a threshold of 0.4 psi on either sensor. Helmets were worn throughout training and the average of the right and left sensors was used as data to approximate levels of explosive blast each participant experienced. The sensitivity of the sensors is based on the technological specifications of the device itself (micro Data Acquisition System, μDAS; Applied Research Associates, Inc., Albuquerque, NM) as well as considerations for signal-to-noise ratios and effects on data interpretation.

Laboratory Methods

Blood Sampling

Blood samples were collected at baseline and at the end of 2 week training, which was 3 days after moderate blast. Blood was collected in PAXgene tubes and stored in a −80°C freezer until the time of batch processing.

RNA-Sequencing

Random fragmentation of complementary deoxyribonucleic acid (cDNA) followed by 5′ and 3′ adapter ligation was used to create a cDNA library. Average fragment length was 150–170 bp. RNA integrity was assessed using Aligent Technologies 2100 Bioanalyzer and the mean value was 8.9 with standard error of 0.05. Samples from 29 participants on day 1 and 10 were sequenced for mRNA using the Illumina HiSeq-2500 Next Generation Sequencing system (Illumina Inc., San Diego, CA). Using this system, we performed RNA-seq to read paired-ends and read 101 bases per each end. Sequencing data used in the study were deposited in the Gene Expression Omnibus (GEO) with GEO ID GSE89866.

Ingenuity Pathway Analysis

Dysregulated genes were further explored using IPA software (build version 389077M, content version 27821452, released 2016-06-14, Qiagen, Redwood City, CA). Two pathways of interest were identified (see section Results for details and **Figures 1**, **2**).

NanoString

A subset of genes examined in RNA-seq data were selected to validate gene expression changes using a direct digital detection system (NanoString Technologies, Seattle, WA). In selecting genes to validate, the extent of dysregulation, biological plausibility, and the position of the protein within the IPA pathway diagrams were considered. Two pathways were identified: one focused on cell death and survival and another focused on basic structure, function, and development. A panel was designed for each pathway to include 50 markers of interest, plus a total of nine reference genes for data normalization (**Tables 2**, **3**). Reference genes met the following criteria: (1) not dysregulated in the RNA-seq data for the same samples; (2) not clearly implicated in traumatic brain injury, blast exposure, or a similar condition; and (4) no published evidence that this was an unstable reference gene in human blood. Probes for the 50 genes of interest and the reference genes were designed and manufactured by NanoString Technologies. NanoString was used to determine the mean copy number of each mRNA probe of interest based on manufacturer's protocol. The standard manufacturer protocol was followed for sample preparation, hybridization, and detection.

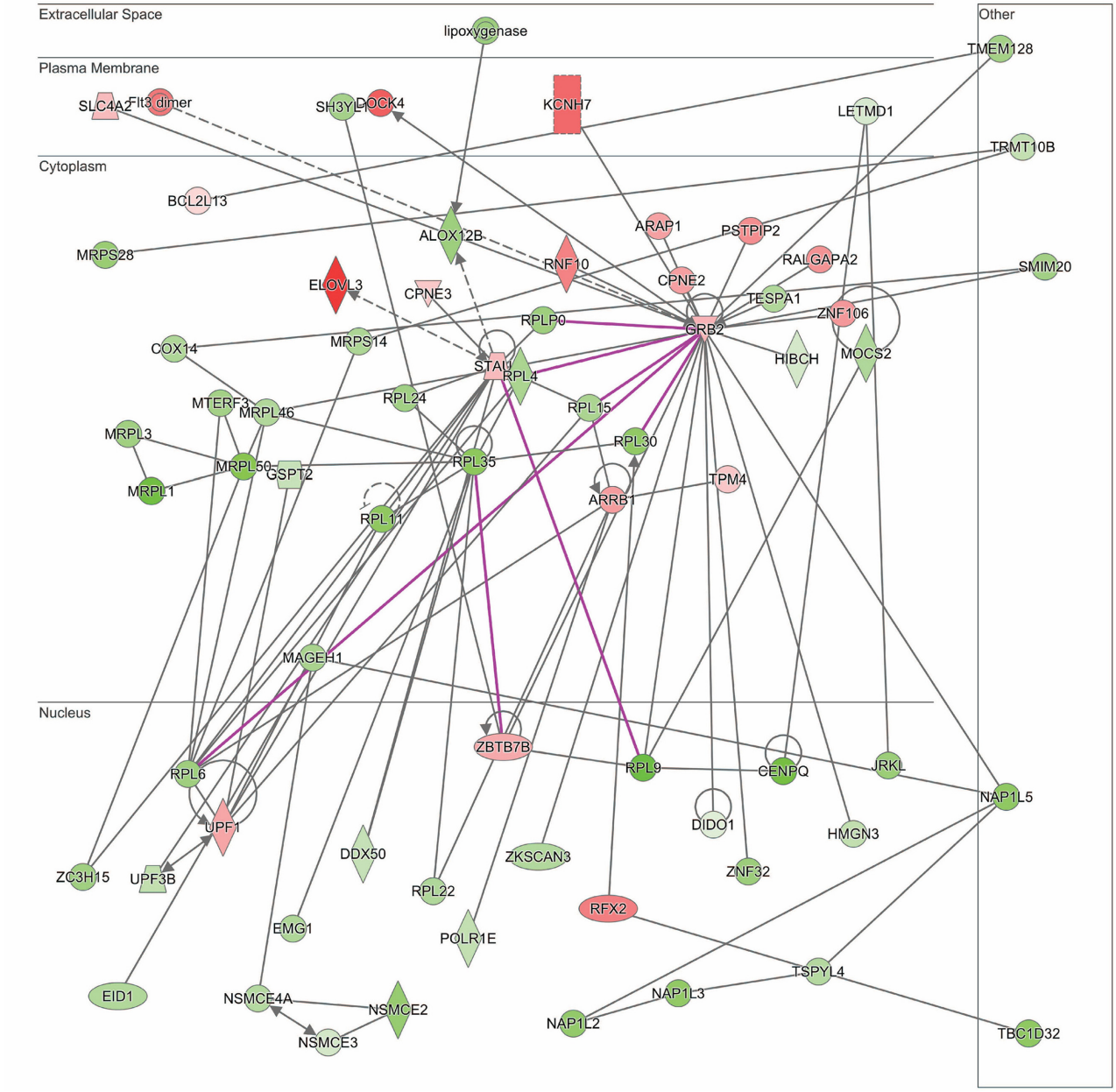

FIGURE 1 | Network shows dysregulated cell death and survival pathway following moderate blast. Genes described in the text included: *UPF1, UPF3B, ARRB1, ZBTB7B, flt3, HIBCH, RPL6, RPL35, MRPL1, MRPL3, MRPL36,* and *MRPL50*. Red indicates increased measurement; green represents decreased measurement; with increased color saturation representing more extreme measurement in dataset. Solid lines represent direct interactions, non-targeting interactions, or correlations between chemicals, proteins, or RNA. Dotted lines represent indirect interactions. Purple lines denote the commonly found relationship among the two merged networks. Arrowed lines represent activation, causation, expression, localization, membership, modification, molecular cleavage, phosphorylation, protein-DNA interactions, protein-RNA interactions, regulation of binding, transcription. Shapes represent molecule type (double circle, complex/group; square, cytokine; diamond, enzyme; inverted triangle, kinase; circle, other; triangle, phosphatase; oval, transcription regulator; trapezoid, transporter). The network was generated through the use of IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).

STATISTICAL ANALYSIS

Overview

The Statistical Package for the Social Sciences (SPSS; version 22; IBM Corporation, Armonk, NY) and NanoString's nSolver Analysis Software (version 3; NanoString Technologies, Seattle, WA) were used for analyses.

RNA-Seq Analysis

The moderate blast exposed cases ($n = 29$) met QC criteria based on the RNA Integrity Number (RIN) and were subsequently

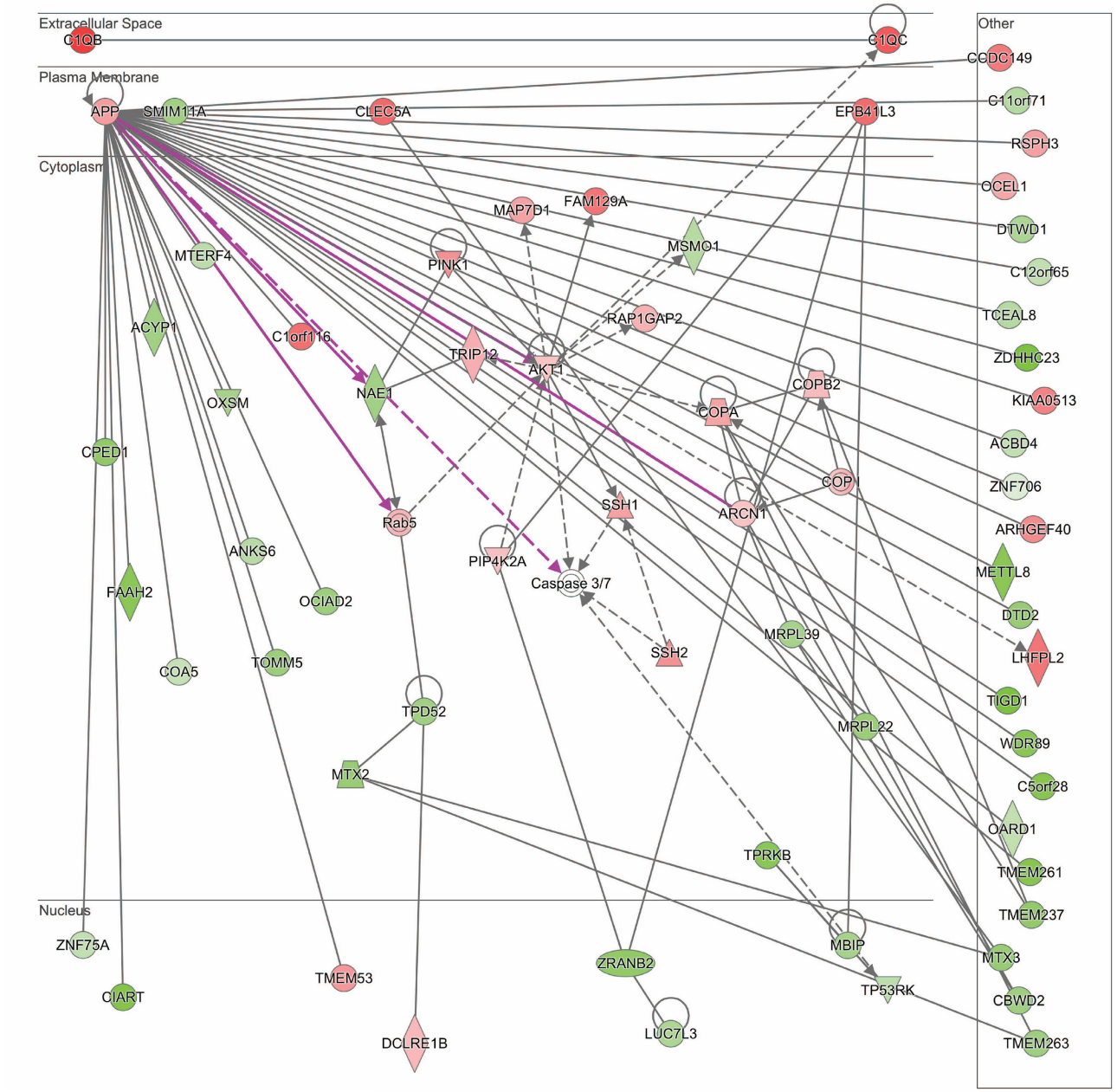

FIGURE 2 | Network shows dysregulated structure, function, and development pathway following moderate blast. Genes described in the text include: *TRIP12, NAE1, AKT1, MBIP, COA5*, and *EPB41L3*. Red indicates increased measurement; green represents decreased measurement; with increased color saturation representing more extreme measurement in dataset. Solid lines represent direct interactions, non-targeting interactions, or correlations between chemicals, proteins, or RNA. Dotted lines represent indirect interactions. Purple lines denote the commonly found relationship among the two merged networks. Arrowed lines represent activation, causation, expression, localization, membership, modification, molecular cleavage, phosphorylation, protein-DNA interactions, protein-RNA interactions, regulation of binding, transcription. Shapes represent molecule type (double circle, complex/group; square, cytokine; diamond, enzyme; inverted triangle, kinase; circle, other; triangle, phosphatase; oval, transcription regulator; trapezoid, transporter). The network was generated through the use of IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).

sequenced. In total, between 52.5 and 75.5 million read counts were completed for each sample; in 94.95% of base calls, an accuracy of at least Q30 was achieved. To establish bioinformatics quality control (QC), FastQC (version 0.11.5, Babraham Bioinformatics, Cambridgeshire, UK) was used. Data were aligned to a reference genome (hg19) using an open-source aligner, STAR (version 2.5) (30). To count the number of reads mapped to genes, HTSeq software was used (version

TABLE 2 | Genes included in the cell death and survival pathway.

