AMPAR Peptide Values in Blood of Nonathletes and Club Sport Athletes With Concussions

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ABSTRACT Objectives: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) peptide, a product of the proteolytic degradation of AMPA receptors in healthy nonathletes and athletes with concussions, is assessed. The detection of AMPAR peptide in conjunction with neuropsychological testing and neuroimaging is undertaken. Subjects: Persons (n = 124, 19–23 years) are enrolled in the pilot-blinded study according to approved Institutional Review Board protocols at Kennesaw State University and DeKalb Medical. Methods: AMPAR peptide plasma assay was performed using magnetic particles-enzyme-linked immunosorbent assay. All participants had neurocognitive tests (ImPACT); selected subjects with concussions were followed-up with magnetic resonance imaging and neurologic consultations. Results: Athletes (n = 33) with clinically defined single or multiple concussions were compared to 91 age and gender matched controls without a history of concussion. AMPAR peptide values of 0.05–0.40 ng/mL for controls and 1.0–8.5 ng/mL for concussions are found. The biomarker sensitivity of 91% and a specificity of 92% (0.4 ng/mL cut off) to assess concussions are calculated. Poorer ImPACT scores correlated with abnormal levels of the biomarker. In athletes with multiple concussions, increased AMPAR peptide values (2.0–12.0 ng/mL) were associated with minor findings on magnetic resonance imaging. Conclusion: AMPAR peptide assay combined with ImPACT and neuroimaging is a promising tool for assessment of concussions. Additional clinical validation studies are required.

INTRODUCTION

Mild traumatic brain injury (TBI) is the most prevalent form of injury in military and civilian settings. Brain injuries caused by explosions have become some of the most common combat wounds suffered in the field. Sports and recreational activities contribute up to 21% of all cases of mild TBI including concussions.[1]

Assessment of concussion regardless of origin is complicated. Many primary concussions go unrecognized or are not reported, particularly when there is no loss of consciousness.[2] Additionally, without sufficient reports of prior incidents, soldiers and competitive athletes are often subjected to multiple concussions. Advanced neuroimaging techniques (diffusion tensor imaging, functional magnetic resonance imaging, and positron emission tomography) that can register minor structural and functional changes are primarily used for research. These modalities are not available in emergent situations or for routine clinical evaluations and have a limited application in persons with metal implants[3] or claustrophobia.[4]

Currently, there is an unmet diagnostic need for a rapid and affordable assay to detect brain-specific biomarkers in the bloodstream following a concussion. A novel biomarker should be able to differentiate subtle brain injury associated with concussions from non–central nervous system-injured individuals. In addition, it could speed up assessment of concussions on the field in competitive contact sports or during combat to assist in determining when an injured athlete should return to play or a soldier may return to duty.

It is well known a family of location-specific glutamate receptors is involved in more than 80% of cortical and subcortical neuronal communications underlying superior mental functions.[5] However, the practical application of glutamatergic mapping in human brain injuries is limited. Recently, it was shown that an ionotropic type of glutamate receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor [AMPAR]) represents a biomarker of neurotoxicity cascade underlying subtle brain injury.[6]

AMPARs are primarily distributed in the forebrain and subcortical pathways including the hippocampus, amygdala, thalamus, hypothalamus, and brain stem.[7–9] This architecture supports the noncortical nature of subtle brain injury. These regions of the brain are predictable sources of biomarkers given the functional spatial–temporal coherence, developmental pathways, and cerebral plasticity subjected to coup–countercoup mild brain injury.[10]

During the acute phase of mild TBI or following axonal injury, a massive release of glutamate, which up-regulates excitotoxic AMPARs has been detected.[11] The GluR1-subunit, of N-terminal AMPAR fragments is rapidly cleaved by extracellular proteases and released into the bloodstream through the compromised blood–brain barrier. This degradation product can be detected directly as AMPAR peptide fragments (molecular weight 5–7 kD) by use of a specific immunoglobulin raised against AMPAR peptide.[11]

The AMPAR peptide has not previously been assessed as a biomarker in subjects with concussions. In this study we

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examine the diagnostic potential of the AMPAR peptide assay in conjunction with neuropsychological testing and neuroimaging to differentiate those with concussions from healthy controls.

