BIOMARKERS OF BRAIN DAMAGE – A COMPLEX CHALLENGE WITH GREAT POTENTIAL

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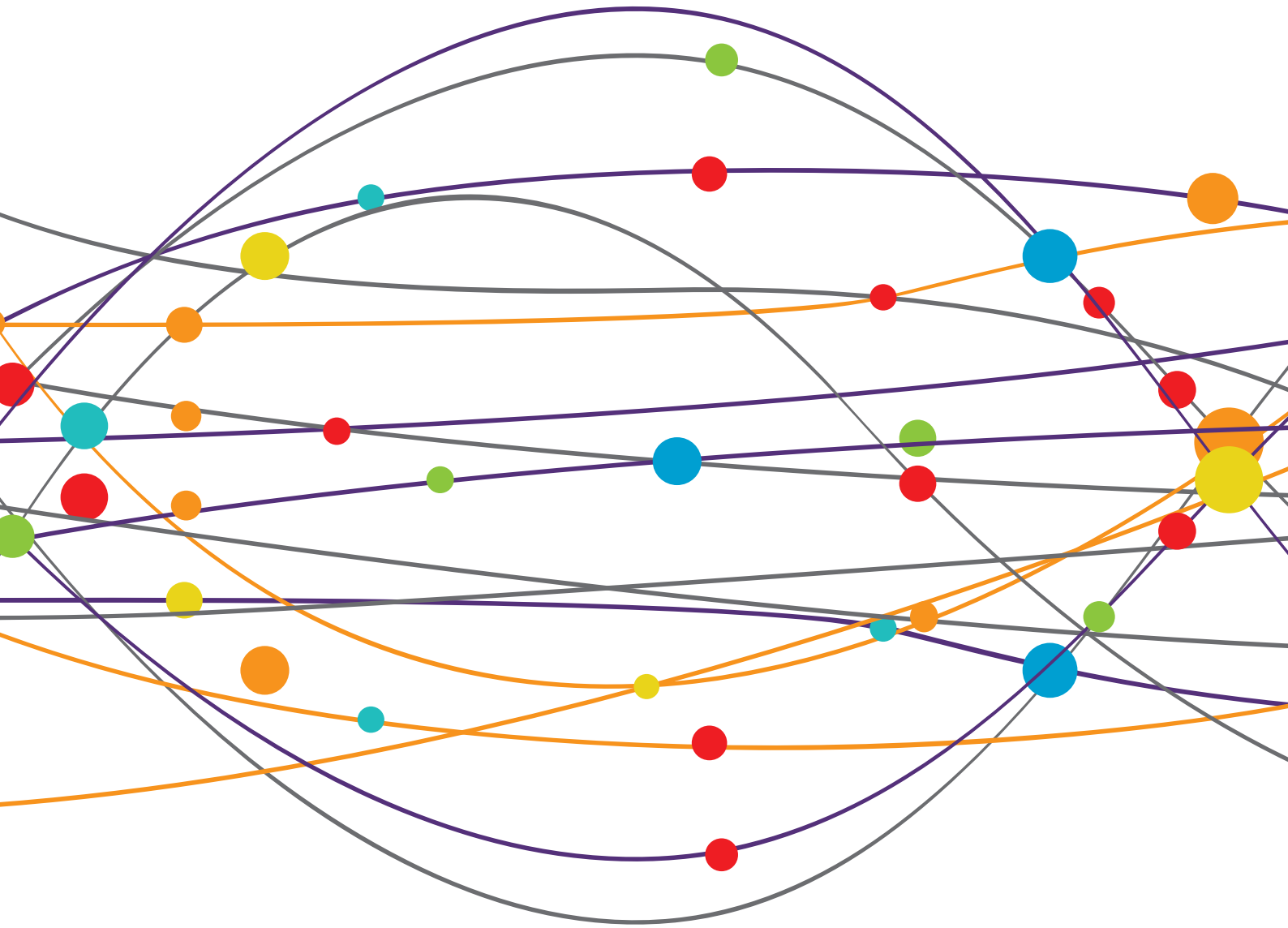

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BIOMARKERS OF BRAIN DAMAGE – A COMPLEX CHALLENGE WITH GREAT POTENTIAL

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Olli Tenovuo is consultant and member of SAB for NeuroTrauma Sciences LLC (Georgia, USA) and ABCDx SA (Geneva, Switzerland). Jean-Charles Sanchez has a research grant to disclose from ABCDx SA, (Geneva, Switzerland). Damir Janigro is the CEO of FloTBI Inc. (Cleveland, USA), a company interested in biomarkers of neurological diseases.

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EDITORIAL
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Editorial: Biomarkers of Brain Damage – A Complex Challenge With Great Potential

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Keywords: brain, injury, biomarker, proteomics, diagnostics

Editorial on the Research Topic

Biomarkers of Brain Damage - A Complex Challenge With Great Potential

The brain has remained as the only organ where blood- or other fluid-based biomarkers have been practically lacking from the commonly used clinical diagnostic tools. There are several reasons for this, such as the complexity of the brain as an organ, the presence of a blood-brain barrier (BBB), extremely small concentrations of many brain-derived proteins in blood, and the difficulty of using cerebrospinal fluid for routine diagnostics. The development of biochemical analytics has enabled measuring of very small amounts of various substances, which has led to a new and promising era for brain biomarker diagnostics.

Despite the vast number of published studies, few biomarkers have thus far entered the clinic for a number of reasons. First, many target molecules are brain-enriched, and not specific for the brain or CNS. Thus, there is a need to perform control studies in several potential patient groups. Second, to what extent the biomarker reflects brain tissue damage, and to what extent the biomarker level depends on the integrity of the BBB and/or the glymphatic function, is a challenging question to clarify. Third, each biomarker has a specific kinetic profile, some appearing rapidly and some slowly depending on the clinical condition, and each with a different half-life possibly related to kidney function. Thus, it may be challenging to interpret what a single level means, giving only a narrow window for the often complex and dynamic pathophysiological events. Fourth, the fairly demanding detection technologies are prone to technical errors, thus results have to be validated in independent laboratories before broader acceptance. Fifth, collecting sufficient numbers of samples from well-characterized subjects with appropriate control groups is a significant effort, often requiring multicenter collaboration.

Despite the aforementioned challenges, there is no question that the forthcoming years and decades will see a revolution in the diagnostics of brain disorders. Biomarkers have potential uses that other methodologies cannot replace. They may give information on injuries in different cell types and separate cellular compartments, such as axonal or synaptic injury. They may be used to monitor treatment responses rapidly. They can give – when used in validated panels – a comprehensive picture about the brain state non-invasively. Not least, they may be used for point-of-care rapid diagnostics, thus opening entirely new possibilities for the clinicians.

This special issue has aimed to collect papers that advance the field. The paper by Janigro et al. reports how to assess BBB permeability and how the BBB influences brain biomarker measurement in peripheral biofluids. These kinds of studies are of utmost importance for the understanding of clinical samples. The paper by Smirl et al. also studied the neurovascular unit, as other articles focused on traumatic brain injury (TBI), by examining the alterations of the neurovascular unit after soccer headings. Four of the papers

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(Hossain et al.; Lagerstedt et al.; Kahouadji et al.; Posti et al.) report the value of different promising biomarkers in assessing acute TBI, and how they relate to imaging findings and outcome. A fifth paper by Huebschmann et al. reports how the levels of a much-studied glial fibrillary acidic protein (GFAP) differ in serum and plasma of older adults. The paper by Guedes et al. extends TBI-related studies beyond proteomics in reporting the use of extracellular vesicles and microRNAs as TBI biomarkers. The paper by Kawata et al. reports how some of the most promising TBI protein biomarkers associate with imaging markers of axonal injury in subjects with repeated head impacts. Although the concept "biomarker" is commonly associated with measurement of biomolecules, in a wider sense all biological measurements are biomarkers; the paper by Haider et al. reports how concussion alters responses of the parasympathetic nervous system. Finally, the paper by Quiroz-Baez et al. discusses the use of extracellular vesicles as biomarkers of neurodegenerative conditions.

We hope that this special issue stimulates further efforts in the field – there is still much to be done. Understanding how different physiological and pathophysiological phenomena affect various biomarkers, and how different biomarkers behave under multiple clinical conditions and their stages will still require intensive research. By all likelihood, the vast brain complexity can rarely be assessed using a single biomarker; we thus assume that the use of various biomarker panels and their interpretation using artificial intelligence approaches will constitute a major change in clinical neurosciences, possibly already during this decade.

AUTHOR CONTRIBUTIONS

OT wrote the draft. All authors revised and approved the final text.

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ORIGINAL RESEARCH
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Admission Levels of Total Tau and β-Amyloid Isoforms 1–40 and 1–42 in Predicting the Outcome of Mild Traumatic Brain Injury

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Background: The purpose of this study was to investigate if admission levels of total tau (T-tau) and β-amyloid isoforms 1-40 (Aβ40) and 1-42 (Aβ42) could predict clinical outcome in patients with mild traumatic brain injury (mTBI).

Methods: A total of 105 patients with mTBI [Glasgow Coma Scale (GCS) ≥ 13] recruited in Turku University Hospital, Turku, Finland were included in this study. Blood samples were drawn within 24 h of admission for analysis of plasma T-tau, Aβ40, and Aβ42. Patients were divided into computed tomography (CT)-positive and CT-negative groups. The outcome was assessed 6–12 months after the injury using the Extended Glasgow Outcome Scale (GOSE). Outcomes were defined as complete (GOSE 8) or incomplete (GOSE < 8) recovery. The Rivermead Post Concussion Symptoms Questionnaire (RPCSQ) was also used to assess mTBI-related symptoms. Predictive values of the biomarkers were analyzed independently, in panels and together with clinical parameters.

Results: The admission levels of plasma T-tau, Aβ40, and Aβ42 were not significantly different between patients with complete and incomplete recovery. The levels of T-tau, Aβ40, and Aβ42 could poorly predict complete recovery, with areas under the receiver operating characteristic curve 0.56, 0.52, and 0.54, respectively. For the whole cohort, there was a significant negative correlation between the levels of T-tau and ordinal GOSE

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score (Spearman $\rho = -0.231$, $p = 0.018$). In a multivariate logistic regression model including age, GCS, duration of posttraumatic amnesia, Injury Severity Score (ISS), time from injury to sampling, and CT findings, none of the biomarkers could predict complete recovery independently or together with the other two biomarkers. Plasma levels of T-tau, Aβ40, and Aβ42 did not significantly differ between the outcome groups either within the CT-positive or CT-negative subgroups. Levels of Aβ40 and Aβ42 did not significantly correlate with outcome, but in the CT-positive subgroup, the levels of T-tau significantly correlated with ordinal GOSE score (Spearman $\rho = -0.288$, $p = 0.035$). The levels of T-tau, Aβ40, and Aβ42 were not correlated with the RPCSQ scores.

Conclusions: The early levels of T-tau are correlated with the outcome in patients with mTBI, but none of the biomarkers either alone or in any combinations could predict complete recovery in patients with mTBI.

Keywords: traumatic brain injury, total tau, β-amyloid 1-40, β-amyloid 1-42, outcome

INTRODUCTION

Traumatic brain injury (TBI), "the silent epidemic," will become a leading cause of disability and death globally by 2030 according to the recent estimation of the World Health Organization (1). Approximately 80–90% of all TBIs presenting to emergency departments are mild (mTBI) (2). Although most of the patients with mTBI show good recovery, a subgroup comprising 15–20% continue to have post-injury symptoms after 1 year (3). Computed tomography (CT), which is the standard tool for the assessment of acute TBI, is not sensitive enough for the long-term outcome prediction of mTBI (4, 5). Furthermore, there is still no clinically validated models for the outcome prediction following mTBI, and the performance of the tested models for mTBI are poor (6).

The process of recovery from mTBI is highly variable and individual. Importantly, there are no validated TBI biomarkers to provide objective measures of the degree of neuronal damage as well as the pathophysiological events following a TBI, which could help the clinician to evaluate the risks for incomplete recovery and to properly recognize patients who will need follow-up care (7–9). Glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase-L1 (UCH-L1), and neurofilament light (NF-L) protein have been reported as promising biomarkers for the outcome prediction of mTBI (10–17).

Recently, also tau protein and β-amyloid isoforms 1-40 (Aβ40) and 1-42 (Aβ42), axon terminal biomarkers, known as the neurodegenerative biomarkers (18, 19), have been studied to explore the association between post-concussion symptoms (PCS) and neuronal damage, especially after repeated mTBIs. Tau is a microtubule-associated protein that is located in the axons of central nervous system (CNS) neurons and serves as a structural element in the axonal cytoskeleton (20–22). Total tau (T-tau) has been reported as a biomarker of injury to thin unmyelinated axons in a human post-mortem study (23). One study reported that elevated levels of plasma tau are associated with repetitive mTBIs in amateur boxers (24). Another study showed a marked increase in the plasma levels of tau in concussed professional ice hockey players (25). Serum tau levels were reported as a significant outcome predictor following severe TBI (26). In addition, admission cerebrospinal fluid (CSF) tau was correlated with long-term outcome in patients with severe TBI (27). Lately, it has been suggested that acute plasma hyperphosphorylated tau protein (P-tau) levels and the P-tau–T-tau ratio outperform T-tau levels for the outcome prediction of TBI (22).

Aβ40 (28) and Aβ42 (29, 30) reflect amyloidogenic amyloid precursor protein (APP) metabolism and have been reported as potential biomarkers of axonal damage in TBI (31). Aβ pathology, primarily consisting of aggregated Aβ42 peptides, is a histologic hallmark of Alzheimer's disease (AD) (32), and TBI has been suggested to be one of the risk factors for AD (33). Aβ pathology (amyloid plaques) have been found in boxers having dementia pugilistica (34) and in a proportion of other contact sport athletes having chronic traumatic encephalopathy (35). Although ventricular CSF levels of Aβ40 and Aβ42 were elevated during the first week after severe TBI (36), no changes in Aβ40 or Aβ42 were reported in mTBI where CSF samples were collected by lumbar puncture (37). However, for repetitive mTBI, post-injury subjective symptoms were associated with the reduction of CSF levels of Aβ40 and Aβ42 (15, 38). It has been reported that plasma levels of Aβ40 and Aβ42 do not have a value for the diagnosis and the prediction of outcome of mTBI (15, 23, 33, 35, 36). Lately, our research group has reported significant relationship between the acute plasma levels of axonal protein biomarker NF-L and the outcome in patients with mTBI (16). There are no studies correlating the admission plasma levels of the other axonal biomarkers such as Aβ40 and Aβ42 with the outcome of mTBI.

The aim of the current study was to correlate the levels of T-tau and Aβ40 and Aβ42 during the first 24 h after admission with outcome in patients with mTBI, using ultrasensitive single molecule array (Simoa) technology (39, 40). We hypothesized that these biomarkers would show some correlation with the outcome in these patients.

METHODS

Study Population

This prospective study was a part of the EU-funded TBIcare (Evidence-based Diagnostic and Treatment Planning Solution for Traumatic Brain Injuries) project. One hundred seven (107) patients with mTBI [Glasgow Coma Scale (GCS) $\geq$ 13] were recruited whose blood samples were available within 24 h from the arrival to the ED of Turku University Hospital, Finland.

Inclusion criteria were lowest GCS $\geq$ 13, age $\geq$ 18 years, clinical diagnosis of TBI, and indications for acute head CT according to the NICE criteria (http://www.nice.org.uk/guidance/cg176).

Exclusion criteria were age < 18 years, blast-induced or penetrating injury, chronic subdural hematoma, inability to live independently due to pre-existing brain disease, TBI or suspected TBI not needing head CT, more than 2 weeks from the injury, not living in the district thereby preventing follow-up visits, not speaking native language, or no consent received.

Analysis of T-Tau and Aβ40 and Aβ42

Plasma T-tau was analyzed using the Human Neurology 4-Plex A assay (N4PA) on an HD-1 single molecule array (Simoa) instrument according to instructions from the manufacturer (Quanterix, Lexington, MA, USA). For T-tau, the lower limit of detection (LLoD) was 0.024 pg/ml, while the lower limit of quantification (LLoQ) was 0.053 pg/ml, and the calibration range was 0.136 pg/ml to 112 pg/ml. Plasma Aβ40 and Aβ42 concentrations were measured using a duplex Simoa immunoassay (Quanterix, Lexington, MA, USA). For Aβ40, the LLoD was 0.045 pg/ml, and the LLoQ was 0.142 pg/ml with a calibration range between 0 pg/ml to 90.0 pg/ml. For Aβ42, the LLoD was 0.142 pg/ml, and the LLoQ was 0.69 pg/ml with a calibration range between 0 and 11.0 pg/ml. The measurements were performed by board-certified laboratory technicians who were blinded to the clinical data. There were no samples below the LLoDs and LLoQs.

TBI Severity and Outcome Grading

For the assessment of TBI severity, the lowest recorded GCS was used either at the scene of accident or emergency department (11, 17). The overall injury severity of the patients was assessed using the Injury Severity Score (ISS) (41). The duration of posttraumatic amnesia (PTA) was assessed at the outcome visit using the Rivermead method (42). The descriptive system proposed by Marshall et al. was used to analyze the CT scans (43), where class 1 corresponds with normal CT, classes 2–4 diffuse injuries, and classes 5–6 CTs with mass lesions.

Outcome

The Extended Glasgow Outcome Scale (GOSE) was used at 6–12 months after the injury to assess the outcome (44). Outcomes were defined as complete recovery (GOSE 8) and incomplete recovery (GOSE < 8). The presence and severity of mTBI-related symptoms were assessed using the Rivermead Post Concussion Symptoms Questionnaire (RPCSQ) (45). Every patient was evaluated by the same experienced neurologist at the Turku Brain Injury Centre.

Time Elapse

Time elapse was defined as the interval between the injury and sampling. Although the samples were obtained within 24 h of admission, they were not always drawn within 24 h after injury. Time elapse was used as a dichotomous variable, less than 24 h or more than 24 h, in the multiparameter prognostic panel analyses.

Ethics Declarations

Ethics Approval and Consent to Participate

The study protocol was approved by the ethical review board of the Hospital District of South-West Finland. A written informed consent was obtained from all patients or from their next of kin.

Statistical Analyses

Demographics of the subjects are presented as mean ± SD or percentages. The Kolmogorov–Smirnov test and visual inspection of data histograms were used to assess the normality of distribution. The levels of T-tau and Aβ40 and Aβ42 were not normally distributed, therefore, nonparametric tests were used in the statistical analyses. Data are presented as medians and interquartile range (IQR). Spearman rank correlation coefficient was used to assess the correlations between the levels of biomarkers and the outcomes. Correlations of biomarker levels with age and gender were analyzed with Pearson's and Spearman rank correlation, respectively. Spearman correlation coefficient was also used to assess the correlation between the levels of T-tau and amyloids in the whole cohort, as well as in the complete and incomplete recovery groups. Mann–Whitney *U* test was used to compare the levels of biomarkers between the outcome groups. A multivariate logistic regression analysis was performed in order to investigate if a biomarker alone or combined with other biomarkers had independent predictive power for the outcome beyond the clinical predictors. A biomarker panel analysis was used to investigate if a combination of biomarkers had better predictive ability than any biomarker alone. The regression analysis included the following variables: age, sex, educational level, ISS, worst GCS, Marshall CT classification, duration of PTA, time elapse, and the levels of T-tau and Aβ40 and Aβ42. Educational level was divided into basic school education, lower level professional, higher level professional, and academic. Marshall CT classification, sex, time elapse, and educational level were taken into account as categorical variables. Marshall class I (denoting CT-negative finding), female sex, time elapse of more than 24 h, and basic school education were used as reference categories in multivariate logistic regression. All other variables were considered to be numerical variables in the analyses. T-tau and Aβ40 and Aβ42 were used in the multivariate logistic regression models independently with the other variables and together in the same models. To study the prognostic ability of the biomarkers, area under the receiver operating characteristic (ROC) curve (AUC) was also used. AUC of 0.8 to 1.0 was considered very good; AUC of 0.7 to 0.8 was considered adequate; and AUC of 0.5 to 0.7 was considered poor (23). A value of $p < 0.05$ was considered statistically significant. For the prediction of dichotomized outcomes, cut-off values were defined using the ROC curve at the clinically compatible sensitivity >90%. For the data analyses, IBM SPSS

Statistics 22 (IBM Corp, Armonk, New York, NY, USA) and MATLAB R2016b (Math Works, Natick, MA, USA) were used. Furthermore, a multiparameter prognostic panel was formed by PanelomiX toolbox (38) using clinical information (age, sex, educational levels, GCS, duration of PTA, ISS, time elapse, CT findings, and GOSE) and the admission levels of T-tau and Aβ40 and Aβ42 for the best prediction of incomplete recovery. Cut-off values were selected to ensure a sensitivity of more than 90%. For the prognostic panels, the partial AUC (pAUC) was used as a local comparative approach that focuses only on a portion of the ROC curve.

RESULTS

Study Subjects

One hundred seven (107) patients with mTBI were recruited, of which GOSE score was available for 105, forming the final study population. There were 72 males (68.6%) and 33 females (31.4%), with a mean age of 47±20 years. The number of patients with CT-positive and CT-negative findings were 54 (51.4%) and 51 (48.6%), respectively. Patient characteristics are shown in **Table 1**. With regard to the outcome, 37 patients (35.0%) had complete recovery, 68 patients (65.0%) had incomplete recovery, and the mortality was 3.8% ($n = 4$). Among patients in whom the exact time of injury was available, the time elapse from injury to blood sampling was 28 ± 35 h ($n = 76$). In patients for whom the exact time of injury was unavailable, 11 patients were sampled within 24 h, and 18 patients were sampled after 24 h from the injury.

TABLE 1 | Patient characteristic.

Age (years)	47.46±20.25
Sex	
Male	72 (68.6%)
Female	33 (31.4%)
Marshall grade	
No visual pathology	51 (48.6%)
Diffuse injury	24 (22.9%)
Diffuse injury with swelling	1 (1%)
Diffuse injury with shift	1 (1%)
Mass lesions	28 (26.7%)
Pupil reactivity	
Unreactive	1 (1%)
Sluggish	2 (1.9%)
Reactive	98 (96.2%)
Missing data	4 (3.8%)
GOSE	
1	4 (3.8%)
2	0
3	6 (5.7%)
4	5 (4.8%)
5	7 (6.7%)
6	14 (13.3%)
7	32 (30.5%)
8	37 (35.0%)
Total	105 (100%)

Demographics are reported in mean ± SD or percentages (%).

The Levels of T-Tau and Outcome

The levels of T-tau were compared between patients with complete recovery (2.65 pg/ml, IQR 3.58 pg/ml) and incomplete recovery (2.8 pg/ml, IQR 7.5 pg/ml) (**Figure 1**), but significant differences were not observed. There was a significant negative correlation between the levels of T-tau and ordinal GOSE score in all patients (Spearman $\rho = -0.231$, $p = 0.018$) (**Table 2**). The level of T-tau was not able to predict the likelihood of complete recovery (AUC 0.56, 95% CI 0.45–0.67) (**Figure 2A**). Gender seemed to have an effect on T-tau (**Table 2**). The levels of T-tau did not differ between the outcome groups, and the levels of T-tau did not correlate significantly with the outcome within the CT-negative subgroup. In the CT-positive subgroup, there was a significant negative correlation between the levels of T-tau and ordinal GOSE score (Spearman $\rho = -0.288$, $p = 0.035$). The levels of T-tau did not correlate with the RPCSQ scores (**Table 2**).

The Levels of Aβ40 and Aβ42 and Outcome

The levels of Aβ40 were not significantly different between patients with complete (16.9 pg/ml, IQR 12.76 pg/ml) and incomplete recovery (17.42 pg/ml, IQR 12.65 pg/ml). The levels of Aβ42 were also not significantly different between patients with complete (16.94 pg/ml, IQR 12.36 pg/ml) and incomplete recovery (15.23 pg/ml, IQR 10.61 pg/ml) (**Figure 1**). There was no significant correlation between the levels of Aβ40 and Aβ42 and the GOSE score (**Table 2**). Aβ40 and Aβ42 were not able to predict the likelihood of complete recovery (AUC 0.52, 95% CI 0.41–0.64 and AUC 0.54, 95% CI 0.43–0.63, respectively) (**Figures 2B,C**).

When patients were divided into CT-positive and CT-negative subgroups, the levels of Aβ40 and Aβ42 did not differ between the outcome groups, nor did the levels correlate significantly with the outcome within these subgroups. The levels of Aβ40 and Aβ42 did not correlate with the RPCSQ scores (**Table 2**).

Combining T-Tau, Aβ40, and Aβ42

Using conventional multivariate logistic regression model, Aβ40 and Aβ42 were not able to predict outcome independently or together with T-tau, or vice versa. We also used the Panelomix tool for evaluating the capacity of these three biomarkers in predicting incomplete recovery. When setting the sensitivity to >90%, we found that the optimal sensitivity and specificity was 92.5% (95% CI, 85.1–98.5) and 27.8% (95% CI, 13.9–41.7), respectively (**Supplementary Figure 1**), when the levels of at least two out of T-tau, Aβ40, and Aβ42 were above 0.55, 20.26, and 23.9 pg/ml, respectively.

Correlation Among the Levels of T-tau, Aβ40, and Aβ42

For the whole population, as well as complete and incomplete recovery subgroups, the levels of T-tau and Aβ40 and Aβ42 were not significantly correlated with each other.

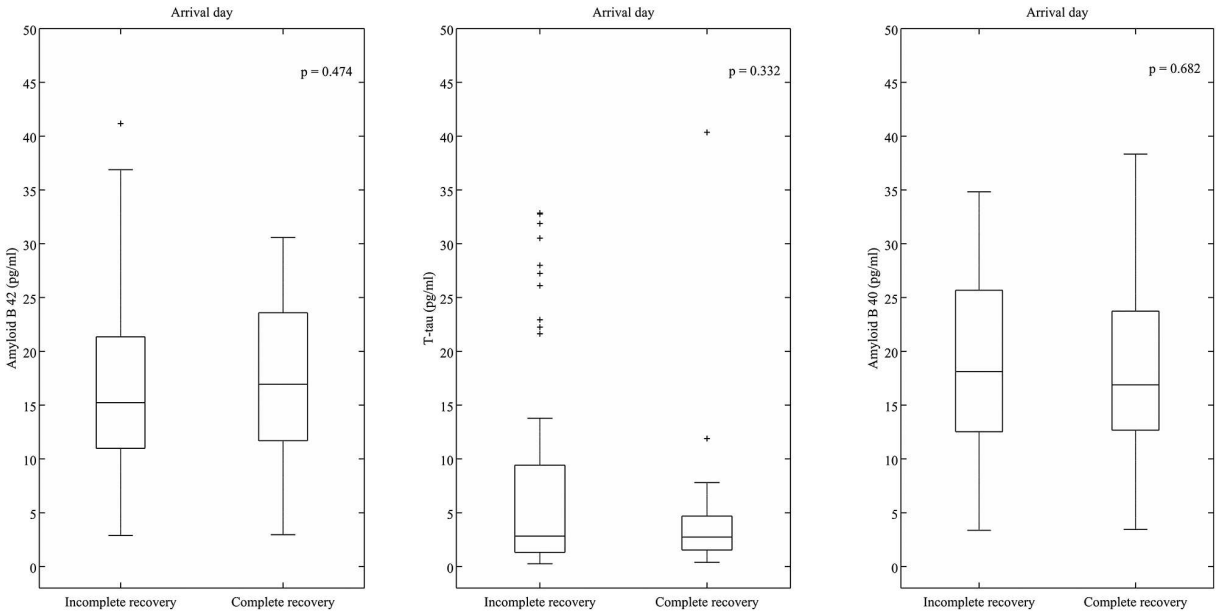

FIGURE 1 | Levels of total tau (T-tau), β-amyloid isoform 1-40 (Aβ40), and β-amyloid isoform 1-42 (Aβ42) in patients with complete (GOS 8) and incomplete (GOS < 8) recovery (y axis is zoomed). Box plots represent medians in picograms per milliliter and interquartile ranges.

TABLE 2 | Correlation between biomarkers and Glasgow outcome scale extended (GOSE), gender, total PRQ, age, and RPCSQ (16 cut-off).

Biomarker	GOSE			Gender			RPCSQ (total)			Age			RPCSQ (16 cut-off)		
	Spearman ρ	*p*-Value	*n*	Spearman ρ	*p*-Value	*n*	Pearson's r	*p*-Value	*n*	Pearson's r	*p*-Value	*n*	Pearson's r	*p*-Value	*n*
Amyloid β40	−0.082	0.410	104	0.034	0.731	104	−0.007	0.948	95	0.180	0.068	104	−0.007	0.946	95
Amyloid β42	0.063	0.525	103	−0.032	0.750	103	−0.015	0.889	94	0.063	0.525	103	−0.028	0.788	94
Tau	**−0.231**	**0.018**	105	**0.252**	**0.010**	105	−0.013	0.900	96	0.013	0.899	105	−0.026	0.799	96

GOSE, Glasgow Outcome Scale extended; RPCSQ, Rivermead Post Concussion Symptoms Questionnaire. Statistically significant findings are in bold.

Best Multiparameter Panel for Outcome Prediction

We also tried to find the best combination for predicting the outcome by combining clinical variables, biomarker levels, and taking into consideration the time from injury to sampling. The best available panel found was for the levels of T-tau taken more than 24 h from the injury and combined with age and ISS. This panel had a sensitivity of 90.8% (95% CI, 83.1–96.9) and a specificity of 57.1% (95% CI, 40–74.3), provided that at least two of these three variables were above their cut-off values (22.5 years for age, 3.5 for ISS, and 12.84 pg/ml for T-tau) (**Supplementary Figure 2**).