Gene symbol	Gene name	Ref seq accession	HKG	log2 fold change	Adjusted *p*-value
RPL9*	Ribosomal Protein L9	NM_000661.4	–	−0.714	0.002
MRPL1*	Mitochondrial Ribosomal Protein L1	NM_020236.3	–	−0.624	0.003
MRPS14*	Mitochondrial Ribosomal Protein S14	NM_022100.1	–	−0.278	0.004
MAGEH1*	MAGE Family Member H1	NM_014061.3	–	−0.295	0.004
BIRC3*	Baculoviral IAP Repeat Containing 3	NM_182962.2	–	−0.507	0.006
ARRB1*	Arrestin Beta 1	NM_004041.3	–	0.279	0.006
TSPYL4*	TSPY Like 4	NM_021648.4	–	−0.247	0.009
TESPA1*	Thymocyte Expressed, Positive Selection Associated 1	NM_001098815.2	–	−0.261	0.010
RPL35*	Ribosomal Protein L35	NM_007209.3	–	−0.431	0.011
MRPL50*	Mitochondrial Ribosomal Protein L50	NM_019051.1	–	−0.461	0.011
MRPL46*	Mitochondrial Ribosomal Protein L46	NM_022163.3	–	−0.261	0.011
ZNF32*	Zinc Finger Protein 32	NM_006973.2	Yes	−0.356	0.013
ZNF106*	Zinc Finger Protein 106	NM_022473.1	–	0.294	0.015
STAU1*	Staufen Double-Stranded RNA Binding Protein 1	NM_017454.2	–	0.193	0.016
TPM4*	Tropomyosin 4	NM_003290.2	–	0.161	0.016
RPL11*	Ribosomal Protein L11	NM_000975.2	–	−0.423	0.017
NSMCE4A*	NSE4 Homolog A, SMC5-SMC6 Complex Component	NM_017615.2	–	−0.244	0.017
RPL30*	Ribosomal Protein L30	NM_000989.2	–	−0.408	0.017
RPL6*	Ribosomal Protein L6	NM_000970.3	–	−0.340	0.018
FLT3*	Fms Related Tyrosine Kinase 3	NM_004119.2	–	0.390	0.019
RNF10*	Ring Finger Protein 10	NM_014868.3	–	0.363	0.020
RPL4*	Ribosomal Protein L4	NM_000968.2	–	−0.290	0.020
ACBD4*	Acyl-CoA Binding Domain Containing 4	NM_024722.2	–	−0.199	0.020
RPL22*	Ribosomal Protein L22	NM_000983.3	–	−0.265	0.021
HMGN3*	High Mobility Group Nucleosomal Binding Domain 3	NM_004242.3	–	−0.201	0.023
RPL15*	Ribosomal Protein L15	NM_001253379.1	–	−0.285	0.023
PGK1	Phosphoglycerate Kinase 1	NM_000291.2	Yes	0.194	0.024
ZBTB7B*	Zinc Finger And BTB Domain Containing 7B	NM_015872.2	–	0.243	0.025
ZC3H15*	Zinc Finger CCCH-Type Containing 15	NM_018471.2	–	−0.335	0.027
TRMT10B	TRNA Methyltransferase 10B	NM_144964.3	–	−0.197	0.027
UPF3B*	UPF3 Regulator Of Nonsense Transcripts Homolog B (Yeast)	NM_080632.2	–	−0.202	0.027
ALAS1	5'-Aminolevulinate Synthase 1	NM_000688.4	Yes	0.243	0.028
RFX2*	Regulatory Factor X2	NM_000635.3	–	0.371	0.029
PSTPIP2*	Proline-Serine-Threonine Phosphatase Interacting Protein 2	NM_024430.3	–	0.327	0.029
MRPL3*	Mitochondrial Ribosomal Protein L3	NM_007208.2	–	−0.330	0.030
ZKSCAN3*	Zinc Finger With KRAB And SCAN Domains 3	NM_001242895.1	–	−0.254	0.031
GUSB	Glucuronidase beta	NM_000181.3	Yes	0.194	0.034
UPF1*	UPF1, RNA Helicase And ATPase	NM_002911.3	–	0.255	0.036
KCNH7*	Potassium Voltage-Gated Channel Subfamily H Member 7	NM_033272.2	–	0.441	0.038
SH3YL1*	SH3 And SYLF Domain Containing 1	NM_001159597.1	–	−0.312	0.038
PARK2*	Parkin RBR E3 Ubiquitin Protein Ligase	NM_004562.2	–	−0.462	0.039
ALOX12B*	Arachidonate 12-Lipoxygenase, 12R Type	NM_001139.2	–	−0.347	0.040
NAP1L2*	Nucleosome Assembly Protein 1 Like 2	NM_021963.3	–	−0.394	0.041
NAP1L3*	Nucleosome Assembly Protein 1 Like 3	NM_004538.4	–	−0.403	0.043
DIDO1*	Death inducer-obliterator 1	NM_001193369.1	–	−0.093	0.045
ARAP1*	ArfGAP With RhoGAP Domain, Ankyrin Repeat And PH Domain 1	NM_001040118.2	–	0.270	0.045
MRPS28*	Mitochondrial Ribosomal Protein S28	NM_014018.2	–	−0.368	0.046
GRB2*	Growth Factor Receptor Bound Protein 2	NM_002086.4	–	0.214	0.048
HIBCH*	3-Hydroxyisobutyryl-CoA Hydrolase	NM_014362.3	–	−0.137	0.049
MOCS2*	Molybdenum Cofactor Synthesis 2	NM_004531.4	–	−0.267	0.049

(Continued)

TABLE 2 | Continued

Gene symbol	Gene name	Ref seq accession	HKG	log2 fold change	Adjusted *p*-value
BCL2L13*	BCL2 Like 13	NM_001270733.1	–	0.180	0.049
NSMCE3*	NSE3 Homolog, SMC5-SMC6 Complex Component	NM_138704.2	–	−0.139	0.070
ALOXE3*	Arachidonate Lipoxygenase 3	NM_001165960.1	–	−0.228	1.000
ABCF1	ATP Binding Cassette Subfamily F Member 1	NM_001090.2	Yes		
DECR1	2,4-Dienoyl-CoA Reductase 1, Mitochondrial	NM_001359.1	Yes		
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	NM_002046.3	Yes		
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	NM_000194.1	Yes		
IPO8	Importin 8	NM_006390.2	Yes		
−93 miR	MicroRNA 93	NR_029510.1	Yes		
TBP	TATA-Box Binding Protein	NM_001172085.1	Yes		

**Validated by NanoString; HKG, house-keeping gene.*

0.6.1p1) (31). DESeq2 (version 1.12.3) (32) was used to identify differentially expressed genes, with cutoff value of false discovery rate (FDR) of 0.05. This cut off value of the false discovery rate is considered to be a conservative balance in minimizing both false positives (type I errors) and false negatives (type II errors) (33, 34).

NanoString Validation Analysis

RNA sequencing data were validated with 50 genes from each network (100 genes total), plus reference genes, using a custom NanoString codeset. Raw data were analyzed using nSolver 3.0 digital analyzer software using standard settings and quality control parameters. Raw data were normalized against reference genes. Gene expression differences before and after blast exposure were measured using *t*-tests, with the Benjamini-Yekutieli FDR method for multiple comparison corrections to conservatively balance the minimization of type I and type II errors (35). Log_2 fold changes and adjusted *p*-values were calculated for samples before and after blast exposure, with statistical significance defined at the level of $p < 0.05$, demonstrating congruency with RNA sequencing findings.

RESULTS

Demographic Results

Participants in the study were male military service members with a mean age of 30.42 and a mean length of service of 9.94 years (**Table 1**). Almost half of participants (46.3%) had a history of >40 prior blast exposures. No significant differences based on demographic information were noted among the cohort [described in previous report by Gill et al. (13)].

RNA-Seq Results

Results of the RNA-seq analysis demonstrated significantly dysregulated gene activity changes following moderate blast exposure. These genes were entered into IPA software (29). IPA analysis identified five significantly dysregulated networks before and after moderate blast exposure (**Table 4**). Two sets of two networks shared overlapping functions and were subsequently merged to form two networks of interest in the present study: (1) Cell Death and Survival and (2) Cell Structure, Function, and Metabolism (**Table 4**). NanoString analysis confirmed 32 significantly differentially expressed genes in the Cell Death and Survival network ($p < 0.05$) and 35 significantly differentially expressed genes in the Cell Structure, Function, and Metabolism network ($p < 0.05$), validating differential expression of these two gene networks following blast exposure. Here, we report on the most significant gene network activity changes in the Cell Death and Survival and the Cell Structure, Function, and Metabolism networks (**Figures 1**, **2**).

Cell Death and Survival Network

One merged pathway centered on cell death and survival. Genes identified in this pathway are defined in **Table 2**. This pathway was comprised of genes implicated in apoptosis, necrosis, autophagy, mitophagy, ferroptosis, survival, regeneration, and recovery, with an IPA score of 42 (**Figure 1**). Significantly dysregulated genes include UPF1, RNA helicase and ATPase *(UPF1),* UPF3 regulator of nonsense transcripts homolog B (*UPF3B*), arrestin β1 (*ARRB1*), zinc finger and BTB domain containing 7B (*ZBTB7B*), fms related tyrosine kinase 3 (*flt3*), 3-hydroxyisobutyryl-CoA hydrolase (*HIBCH*), ribosomal proteins (*RPL6, -L35*), and mitochondrial ribosomal proteins (*MRPL1, -L3, -L36,* and *-L50*). Major hubs within this network include growth factor receptor bound protein 2 (*GRB2*) and staufen double-stranded RNA binding protein 1 (*STAU1*).

Cell Structure, Function, and Metabolism Network

The second merged pathway focused on development, metabolism, and cell structure/function. Genes identified in this pathway are defined in **Table 3**. This pathway consisted of genes involved in cytoskeleton, organelles, cellular metabolism, lipid metabolism, heat shock, cell motion, cell growth, and differentiation, with an IPA score of 41 (**Figure 2**). Significant genes within this network include tripartite motif containing 12 (*TRIP12)*, NEDD8 activating enzyme E1 subunit 1 (*NAE1*), cytochrome C oxidase assembly factor 5 (*COA5*), and erythrocyte membrane protein band 4.1-like 3 (*EPB41L3*). AKT serine/threonine kinase 1 (*AKT1*), amyloid precursor

TABLE 3 | Genes included in the structure, function, and development pathway.