METHODS

This article summarizes data from a and T1 prospective, blinded study performed from September 2011 to March 2012 at two clinical sites. The local Institutional Review Boards for each site approved the study, and written informed consent was obtained from each enrolled subject. Participants with semiacute (1–2 weeks) concussion and healthy controls were recruited at Kennesaw State University (KSU). All participants completed a standard questionnaire detailing medical history, medication use, and history of any prior concussion, in addition to informed consent.

Subjects

A total of 84 club sport athletes (56 male, 28 female; ages 20.8 ± 1.8 years) and 40 nonathletes without a history of concussion (21 male, 19 female; ages 22.0 ± 4.1 years) were included in the pre-season (baseline) study (n=124). The majority of participating athletes represented the KSU Sports & Recreation Department, where rugby (23%), soccer (23%), lacrosse (16%), and cheerleading (14%) occupy about 76% of the athletic curricula.

Among the athletes, 33 had clinically confirmed single (n = 20) or multiple (n = 13) concussions registered by a certified athletic trainer on the field and confirmed by an experienced neurologist within 1–2 weeks of the event. The 40 non-athletes and 51 athletes without a history of recent concussions (eg, within the previous year) formed the control group (n = 91). Data from all participants were included in the final study analysis.

Neurocognitive Testing

All study participants had baseline neurocognitive testing (ImPACT, version 2.1). In addition, the postconcussion scale embedded in ImPACT testing was offered to all participants at baseline to determine the presence of 1 to 22 commonly reported symptoms after concussions.[12]

Clinical and Radiological Procedures

Selected athletes (n = 3) underwent a standard neurological and general medical evaluation by an experienced neurologist.

Standard MRI included: axial fluid attenuation recovery and T1-sagittal, T2-weighted axial that shows areas of permanent axonal damage and the total number of lesions, respectively. MRI images were obtained on a clinical MRI scanner (Siemens 1.5 Tesla). General Electric (GE) Centricity software was used for image analysis.

Sample Collection and Storage

At the time of enrollment, each study subject had a single blood draw. Selected subjects had two additional blood samples drawn during a follow up-visit (within 6 months of the first sample withdrawal). Blood samples (5 mL) were drawn by venipuncture into vacuum tubes (Becton Dickinson) containing sodium ethylenediaminetetraacetic acid, placed on ice. Plasma drawn from each participant was separated by centrifugation at 3,000 rpm for 5 minutes at 4°C. Sample processing was completed within 30 minutes of blood collection to protect the AMPAR peptide from depletion by endogenous proteases. Hemolyzed plasma was not accepted for the study because of cross-reaction with hemoglobin.

Processed samples were stored as multiple 0.5-mL aliquots (n = 6) at −80°C at KSU Lab.

AMPAR Peptide Detection in Plasma

Aliquots from each batch of samples were analyzed for AMPAR peptide (GRACE Labs, LLC, Decatur, GA) by a blinded investigator. Once an aliquot was thawed for testing and used, remaining amounts were discarded.

Briefly, 20 μL plasma samples, five calibrators, negative/positive controls in duplicates, and 80 μL of working mixture consisting of magnetic particles with covalently attached specific antibodies against AMPAR peptide were added to the microtiter plate. The mixture was incubated for 2 minutes at 37°C; AMPAR antibodies labeled with horseradish peroxidase solution were then added for 20 minutes at 37°C. After the bound magnetic particles were washed with a buffer using a magnetic separator, the reaction was revealed by pipetting 100 μL ready-to-use tetramethyl benzidine substrate into each well of the microtiter plate. The color reaction was developed for 8 minutes. At 25°C, the reaction was stopped with acid solution (100 μL), and monitored at 490/630 nm on a microplate reader (Bio Tek ELx800, BioTek Instruments).