DISCUSSION

This prospective, observational study including patients with CT-positive and CT-negative mTBI investigated the performance of the blood protein biomarkers T-tau, Aβ40, and Aβ42 for the outcome prediction during the first 24 h after admission, utilizing modern highly sensitive immunoassays in a well-characterized cohort. We found that T-tau was significantly correlated with the outcome in the whole population as well as in the subgroup of patients with CT-positive mTBI. However, the levels of T-tau, Aβ40, and Aβ42 were not significantly different between the patients with complete and incomplete recovery, and the levels of T-tau, Aβ40, and Aβ42 were not able to give any useful prediction about the likelihood of complete recovery. Moreover, none of the biomarkers was correlated with the symptom severity as assessed with the RPCSQ scores. Yet, a multiparameter panel method suggested that levels of T-tau may have predictive value when sampled >24 h from the injury and combined with age and ISS, obtaining a sensitivity of 90.8% and a specificity of 57.1% for predicting incomplete recovery.

Earlier studies reported that serum tau had limited value for the diagnosis of intracranial injury and the outcome prediction of mTBI (46, 47), which is in agreement with our results. Recently, TRACK-TBI investigators used another high-sensitive assay platform and reported that acute P-tau levels and the P-tau–T-tau ratio outperformed T-tau levels in the outcome prediction of TBI (22). As only the levels of T-tau were measured in

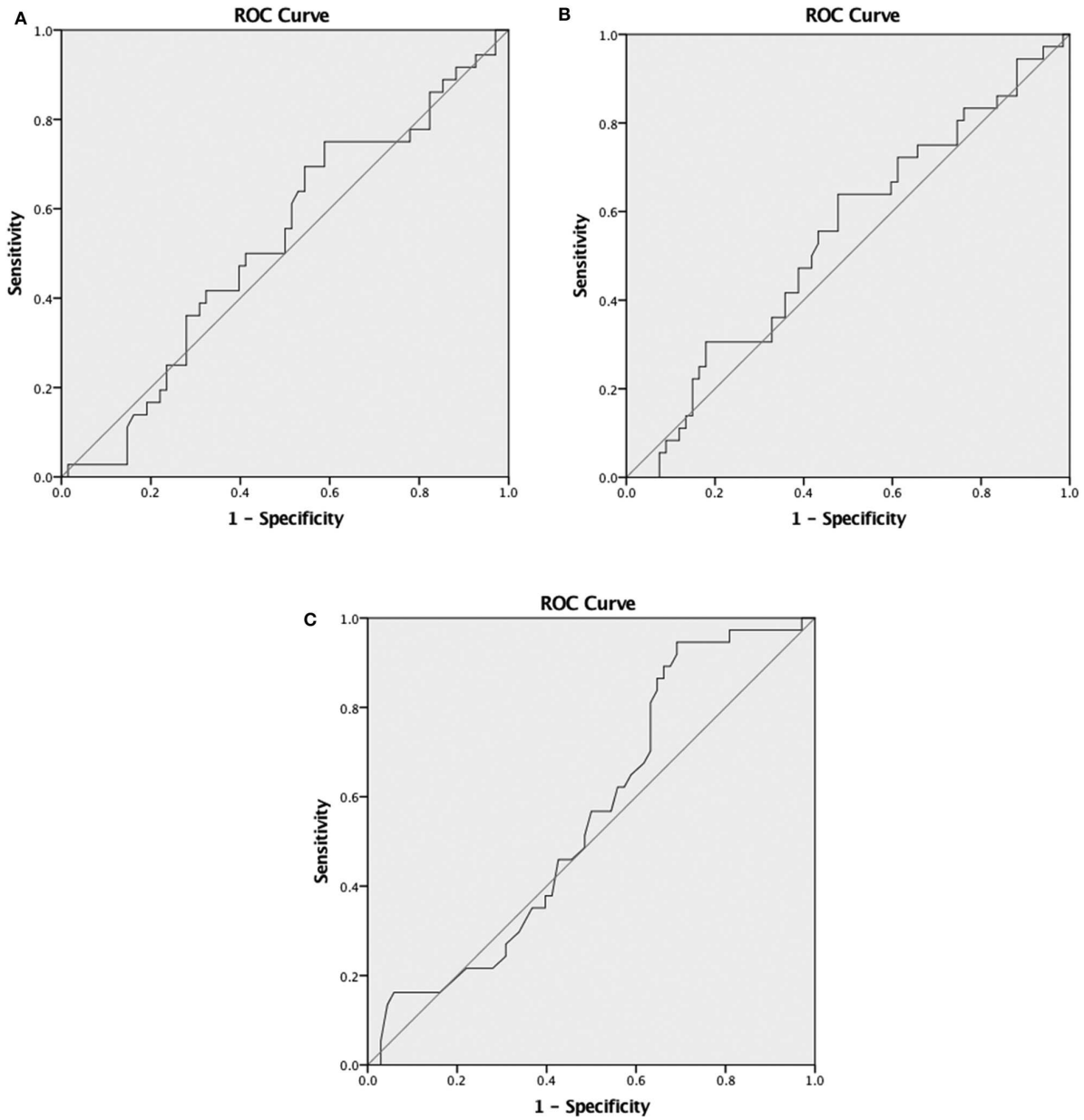

FIGURE 2 | (A) Receiver operating characteristic (ROC) curves for predicting complete recovery (GOS 8). Area under the curve (AUC) for T-tau, 0.56 (95% CI 0.45–0.67). **(B)** ROC curves for predicting complete recovery (GOS 8). AUC for Aβ40, 0.52 (95% CI 0.41–0.64). **(C)** ROC curves for predicting complete recovery (GOS 8). AUC for Aβ42, 0.54 (95% CI 0.43–0.63).

our study, the results might have been different if also P-tau was measured. Since tau is mainly expressed in unmyelinated cortical axons (15), the inability of the admission levels of plasma T-tau to differentiate complete and incomplete recovery may support the concept that in most of the cases of mTBI, mainly subcortical myelinated axons of the white matter are injured (15, 16, 48). Another possible explanation is that the eventual injury of cortical axons is a slower process, not reflected in blood levels of T-tau during the time frame used in this study.

Our study findings of Aβ40 and Aβ42 are in line with the results of the previous studies (15, 23, 33, 35, 36), where the levels of Aβ40 and Aβ42 did not correlate with the outcome as well as the levels were unable to predict complete and incomplete recovery.

A recent study reported that there was no significant relationship between the plasma levels of T-tau and Aβ42 and neurocognitive tests following mTBI (49). The study used late levels of T-tau, which is why our results cannot be compared with those data.

There are limitations in our study. First, we had data on T-tau and Aβ40 and Aβ42 available only at a single timepoint—within 24 h after admission. A kinetic study with serial sampling would

allow estimation of the total efflux of a biomarker and timing of the peak values, which could reveal more information about the outcome prediction abilities of the studied biomarkers (50). Tau has been reported to be a long-term biomarker having the peak value within the first hour after the initial injury and a second peak after 36 h following mTBI (25). Aβ42 becomes significantly elevated within the first 24 h after injury and remains quite stable for ca. 6 days (18), although, there are contraindicatory studies reporting no significant elevation of Aβ40 and Aβ42 following mTBI (48). Indeed, we found that the levels of T-tau seemed to perform best when taken >24 h from the injury and combined with clinical variables. The outcome prediction abilities of the studied blood biomarkers could be negatively driven by the variability in timing of sample collection in relation to injury between patients. The most accurate diagnostic time windows for the biomarkers might have been missed; however, the time from injury to sampling was taken into account as a covariate in the analysis. Second, the variability in assessing the GOSE between 6 and 12 months after the injury should be considered as a limitation of the study. This limitation has been elaborately discussed in one of our recently published biomarker studies utilizing the same study cohort (16). Third, our patients with mTBI had more severe injuries than an average mTBI population who are seen at the ED. This is because there was a recruitment bias favoring those patients who required in-hospital treatment. This is why many patients of our mTBI cohort had abnormalities on CT. In addition, some patients—although having GCS in the mild category—had PTA for >24 h, which according to many classifications indicate a more severe TBI. These issues reflect the problems in defining an acute TBI by severity, nicely shown also in the CENTER-TBI study (51), where about one-third of cases treated at the ICU had mTBI based on GCS (52). Thus, when interpreting our results, the nature of our study population has to be taken into account. Additionally, in our study, the duration of PTA was assessed retrospectively at the outcome visit, which is considered to be less reliable than prospective evaluation. When comparing our results with earlier studies, it is important to note that none of our patients had a sports-related repetitive injury as the injury mechanism, and CSF samples were not collected.

A strength of our study is the use of ultrasensitive single molecule array (Simoa) technology. Especially for T-tau, the concentrations are very low in the peripheral blood and are thus almost impossible to measure precisely by most of the immunoassays (18). In addition, our patient cohort was prospectively collected and well characterized.

In this study, we studied biomarkers that mainly originate from axon terminals. However, they apparently represent a different kind of axonal damage, and thus, we sought to investigate their outcome prediction ability in a panel analysis. Since mTBI is a complex cascade of neurometabolic changes (25), therefore, developing a prediction model including the blood biomarkers of different cellular origins is an emerging need. It has recently been reported that panels of biomarkers from different cellular origins outperform single proteins' ability to detect patients with a need for head CT scanning after TBI (53). It has also been reported that a serum biomarker panel consisting of proteins of different cellular origins improved outcome prediction in TBI, where 70% of the cohort had severe TBI (50).

CONCLUSIONS

The main finding of the current study was that the admission levels of T-tau were significantly correlated with the outcome in patients with mTBI. Neither T-tau, Aβ40, or Aβ42 alone or their different combinations could predict complete recovery in patients with mTBI. Our study showed that T-tau may have potential in outcome prediction of mTBI, but more studies are needed using larger sample sizes, serial sampling method, and possibly including P-tau and P-tau/T-tau ratio. Panels of biomarkers of different cellular origins are recommended to be utilized as they appear to outperform single biomarkers in outcome prediction.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Southwest Finland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IH, JP, RT, MM, and OT conceived and designed the study. JP, RT, AK, H-RM, JT, and OT recruited the patients. JP, RT, AK, H-RM, JT, IH, and OT designed the data collection at Turku University Hospital. MM conducted the statistical analyses with contributions from IH, LA, and J-CS. JG, HZ, and KB supervised the biomarker analyses. IH drafted the manuscript with critical contributions from RT, OT, and JP. MM, LA, JF, MG, PH, AK, H-RM, DM, VN, JT, KH, DW, JG, KB, J-CS, and HZ contributed to the revision of the manuscript. IH and JP take the responsibility for the paper as whole.

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Hossain et al. (54) and (55) Results of this study have been presented as an oral presentation in the 13th World Congress On Brain Injury (IBIA 2019) in Toronto on March 16, 2019 and as an oral presentation in the European Association of Neurosurgical Societies Annual Meeting (EANS 2019) in Dublin on September 25, 2019.

SUPPLEMENTARY MATERIAL

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Interleukin 10 and Heart Fatty Acid-Binding Protein as Early Outcome Predictors in Patients With Traumatic Brain Injury

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Background: Patients with traumatic brain injury (TBI) exhibit a variable and unpredictable outcome. The proteins interleukin 10 (IL-10) and heart fatty acid-binding protein (H-FABP) have shown predictive values for the presence of intracranial lesions.

Aim: To evaluate the individual and combined outcome prediction ability of IL-10 and H-FABP, and to compare them to the more studied proteins S100β, glial fibrillary acidic protein (GFAP), and neurofilament light (NF-L), both with and without clinical predictors.

Methods: Blood samples from patients with acute TBI (all severities) were collected <24 h post trauma. The outcome was measured >6 months post injury using the Glasgow Outcome Scale Extended (GOSE) score, dichotomizing patients into: (i) those with favorable (GOSE≥5)/unfavorable outcome (GOSE≤4) and complete (GOSE = 8)/incomplete (GOSE≤7) recovery, and (ii) patients with mild TBI (mTBI) and patients with TBIs of all severities.

Results: When sensitivity was set at 95–100%, the proteins' individual specificities remained low. H-FABP showed the best specificity (%) and sensitivity (100%) in predicting complete recovery in patients with mTBI. IL-10 had the best specificity (50%) and sensitivity (96%) in identifying patients with favorable outcome in patients with TBIs of all severities. When individual proteins were combined with clinical parameters, a model including H-FABP, NF-L, and ISS yielded a specificity of 56% and a sensitivity of 96% in predicting complete recovery in patients with mTBI.

In predicting favorable outcome, a model consisting IL-10, age, and TBI severity reached a specificity of 80% and a sensitivity of 96% in patients with TBIs of all severities.

Conclusion: Combining novel TBI biomarkers H-FABP and IL-10 with GFAP, NF-L and S100β and clinical parameters improves outcome prediction models in TBI.

Keywords: biomarker, heart fatty-acid binding protein, interleukin 10, panel, protein, outcome, traumatic brain injury

INTRODUCTION

Patients with TBI may suffer from different levels and persistence of cognitive, behavioral, emotional, and physical impairments (1, 2). These impairments are frequent in patients with moderate and severe TBI, while, in cases of mild TBI (mTBI), most of the patients recover within weeks to months after the injury. However, a significant subgroup of patients with mTBI shows incomplete recovery (3–5). Despite these post-traumatic symptoms, structural brain damage in this population is often not seen using current clinical imaging modalities. The problem is significant worldwide, as mTBI accounts for 80–90% of all cases with TBI (6).

Different blood-based biomarkers have been suggested as outcome predictors of TBI to improve clinicians' abilities to optimize clinical care. Among the most studied biomarkers are the astroglial proteins S100 calcium-binding protein B (S100β), glial fibrillary acidic protein (GFAP), and the axonal protein neurofilament light (NF-L) (7–13). Other proteins, such as the anti-inflammatory protein interleukin 10 (IL-10) and the brain injury marker heart fatty acid-binding protein (H-FABP), have recently gained in interest as diagnostic tools for TBI, but little is known about their abilities as outcome predictors (14, 15).

To date, most biomarker studies have investigated proteins' individual prediction abilities. Considering the pathophysiological complexity of TBI, single biomarkers tend to have low prediction capacities, and they may therefore not be optimal for clinical use (16). To improve the accuracy, combining several biomarkers together or combining biomarker(s) with clinical parameters have been suggested, thus producing models of several predictive markers (16, 17). These kinds of models have already shown to significantly increase the predictive power compared to single markers in different diseases, such as aneurysmal subarachnoid hemorrhage, lung cancer, and sleeping sickness (18–21). Recent studies have also highlighted the beneficial use of panels as diagnostic tools for mTBI (22, 23) and for predicting the need for acute head imaging following TBI (24). Furthermore, combining inflammatory proteins together with brain-derived ones may improve the ability to predict outcome after TBI (25, 26).

The aim of the present study was to compare the proteins IL-10 and H-FABP to the well-studied proteins S100β, GFAP, and NF-L for their individual ability to predict patients who will have favorable long-term outcome analyzing patients on a clinically meaningful allocation basis: patients with mTBI and patients with TBIs of all severities. In order to demonstrate the added value of blood biomarkers in addition to clinical variables, we analyzed the biomarkers in isolation and with clinical variables recorded upon admission. Furthermore, combinations of predictive markers were evaluated in an attempt to increase this prediction ability.

MATERIALS AND METHODS

Study Population and Clinical Variables

In this single-center study, patients were recruited at Turku University Hospital (a tertiary care university hospital with a combined primary and tertiary care emergency department) in Finland between the years 2011–2013. All the consecutive patients with TBI were evaluated for eligibility to be recruited in the study by the research team between 8 a.m. to 10 p.m. To be included in the study, the following inclusion criteria needed to be fulfilled; age $\geq$18 years, hospital admission within 24 h after trauma, clinical diagnosis of a TBI with an indication for a head computed tomography (CT) scan according to the NICE criteria (27) as judged by an emergency physician on call, and outcome data at 6–12 months after injury had to be available. Exclusion criteria were penetrating or blast-induced injury, chronic subdural hematoma, inability to live independently due to a previous brain disease, no performed CT scan, or no written consent.

The ethical review board of Hospital District of South-West Finland approved the study protocol (decision 68/180/2011). Written informed consent was obtained from all participants or their legal representatives prior to inclusion.

The outcomes were assessed by single experienced neurologist (OT) using the Glasgow Outcome Scale Extended (GOSE) (28). The total injury burden was assessed with Injury Severity Score (ISS) (29).

Head Imaging and Traumatic Brain Injury Severity Classifications

Head CT scans were classified according to the Marshall classification system (30). Neuroradiologists at the Turku University Hospital and a senior neurosurgeon (JPP) double-read the CT scans.

In addition to using the lowest Glasgow Coma Scale (GCS) score before possible intubation, either at the scene of accident or emergency department (31), TBI severity was also classified using

an aggregate covariate combining the lowest GCS and the length of post-traumatic amnesia[1].

Protein Measurement

Serum samples were drawn within 24 h after trauma. However, as these different time points did not appear to correlate with biomarker levels, all of them were considered as a common time point. Only admission samples were assessed. After obtaining the blood samples, the samples were centrifuged and stored at −70 °C. The proteins GFAP and NF-L were measured using the Human Neurology 4-plex A assay (N4PA) on HD-1 single molecule array (Simoa) device from Quanterix (Lexington, MA, USA). The lower limit of quantification (LLoQ) for each kit was 0.104 pg/mL for NF-L and 0.221 pg/mL for GFAP. The protein S100β was measured using the EZHS100B-33K kit from Millipore (Millipore, Billerica, MA, USA) with an LloQ of 2.74 pg/mL. H-FABP and IL-10 were analyzed using the K151HTD and K151QUD kits, respectively, Meso Scale (Meso Scale Diagnostics, Rockville, MD, USA). The LloQ for H-FABP was 0.137 ng/mL and for IL-10 0.298 pg/mL. All proteins were measured according to manufacturers' recommendations by board-certified laboratory technicians who were blinded to clinical data.

Statistical Analysis

Statistical analyses were performed to evaluate the proteins' outcome prediction ability defined by the GOSE score. The patients were dichotomized in two different groups: (a) the first one included TBI patients of all severities (worst GCS 3–15, $n = 88$), while the second one (b) included only the subgroup of mTBI (worst GCS 13–15, $n = 49$) patients. The GOSE was dichotomized into (a) 1–4 for unfavorable outcome or 5–8 for favorable outcomes for all severity patients and (b) 1–7 for incomplete recovery or 8 for complete recovery for mTBI patients. These dichotomizations were chosen because the favorable/unfavorable outcome is relevant for TBIs in general (especially moderate—severe TBIs) and complete/incomplete for those with mTBI. Non-parametric tests were used because all proteins were non-normally distributed, as indicated by the Kolmogorov-Smirnov test ($p < 0.05$). Therefore, to evaluate the proteins' differences between groups, the Mann-Whitney U test was performed using IBM SPSS software, version 24.0 (SPSS Inc., Chicago, IL, USA). The proteins' outcome prediction capacities were evaluated using the partial area under a receiver operating characteristic cure (pAUC), which allowed us to focus only in the interest region of the ROC curve. Analysis were computed using TIBCO Spotfire S+® version 8.2 software (TIBCO Software Inc., Palo Alto, CA, USA). Model selections were done using the PanelomiX toolbox based on the iterative combination of biomarkers/variables and thresholds method (ICBT) (32). To make the process faster, several biomarkers (H-FABP, IL-10, S100β, NF-L, and GFAP)/variables (age, Marshall grade, Injury Severity Score, Severity, and GCS) were tested together. PanelomiX used the random forest method to select the different thresholds. Cross validation and ROC analysis were used to evaluate the performance of the model. In this manuscript, a maximum number of three biomarkers or clinical parameters in each model were investigated. The PanelomiX tool established the best cut-off for each single biomarker, and the best combinations were investigated when the sensitivity was set at 95–100% in order to reduce the false negative cases.

[1]https://www.healthquality.va.gov/guidelines/Rehab/mtbi/mTBICPGFullCPG50821816.pdf

RESULTS

Study Population

A total of 88 TBI patients with blood samples available within 24 from the time of injury were included in this study, and 69% of the patients were male. The most common causes of trauma were falls and traffic accidents. The distributions of severity, imaging findings, and outcome are shown in **Table 1**.

Complete Recovery – Patients With mTBI

We first investigated the proteins' individual capacities to predict complete recovery in patients with mTBI. All these proteins tended to be higher in patients with incomplete recovery (GOSE ≤ 7, $n = 24$) compared to those with complete recovery (GOSE 8, $n = 25$), although these findings were without significance. The proteins were investigated when the sensitivity was set at 95–100%. The individual specificities remained low. The best performing protein was H-FABP, reaching 4% specificity and 100% sensitivity. NF-L and S100β had somewhat higher specificities of 12% but lower sensitivities of 96% (**Table 2**).

Next, combinations of proteins to increase the ability to predict complete recovery were evaluated. The best two-protein panel was a combination of H-FABP and NF-L, reaching a specificity of 40% and sensitivity of 96%, thus resulting in a 36-percentage point increase in specificity compared to the best performing single protein H-FABP. A panel comprising three proteins—H-FABP, GFAP, and NF-L—was further capable of increasing the specificity to 44% with 96% sensitivity (**Table 3**).

Combining individual proteins with clinical parameters was also evaluated to predict complete recovery (**Table 3**). Combination of H-FABP, the most specific molecule in isolation, with clinical parameters showed an improved performance over proteins alone. Maintaining a value of 96% sensitivity, the combination of H-FABP, NF-L, and TBI severity reached a specificity value of 56%, which is far better than for H-FABP alone (**Table 3**). The individual outcome prediction capacities of clinical parameters are presented in **Supplementary Table 1**.

Favorable Outcome – Patients With TBIs of All Severities

H-FABP, IL-10, S100β, NF-L, and GFAP were evaluated for their individual capacities to predict favorable outcome in patients with TBIs of all severities. The blood levels of all proteins studied were significantly higher in patients with unfavorable outcome (GOSE ≤ 4, $n = 28$) compared to those with favorable outcome (GOSE ≥ 5, $n = 60$) ($p \leq 0.001$). Again, the ability of each biomarker for the detection of favorable outcome was evaluated when the protein reached 95–100% sensitivity. According to these criteria, the best performing protein was IL-10, with a

TABLE 1 | Characteristics of the study patients.

	TBI all severities				Mild TBI			
	Complete recovery		Favorable outcome		Complete recovery		Favorable outcome	
	GOSE 8	GOSE 1–7	GOSE 5–8	GOSE 1–4	GOSE 8	GOSE 1–7	GOSE 5–8	GOSE 1–4
	(*n* = 25)	(*n* = 63)	(*n* = 60)	(*n* = 28)	(*n* = 25)	(*n* = 24)	(*n* = 45)	(*n* = 4)
Age								
Mean (SD)	40.9 (20.27)	51.13 (18.39)	44 (18.68)	57.25 (18.02)	40.9 (20.3)	47.5 (18.7)	43.4 (19.6)	52.8 (20.9)
Gender								
Male, n (%)	17 (68)	44 (69.8)	43 (71.7)	18 (64.3)	17 (68)	9 (37.5)	31 (68.9)	1 (25)
Marshall Grade, *n* (%)								
No visual pathology (grade 1)	17 (68)	18 (28.6)	33 (55)	2 (7.1)	17 (68)	11 (45.8)	28 (62.3)	0 (0)
Diffuse injury (grade 2)	4 (16)	11 (17.5)	11 (18.3)	4 (14.3)	4 (16)	9 (37.5)	10 (22.2)	3 (75)
Diffuse injury with swelling (grade 3)	0 (0)	1 (1.6)	0 (0)	1 (3.6)	0 (0)	0 (0)	0 (0)	0 (0)
Diffuse injury with shift (grade 4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Evacuated mass lesion (grade 5)	1 (4)	22 (34.9)	9 (15)	14 (50)	1 (4)	1 (4.2)	2 (4.4)	0 (0)
Non evacuated mass lesion (Grade 6)	3 (12)	11 (17.5)	7 (11.7)	7 (25)	3 (12)	3 (12.5)	5 (11.1)	1 (25)
Injury Severity Score (ISS), *n* (%)								
Minor 1–8	16 (64)	13 (20.6)	27 (45)	2 (7.1)	16 (64)	10 (41.7)	25 (55.6)	1 (25)
Moderate 9–15	4 (16)	15 (23.8)	13 (21.6)	6 (21.4)	4 (16)	8 (33.3)	10 (22.2)	2 (50)
Serious 16–24	4 (16)	16 (25.3)	12 (20)	8 (28.6)	4 (16)	4 (16.7)	7 (15.6)	1 (25)
Severe 25–49	1 (4)	17 (26.9)	7 (11.7)	11 (39.3)	1 (4)	2 (8.3)	3 (6.7)	0 (0)
Critical 50–74	0 (0)	1 (1.6)	1 (1.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Maximum 75	0 (0)	1 (1.6)	0 (0)	1 (3.6)	0 (0)	0 (0)	0 (0)	0 (0)
***Severity, *n* (%)**								
Very mild 1	1 (4)	0 (0)	1 (1.7)	0 (0)	1 (4)	0 (0)	1 (2.2)	0 (0)
Mild 2	23 (92)	27 (42.9)	45 (75)	5 (17.9)	23 (92)	18 (75)	39 (8.7)	2 (50)
Moderate 3	0 (0)	12 (19.1)	7 (11.7)	5 (17.9)	0 (0)	4 (16.6)	3 (6.7)	1 (25)
Severe 4	1 (4)	10 (15.9)	5 (8.2)	6 (21.4)	1 (4)	1 (4.2)	1 (2.2)	1 (25)
Very severe 5	0 (0)	12 (19.1)	1 (1.7)	11 (39.3)	0 (0)	0 (0)	0 (0)	0 (0)
Unknown	-	2 (3.1)	1 (1.7)	1 (3.6)	0 (0)	1 (4.2)	1 (2.2)	0 (0)
GCS, *n* (%)								
Mild 13–15	25 (100)	24 (38.1)	45 (75)	4 (14.2)	25 (100)	24 (100)	45 (100)	4 (100)
Moderate 9–12	0 (0)	24 (38.1)	12 (20)	12 (42.9)	0 (0)	0 (0)	0 (0)	0 (0)
Severe 3–8	0 (0)	15 (23.8)	3 (5)	12 (42.9)	0 (0)	0 (0)	0 (0)	0 (0)
Time from injury to blood sampling								
Mean, hours (SD)	6.2 (5.7)	9.9 (6.1)	9.8 (6.1)	12.3 (6.6)	6.2 (4.8)	12.9 (5.7)	9 (10.3)	14 (4.6)

**Severity combined from GCS and duration of posttraumatic amnesia, see ref[1].*

sensitivity reaching 96% and specificity of 50%. The proteins H-FABP and GFAP performed similarly, with sensitivities of 96% for both and specificities of 30% and 28%, respectively (**Table 4**).

The proteins' performances were also evaluated when they were combined in panels and when they were combined with clinical parameters. Individual performances of clinical parameters are presented in **Supplementary Table 2**.

The best combination using two proteins was IL-10 and H-FABP. This panel was capable of reaching 96% sensitivity and 58% specificity. This combination increased the specificity with 8 percentage points compared to the best single molecule IL-10. The best performing panel combining three proteins included IL-10, H-FABP, and GFAP. This panel managed to maintain the sensitivity at 96% and to increase the specificity up to 63% (**Table 5**).

The combination of individual proteins with clinical parameters improved the predictive ability compared to predictions using only protein biomarkers. Combining IL-10, the most specific protein, with patient's age and TBI severity reached 80% specificity with 96% sensitivity, which produces an increase in the specificity of 30 percentage points when comparing with the best single protein marker (**Table 5**).

DISCUSSION

Reliable early prediction of the patient's outcome in TBI can help clinicians in their decision-making and thereby optimize the care cost-effectively. Different blood biomarkers have previously been suggested as objective outcome predictor tools. This prospective, observational study of patients with acute TBI showed the potential benefits of using novel TBI biomarkers IL-10 and H-FABP but also previously studied biomarkers GFAP (13, 33), S100β (34), and NF-L (11,

33, 35) as individual predictive biomarkers for promising outcome prediction; this also showed that models including previously known robust clinical predictors may further improve the accuracy.

According to the results presented in this manuscript we can highlight that blood biomarkers have a strong outcome-predictive capacity in patients with TBI and more importantly that the combination with other biomarkers or clinical parameters enhances this precision.

The present study has shown that, in mTBI patients, among all the tested proteins, H-FABP exhibits the best capacity in discriminating patients with complete and incomplete recovery. This protein was also selected among the most promising ones when the predictive capacity of panel of proteins was evaluated; the combination of GFAP, H-FABP, and NF-L reached a specificity value of 40% when the sensitivity was set at 95–100%. Furthermore, as previously stated, inclusion of different clinical covariates showed to importantly augment the outcome prediction capacity of the proteins. Models including H-FABP, NF-L, and ISS yielded a specificity of 56%

In regard to patients with TBIs of all severities, IL-10 has the best capacity to discriminate favorable and unfavorable outcome; however, similarly to the mTBI population, the specificities of all proteins studied remained ≤50% when studied in isolation. Based on these findings, we again studied the proteins in panels fixing the sensitivity at 95–100%, which allowed us to find that the combination of GFAP, H-FABP, and IL-10 reached a specificity value of 63%. Once again, the inclusion of clinical covariates drastically improved the results reaching a specificity of 80% when IL-10, age and TBI were combined together.