Gene symbol	Gene name	Ref Seq accession	HKG	log2 fold change	Adjusted *p*-value
LHFPL2*	Lipoma HMGIC Fusion Partner-Like 2	NM_005779.2	–	0.401	0.001
TMEM261*	Transmembrane Protein 261	NM_001318058.1	–	−0.465	0.003
TRIP12*	Thyroid Hormone Receptor Interactor 12	NM_004238.1	–	0.231	0.003
ZDHHC23	Zinc Finger CCHC-Type Containing 23	NM_173570.3	–	−0.521	0.004
OCIAD2	Ovarian Carcinoma Immunoreactive Antigen-Like Protein 2	NM_152398.2	–	−0.350	0.005
FAAH2	Fatty Acid Amide Hydrolase 2	NM_174912.3	–	−0.499	0.006
TPRKB*	TP53RK Binding Protein	NM_016058.2	–	−0.443	0.006
EPB41L3*	Erythrocyte Membrane Protein Band 4.1 Like 3	NM_012307.2	–	0.431	0.006
NAE1*	NEDD8 Activating Enzyme E1 Subunit 1	NM_001018159.1	–	−0.324	0.007
SSH1*	Slingshot Protein Phosphatase 1	NM_018984.3	–	0.269	0.010
RAP1GAP2*	RAP1 GTPase Activating Protein 2	NM_015085.4	–	0.219	0.011
C1QB*	Complement Component 1, Q Subcomponent, B Chain	NM_000491.3	–	0.584	0.011
OARD1*	O-Acyl-ADP-Ribose Deacylase 1	NM_145063.2	–	−0.205	0.012
COPB2*	Coatamer Protein Complex Subunit Beta	NM_004766.2	–	0.193	0.012
TIGD1*	Tigger Transposable Element Derived 1	NM_145702.1	–	−0.531	0.012
CLEC5A*	C-Type Lectin Domain Family 5 Member A	NM_013252.2	–	0.437	0.014
KIAA0513*	KIAA0513 Ortholog	NM_014732.3	–	0.361	0.015
MRPL39*	Mitochondrial Ribosomal Protein L39	NM_017446.3	–	−0.304	0.015
DCLRE1B*	DNA Cross-Link Repair 1B	NM_022836.3	–	0.198	0.017
MBIP*	MAP3K12 Binding Inhibitory Protein 1	NM_001144891.1	–	−0.305	0.017
TP53RK*	TP53 Regulating Kinase	NM_033550.3	–	−0.213	0.018
COPA*	Coatamer Protein Complex Subunit Alpha	NM_004371.3	–	0.259	0.019
ACYP1*	Acylphosphatase 1	NM_001107.3	–	−0.329	0.019
ZRANB2*	Zinc Finger RANBP2-Type Containing 2	NM_005455.4	–	−0.404	0.019
MTX2*	Metaxin2	NM_006554.4	–	−0.369	0.020
ABCD4*	ATP Binding Cassette Subfamily D Member 4	NR_003256.2	–	−0.199	0.020
MRPL22*	Mitochondrial Ribosomal Protein L22	NM_014180.3	–	−0.361	0.023
PGK1	Phosphoglycerate Kinase 1	NM_000291.2	Yes	0.194	0.024
SSH2*	Slingshot Protein Phosphatase 2	NM_033389.3	–	0.315	0.024
FAM129A*	Family with sequence similarity 129, member A	NM_052966.2	–	0.403	0.025
C12orf65*	Chromosome 12 open reading frame 65	NM_152269.4	–	−0.204	0.026
ALAS1	5'-Aminolevulinate Synthase 1	NM_000688.4	Yes	0.243	0.028
OXSM*	3-Oxoacyl- Acyl Carrier Protein Synthase, Mitochondrial	NM_017897.2	–	−0.306	0.028
COA5*	Cytochrome C Oxidase Assembly Factor 5	NM_001008215.2	–	−0.188	0.030
ZNF706*	Zinc finger protein 706	NM_001042510.1	–	−0.109	0.031
RSPH3*	Radial Spoke 3 Homolog	NM_031924.4	–	0.272	0.032
TMEM237*	Transmembrane Protein 237	NM_001044385.1	–	−0.394	0.032
MTX3*	Metaxin3	NM_001010891.4	–	−0.370	0.033
PIP4K2A*	Phosphatidylinositol-5-Phosphate 4-Kinase Type 2 Alpha	NM_005028.3	–	0.181	0.033
APP*	Amyloid Precursor Protein	NM_000484.3	–	0.275	0.034
GUSB	Glucuronidase Beta	NM_000181.3	Yes	0.194	0.034
TPD52*	Tumor Protein D52	NM_005079.2	–	−0.330	0.038
CIART*	Circadian Associated Repressor of Transcription	NM_144697.2	–	−0.475	0.038
AKT1*	AKT Serine/Threonine Kinase 1	NM_001014432.1	–	0.170	0.039
MAP7D1*	MAP7 Domain Containing 1	NM_018067.3	–	0.267	0.040
ANKS6*	Ankyrin Repeat and Sterile Alpha Motif Domain Containing 6	NM_173551.3	–	−0.236	0.041
MSMO1*	Methylsterol Monooxygenase 1	NM_001017369.1	–	−0.238	0.041
LUC7L3*	LUC7 Like 3 Pre-MRNA Splicing Factor	NM_006107.2	–	−0.279	0.042
TCEAL8*	Transcription Elongation Factor A Like 8	NM_153333.2	–	−0.243	0.045
TOMM5*	Translocase Of Outer Mitochondrial Membrane 5	NM_001001790.2	–	−0.354	0.046
ARCN1*	Archain 1	NM_001655.4	–	0.171	0.047

(Continued)

TABLE 3 | Continued

Gene symbol	Gene name	Ref Seq accession	HKG	log2 fold change	Adjusted *p*-value
TMEM263	Transmembrane Protein 263	NM_152261.2	–	−0.342	0.049
RAB5A*	RAS-Associated Protein RAB5A	NM_004162.4	–	0.182	0.136
ABCF1	ATP Binding Cassette Subfamily F Member 1	NM_001090.2	Yes		
DECR1	2,4-Dienoyl-CoA Reductase 1	NM_001359.1	Yes		
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	NM_002046.3	Yes		
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	NM_000194.1	Yes		
IPO8	Importin 8	NM_006390.2	Yes		
miR-93		NR_029510.1	Yes		
TBP	TATA-Box Binding Protein	NM_001172085.1	Yes		

**NanoString validation; HKG, house-keeping gene.*

TABLE 4 | IPA Network Scores.

Network	IPA network score
Metabolic	45
Cell death and survival	42
Post-translational modification	42
Cancer, cell death and survival	42
Immunological diseases	37
Merged networks	
Cell death and survival	42
Cell structure, function, and metabolism	41

Network scores are numerical values used to rank fit of molecules to the network. The scores are calculated using an algorithm based on Fisher's Exact Test. Eligible molecules are compared to the Ingenuity Knowledge Base of over 1 million molecules curated from literature findings. Highly interconnected genes imply significant biological function.

protein (*APP*), and MAP3K12 binding inhibitory protein 1 (*MBIP*) are the major hubs in this network.

DISCUSSION

Activity changes in two gene networks were found after moderate blast exposure in military personnel engaged in training. Differentially regulated networks after blast included (1) Cell Death and Survival (**Figure 1**) and (2) Cell Structure, Function, and Development (**Figure 2**). Genes within these two networks relate to ubiquitination, nonsense mediated decay (NMD), apoptosis, as well as activity related to ribosomes, mitochondria, and inflammation. Findings provide novel insights for understanding the biological changes that occur following blast, which for some individuals, may result in biological changes that increase their risk for neurological or behavioral symptoms and deficits. These findings may ultimately contribute to characterizing the cellular mechanisms of blast exposure to improve diagnosis, monitoring, and prognosis of military personnel exposed to blast.

In this study, genes related to ubiquitination were increased in activity following blast exposure, including tripartite motif containing 12 (*TRIP12)*, which is an E3 ubiquitin-protein ligase involved in ubiquitin fusion degradation. Protein ubiquitination initiates the removal of oxidized and misfolded proteins following injury, and its processes can protect neurons from reactive oxidative species (ROS) that accumulate following blast exposure in pre-clinical models (36). Our findings provide further evidence of increased UCH-L1, the primary protein for ubiquitination, following repeated low-level blast (12). This finding suggests that there may also be overlap with the biological mechanisms related to recovery from TBIs in civilians, as UCHL1 increases are one of the most often reported changes following a TBI (37, 38). However, these findings are in contrast to another previous report in which the activity of genes related to ubiquitin were lower in activity in military personnel with TBIs, with many related to blast exposures, and chronic symptoms (11). Therefore, it may be that ubiquitin activity is critical to acute recovery from biTBIs, and that in some individuals, there is a reduction in activity that may place them at higher risk for chronic symptoms. In support of this, pre-clinical studies show that reductions or inactivation of ubiquitin activity results in poor outcomes, including behavioral deficits, possibly indicating long-term neurodegenerative processes (39).

Genes that may relate to neuronal recovery were altered in activity following a moderate blast. Specifically, we report gene activity changes within the NMD pathway, including *UPF1* and *UPF3B*, which are responsible for neuronal specific cell development and repair through a reciprocal pattern of activity (40). Previous studies show an interaction in the activity of these two genes, such that when one gene is less active, the other gene will compensate, preserving the activity of this network; our findings mirror this. Here we report that *UPF1* was increased in activity, whereas *UPF3B* was downregulated. These findings suggest that in response to the blast, injury mechanisms may have been initiated (inflammation, aberrant cellular formation, and cell death), and this initiation may result in an upregulation of *UPF1*, in an effort to preserve the activity of the NMD pathway. Subsequently, here we report that the expression of *UPF3B* is suppressed, hindering possible detrimental neurological effects. These findings suggest complex gene-activity changes following blast exposure that may be occurring to promote recovery; additional studies are needed to increase understanding of the temporal relationship of these changes and their relation to neuronal recovery.

A downregulated gene within the structure, function, and development pathway was *NAE1* (NEDD8 Activating Enzyme E1 Subunit 1), a protein associated with the neddylation pathway. Vogl et al. showed that neddylation was a critical regulator of dendritic spine development, reporting that in *NAE1* knockout mice, there were cognitive deficits as well as synaptic and neurotransmitter impairments (41). The down regulation observed in our military population could suggest similarly that exposure to blast hinders the neddylation pathway, which might suggest a marker of injury resulting directly from blast exposure. Additionally, recent *in vitro* work suggests Il-1β may inhibit NEDD8 and neddylation in conjunction with increased ubiquitination; while activation of NEDD8 downregulates the nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) pathway (42). This finding is of interest because we also found that genes within the NF-κB network show activity changes, with most genes becoming more active. The NF-κB network is a dominant activator of the immune system following TBI, and this activity is essential because it initiates secondary injury mechanisms required for neuronal recovery. However, if activity of this pathway is too high, or too long-lasting, it can be detrimental to neuronal recovery (43). One such gene is *ARRB1*(arrestin β1), which is increased following blast exposure. This gene has been reported to play a role in the beta-adrenergic receptor kinase (BARK) mediated desensitization of beta-adrenergic receptors. In TBI patients, catecholamine surge after injury has been linked to immunosuppression and greater mortality risk that is reversed through β-blocker treatment (44). *ZBTB7B* (zinc finger and BTB domain containing 7B) is also upregulated after blast and linked to reductions in CD8-cytotoxic activity (45), which could be a mechanism to prevent further cellular damage after blast injury.

In addition to the activation of genes within the NF-κB network, *AKT1* is another immune-related gene with increased activity. *AKT1* is a hub with 14 connections in the structure, function, and development network. *AKT1* encodes for a serine-threonine protein kinase (AKT1), which is known to regulate a vast number of cellular processes including neuronal survival, glucose uptake, protein and fatty acid synthesis, cell proliferation, and the previously mentioned role in apoptosis (46, 47). Additionally, *AKT1* may function in the inflammatory response as an upstream activator of NF-κB (48). Interestingly, in this population, significantly elevated levels of the cytokines tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) have been reported during the acute period following moderate blast (49). This finding is relevant because NF-κB is recognized as a master regulator of cytokines including TNFα and IL-6 (47, 50). The NF-κB pathway has been implicated in the regulation of proinflammatory cytokines during meningitis (51) and in blood-brain barrier permeability (52). Moreover, the NF-κB pathway has been found to be dysregulated in clinical studies of acute and subacute TBI (7–10). Upregulation of *AKT1* in this sample suggests activation of the NF-κB pathway; a finding that supports these prior studies, although the specific role of *AKT1* in blast effects on the central nervous system remains to be examined.

Also showing increased activity in this study was the *Flt3* (dimer), which encodes for a receptor tyrosine kinase and is also related to NF-κB pathway. *Flt3* is implicated in multiple signaling pathways including regulation of the proliferation and survival of hematopoietic cells, which ultimately relates to the number of intermediate monocytes (53). Because intermediate monocytes promote production of inflammatory cytokines within the NF-κb network, including TNF-α and Il-1β (54), these findings suggest the possibility of a pro-inflammatory response through increased production of intermediate monocytes.