AMPAR peptide concentrations in plasma were determined by plotting absorbance values on a calibration curve constructed from the absorbance units of each calibrator and their known concentrations. The intra-assay coefficient of variation was 5.1%–6.2%, and the interassay coefficient of variation was 5.7%–9.5%.

Statistical Analysis

Differences between groups were assessed using descriptive statistics and standard tests of significance. Univariate statistical analyses with 95% confidence interval (CI) were calculated. Analyses were performed on the study subjects using R Statistical Package (http://www.r-project.org/). Continuous independent variables were compared by the use of the one-way analysis of variance followed by post-hoc test.

A receiver operator characteristic (ROC) curve was used to calculate the cutoff value for optimal sensitivity and specificity.[13] ROC curves (sensitivity vs. 1-specificity) for concussion vs. controls by varying the cutoff value were built. The gold standard used for constructing the ROC curve was based on the diagnoses. We used the partial area under the ROC curve for the region with specificity between 0.75 and 0.95, as a global

TABLE I. Enrolled Subjects Demographic Data

Group	N	Age	Gender M/F	Distribution			
				African–American	Caucasian	Hispanic	Asian
Concussion	33	21.0 ± 3.3	24/9	3	26	3	1
Control	91	21.0 ± 3.0	66/25	12	73	4	2

TABLE II. ImPACT Test Results (Baseline) Taken by Study Participants

Parameter	Cutoff[a]	ImPACT Result[c]		t-Test
		Controls N = 91	Concussion N = 34	
Verbal Memory	64.5	89.8 ± 7.9	87.9 ± 9.5	$p = 0.37$
Visual Memory	46	78.9 ± 11.1	72.2 ± 13.1	$p = 0.021$
Processing Speed	23.5	41.6 ± 5.5	39.9 ± 6.3	$p = 0.21$
Reaction Time[b]	0.78	0.59 ± 0.08	0.59 ± 0.06	$p = 0.06$
Cognitive Efficiency	—	0.40 ± 0.11	0.34 ± 0.10	$p = 0.007$

[a]At 80% sensitivity.[9] [b]For this variable, a higher score represented poorer performance, for remaining variables, a lower score represented worse performance. [c]Data presented as mean ± SD.

TABLE III. AMPAR Peptide Reference Values

AMPAR Peptide (ng/mL)	Controls (n = 91)		
	N	% Absolute	% Population
<0.1	12	13.2	13.2
0.1–0.2	31	34.1	47.3
0.2–0.3	44	48.4	95.7
>0.4	4	4.3	100

measure of the diagnostic effectiveness of the AMPAR peptide assay. To test the global null hypothesis that the AMPAR peptide does not have adequate accuracy to differentiate concussions from controls, we evaluated whether the area under curve was at least 0.8.

RESULTS

The average age, gender, and race distribution is presented in Table I for each group. ImPACT testing showed all four neurocognitive composite scores above the 30th percentile for age-normative parameters (Table II). The athletes with concussion(s) had significantly lower visual memory scores ($p = 0.02$, independent samples t-test, Welch test, 95% CI) and cognitive efficiency index ($p = 0.007$, independent samples t-test, Welch test, 95% CI). Baseline assessment of the postconcussion scale embedded in ImPACT showed that athletes, independently of number of concussions and the time since last injury, complained about poor memory and trouble concentrating, problems with falling asleep and drowsiness, persistent fatigue, and headaches.

Samples from apparently healthy males ($n = 66$) and apparently healthy females ($n = 25$), in the clinically relevant age range of 19–23 years, were evaluated with the AMPAR peptide assay. The distributions of enrolled control population ($n = 91$) according to AMPAR peptide levels are presented in Table III. The reference interval calculated from the samples (96%) was found to be 0.05–0.40 ng/mL for both genders. In control group, 4.3% persons had AMPAR peptide levels of >0.40 ng/mL (Table III).

