Neurochemical aftermath of repetitive mild traumatic brain injury

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ABSTRACT

Importance: Evidence are accumulating that repeated mild traumatic brain injury (mTBI) incidents can lead to persistent, long-term debilitating symptoms, and in some cases a progressive neurodegenerative condition referred to as chronic traumatic encephalopathy (CTE). However, there are no objective tools to examine to which degree persistent symptoms after mTBI are due to neuronal injury.

Objective: To determine whether persistent symptoms after mTBI are associated with brain injury as evaluated by cerebrospinal fluid (CSF) biochemical markers for axonal damage and other aspects of central nervous system (CNS) injury.

Design, settings, and participants: A multicenter cross-sectional study involving professional Swedish ice hockey players who have had repeated mTBI, and post-concussion symptoms for over 3 months, and fulfilling the criteria for post-concussion syndrome (PCS) according to the Diagnostic and Statistical Manual of Mental Disorder-IV, as well as neurologically healthy controls. The participants were enrolled between January 2014 and February 2016. All players were also assessed with Rivermead Post-Concussion Symptoms Questionnaire (RPQ) and magnetic resonance imaging.

Main Outcomes and Measures: Neurofilament light protein (NF-L), total-tau (T-tau), glial fibrillary acidic protein (GFAp), amyloid-β (Aβ1-42), phosphorylated tau (P-tau), and neurogranin (Ng) concentrations in CSF.

Results: A total of 31 participants (16 with PCS, and 15 controls) were assessed. Of 16 players with PCS, nine had PCS symptoms > 1 year, while the remaining seven returned to play within a year. NF-L were significantly increased in players with PCS > 1 year compared with players whose PCS resolved within 1 year, as well as controls ($P = .035$ and $P = .021$, respectively). Furthermore, NF-L concentrations correlated with RPQ scores and lifetime concussion event ($\rho = 0.58$, $P = .020$ and $\rho = 0.52$, $P = .040$, respectively). Overall, players with PCS had significantly lower CSF Aβ1-42 levels compared to controls ($P = .048$).

Conclusions and Relevance: Increased CSF NF-L and reduced Aβ1-42 were observed in PCS, suggestive of axonal white matter injury and amyloid deposition. Measurement of these biomarkers may be an objective tool to assess the degree of CNS injury in individuals with PCS, and to distinguish individuals who are at risk of developing CTE.

Introduction

Traumatic brain injury (TBI) represents a leading cause of mortality and morbidity worldwide, with 1.6–3.6 million sports-related TBIs occurring annually alone in the Unites States and additional large numbers occurring in civilian and military personnel.[1] Mild TBI (mTBI), commonly also referred to as concussion in the literature, is a type of mTBI that is caused by rapid acceleration, deceleration and rotational forces to the head, which cause the brain to deform, resulting in stretching of individual neurons, glial cells and blood vessels.[2]

Symptoms of mTBI usually resolve within days to weeks, but in 10-15% of individuals neurological symptoms persist for more than three months.[3] The presence of neurological symptoms lasting more than three months in individuals who have suffered from mTBI is referred to as post-concussion syndrome (PCS).[3] A proportion of individuals exposed for repeated mTBI episodes may develop a progressive neurodegenerative condition referred to as chronic traumatic encephalopathy (CTE).[4,5] CTE shares similar histopathological changes with other neurodegenerative disorders, in particular Alzheimer's disease (AD), including hyperphosphorylation of tau protein in neurofibrillary tangles and in a proportion of cases also deposition of amyloid β (Aβ) in diffuse plaques.[6,7]

The relationship between repeated mTBIs and development of PCS is poorly understood, specifically to which degree such long-lasting symptoms are due to neuronal damage, and to which degree psychogenic or psychosocial mechanisms contribute. Notably, there are no objective biomarkers to quantify neuronal damage

or other types of central nervous pathology in individuals with PCS. At present, PCS diagnosis is mainly based on self-reporting clinical symptoms, while CTE diagnosis can only be made post-mortem.[4,5,8,9] Further, the relation between PCS and future development of CTE is unknown.

The cerebrospinal fluid (CSF) is in direct contact with the brain parenchyma and is a suitable biofluid to monitor biochemical changes in the central nervous system (CNS). CSF biomarkers reflecting amyloid and tau pathology have been validated extensively in the context of AD.[10-14] Recently, independent reports have shown that neurodegenerative disorders, mainly AD, also are associated with increased levels of CSF neurogranin (Ng), which reflect synaptic degeneration and loss,[15-17] as well as increased CSF neurofilament light protein (NF-L), reflecting injury to large-caliber myelinated axons in the white matter.[18,19]

Considering that CTE shares many neuropathological changes with AD, we hypothesized that PCS would also display similar pathophysiology, at least in CSF. We specifically tested the following hypotheses: i) PCS is associated with axonal injury and astrogliosis, as reflected by increased CSF concentrations of the axonal proteins total-tau (T-tau) and NF-L, as well as the astroglial protein glial fibrillary acid protein (GFAp), and ii) PCS is associated with increased amyloid burden and tau pathology, as well as synaptic loss, as reflected by altered CSF concentrations of the 42 amino acid variant of Aβ (Aβ1-42), phosphorylated tau (P-tau), and the synaptic biomarker Ng.