Our findings of changes in activity of apoptosis-related genes following blast are of interest because preclinical models show that blast exposure results in astrocytic and microglial activation, oxidative stress, axonal and vascular damage, and inflammation, which ultimately contribute to programmed cell-death (55–58). Specifically, we found activation of caspase complexes, a family of cysteine-dependent proteases, which have been previously associated with neuronal and oligo-dendroglial cell death in both pre-clinical and human brain injuries (59). Otherwise referred to as apoptosis executioners, caspase-3 and -7 are both indirectly activated by *MBIP*, a major hub of the cell structure, function, and metabolism network. Increased expression in caspase-3 and -7 complexes have also been previously linked to TBIs in pre-clinical models (60, 61) and to mortality in patients with severe TBIs (62). We also report increased activity of other apoptosis genes following blast including *EPB41L3*, or erythrocyte membrane protein band 4.1-like 3, and *EPB41L3*, a tumor suppressor gene strongly expressed in the brain that promotes apoptotic pathways and inhibits cellular proliferation (63). These findings suggest that moderate blast results in expression of apoptosis inducing genes, and that mitigating these activities may be protective.

Lastly, several mitochondrial genes and genes connected with the mitochondrial gene network are dysregulated, including *COA5, HIBCH, RPL6, RPL35*, as well as mitochondrial ribosomal genes *MRPL50, MRPL1, MRPL3*, and *MRPL46*. Although the function of mitochondria is not yet well-understood in blast exposures, it is worth noting that mitochondrial dysfunction has been implicated in preclinical TBI pathology. Previous studies have indicated that following TBI an influx of intracellular calcium leads to disruption of the mitochondrial membrane potential, impairing ATP production and creating ROS, activating cell death pathways and leading to neuronal damage associated with cognitive impairments (64, 65). The biological mechanisms specific to blast effects on the central nervous system in the context of mitochondrial genes are not yet known.

Alterations in these gene expression pathways may have translational implications for blast-related neuropathology if replicated. In this military training population, dysregulation in gene expression pathways related to ubiquitination, NMD, apoptosis, inflammation, and ribosomal and mitochondrial activity were observed, suggesting a role for these pathways following acute blast exposures. Examination of related, downstream proteins may indicate potential diagnostic or prognostic biomarkers. Mapping temporal changes in gene expression, downstream proteins, and symptomology may shed light on the role of these pathways in underlying neuropathological processes and clinical outcomes.

Limitations

Although these initial findings provide novel insights into gene-activity changes following blast, this study has a number of limitations. First, the secondary data analysis in this study precluded the comparison of control personnel who were engaged in blast training, but did not sustain a moderate blast; therefore, gene expression changes related to normal daily training activities or other activities of daily living such as circadian rhythm or diet cannot be determined. In addition, a sub-portion of the moderate blast cohort were experienced trainers with previous blast exposures, and thus were not naïve to blast. However, this sample represents typical training cohorts. Second, blast exposure may affect cell types throughout the body in addition to brain-related pathways. Preclinical models demonstrate that the CNS is affected by blast exposure (66, 67); however, at this time translating this information to clinical populations presents difficulty as accessing the CNS requires intensive, invasive procedures. Third, due to the exploratory nature of this work, clinical symptomology measures were not collected.

These limitations represent important considerations for future work. Additional studies in clinical blast exposure will need to collect samples from unaffected control cohorts, not only a priori matched samples, in order to differentiate possible causative and confounding agents such as blast exposure vs. training effects. We are addressing this design limitation in future studies. Next, considering the limitation in directly studying the CNS in clinical populations, an exciting future direction for this work would be the ability to measure brain-specific peripheral biomarkers, in order to differentiate brain-related pathways from other cellular processes influenced by blast exposures. Finally, the significant gene expression changes found in this study warrant further research into possible symptomology that may be experienced following blast exposure utilizing validated clinical measures and mapping these symptoms to biomarker changes. Notably, this study identified altered gene expression pathways, which may be the focus of more specific biological mechanisms significant to blast exposure in future studies.

CONCLUSION

The initial findings reported here show that there are robust gene activity changes following a moderate blast exposure in a sample of military personnel. Notably, this study's findings are important to distinguish the effects of blast exposure without the co-occurring impact of blunt-force injuries that take place in combat stations. Findings from this study suggest that additional studies are needed to examine gene-activity related to blast exposure, in order to examine the impact of previous blast exposures on biomarker changes, relationships to neurological impacts, and subsequent clinical symptoms. Thus, these findings provide novel insights into gene network dysregulation observed following objectively measured blast exposure that warrant future clinical studies to advance the understanding of neuropathology related to blast exposure.

DATA AVAILABILITY STATEMENT

Sequencing data used in the study were deposited in the Gene Expression Omnibus (GEO) with GEO ID GSE89866.

ETHICS STATEMENT

This study protocol was reviewed and approved by the Institutional Review Boards at the Naval Medical Research Center and Walter Reed Army Institute of Research (NMRC#2011.0002; WRAIR#1796). All subjects gave written informed consent prior to participation in the study.

AUTHOR CONTRIBUTIONS

KE, JG, NO, and H-SK contributed to the conception or design of the study. KE, VM, SY, Y-EC, and CL contributed to lab analysis, interpretation of the data, and drafting of the work. KE, SY, Y-EC, CL, KD, WC, PW, SA, ML, AY, AT, and JG contributed to the collection, interpretation of the data, and drafting and editing of the work. All authors contributed to critical revision of the manuscript, read and approved the submitted version, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Delayed Symptom Onset Following Pediatric Sport-Related Concussion

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Objective: (1) To examine the prevalence of delayed symptom onset (DSO) among pediatric sport-related concussion (SRC) patients as well as the effect of symptom onset on initial symptom severity, length of recovery, and development of delayed recovery; (2) to evaluate the impact of symptom onset on sideline management.

Methods: We conducted a prospective study of pediatric SRC patients (<20 years of age) evaluated at a multi-disciplinary concussion program. Patients underwent initial medical assessment by a single neurosurgeon and a structured interview by a research assistant. Patients were classified as experiencing early symptom onset (symptom onset <15 min from the time of the suspected injury; ESO) or DSO (≥15 min from the time of the suspected injury).

Results: A total of 144 SRC patients (61.1% male; mean age 14.6 years, SD 1.8) evaluated a median of 5.0 days (IQR 4.0, 9.0) post-injury were included in the study. Among these patients, 120 (83.3%) reported experiencing ESO while 24 (16.7%) experienced DSO following injury. Among those that experienced DSO the median length of time from the suspected injury to symptom onset was 60.0 min (IQR 20.0, 720.0). No significant differences were observed in symptom severity at initial medical assessment (median Post-Concussion Symptom Scale score 20.0 vs. 18.0, $p = 0.35$), length of physician-document clinical recovery (median 22.0 vs. 24.0 days; $p = 0.46$) or the proportion of those who developed delayed physician-documented clinical recovery (34.4 vs. 42.1%, $p = 0.52$) among patients with ESO or DSO. Patients who reported experiencing ESO were significantly more likely to be immediately removed from play at the time of their suspected injury compared to those who experienced DSO (71.6% vs. 29.2%; $p < 0.0001$).

Conclusions: This study suggests that an important proportion of children and adolescents who sustain an acute SRC experience DSO. DSO is associated with lower rates of immediate removal from play at the time of suspected injury. Secondary study findings highlight the need for improved sport stakeholder concussion education and standardized concussion protocols that mandate the immediate and permanent removal of all youth with a suspected concussion until they undergo medical assessment.

Keywords: sports-related concussion, pediatric, symptom onset, delayed symptoms, clinical outcomes

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BACKGROUND

Concussion is a condition caused by the transmission of biomechanical forces to the brain leading to temporary impairments in neurological functioning that often resolve spontaneously (1). Advanced laboratory and neuro-imaging studies suggest that concussion is associated with acute alterations in cellular metabolism, white matter tract injury, and disruptions in cerebral blood flow regulation that may confer an increased risk to additional trauma (2). Athletes who sustain an acute concussion and continue to participate in sports are at risk of sustaining additional injuries that may be associated with more severe or prolonged symptoms or in rare cases, fatal or disabling injuries related to second impact syndrome or diffuse cerebral edema (3, 4).

In order to prevent these adverse outcomes, current national, and international guidelines recommend that all youth athletes who sustain a suspected concussion during a sporting activity be immediately removed from play and undergo standardized sideline assessment by a licensed healthcare professional (1, 5–7). Where licensed healthcare professionals are not available, urgent referral to a physician for medical assessment is recommended (1). Despite the development of additional sideline screening tools and standardized concussion protocols, proper recognition, and removal of athletes with suspected concussion continues to remain a challenge, and is highly dependent on the athlete's timely recognition and reporting of their own symptoms. Although previous studies have examined clinical outcomes among athletes who continued to play despite a symptomatic sport-related concussion (SRC) (8, 9), little research attention has been paid to athletes who experience delayed symptom onset (DSO) and may be at risk of additional injury during continued sport participation (10–12). One prospective study among collegiate athletes found that athletes who underwent delayed removal from play were more likely to have reported DSO (13). To date, no prospective studies have examined the prevalence of DSO among children and adolescents with acute SRC and its effect on clinical outcomes and athlete sideline management.

Therefore, the primary objectives of this study were to examine the prevalence of DSO among pediatric SRC patients referred to a multi-disciplinary concussion program and evaluate the effect of self-reported symptom onset on clinical outcomes such as initial symptom severity, length of clinical recovery, and risk of delayed clinical recovery. The secondary objective was to describe patients' symptom onset, sideline assessment and management after their concussion.

METHODS

Study Design and Recruitment

Pediatric SRC patients included in this study were prospectively recruited from the Pan Am Concussion Program in Winnipeg, Manitoba. The Pan Am Concussion Program is a government-funded, multi-disciplinary pediatric concussion clinic that receives referrals for acute SRC patients from primary care, sports, and emergency medicine physicians, and through standardized sport-specific concussion protocols (11). Inclusion criteria for the study included: (1) age 19 years or younger; (2) physician diagnosis of concussion; (3) injury occurring during a sport or recreational activity; and, 4) symptomatic at the time of initial assessment. Exclusion criteria included: (1) no definitive injury date; (2) self-reported symptom onset >1 week from the date of suspected injury; (3) diagnosed with persistent post-concussion symptoms (symptoms >28 days) at initial visit; and, (4) patients who reported transient non-specific symptoms during sporting activities but who did not meet the clinical criteria for concussion as assessed by the physician. Following initial medical assessment by a neurosurgeon, eligible patients and their parents underwent informed assent and consent, respectively, by a research assistant. Afterwards, consented patients completed a structured 20-min oral interview that included open and closed-ended questions that allowed researchers to collect details regarding the mechanism of injury, symptom onset, whether, and how timely they were removed from play, how truthful they were during sideline assessment, whether they returned to play, and if they sustained additional instances of body or head contact. The research assistant recorded the responses on the structured data collection form. In the event that a sideline assessment was performed, the treating physician and research assistants did not have access to this data. No data was collected from sideline assessments provided by sideline healthcare providers (e.g., athletic therapists). The study was approved by the University of Manitoba Bannatyne Research Ethics Board.

Clinical Assessment and Management

At the time of initial medical assessment at the concussion program, all patients completed a standardized data collection form that included demographic data, past medical history, and the Post-Concussion Symptom Scale (PCSS), a valid and reliable post-concussion symptom inventory (14, 15). All patients subsequently underwent assessment by a single neurosurgeon including a clinical history and physical examination that included evaluation of cranial nerve, motor, sensory, reflex, cerebellar, balance, vestibulo-ocular, and cervical spine functioning. Following medical diagnosis of SRC, patients were seen in follow-up on a weekly basis or as dictated by their rate of recovery and sport schedules. In general, patients met the criteria for physician-documented clinical recovery when they were asymptomatic or back to their pre-injury neurological status at rest, were tolerating full-time school without symptoms, and had a normal physical examination including no evidence of vestibulo-ocular or cervical spine dysfunction (16, 17). Patients were also required to complete the International Consensus on Concussion in Sport graduated Return-to-Play protocol that was specific to their sport where applicable (18). Based on the discretion of the neurosurgeon, some patients underwent graded aerobic treadmill testing and/or neuropsychological testing to confirm clinical recovery. These supplemental tests were considered in patients with more complex medical histories and pre-existing conditions that present with concussion-like symptoms (e.g., migraine, mental health disorders, previous

concussions) and those returning to sports with a higher risk of concussion (e.g., hockey, football).