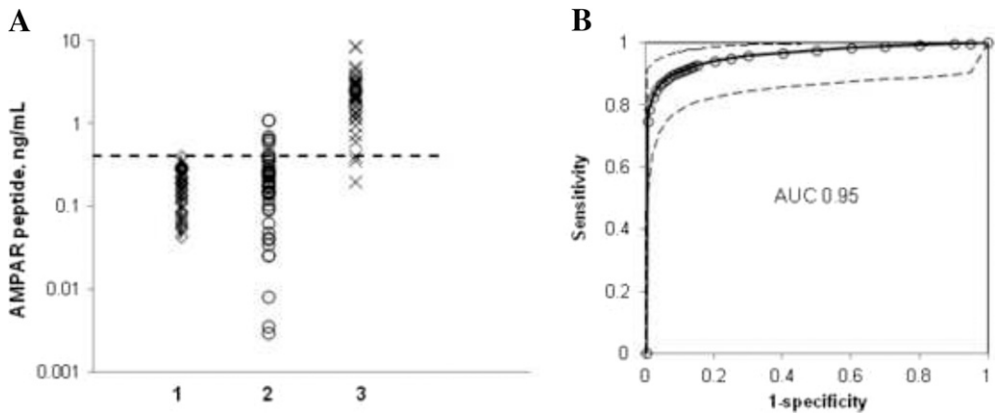

FIGURE 1. Distribution of plasma AMPAR peptide concentrations in A: (1) nonathletes ($n = 40$); (2) athletes with no history of recent concussions ($n = 51$); and (3) concussions ($n = 34$). ROC curve for plasma AMPAR peptide (B). AUC is 0.95 calculated for the biomarker potential to distinguish concussions vs. controls.