Briefly, the current results indicate that of all studied proteins H-FABP has the best capacity to predict complete recovery and IL-10 to predict favorable outcome. In all two- and three-biomarker panels, either H-FABP and IL-10 were included. These markers have previously been shown efficient as CT scan triage tool for patients with mild TBI (22).

The protein H-FABP is a small cytoplasmic protein that leaks out from injured endothelial cells and neuronal cell bodies. It is a well-known myocardial infarction biomarker, and, even if it is not a TBI specific marker, increased levels have previously

TABLE 2 | Performance of single proteins when differentiating between complete (GOSE 8) and incomplete (GOSE ≤ 7) recovery in patients with mTBI.

	% pAUC (95% CI)	Threshold	%SP (95% CI)	95–100 %SE (95% CI)
H-FABP	0.2 (0.0–0.7)	44.9	4.0 (0.0–12)	100 (100–100)
NF-L	0.1 (0.0–1.2)	4.85	12 (0.0–24.1)	95.8 (87.5–100)
S100β	0.1 (0.0–1.0)	23.17	12 (0.0–28.0)	95.8 (87.5–100)
IL-10	0.0 (0.0–0.7)	–	0.0 (0–0)	100 (100–100)
GFAP	0.0 (0.0–0.6)	–	0.0 (0–0)	100 (100–100)

Biomarkers are shown according to their specificity obtained at 95–100% sensitivity. pAUC, partial area under the curve; SP, specificity; SE, sensitivity; IL-10, interleukin 10; H-FABP, heart fatty-acid binding protein; GFAP, glial fibrillary acidic protein; NF-L, neurofilament light; S100β, S100 calcium-binding protein B. All threshold concentrations are in pg/mL except for H-FABP which is in ng/mL.

TABLE 4 | Performance of single proteins when differentiating between favorable (GOSE 5–8) and unfavorable (GOSE 1–4) outcomes in all severity patients.

	% pAUC (95% CI)	Threshold	%SP (95% CI)	95-100 %SE (95% CI)
IL-10	1.4 (0.7–3.0)	0.39	50.0 (36.7–63.3)	96.4 (89.3–100)
H-FABP	1.1 (0.6–2.7)	4.31	30.0 (18.3–41.7)	96.4 (89.3–100)
GFAP	0.8 (0.3–2.3)	415	28.3 (16.7–40.0)	96.4 (89.3–100)
NFL	0.5 (0.2–4.4)	5.47	10.0 (3.3–18.3)	100 (100–100)
S100β	0.1 (0–1.4)	23.17	6.7 (1.7–13.3)	96.4 (89.3–100)

Biomarkers are shown in order according to their specificity obtained at 95–100% sensitivity. Mann U, Mann-Whitney U-test; pAUC, partial area under the curve; SP, specificity; SE, sensitivity; IL-10, interleukin 10; H-FABP, heart fatty-acid binding protein; GFAP, glial fibrillary acidic protein; NF-L, neurofilament light; S100β, S100 calcium-binding protein B. All threshold concentrations are in pg/mL except for H-FABP, which is in ng/mL.

TABLE 3 | Panels performed in patients with mTBI, including proteins (H-FABP, IL-10, GFAP, S100β, and NF-L) or proteins and clinical parameters: Marshall grade, severity, injury severity score, and age.

Panel	Markers cut-off	n GOSE 8	n GOSE <7	% SP (95% CI)	% SE (95% CI)
2 Parameters (only proteins)	H-FABP (6.26) NF-L (15.9)	25	24	40.0 (20.0–60.0)	95.8 (87.5–100)
3 Parameters (only proteins)	H-FABP (5.01) NF-L (13.46) GFAP (2457.5)	25	24	44.0 (24.0–64.0)	95.8 (87.5–100)
2 Parameters (proteins/clinical parameters)	H-FABP (6.26) NF-L (15.9)	25	24	40.0 (20.0–60.0)	95.7 (87.0–100)
3 Parameters (proteins/clinical parameters)	H-FABP (4.30) NF-L (13.46) Severity (2.5)	25	24	56.0 (36.0–76.0)	95.7 (87.0–100)

Complete recovery GOSE 8 and incomplete recovery GOSE ≤ 7; SP, specificity; SE, sensitivity. All protein cut-off concentrations are in pg/mL except for H-FABP, which is in ng/mL.

TABLE 5 | Panels including only proteins (H-FABP, IL-10, GFAP, S100β, and NF-L) or proteins and clinical parameters (Marshall grade, severity, injury severity score, age, and GCS).

Panel	Markers cut-off	n GOSE≥5	n GOSE≤4	% SP (95% CI)	% SE (95% CI)
2 Parameters (only proteins)	IL-10 (0.38) H-FABP (4.31)	60	28	58.3 (45.0–71.7)	96.4 (89.3–100)
3 Parameters (only proteins)	IL-10 (0.38) H-FABP (4.31) GFAP (145.13)	60	28	63.3 (51.7–75.0)	96.4 (89.3–100)
2 Parameters (proteins/clinical parameters)	NF-L (41.5) Severity (2.5)	59	27	72.9 (61–84.7)	96.3 (88.9–100)
3 Parameters (proteins/clinical parameters)	IL-10 (0.44) Age (61) Severity (2.5)	59	27	79.7 (69.5–89.8)	96.3 (88.9–100)

Favorable outcome GOSE ≥5 and unfavorable outcome GOSE ≤4; SP, specificity; SE, sensitivity. All protein cut-off concentrations are in pg/mL except for H-FABP, which is in ng/mL.

been shown in patients with severe TBI and poor outcome (36–39). IL-10 or human cytokine synthesis inhibitory factor is a potent anti-inflammatory cytokine mainly produced by macrophages, B cells, and dendritic cells. Similarly, it is not a brain-specific marker, but the levels have also been shown to be increased in patients with severe TBI and poor outcome (40). Literature on the prognostic value of both H-FABP and IL-10 is scarce. The previous reports on the outcome prediction ability of IL-10 after TBI are contradictory, though it has shown some potential in the prediction of mortality—the past studies have been very heterogenic in their methods (41). Intriguingly, the best-performing three-biomarker panel in discriminating patients with favorable and unfavorable outcome in patients with TBIs of all severities (GFAP, H-FABP, and IL-10) was the same that we have previously reported to be the best panel in detecting patients with CT-positive findings in the groups of patients with TBIs of all severities and patients with mTBIs without extracranial injuries (24). Because all these studies are from the same patient population, further studies should confirm if these combinations perform best also in other patients with acute TBI.

Several different pathophysiological processes can affect the outcome of TBI, such as apoptosis, blood brain barrier damage, and neuroinflammation (42). TBI is a complex pathophysiological disease where single markers may never be accurate enough for clinical applications (43). Combining biomarkers from different pathophysiological pathways has previously been shown to increase the overall accuracy (16, 24). Among other clinical parameters, such as age, Marshall grade, or ISS, we chose to include both the GCS score and TBI severity (an aggregate covariate) to the predictive models consisting of clinical covariates and biomarkers because we hypothesized that it would better reflect the importance of PTA in combination with the GCS score. As seen in the results, TBI severity is included more often in the predictive models than the GCS score alone. The prediction capacity of biomarkers is usually evaluated using the total AUC value; however, this entire value of ROC curve evaluates regions that usually are not relevant to clinical applications. Therefore, in order to avoid this drawback we decided to focus on pAUC, evaluating specificity values only when the sensitivity was fixed between 95 and 100% (44).

The model selection method used in this manuscript, PanelomiX, has been shown to have several advantages over other already known selection methods, such as random forest, support vector machine or logistic regression. It has provided us the selection of best biomarkers specifying their optimal cut-off points (obtained by cross-validation). Obtained results are easy to interpret for the clinicians, which is another important advantage over other traditional black-box methods, and furthermore the prediction performances of panels obtained using PanelomiX tool are similar to that obtained with support vector machine among others.

This study has several limitations. Blood samples were collected within the first 24 h after trauma, which could be too late for the measurement of several biomarkers as S100β and GFAP (24, 45). The optimal biomarker panel is probably greatly dependent on both the time from injury and type of injury, and this is why further studies are necessary to clarify which biomarkers or biomarker combinations perform best at different points of time in different TBI populations. There is also some evidence that the clinical usability of biomarkers may depend on the age of the patient (46). Furthermore, the cohort used here included only 88 patients with TBI, making multivariate analysis of the molecules inconvenient according to the Monte Carlo study (47). The fairly small study population also increases the risk of over-fitting bias, which is why the results should be verified and validated in a larger cohort. Our study cohort included patients with TBI of all the severities where 56% had mTBI and 17% had severe TBI. For the whole cohort, the results may be partly driven by the more severe cases of TBI due to their higher biomarker levels, and, on the other hand, our cases of mTBI were more severe than patients with mTBI as a whole

since most of our cases had been admitted to neurosurgical ward. We chose to study patients with mTBI and TBIs of all severities independently because (i) patients with mTBI are a distinctive group of patients with specific diagnostic needs, and (ii) examining the outcome prediction ability in patients with all severities is important because initial severity of a TBI is not always clear due to possible confounding factors (such as intoxication, hypoglycemia, and neurological diseases).

Moreover, using GOSE as an outcome measure is a relatively insensitive way to detect subtle impairments in patients with mTBI or in TBI in general since it will miss many important and subtle deficits and it may be affected also by factors not directly related to the anatomical brain injury itself. The usability of IL-10 and H-FABP should be studied using a higher number of patients with different clinical characteristics.

CONCLUSION

The novel proteins IL-10 and H-FABP in TBI diagnostics show promise in detecting patients with either favorable outcome or complete recovery following TBI. Combining levels of these proteins and NF-L with clinical covariates may assist in clinical decision-making at the emergency department and stratification for different monitoring and treatment algorithms.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this article are not publicly available. Requests to access the datasets should be directed to leire.azurmendi@unige.ch.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by 68/180/2011. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LL, LA, and J-CS conceived and designed the study with critical contributions from OT and JP. OT, JP, AK, RT, H-RM, and JT recruited the patients and collected the data with critical contributions from IH. LL, LA, J-CS, HZ, and KB were responsible for analytical biomarker assessments. LL, LA, and J-CS conducted the statistical analyses. LL, LA, OT, JP, and J-CS drafted the manuscript. All authors substantially contributed to the revision of the manuscript. OT, VN, MG, PH, JP, and J-CS supervised the study. JP and J-CS take the responsibility for the paper as whole.

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REVIEW
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Extracellular Vesicle Proteins and MicroRNAs as Biomarkers for Traumatic Brain Injury

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Traumatic brain injury (TBI) is a heterogeneous condition, associated with diverse etiologies, clinical presentations and degrees of severity, and may result in chronic neurobehavioral sequelae. The field of TBI biomarkers is rapidly evolving to address the many facets of TBI pathology and improve its clinical management. Recent years have witnessed a marked increase in the number of publications and interest in the role of extracellular vesicles (EVs), which include exosomes, cell signaling, immune responses, and as biomarkers in a number of pathologies. Exosomes have a well-defined lipid bilayer with surface markers that reflect the cell of origin and an aqueous core that contains a variety of biological material including proteins (e.g., cytokines and growth factors) and nucleic acids (e.g., microRNAs). The presence of proteins associated with neurodegenerative changes such as amyloid-β, α-synuclein and phosphorylated tau in exosomes suggests a role in the initiation and propagation of neurological diseases. However, mechanisms of cell communication involving exosomes in the brain and their role in TBI pathology are poorly understood. Exosomes are promising TBI biomarkers as they can cross the blood-brain barrier and can be isolated from peripheral fluids, including serum, saliva, sweat, and urine. Exosomal content is protected from enzymatic degradation by exosome membranes and reflects the internal environment of their cell of origin, offering insights into tissue-specific pathological processes. Challenges in the clinical use of exosomal cargo as biomarkers include difficulty in isolating pure exosomes, variable yields of the isolation processes, quantification of vesicles, and lack of specificity of exosomal markers. Moreover, there is no consensus regarding nomenclature and characteristics of EV subtypes. In this review, we discuss current technical limitations and challenges of using exosomes and other EVs as blood-based biomarkers, highlighting their potential as diagnostic and prognostic tools in TBI.

Keywords: exosomes, concussion, neurodegeneration, neuroinflammation, extracellular vesicles

INTRODUCTION

Traumatic brain injury (TBI) is a major cause of disability worldwide, affecting an estimated 10 million people annually, representing a growing burden to public health (1, 2). TBI may be caused by a bump, blow or jolt to the head or a penetrating head injury that causes structural damage or disrupts normal brain function (3). TBI severity can range from mild to severe and is determined based on clinical factors including presence and duration of loss of consciousness, post-traumatic amnesia, mental state alterations and neuroimaging findings (3). Mild TBI (mTBI) is the most frequent type, affecting all demographics (4). Clinical management of mTBI is challenging as diagnosis may be difficult and clinical presentation and recovery varies among individuals. Moreover, even in milder cases, TBI may trigger neurodegenerative changes and place survivors at risk of developing chronic neurological and behavioral symptoms (5, 6), and affecting quality of life and functioning of individuals within family and society (7).

TBI is increasingly seen as a chronic disorder that may affect long-term health (7). Factors underlying individual susceptibility to develop TBI-related neurodegenerative changes and persistent or late-in-life symptoms are still largely unknown (7, 8). Lifestyle, sex, genetic, and social factors, medical history, including previous head injuries, are all likely important determinants in TBI recovery (7, 9). Indeed, sustaining multiple TBIs has been linked to lasting or worsening neurobehavioral symptoms, placing populations such as service members and contact-sport athletes at a higher risk for worse outcomes following a TBI (10–12). The heterogeneous nature of TBI and limited understanding of underlying pathology represents a challenge to the development of effective therapeutic strategies. Thus, the clinical need for diagnostic and prognostic tools for TBI has prompted several studies aimed at identifying biomarkers to inform clinical interventions and identifying those most at risk for poor recovery and chronic sequelae (13–17). Candidate biomarkers measured in serum or plasma (i.e., blood-based biomarkers) and other bodily fluids have been explored by several research groups and offer safe and inexpensive methods to monitor brain injury (13–20). Most studies have focused on proteins derived from damaged neurons and astrocytes (13, 17, 21–23). Other candidate biomarkers include markers of inflammatory responses and vascular injury (14, 24, 25), as well as circulating microRNAs (miRNAs), which are small non-coding RNAs with major roles in the regulation of gene expression (26, 27). Studies have shown the potential of biomarkers to inform clinical decisions and predict short-term outcomes such as return-to-play in sport-related concussion (14, 17, 23, 28).

In recent years, exosomes have sparked interest in the scientific community for their emerging role in cell-to-cell communication involved in physiological and pathological processes throughout the body. Exosomes are part of the broader population of extracellular vesicles (EVs), that also includes microvesicles and apoptotic bodies (29). However, no consensus has been reached among experts regarding characteristics of EV subtypes as this is a fast evolving and relatively new research field, and specific markers are still being defined (30–32). The terms exosome and EV are commonly used interchangeably in the literature to refer to vesicles formed by a lipid bilayer that contains cargo including proteins (e.g., cytokines and growth factors), nucleic acids and lipids (30, 32). Here, we follow the International Society for Extracellular Vesicles (MISEV) recommendations and adopt the term EV as the generic term for lipid bilayer-delimited particles released from cells (31, 32). Thus, the term EV will be used in this review to describe study findings and general concepts, applying the term exosome only when necessary to describe the specific subtype of EV.

Candidate biomarkers of TBI, including proteins and miRNAs previously identified in serum or plasma samples, have also been found in EVs isolated from peripheral blood (33, 34). Moreover, efforts have been made to identify the proteomic signature and RNA expression profiles in EVs derived from specific cell types (35–37). This review focuses on the evidence of EVs as a promising new family of biomarkers for TBI as well as challenges in the field. We address the emerging insights into roles played by EVs in the central nervous system (CNS), linking them to TBI-related neuropathology.

TBI-RELATED SYMPTOMS AND ASSOCIATED DISORDERS

TBI can result in highly variable symptoms among individuals that are typically related to physical, cognitive, and affective domains (38, 39). Headache is the most common physical symptom in individuals with mTBI (40, 41). Sleep disruption and fatigue are also frequently reported following TBI; incidents estimates indicate that post-TBI, between 21 and 73% of individuals experience fatigue, which can persist for years after initial injury (42). Some form of sleep disturbance is reported by 50% of individuals following TBI. Moreover, prevalence rates of sleep disorders in TBI patients are elevated compared to the general population, with two times the risk for periodic limb movements, three times the risk for insomnia and hypersomnia, and 12 times the risk for sleep apnea (43). Other common physical symptoms include dizziness (41, 44, 45), nausea (41), light/noise sensitivity (46), chronic pain (47), and in moderate-to-severe TBI, seizures (48).

Acute moderate to severe TBI is characterized by impaired consciousness and post-traumatic amnesia (PTA) (3, 49). In mTBI, loss of consciousness and PTA might not occur. Subacute and chronic cognitive symptoms are common, persisting in ∼31–63% of individuals who sustained a TBI (50–52). The most prevalent chronic cognitive deficits are memory, executive functioning, attention, and processing speed, especially among those with a history of multiple mTBIs or moderate-to-severe TBI (51, 53, 54). Symptoms following TBI, even mTBI, can be long-lasting: more than half of patients who incurred a TBI reported experiencing three or more symptoms 1 year after injury (55). In mTBI, the collection of neuropsychological symptoms (i.e., a constellation of neurological, cognitive, and affective symptoms) is often referred to as post-concussion syndrome (PCS). However, this term is controversial and is not universally accepted because these symptoms are not specific to concussion

patients and can be found in patients with moderate and severe TBI (55–58).

TBI is a risk factor for the development of several psychiatric disorders. Post-traumatic stress disorder (PTSD) and TBI are often comorbid, with this relationship mostly studied in military populations. In two studies of over 2,500 US military personnel, 44% of individuals who reported loss of consciousness during deployment also met criteria for PTSD (59); and combat-related mTBI increased the risk for PTSD more than 2-fold (60). Major depressive disorder (MDD) and generalized anxiety disorder (GAD) are frequently comorbid with TBI; 27 and 11% of individuals with TBI are also diagnosed with MDD or GAD, respectively (61, 62).

TBI, including mild cases, can lead to neurogenerative changes. Moderate to severe TBI has been linked to earlier onset of Alzheimer's disease (AD) and dementia (5, 6, 63–66). A recent meta-analysis concluded that previous head injury increases the risk factor for any dementia by 63% and AD by 51%, but only for males (67). Moreover, mTBIs have been recently associated with a 2-fold increased risk of developing dementia in Veterans (5). Multiple mTBIs are linked to elevated risk of developing progressive neurodegenerative disease associated with neurological and cognitive impairments (6). TBI, including mTBI, has also been associated with increased risk for Parkinson's disease (68, 69). Sustaining multiple mTBIs is linked to elevated risk of developing progressive neurodegenerative disease associated with neurological and cognitive impairments (6).

The link between TBI and a wide range of cognitive, psychiatric, and neurological symptoms and disorders is marked, but the biological processes underlying this association are still largely unknown. As discussed in the following sections, TBI results in a cascade of cellular and molecular events that lead to cell death, neurovascular injury and inflammation (3, 6). Several research groups have been able to isolate EVs from the peripheral blood of TBI patients and measure their content (33, 35, 37). Analyzing levels of specific proteins, miRNAs, and other signaling molecules in EVs at different timepoints after TBI, while examining relationships with specific symptoms, could lead to the development of novel therapeutic strategies in TBI, and biomarkers that predict risk of developing specific symptoms after a head injury. This approach may ultimately lead to clinical interventions for those most at risk, prior to the onset of symptoms and underlying pathological processes. Associations between EV biomarkers and symptom severity are described in the following sections (33, 35). Next, we discuss major mechanisms underlying TBI neuropathology.

NEUROPATHOLOGY OF TBI

The pathology of TBI is complex, heterogeneous, and comprised of both immediate and delayed elements. Morphologically, brain injury can be divided into focal and diffuse injury. Focal injury is due to a severe and direct impact on the brain, including cortical and subcortical contusions and lacerations as well as hemorrhage and hematoma (70, 71). Diffuse injury is caused by stretching and tearing of brain tissue and includes axonal and microvascular injury (70, 71). Diffuse axonal injury (DAI) is a form of diffuse injury caused by acceleration and deceleration forces that lead to the shearing of axons (70, 72). DAI is a key pathological process in mTBI, reflecting the vulnerability of white matter axons to rapid head acceleration/deceleration caused by a hit to the head. DAI is believed to break the axonal cytoskeleton, affecting axonal transportation, which leads to neurodegeneration (70, 72, 73).

TBI neuropathology consists of a primary injury, which ranges from mild to severe, that is a direct consequence of the traumatic insult and the effects of the mechanical forces on the brain tissue, directly damaging neurons, glial cells and vasculature in focal or diffuse patterns (71) (**Figure 1**). Secondary injury results from a cascade of molecular and cellular events triggered by the primary injury and includes responses such as edema, hypoxic-ischemic injury, vascular injury, hypometabolism, and neuroinflammation (70, 71).

Microglia are resident myeloid cells in the brain that clean debris and dying cells, among other housekeeping functions (74). Microglia mediate host defense against infectious pathogens, CNS tumors, and proteins such as amyloid β (Aβ) (75, 76). In response to TBI, microglia, as well as astrocytes, become active, changing morphology and initiating an inflammatory cascade by secreting cytokines, chemokines, and growth factors (76, 77). The inflammatory response following TBIs starts within minutes of the injury (78). Resident brain microglia are the first to activate and migrate toward the focal injury (79, 80). Within hours of injury, neutrophils arrive at the injury site to begin clearance, followed by macrophages 1–2 days later (81). After a TBI, the levels of various cytokines undergo a pronounced increase, which typically peaks hours or days after the injury (82, 83).

Blood-brain barrier (BBB) disruption frequently occurs after TBI and can last from days to years after head trauma (84, 85). Increased BBB permeability is considered a key mechanism in TBI secondary injury, and is involved in prolonged inflammatory responses, delayed neuronal dysfunction, and cell death (85). Additionally, damage to cell lining of the BBB leak compounds usually confined within the brain into the periphery, exposing innate, and adaptive immune cells to neurological antigens. Some researchers have suggested that acute TBI may trigger brain tissue-targeting autoimmunity (86), and indeed, acute TBI patients have developed autoreactive antibodies and T-cells within the periphery that are capable of detecting and reacting with brain-derived components years after the initial injury (81). Components of the BBB (i.e., astrocytes, pericytes and endothelial cells) are susceptible to the effects of the injury (87), but underlying molecular changes that lead to BBB disruption following TBI are not completely known.

Pathophysiological mechanisms of TBI that result in AD-like neurodegenerative processes remain poorly understood, but neuroinflammation leading to neurodegeneration is a likely candidate. AD pathology is characterized by intra-neuronal neurofibrillary tangles of hyperphosphorylated tau (p-tau) and deposits of extracellular Aβ, which likely relate to the dysfunction of brain clearance mechanisms (88, 89). Aβ deposition is regulated by an equilibrium between Aβ production and

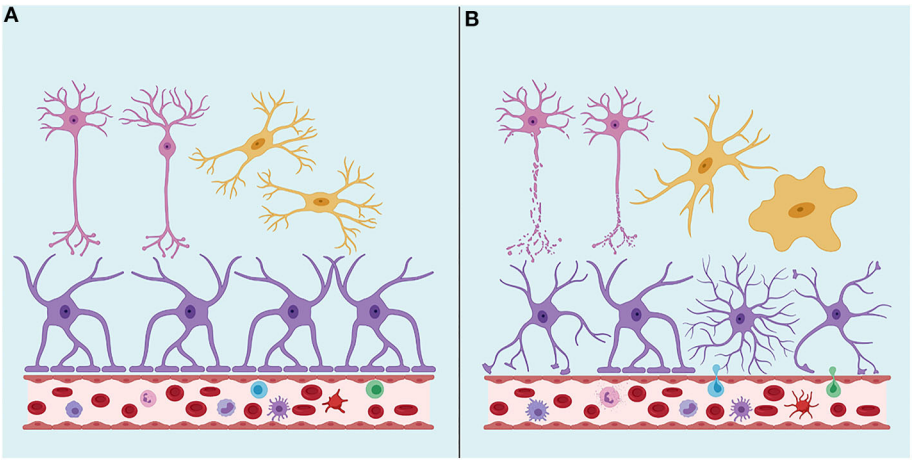

FIGURE 1 | (A) Under physiological conditions, the Brain-Blood Barrier (BBB) creates a restrictive barrier between central nervous system and circulating blood contents. BBB is formed by astrocyte endfeet, pericytes and tight junctions among endothelial cells. **(B)** After a traumatic brain injury (TBI), BBB may become dysfunctional. TBI may also lead to axonal shearing, activation of microglia, astrocytes, and peripheral immune cells, often resulting in neuroinflammation, edema, neuronal hyperexcitability, and cell death. Neurons are represented in pink, astrocytes in purple and microglia in yellow. Created with Biorender.com.

clearance. Chronic traumatic encephalopathy (CTE) and CTE-like neurodegeneration involve the progressive buildup of p-tau and neurofibrillary tangles (NFT). Neuroinflammation, with the presence of activated microglia and astrocytes, has also been implicated in AD as well CTE. Similarly, long-lasting increases in microglia and astrocyte reactivity, in addition to elevated levels of proteins associated with neurodegeneration (e.g., tau, Aβ42, and Aβ40), have also been described in TBI (90–92). In TBI, as well as in neurodegenerative diseases, microglia and astrocyte activation is a double-edged sword. Microglia and astrocyte activation can elicit protective mechanisms, but their persistent activation can also trigger deleterious processes and worsen tissue injury (75, 93). At a certain timepoint during disease progression, glial cells assume a useful role, then progress into a dysfunctional cell that ultimately becomes harmful.

Further understanding the neuropathology of TBI and mechanisms underlying long-term consequences of head injuries is fundamental to develop novel and effective clinical interventions. Investigating the emerging role of EVs in TBI and related pathologies may fill important knowledge gaps. However, as discussed next, the term EV encompasses a variety of vesicle types, which are yet to be fully characterized and may play distinct roles in TBI.

HETEROGENEITY OF EXTRACELLULAR VESICLES

EVs are heterogeneous in size, content, biogenesis, and membrane composition, which suggests variability in biological function. Terms used to classify EVs include exosomes, ectosomes (microvesicles or microparticles), apoptotic bodies and oncosomes (29, 94). EVs include different populations of vesicles that can be categorized according to their biogenesis mechanisms in exosomes (derived from endocytic membranes) and ectosomes (assembled in the plasma membrane) (95). Exosomes are smaller (30–150 nm) than ectosomes (50–1,000 nm), but size alone does not determine the population which adds to the challenge of distinguishing EV subtypes (29, 31, 96, 97). Exosome precursors are called intraluminal vesicles (ILVs) which are formed via the inward budding of the membrane of endocytic cisternae. The accumulation of ILVs in the endocytic cisternae forms multivesicular bodies (MVBs). When MVBs fuse with the plasma membrane, the released ILVs are called exosomes. EV biogenesis has been reviewed in detail elsewhere (95). Apoptotic bodies are vesicles that are also shed from the plasma membrane during apoptosis (98). The term oncosomes (100–400 nm) is applied to vesicles that carry abnormal macromolecules such as oncogenic proteins (99).

Exosomes have membranes abundant in tetraspanins (e.g., CD9, CD63, CD81) that are important for trapping membrane and luminal proteins and lipids (e.g., cholesterol and sphingomyelin) (94, 95, 100). Exosomal membrane also contains adhesion proteins (e.g., L1 cell adhesion molecule, L1CAM, which is considered a neuron-specific marker), integrins, heat shock proteins (HSPs), tumor susceptibility gene 101 protein (Tsg101), and ALG-2-interacting protein X (Alix), among others (31). The membrane of ectosomes is rich in glycoproteins, metalloproteinases and some receptors (101). Exosomes and ectosomes contain many proteins and nucleic acids (mRNAs, miRNAs and other non-coding RNA) in their lumina (102). Exosome cargo is enriched for miRNA and their membrane offers protection against RNAases that degrade free RNA, providing higher stability for miRNAs in body fluids and during experimental manipulation (102).

Exosome and ectosomes are both found in extracellular fluids, such as blood, and may be produced by the same cell types (103, 104). EVs with a similar size as exosomes can bud at the plasma membrane, and exosomes themselves are a heterogeneous population with variable sizes (100). Thus, determinations of size and density of exosomes should not be used as the only criteria to determine the presence of exosomes in a sample. Because exosome membrane is enriched in tetraspanins, they are frequently used as exosome markers (31, 100). However, tetraspanins might also be present in other subpopulations of EVs (100). Additionally, EVs derived from distinct cell types differ not only in their cargo content but also in membrane proteins, allowing for the use of antibodies against specific protein markers to enrich samples for EVs that originated from specific cells (100).

The existence of cell-derived vesicles has been known for decades. Platelet-derived vesicles were described by Wolf in 1967 (105). He reported that plasma free of platelets contains a material he called platelet-dust, which he isolated by ultracentrifugation and that contained coagulant properties. The term exosomes was first proposed by Trams et al. (106), referring to vesicles "exfoliated" from neoplastic cell lines. Examining these vesicles under the electronic microscope, Trams et al. (106) reported an average diameter ranging from 500 to 1,000 nm and the frequent presence of a second population of vesicles 40 nm in diameter. In 1987, the term exosomes was used to describe vesicles released from the plasma membrane and originated from multivesicular bodies that fused with the plasma membrane of reticulocytes in cell culture (107). Subsequent studies showed the release of exosomes from different cell types and the presence of MHC class-II on the membrane of these vesicles (108–111). Exosomes released from human and murine B lymphocytes induced antigen-specific MHC class-II restricted T cell responses, suggesting a role for exosomes in antigen presentation *in vivo* and in immunological responses (108).