Definitions and Outcome Measures

The diagnosis of SRC was made by the neurosurgeon based on definition set forth by the International Consensus on Concussion in Sport (18). SRC was diagnosed in patients who reported sustaining a traumatic force or blow to the head, neck, face, or body and presented with new concussion-like symptoms identified on clinical interview or the PCSS including symptoms such as headaches, sensitivity to light or sound, dizziness, or difficulty with remembering or concentrating that could not be attributed to a more appropriate medical diagnosis.

At the time of this study, the authors were not aware of any standardized definitions for delayed symptom onset (DSO) following SRC. Therefore, for the purposes of this study we defined DSO as the onset of initial self-reported concussion-like symptoms $\geq$15 min from the time of the suspected injury. Patients who experienced the onset of concussion-like symptoms $<$15 min from the time of the suspected injury were classified as those with early symptom onset (ESO). Fifteen minutes was chosen as a cut-off time point because this is the estimated amount of time it would take for a licensed healthcare professional to remove an athlete with a suspected concussion from play and perform a sideline assessment that does or does not include the use of the Sport Concussion Assessment Tool 5 (SCAT5) or Child SCAT5 (19).

Length of physician-documented clinical recovery was defined as the time from the date of injury to the date of the final follow-up visit in which clinical recovery was documented by the treating neurosurgeon. Because the majority of pediatric concussion patients recover within 1 month, delayed physician-document clinical recovery was defined as a patient who achieved physician-documented clinical recovery $>$28 days post-injury (20).

Data Analysis

Normally distributed data were summarized as means and standard deviations and were compared using an unpaired *t*-test. Skewed continuous data were reported as the median with the interquartile range (IQR) and were compared using the rank sum test. Proportions were calculated for dichotomous or polychotomous data and differences were compared using a chi squared test or Fisher's exact test as appropriate. A $p < 0.05$ was defined as statistically significant.

A sensitivity analysis was performed to determine if there were any systematic differences in baseline characteristics between those patients who were followed to physician-documented clinical recovery and those who were lost to follow-up. A second sensitivity analysis was conducted to look at time to recovery or lost to follow-up by symptom onset status.

RESULTS

From September 2016 to April 2018, 152 SRC patients were approached to participate in the study; however, eight were excluded due to non-sport related mechanisms of injury ($n = 2$), clinically asymptomatic at time of initial assessment ($n = 2$), no definitive injury date ($n = 2$), presenting with PPCS ($n = 1$), and self-report of symptom onset more than 1 week from the time of suspected injury ($n = 1$). No patient who met study eligibility and was approached declined to participate.

Overall, 144 SRC patients (61.1% male; mean age 14.6 years, SD 1.8) were included in the study. The median time from injury to initial medical assessment at the concussion program was 5.0 days (IQR 4.0, 9.0) and the median PCSS score at initial presentation was 20.0 (IQR 10.3, 32.0). Additional baseline characteristics are summarized in **Table 1**.

Among these patients, 120 (83.3%) reported experiencing ESO while 24 (16.7%) experienced DSO following injury. Among those who experienced DSO the median length of time from the suspected injury to symptom onset was 60.0 min (IQR 20.0, 720.0). Sports-related concussion patients who experienced DSO underwent initial medical assessment at the concussion program a median of 6.5 days (IQR 2.0, 15.5) post-injury while those with ESO were assessed a median of 5.0 days (IQR 2.0, 19.0) post-injury ($p = 0.52$). No significant differences were observed between those with ESO and DSO when considering baseline characteristics including age, sex, personal and family medical histories, setting of sport, and sport played at the time of concussion. There were no significant differences in median symptom severity at initial medical assessment between those with ESO vs. DSO (20.0 vs. 18.0, $p = 0.35$).

At the end of the study period, 115 (79.9%) patients achieved physician-documented clinical recovery, 26 (18.1%) were lost to follow-up, and 3 (2.1%) remained in treatment. The median length of physician-documented recovery for the entire cohort was 22.0 days (IQR: 16.0, 34.0). Among those who achieved physician-documented clinical recovery, 41 (35.7%) experienced delayed physician-documented clinical recovery and symptom onset was not associated with the development of delayed physician-documented clinical recovery (ESO: 34.4 vs. DSO: 42.1%, $p = 0.52$). There was also no significant difference in days until physician-documented recovery among those who experienced ESO or DSO (22.0 vs. 24.0 days; $p = 0.46$) (**Table 2**).

Overall, 26 participants (18.1%) were lost to follow-up (DSO 5, ESO = 21); however, the proportion of lost to follow-up did not significantly differ between the two groups ($p = 0.70$). The only significant differences in baseline characteristics was significantly higher proportion of family history of psychiatric disorders among those lost to follow-up ($p = 0.049$) and significantly longer time between injury and initial consult ($p = 0.04$). A sensitivity analysis was conducted where the length of time from injury to medical clearance for those who received a physician diagnosed medical clearance was combined with the length of time from injury to the last appointment date for those who were lost to follow-up. Those with DSO were medically cleared or attended their last follow-up appointment at a median of 23.0 days (IQR: 17.0, 32.0) after their injury, and those with ESO were medically cleared or attended their last follow-up appointment at a median of 21.0 days (IQR: 15.0, 32.0) following injury ($p = 0.53$).

TABLE 1 | Baseline characteristics of sport-related concussion patients with early and delayed symptom onset.

	Total	DSO	ESO	*P* value[a]
	***N* = 144**	***N* = 24 (16.7%)**	***N* = 120 (83.3%)**	
Mean age (SD)	14.6 (1.8)	14.2 (2.0)	14.68 (1.7)	0.24*
Male	88 (61.1%)	15 (62.5%)	73 (60.8%)	0.88†
Median days from injury to medical assessment (IQR)	5.0 (2.0, 17.4)	6.5 (2.0, 15.5)	5.0 (2.0, 19.0)	0.52‡
Median PCSS score (IQR) at initial medical assessment	20.0 (10.3, 32.0)	18.0 (8.0, 26.0)	20.0 (11.0, 32.8)	0.35‡
Loss of consciousness	14 (9.7%)	0 (0.0%)	14 (11.7%)	0.13§
Post-traumatic amnesia	34 (23.6%)	3 (12.5%)	31 (25.8%)	0.20†
History of previous concussion	58 (40.3%)	9 (37.5%)	49 (40.8%)	0.76†
History of headache or migraine	10 (6.9%)	1 (4.2%)	9 (7.50%)	1.00§
History of a learning disorder or ADHD	6 (4.2%)	1 (4.2%)	5 (4.2%)	1.00§
History of depression	4 (2.8%)	0 (0.0%)	4 (3.3%)	1.00§
Family history of mental health disorder	34 (23.6%)	4 (16.7%)	30 (25.0%)	0.43§
Concussions occurring during a game[a]	116 (80.6%)	19 (79.2%)	97 (80.8%)	0.85†
Concussions occurring during a contact sport[b]	137 (95.1%)	23 (95.8%)	114 (95.0%)	1.00†
SPORT PLAYED AT TIME OF CONCUSSION				
Hockey	61 (42.4%)	10 (41.7%)	51 (42.5%)	
Football	24 (16.7%)	4 (8.3%)	20 (16.7%)	
Soccer	18 (12.5%)	4 (8.3%)	14 (11.7%)	
Basketball	11 (7.6%)	0 (0.0%)	11 (9.2%)	
Other[c]	30 (20.8%)	6 (25.0%)	24 (20.0%)	

[a] *Comparison between ESO versus DSO (*Two sample t-test; †Chi-square test; ‡Two sample Mann-Whitney rank sum; §Fishers exact test).*
[b] *Compared to concussions that occurred during an organized practice or a supervised gym class.*
[c] *Contact sports: basketball, dodgeball, football, hockey, judo, ringette, rugby, soccer, volleyball, waterpolo, and wrestling.*
Non-contact sports: dance, gymnastics, snowboarding, and speedskating.
SD, standard deviation; IQR, interquartile range; ADHD, attention-deficit-hyperactivity disorder; PCSS, Post-Concussion Symptom Scale; ESO, early symptom onset; DSO, delayed symptom onset.

At the time of suspected injury, 86 (71.6%) of athletes who experienced ESO underwent immediate removal from play, 13 (10.8%) underwent delayed removal and 21 (17.5%) remained in the same game or practice. Among those who experienced DSO, 7 (29.2%) underwent immediate removal, 3 (12.5%) underwent delayed removal and 14 (58.3%) remained in the same game or practice after the time of the suspected injury. Overall, SRC patients who reported experiencing ESO were significantly more likely to be immediately removed from play compared to those who experienced DSO (71.6 vs. 29.2%; $p < 0.0001$). Additional data regarding sideline assessment and management for this cohort is presented in **Figures 1**, **2**.

DISCUSSION

This study provides important insight into the heterogeneity in symptom onset following pediatric SRC and the relationship between delayed symptom onset and clinical outcomes and athlete sideline management.

In this prospective study of pediatric SRC patients who were evaluated at a multi-disciplinary concussion program a median of 5 days post-injury, 16.7% reported experiencing delayed onset of post-concussion symptoms following injury. Among those who experienced DSO, the median duration of time from the suspected concussive injury to symptom onset was

TABLE 2 | Symptom severity and recovery outcomes in sport-related concussion patients with early and delayed symptom onset.

	Total	DSO	ESO	*P* value[a]
	***N* = 115**	***N* = 19 (16.7%)**	***N* = 96 (83.3%)**	
Days to physician documented clinical recovery (median, IQR)	22.0 (16.0, 34.0)	24.0 (17.0, 32.0)	22.0 (15.0, 34.0)	0.465*
Experienced delayed physician diagnosed delayed recovery (>28 days)	41 (35.7%)	8 (42.1%)	33 (34.4%)	0.520†

[a]*Comparison between ESO vs. DSO (*Two sample Mann-Whitney rank sum; †Chi-square test).*
Analysis completed for patients with complete medical follow-up.
IQR, interquartile range; PCSS, Post-Concussion Symptom Scale; ESO, early symptom onset; DSO, delayed symptom onset.

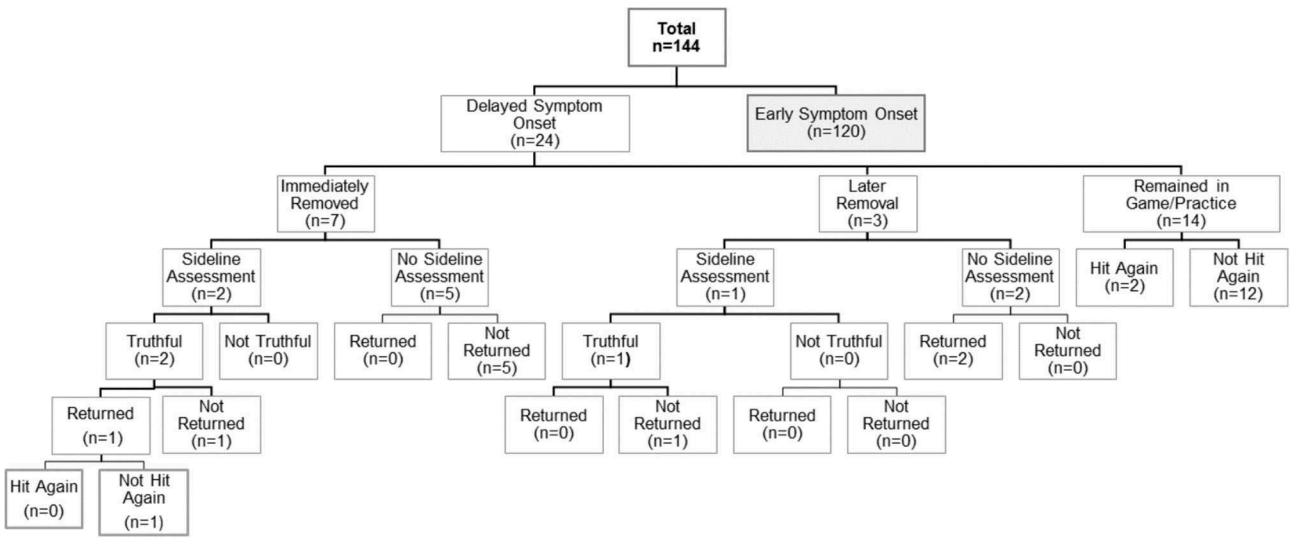

FIGURE 1 | Summary of self-reported sideline management among sport-related concussion patients with delayed symptom onset.