Methods

Study population

In this multicenter cross-sectional study, we enrolled 16 male professional ice hockey players with prolonged post-concussive symptoms for over three months and 15 neurologically healthy controls between January 2014 and February 2016. The study was approved by the Ethics Committee for Medical Research at the University of Gothenburg, Sweden. Written informed consent was obtained from all participants.

Selection of participants

The diagnosis of concussion was made according to the latest diagnostic guidelines on sports-related concussion and players with concussion were managed according to these guidelines.[20,21] The diagnosis of PCS was based on Diagnostic and Statistical Manual of Mental Disorder-IV (DSM- IV) criteria.[22] The inclusion criteria were: 1) persistent post-concussion symptoms for more than three months following mTBI, 2) no contraindications to lumbar puncture (LP) (decreased platelet count (< 50 x 10^9/L), focal neurologic sign, papilledema, reduced consciousness, infection at puncture site), and 3) no evidence of structural damage on conventional magnetic resonance imaging (T1/T2 and flair).

The inclusion criteria for control subjects were: 1) age > 18 years, 2) no history of known head trauma, 3) no history of neurological or psychological condition, and 4) no contradictions to LP as stated above.

At inclusion, the participants underwent neuropsychological assessment with Rivermead Post Concussion Questionnaire (RPQ)[23], and the PCS group was also followed with repeated RPQ assessment at the end of the study. One of the players agreed to undergo repeated LPs at inclusion, 11 months, 17 months and 23 months after the injury.

Biochemical procedures

CSF was collected in polypropylene tubes by LP through the L3-4 or L4-5 interspace. All CSF samples were stored at −80 °C pending analysis. The participants were examined physically and neurologically before LP. All were healthy and showed no signs of focal neurologic injury.

CSF NF-L concentrations were measured using a commercial ELISA (NF-light® ELISA, Uman Diagnostics, Umeå, Sweden) as described previously.[24] CSF GFAp concentrations were measured using a previously described in-house ELISA procedure.[25] CSF concentrations of Aβ1-42, T-tau, and P-tau were measured with INNOTEST (Fujirebio Diagnostics, Japan). CSF Ng concentrations were measured using an in-house developed ELISA employing two monoclonal antibodies. In brief, the monoclonal antibodies were developed by immunizing 8-week-old Balb/c mice with the KLH-conjugated peptide Ng52–75 or Ng 63-75 (Caslo, ApS Denmark) in complete Freund's adjuvant (Sigma). Ng22 (with the epitope 63-75) and Ng2 (with the epitope 52-63) were selected for the final assay set up and the method protocol was developed as follows. Nunc maxisorp 96-well microliter plates were coated with 3μg/mL of Ng22 in bicarbonate buffer (pH 9.6) and incubated over night at + 4°C. After washing with PBS (0.01 M phosphate buffer, 0.14 M NaCl, pH 7.4)-Tween

(0.05%) the plates were blocked with 200 µL PBS-Tween (0.05%) with 1% BSA. After the second wash, 100 µL of calibrators (full length 1-78 neurogranin (Caslo)), quality control CSF samples and CSF samples was loaded in each well and incubated over night at + 4°C. After washing, 100 µL of the detection antibody, biotinylated Ng2, diluted to 2.7µg/mL in PBS-Tween (0.05% BSA) with 1% BSA, was incubated at room temperature for 1 hour, shaking. After washing, 100µL Enhanced Streptavidin-HRP (Kem-En-Tec Diagnostics) was added in a 1:20000 dilution in PBS-Tween (0.05%) with 1% BSA and incubated for 30 min. After a final wash, 100 µL of substrate (TMB One Substrate, Kem-En-Tec Diagnostics) was incubated in the dark for 20 minutes and the reaction was quenched with 100 µL 2 M H_2SO4. The plate was read at 450 nm (reference wavelength 650 nm) using an ELISA plate reader (Vmax, Molecular Devices, USA) and the calibration curve was plotted using a 4-parameter curve and calculations were made using SoftMax. The assay ranged between 6400 pg/mL and 50 pg/mL, where 50 pg/mL was set as the limit of quantification. Within and between plate coefficients of variability were < 5% and < 13%, respectively.

All samples were analyzed at the same time using the same batch of reagents by board-certified laboratory technicians who were blind to clinical information.