The interest in exosomes, and more recently other EV types, has increased during the last decade, resulting in an extensive and rapidly growing literature, making it challenging to separate evidence-based information from assumptions and hypothesis. A wealth of information regarding exosomes and other EVs can be found in online resources such as ExoCarta (http://www.exocarta.org) (112) and Vesiclepedia (http://www.microvesicles.org). In an effort to establish minimal requirements for the definition of EVs and their functions, the International Society for Extracellular Vesicles (ISEV) has published a set of guidelines (31, 113). Nevertheless, terminology and classification of EVs, including the size range associated with specific EV types, is highly variable in the literature. Further understanding of EV roles in healthy tissues and pathological processes, in addition to technical advancements in the field, may shed light on the functional significance of EV heterogeneity and allow further characterization of distinct vesicle subpopulations. Concentrations and content of specific EV subpopulations could be analyzed in TBI patients, examining relationships between biomarker levels in each EV subpopulation and TBI recovery.

EXTRACELLULAR VESICLES IN THE CENTRAL NERVOUS SYSTEM AND NEUROLOGICAL DISEASES

The secretion of EVs used to be understood to be a means of elimination of proteins and unwanted molecules from the cells (114). Currently, EVs are considered promising biomarkers and delivery systems for therapeutics and a new form of cell-to-cell communication with roles in an expanding list of diseases and conditions such as cancer, inflammatory bowel diseases, obesity and diabetes, rheumatoid arthritis, and neurological diseases (115). In TBI, possible roles for EVs are only beginning to be explored. Studies investigating EVs in TBI will be discussed in the next section. Here, we briefly discussed evidence suggesting a role for EVs in the brain and neurogenerative diseases, which provides insight into the possible relevance of EVs in TBI.

EVs are released by all major cells in the CNS, including neurons, astrocytes, microglia and oligodendrocytes (116–118). Roles of EVs in brain physiology and disease are only beginning to be understood. Studies have suggested roles for EVs in elimination of waste (119) and cell-to-cell communication (119–121). A subpopulation of MHC class -II-negative microglia has been shown to internalize EVs secreted by oligodendrocytes *in vitro*, which suggests a role for EVs in the pathogenesis of autoimmune diseases that include the transfer of antigens from oligodendrocytes to immune cells (119). A bidirectional communication between neurons and oligodendrocytes involving EVs has also been reported: the release of the glutamate by neurons regulates the secretion of EVs by oligodendrocytes, which are internalized by neurons (122).

In AD, EVs have been hypothesized to be involved in the lateral and long-distance propagation of tau as well as in a number of mechanisms associated with AD pathogenesis as previously reviewed elsewhere (123, 124). Importantly, proteases that contribute to the biogenesis of Aβ fragments have been found in EVs (125–127). Nevertheless, while EVs are likely associated with the progression of AD, they might also be part of protective mechanisms as they are a part of clearance processes in the brain (128, 129). Indeed, EV surface carries insulin-degrading enzyme, which also degrades Aβ (128). EVs are also believed to be a potential source of biomarkers for AD, as well as other neurodegenerative diseases such Parkinson's disease, CTE and Creutzfeldt-Jacob disease. Proteins such as Aβ, tau, α-synuclein and prions are found in EVs (123, 130, 131). Similarly, elevated levels of molecules such as Aβ and tau in EVs might serve as biomarkers for neurodegenerative changes after TBI. Levels of miRNAs in EVs may also be used to reveal underlying signaling mechanisms and serve as biomarkers. Accordingly, changes in miRNA expression, including EV miRNAs, have been linked to aging and age-related diseases (132), and targeted inhibition of miRNAs may have therapeutic effects (133).

As previously discussed, neuroinflammation characterized by glial activation and cytokine release is an important element of neurodegenerative diseases and TBI pathology in acute and chronic phases. Following TBI, peripheral blood levels of diverse

cytokines undergo a pronounced increase, which typically peaks hours or days after injury (82, 83). Higher acute blood levels of interleukin (IL)-6 (134–137), IL-10 (134, 138, 139), TNF-alpha (137, 140, 141), as well as other cytokines (83) after TBI have been linked to poor outcomes. Similarly, in chronic TBI, increased blood levels of IL-6 and TNF-α relate to TBI symptoms in military personnel (137, 142). Interestingly, recent studies suggest that cytokines mediate cell-to-cell signaling not only as a soluble factor, but also via a system mediated by EVs (35, 143). Cytokines associated with EVs (surface-bound and encapsulated) are biologically active (143). Researchers have hypothesized that cytokines found on the EV surface may interact with cell-specific receptors facilitating cell-to cell communication (144). A recent study investigating eight different biological systems (e.g., tonsillar explants, amnion explants, T cells, monocytes) suggested that cytokines are released in a soluble (free) form or associated with EVs depending on the physiological context. Authors suggested that systems involving long-distance communication tend to release more EV-associated cytokines (143). Accordingly, EVs are likely implicated in long-distance communication between brain and peripheral tissues (101).

Studies in TBI and AD found higher inflammatory protein markers in EVs isolated from peripheral blood, suggesting a role of EVs in neuroinflammation (35, 145). EVs secreted from monocytes are also thought to influence neuroinflammation by facilitating the exchange of miRNA and proteins (146). Differential regulation of miRNAs associated with peripheral circulating EVs have been described and will be discussed in the following section. Moreover, EV encapsulated miRNAs can deliver genetic material to recipient cells, impacting their gene expression (147). Determining levels of inflammatory proteins and miRNAs in EVs may be used to reveal underlying signaling pathways and serve as biomarkers of specific disease mechanisms.

In TBI and other neuroinflammatory conditions, central inflammation as well as responses from the peripheral immune system are observed (148). As EVs can cross the BBB, EVs originated from the CNS can be isolated from the peripheral circulation (35, 145). Antibodies against proteins located in the EV membrane can be used to isolate EVs of specific cell types from serum, plasma, and other bodily fluids (35, 37, 145), allowing the investigation of mechanisms involving distinct brain cell types in a minimally invasive manner (**Figure 2**). Several studies have successfully measured inflammatory proteins in neuron-derived (NDE) and astrocyte-derived (ADE) EVs isolated from peripheral blood (35, 37, 149, 150). Neural cell adhesion molecules NCAM and L1CAM (CD171) have been used as targets to select NDE due to their relatively specific expression in neural tissue on derived from cultured neurons (35, 151). To enrich EV samples for ADEs, glutamine aspartate transporter (GLAST) antibody has been used (150). A study in AD patients reported higher levels of classical and alternative complement pathway proteins in ADE when compared to matched controls, suggesting the existence of signaling mechanisms involving inflammatory mediators released by activated astrocytes via EVs (150). This approach could also shed light on the roles played by distinct cell types in the body in response to a TBI as well as distinguish between the central or peripheral origin of proteins and miRNAs found in blood, in TBI and other diseases.

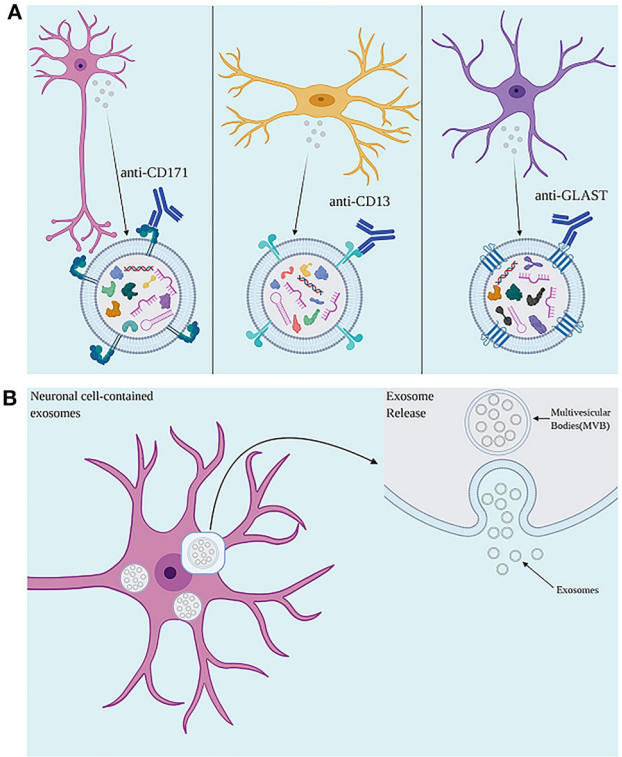

FIGURE 2 | (A) Use of antibodies against cell surface proteins present on exosomes allows for isolation of neuronal-, microglial-, and astrocyte-derived exosomes. **(B)** The accumulation of intraluminal vesicles (ILVs), exosome precursors, forms Multivesicular bodies (MVBs). MVBs fuse with the plasma membrane, releasing exosomes to the extracellular environment. Created with Biorender.com.

In addition to levels of specific proteins and signaling molecules in EV cargo, studies have evaluated changes in EV concentration in the blood after TBI (35, 149). Decreases in the concentration of NDEs have been reported in the acute but not chronic phase of TBI as measured by particle counts and concentrations of EV markers (149). Accordingly, another study found no significant differences in EV counts when comparing participants with chronic TBI to those with no TBI history (35). Higher EVs counts in acute, but not chronic TBI phases, may reflect mechanisms triggered shortly after injury and that are no longer present at later timepoints. Pathological processes triggered by a TBI vary according to aspects such as time after the injury and its severity (140). Biomarkers that are informative at earlier timepoints and for severe TBIs may not be reliable in chronic or milder injuries due to factors such as lower concentrations. Prospective studies examining longitudinal changes could inform temporal profiles of EV concentration in the peripheral blood. Moreover, as previously discussed, EV populations are very heterogenous. Different EV subpopulations that could likely be characterized by distinct membrane markers and functional roles could be released at

different rates depending on factors such as time after TBI, severity of injury, or presence of comorbidities. Study of EV populations in the brain is a new field even though it has witnessed fast technical advancement. EV biogenesis, including protein and miRNA packing, secretion, and their roles in cell signaling are still poorly understood in health and disease. Future studies will likely benefit from technological development in the field to elucidate on the role of EVs in brain pathology. Expanding knowledge on basic mechanisms involved in EV cargo-loading and biogenesis, as well as characterizing distinct EVs subpopulations, are warranted to better understand TBI pathology and unleash their full clinical potential.

EXTRACELLULAR VESICLES IN TBI

Biomarker studies in TBI have focused on plasma and serum levels of proteins found in brain cells such as tau, p-tau, and neurofilament light chain (NfL) (152, 153); glial fibrillary acidic protein (GFAP), that is released from astrocytes (13); and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1), a brain-specific deubiquitinating enzyme (154). NfL, GFAP, tau and UCHL1 have been linked to TBI severity, poor recovery, as well as PCS and PTSD symptomology in a variety of populations, including civilians (23, 155, 156), athletes (157, 158) and military personnel (11, 90, 159). Other studies have evaluated levels of inflammatory markers (134–137) as previously discussed in this article. Challenges of measuring blood-based biomarkers include low concentrations in the peripheral circulation. That is the case of tau, which requires highly sensitive platforms to be reliably measured in blood (160). Low levels of brain-derived biomarkers in blood may be attributed to factors such as proteolytic degradation and low permeability of the BBB (160). Furthermore, clearance of interstitial proteins depends in part on the glymphatic system (161), which is dysfunctional after TBI (89), likely contributing to discrepancies between levels of biomarker proteins in the brain and blood.

While circulating concentrations of proteins in blood have important diagnostic potential, it is possible that these same proteins within EVs may be more reflective of biological underpinnings of TBI (162). Specifically, EVs have been linked to important biological functions, such as cell-to-cell signaling pathways associated with inflammatory responses and removal of aggregated and misfolded proteins within the brain (129, 143). Moreover, EVs can cross the BBB and their membrane provides protection to proteins and nucleic acids, likely reducing their degradation in the peripheral circulation (163–166).

Here, we have reviewed studies investigating EVs in TBI. We have also included studies using animal models. Currently available animal models of TBI have limitations, especially for mTBI, which include anatomical differences between the brain of humans and non-human mammals (167–169). These limitations contribute to the challenge of translating new therapeutic approaches from bench to the clinic. Moreover, animal models are limited in their ability to mimic the complex symptomatology of TBI in humans that includes cognitive and affective changes (12, 49, 167). Despite limitations, animal models allow the dissection of injury mechanisms and use of genetic manipulation, providing opportunities to develop novel therapies and evaluate them before human testing. A summary of clinical and pre-clinical studies is provided in **Table 1**, a selection of studies has been discussed below to illustrate approaches that have been used to investigate EVs in TBI.

Serum and Plasma Extracellular Vesicles

Studies have examined levels of biomarkers in EVs isolated from either serum or plasma, without enriching samples for specific EV subtypes. Mondello et al. (171) explored longitudinal trajectories of serum EV levels of proteins and their free-circulating counterpart in moderate-to-severe TBI for up to 5 days after injury. Authors found differences in dynamics of free-circulating and EV proteins. Total tau (t-tau) and UCH-L1 levels in EVs were substantially increased immediately after injury and quickly dropped. For EV UCHL1, two distinct groups were identified, with early increase in UCHL1 levels in both. In one of the groups, a decline of EV UCHL1 levels was observed in the first 2 days. The second group had substantially higher early concentrations of EV UCHL1 and a subsequent decrease, which was followed by a secondary peak with very high concentrations. This trajectory strongly predicted early mortality (within 3 days). Higher levels of EV NfL and GFAP were observed in those with diffuse injury when compared to those with focal lesions. Correlations between EV and free-circulating levels of t-tau and UCHL1 were initially weak, and worsened at later time-points. Alternatively, correlations for NfL and GFAP were strong and improved overtime. These findings highlight the complexity of the relationship between free and EV levels of proteins, and the need for studies comparing both.

In sports-related mTBI, Stern et al. (172) found that tau in plasma EVs was elevated in former National Football League (NFL) players who sustained mild repetitive TBIs (rTBIs) when compared to controls, suggesting its potential use as a predictive biomarker of CTE. Similarly, Kenney et al. (33) analyzed plasma EV levels of t-tau and p-tau in Veterans with a history of military-related mTBI. Higher EV levels of t-tau and p-tau were found in Veterans with rTBI compared to Veterans with two or less mTBIs, or no mTBI. Kenney et al. (33) also found that higher levels of EV t-tau and p-tau were correlated with more severe PCS and PTSD symptoms, whereas Stern et al. (172) observed that the number of tau-positive plasma EVs correlated with worse cognitive function, but not measures of mood and behavior. Kenney et al. (33) also compared cases of mTBI with loss of consciousness (LOC)/PTA; mTBI with alteration of consciousness (AOC) only, without LOC or PTA; and controls without history TBI, but found no significant differences in concentrations of t-tau or p-tau. These findings suggest that elevations in EV t-tau and p-tau are linked to history of multiple lifetime mTBIs, rather than presence of LOC/PTA after the injury. Future studies should include multiple timepoints, evaluations of cognitive function, mood, and neurobehavioral symptoms. Additional studies are also warranted to confirm the potential of t-tau and p-tau to predict severity of TBI symptoms in individuals with chronic rTBI, and risk for CTE and other tauopathies (6, 71).

TABLE 1 | Extracellular vesicles studies in traumatic brain injury.

Reference	Organism	Cohort	Focus	Measured exosomal cargo*
Kawata et al. (170)	Human	Sports Related Concussion (acute and post-acute)	Plasma and brain, neuron, astrocyte, microglia- derived EVs	NfL, tau, SNAP25, GFAP, MBP
Mondello et al. (171)	Human	Moderate-to-severe TBI (acute through sub-acute)	Serum exosomes	**GFAP**, NfL, **total tau, UCHL1**
Goetzl et al. (149)	Human	Sports Related Concussion (acute and post-acute mTBI)	Plasma, neuron-derived exosomes	**UCHLI, Aβ42, AQP4, and many others**
Winston et al. (37)	Human	Military-related mTBI (post-deployment sampling)	Neuronal- and astrocyte- derived exosomes	**Aβ42**, **NRGN**, NfL, total tau, p-T180-tau, PS396-tau
Kenney et al. (33)	Human	Military-related chronic repetitive mTBI	Plasma exosomes	**p-tau, total tau**
Gill et al. (35)	Human	Military-related chronic mTBI	Plasma neuron-derived exosomes	**tau**, **Aβ42**, TNF-alpha, IL-6, **IL-10**
Stern et al. (172)	Human	Sports Related Concussion (acute and post-acute)	Plasma exosomes	**tau**
Muraoka et al. (173)	Human	Sports-related TBI (post-acute)	CSF, EVs	**p-tau, total tau**
Goetzl et al. (174)	Human	Acute TBI	Plasma and serum neuron-derived exosomes	**SYNPO**, NSE, mitochondrial cytochrome c oxidase
Wang et al. (175)	Human		Exosomes	**p-tau, total tau**
Ghai et al. (34)	Human	Blast related chronic military TBI	Plasma EV's	32 miRNAs in plasma; **45 miRNAs in EVs**, concentrations of C-reactive protein (CRP) and membrane metalloendopeptidase (MME) elevated in chronic mTBI samples
Ko et al. (176)	Human, mouse		Brain-derived EVs	
Ko et al. (177)	Human, mouse		Brain-derived EVs	
Wang et al. (34)	Rat	mTBI	Plasma exosomes	**50 miRNAs differentially expressed: 30 up** (miR-9a-3p, miR-29b-3p, miR-106b-5p, miR-124-3p, miR-142-3p, miR-181c-3p, miR-195-3p, miR-328a-5p, miR-361-3p, miR-374-5p, miR-434-3p, miR-532-5p, and others), **19 down** (miR-145-3p, miR-221-5p, miR-28-3p, miR-96-5p, miR-9a-5p, and others)[+]
Hazelton et al. (178)	Mouse	Acute TBI	EVs	Selective targeting of macrophage/monocyte populations
de Rivero Vaccari et al. (179)	Rat		Neuron-derived exosomes	
Ge et al. (180)	Mouse	rmTBI	Microglial exosomes	miR-124-3p
Huang et al. (36)	Mouse	rTBI	Microglial exosomes	miR-124-3p
Di et al. (181)	Mouse	rTBI	Microglial exosomes	miR-124-3p
Yang et al. (182)	Rat		Inflammation/neuroprotection/therapeutic value (exosomal miR-124)	miR-124-3p
Harrison et al. (183)	Mouse		EVs	**miR-21, miR-212, miR-146, miR-7a, and miR-7b**
Kim et al. (184)	Mouse		MSC-derived exosomes	
Zhang et al. (185)	Rat		Plasticity/neuroprotection/therapeutic value (MSC-derived exosomes)	

(Continued)

TABLE 1 | Continued

Reference	Organism	Cohort	Focus	Measured exosomal cargo*
Ni et al. (186)	Mouse		Inflammation/neuroprotection/therapeutic value (BMSC-derived exosomes)	
Sun et al. (187)	Rat		Therapeutic value (NSC-derived EVs)	
Wang et al. (188)	Mouse		Astrocyte-derived exosomes	
Zhang et al. (189)	Rat		Therapeutic value (cell-free exosomes generated by human BMSCs cultured under conventional or 3D conditions)	

*Summarized work comprises biomarker, mechanistic as well as therapy-focused publications in clinical studies (top) and animal models of TBI (bottom). TBI, Traumatic brain injury; rTBI, repetitive TBI; EVs; CSF; MSCs, mesenchymal stem cells; BMSCs, Bone-marrow derived mesenchymal stem; NCS, neural stem cells; 3D, 3-dimensional; miR, microRNA; NfL, neurofilament light chain; UCHL1, Ubiquitin C-Terminal Hydrolase L1; Aβ42; AQP4, Aquaporin-4; NRGN, Neurogranin; p-tau, phosphorylated tau; TNF-alpha, Tumor necrosis factor-alpha; IL-6, Interleukin 6; IL-10, Interleukin 10; SYNPO, Synaptopodin; NSE, neuron-specific enolase. +Underlined miRNAs were noted as more relevant to study. *Molecules measured in exosome cargo are described when applicable (Statistically significant analyses marked in bold).*

Tau is a microtube-associated protein, with multiple isoforms generated by alternative splicing (190). Tau phosphorylation regulates tau function, but hyperphosphorylated tau forms aggregates and intraneuronal neurofibrillary tangles that result in neurodegenerative changes (129). Mechanisms underlying neurodegenerative changes in TBI are poorly known, but they may share elements with tauopathies. In neurodegenerative diseases, increased extracellular levels of tau could be attributed to passive release of tau from dead or dying neurons (191). However, EV-mediated secretion of tau in tauopathies has been shown (129, 191). In mild AD, EV-associated relative to free tau is elevated in CSF (129, 191). Challenges in the study of tau as a biomarkers include the low levels of tau in peripheral circulation, which requires high-sensitivity platforms to obtain reliable measurements (150, 157). Furthermore, tau is also expressed in peripheral tissues such as muscle, liver and kidney (192). Sample enrichment for NDEs could improve tau measurements in peripheral blood and allow the analysis of levels of tau derived from CNS, rather than other tissues. Studies that measured levels of biomarkers in NDEs and ADEs are discussed next.

Plasma Neuron-Derived and Astrocyte-Derived Extracellular Vesicles

Gill et al. (35) evaluated the levels of tau, Aβ40, Aβ42, IL-6, IL-10, and TNF-alpha in plasma NDEs. EVs were enriched for neuronal injury by using an immunoprecipitation method with L1CAM (CD171) antibody. Elevated levels of NDE tau, Aβ42, and IL-10 were found in Veterans with chronic mTBI compared to controls, with elevations in tau being the most related to PCS symptoms endorsed within the mTBI group (35). Despite relatively small sample size, Gill et al. (35) showed that protein markers of neurodegeneration can be measured in NDEs isolated from the blood of chronic mTBI patients, which is associated with the severity of symptoms, suggesting the potential of NDEs as prognostic biomarkers in chronic mTBI. Prospective studies are needed to further examine longitudinal changes in NDE, and their potential as prognostic markers for PCS.

Elevated levels of plasma NDE Aβ42 have also been detected in service members with mTBI exposure at less remote timepoints in a study by Winston et al. (37). In this study, plasma NDE as well as ADE proteins were measured in service members within 3–6 months of deployment (37). EVs were precipitated and enriched for NDE and ADE by using L1CAM and GLAST antibody, respectively, using magnetic beads to immunocapture the proteins that were selected by fluorescent activated cell sorting (FACS). Plasma NDE and ADE levels of Aβ42, Aβ40, neurogranin (NRGN), NfL, t-tau, p-T180-tau, and PS396-tau were compared in service members with deployment-related mTBI to controls with no mTBI history. Higher levels of Aβ42 in plasma NDE and ADE, and lower levels of NRGN in NDE and ADE were found in service members with mTBI exposure; however, no differences in Aβ40, t-tau, NfL, p-T180-tau, and PS396-tau were observed. NDE and ADE levels of Aβ42 and NRGN distinguished service members with mTBI from those with no TBI with moderate sensitive and accuracy (37). Winston et al. (37) also observed that plasma NDE cargo proteins from mTBI samples, but not ADE cargo proteins, were toxic to neuron-like recipient cells *in vitro*.

Goetzl et al. (149) found that levels of proteins in the cargo of plasma NDEs distinguish between acute and chronic sports-related mTBI. Immunoprecipitation in association with L1CAM antibody was also used to enrich samples for neuronal origin. Plasma NDE were collected from athletes within 1 week of sports related TBI, at 3 months or longer following the last of 2–4 mTBIs (chronic mTBI), and in athletes with no prior history of TBI. Plasma NDE proteins assessed between the 3 groups included neurofunctional proteins (Rab-10; annexin VII; UCHL1; AII-spectrin fragments; claudin-5; sodium-potassium-chloride cotransporter-1; Aquaporin-4, AQP4; Synaptogyrin 3, SYNGR3), and neuropathological proteins (Aβ42; P-T181-tau; P-S396-tau; IL-6; prion cellular protein, PRPc). NDE levels of the functional brain proteins were significantly altered relative to controls in acute but not chronic mTBI. In acute and chronic mTBI, elevated NDE levels of neuropathological proteins were observed. The same set of proteins was subsequently

assessed by Goetzl et al. (193) in a study of military-related chronic TBI. Plasma NDE protein levels were compared among Veterans assigned into groups based on TBI history and current cognitive impairment (CI). Plasma NDE levels of PRPc, SYNGR3, P-T181-tau, P-S396-tau, Aβ42, and IL-6 were significantly elevated in Veterans with TBI and CI compared with controls with TBI but no CI. Among Veterans without TBI, subjects with CI had significantly elevated levels of PRPc, SYNGR3, P-T181-tau, and Aβ42, in comparison to controls without CI. Taken together, these findings suggest that neuronal Aβ peptides and P-tau species remain elevated for decades after TBI, may be associated with TBI-related cognitive alterations and neurodegenerative changes, and should be considered as potential therapeutic targets.

Extracellular Vesicle miRNAs

MiRNAs are small, about 21 nucleotides long, non-coding RNAs that function as gene regulators at the post-transcriptional level in eukaryotic cells (194, 195). Pre-miRNA are hairpin-loop precursors that are 60–90 nucleotides long and cleaved into miRNA duplex by the ribonuclease III in the cytoplasm. The mature miRNA negatively regulates gene expression by targeting messenger RNA (mRNA) (196). In TBI, miRNAs have attracted interest as possible biomarkers, and as therapeutic targets.

MiRNAs have been linked to inflammation in several human diseases (197). In a recent study, (34) isolated miRNA from plasma and plasma-derived EVs from Veterans with blast-related mTBI, which were analyzed by using next generation sequencing (NGS). Analysis revealed that 45 and 32 miRNAs were differentially regulated in EVs and plasma, respectively. Pathways functionally associated with differentially regulated miRNAs involved neuroinflammation, BBB integrity, vascular modeling, and neuronal function. Future studies should investigate miRNA changes in response to mTBI caused by other mechanisms, such as blunt head trauma, and at different timepoints after injury.

In a 2018 study, Ko et al. (176) identified a miRNA based panel biomarker to diagnose TBI, both in a mouse model and human TBI. MiRNAs associated with EVs positive for GluR2 (an AMPA receptor subunit) were isolated from plasma of mice exposed to blast overpressure injury. MiRNA profiling in combination with machine learning were used to generate a biomarker panel of seven miRNA (miR-129-5p, 212-5p, miR-9-5p, miR-152-5p miR-21 miR-374b-5p, miR-664-3p) capable of distinguishing TBI patients from healthy controls with high accuracy (176). In a subsequent study, miRNA profiling of GluR2+ EVs across various injury types, severity, and times, allowed Ko to identify distinct TBI signatures across different injury models and post-injury time points and biomarker panels capable of classifying specific states of injury (177). A panel of eight miRNAs were identified for injured mice vs. sham mice. Four were differentially regulated in TBI patients when compared to healthy controls, (miR-203b-5p, miR-203a-3p, miR-206, miR-185-5p) (177).

In a 2016 study, Harrison et al. (183) examined the miRNA cargo of brain-derived EVs isolated from brain injured mice and controls. Decreased expression of miR-212, and increased expression of miR-21, miR-146, miR-7a, and miR-7b were observed in injured mice at 7 days after controlled cortical impact (CCI) relative to controls, with miR-21 showing the largest change between the groups (183). Notably, the authors found that the expression of miR-21 was largely localized to neurons near the lesion site and, notably, that adjacent to these miR-21-expressing neurons were activated microglia (183). This study reveals potential mechanisms of cell-to-cell communication as the increase in miR-21 in EVs with the elevation of miR-21 in neurons, suggests that miR-21 is secreted from neurons as EV cargo.

Microglial Extracellular Vesicles

History of rTBI is believed to make the brain more susceptible to pathological processes as a consequence of a head trauma, which might be at least partially mediated by microglial cells (70, 198). After a brain injury, microglia are hypothesized to remain in a heightened inflammatory status or primed. The primed microglia have a lower threshold for response to events that disrupt the brain physiology (199). Moreover, recurrent head trauma has been linked to the postmortem diagnosis of CTE in contact-sports athletes and in the military. Neuroinflammation is observed in CTE brains, with large increases in the number of activated microglia in the white matter (200). Microglia-derived EVs (MDEs) have been linked to AD. As previously discussed, microglia activation may have beneficial effects in earlier stages after injury, but later become detrimental. However, the role of miRNAs in microglial EV on regulation of TBI-neurodegeneration is still unclear.

In a mouse model of rTBI, analysis of MDE miRNAs revealed that miR-124-3p played a protective role in TBI-related recovery processes by promoting M2 polarization in microglia and repressing neuroinflammation (36). In support of these findings, Yang et al. (182) showed that EV miR-124 treatment enhanced hippocampal neurogenesis and functional recovery by promoting the M2 polarization of microglia, the effect of which was produced through inhibition of the Toll-like receptor 4 (TLR4 pathway). In a subsequent study, Li et al. (181) showed that increased miR-124-3p in MDE promoted neurite outgrowth via miR-124-3p transfer into neurons, thereby inhibiting neuronal autophagy and protecting again against nerve injury.

In a 2020 study of rTBI, miR-124-3p levels in MDE were found to be significantly altered in the acute, sub-acute, and chronic phases following the injury (180). Intravenous administration of MDE with upregulated miR-124-3p alleviated neurodegeneration in repetitive scratch-injured neurons, the effects of which were exerted by miR-124-3p targeting RelA, an inhibitory transcription factor of apolipoprotein E (ApoE) that promotes β-amyloid proteolytic breakdown, thereby inhibiting β-amyloid abnormalities (180).