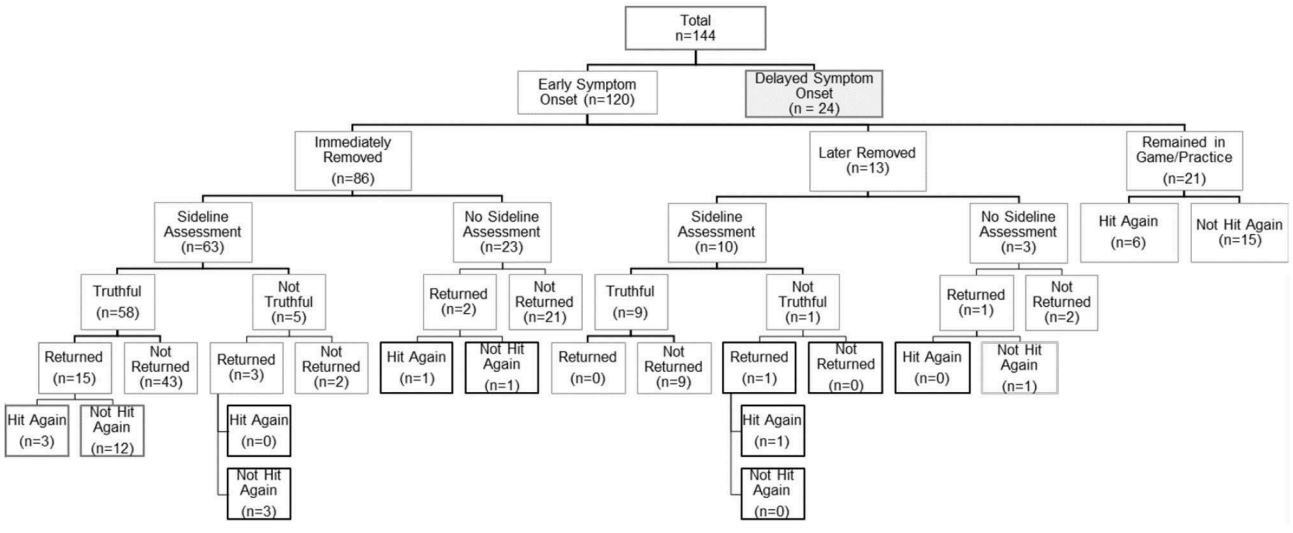

FIGURE 2 | Summary of self-reported sideline management among sport-related concussion patients with early symptom onset.

60 min. Despite evaluating a number of baseline demographic and clinical variables, no specific risk factors for DSO were identified. Although patients who experienced DSO underwent initial medical assessment at the concussion program a median of 1.5 days later than those with ESO, no significant differences in initial symptom burden were observed between the two groups. Overall, there were no significant differences in the length of physician-documented recovery or the proportion of patients who experienced delayed physician-documented clinical recovery among those with ESO and DSO.

Although SRC has been historically viewed as a condition that typically results in the rapid onset of short-lived neurological impairment following the transmission of biomechanical forces to the head (1), limited studies have examined the onset of symptoms following this injury and its effect on clinical outcomes. In a study of collegiate athletes, Duhaime et al. (10) found that symptom onset among 44 acute concussions was immediate in 54%, delayed in 25% (no standardized definition) and unknown in 20%. In a previous retrospective study of SRC patients presenting to our multi-disciplinary pediatric concussion program, 28.1% of acute SRC patients reported that the majority of their symptoms started more than 1 h post-injury (11). In addition, Morgan et al. (12) performed a retrospective case-control study of pediatric SRC patients evaluated at a tertiary sport concussion clinic who recovered within 3 weeks post-injury and those that developed persistent symptoms lasting >3 months. They found that 2.5% of patients who recovered within 3 weeks and 25% of patients who developed persistent symptoms experienced delayed symptom onset (symptom onset >3 h post-injury), and that delayed symptom onset was associated with a greater odds of developing persistent symptoms lasting >3 months.

In addition to examining the effect of symptom onset on traditional clinical outcomes such as initial symptom burden, length of recovery, and the risk of developing delayed recovery, this study also provides important insight into how symptom onset may complicate the sideline assessment and management of children and adolescents with suspected acute SRC. Current international and national guidelines recommend that youth athletes with a suspected concussion be immediately removed from play and undergo sideline assessment by a licensed healthcare professional (1, 5–7). In instances where a licensed healthcare provider is not available, urgent referral to a physician for medical assessment is recommended (1). Although sideline tools such as the ChildSCAT5 and SCAT5 contain objective measures of cognitive and balance functioning that demonstrate some sensitivity to acute concussion (21), experts warn that these tests may be normal in the setting of acute concussion, leaving sideline healthcare professionals and sport stakeholders to rely heavily on the athlete's recognition and reporting of their concussion-like symptoms to determine the appropriate management of the athlete. Previous studies suggest that athletes who undergo delayed removal from play, following a symptomatic SRC, experience more severe symptoms and take longer to achieve clinical recovery (8, 9). In instances where an athlete has sustained an injury that is sufficient to cause a concussion but results in DSO, there is a similar risk that the athlete will remain in play or be returned to play in an asymptomatic state which may be associated with increased vulnerability to additional injury and could lead to more severe or prolonged symptoms as well as more severe forms of traumatic brain injury (3, 4). In a recent study of collegiate athletes, Asken et al. (13) found that those athletes who were immediately removed from their sporting activity reported less severe initial concussion symptoms, experienced a shorter duration of symptoms, and were at lower risk of taking >14 and >21 days to recovery from their injury. Among this cohort, 36% reported experiencing DSO (no standardized definition of delayed symptoms was provided). Although a significantly higher proportion of athletes who underwent delayed removal from their sporting activity were found to experience delayed (48.1%) vs. immediate (15.1%) symptom onset, there was no main effect of symptom onset or interaction between symptom onset and removal from sport status on recovery outcomes. In the present study, SRC patients who experienced ESO were significantly more likely to be immediately removed from play compared to those who experienced DSO; however, the impact of removal status on patient outcomes was difficult to assess in this sample given the heterogeneity in sideline assessment and management reported by these athletes. Indeed a significant proportion of athletes included in this study reported experiencing concussion symptoms but were not removed from play while others who did report experiencing concussion symptoms and were evaluated on the sidelines were also returned to play.

The results of this study have important implications for concussion education and public health policy in Canada. These findings suggest that an important proportion of youth athletes who sustain acute concussion experience DSO, which can lead to challenges in early identification and removal of these athletes from sports. This study also suggests that a notable proportion of youth athletes with suspected concussion are being allowed to return to the same sport activity where they are at risk of being exposed to additional and potentially life-threatening brain injuries. To address these knowledge gaps in Canada, Parachute has partnered with the Public Health Agency of Canada and national leaders in sport to develop the *Canadian Guideline on Concussion in Sport* (7). This guideline recommends that all sport stakeholders including athletes, parents, coaches, teachers, and officials review a standardized *Pre-season Concussion Education Sheet* that provides information on the signs and symptoms of concussion, indicates that concussion symptoms can develop immediately and in a delayed fashion following injury, and recommends that all youth with a suspected concussion be immediately and permanently removed from play until they undergo medical assessment regardless of the results of sideline assessment. In those athletes who sustain an injury but do not report symptoms or demonstrate signs of concussion, the guideline recommends that the athlete may be returned to play but should be followed for delayed symptom. Since the release of this guideline in 2017, Sport Manitoba and other community leaders have begun work to develop harmonized sport-specific concussion protocols for school divisions and youth sport organizations throughout the province that encourage all

sport stakeholders to undergo pre-season education using this national resource (22–24). However, at the time of the present study there were no provincial sport- or school protocols mandating pre-season concussion education for youth sports, which may have contributed to the inconsistent and sub-optimal sideline management that some athletes received during this study period.

This study has several important limitations. First, much of the collected data included in this study was based on patient self-report and therefore is subject to recall bias and can also be impacted by post-traumatic amnesia in some instances. Data on symptom onset and sideline care depended entirely on accurate recognition and reporting by patients as well as collateral history provided by parents. Patient impressions regarding what constituted a sideline assessment or an unstructured encounter with a coach or parent may have differed between study subjects. Given that all patients were in the presence of one of their parents during initial assessment by the physician and structured interview by the research assistant, this may have influenced how patients reported the events of their injury. This is a common limitation of concussion research and without obtaining collateral history from sideline sport stakeholders (i.e., team trainers or coaches) or utilizing technology such as video replay, it is impossible to create an accurate reconstruction of injury events and sideline management among this cohort. Second, 18.1% of participants were lost to follow-up. This limitation is common among those conducted at tertiary concussion clinics; however, a sensitivity analysis revealed that the baseline characteristics among those lost and not lost to follow-up were similar with the exception of those who were lost to follow-up were more likely to have family history of psychiatric disorders. Timing of symptom onset was not associated with being lost to follow-up. Finally, study participants were recruited from a multi-disciplinary pediatric concussion program and therefore may represent a patient population with more severe injuries. It is possible that the proportion of youth with DSO vs. ESO in this study may not be reflective of the general pediatric SRC population; however, these results are likely an accurate reflection of SRC patients evaluated at tertiary concussion clinics.

In conclusion, this prospective study confirms that children and adolescents who suffer an SRC can experience delayed onset of self-reported concussion symptoms following injury. Although SRC patients who experienced DSO were less likely to be immediately removed from play, we observed no significant differences in symptom burden at initial assessment, length of physician-documented clinical recovery, or the development of delayed physician-documented clinical recovery. The sideline care of youth athletes with suspected concussion would benefit from the development of accurate and reliable sideline diagnostic tools that do not rely on athlete symptom reporting and can be administered in sport settings with limited access to sideline healthcare professionals. Until such tests are available, additional work is needed to improve concussion education among all sport stakeholders and reinforce the message that all youth athletes with a suspected concussion during sport or recreational activities should be immediately and permanently removed from play until they undergo medical assessment.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available because the participants did not consent to their data being publically available. Requests to access the datasets should be directed to Shelly Rempel-Rossum at Shelly.rempel-rossum@umanitoba.ca.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Research Ethics Board Guidelines, University of Manitoba Bannatyne Campus Research Ethics Board with written informed consent from all subjects. All subjects gave written consent in accordance with the Declaration of Helsinki. The protocol was approved by the University of Manitoba Bannatyne Campus Research Ethics Board.

AUTHOR CONTRIBUTIONS

KR and ME conceptualized and designed the study, carried out the data collection and analysis, drafted the initial manuscript, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. AO and ES carried out data collection and analysis, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. All authors agree to be accountable for all aspects of the work ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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Performance on the DANA Brief Cognitive Test Correlates With MACE Cognitive Score and May Be a New Tool to Diagnose Concussion

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Nearly 380,000 U.S. service members between 2000 and 2017 were, and at least 300,000 athletes annually are, diagnosed with concussion. It is imperative to establish a gold-standard diagnostic test to quickly and accurately diagnose concussion. In this non-randomized, prospective study, we examined the reliability and validity of a novel neurocognitive assessment tool, the Defense Automated Neurobehavioral Assessment (DANA), designed to be a more sensitive, yet efficient, measure of concussion symptomatology. In this study, the DANA Brief version was compared to an established measure of concussion screening, the Military Acute Concussion Evaluation (MACE), in a group of non-concussed service members. DANA Brief subtests demonstrated low to moderate reliability, as measured by intra-class correlation coefficient (ICC; values range: 0.28–0.58), which is comparable to other computerized neurocognitive tests that are widely-implemented to diagnose concussion. Statistically significant associations were found between learning and memory components of the DANA Brief and the diagnostic MACE cognitive test score (DANA Brief subtests: CDD: $R^2 = 0.05$, $p = 0.023$; CDS: $R^2 = 0.10$, $p = 0.010$). However, a more robust relationship was found between DANA Brief components involving attention and working memory, including immediate memory, and the MACE cognitive test score (DANA Brief subtests: GNG: $R^2 = 0.08$, $p = 0.003$; PRO: $R^2 = 0.08$, $p = 0.002$). These results provide evidence that the DANA Rapid version, a 5-min assessment self-administered on a hand-held portable device, based on the DANA Brief version, may serve as a clinically useful and improved neurocognitive concussion screen to minimize the time between injury and diagnosis in settings where professional medical evaluation may be unavailable or delayed. The DANA's portability, durability, shorter test time and lack of need for a medical professional to diagnose concussion overcome these critical limitations of the MACE.