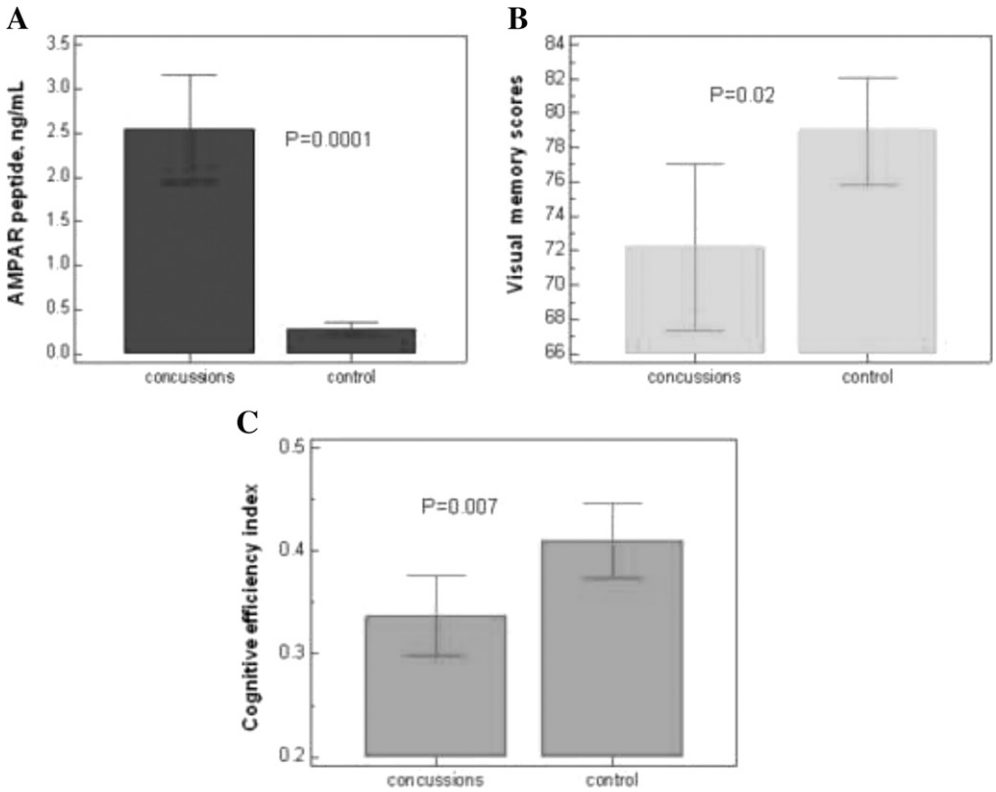

FIGURE 2. Independent samples *t*-test of (A) AMPAR peptide in plasma of subjects enrolled, (B) visual memory scores, and (C) cognitive efficiency index of ImPACT test for concussions and all controls.

The AMPAR peptide plasma concentrations for each group are shown in Figure 1A. There were no adverse events from performing the AMPAR peptide assay. Thirty athletes with concussions had an increased AMPAR peptide level with a median concentration of 2.15 ng/mL (0.96–8.49 ng/mL). Three subjects with concussions had low AMPAR peptide concentrations (0.2–0.36 ng/mL). Nonathletes ($n = 40$) had a median value of 0.19 ng/mL (0.043–0.40 ng/mL). A group of athletes with no history of recent concussions ($n = 44$) had a median value of 0.21 ng/mL (0.003–1.10 ng/mL), whereas seven individuals from the same group showed increased AMPAR peptide values (0.44–1.10 ng/mL). Group comparison of median values of the AMPAR peptide concentration showed significant differences ($p < 0.0001$, independent samples *t*-test, Welch test, 95% CI) for the concussion group compared with all controls (Fig. 1B). AMPAR peptide values within controls belong to the same distribution ($p < 0.001$, one-way analysis of variance, Levene's test for equality of variances).

There was a moderate correlation between high AMPAR peptide levels in concussion group (Fig. 2A) and poor visual memory scores (Fig. 2B) or reduced cognitive efficiency index (Fig. 2C).

Operating characteristics of the AMPAR peptide are depicted in Table IV. The predictive values and likelihood ratios at specific cutoff points were chosen to approximate a sensitivity of 82%, 91%, 98%, and specificities of 83%, 92%, 98%, respectively. A sensitivity of 91% and a specificity of 92% with a positive predictive value of 82% for the AMPAR peptide assay to diagnose concussions are achieved at optimal cutoff value of 0.4 ng/mL. Considering a pretest probability

TABLE IV. Operating Characteristics of AMPAR Peptide Assay at Different Cutoff Values

	AMPAR Peptide Assay Performance at Respective Cutoffs		
Parameter	0.3 ng/mL	0.4 ng/mL	1.0 ng/mL
Sensitivity, % (95% CI)	97.1 (84.7–99.9)	91.2 (76.3–98.1)	82.4 (65.5–93.2)
Specificity, % (95% CI)	83.5 (74.3–90.5)	92.3 (84.8–96.9)	97.8 (92.3–99.7)
Positive Predictive Value, % (95% CI)	68.8 (53.8–81.3)	81.6 (65.7–92.3)	93.3 (77.9–99.2)
Negative Predictive Value, % (95% CI)	98.7 (93.0–99.9)	96.6 (90.3–99.3)	93.7 (86.8–97.7)
Positive Likelihood Ratio (95% CI)	5.89 (3.69–9.39)	11.9 (5.77–24.34)	37.47 (9.43–148.9)
Negative Likelihood Ratio (95% CI)	0.04 (0.01–0.24)	0.10 (0.03–0.28)	0.18 (0.09–0.37)