Studies analyzing the content of MDEs cargo have performed the enrichment from cultured microglia (180), instead of peripheral blood samples. To our knowledge, no study have examined MDEs in clinical samples, likely for a lack of antibodies shown to distinguish EVs derived from microglia, from those derived from peripheral macrophages. Cell surface markers used

to identify myeloid cells in the CNS are expressed by microglia as well as macrophages (201). Evidence suggests a role of MDEs in neurodegeration and neuroinflammation, making microglial EVs a likely candidate biomarker in TBI.

METHODOLOGICAL CHALLENGES

In this section we will discuss some of technical challenges such as biomarker source, isolation methods, and diversity of EV populations encountered in EV-based biomarker research using clinical samples in TBI.

Source

Blood is a source for biomarkers, and is frequently used in clinical diagnostics (202, 203). Exosomes have been shown to maintain the majority of their protein and nucleic contents in serum and plasma, with fresh plasma considered the best source of intact exosomes (204). Muller et al. (204) concluded that both plasma and serum are equally comparable sources of EVs when evaluating total protein recovery and morphology of isolated EVs. However, when looking at fresh vs. frozen plasma, fresh plasma yielded less protein aggregation (more purity) and morphologically intact EVs (35). Although a single freeze-thaw cycle with a storage of 1 year did not affect size and concentration of EVs in the study by Yuana et al. the authors noted changes to the membrane phospholipid distribution suggesting increase in coagulation (205), whereas Muller et al. (204) found increase in protein aggregation after thaw/freeze cycles. Additional studies have also shown that exosomes stored at −80 or −20°C in plasma are more stable yielding higher recovery compared to storage at 4°C after 90 days of storage (206, 207).

Yet plasma remains the most heterogenous of all body fluids, being abundant in platelets, albumin, lipoproteins, fibrinogens and many other proteins, also making it the most challenging source for exosomal purity. Complicating matters more, much of blood plasma and serum repository samples follow different collection and handling procedures. For example, because many EVs are platelet derived, it is ideal to have platelet poor plasma (PPP) or platelet free plasma (PFP) so that samples can be used in other cell-focused exosome research. Pre-handling of the blood that includes the process of venipuncture, time between blood draw and initial centrifugation, and subsequent centrifugation speeds are all important factors that will affect EV recovery and purity. Lacroix et al. (208) investigated these factors and concluded that using larger needle size, discarding initial few milliliters of blood, decreasing time delay for initial blood processing under 2 h, introducing two subsequent centrifugations of 2,500 g for 15 min yielded better recovery of EVs from plasma (208).

Bypassing these steps enriches the collected plasma with platelets, making non-platelet EVs isolation increasingly difficult. One common technique pretreats plasma with thrombin to remove platelets, however, a recent study investigated the differences between centrifugation vs. thrombin methods and found substantial loss of vesicles in the fibrin clot when treated by the thrombin (209). Specifically, the authors demonstrated the fibrin clot to become activated by thrombin treatment, leading to entrapment of EVs in the clot and a reduction of total sample EVs. As previously described, repeated low speed centrifugation allows higher recovery of vesicles (208). Briefly, the initial vial of blood should be discarded to avoid release of platelets activated by venipuncture, and the collection tubes are then centrifuged at 3,000 g × 15 min to obtain PPP. When looking at neuronal biomarkers, researchers must be cautious in interpreting results when improper collection or processing measures were used.

Isolation Method

As the field of extracellular vesicle research exponentially grows, experts often debate the "best" isolation technique (97). While each method unequivocally offers certain advantages, the selection of one over another is driven by the aims of the project. The choice of isolation technique is dictated by a variety of factors, including sensitivity, specificity, cost, personnel, sample, and time constraints. For example, a researcher looking to characterize exosome morphology may select multi-step ultracentrifugation, whereas a researcher conducting clinical trials would be more inclined toward a large throughput method, such as polymer-based precipitation or high-throughput size exclusion chromatography (SEC) (210, 211). When analyzing hundreds of patient samples, methods such as ultracentrifugation of microfluidics can be cumbersome. Current TBI research has used a combination of polymer-based precipitation and immunoaffinity methods to pull down neuronal exosomes. A detailed table comparing different separation or isolation methods described in position statement by MISEV suggests that high recovery and high specificity may not yet be achievable (100). While polymer solutions allow for isolation of EVs from relatively small sample volumes with high recovery, it has low specificity (100) leading to co-precipitation of co-isolated contaminants such as non EV proteins and polymer requiring post-isolation clean-up or purification methods. A survey among EV researchers showed that most common additional purification steps post EV isolation included ultracentrifugation and density gradient centrifugation (212). Without additional cleaning steps, it is difficult to characterize the morphology and composition of the derived.

Due to the heterogeneity of sample preparation and challenges related to co-isolated contaminates, the MISEV has a suggested set of minimal reporting guidelines. For example, to characterize EVs they recommend demonstrating presence of: (1) non-tissues specific tetraspanins (e.g., CD81, CD63), (2) membrane proteins (e.g., TSG101, ALIX), and minimal presence or absence of (3) source specific contaminants (albumin, APOA1/2). (100). Several studies in TBI demonstrated presence of EV markers using western blot (e.g., CD63, CD9, HSP70) (213, 214) and Chen et al. (214) utilized western blotting (WB) to show presence of GJA1-20K from astrocyte-derived EVs which facilitated neuronal recovery. MISEV also recommends characterization of EV morphology using transmission electron microscopy and characterization of EV size and concentration, most often conducted using nanoparticle tracking analysis (100). The main aim is identification of biomarkers with good specificity, sensitivity, and reproducibility. The latter may pose the biggest challenge due to variations in the sample processing, incubation times, plasma pre-cleaning steps, and variability in protocols across different laboratories.

Perhaps one of the most exciting directions for identifying TBI biomarkers is diversity within EV populations. Recent publications (215, 216) discuss limitations of previously established notions of an "exosome" and the importance of distinguishing EV subtypes. Different isolation methods can eliminate a subset of exosomes, whether smaller or larger, that contains important diagnostic information. Experts debate whether subtype classification should be done through biogenesis pathways or EV size (144), however, isolation of specific exosomal categories is still being developed and classification is actively being determined.

DISCUSSION

TBI is a heterogenous injury with highly variable clinical presentation and recovery patterns. TBI can lead to lasting or late-in-life neurobehavioral sequelae, cognitive and affective symptoms, and is associated with increased risk of developing neurodegenerative diseases. Reliable biomarkers for TBI could improve diagnosis and therapeutic monitoring of individuals who have sustained head injuries. Determining those who are most at risk for neurodegenerative processes and chronic symptoms after a TBI is essential, and identifying underlying mechanisms may provide necessary insights for developing clinical interventions prior to the onset of non-reversible pathological changes.

EVs have been successfully isolated from human serum and plasma from TBI patients, allowing the quantification of proteins and RNAs in their cargo. Blood-based EV biomarkers confer advantages when compared to free proteins and miRNAs, as EVs cross the BBB and their membrane protects the cargo from degradation. Moreover, antibodies against proteins located in the EV membrane can be used to isolate EVs of specific cell types in the peripheral circulation. This approach can be applied to improve measurements for proteins such as tau, which is found at low concentrations in the peripheral circulation, and can be released by the brain as well as peripheral tissues. It also provides a powerful tool to distinguish peripheral and central pathological processes, shedding light on mechanisms associated with neuroinflammation and peripheral immune responses in TBI. Finally, identifying proteins and miRNAs originated from distinct cell types of the brain could improve our understanding of how specific cell types respond to the injury, and underlying signaling mechanisms. Moreover, abundant evidence suggests a role of EVs in the physiological and pathological processes in the CNS, including cell-to-cell signaling between distinct cell types in the brain and clearance processes, eliminating unwanted biological material.

In neurodegenerative diseases, EVs are thought to contribute to the spread of pathogenic proteins, including lateral and long-distance propagation of tau. Mechanisms involving EVs in neurogenerative diseases may provide insight into the possible relevance of EVs in TBI pathology, which is still poorly understood. Neurodegeneration and neuroinflammation are major elements in the neuropathology of TBI as well as neurological diseases. Biomarkers of AD such as Aβ42, t-tau, p-T180-tau, and PS396-tau among others have also been found in EVs isolated from TBI patients at higher levels than controls. Studies have shown higher levels of EV tau and Aβ42 in populations with history of multiple mTBIs, which were linked to the severity of neurobehavioral symptoms. NfL, which is considered a marker of neuronal injury and degeneration, is elevated in many neurodegenerative diseases. Moreover, higher levels of EV nfL and GFAP, an astrocyte marker, were associated with diffused injury when compared to focal lesions in patients with moderate-to-severe TBI.

Monitoring brain injury and associated symptoms using blood-based biomarkers is a safe and relatively inexpensive method. A fast-growing literature suggests the potential of EVs isolated from peripheral blood as TBI biomarkers. Nevertheless, the study of EVs in health and disease is still in its infancy; there are technical limitations and a lack of standards regarding terminology and vesicle characterization. Future studies may benefit from technological development in the field to shed light on the role of EVs in brain pathology.

AUTHOR CONTRIBUTIONS

VG and JG contributed to the conception of the review, coordinated writing efforts, and edited the final article version. VG wrote the Introduction. JL and VG wrote the TBI-related Symptoms and Associated Disorders. VG and JA wrote the Neuropathology of TBI. VG wrote Heterogeneity of EVs, EVs in the Central Nervous System and Neurological Diseases, and the Discussion. CD and VG wrote the Extracellular Vesicles in TBI. DS wrote the Methodological Challenges. CD, JL, SM, and VG constructed the table. JA produced the figures. 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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An Acute Bout of Soccer Heading Subtly Alters Neurovascular Coupling Metrics

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Objective: The current investigation examined how a bout of soccer heading may impact brain function.

Design: Semi-randomized crossover cohort.

Setting: Controlled soccer heading.

Participants: Seven male soccer players (24.1 ± 1.5 years).

Intervention: 40 successful soccer headers were performed in 20 min (25 m, launch velocity ~80 km/h). X2 xPatch recorded linear and rotational head accelerations during each impact. A contact control *"sham"* condition – ball made body contact, but not by the head; and a no activity time *"control"* condition were also completed.

Main Outcome Measures: Posterior and middle cerebral artery (PCA and MCA, respectively), cerebral blood velocity (CBV) was recorded during a visual task (neurovascular coupling: NVC) alongside SCAT3 symptoms scores pre/post a controlled bout of soccer heading.

Results: Cumulative linear and rotational accelerations were 1,574 ± 97.9 g and 313,761 ± 23,966 rads/s^2, respectively, during heading and changes in SCAT3 symptom number (pre: 2.6 ± 3.0; post: 6.7 ± 6.2, $p = 0.13$) and severity (pre: 3.7 ± 3.6, post: 9.4 ± 7.6, $p = 0.11$) were unchanged. In the PCA, no NVC differences were observed, including: relative CBV increase (28.0 ± 7.6%, $p = 0.71$) and total activation (188.7 ± 68.1 cm, $p = 0.93$). However, MCA-derived NVC metrics were blunted following heading, demonstrating decreased relative CBV increase (7.8 ± 3.1%, $p = 0.03$) and decreased total activation (26.7 ± 45.3 cm, $p = 0.04$).

Conclusion: Although an acute bout of soccer heading did not result in an increase of concussion-like symptoms, there were alterations in NVC responses within the MCA during a visual task. This suggests an acute bout of repetitive soccer heading can alter CBV regulation within the region of the brain associated with the header impacts.

Keywords: repetitive football/soccer heading, sub-concussive impacts, cerebral blood flow, sport concussion assessment tool 3, SCAT3

INTRODUCTION

With over 265 million players across the globe, soccer is the world's most popular sport (1). While infrequently resulting in a concussion, the subconcussive impacts associated with heading the soccer ball have been speculated to lead to potential injuries such as increases in intracranial pressure leading to retinal bleeding (2), hyphema (3), and orofacial and dental injuries (3). It has been estimated soccer players use their head to make contact with a ball ∼6–16 times per game, which extrapolates to 3,500–8,500 lifetime subconcussive exposures [Reviewed in: (4, 5)] for the average amateur soccer player. While these values represent an average exposure based on the broader literature, the total number of heading events for any given player will be highly affected by player position and level of play. Furthermore, retrospective analyses have demonstrated increased rates of suspected neurodegenerative disorders among elite soccer players (6–9). Pertinently, the degree of exposure necessary to induce retinal bleeding (2), concussions (5), and any direct association to the subsequent development of chronic traumatic encephalopathy or other neurodegenerative brain diseases remains unclear (10).

Examining the role of cerebral blood flow (CBF) regulation and neurovascular coupling (NVC) responses has provided further understanding of the pathophysiological underpinnings related to both subconcussive and concussive impacts (11–13). Specifically, NVC reflects the ability to regulate CBF in response to the metabolic demand within discrete regions of the brain when they are activated (11, 14, 15). One of the most common ways to assess the NVC response is to track increases in CBF in a posterior cerebral artery (PCA) during a visual task, as these are the primary conduit vessel which supplies the visual cortex in the occipital lobe (16). Given the nature of soccer heading, there is the potential for alterations in CBF within both the frontal/temporal (supplied by middle cerebral arteries: MCA), as well as occipital (supplied by PCA) lobes of the brain (17, 18). Furthermore, the neurometabolic cascade present following a concussive impact, results in a rapid depletion of energy stores in the brain (19, 20). In the early stages of this period (hours), it is expected there will be an uncoupling of the CBF response to metabolic demands. Previous research has suggested there will be basal CBF reductions at this point (19, 20), which in turn results in larger than normal CBF increases required to match metabolic demands associated with a given cognitive task. How this response is affected by a controlled bout of subconcussive impacts is currently unknown.

The purpose of this study was to evaluate the NVC response following an acute bout of soccer heading to enhance our understanding of neurophysiological changes associated with controlled subconcussive impacts. It was hypothesized soccer heading would lead to an augmented NVC response, whereas these effects would not be observed either during contact with the soccer ball to other body regions (*sham*), nor during a non-contact *control* session.

MATERIALS AND METHODS

Participants

This prospective cohort study enrolled 7 male soccer players (mean age 24.1 ± 1.5 years; mean body mass index 25.5 ± 1.6 kg/m^2) with 5 + years of experience playing at the senior club or university level. Exclusion criteria included: any significant self-reported history of cardiorespiratory, cerebrovascular, neurological, severe neurodevelopmental disorders, or <5 years of experience playing soccer at an elite level. No participants were excluded on these grounds. All subjects were familiarized with testing procedures, provided written informed consent, and abstained from caffeine, exercise, and alcoholic beverages for 12 + h before testing. This study was approved by the University of British Columbia clinical research ethics board (H14-00368).

The current results are part of a larger investigation into the effects of a controlled bout of soccer heading which included assessments of blood biomarkers (21), cerebral autoregulation, cardiovascular/autonomic function, balance metrics and executive function tasks. Participants were compensated $50 CAD for each testing session, total compensation of $150 CAD across the study.

Study Design

Participants were evaluated using a pre-test, exposure, post-test design using a NVC response protocol as described previously (16). Briefly, participants completed eight cycles of 20 sec eyes-closed, 40 sec eyes-open to a complex visual search paradigm to augment metabolic demand. Following stimulation, PCA and MCA velocity increases relative to baseline were measured using transcranial Doppler (TCD) ultrasound to characterize the NVC response.

This NVC protocol was completed prior to and ∼10–15 min following a 20-min exposure to three different conditions. Testing for each condition outlined below was done on separate days in a pseudo-random order, an average of 26.1 ± 25.2 days apart:

i) *Heading* – participants stood ∼25 m from a JUGS machine (JUGS International, Taulatin, Oregon, USA) and performed 40 successful soccer headers in 20 min, with ∼30 sec separating each trial. If a trial was unsuccessful (i.e., there was no contact by the head with the ball), another soccer ball was launched within the 30-sec window and repeated until a successful header occurred. The soccer balls employed were FIFA regulation size 5 ball, inflated to 13 psi and propelled from the JUGS machine at 77.5 ± 3.7 km/h recorded via Bushnell Velocity Speed Gun (Bushnell Outdoor Products, Richmond Hill, Ontario, Canada) in the heading group. All successful heading attempts were completed with this ball launch speed, the unsuccessful heading attempts were a result of the player not making head contact with the ball, and not a result of a reduced launch velocity. This protocol was designed to mimic one of the most consistent and controlled situations in soccer play which is likely to result in a heading situation in game play, the corner kick.
ii) *Sham* – This condition was identical to the heading condition except participants contacted the ball with any part of the body other than the head. This condition was performed to determine if any potential differences in the NVC response were due to making contact with the ball in general, potential *"whiplash-like"* effects (22) or if head contact was required for NVC alterations.
iii) *Control* – This condition is identical to the previous two, except no soccer balls were launched. Participants went to the testing area, completed 20 min of quiet rest and then returned to the laboratory. This was completed to control for any non-specific time effects on NVC responses.

Lab-Based Instrumentation

During the laboratory assessments of NVC, participants were equipped with a three-lead electrocardiogram (ECG). Blood pressure was measured using finger photoplethysmography, with a brachial cuff to adjust finger and brachial artery height differences (Finometer; Finapres Medical Systems, Amsterdam, The Netherlands). This method has been shown to reliably assess the dynamic changes in beat-to-beat blood pressure and correlates well with intra-arterial measurements (23, 24).

2-MHz transcranial Doppler (TCD) ultrasound probes (Spencer Technologies, Seattle, WA, USA) were placed over the temporal acoustic windows to obtain cerebral blood velocity (CBV) in the vessel of interest, providing an index of CBF (25, 26). The P-1 segment of the PCA and M-1 segment of the MCA were insonated and optimized according to their signal depth, waveform, and CBV, and were confirmed with unilateral carotid compression and visual stimulation tests (25, 27). Once the cerebral arteries were confirmed, the probes were secured and locked in place with a headband (Spencer Technologies, Seattle, WA, USA). Our research group has previously demonstrated this method of indexing CBF (via CBV) to be highly reliable and reproducible in a similarly aged population, as noted via within-subject coefficient of variations of ∼2–3% (28). End-tidal partial pressure of carbon dioxide ($P_{ET}CO_2$) was sampled by mouthpiece and monitored with an online gas analyzer (ML206; AD Instruments, Colorado Springs, CO, USA), calibrated with a known gas concentration before each collection. All data were time-aligned and collected at a sampling frequency of 1,000 Hz via an 8-channel PowerLab (AD Instruments, Colorado Springs, CO, USA) and stored for offline analysis using commercially available software (LabChart version 7.1; AD Instruments, Colorado Springs, CO, USA).

Six degree-of-freedom linear and angular head accelerations were captured at 1,000 Hz using a xPatch (X2 Biosystems; Seattle, WA) placed over the right mastoid process of participants during header and sham conditions. Acceleration events were recorded when forces exceeded the 10 *g* linear threshold. Peak linear (PLA) and peak rotational (PRA) acceleration and average impact duration were recorded for each detected event. Impacts which did not reach the 10 *g* threshold were recorded as 0 *g* for interpretations of average/cumulative impact exposure levels.

The Sport Concussion Assessment Tool – 3rd edition (SCAT3) was used to record the total number and severity of concussion symptoms before and after all exposure conditions (29). The SCAT3 includes 22 symptoms of somatic, cognitive and neurobehavioural nature with each symptom ranked on a Likert scale from 0 (absent) to 6 (severe). Total number of symptoms (range 0–22) were recorded, and total symptom severity was determined by summing the severity for each reported symptom (range 0–132). Participants were contacted in the evening following the soccer heading condition and asked to report any persistent symptoms. No players reported any symptoms at this follow up.

Data Processing

Using the R-R intervals from the electrocardiogram for gating, beat-to-beat heart rate, mean blood pressure, and mean PCA and MCA CBV were processed in LabChart Scope View (AD Instruments, Colorado Springs, CO, USA). Data from each trial were aligned to stimulus onset (eyes open command), then averaged to generate one response per subject, per testing session. As per previous research (30), the average of the 5 sec preceding initiation (i.e., the last 5 sec of eyes closed), are reported as the baseline CBV in both the PCA and MCA. The peak velocity observed in each respective vessel obtained during the 30 sec following the eyes open command is reported in both absolute and relative terms. Area-under-the-curve to 30 sec (AUC_{30}) for CBV in the PCA and MCA relative to baseline was used as an index of total activation for each paradigm.

Statistical Analysis

Statistical analyses were conducted with SPSS v.25.0 (IBM Corp, Armonk, NY). A 3 (condition: Heading, Sham, Control) by 2 (time: pre, post) repeated measures ANOVA was conducted to examine the effect of condition and time on the neurovascular coupling measures. Where hypothesized, *a priori* simple main effects were investigated with Bonferroni corrections for condition or time. Due to the small sample size, Benjamini-Hochberg adjustments were also performed on the simple main effects to investigate the false discovery rate and potential influence of Type II errors. Data are presented as means SD. Significance was set *a priori* at $p < 0.05$.

TABLE 1 | Head impact data for all conditions as recorded with the xPatch sensor.

Metric	Heading	Sham	Control	*p*-value
Total impacts (>10 g)	39.0 ± 1.5	0.4 ± 0.8	0 ± 0	<0.001
Average PLA (g)	40.7 ± 3.6	3.2 ± 5.6	0 ± 0	<0.001
Cumulative PLA (g)	1,574.7 ± 97.6	5.0 ± 9.6	0 ± 0	<0.001
Average PRA (rad/s^2)	8,097.8 ± 807.3	827.9 ± 1,424.5	0 ± 0	<0.001
Cumulative PRA (rad/s^2)	313,760.6 ± 23,966.4	1,284.8 ± 2,453.2	0 ± 0	<0.001
Average duration (ms)	16.4 ± 1.0	0.9 ± 1.9	0 ± 0	<0.001

Data are presented as mean ± SD. PLA, Peak Linear Acceleration; PRA, Peak Rotational Acceleration.

RESULTS

xPatch Impact Sensor

During the heading condition, 98% of the successful headers were recorded over 10 *g* with an average of 40.7 ± 3.6 *g* linear acceleration and 8,097.8 ± 807.3 rad/s^2 rotational acceleration. Combined this equates to a cumulative impact exposure of 1,574.7 ± 97.9 *g* and 313,760.6 ± 23,966.4 rad/s^2 for linear acceleration and rotational acceleration, respectively, during the acute soccer heading exposure (**Table 1**). In comparison, although the sham condition did not involve any contact occurring directly to the head, 1% of these impacts registered >10 *g* on the xPatch (average linear acceleration of 3.2 ± 5.7 *g*). The average rotational acceleration during sham trials was 827.9 ± 1,424.5 rad/s^2 rotational acceleration for cumulative impact exposures of 1,574.7 ± 97.9 *g* (linear) and 1,284.8 ± 2,453.2 rad/s^2 (rotational: **Table 1**). As per study design, there were no impacts recorded >10 *g* during the control condition.

Sport Concussion Assessment Test

SCAT3 metrics were recorded prior to and immediately following all conditions in all participants. There were no differences in symptoms or severity of symptoms reported on the SCAT3 preceding the heading, sham or control conditions *($p > 0.80$).* After the soccer heading, there was a greater severity of concussion-like symptoms compared with sham and control trials *($p = 0.03$)* and a trend toward an increased number of symptoms reported *($p = 0.08$).* Overall, 71% of participants reported an increase in both the number and severity of concussion-like symptoms after soccer heading, however these alterations did not reach statistical significance (symptom number: pre 2.6 ± 3.0, post 6.7 ± 6.2, $p = 0.13$; symptom severity: pre 3.7 ± 3.6, post 9.4 ± 7.6, $p = 0.11$) (**Figure 1**). In contrast to the heading trials which showed increased symptoms, all participants in the sham heading condition and 86% of participants in the control condition reported fewer number of concussion-like symptoms and lessened severity following those conditions.

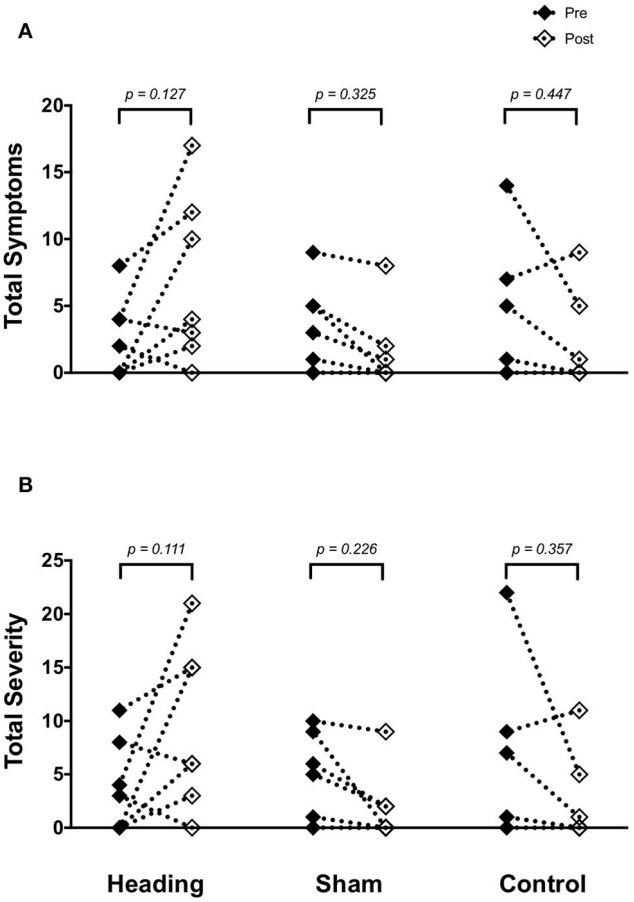

FIGURE 1 | SCAT3 symptom metrics **(A)** across all three experimental conditions: heading, sham and control; and the total severity of symptoms reported on the SCAT3 **(B)** across the conditions. *p*-values represent *a priori* simple effects comparisons. Note: all individual data is reported however, some subjects reported similar values for number and severity of symptoms and are overlapped (i.e., 0 for both pre- and post-control/sham condition).

Neurovascular Coupling Response

At pre-condition baseline, there were no differences observed in any cerebrovascular measures of interest (baseline CBV, peak CBV, total activation indexed via AUC_{30} or relative increase from eyes-closed baseline) in brain regions supplied by either the PCA (all $p > 0.42$) or MCA (all $p > 0.40$).

Within the posterior cerebral circulatory region, there were similarly no differences in baseline PCA CBV ($p = 0.67$), peak PCA CBV ($p = 0.67$), or total activation as indexed via PCA AUC_{30} ($p = 0.93$; **Figure 2**). Consistent with this observation, there were also no significant changes following either the sham or control exposure detected for baseline PCA CBV (sham: $p = 0.57$; control: $p = 0.87$), peak PCA CBV (sham: $p = 0.57$; control: $p = 0.92$), or PCA AUC_{30} (sham: $p = 0.74$; control: $p = 0.86$). There were also no changes observed in the middle/anterior cerebral circulatory region following the heading, sham, or control experimental conditions for either the baseline MCA CBV (heading: $p = 0.75$; sham: $p = 0.82$; control: $p = 0.84$) or

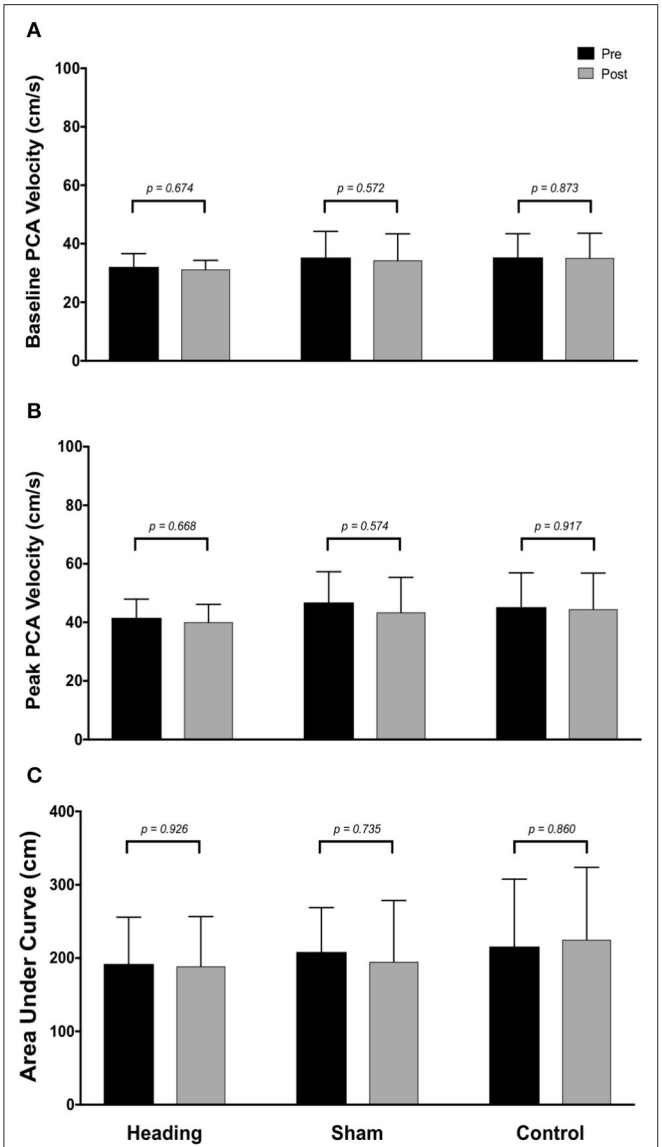

FIGURE 2 | Summary of neurovascular coupling (NVC) responses to visual stimulation in the posterior cerebral artery (PCA) across participants completing the heading, sham, or control condition: **(A)** eyes-closed PCA blood velocity (baseline PCA*v*); **(B)** maximum increase in PCA*v* following visual stimulation; **(C)** area under the curve during the first 30 sec after stimulus onset. Columns represent average values of the group; error bars represent standard deviation. *p*-values represent *a priori* simple effects comparisons.