Keywords: mTBI, concussion, DANA, MACE, military, neurocognitive assessment

INTRODUCTION

According to the Defense and Veterans Brain Injury Center, US service members (a person serving in the armed forces) sustained nearly 380,000 cases of traumatic brain injury (TBI) between 2000 and 2017. Of these, 82.3% were classified as mild (1). According to the Department of Defense guidelines, mild traumatic brain injury (mTBI), also called concussion, is diagnosed when an injury event has occurred and the individual experiences one or more of the following: (1) an alteration of consciousness lasting <24 h, (2) loss of consciousness (LOC) from 0 to 30 min, or (3) post traumatic amnesia from 0 to 1 days (2).

Concussion is also common among athletes. According to the Centers for Disease Control and Prevention (CDC), at least 300,000 athletes are diagnosed with concussions per year (3). Sport-related concussions are defined as a mTBI induced by biomechanical forces and, because of their potential to have rapidly changing and sometimes unpredictable clinical symptoms, are often difficult to diagnose (4, 5). Exposure to these biomechanical forces triggers a complex neurometabolic cascade, described by Giza and Hovda as a series of microcellular events, including ionic shifts, abnormal energy metabolism, diminished cerebral blood flow, and impaired neurotransmission (6). Because of the underlying pathophysiology of mTBI, conventional imaging (i.e., CT, MRI) is not considered diagnostic and frequently does not provide useful clinical information. Additionally, reliance on athlete/patient self-report is itself problematic. Delaney et al. report that 63–70% of contact sport athletes reported symptoms; however, only 20–23% realized that these symptoms constituted concussion (7). In some cases, concussion symptoms are known but not reported for various reasons. McCrea et al. reported on confidential surveys of 1,500 high school football players, 40% of which acknowledged the presence of concussive symptoms but deliberately failed to disclose the information out of fear of being removed from play (8).

A concussion can result in a variety of acute symptoms, including, but not limited to, nausea, headache, dizziness, fatigue, balance problems, sensitivity to light, and memory and concentration deficits (9, 10). Typically, symptom recovery occurs within 7–10 days post-injury (11, 12). Recovery of cognitive function, however, can take anywhere from 1 to 3 months (13, 14). It is, therefore, possible that individuals may present as clinically recovered (asymptomatic), but still have lingering cognitive deficits. In the absence of a gold-standard biomarker for concussion, clinicians often utilize neurocognitive testing methods to diagnose and determine recovery status (4). The utility of objective neurocognitive testing is well-established, with several studies showing measurable decrements in performance during the initial hours to weeks post-injury (15–17). Additionally, resolution of measured cognitive impairment via objective assessment tends to be on the magnitude of 2–14 days, which is similar to symptom recovery (11).

The United States Department of Defense currently uses the Military Acute Concussion Evaluation (MACE) as its designated method for evaluating suspected concussion (18–20). The MACE is composed of three parts: history of the injury, cognitive examination, and neurological screening. The cognitive component of the MACE includes questions that are heavily weighted toward memory functions. The aggregate of these subtests generates the MACE cognitive test score, which can help to determine cognitive impairment. There are normed cut-off values for the MACE which, when abnormal, are helpful in clinical evaluation. Normal values, however, cannot rule out the presence of concussion, which remains a clinical diagnosis based upon the history of events surrounding the injury. Also, if the MACE is administered more than 12 h after a concussive event, the cognitive examination section has been shown to lack sensitivity and specificity, thereby limiting its utility for early and accurate identification of concussion (20, 21). Additionally, the MACE must be administered by a trained professional, decreasing its functionality as a quick and easy method to diagnose concussion and track its recovery.

Unlike the MACE, the Defense Automated Neurobehavioral Assessment (DANA) is a durable and portable neurocognitive assessment tool that can be self-administered on a handheld device, making it well-suited for military operations and/or extreme environments as well as the sideline of a sports field. The DANA has three test battery versions: the DANA Rapid (5 min), DANA Brief (15 min), and DANA Standard (45 min). Each test varies in subtest composition and duration. The DANA Rapid focuses on basic reaction time measures (one of the more sensitive measures of impairment after concussion) and is the shortest of the three batteries (5 min). The DANA Brief builds on the Rapid and consists of a 15-min battery of tests plus additional psychological screening tools for post-traumatic stress disorder (PTSD), depression, and insomnia. The DANA Standard is the most comprehensive of the versions, containing the DANA Standard subtests as well as additional neurocognitive and psychological tests, and takes approximately 45 min to administer (22).

Since it is well established that cognitive impairment as a result of concussion is quite heterogeneous, previous studies have suggested concussive cognitive sequelae tend to follow a subcortical phenotype characterized by difficulties in attention, processing speed, executive function and memory recall (vs. encoding). While the MACE assesses attention, learning and memory items, the DANA extends the breadth of cognitive task domains that are assessed, with the inclusion of measures of reaction time, visuomotor processing speed, and measures of executive function (i.e., cognitive control). A more detailed discussion of the individual components in the MACE and DANA is presented in the Methods section below.

Because of the clinical ramifications of undiagnosed concussion, which can lead to further brain injury, it is imperative to establish a gold-standard diagnostic test to quickly and accurately diagnose this injury. A gold-standard neurocognitive test should ideally rely on purely objective measures and be rapidly self-administrable in diverse environments where professional medical evaluation is unavailable or delayed. This would benefit service members, professional athletes, and other

individuals who have sustained a concussion by minimizing the time between injury and diagnosis.

This study was a prospective, non-randomized study comparing results from the DANA Brief subtests to the MACE cognitive test score in a group of non-concussed military service members in a remote, deployed environment. The primary objective of this study was to determine which individual of DANA Brief subtests were most correlated with the MACE cognitive test score in a remote, isolated, austere environment. We hypothesized that subtests related to memory on the DANA Brief [Code Substitution Simultaneous (CDS) and Code Substitution Delayed (CDD)] would be significantly correlated with the MACE cognitive test score. This hypothesis was based on the fact that the CDS and CDD assess short-term memory, where roughly 57% of the MACE cognitive test score is memory-task dependent. The investigators acknowledged that the cognitive constructs assessed by the CDS and CDD are diverse. Specifically, the CDS involves visual processing speed and attention. Further, the memory paradigm assessed in the CDS and CDD is primarily one of incidental learning and memory, as opposed to the more intentional learning that is screened as part of the MACE cognitive subtests. Nonetheless, we hypothesized that there would be greater overlap between the CDS and CDD DANA Brief subtest scores and the MACE cognitive test score, than between the DANA Brief simple reaction time subtests [Simple Reaction Time (SRT1 and SRT2)] and the MACE cognitive test score. A secondary objective of this study was to examine successive iterations of performance on the DANA Brief in the same group of volunteers. We also hypothesized that repeated administrations of the DANA Brief would result in improvements in an individual's performance due to a learning curve.

MATERIALS AND METHODS

Study Volunteers

This study was approved by the Institutional Review Board of the United States Army Medical and Materiel Command, which had regulatory oversight over all human subjects research in Afghanistan. A total of forty male United States Marines from an infantry unit deployed to Afghanistan volunteered to participate in the study (mean age $\pm$ SD $= 22.6 \pm 3.8$). Inclusion criteria were defined as active duty military service members who had a Glasgow Coma Scale score of at least 15 at the time of consent and who had not experienced blast exposure, collisions, rollovers or direct blows to the head within the past 24 h (2, 22). Exclusion criteria included sustaining a concussion within 3 months prior to study participation.

Military Acute Concussion Evaluation (MACE)

The MACE is a three-part, 15-min test battery based on the SAC, and is the primary screening tool used in military populations for the acute evaluation of concussion. The first part consists of open-ended and yes or no clinical history questions, such as how the head injury occurred, what symptoms were experienced, and whether there was a LOC. The second part is a scored cognitive exam with four subtests: orientation (5 points); immediate verbal memory (15 points; recall of a 5-item word list presented over three consecutive learning trials); concentration (5 points; reverse digit sequencing and reversal naming of calendar months); and delayed memory (5 points; recall of the previous 5-item word list). From this exam, a total score out of 30 points, called the MACE cognitive test score, is generated based on adding the scores from each subtest. The mean MACE cognitive test score in military populations is 28, and a score of <25 points (2 standard deviations from the mean) represents potentially clinically relevant cognitive impairment. A neurologic exam follows the cognitive exam with special focus on examining pupil reactivity, speech fluency, and motor deficits (18).

Defense Automated Neurobehavioral Assessment (DANA) Brief

The DANA Brief consists of seven subtests, including four from the DANA Rapid: Simple Reaction Time (SRT) assessing basic reaction time (administered twice, once at the beginning of the test battery and once at the end, giving two subtests, SRT1 and SRT2), Procedural Reaction Time (PRO) measuring attention and processing speed, and Go/No-Go (GNG) which assesses speed, accuracy, and response omissions and commissions. Additional subtests on the DANA Brief include Code Substitution Simultaneous (CDS) assessing visual scanning, processing speed, attention, learning and immediate memory; Spatial Discrimination (SPD) which measures spatial manipulation, and Code Substitution Delayed (CDD) which assesses visual recognition memory. It also includes a Patient Health Questionnaire (PHQ), a Primary Care PTSD screen (PC-PTSD), and an Insomnia Screening Index (ISI).

Three scores are calculated and reported for each DANA subtest: mean reaction time (RT; ms), RT correct (RT of correct responses; ms), and a mean throughput score, which is calculated using the following equation:

$$Mean\ throughput\ score\ = \frac{\%\ correct\ responses}{mean\ RT\ correct} \quad (1)$$

Test Administration

The MACE was administered to forty non-concussed service member volunteers by a clinician in a remote, deployed environment medical tent, which was the standard medical treatment (Role 1) field facility. Immediately following the MACE administration, the DANA Brief was self-administered on a commercially available handheld computer with a touch screen (Trimble Nomad). The total administration time for both the MACE and DANA Brief was 30 min. All forty volunteers were re-assessed on a single follow up day, 24 h after baseline assessment, for the second iteration of testing. This testing session consisted of a repeat administration of the MACE and DANA Brief. Twenty of the forty volunteers were assessed again on a subsequent follow-up day, 48 h after baseline assessment, for a total of three MACE and DANA Brief test iterations. Testing order was not randomized (the MACE was always administered prior to the DANA Brief).

TABLE 1 | Group summary statistics and reliability, as measured by intra-class correlation coefficient (ICC), for DANA Brief mean throughput scores and the MACE cognitive test scores for each test iteration.

N	Test Iteration			
	1	2	3	
	40	40	20	
DANA Brief Subtest	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**ICC (95% CI)**
CDD	54.76 ± 14.86	54.92 ± 13.68	41.94 ± 18.18	0.28 (0.09, 0.49)
CDS	49.42 ± 8.62	51.77 ± 7.79	48.39 ±8.45	0.58 (0.40, 0.73)
GNG	121.47 ± 15.72	121.78 ± 17.17	102.10 ± 20.46	0.58 (0.40, 0.73)
PRO	100.99 ± 15.18	104.52 ±13.37	98.84 ± 17.01	0.58 (0.40, 0.73)
SPD	35.65 ± 7.82	39.51 ± 9.20	37.06 ± 11.46	0.58 (0.40, 0.73)
SRT1	200.70 ± 24.03	194.82 ± 26.29	180.43 ± 30.20	0.58 (0.40, 0.73)
SRT2	192.56 ± 24.99	189.05 ± 28.24	156.28 ± 43.79	0.58 (0.40, 0.73)
MACE Cognitive Test	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	
MCTS	27.10 ± 2.06	27.63 ± 1.73	26.90 ± 2.94	0.58 (0.40, 0.73)

DANA Brief subtests: CDD, Code Substitution Delayed; CDS, Code Substitution Simultaneous; GNG, Go/No-Go; PRO, Procedural Reaction Time; SPD, Spatial Discrimination; SRT1, Simple Reaction Time (first administration); SRT2, Simple Reaction Time (second administration); MCTS, MACE cognitive test score.