Statistical analysis

For the PCS versus controls comparison, the Mann-Whitney *U* test and independent sample *t* tests were used. Dunn's correction was performed for all multiple comparisons. The Spearman's rank correlation coefficient (r_s) was used for analyses of correlation between changes in various biomarker levels and lifetime concussion

event, as well as RPQ score. All tests were two-sided and statistical significance was determined at $P < .05$. All statistical calculations were performed using GraphPad Prism 6.0 (GraphPad Inc., San Diego, CA).

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Results

Characteristics of the subjects

Sixteen male players with PCS (median age, 31 years; range, 22-53) and 15 male neurologically healthy controls (median age, 25 years; range, 21-35) were enrolled between January 2014 and February 2016 (Table 1). Nine of the 16 players had persistent PCS symptoms for more than one year and retired from the game, while seven players returned to the game within one year from the injury.

Axonal white matter damage in players with persistent PCS

CSF concentrations of NF-L were increased in the PCS group as compared with the control group, but the increase did not reach statistical significance (Figure 1A). However, the concentrations of NF-L were significantly higher in the subgroup of players with PCS > 1 year vs. PCS < year (P = .035), as well as compared to the control group (P = .021) (Figure D). No significant differences were observed in the concentrations of T-tau or GFAp (Figure 1B,C, and E).

Emerging CSF amyloid pathology in PCS

CSF concentration of Aβ1-42 was significantly (P = .048) lower in the PCS group as compared to controls (P = .048) (Figure 2A). The subgroup of players with PCS > 1 year had lower Aβ1-42 concentrations compared with players whose symptoms resolved within 1 year (P = .091), although the change was not statistically significant after correcting for multiple comparisons (Figure 2D). There was a trend towards lower concentrations of P-tau and Ng at group level, the differences were not significant though (Figure 2B, C, and F).

Reduced CSF Aβ1-42, and increased Ng and NF-L over time

Mean CSF NF-L concentration remained more than 2-fold increased compared to the mean of the controls in one of the player who underwent repeated lumbar punctures (Figure 3A). In addition, the player displayed reduced concentrations of Aβ1-42 compared to the controls, while the concentrations of Ng were increased (Figure 3B) at all sampling time points. There was also a trend toward higher concentrations of CSF P-tau compared to controls (Figure 3B). The concentrations of other measured biomarkers were essentially unchanged (Figure 3B).

CSF NF-L correlated with symptom severity in PCS

As expected, the PCS group had higher RPQ score (median, 22; range (8-35) compared to the controls (median, 0; range, (0-0), $P < .0001$). CSF NF-L concentrations correlated with RPQ scores ($\rho = 0.58$, $P = .020$) (Figure 4A). There was no significant relationship between the concentrations of other measured biomarkers and RPQ (Figure 4B-F).

CSF NF-L and P-tau correlate with lifetime concussion event

CSF NF-L correlated with lifetime concussion event ($\rho = 0.52$, $P = .040$) (Figure 5A). There was also a significant correlation between CSF P-tau and lifetime concussions ($\rho = 0.55$, $P = .030$) (Figure 5E). No significant relationships were observed between the lifetime concussion events and concentrations of the other biomarkers in the study (Figure 5B-D and F).

Discussion

Symptoms of mTBI usually resolve within days to weeks.[3] However, a subgroup of individuals with mTBI, mainly repeated mTBI, display persistent physical, cognitive, and behavioral impairment for months, referred to as PCS.[3] The relation between PCS and development of future CTE is unknown. Thus, improved methods of characterizing neurodegenerative processes triggered by repeated mTBI, and identifying individuals at risk of developing PCS or progressive neurodegeneration are needed. To our knowledge, this is the first study to investigate CSF biomarkers reflecting axonal white matter injury, amyloid burden, tau pathology, and synaptic loss in professional ice hockey players who have suffered repeated mTBI and fulfilled the criteria for PCS. We found that i) a subgroup of individuals with PCS (those with chronic symptoms forcing them to retire) displayed increased concentrations of CSF NF-L compared with those whose PCS resolved within one year, as well as controls, ii) CSF concentrations of NF-L correlated with RPQ scores and lifetime concussion events, iii) overall, the PCS group had lower CSF Aβ1-42 concentrations compared with controls, and iv) there were no PCS-related changes in CSF T-tau, GFAp and Ng concentrations.