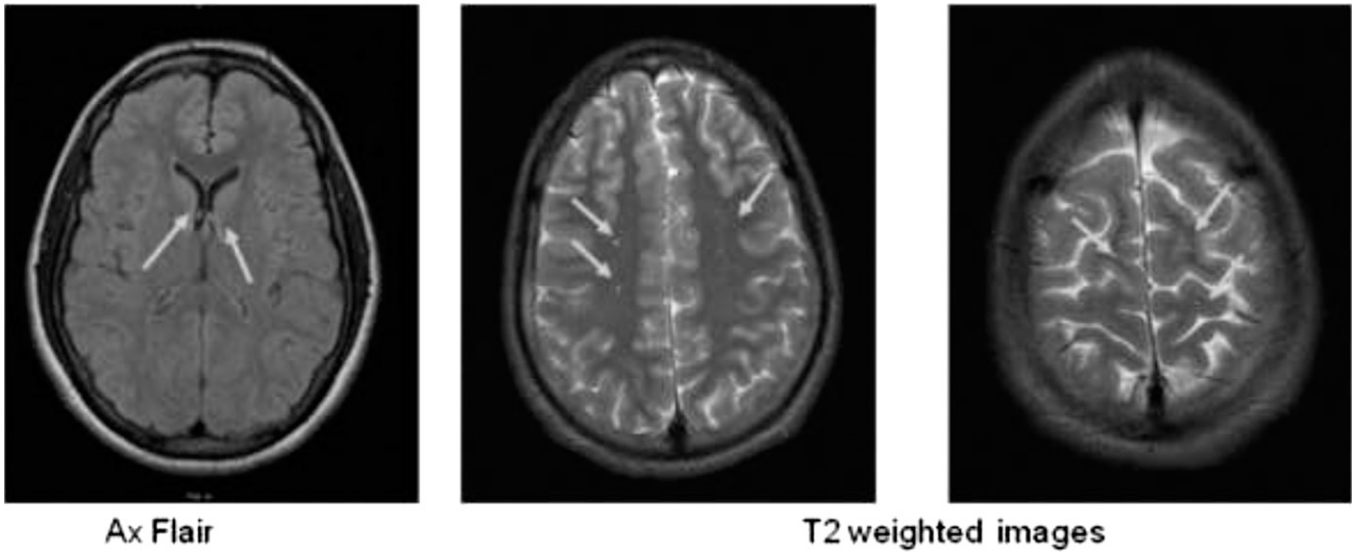

FIGURE 3. Basic 1.5T MRI of individual with concussions performed within 1 month after concussion. Arrows in Axial Flair and T2-weighted images (TE/TR = 103/4790 ms; FOV 424 × 23 mm; slice thickness = 5 mm, flip angle = 150°, matrix 0/512/224/0) depicted areas of micro bleeding and slightly increased number of enlarged high-convexity white matter VRS.

of concussions of 0.37, positive and negative posttest probabilities resulted in 96% (95% CI, 88%–98%) and 4% (95% CI, 2%–8%), respectively.

The trade-offs between true-positive and false-positive rates are shown by presenting the data as a traditional ROC curve (Fig. 1B). The proportional areas under curve comparing concussions vs. controls yielded a close-up value of 0.95.

Based on poor ImPACT scores and abnormally high AMPAR peptide values, 3 athletes with multiple concussions were recommended for neurological consultation and MRI. There were no abnormal signals in the typical locations for axonal shearing damage in 1.5T images for all three subjects. No significant motor, sensory, or cognitive deficits on their neurological exams were noted. These athletes had a history of multiple concussions with persistent headache and mild cognitive dysfunction. The symptoms appeared to be resolved within 3 weeks for two athletes, with corresponding reduction of AMPAR peptide values. The individual who maintained high AMPAR peptide values for 6 months after injury (5.2 ng/mL) showed minor changes on MRI with slightly increased number of enlarged high-convexity white matter Virchow–Robin spaces (VRS) (Fig. 3).

DISCUSSION

Biomarkers of excitatory neurotransmitter receptors may assist in the assessment of subtle, or asymptomatic, dendritic or axonal injuries. It has been proposed that biomarkers of neurotoxicity and abnormal spiking activity may be useful in the assessment of concussions.[6] It is known that AMPA excitatory receptors are located on dendrites and axons of neurons,[14] and regulate glutamatergic neurotransmission.[15]

This study focused on the diagnostic potential of the AMPAR peptide assay to differentiate subjects with concussions from controls. The analysis of AMPAR peptide value variance in subjects with concussions and all controls yielded statistically significant ($p < 0.0001$) increases in AMPAR peptide values in those with concussions.