Statistical Analysis

For each DANA Brief subtest, a repeated measures ANOVA was used to assess the association between each DANA Brief subtest mean throughput score (subtest_score) and volunteer's corresponding MACE cognitive test score (MCTS). The full model of each DANA brief subtest included the following dependent and independent variables: DANA Brief subtest mean throughput score (continuous variable), iteration (categorical variable), age (continuous variable) and the interaction term between DANA Brief subtest score and iteration (Equation 2).

$$MCTS = \beta_0 + \beta_1 subtest\_score + \beta_2 iteration + \left(\beta_3 subtest\_score * iteration\right) + \beta_4 age + \beta \quad (2)$$

For each DANA Brief subtest, the final mixed model was determined by a backward model selection and included the following dependent and independent variables: MCTS (continuous variable), DANA Brief subtest mean throughput score (continuous variable) and age (continuous variable) (Equation 3). Reliability of the final mixed model was evaluated based on the intraclass correlation coefficient (ICC) in the final mixed model with 95% confidence intervals calculated based on a single rater, absolute agreement, one-way random effects model. Validity was assessed based on the correlation between each of the DANA Brief subtests and the MACE cognitive test score. Eta-squared was calculated to obtain effect size, which measures the strength of the relationship between DANA Brief subtests and the MACE cognitive test score. Statistical analyses were performed using SAS 9.4.

$$MCTS = \beta_0 + \beta_1 subtest\_score + \beta_2 age + \beta \quad (3)$$

Results

Group summary statistics for DANA Brief mean throughput scores and the MACE cognitive test scores for each test iteration are reported in **Table 1**. DANA Brief performance was not significantly different between baseline and testing 24 to 48-h later (**Table 2**). DANA Brief subtests demonstrated low to moderate reliability, with a range of ICCs from 0.28 to 0.58 (**Table 1**). The association between CDD ($R^2 = 0.05$, $p = 0.023$), CDS ($R^2 = 0.10$, $p = 0.010$), GNG ($R^2 = 0.08$, $p = 0.003$), PRO ($R^2 = 0.08$, $p = 0.002$) DANA Brief mean throughput scores and the corresponding MACE cognitive test score were all statistically significant; however, SPD, SRT1 and SRT2 mean throughput scores were not significantly associated with the MACE cognitive test score. Eta-squared values for the DANA Brief subtests ranged from 0.4 to 9.5%, where CDS ($\eta^2 = 9.5\%$) and GNG ($\eta^2 = 7.4\%$) had the highest effect sizes, explaining the proportion of total variance can be accounted for by DANA Brief subtests in the final mixed model. Results are reported in **Table 3**.

DISCUSSION

While there is no gold-standard test for the assessment of concussion, any new tool has to be equivalent or better than established screening tools such as the MACE. This study examined the reliability and validity of a novel measure (the DANA Brief), designed to be a more sensitive, yet efficient, measure of cognitive performance following a concussion. In this non-randomized, prospective study, the DANA Brief was compared to an established measure of concussion screening (the MACE).

Low to moderate test-retest reliability of the DANA Brief subtests is an unsurprising reflection of ICCs commonly reported in studies of widely-implemented computerized neurocognitive tests (CNTs) to assess and diagnose concussion. The present study found ICC values that range from 0.30 to 0.63. Although results of repeat testing in non-concussed individuals should not change in theory, some inherent level of measurement error can be expected in any test and is reflected by low reliability (23). Reliability values associated with CNTs such as the DANA

TABLE 2 | Results of comparison between the DANA Brief subtest mean throughput scores and MACE cognitive test scores in the linear mixed model (Equation 2).

Variables	β	*p*-value	η^2 (%)	R^2
CDD	0.013	0.642	2.8	0.03
Iteration	−0.249	0.715	−1.7	
Iteration*CDD	0.009	0.480	−1.0	
Age	0.050	0.475	0.8	
CDS	0.085	0.047*	8.4	0.08
Iteration	0.865	0.435	−2.0	
Iteration*CDS	−0.015	0.493	−1.0	
Age	0.086	0.209	3.0	
GNG	0.054	0.017*	7.2	0.07
Iteration	1.831	0.125	−1.2	
Iteration*GNG	−0.013	0.210	−0.9	
Age	0.102	0.161	3.0	
PRO	0.073	0.002*	7.9	0.08
Iteration	2.218	0.066	−0.1	
Iteration*PRO	−0.021	0.078	1.0	
Age	0.076	0.249	3.0	
SRT1	0.024	0.096	2.3	0.03
Iteration	1.934	0.146	−0.5	
Iteration*SRT1	−0.009	0.208	1.4	
Age	0.052	0.456	1.1	
SRT2	0.018	0.250	4.0	0.04
Iteration	0.932	0.460	0.3	
Iteration*SRT2	−0.004	0.594	0.8	
Age	0.046	0.500	0.7	
SPD	0.082	0.068	0.5	0.01
Iteration	1.537	0.032*	−0.92	
Iteration*SPD	−0.037	0.053	0.1	
Age	0.069	0.351	2.5	

*β, validity; η^2, effect size. DANA Brief subtests: CDD, Code Substitution Delayed; CDS, Code Substitution Simultaneous; GNG, Go/No-Go; PRO, Procedural Reaction Time; SPD, Spatial Discrimination; SRT1, Simple Reaction Time (first administration); SRT2, Simple Reaction Time (second administration); *p < 0.05.*

TABLE 3 | Results of comparison between the DANA Brief subtest mean throughput scores and MACE cognitive test scores in the final linear mixed model (Equation 3).

Variables	β	*p*-value	η^2 (%)	R^2
CDD	0.027	0.023*	3.5	0.05
Age	0.051	0.461	0.9	
CDS	0.064	0.010*	9.5	0.10
Age	0.086	0.203	3.0	
GNG	0.030	0.003*	7.4	0.08
Age	0.097	0.179	2.9	
PRO	0.040	0.002*	6.9	0.08
Age	0.077	0.243	1.9	
SRT1	0.007	0.267	0.8	0.02
Age	0.053	0.449	0.6	
SRT2	0.010	0.071	2.7	0.04
Age	0.048	0.482	0.4	
SPD	0.017	0.463	0.4	0.02
Age	0.068	0.358	2.0	

*β, validity; η^2, effect size. DANA Brief subtests: CDD, Code Substitution Delayed; CDS, Code Substitution Simultaneous; GNG, Go/No-Go; PRO, Procedural Reaction Time; SPD, Spatial Discrimination; SRT1, Simple Reaction Time (first administration); SRT2, Simple Reaction Time (second administration); *p < 0.05.*

Brief may be biased by various factors. Given the conflicting implications of moderating variables in the present study, future studies would benefit from a larger sample size to increase power and allow for the comparison of these variables to better understand the basis of the DANA Brief subtest ICCs reported in this study.

In addressing the validity of the DANA Brief, of particular interest was the relationship between measures of incidental visual memory and recognition, inherent in several DANA Brief subtests and the MACE cognitive test. In this preliminary investigation, we found evidence to support our hypothesis regarding the correlation between learning and memory components of the DANA Brief (i.e., CDD, CDS) and the MACE cognitive test score. However, a more robust relationship was found between the GNG and PRO subtests and the MACE cognitive test score. This is not surprising given that several components of the MACE cognitive test score involve attention and working memory, including immediate memory. It is well established that attentional control and focus to task influences initial encoding and immediate memory performance. This is relevant in the current study, as 50% of MACE cognitive test score points come from immediate memory. Further, studies have suggested that the primary memory problem in concussed individuals tends to be one of initial encoding, requiring greater attentional demand, as opposed to delayed retrieval (24). Taken together, findings that DANA Brief measures of more direct reaction time and processing speed (SRT1 and SRT2) were not significantly associated with the MACE cognitive test score, and that GNG and PRO subtests explain the most variance in the final mixed model, suggests the DANA Brief is an instrument tapping an additional, unique cognitive construct relevant in the context of concussion/mTBI. These results, in the context of other study findings, further support preliminary evidence that the DANA Rapid version may serve as a clinically useful and improved neurocognitive concussion screen where professional medical evaluation is unavailable or delayed.

From a practical standpoint, the clinical definition of concussion has, historically, relied upon observable signs (e.g., LOC); however, we also know that a high percentage of concussions (90%) do not present with LOC, and the U.S. Department of Defense uses either an alteration of consciousness or LOC in its definition of concussion (11, 25, 26). Accordingly, there is a need for instruments that assess beyond observable symptoms. Based on our findings, the DANA Brief appears to meet this need. It can be used acutely in the remote and austere setting, and provides accurate assessment, including the assessment of cognitive constructs above and beyond those of other established measures (i.e., the MACE). Current findings

provide at least preliminary evidence that the DANA Rapid may be a reasonable alternative to the more extended DANA Brief. Specifically, although the DANA Rapid excludes memory-based subtests, it includes the GNG and PRO, which appear to be more sensitive in the current analysis. It also includes two administrations of SRT, a measure of basic reaction time, which is one of the more sensitive measures of impairment after concussion.

Regarding our second hypothesis, since repeat testing over multiple days did not result in significantly improved DANA subtest performance, we did not find support for practice effects with repeated administration of the DANA Brief. It is unclear whether this was a function of the specific cognitive constructs assessed with the DANA Brief, as the extant neuropsychological literature suggests that practice effects do not respect any one domain or type of test (27). This is not a negative finding, as the goal of psychometric test development, used in serial monitoring, is to avoid practice effects (28).

The current findings have clear implications for the methodology or clinical practice of concussion assessment in multiple acute settings (e.g., emergency room, athletic field sideline). There is a need for future studies to assess the utility of the DANA Brief over longer injury and recovery intervals, as well as in concussed individuals. As discussed by McCrea, et al., the effect sizes of brief test instruments tend to diminish within the first week after injury (11). While this aligns with typical recovery curves in patients with uncomplicated mTBI, there is a subset that will continue to experience symptoms, including those in the cognitive domain. Therefore, showing sensitivity beyond these brief intervals, especially in concussed vs. non-concussed individuals, would further extend the utility of the DANA Brief. Future studies should counterbalance the administration of the DANA and the MACE to eliminate the potential of an order effect. In addition, there is certainly a need for generalizability, which may also be addressed in future studies by the use of a larger sample size. Studies have shown differential recovery curves, with interactions noted between age, gender, and symptom subset (29, 30). Including females, non-military, and age-varied participants would be worthwhile in this regard, as it would address the primary limitations of the current study. Although the cognitive assessment portion of the recently released MACE 2 is the same as is used in the MACE, the MACE 2 incorporates additional clinical assessment, including vestibular-ocular-motor screening. Therefore, more comprehensive future studies should compare the DANA and MACE 2. Finally, future studies would benefit from a larger sample size to increase power and reliability coefficients, bolstering the interpretation of diagnostic data.

While current findings provide at least preliminary evidence that the DANA Rapid version may serve as a clinically useful and improved neurocognitive concussion screen where professional medical evaluation is unavailable or delayed, future studies are needed to validate this potential by addressing identified study limitations. The use of the DANA Rapid in multiple acute settings would benefit service members, athletes and other individuals with concussion by minimizing the time between injury and diagnosis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of the United States Army Medical and Materiel Command. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

JT conceived of the study. JT and AM designed the study. JT and SF were responsible for the acquisition of data. JP designed and reviewed the biostatistical analysis, which was conducted by JW. JP and BB interpreted the data. JP was the primary author responsible for the collaborative effort of drafting (JP, BB, EO'C, and AP) and revising (JP, BB, AM, SF, JW, and JT) the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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