NF-L is a structural protein that is highly expressed in the large-caliber myelinated subcortical axons of the white matter.[18,19] The findings that NF-L is elevated in CSF of professional athletes with PCS, and CSF concentrations of NF-L correlated with RPQ scores, add support for the hypothesis that axonal white matter injury is a primary determinant of outcome following TBI.[26-28] Furthermore, NF-L concentrations correlated with lifetime concussion event in this study. This result is

also consistent with earlier studies on amateur boxers with repeated trauma to the head, where the levels of NF-L in CSF increased after bouts, even without knockouts.[29,30] Additionally, a case study on a boxer with mTBI leading to a knock-out showed increased CSF NF-L levels over many months following the trauma.[31] Taken together, the findings of this study support the hypothesis that repetitive mTBI may lead to chronic axonal white matter injury. CSF NF-L correlates with plasma NF-L,(Ref: Gisslen M et al., EBioMed 2015 eller 2016; Kuhle 2016 CCLM) which suggests that this marker could be more broadly applied in future mTBI and PCS studies.

Tau is a normal axonal protein that is responsible for microtubule assembly and stability, and mainly expressed in unmyelinated cortical axons.[11] In this study, the concentration of CSF T-tau was unchanged. Considering the pathophysiology of mTBI, it is plausible to assume that shorter unmyelinated axons, which do not travel long distance, may only be locally teared as a result of the acceleration and deceleration. Indirectly, the unaltered T-tau levels further support the hypothesis that TBI mainly injures long axons.

Evidence suggests that athletes who have been exposed to repetitive head trauma are at increased risk of developing neurodegenerative changes such as tau pathology and Aβ deposition.[4,5,8,9] Numerous studies have shown that reduced CSF Aβ1-42 levels correlate tightly with positive amyloid PET finding[32], and a recent study also suggest that the reduction in CSF Aβ1-42 may be an earlier indicator of cerebral β-amyloid deposition than amyloid PET.[33] The finding that CSF Aβ1-42 was lower in players with PCS compared to controls, and the lowest levels of Aβ1-42 were observed in

players with PCS > 1year, who were also forced to retire are suggestive of potential early amyloid deposition following mTBI. Our findings are also consistent with post-mortem studies of patients with mTBI, as well as a recent imaging study of patients with severe TBI.[34] The exact mechanism of what triggers Aβ production is not fully understood. It has been suggested that axonal damage produced at the time of injury may act as an initial trigger for Aβ production and accumulation of amyloid pathology.[35] Additionally, animal models and human autopsy studies provide evidence that Aβ is produced at the site of axonal injury shortly after TBI. [35]

Trauma to the axons may also cause hyperphosphorylation of tau protein and aggregation into neurofibrillary tangles, which is a histological feature of repetitive mTBI or CTE. However, in this study, the concentration of CSF P-tau remained statistically unchanged. A plausible assumption would be that tau pathology may appear at a later stage or that tau pathology is not as widespread following mTBI or alternatively P-tau measured in CSF is not sensitive enough to reflect it. Despite the unchanged concentrations between the PCS and control group, P-tau correlated with the lifetime accumulated concussion score. However, given the modest sample size of this study, this should be interpreted with caution. Longitudinal studies with larger sample sizes and repeated lumbar puncture are needed to clarify any causal relationship between repetitive mTBI and hyperphosphorylation of tau. Such studies should also examine the concentrations of CSF Aβ42 and P-tau in the context of genotype, particularly *APOE*,[36] which was not assessed here.

Neurogranin is a post-synaptic protein, which has shown diagnostic utility for early symptomatic AD.[15-17] In this study, the concentrations of Ng were essentially

unaltered in the PCS group. However, in one of the players with persistent PCS who underwent repeated LPs, the mean levels of Ng remained up to 2-fold elevated compared with mean of the control group. It is plausible to assume that, post-synaptic loss may only be evident in individuals with severe PCS.

In conclusion, increased CSF NF-L and reduced Aβ1-42 was observed in PCS, suggestive of axonal white matter injury and amyloid deposition. Measurement of these biomarkers may be an objective tool to assess the degree of CNS injury in individuals with PCS, and to distinguish individuals who are at risk of developing CTE.

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Author Contributions: Drs Shahim and Blennow had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Shahim, Tegner, Zetterberg, Blennow.

Acquisition of data: Shahim, Tegner, Gustafsson, Gren, Olsson, Ärlig.

Statistical analysis: Shahim, Zetterberg.

Drafting of the manuscript: Shahim, Zetterberg, Blennow.

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Critical Revision of the manuscript for important intellectual content: All authors.

Administrative, technical or material support: Tegner, Gustafsson, Lehto, Höglund, Portelius, Engström, Blennow.

Obtained funding: Shahim, Zetterberg, Blennow.

Study Supervision: Zetterberg, Blennow.