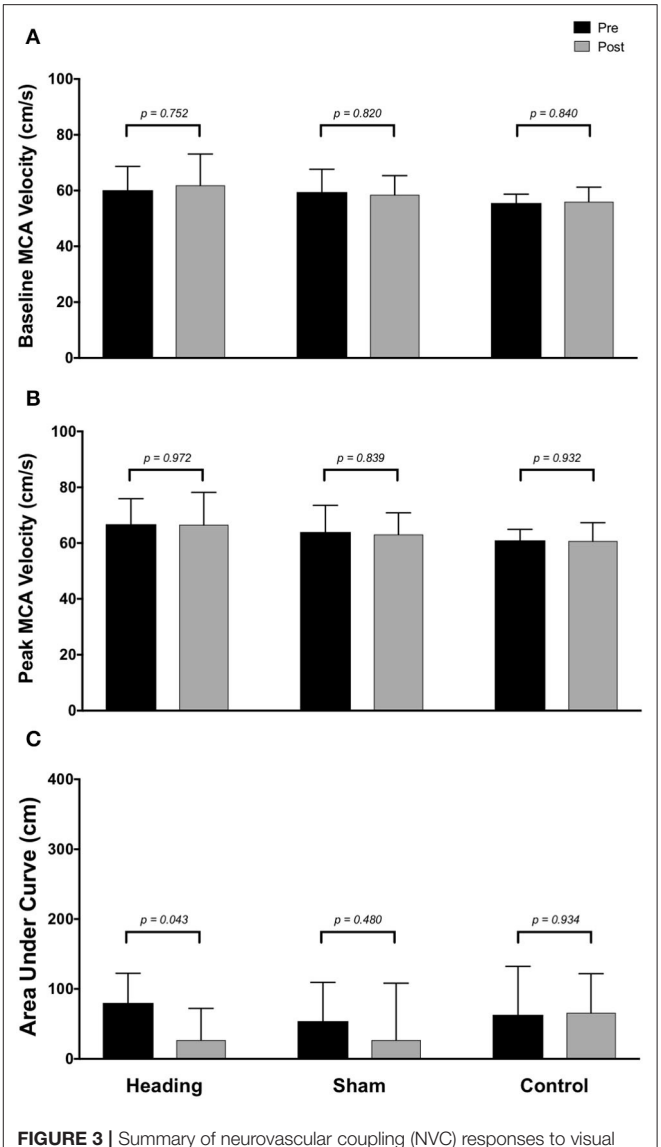

FIGURE 3 | Summary of neurovascular coupling (NVC) responses to visual stimulation in the middle cerebral artery (MCA) across participants completing the heading, sham, or control condition: **(A)** eyes-closed MCA blood velocity (baseline MCA*v*); **(B)** maximum MCA*v* following visual stimulation; **(C)** area under the curve during the first 30 sec after stimulus onset. Columns represent average values of the group; error bars represent standard deviation. *p*-values represent *a priori* simple effects comparisons.

peak MCA CBV (heading: $p = 0.97$; sham: $p = 0.84$; control: $p = 0.93$) (**Figure 3**).

In contrast, the total activation in the regions of the brain supplied by the MCA (as indexed with MCA AUC_{30}) was reduced 67% following the acute bout of controlled soccer heading (pre: 80.0 ± 42.4 cm; post: 26.7 ± 45.3 cm, $p = 0.04$). This observation was not detected in the MCA AUC_{30} following sham ($p = 0.48$) or control exposure ($p > 0.93$) conditions (**Figure 3**). Consistent with this observation, there was a reduction in the relative increase of MCA CBV from baseline to peak velocity (11.1 ± 1.5% pre-heading; 7.9 ± 3.1% post-heading, $p = 0.03$) following the controlled soccer heading condition, which was not observed in sham ($p = 0.91$) or control ($p = 0.47$; **Figure 4**).

There were no between group differences for MAP, HR, or $P_{ET}CO_2$ noted either before or after any of the experimental conditions (**Table 2**).

DISCUSSION

This is the first study to investigate how NVC metrics are affected by an acute bout of controlled soccer heading. The key findings

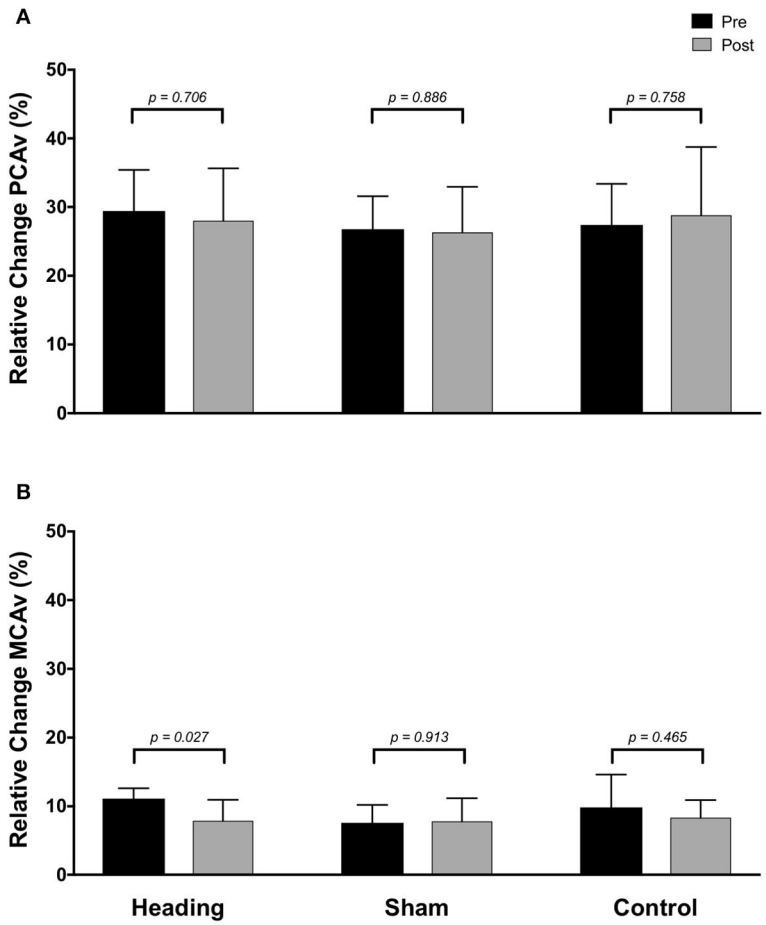

FIGURE 4 | Relative increase from baseline to peak velocity of blood flow upon visual stimulation following heading, sham, or control exposures as measured in the **(A)** posterior cerebral artery and **(B)** middle cerebral artery. *p*-values represent *a priori* simple effects comparisons.

TABLE 2 | Resting physiological parameters pre- and post-experimental condition exposures.

Metric	Heading		Sham		Control		*p*-value		
	Pre	Post	Pre	Post	Pre	Post	Condition	Time	Condition x time
MAP (mmHg)	95.8 ± 6.2	91.9 ± 4.4	94.2 ± 6.4	90.1 ± 3.2	91.9 ± 6.2	92.6 ± 5.3	0.604	0.155	0.439
HR (bpm)	74.9 ± 4.4	81.9 ± 6.3	75.1 ± 13.8	83.2 ± 15.6	76.6 ± 14.1	73.3 ± 11.7	0.649	0.284	0.378
$P_{ET}CO_2$ (mmHg)	36.8 ± 2.2	34.7 ± 2.3	36.0 ± 3.5	35.0 ± 3.0	36.8 ± 3.9	35.6 ± 4.2	t0.736	0.09	0.67

Data are presented as mean (SD). MAP, mean arterial pressure; HR, heart rate; $P_{ET}CO_2$, partial pressure of end-tidal carbon dioxide.

from this study are 3-fold: (1) There was a trend toward an acute increase in concussion-like symptoms following an acute bout of soccer heading; (2) There were no PCA NVC response alterations; and (3) There was a reduction in total NVC activation within the MCA. Collectively, these findings suggest there are subtle NVC response alterations in the frontotemporal (supplied from the MCA) but not occipital (supplied from the PCA) regions of the brain following soccer heading.

To the authors' knowledge, the current investigation protocol included the greatest cumulative impact exposure for a controlled acute soccer heading study to date. Previous research has either used launch velocities of 40 km/h (31–39); 50 km/h (40); 65 km/h (31, 40); 80 km/h (40, 41); 90 km/h (42) and fewer soccer headers: 5 (40); 10 (31, 34, 36, 37); 12 (32, 33, 35, 41); 20 (39, 42). Despite the greater cumulative head impact exposure, the findings revealed only subtle alterations to the NVC response that were restricted to the MCA. This effect could be related to the acute nature of the sub-concussive exposure in the current study. Previous research has demonstrated differing effects on neuropsychological function based on recent vs. long-term exposures (years) to soccer heading (43, 44). However, prior research from our group has shown the PCA NVC

response remains unchanged following a full season of elite contact-sport participation (13), and is disrupted following acute sport-related concussions (12). Consistent with this notion are the findings from Svaldi et al. (45, 46) who have examined cerebrovascular reactivity responses to carbon dioxide using magnetic resonance imaging measures (MRI) in female high school and collegiate soccer players. They used MRI scans at pre-season, during the first 5 weeks of the season, in the second half of the season and 1–2 months after the completion of the season while also tracking head impact exposures with the xPatch (22, 45) and demonstrated deficits in cerebrovascular reactivity in those participants with the highest cumulative accumulation of linear head impacts (those experiencing 14,487 *g* vs. 4,511 g over the season). In conjunction with the current findings, these results suggest soccer heading can dysregulate CBF control mechanisms.

Alterations observed in the MCA (**Figure 3**) following soccer heading were not replicated in the posterior cerebral circulation (**Figure 2**). One potential explanation for this finding is the relative proximity of the affected territory to the ball contact site. The MCA supplies a large anterior region of the cortex including the prefrontal cortex. Previous studies using positron emission tomography (PET) and single-photon emission computerized tomography demonstrated decreased CBF to frontal and temporal brain regions among non-athletes following mild to moderate TBI (47–49). Further, fMRI research has demonstrated decreased prefrontal cortex CBF during a working memory task among athletes after sustaining a concussion despite normal structural imaging results (50). Taken together, it is plausible an acute bout of soccer heading caused a focal change in NVC which could be due to biochemical derangement to MCA supplied brain regions. A potential mechanism of this NVC dysregulation could be related to the release of vasoactive materials from damaged neurons (i.e., CO_2, NO, adenosine, and arachidonic acid metabolites), release of vasoactive signals from activated astrocytes, and/or direct signaling disruption within the neurovascular unit [reviewed in: (51, 52)]. These findings provide evidence that future research is warranted into additional investigations examining the effects of soccer heading on cerebrovascular regulation with respect to both acute and chronic exposure levels.

Limitations

There are several limitations to this study. This small sample size potentially contributed to a reduced number of significant findings, particularly regarding number of concussion-related symptoms and symptom severity in the heading trial. However, given the magnitude of the head impact exposure used and the comparable sample size to previously published studies in this area (36, 40), we are confident our findings provide a meaningful contribution to this field of research. Furthermore, when the data were assessed for the false discovery rate with Benjamini-Hochberg adjustments, there were no changes to the significance reported in the results thus limiting the potential influence of Type II errors. The sham condition in the current investigation was designed to understand the effects of contact by the ball to the body, however what was not considered were the forces experienced by players when the attempt to perform a header but do not contact the ball (i.e., accelerations due to head movements, jumping, and landing). Therefore, future investigations could also include an additional sham condition which reflects this aspect of game play. Additionally, we only included male participants which limits the generalizability of our findings, as female soccer athletes sustain a greater rate of concussions per game played than male soccer athletes (53). Furthermore, females have been shown to have deficits in cerebrovascular reactivity to carbon dioxide associated with increases in head impact exposures (45, 46, 54). The research question and study design precluded the blinding of participants to exposure condition during each testing session. As such, the subjective self-reported symptoms within the SCAT3 may have led to participants being more likely to report symptoms of concussion knowing they had just been exposed to the acute bout of repeated head impacts. Potential bias introduced by the inability to blind was minimized by relying on objective outcomes (NVC response) and validated questionnaires (55). Finally, the game play situation which was mimicked in the current design (corner kick) is not entirely reflective of all potential scenarios which may result in a header, and future investigations could either employ a more diverse series of ball speeds to reflect other situations (cross, referral from the backfield) or perform a similar series of neurovascular coupling assessments across a season of play with measures performed during pre-season, mid-season and post-season with comparisons made based on player positions and number of headers they experienced as a result of game play.

A common limitation with the use of TCD is it only provides a measure of CBV within the MCA and PCA, but not CBF directly. Recently, high resolution MRI studies (56, 57) have revealed cerebral artery diameter is relatively constant when CO_2 is within 8 mmHg of eucapnia. In the current study, end-tidal CO_2 was maintained during all the visual stimulation protocols (**Table 2**), which supports the notion that CBV data presented in the current study provides a representative index of alterations in CBF. Finally, despite the excellent temporal resolution of TCD assessment of CBV, the spatial resolution is limited. Therefore, we were unable to measure changes in CBV through any of the smaller perforating branches off of the major cerebral arteries. We were therefore unable to directly quantify localized CBF, which may provide additional pathophysiological insight.

CONCLUSION

This study revealed exposure to a controlled bout of subconcussive head impacts in the form of soccer heading is sufficient to change the NVC response as measured in the MCA, but not the PCA. This finding was present irrespective of a significant increase in concussion symptom number or severity following the bout of soccer heading. These findings add to our knowledge regarding the pathophysiologic mechanisms underpinning head injury in contact sports. Further investigations are warranted to investigate NVC changes within focal brain regions following a controlled bout of soccer heading to better understand the regional specificity associated with these head impacts. Furthermore, additional investigations

into the cumulative effects across a soccer career of heading the ball, with respect to alterations in the NVC response are warranted.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UBC Clinical Research Ethics Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JS, PD, and AW designed the study. JS, AW, KB, JD, JB, MK, and CW collected data. JS and DP analyzed data. All authors contributed to the writing and final approval of the manuscript.

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S100B Blood Level Determination for Early Management of Ski-Related Mild Traumatic Brain Injury: A Pilot Study

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Background: Mild traumatic brain injury (mTBI) management in emergency departments is a complex process involving clinical evaluation, laboratory testing, and computerized tomography (CT) scanning. Protein S100B has proven to be a useful blood biomarker for early evaluation of mTBI, as it reduces the required CT scans by one-third. However, to date, the ability of S100B to identify positive abnormal findings in the CT scans of patients suffering from mTBI caused by ski practice has not been investigated. Thus, the primary aim of this study was to investigate the diagnostic performance of S100B as an mTBI management biomarker in patients with ski-related mTBI.

Materials and Methods: One hundred and thirty adult mTBI patients presenting to the emergency department of Hôpital du Valais in Sion, Switzerland, with a Glasgow Coma Scale (GCS) score of 13–15 and clinical indication for a CT scan were included in the study. Blood samples for S100B measurement were collected from each patient and frozen in 3-hour post-injury intervals. CT scans were performed for all patients. Later, serum S100B levels were compared to CT scan findings in order to evaluate the biomarker's performance.

Results: Of the 130 included cases of mTBI, 87 (70%) were related to ski practice. At the internationally established threshold of 0.1 μg/L, the receiver operating characteristic curve of S100B serum levels for prediction of abnormal CT scans showed 97% sensitivity, 11% specificity, and a 92% negative predictive value. Median S100B concentrations did not differ according to sex, age, or GCS score. Additionally, there was no significant difference between skiers and non-skiers. However, a statistically significant difference was found when comparing the median S100B concentrations of patients who suffered fractures or had polytrauma and those who did not suffer fractures.

Conclusion: The performance of S100B in post-mTBI brain lesion screenings seems to be affected by peripheral lesions and/or ski practice. The lack of neurospecificity of the biomarker in this context does not allow unnecessary CT scans to be reduced by one-third as expected.

Keywords: S100B, mTBI (mild traumatic brain injury), ski, ski accidents, biomarker

INTRODUCTION

Mild traumatic brain injury (mTBI) is a complex injury that causes a wide range of symptoms and disabilities. It constitutes a major public health concern, as it leads to a significant amount of morbidity and mortality worldwide. Although there are differences in criteria used to categorize traumatic brain injuries (1), these injuries are usually stratified using the Glasgow Coma Scale (GCS), which differentiates between severe (score of 3–8), moderate (score of 9–12), and mild brain (mTBI) injuries (score of 13–15). About 70–90% of all treated brain injuries are mTBIs, with an incidence estimated at 100–300 per 100,000 people (2, 3). The two leading causes of mTBI-related hospital admissions in Europe and the US are road traffic accidents and falls (3, 4). Different mechanisms of mTBI, associations with peripheral bone fractures and sport practice could influence patients' management in the emergency department (ED). In Allouchery et al.'s (5) study of 1,449 mTBI patients admitted to Clermont-Ferrand's adult ED, 59% were linked to a domestic fall, 14% to a road accident, and 6% to a sport-related accident. The proportions of these causes may vary depending on the location of the hospital. For example, in hospitals located near major ski resorts, winter-sport-related head trauma constitutes an important portion of emergency admissions. Indeed, in the 2018 Club Alpin Suisse (CAS) report, the Swiss mountain emergency medicine organizations Garde aérienne Suisse de sauvetage (Rega), Secours Alpin Suisse (SAS), and Organisation Cantonale Valaisanne de Secours (OCVS) registered 3,211 accidents, 729 of which were related to winter sports (6).

Computerized tomography (CT) scans of the brain are essential for diagnostic work-up, classification, prognostication, and follow-up of mTBI patients (7). Additionally, such scans enable detection of patients who require neurosurgical care. Intracranial complications occur in 10% of mTBI patients, and 1% of cases require neurosurgical intervention, with a 0.1% fatality rate (8). Screening all mTBI patients would be expensive, and the ionizing radiation involved in screening incurs potential risks (9). Limiting unnecessary CT scans is essential to reduce overcrowding in EDs, avoid exposing patients to radiation, and achieve significant financial savings (10). The decision to perform imaging in cases of head trauma depends on multiple factors, including clinical symptoms and measured blood biomarkers (11). Although clinical parameters, including headache, nausea, vomiting, amnesia, and seizures, may be correlated with positive CT scans, the number of negative CTs is not significantly reduced when they are present (12–14).

S100 calcium-binding protein B (S100B) is the most commonly studied blood biomarker for its ability to predict abnormal findings in the CT scans of mTBIs in children and adults (15, 16). Since 2013, S100B has been implemented in Scandinavian countries for management of mTBI (17). Upon admission, measurement of S100B serum may reduce 30% of unnecessary negative CT scans (16, 18–20). In addition, there is a growing interest of S100B amongst other head trauma biomarkers in the management of sport-related concussions (21), as consequences of repeated mTBIs have been linked to brain lesions similar to those observed in dementia (22–24).

As of today, S100B is the only blood biomarker used in routine mTBI screening guidelines in Europe and the ∼30% CT scans reduction has been established in routine use (5, 17, 20). However, we do not observe the same performances in our practice in the ED of Valais Hospital in Sion, Switzerland, where a relatively high percentage of brain injury admissions is ski-related. S100B has never been studied in the context of ski-related mTBI, and there is no literature that provides data on the influence of ski practice on serum S100B concentrations. We conducted this study to evaluate the diagnostic performances of serum S100B measurement to identify CT scan abnormalities (i.e., positive CT scans) in a cohort of mTBI patients in which ski-related accidents represent a significant percentage. The main outcome of this study is to assess the CT scan reduction allowed by S100B in this population of patients.

MATERIALS AND METHODS

Study Design

This prospective study was carried out from February 2018 to April 2019 at the Department of Emergency Medicine at Valais Hospital (*Centre Hospitalier du Valais Romand*) in Sion, Switzerland. The study was approved by CER-VD and conducted in accordance with the ethical principles for medical research outlined in the Helsinki Declaration. Patients provided an informed consent. Adult (18 years old and older) mTBI patients with a clinical indication for a CT scan, as described in the Canadian CT Head Rule (25) were included. mTBI patients were defined as patients suffering from a head trauma with a GCS score of 13–15, as determined by the attending physician. All patients underwent a CT scan and venipuncture for subsequent S100B and creatine kinase (CK) blood measurements. The time interval for S100B sampling was set at 3 h post-trauma, as described by Biberthaler et al. (18) and Laribi et al. (26).

Data Collected

Data collected were: sex, age, GCS score, mechanism of injury (occurred during alpine skiing, other sport-related accident, road accident, domestic accident, alcohol intoxication and other), the

wear of a safety helmet, ski session duration, presence of fractures and if present, the number of fractures, and whether the patients were described as polytraumatized according to Lew et al. (27).

S100B and Creatine Kinase Assays

Venous blood samples were centrifuged at 2,100 g for 15 min and stored at −20°C until analysis. The S100B results did not influence patients' clinical management.

Serum S100B concentrations were determined by an electrochemiluminescence immunoassay using a Roche Diagnostics Cobas e411® instrument (Meylan, France). The assay time was 18 min, the sample volume was 20 μL, and the lower detection limit was 0.005 μg/L. Concentrations of up to 39 μg/L could be measured without dilution. Typical within-assay precision was below 5%. The results are reported in micrograms per liter and rounded to two decimal places. Total CK activity concentration assays were performed using a Vista® analyzer (Siemens, Munich, Germany) following the manufacturer's recommendations.

Cranial Computed Tomography Scans

An emergency cranial CT scan was performed using a GE Healthcare Revolution GSI® according to the following protocol: helical mode with a slice thickness of 2.25 mm, an interval of 1.25 mm, 120 kV, and a maximum of 280 mA from C1 to the top of the head with additional bone window reconstructions. All CT scans were analyzed by a radiologist. To determine whether patients had a trauma-relevant intracranial lesion, radiological parameters were recorded. The patients were divided into two groups: normal CT scan (CT–) for mTBI patients with no signs of trauma-relevant intracranial lesions and abnormal CT scan (CT+) for mTBI patients with at least one pathophysiological trauma-relevant intracranial lesion. CT scans were considered positive if any signs of cranial (skull fracture) or intracranial pathology (hematoma, air, or contusion) were present, subgaleal hematomas were also considered positive to prevent disregarding abnormalities that may influence S100B levels.

Statistics

Statistical analyses were performed using Stata software, version 15 (StataCorp, College Station, US). The tests were two-sided, with the Type I error set at 5%. Continuous data were expressed as the mean ± standard deviation (SD) or median (interquartile range) according to the statistical distribution. The assumption of normality was assessed using the Shapiro-Wilk test. An analysis of variance (ANOVA) or Kruskal-Wallis test (when ANOVA assumptions were not met) were performed to compare continuous parameters (e.g., S100B serum) between the independent groups. The assumption of homoscedasticity was verified using Bartlett's test. Then, to evaluate the ability of S100B serum to identify CT+ patients, the receiver operating characteristic (ROC) curve was plotted. The area under the curve was estimated with a 95% confidence interval.

Univariate analyses were completed using several inferential statistical tests. Comparisons involving categorical variables were performed with the chi-squared or Fisher's exact tests, while the relationships between continuous variables were explored using either Pearson's or Spearman's correlation coefficient, depending on the statistical distribution. The correlation results were illustrated with a color-coded heatmap.

Multivariate analyses, specifically multiple linear regressions, were carried out to investigate the influence of different variables on S100B levels. The normality of residuals was examined as mentioned above, and logarithmic transformation of S100B serum concentrations was proposed to achieve the normality assumption. Sensitivity analyses were performed to guarantee the robustness of the results.

RESULTS

Patient Characteristics

Between February 2018 and April 2019, a total of 130 patients admitted for head trauma in the ED were recruited for this study. S100B serum assays were performed within 3 h of the injury. The sample included 81 males (62%) and 49 females (38%), with a 1.6 sex ratio (male/female). The mean age was 44.8 years (SD: 20.4). In total, 108 (83%) patients had a GCS score of 15 at admission, and 22 (17%) had a GCS score of 13 or 14. Of the 130 patients, 90 (69%) had a sport-related accident, 87 of them were ski-related. The helmet was used by 76 of the 87. The mean ski session duration was 2 h and 18 min (SD: 1 h and 42 min). Thirty-three (25%) patients had abnormal findings in the initial CT scan (CT+; **Table 1**). The pathophysiological trauma-relevant findings were as follows: subgaleal hematoma (45%), subarachnoid hemorrhage (24%), basal skull fracture (18%), intraparenchymal hemorrhage (9%), and intracranial hemorrhage (3%). The median serum S100B level was 0.21 μg/L (min: 0.05; max: 1.42; IQR: 0.14–0.35).

S100B Performance for Identifying Abnormal Findings in CT Scans

The area under the curve (AUC) was calculated to evaluate the ability of S100B serum concentrations to diagnose CT+ patients. For all patients, AUC was 0.71 (95% CI; 0.60–0.81; $p < 0.001$; **Figure 1**). At the internationally established cutoff of 0.1 μg/L (16, 18, 28), sensitivity was 97% (95% CI; 84.2–99.9), specificity was 11% (95% CI; 5.8–19.4), the positive predictive value was 27% (95% CI; 19.3–36.1), and the negative predictive value was 92% (**Table 2**). The best threshold to achieve sensitivity of 100% (95% CI; 89.4–100) was 0.08 μg/L, with 7% specificity (95% CI; 2.3–13). The best threshold to achieve specificity of 31% (95% CI; 21.9–41.1) was 0.14 μg/L, with 91% (95% CI; 76–98) sensitivity.

Among the group of skiers (87 patients), AUC was 0.70 (95% CI; 0.56–0.84; $p < 0.001$). At a cutoff of 0.1 μg/L, sensitivity and specificity were 95% (95% CI; 75.1–99.9) and 10% (95% CI; 4.3–20.3), respectively. Positive predictive value was 24.1% (95% CI; 15.1–35.0) and negative predictive value was 87.5% (95% CI; 47.3–99.7). The best threshold for achieving sensitivity of 100% (95% CI; 83.2–100) was 0.08 μg/L, with 6% (95% CI; 2.0–15.0) specificity. The best threshold for achieving specificity of 33% (95% CI; 22.0–45.0) was 0.14 μg/L, with 85% (95% CI; 62.0–97.0) sensitivity.

TABLE 1 | Demographic characteristics, clinically relevant information, and radiological findings for the whole study population.

	Data
Total	130
Sex ratio (M/F)	1.6
Mean age in years (SD)	44.8 (20.4)
GCS at admission	
15	108 (83%)
13–14	22 (17%)
Positive CT scan	33 (25%)
Subgaleal hematoma	15 (45%)
Subarachnoid hemorrhage	8 (24%)
Basal skull fracture	6 (18%)
Intraparenchymal hemorrhage	3 (9%)
Intracranial hemorrhage	1 (3%)
Contusions at admission	59 (45%)
Fractures at admission	34 (26%)
Fracture = 1	26 (20%)
Fractures ≥ 2	8 (6%)
Polytrauma	12 (9%)
Injury mechanism	
Road accident	13 (10%)
Ski-related accident	87 (67%)
Other sport-related accident	3 (2%)
Domestic accident	14 (11%)
Alcohol intoxication	5 (4%)
Other	6 (5%)

GCS, Glasgow Coma Scale; CT scan, computed tomography scan; fractures = 1, patients that suffered only one fracture; fractures ≥ 2, patients that suffered at least two fractures; SD, standard deviation.

TABLE 2 | Contingency table of S100B serum (0.1 μg/L threshold) concentration according to cranial tomography findings.

S100B	CT+	CT–	
> 0.1 μg/L	32	87	Positive predictive value 27%, (19.3–36.1)
≤ 0.1 μg/L	1	10	Negative predictive value 92%, (61.5–99.8)
Total	33	97	130
	Sensitivity, 97% (84.2–99.9)	Specificity, 11% (5.8–19.4)	

CT–, normal CT scan for mTBI patients with no signs of trauma-relevant intracranial lesions; CT+, abnormal CT scan for mTBI patients with at least one pathophysiological trauma-relevant intracranial lesion.

Clinical Factors' Influence on Serum S100B Levels

The median concentrations of serum S100B did not differ according to sex, age, and GCS score (13 vs. 14 and 15) (**Table 3**).

A statistically significant difference was observed when comparing the median concentrations of patients with a negative CT scan (CT–) and that of abnormal CT scan (CT+) patients ($p < 0.001$). S100B medians were 0.18 μg/L (min: 0.05; max: 1.21; IQR: 0.13–0.28) and 0.31 μg/L (min: 0.08; max: 1.2; IQR: 0.17–0.62), respectively (**Table 3**).

To investigate whether peripheral traumatic injuries affect serum S100B concentrations, patients were sorted into groups depending on the number of fractures they suffered and whether they were categorized as polytrauma patients. The median S100B concentration was 0.20 μg/L (min: 0.05; max: 1.42; IQR: 0.13–0.34) for patients without fractures and 0.27 μg/L (min: 0.08; max: 1.32; IQR: 0.16–0.44) for patients that suffered at least one fracture. There was a statistically significant difference between the two values ($p = 0.036$). The medians for non-polytrauma patients and polytrauma patients were 0.20 μg/L (min: 0.05; max: 1.42; IQR: 0.14–0.35) and 0.52 μg/L (min: 0.08; max: 1.10; IQR: 0.27–1.10), respectively. Again, the difference was statistically significant ($p = 0.004$; **Table 3**).