Data analyses provided a preliminary cutoff value of 0.4 ng/mL. Several athletes (7 of 91) with no history of recent concussions showed an increase AMPAR peptide of 0.44–1.10 ng/mL, perhaps due to a prior unreported concussion. Even with this false-positive levels, a statistically significant increase in AMPAR peptide in concussions compared with controls ($p < 0.0001$) has been observed. An association between AMPAR peptide values and decrease in visual memory scores or reduced cognitive efficiency index for athletes with concussions was found. It could be hypothesized that the glutamate synaptic transmission dysfunction may contribute to the development of cognitive deficits[16] and alteration of plasticity in visual system at the subcortical level.[17]

In three subjects who presented with concussions, the AMPAR peptide concentrations were below the 0.4 ng/mL threshold. We treated these results as false negative. Measurement of the AMPAR peptide with a preliminary threshold of 0.4 ng/mL for persons with concussions because of sporting activities, car accidents, or blasts may have a potential clinical indications; ie, to assist in the emergent diagnosis of mild TBI vs. non-TBI before other diagnostic procedures. This premise is supported by the predictive value of the test for recognizing individuals with concussions (91% at 0.4 ng/mL cutoff; likelihood ratio, 11.9) and preliminary assessment of correlating biomarker with ImPACT scores. Conversely, the test could be used for ruling out individuals without

concussions. If the test is negative at a preliminary cutoff point of 0.4 ng/mL, the posttest probability of concussions would be low (about 4%).

In this pilot study, the biomarker was measured in a single blood draw taken on enrollment from all participants. However, three 3 athletes with persistent symptoms had additional AMPAR peptide assays within 6 months after the initial enrollment. One of these three had an increased number of dilated white matter VRS depicted on Axial T1 and T2-weighed MRI scans (Fig. 3), perhaps due to shear–strain injury.[18] Further investigations of the biomarker in acute concussions applying advanced 3T MRI modalities (functional magnetic resonance imaging, diffusion tensor imaging, and diffusion weighted image) may clarify the nature of AMPAR peptide changes in diffuse axonal injury.

These results suggest that the AMPAR peptide could serve as a brain-specific biomarker to differentiate concussed athletes from nonconcussed individuals. Combined with neuropsychological testing and advanced neuroimaging, the biomarker has a diagnostic potential to assess concussions. Further studies of the AMPAR peptide assay should be devoted to evaluating this clinical indication for subjects with acute mild TBI.

STUDY LIMITATIONS

The pilot study examined the diagnostic potential of the AMPAR peptide assay in conjunction with neuropsychological testing and neuroimaging to differentiate those with concussions from healthy controls. In this study, the pre-season, or baseline assessment of AMPAR peptide values in club sport athletes relatively nonathletes control has been undertaken. Future investigations analyzing the in-season (or after season) AMPAR peptide concentration in these athletes and comparing them with preseason values will help estimate the potential of false-positive AMPAR peptide assays.

An additional study with increased sample size is needed performance characteristics. According to a guideline for the minimum number of cases to include in such a study,[19] the smallest proportions of negative or positive cases in each group with proportion of positive cases in the population about 0.30 (30%) should be >67.

A body of advanced MRI-based analyses of micro-lesion size should be performed simultaneously with biomarker detection to estimate the preliminary correlation between damaged area and AMPAR peptide levels. There may be a significant time lag between blood draw and MRI (24–72 hour), which could confound findings. A study of a subpopulation of subjects who presented early after concussion (within 3 hours) and who had rapid advanced imaging to gain a first glimpse into the relation between dynamic changes in brain tissue and its effect on biomarker levels would be needed.

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