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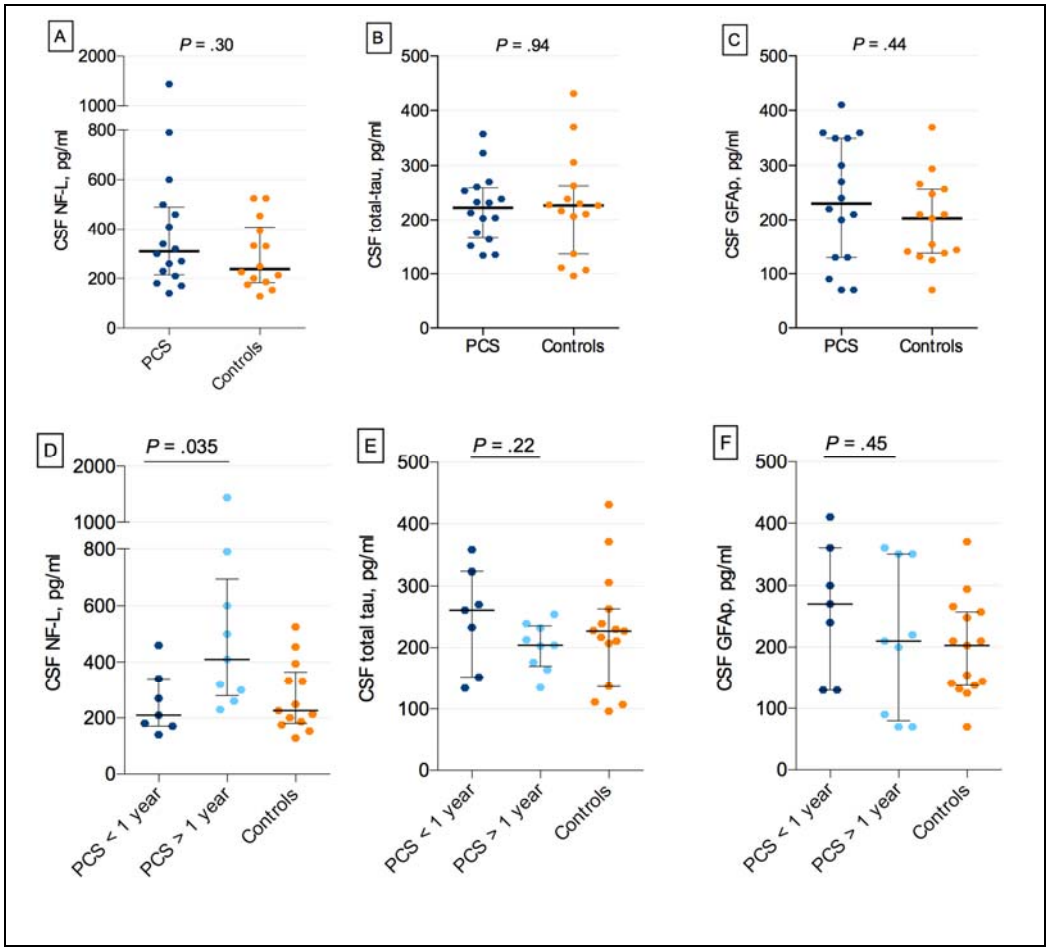
Figure legends

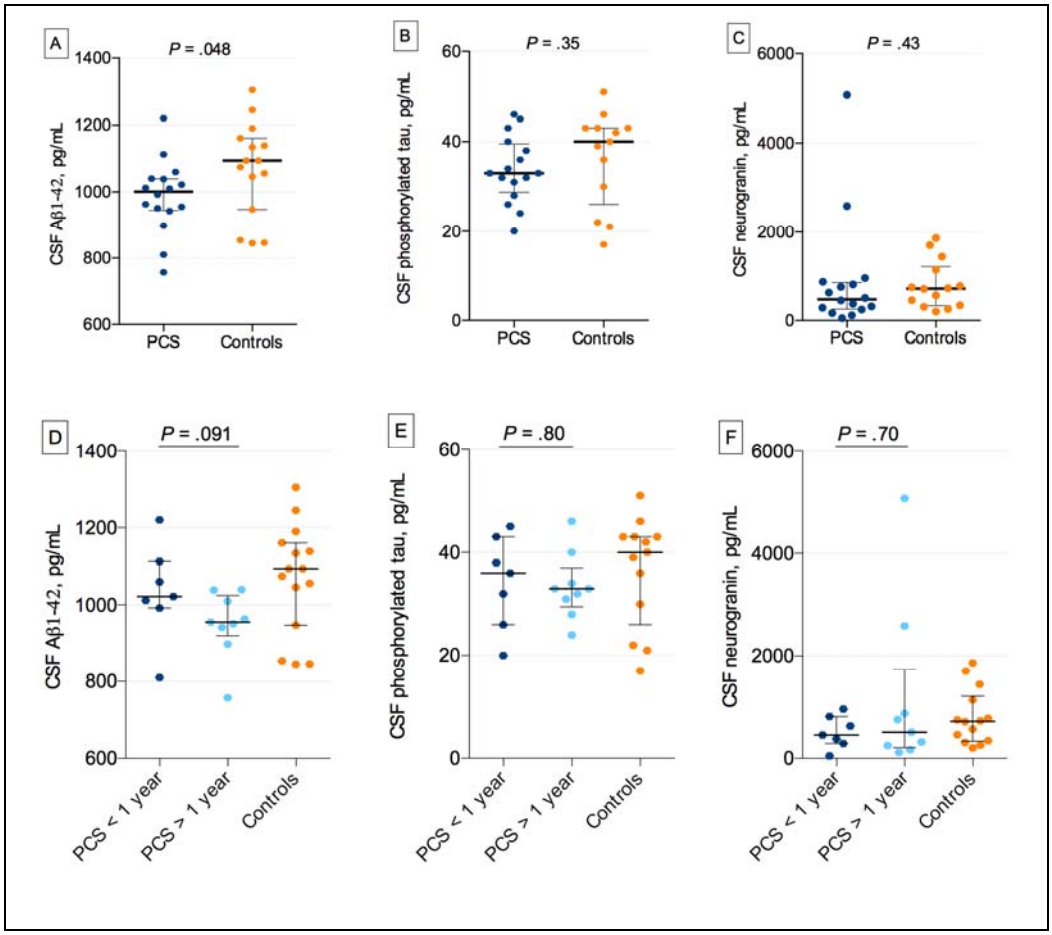
Figure 1. CSF biomarkers reflecting axonal injury biomarkers