The medians for patients with ski-related accidents and non-skiers were compared to determine whether ski practice has an effect on S100B measurements. There was no significant difference between the two subgroups. The use of a helmet did not impact S100B medians (**Table 3**).

A multivariate linear model was used to investigate the influence of different variables, such as age, sex, GCS score, number of fractures, and polytrauma, on S100B levels (see *p*2 in **Table 3**). The model revealed that age, sex, and GCS score did not have an impact on S100B levels. Among the variables that showed a difference when the S100B medians were compared (i.e., fractures and polytrauma), the only statistically significant association was observed in cases with two or more fractures ($p = 0.009$; **Table 3**).

To investigate the possible peripheral release of S100B due to tissue damage other than head trauma, including muscle damage, serum CK was measured. The Spearman's correlation coefficients between serum CK and serum S100B levels were 0.07 ($p = 0.48$) for the whole population, 0.19 ($p = 0.11$) for the skier group, 0.13 ($p = 0.50$) for patients that suffered only one fracture, and 0.57 ($p = 0.19$) for patients that suffered at least two fractures (**Figure 2**).

DISCUSSION

This prospective pilot study was the first to analyze the utility of S100B measurement for early management of mTBI patients in emergency departments in close proximity to ski stations. S100B is a well-established blood biomarker for mTBI screening in patients admitted in the ED. It is considered useful due to its negative predictive value and ability to achieve an approximate 30% reduction in unnecessary CT scans for these patients. In the present study, the diagnostic performance of serum S100B, which was always identified as significant by ROC curves, showed that, at a threshold of 0.1 μg/L, the sensitivity was 97% due to one patient from the skier group who had a negative S100B value and a positive CT scan showing a unique minimal hemorrhagic focus, which did not require neurosurgical care and did not have an impact on the clinical outcome, the patient was discharged after being held for observation. For this patient, the time interval for S100B sampling was >3 h

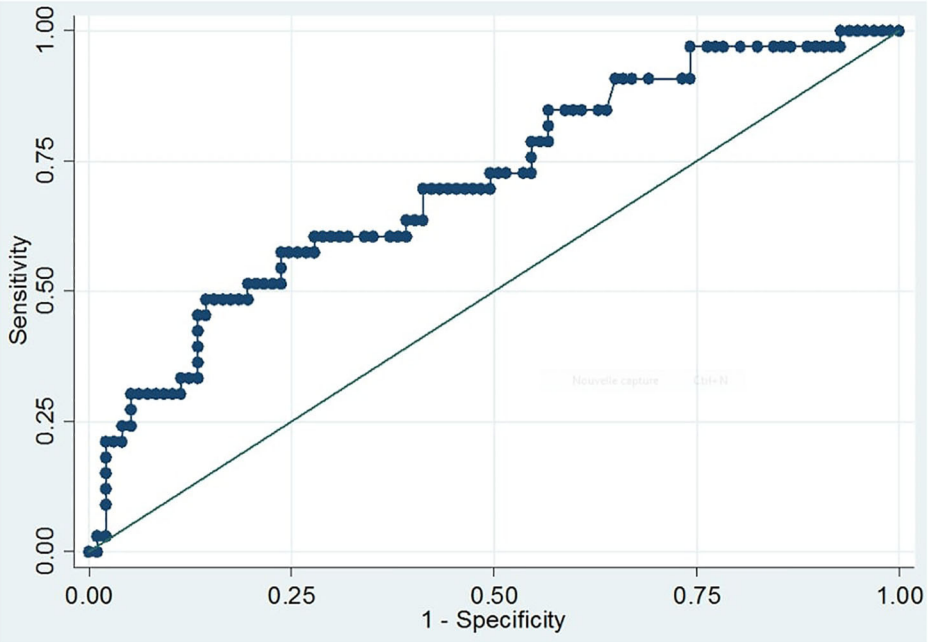

FIGURE 1 | ROC curve of S100B serum levels for prediction of intracranial injury revealed by CT scans. The curve depicts the sensitivity and one-specificity values calculated for each individual serum S100B concentration with respect to the radiological findings of the initial CT scan. The quality of the discriminative potential is expressed by the AUC, which was 0.71 (95% CI, 0.60–0.81; $p < 0.001$).

and the value was confirmed by a second measurement of the same sample. We also found that, at a threshold of 0.1 μg/L, serum S100B levels would reduce unnecessary CT scans by only 10%. Allouchery et al. (5) observed a 30% scans reduction using French recommendation for CT Scan (29), while Minkkinen et al. (30) reported a 27.4% CT scans reduction when using S100B in adherence to the Scandinavian guidelines for mTBI with low risk of lesions. Theoretically, including subgaleal hematomas which do not constitute an intracranial injury would have reduced false positives in this study. The increased number of false-positive patients in this population could be associated with selection bias due to the hospital's geographic location and the nature of the accidents leading to ED admission. In Allouchery et al.'s (5) study, 862 (59.5%) patients of the whole population (n = 1,449) were admitted to the ED due to mTBI after a domestic fall. In contrast, in the present study, only 14 (10.8%) of the 130 patients in our study population suffered from domestic falls. We hypothesize that the lack of specificity of S100B for positive CT scans in the present study is linked to the underrepresentation of domestic accidents in comparison to studies conducted in urban areas, which lead to less complicated mTBIs and less increased S100B levels. The inclusion of these mTBI patients may vary depending on the hospital's decision algorithm to perform CT scans. The Canadian CT Head Rule may explain a lower specificity. In the present study's population, most mTBI admissions were related to ski accidents (66.9% of our population) rather than domestic falls. The ROC curve analysis showed a 2-fold specificity reduction in the skier group compared with the specificity for the whole population. This suggests that ski practice contributes to the increase in serum S100B, thus increasing false-positive S100B measurements when screening for mTBIs.

This hypothesis has been verified in various other sports (21), but not in ski practice. However, comparison of the median S100B concentrations between the skier and non-skier groups did not confirm this hypothesis. However, we cannot exclude this hypothesis based solely on median comparison, the non-skier group was not a valid control group as the patients in the two groups did not suffer the same injuries, ski practice is not the only variable that differs between the two groups, and the number of patients included in the skier and non-skier groups (87 and 43) do not provide sufficient statistical power. A valid way to investigate the relation of S100B to ski practice would be to measure S100B in a control group before and after ski practice.

Finally, previous studies have found that serum S100B concentrations were higher in the elderly population (31) (over 65 years old) and a threshold of 0.1 μg/L allowed for more false positives (26). However, this was not confirmed by our results. The poorer specificity of S100B observed in older patients by Allouchery et al. (5) was observed in all patients in the present study. This could also could be explained by the contribution of S100B from adipose tissue due to lipolysis or chondrocyte membrane rupture during ski practice (21).

Serum S100B in this present population is clearly affected by the presence of multiple fractures and polytrauma. Numerous studies suggest an extracerebral release of S100B in acute bone fractures patients with or without cerebral injuries (32–34). Though our multivariate model shows a stronger

TABLE 3 | Median concentrations of S100B according to demographic characteristics, clinical evaluation, radiological findings, and mechanism of injury.

		n	S100B median (μg/L) (min; max; IQR)	*p*	*P2*
Demographics					
Male		49	0.19 (0.05; 1.32; 0.14–0.37)	NS	NS
Female		81	0.22 (0.07; 1.42; 0.14–0.34)		
< 65 years old		101	0.21 (0.05; 1.42; 0.14–0.35)	NS	NS
≥ 65 years old		29	0.21 (0.08; 1.00; 0.15–0.34)		
Clinical and radiological evaluation					
GCS score of 13–14		22	0.26 (0.05; 1.42; 0.11–0.38)	NS	NS
GCS score of 15		108	0.20 (0.07; 1.32; 0.14–0.35)		
CT–		97	0.18 (0.05; 1.42; 0.13–0.29)	< 0.001	NA
CT+		33	0.35 (0.08; 1.32; 0.17–0.53)		
No fractures (F0)		96	0.20 (0.05; 1.42; 0.13–0.34)	F1F2 vs. F0: 0.036	F1F2 vs. F0: NS
Fractures	F1F2	34	0.27 (0.08; 1.32; 0.16–0.44)	F1 vs. F0: NS	F1 vs. F0: NS
	F1	26	0.23 (0.09; 0.85; 0.16–0.85)	F2 vs. F0: 0.041	F2 vs. F0: 0.009
	F2	8	0.96 (0.08; 1.32; 0.31–1.2)	F2 vs. F1: 0.048	F2 vs. F1: 0.009
Non-polytrauma		118	0.20 (0.05; 1.42; 0.14–0.35)	0.004	NS
Polytrauma		12	0.52 (0.08; 1.32; 0.27–1.10)		
Injury mechanism					
Non-skiers		43	0.27 (0.05; 1.42; 0.14–0.41)	0.19	NA
Skiers		87	0.19 (0.05; 1.21; 0.13–0.34)		
Using a helmet		76	0.17 (0.07; 1.21; 0.10–0.52)	NS	NA
Not using a helmet		11	0.20 (0.05; 1.2; 0.14–0.30)		

A p-value of ≤ 0.05 is considered statistically significant. The multivariable analysis did not include non-significant results of the univariate analysis and did not include CT+. p, p-value of the median comparison; p2, p-value of the multivariate model; NA, Non Applicable; NS, Non Significant; GCS, Glasgow Coma Scale; CT–, negative computed tomography scan; CT+, positive computed tomography scan; F0, patients without fractures; F1, only one fracture; F2, two fractures or more; F1F2, F1 and F2 patients.

link between S100B and fractures than between S100B and polytrauma. The factor that most influenced S100B level was the presence of at least two fractures, as observed in the multivariate analysis. The hypothesis of an extracerebral S100B release is supported by the statistically significant difference in median serum S100B concentrations when comparing the no fracture and fracture subgroups and the improvement of the correlation factor between CK and S100B for patients with more fractures. Our results suggest that S100B should not be used as a head trauma biomarker when patients suffer from bone fractures. Although serum CK is not a valid biomarker for bone fracture exploration, we retained it as a practical and accessible biomarker to approximate peripheral traumatic lesions. In addition, we expected lower S100B levels in skiers using a helmet. However, the use of a helmet did not yield lower levels of S100B in this study. This can be explained by the S100B release consequent to extracranial injuries counterbalancing the potential protective effects of the helmet. Similar results were described for bicycle-related trauma (35). Another hypothesis is that in skiers, mTBI is consecutive to a combination of direct head impact, which should be protected by the helmet, and blunt head injury via acceleration-deceleration phenomena which would not be affected by the use of a helmet. Neural tissue is susceptible to injury from shearing stresses which are less tolerated than uniform compressive and tensile forces (36).

The investigation of other potential mTBI biomarkers, such as glial fibrillary acidic protein (GFAP), heart fatty acid binding protein (H-FABP), and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), may enable the development of more neurospecific tools, bringing new perspectives for mTBI management. In Yue et al.'s (37) study of suspected TBI patients, blood GFAP concentrations allowed the prediction of pathological findings in MRI in patients with a negative CT scan. In Lagerstedt et al.'s (38) study of mTBI patients with a GCS score of 15, a panel combining H-FABP, GFAP, and interleukin 10 (IL-10) yielded a 52% specificity (38). Posti et al. (39) tested a panel of three biomarkers—S100B, tau, and H-FABP—with 100% sensitivity and 46.4% specificity. Thus, use of a biomarker panel may have the potential to reduce unnecessary CT scans, and could be particularly interesting in mTBI consecutive to a complicated mechanism and/or when fractures are present. The S100B protein is to this day the only brain trauma biomarker used in routine in Europe and this study was conducted to explain the limited performances of S100B in emergency departments where ski-related accidents represent a significant percentage. Although, testing other biomarkers would have been interesting, test kits are not yet available in routine kits with the proper CE marking.

LIMITATIONS

The main limitation of our study is the relatively small number of patients included. However, S100B is a robust biomarker and the ∼30% CT scan reduction was confirmed by numerous studies regardless of the population size (5, 16, 18, 19). Also, a

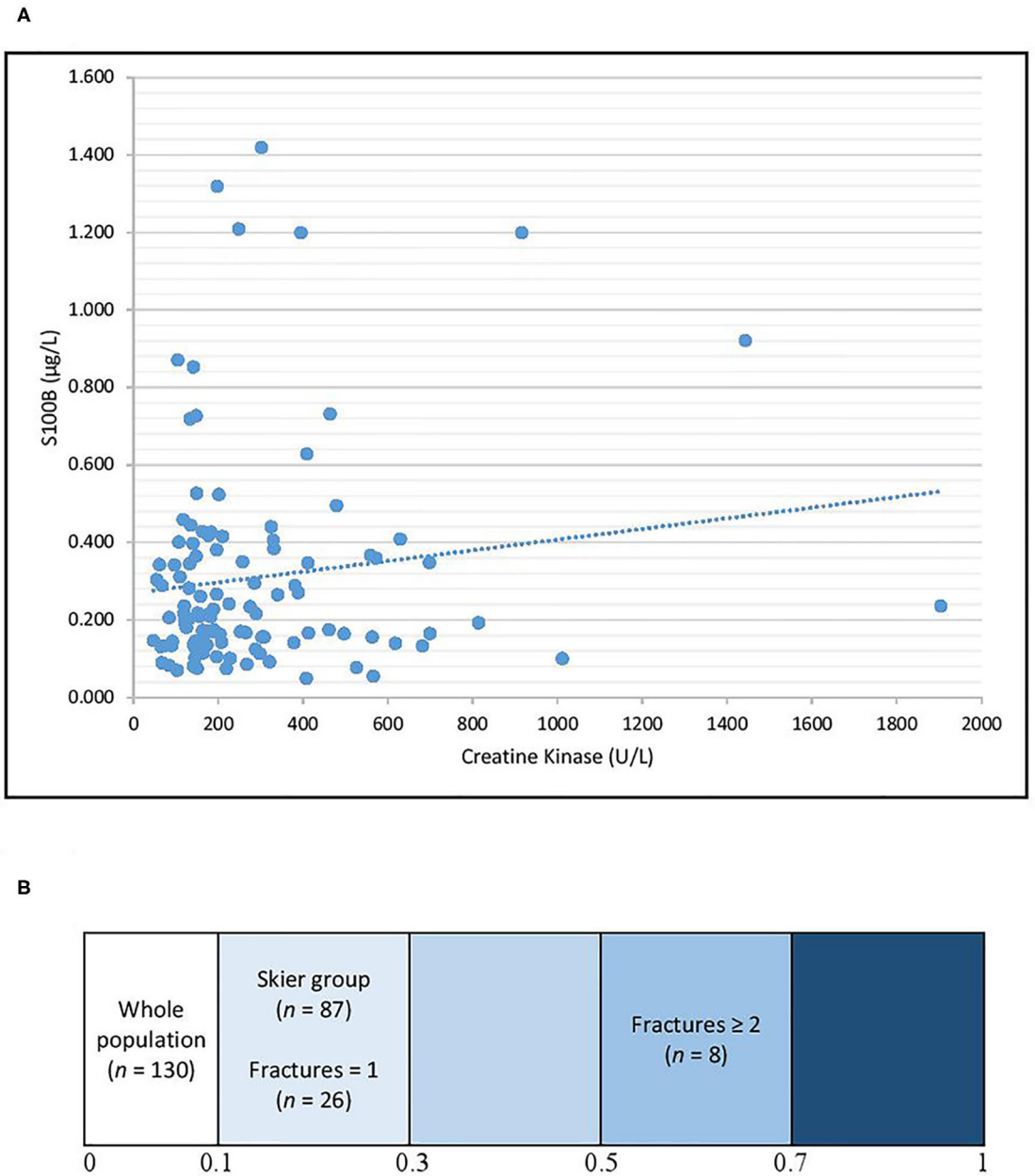

FIGURE 2 | Correlation between S100B and CK for the whole study population **(A)** and heatmap representing the Spearman's correlation coefficients for different subgroups **(B)**. The Spearman's correlations coefficients were 0.07 for the whole population, 0.19 for the skier group, 0.13 for patients that suffered only one fracture, and 0.57 for patients that suffered at least two fractures.

multicenter design would have provided a preferable basis for subsequent generalization of our findings. Regarding the data collection and interpretation, traceability concerning patients' inclusion process has not been documented, this constitutes a potential selection bias. Additionally, there was a lack of documentation of some clinical variables justifying the use of a CT scan such as the duration of loss of consciousness and posttraumatic amnesia. Finally, the inclusion of subgaleal hematomas in the abnormal CT scans may influence the comparability to other studies which don't include this CT finding, although, we did not observe a statistical difference in the interpretation of our data when including or excluding subgaleal hematomas from the positive CT scans.

CONCLUSION

S100B is a well-established blood biomarker for early mTBI management. When used for clinical evaluation, it enables a

significant reduction in the number of CT scans performed. Our study shows that the utility of S100B could vary depending on multiple confounding factors, such as sport practice and bone fractures. For ski-related mTBIs, S100B does not allow a significant reduction of unnecessary CT scans.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CER-VD. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SK and PS analyzed and interpreted the data and wrote the initial version of the manuscript. DB and VS designed the study and assisted with interpretation of the data and writing of the manuscript. LP, JC, and VF supervised the trial and data collection. CO, JD, and LA carried out assays. BP provided statistical advice for the study design and analyzed the data. All authors contributed to the article and approved the submitted version.

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Comparing Glial Fibrillary Acidic Protein (GFAP) in Serum and Plasma Following Mild Traumatic Brain Injury in Older Adults

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Objective: Identification and validation of blood-based biomarkers for the diagnosis and prognosis of mild traumatic brain injury (mTBI) is of critical importance. There have been calls for more research on mTBI in older adults. We compared blood-based protein marker glial fibrillary acidic protein (GFAP) concentrations in serum and in plasma within the same cohort of older adults and assessed their ability to discriminate between individuals based on intracranial abnormalities and functional outcome following mTBI.

Methods: A sample of 121 older adults [≥50 years old with head computed tomography (CT), $n = 92$] seeking medical care for a head injury [Glasgow Coma Scale scores of 14 ($n = 6$; 5.0%) or 15 ($n = 115$; 95.0%)] were enrolled from the emergency department (ED). The mean time between injury and blood sampling was 3.4 h ($SD = 2.1$; range = 0.5–11.7). Serum GFAP concentration was measured first using the Human Neurology 4-Plex Assay, while plasma GFAP concentration was later measured using the GFAP Discovery Kit, both on an HD-1 Single molecule array (Simoa) instrument. Glasgow Outcome Scale-Extended was assessed 1 week after injury.

Results: Both serum and plasma GFAP levels were significantly higher in those with abnormal CT scans compared to those with normal head CT scans (plasma: $U = 1{,}198$, $p < 0.001$; serum: $U = 1{,}253$, $p < 0.001$). The ability to discriminate those with and without intracranial abnormalities was comparable between serum (AUC = 0.814) and

plasma (AUC = 0.778). In the total sample, GFAP concentrations were considerably higher in plasma than in serum (Wilcoxon signed-rank test $z = 0.42$, $p < 0.001$, $r = 0.42$). Serum and plasma GFAP levels were highly correlated in the total sample and within all subgroups (Spearman's *rho* range: 0.826–0.907). Both serum and plasma GFAP levels were significantly higher in those with poor compared to good functional outcome (serum: $U = 1{,}625$, $p = 0.002$; plasma: $U = 1{,}539$, $p = 0.013$). Neither plasma (AUC = 0.653) nor serum (AUC = 0.690) GFAP were adequate predictors of functional outcome 1 week after injury.

Conclusions: Despite differences in concentration, serum and plasma GFAP levels were highly correlated and had similar discriminability between those with and without intracranial abnormalities on head CT following an mTBI. Neither serum nor plasma GFAP had adequate discriminability to identify patients who would have poor functional outcome.

Keywords: traumatic brain injuries, glial fibrillary acidic protein, plasma, serum, computed tomography

INTRODUCTION

Glial fibrillary acidic protein (GFAP) concentration is increased following a traumatic brain injury (TBI), in studies of patients with predominantly mild to moderate injuries (1–6). There is some evidence that GFAP is associated with, and may be able to predict, unfavorable outcome following TBI (7–10). GFAP is a 50 k-Da intermediate filament protein that is highly abundant in the cytoskeleton of astrocytes (11, 12). Following plasma membrane damage secondary to neurotrauma, GFAP is released into the interstitial fluid, and enters the bloodstream by crossing the blood-brain barrier, which is compromised following TBI (13–15) or via the glymphatic system (14, 16). GFAP has been shown to be detectable within 1 h of injury (3, 17, 18), continues to rise and appears to peak within 20–24 h (3, 18), and then declines over 72 h (3), with a biological half-life of 24–48 h (19). Many studies have examined the utility of GFAP for identifying patients with intracranial abnormalities following TBI (20). GFAP is considered useful for this purpose given that it is specific to brain injury (7, 21–23) and has a relatively long half-life compared to other biomarkers (19). The Food and Drug Administration (FDA) recently approved GFAP and ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) for use in the emergency department (ED) to screen for traumatic intracranial abnormalities and aid clinical decisions regarding acute head CT scanning (24).

A large body of evidence from samples obtained from patients with mild to moderate TBIs suggests that GFAP in both serum (1–3, 18, 25, 26) and plasma (4–6, 27) can discriminate between those with and without acute traumatic abnormalities on head CT, outperforming both UCHL-1 (4) and S100B (2). Despite extensive research examining GFAP, a comprehensive, direct comparison of serum and plasma GFAP levels from the same patient sample is lacking. The purpose of the present study is to compare serum and plasma GFAP levels following mTBI within the same sample of subjects using two different and widely used assays. We chose to examine a convenience sample of older adults with mild TBIs (mTBI) because (i) there have been calls for more research focused on mTBI in older adults (28, 29), (ii) they have pre-existing neurological conditions that can influence biomarker results (30), and (iii) there is evidence that they have higher levels of GFAP following injury than younger adults, as well as generally have more prolonged recoveries (31, 32). We hypothesized that plasma and serum GFAP levels would (i) be highly correlated, (ii) have similar ability to discriminate between those with and without acute traumatic intracranial abnormalities on head CT, and (iii) have similar associations with functional outcome in older adults with a mTBI.

MATERIALS AND METHODS

Participants

The data used for secondary analyses in the present study were part of a larger prospective study that aimed to clinically validate the Scandinavian Guidelines for Initial Management of Minimal, Mild, and Moderate Head Injuries in Adults in the Emergency Department (ED) of the Tampere University Hospital (33). Tampere University Hospital is the only neurosurgical referral hospital in the district, and the ED provides health services for approximately 470,000 residents from 22 municipalities, both urban and rural. All adult patients aged 18 or older, with an acute traumatic head injury, seen within 24 h of injury, were eligible for inclusion. The minimum criteria for TBI were as follows: either blunt injury to the head or acceleration/deceleration type injury resulting in witnessed loss of consciousness, disorientation, or amnesia and an initial Glasgow Coma Scale (GCS) score of 13–15. Over a 1-year period from November 2015 to November 2016, 325 patients provided written consent to be included in the study and 225 had both serum and plasma analyzed for GFAP. From these 225, we selected a sample of 121 older adults (≥50 years; 51.2% men) with a suspected mTBI, based on a Glasgow Coma Scale (GCS) of 14–15 upon presentation to the ED, who had blood drawn within 12 h of injury for the present study. The sample for the present study was limited to older adults in

part because (i) nearly all patients who presented to the ED who had abnormal head CT scans in the cohort were, coincidentally, older adults; (ii) GFAP has been shown to increase with age (32, 34); and (iii) GFAP was recently approved to screen for intracranial abnormalities (24), making this a convenience study of older adults.

Functional Outcome Assessment

Participants were administered the Finnish-language Glasgow Outcome Scale-Extended (GOS-E) (35) at 1 week post-injury by a trained research nurse. The 1-week time point was selected for the original S100B validation study to assess if the patients developed early complications that could have been avoided with initial head CT scanning in the ED. The GOS-E ranges from 1 to 8, with higher ratings corresponding to better functional outcome following injury during this subacute time period. The GOS-E was dichotomized with GOS-E of 7 and 8 considered *Good Outcome* and a GOS-E of 6 or lower considered *Poor Outcome*.

Computed Tomography

Non-contrast head CT was performed with a 64-row CT scanner (GE, Lightspeed VCT, WI, USA). Clinical judgment was used to decide whether to perform head CT, but decisions mainly adhered to the Scandinavian Guidelines (36). We did not rely on the interpretation from the on-call radiologist at the time of injury. A neuroradiologist, for research purposes, reviewed and systematically coded all CT findings based on the Common Data Elements (37). To verify the reliability of the head CT findings, an independent neuroradiologist re-interpreted 10% of the CT-scanned patients from the original prospective cohort (33) with the same common data elements. The interrater intraclass correlation for a normal vs. abnormal head CT was 0.879 (95% CI = 0.719–0.948, $p < 0.001$), indicating excellent agreement. Pre-existing and acute traumatic lesions were coded. The following traumatic lesions were considered as intracranial abnormalities on head CT: skull fracture, epidural hematoma, extraaxial hematoma, subdural hematoma, traumatic subarachnoid hemorrhage, vascular dissection, traumatic aneurysm, venous sinus injury, midline shift, cisternal compression, fourth ventricle shift/effacement, contusion, intracerebral hemorrhage, intraventricular hemorrhage, diffuse axonal injury, traumatic axonal injury, penetrating injuries, craniocervical junction/brainstem injury, edema, brain swelling, ischemia/infarction/hypoxic-ischemic injury. Based on head CT, participants were divided into those who did not undergo head CT, those with intracranial abnormalities on head CT, and those without intracranial abnormalities on head CT.

Blood Sampling and Analytics

Venous blood samples were collected within 12 h of injury. The blood samples were collected in Tampere between November 2015 and November 2016. Serum GFAP levels were measured first on March 12, 2018 in a research laboratory in Mölndal, Sweden using the Human Neurology 4-Plex Assay (Quanterix, Billerica, MA) on an HD-1 Single molecule array (Simoa) instrument according to instructions from the manufacturer (Quanterix, Billerica, MA). The lower limit of detection for GFAP was 0.221 pg/mL and the lower limit of quantification was 0.467 pg/mL. Calibrators were run in duplicates while samples were run in singlicates. Two quality control samples were run in duplicates in the beginning and the end of each run, showing a repeatability of 5.8% and intermediate precision of 5.8% at 79.8 pg/mL, and a repeatability of 4.9% and intermediate precision of 6.2% at 87.5 pg/mL.

Plasma GFAP levels were analyzed on September 14–15, 2019, again in Mölndal, Sweden using the GFAP Discovery Kit (Quanterix, Billerica, MA) on an HD-1 Simoa instrument according to instructions from the manufacturer (Quanterix, Billerica, MA). The lower limit of detection for GFAP was 0.211 pg/mL and the lower limit of quantification was 0.686 pg/mL. Calibrators were run in duplicates while samples were run in singlicates. Samples were run with a 4-fold dilution and results have been compensated for this dilution. Two internal quality control samples were run in duplicates in the beginning and end of each run. For a quality control sample with a concentration of 76.3 pg/mL, repeatability was 7.6% and intermediate precision was 11.3%, whereas for a quality control sample with a concentration of 204.2 pg/mL, repeatability was 6.8% and intermediate precision was 12.8%.

Ethical Approval

Ethics approval was obtained from the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (ethical code: R15045). Written informed consent was obtained from all included study participants after participants were provided with necessary information about the study in both oral and written form.

Statistical Analyses

All analyses were conducted for the total sample ($N = 121$) and designated subgroups based on head CT findings and functional outcome. Non-parametric tests were used given that both serum and plasma GFAP levels were non-normally distributed. Within-person comparisons of serum and plasma GFAP levels were conducted using Wilcoxon signed-rank tests, with effect size r calculated for each analysis by dividing the z-statistic by the square root of the sample size (38). This effect size can be interpreted as small ($r = 0.10$), medium ($r = 0.30$), and large ($r = 0.50$) (39). Cohen's d has also been reported and can be interpreted as small ($d = 0.20$), medium ($d = 0.50$), and large ($d = 0.80$) (39), but this effect size assumes normality and may not accurately reflect the magnitude of difference between groups. Non-parametric correlations (i.e., Spearman's *rho*) were also calculated to examine the relationship between age and GFAP levels, time to blood sampling and GFAP levels, and GFAP levels across serum and plasma. Mann-Whitney U tests were conducted to compare participants based on head CT findings (i.e., positive vs. negative) and outcome (i.e., poor vs. good), with effect size r again calculated to quantify the magnitude of the effect. Receiver Operator Characteristic (ROC) curve analyses were conducted to determine the sensitivity and specificity of the serum and plasma GFAP levels at discriminating between participants with and without intracranial abnormalities on head CT and participants with good and poor outcome based on GOS-E. The Area Under

TABLE 1 | Injury characteristics of the sample and imaging findings.