Concentrations of neurofilament light (NF-L) protein were increased in post-concussion syndrome (PCS) group vs. controls (A). There was no significant difference in the level of total-tau (T-tau) (B) and glial fibrillary acidic protein (GFAp) (C). Players with PCS > 1 year had increased concentration of NF-L compared with players whose PCS resolved within 1 year after injury, as well as controls (D). There was no significant difference in the levels of T-tau (E) and GFAp (F) at either group or subgroup level, as well as compared to controls. The p-values are corrected for multiple comparisons. One of the 15 control subjects had increased CSF NF-L concentration (1491 pg/mL) for unknown reasons and was excluded from

further statistical analyses. Values are presented as medians; error bars indicate interquartile range.

Figure 2. CSF biomarkers reflecting amyloid and tau pathology and synaptic loss



Post-concussion syndrome (PCS) group had significantly (*P* = .048) lower amyloid-β 1-42 (Aβ 1-42) than control group (A). Also, concentration of Aβ 1-42 (C) were lower in players with PCS > year vs. PCS < 1 year, however not significant (*P* = .091). There was no difference in the levels phosphorylated tau at either group (B) or subgroup level (D). There was also no significant difference in the concentrations of neurogranin at either group (C) or subgroup level (F). The p-values are corrected for multiple comparisons. Values are presented as medians; error bars indicate interquartile range.

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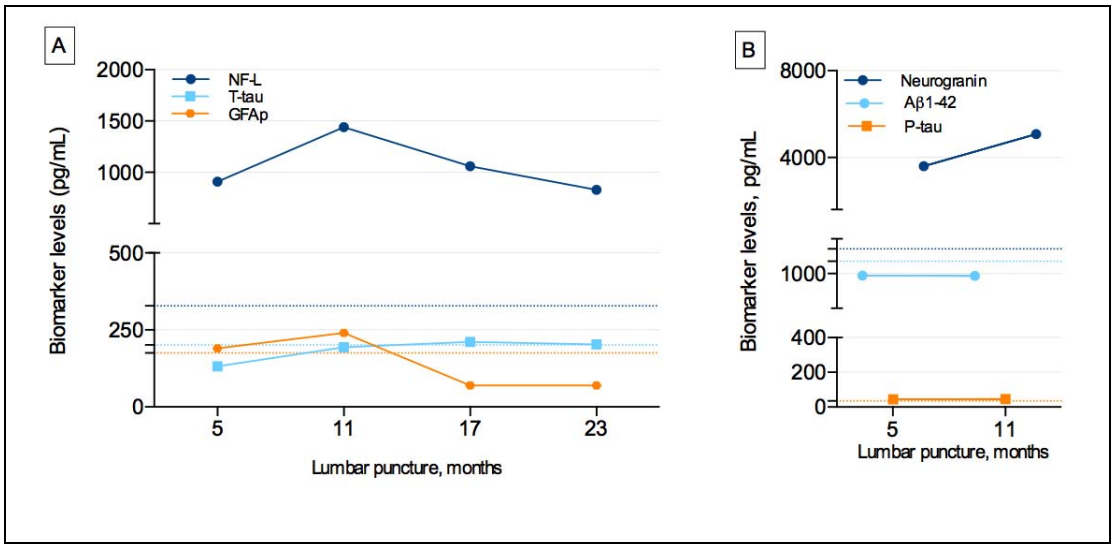
Figure 3. Longitudinal biomarker changes



One of they players was followed with repeated lumbar punctures; 5, 11, 17 and 23 months last concussion. In this player the mean concentration of neurofilament light protein was elevated compared with the mean of the control group, and remained elevated over time, while the levels of total-tau and glial fibrillary acidic protein were unchanged (A). Mean concentrations of amyloid-β 1-42 were lower at two following time points compared with mean of the control group (B). Also, the mean concentration of neurogranin was elevated compared with the mean of the control group (B). Dashed lined indicate mean + standard deviation of the control group.

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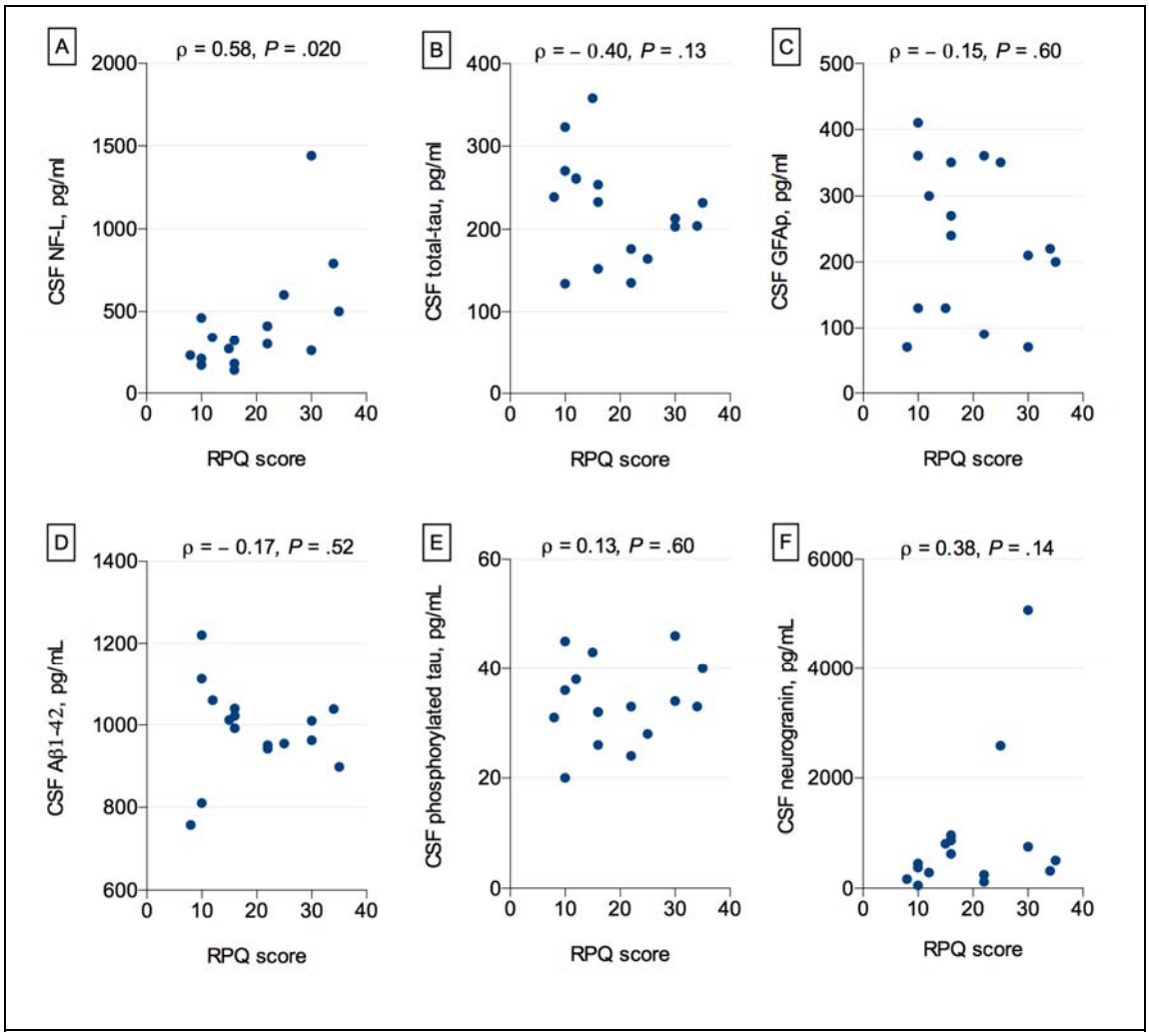

Figure 4. Relationship between biomarker levels and symptom severity

Concentrations of neurofilament light protein correlated (ρ = 0.58, *P* = .020) with Rivermead Post-concussion Symptoms Questionnaire (RPQ) score (A). There was no significant correlation between any of the other biomarkers and RPQ score (B-F).

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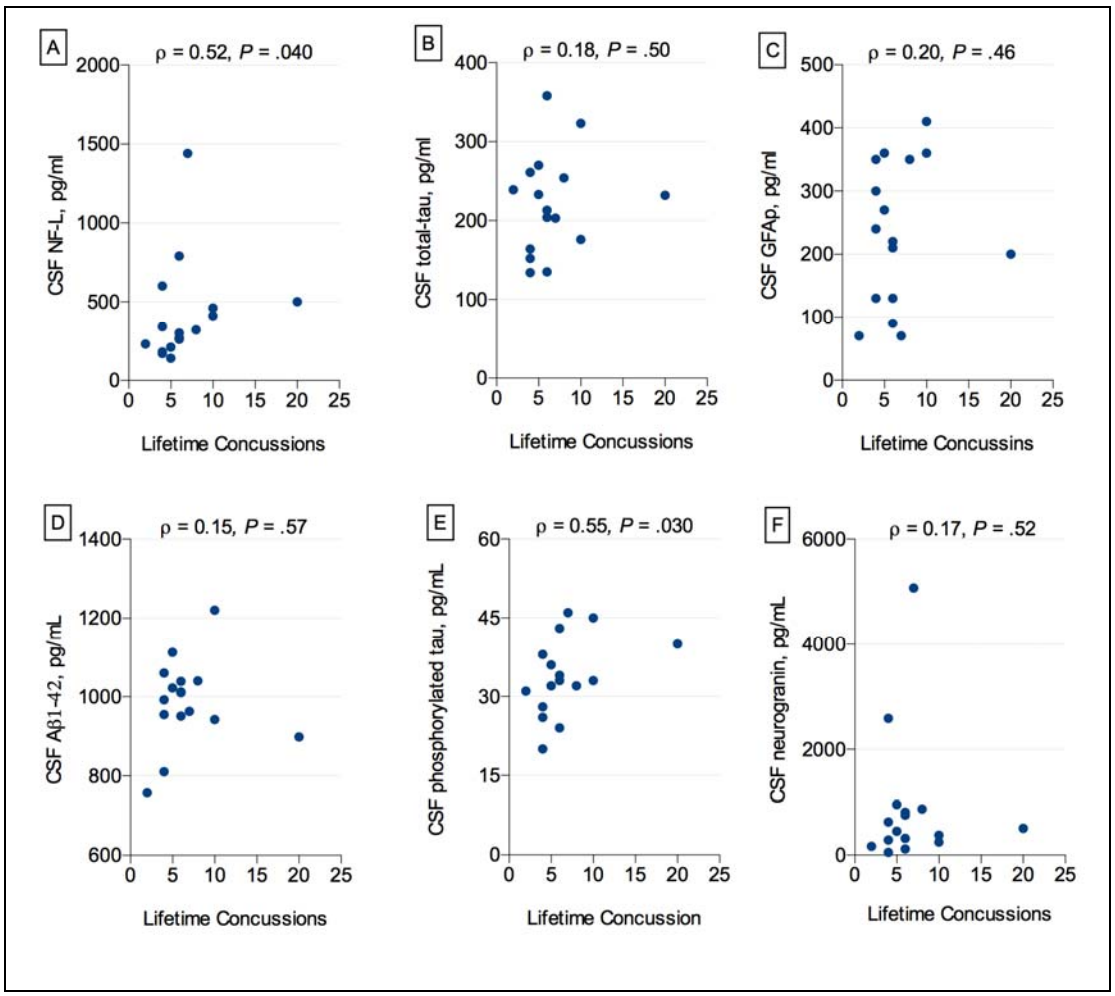
Figure 5. Relationship between biomarker concentrations and lifetime number of concussions



Neurofilament light protein correlated (ρ = 0.52, *P* = .040) with lifetime concussion event (A). There was also a significant correlation between phosphorylated tau and lifetime concussion event (E). There was no significant relationship between the lifetime concussion event and other biomarkers in the study (B-D, and F).

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Table 1. Demographic and clinical characteristics of participants at inclusion[a]

Variable	PCS (n = 16)	Controls (n = 15)
Age, year	31 (22-53)	25 (21-35)
Sex, male No. (%)	16 (100)	11 (73)
Time since last concussion, month	4 (3-144)	0
Lifetime number of concussions	6 (2-20)	0
Post-lumbar headache	2	4
Total Rivermead Post-Concussion Symptoms Questionnaire score, range, 0-64	22 (8-35)	0 (0-0)

Abbreviations: PCS = post-concussion syndrome

[a]All continuous variable are shown as median (range) unless denoted otherwise.

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