	Yes	No	Unknown
	n, %	*n*, %	*n*, %
Loss of consciousness-witnessed/suspected	45, 37.2%	58, 47.9%	18, 14.9%
Post-traumatic seizure	0, 0%	105, 86.6%	16, 13.2%
Post-traumatic amnesia	41, 33.9%	71, 58.7%	9, 7.4%
Focal neurological deficit	11, 9.1%	108, 89.3%	2, 1.7%
Vomited 2 times or more	2, 1.7%	109, 90.1%	10, 8.3%
Headache	50, 41.3%	62, 51.2%	9, 7.4%
Alcohol intoxication at time of injury	39, 32.2%	75, 62.0%	7, 5.8%
Neurosurgery (craniotomy)	2, 1.7%	119, 98.3%	–
Other surgery	0, 0%	121, 100%	–
Acute traumatic lesion on head computed tomography	22, 18.2%	78, 64.5%	29, 24.0%

No patient had an isolated skull fracture.

the Curve (AUC) was calculated for each ROC analysis, under a non-parametric assumption, with an accompanying standard error (*SE*) and 95% confidence interval (CI). AUC values were interpreted as acceptable (AUC = 0.70–0.79), excellent (AUC = 0.80–0.89), and outstanding (AUC $\geq$ 0.90) at discriminating between groups (40). Statistical analyses were conducted using SPSS version 24 and the MedCalc Statistical Software version 19.17 (Bland-Altman Plot, ROC analyses, and Passing Bablock regression). Bland-Altman analyses were used to illustrate the agreement between the two quantitative measurements. Passing Bablock regression is a non-parametric method for estimating a linear regression line and testing whether the intercept is zero and the slope is one, which would illustrate that two measurement systems were yielding the same values.

RESULTS

Patient Characteristics, Blood Sampling, and Imaging Findings

The total sample (n = 121; 51.2% men) had a mean age of 75.1 years old (SD = 11.9) and a median age of 76.0 with an interquartile range (IQR) of 68.0 to 84.5 (full age range = 50.0–100.0). All participants had GCS scores of 14 (n = 6; 5.0%) or 15 (n = 115; 95.0%) in the ED. The mean time to blood sampling was 3.4 h (SD = 2.1; Md = 2.9, IQR = 1.9–4.5, range = 0.5–11.7). The injury characteristics of the total sample and imaging findings are presented in **Table 1**. Intracranial abnormalities were identified in 18.2% (n = 22) of the total sample and 23.9% of those who underwent head CT (n = 92). The imaging findings for those who underwent head CT were as follows: skull fracture (n = 2; 2.2%), extra-axial hematoma (n = 18; 19.6%), acute subdural hematoma (n = 10; 10.9%), traumatic subarachnoid hemorrhage (n = 9; 9.8%), intraventricular hemorrhage (n = 1; 1.1%), midline shift (supratentorial) (n = 2; 2.2%), contusion (n = 4; 4.3%), and traumatic axonal injury (n = 3; 3.3%). No patient had an isolated skull fracture.

Participants were divided into subgroups based on head CT findings, including positive head CT (n = 22; 40.9% men; M = 81.1 years old, SD = 9.4, IQR = 72.8–89.0, range = 61.0–96.0), negative head CT (n = 70; 47.1% men; M = 75.1 years old, SD = 12.2, IQR = 66.8–84.3, range = 50.0–100.0), and head CT not conducted (n = 29; 69.0% men; M = 70.4 years old, SD = 11.1, IQR = 61.0–79.0, range = 50.0–91.0). Participants were also divided into subgroups based on GOS-E into good outcome (n = 31; 58.1% men; M = 71.3 years old, SD = 11.0, IQR = 66.0–78.0, range = 50.0–91.0) and poor outcome (n = 76; 46.1% men; M = 76.5 years old, SD = 11.7, IQR = 70.3–85.8, range = 50.0–96.0). The distribution of GOS-E scores for the entire sample was as follows: 1: n = 0, 0%; 2: n = 1, 0.8%; 3: n = 17, 14.3%; 4: n = 28, 23.5%; 5: n = 2, 1.7%; 6: n = 6, 5.0%; 7: n = 22, 18.5%; 8: n = 31, 26.1%; and missing: n = 12, 10.1%. Two participants sustained repeat head injuries within 1 week of their initial presentation to the ED and were excluded from the 1-week functional outcome analyses.

Findings in the Total Sample

Descriptive statistics for serum and plasma GFAP concentrations in the total sample are presented in **Table 2**. GFAP concentration values were considerably greater in plasma than in serum for the total sample (z = 0.42, p < 0.001, r = 0.42, medium to large effect size). Serum and plasma GFAP concentrations in the total sample were highly correlated, but the values were not redundant (rho = 0.886).

A Bland-Altman plot is presented in **Figure 1**, with the circles illustrating the differences between the plasma minus serum values of GFAP (N = 121; M = 138.9, 95% CI = 47.3–230.6; p < 0.004; lower limit = −859.1, 95% CI = −1,016.2 to −702.0; upper limit = 1,137.0, 95% CI = 979.9–1,294.0; Coefficient of Repeatability = 1,030.5, 95% CI = 915.4–1,179.0). The horizontal dotted lines represent the limits of agreement, in this case defined as the mean difference between plasma and serum GFAP values plus and minus 1.96 times the SD of the differences. The line of equality is the dotted horizontal line at 0.0. The mean difference between the two methods is the solid black line (138.9), and the error bars for that line represent the 95% confidence interval for the mean difference. Because the 95% confidence interval does not overlap the line of equality (0.0), there is a systematic difference between plasma and serum GFAP.

A scatter diagram with the regression line and confidence bands is presented in the upper part (part A) of **Figure 2**. The Passing and Bablock regression equation is $y = -31.72 + 1.30x$; the 95% confidence interval for the intercept value (−31.72) is −50.89 to 7.63 and for the slope (1.30) is 1.10–1.44, revealing a proportional difference between plasma and serum GFAP. The Cusum test, used to evaluate how well a linear model fits the data, revealed no significant deviation from linearity (p = 0.49). The residual plot in the lower part (part B) of **Figure 2** presents the distribution of differences between predicted values and observed values about the fitted regression line.

In the total sample, 64.5% of patients had higher plasma than serum concentrations. We calculated a difference score between plasma and serum GFAP concentrations by subtracting serum

TABLE 2 | Comparison of GFAP concentrations in serum and plasma in the total sample and head CT subgroups.

	Total sample ($N = 121$)		No head CT ($n = 29$)		Normal head CT ($n = 70$)		Abnormal head CT ($n = 22$)	
	Plasma	**Serum**	**Plasma**	**Serum**	**Plasma**	**Serum**	**Plasma**	**Serum**
Descriptive statistics (pg/mL)								
Mean	635.9	497.0	400.3	401.4	459.1	344.5	1509.2	1108.2
Median	326.8	272.1	207.3	217.1	315.9	263.0	928.6	787.8
Standard deviation	972.6	700.5	790.1	867.2	535.7	412.4	1627.8	872.3
Interquartile range	189.9–595.1	157.2–503.3	137.1–337.0	122.3–302.1	176.3–564.9	147.8–433.5	411.0–2035.6	353.5–2096.6
Range	52.2–6838.6	32.6–4813.8	52.2–4420.0	32.6–4813.8	63.4–4027.7	48.7–3382.4	139.6–6838.6	114.4–2640.7
Difference score (plasma – serum)								
Mean	138.9		−1.1		114.6		401.0	
Median	28.0		0.65		37.7		230.1	
Standard deviation	509.2		90.7		237.9		1088.0	
Interquartile range	−15.5 to 140.4		−21.6 to 39.6		−9.0 to 144.4		−54.7 to 612.0	
Range	−1612.0 to 4197.8		−393.8 to 129.4		−118.6 to 1328.7		−1612.0 to 4197.83	
Percentage with plasma > serum	64.5%		55.2%		68.6%		63.6%	
Group comparisons								
Wilcoxson signed ranks test (z)	4.62 ($p < 0.001$)		0.75 ($p = 0.456$)		4.46 ($p < 0.001$)		2.09 ($p = 0.036$)	
Effect sizes (r)	0.42		0.14		0.53		0.45	
Effect sizes (Cohen's d)	0.16		0.001		0.24		0.31	
Spearman correlations								
Age and GFAP level	0.495**	0.540**	0.489**	0.540**	0.508**	0.631**	−0.028	−0.061
Time to blood sampling	0.243**	0.206*	0.315	0.308	0.158	0.074	0.455*	0.387
Values from two assays	0.886**		0.907**		0.862**		0.826**	

*$p < 0.05$ and **$p < 0.01$; CT, computed tomography; GFAP, glial fibrillary acidic protein.

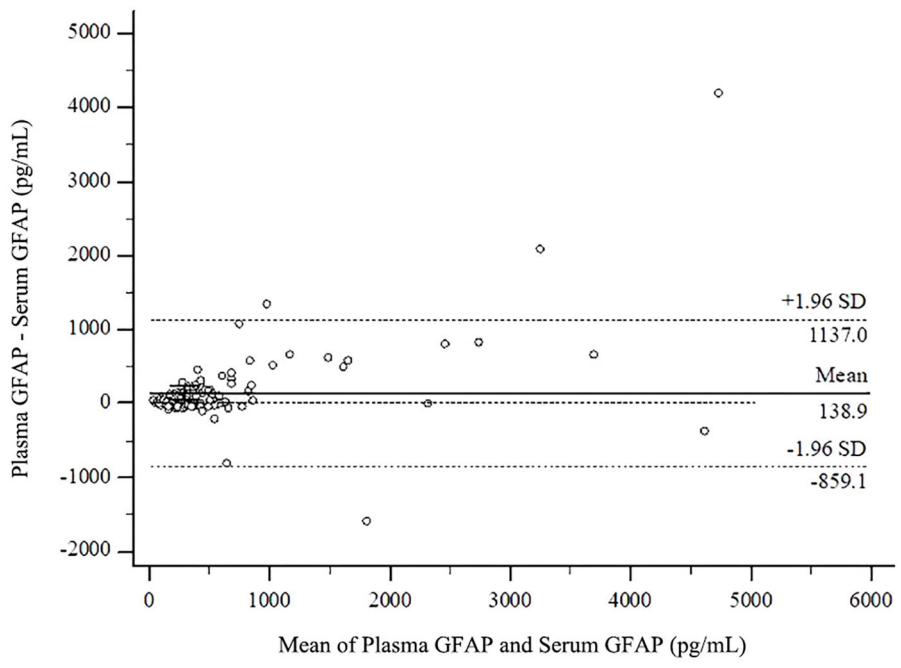

FIGURE 1 | Bland-Altman plot comparing plasma and serum values in the total sample. GFAP, glial fibrillary acidic protein.

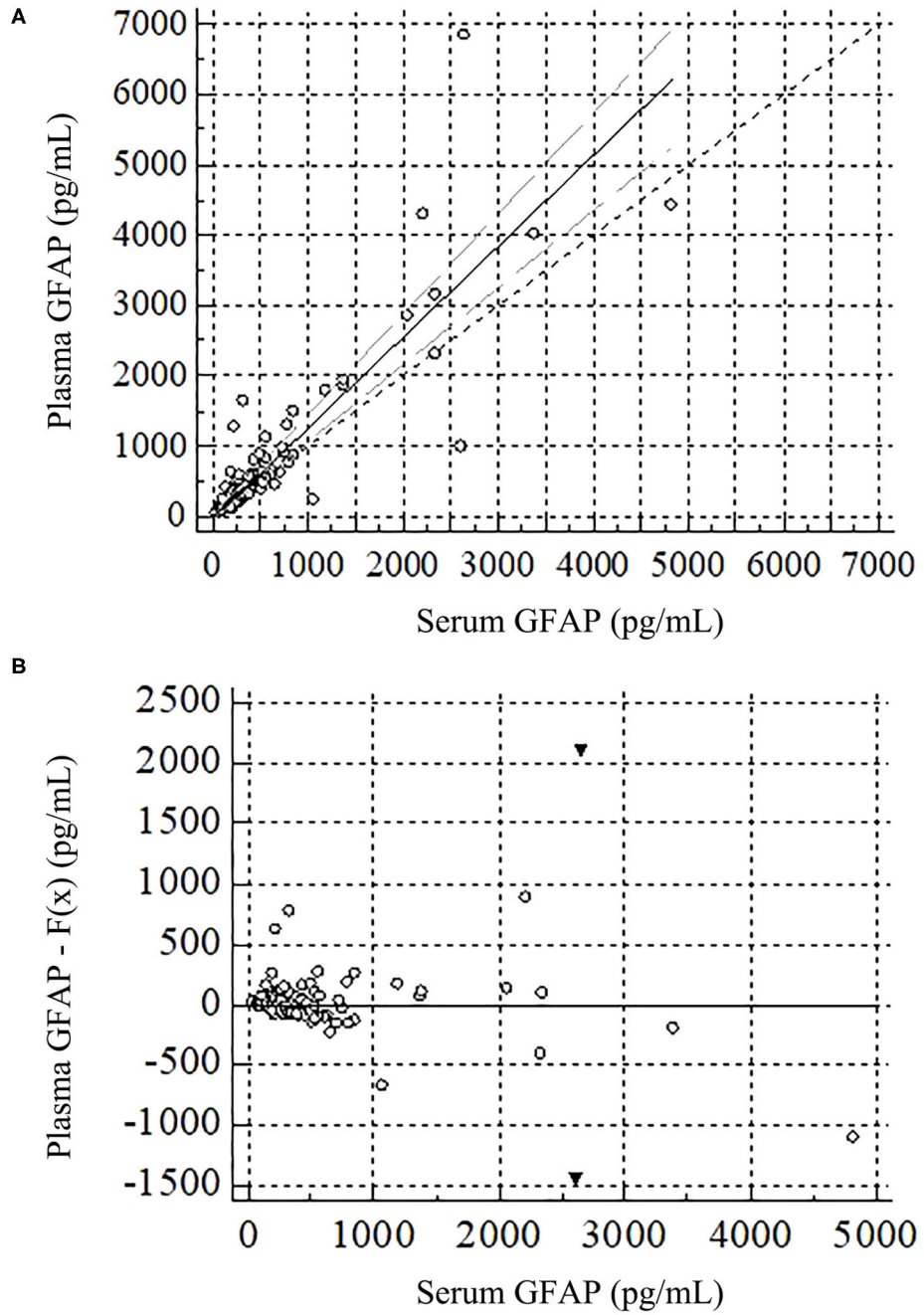

FIGURE 2 | Passing and Bablock regression for plasma and serum GFAP in the total sample. GFAP, glial fibrillary acidic protein. **(A)** Scatter diagram with regression line, confidence intervals, and diagonal line is in the upper figure. **(B)** This residuals plot, in the lower figure, presents the distribution of difference around the fitted regression line, allowing a visual evaluation of the goodness of fit of the linear model. The residuals are the differences between the predicted values and the observed values for the dependent variable. The black triangles represent two extreme values.

concentration from plasma concentration. The median difference score in the total sample was 28.0 pg/mL. Among difference scores, 17 outliers were identified, defined as GFAP difference scores that were >1.5 times the *IQR*. The characteristics of these participants are presented in **Table 3**. Twelve had abnormal head CT scans, 4 had normal head CT scans, and 1 did not undergo a head CT scan.

Findings by Head CT Group

Comparing Normal and Abnormal Head CT Groups

Serum and plasma GFAP concentrations were first compared *between* the subgroups with normal and abnormal head CT scans (see **Table 2** and **Figure 3**). Both serum and plasma GFAP levels were significantly higher in those with abnormal head CT scans compared to those with normal head CT scans (serum: $U =$

TABLE 3 | Characteristics of subjects who were outliers on their GFAP difference scores.

Age	Gender	Mechanism of injury	Injury to blood sampling (hours)	CT result	GOS-E outcome	Plasma GFAP (pg/mL)	Serum GFAP (pg/mL)	GFAP difference (plasma – serum)
80	Male	GLF	1.3	Abnormal	Poor	1,001.8	2,613.8	−1,612.0*
89	Female	GLF	2.9	Abnormal	Poor	243.5	1,064.1	−820.6*
75	Male	Sport	7.2	Not Done	Poor	4,420.0	4,813.8	−393.8
88	Female	GLF	4.5	Abnormal	Poor	886.4	492.6	393.8
92	Female	GLF	1.4	Abnormal	Poor	630.2	188.6	441.6
89	Female	GLF	4.8	Abnormal	Poor	1,863.0	1,372.2	490.7
81	Female	GLF	3.8	Normal	Poor	1,288.3	788.3	500.0
83	Female	Fall	5.3	Abnormal	Poor	1,941.2	1,382.7	558.5
77	Male	GLF	2.9	Abnormal	Poor	1,792.7	1,191.1	601.5
61	Male	GLF	5.1	Abnormal	Poor	1,490.1	846.6	643.4
89	Female	GLF	3.6	Normal	Poor	4,027.7	3,382.4	645.3
68	Female	Fall	3.3	Abnormal	Poor	2,857.2	2,057.9	799.3*
95	Male	GLF	5.9	Abnormal	Poor	3,152.8	2,341.0	811.8*
69	Male	GLF	3.1	Normal	Good	1,283.3	218.4	1,064.8*
78	Female	GLF	5.4	Normal	Poor	1,651.9	323.2	1,328.7*
72	Male	GLF	3.8	Abnormal	Poor	4,296.4	2,212.9	2,083.5*
89	Female	GLF	8.3	Abnormal	Poor	6,838.6	2,640.7	4,197.8*
						M = 2,333.2	*M* = 1,643.0	*M* = 690.3
						Md = 1,792.7	*Md* = 1,372.2	*Md* = 601.5

Outliers were defined as GFAP difference values that were >1.5 times the IQR. Extreme outliers (GFAP difference values >3 times the IQR) are denoted with an asterisk. CT, computed tomography; GFAP, glial fibrillary acidic protein; GLF, ground-level fall; GOS-E, Glasgow Outcome Scale-Extended.

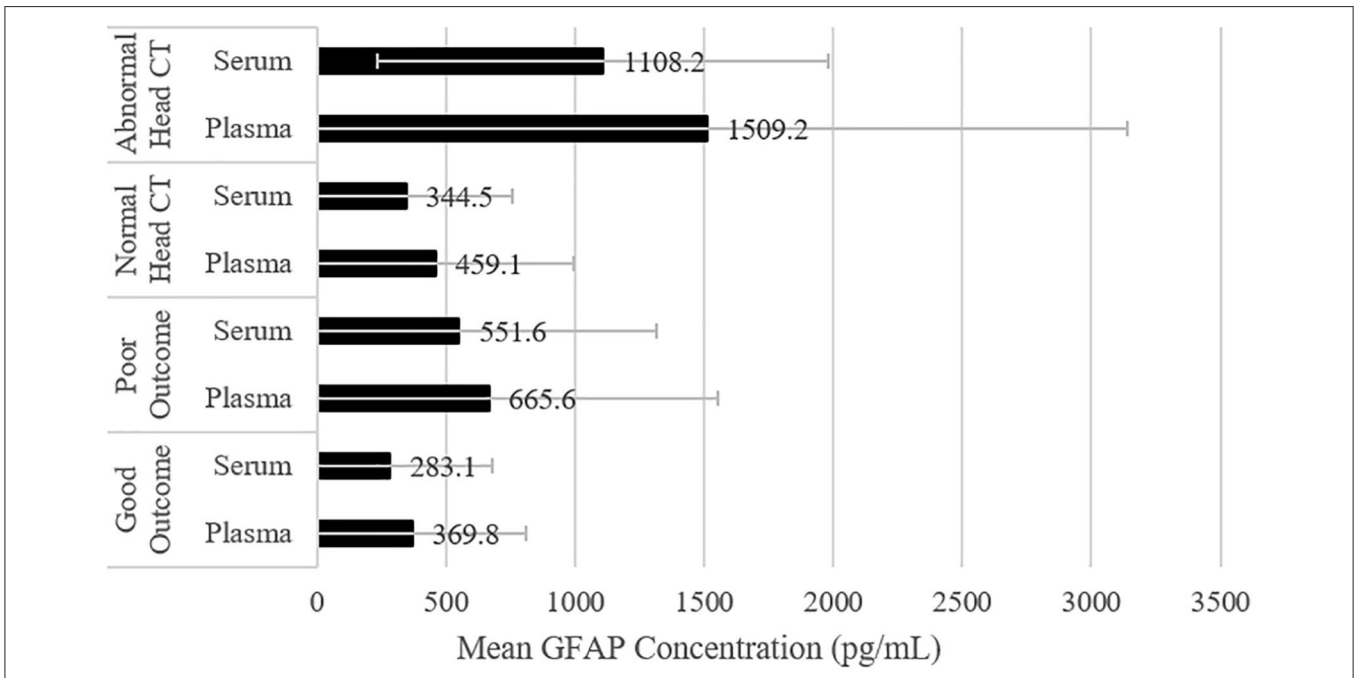

FIGURE 3 | Comparing plasma and serum GFAP levels among subgroups. The mean values are displayed, and the error bars represent one standard deviation. CT, Computed Tomography; GFAP, Glial Fibrillary Acidic Protein.

1,253, $p < 0.001$; plasma: $U = 1{,}198$, $p < 0.001$). ROC curves were computed for serum and plasma GFAP. GFAP levels in serum had slightly greater discriminability than GFAP levels in plasma for detecting intracranial lesions on head CT. The AUC for serum GFAP was 0.814 ($SE = 0.057$, 95% CI = 0.719–0.887, $p < 0.001$) and for plasma GFAP was 0.778 ($SE = 0.059$, 95% CI =

0.679–0.858, $p < 0.001$). The AUC for each fluid was within the 95% CI of the AUC for the other fluid, and a pairwise comparison of the ROC curves revealed no statistically significant difference ($z = 0.772$, $p = 0.440$).

Comparing Concentrations Within Subgroups

Serum and plasma GFAP concentrations were then compared *within* head CT subgroups. Stated differently, the concentrations from the two assays were compared for each subgroup. GFAP concentration values were considerably greater in plasma than in serum for the subgroups that had normal ($z = 4.46$, $p < 0.001$, $r = 0.53$, large effect size) and abnormal head CT scans ($z = 2.09$, $p = 0.036$, $r = 0.45$, medium to large effect size). There was no significant difference between GFAP concentration values in plasma and serum for the subgroup that did not undergo head CT ($z = 0.75$, $p = 0.456$, $r = 0.14$, small effect size). Descriptive statistics for GFAP concentration values and difference scores for the CT subgroups are presented in **Table 2**. GFAP concentration values for these subgroups are presented visually in **Figure 3**. The correlations (*rho*) between plasma and serum GFAP concentrations within all head CT subgroups were high, but not redundant, and ranged from 0.826 to 0.907. There were similar significant medium positive correlations between both serum and plasma GFAP and age in the subgroup that had normal head CT scans and the subgroup that did not undergo a head CT scan. However, there was no significant correlation between either serum or plasma GFAP and age in the subgroup with abnormal head CT scans (see **Table 2**).

Findings by Functional Outcome Group

Comparing Good and Poor Functional Outcome Groups

Serum and plasma GFAP concentrations were first compared *between* the subgroups with good and poor outcome (see **Table 4** and **Figure 3**). Both serum and plasma GFAP levels were significantly higher in those with poor compared to good outcome (serum: $U = 1{,}625$, $p = 0.002$; plasma: $U = 1{,}539$, $p = 0.013$). ROC curves were computed for serum and plasma GFAP. GFAP levels in serum yielded a slightly higher AUC than in plasma for differentiating between those with good vs. poor outcome; however, neither serum (AUC = 0.690, $SE = 0.054$, 95% CI = 0.584–0.796, $p = 0.002$) nor plasma GFAP (AUC = 0.653, $SE = 0.060$, 95% CI = 0.537–0.770, $p = 0.013$) met AUC cutoffs for acceptable discrimination between good and poor outcome groups. The AUC for each fluid was within the 95% CI of the AUC for the other fluid, and a pairwise comparison of the ROC curves revealed no statistically significant difference ($z = 1.02$, $p = 0.308$).

Comparing Concentrations Within Subgroups

Serum and plasma GFAP concentrations were then compared *within* functional outcome subgroups. Stated differently, the concentrations from the two assays were compared for each outcome subgroup. GFAP concentration values were considerably greater in plasma than in serum for subgroups with good ($z = 2.62$, $p = 0.009$, $r = 0.47$, medium to large effect size) and poor outcome ($z = 3.50$, $p < 0.001$, $r = 0.40$, medium to large effect size). Descriptive statistics for GFAP concentration values and difference scores for the outcome subgroups are presented in **Table 4**, and GFAP concentration values for these subgroups are presented visually in **Figure 3**. The correlations (*rho*) between serum and plasma GFAP concentrations in both the good (*rho* = 0.846) and poor (*rho* = 0.865) outcome subgroups were high, but not redundant. There were similar, significant, medium positive correlations between both serum and plasma GFAP and age in both outcome subgroups (see **Table 4**).

TABLE 4 | Comparison of GFAP concentrations in plasma vs. serum in outcome subgroups.

	Good outcome ($n = 31$)		Poor outcome ($n = 76$)	
	Plasma	**Serum**	**Plasma**	**Serum**
Descriptive statistics (pg/mL)				
Mean	369.8	283.1	665.6	551.6
Median	253.9	210.6	337.3	300.5
Standard deviation	441.9	397.5	887.0	761.6
Interquartile range	117.1–417.9	119.2–314.5	221.9–621.1	192.4–551.1
Range	63.4–2318.7	48.7–2331.8	52.2–4420.0	32.6–4813.8
Difference score (plasma – serum)				
Mean	86.7		113.9	
Median	25.5		38.5	
Standard deviation	205.4		408.7	
Interquartile range	−13.2 to 125.8		−26.0 to 168.2	
Range	−94.4 to 1,064.8		−1,612.0 to 2,083.5	
Percentage with plasma > serum	61.3%		67.1%	
Group comparisons				
Wilcoxson signed ranks test (z)	2.63 ($p = 0.009$)		3.50 ($p < 0.001$)	
Effect sizes (r)	0.47		0.40	
Effect sizes (Cohen's d)	0.21		0.14	
Spearman correlations				
Age and GFAP level	0.559**	0.756**	0.435**	0.406**
Time to blood sampling	0.095	0.196	0.222	0.128
Values from two assays	0.846**		0.865**	

***p < 0.01; outcome was defined based on the Glasgow Outcome Scale-Extended (GOS-E), which ranges from 1 to 8, with higher scores indicating better functional outcome. Good outcome was defined as a GOS-E score of 7 (lower good recovery) or 8 (upper good recovery). Poor outcome was defined as a GOS-E score of 6 or lower.*

DISCUSSION

Four Main Findings

This is the first study, to our knowledge, to compare serum and plasma levels of GFAP in a sample of older adults who sustained mTBIs. There were four main findings. First, plasma GFAP levels were significantly higher than serum GFAP levels in the total sample and nearly all subgroups. Second, serum and plasma GFAP levels were highly correlated, but not redundant, in the total sample and all subgroups. Third, GFAP levels measured in both serum and plasma were significantly higher in the subgroup with abnormal head CT scans compared to the subgroup without

findings on a head CT scan—with similar ability to discriminate between patients with and without intracranial abnormalities. Finally, GFAP levels in both serum and plasma were significantly higher in the subgroup with poor functional outcome compared to the subgroup with good functional outcome, but both had comparably poor ability to discriminate between those with good and poor functional outcome, warranting larger studies in the future to better determine if these biomarkers combined with others may be of greater value for predicting functional outcome in this population.

GFAP and Abnormal Head CTs

There is strong evidence that GFAP can be used as a biomarker for identifying people with abnormalities on head CT (1–6, 18, 25–27), and the FDA recently approved GFAP and UCH-L1 for this purpose in the ED setting (24). GFAP outperforms both UCHL-1 (4) and S100B (2) for discriminating between those with normal vs. abnormal head CT scans. In the present study, serum and plasma GFAP had a similar ability to discriminate between those with and without intracranial abnormalities (AUC serum = 0.814; AUC plasma = 0.778). The AUC values in the present study are fairly similar to those reported in other studies (1–6, 18, 25–27, 41) (range = 0.74–0.94) that have examined how effectively GFAP discriminates between patients with and without intracranial abnormalities following TBI (in samples of patients with predominantly mild injuries).

GFAP and Age

There is evidence that GFAP is elevated differentially in older adults following TBI (31, 32). In the present study, there were positive correlations between GFAP and age, in both serum and plasma, in the total sample and in nearly all subgroups. Thus, GFAP levels are greater with greater age. In most subgroups, the correlations between GFAP and age were modestly larger in serum than in plasma. One possible explanation is that fibrinogen and other clotting factors in plasma influence the correlation between GFAP and age (42). Interestingly, there was no correlation between GFAP levels and age in the subgroup with abnormal head CT scans. Past researchers examining GFAP levels following TBI (93.3% mild in severity) found that among those with intracranial abnormalities (i.e., CT positive), GFAP concentrations did not significantly differ between younger (<65 years old) and older (≥65 years old) adults (32). However, among the total sample and those with normal head CT scans, older adults had significantly higher median GFAP levels than younger adults (32). Therefore, it is possible that neurotrauma resulting in macroscopic intracranial abnormalities reduces or even obliterates the association between GFAP levels and age, although this is speculative and cannot be evaluated in the present study.

GFAP and Functional Outcome

There is modest evidence that GFAP is associated with functional outcomes following TBI (7–10). Prior studies have reported adequate ability (i.e., AUC ≥ 0.70) of GFAP for discriminating between those with favorable and unfavorable outcomes following TBI (4, 8, 27, 43). However, inclusion of patients exclusively with moderate-to-severe TBI (8) and less stringent definitions of good vs. poor outcome (e.g., GOS-E >4 = good outcome) (4, 27, 43) may have contributed to those findings. In the present study, we found that those with worse outcome had greater levels of GFAP in both serum and plasma. However, our AUC values were not significant, illustrating that GFAP could not adequately discriminate between these groups. Our study, however, had a very limited outcome assessment—we examined global outcome at 1-week post-injury. It remains unclear whether GFAP, in isolation, is clinically useful for predicting prognosis or functional outcome following mTBI. In a study of adults with mostly mild injuries, although GFAP was associated with functional outcome, GFAP did not predict unfavorable outcome in a multivariate regression model in which age, GCS score, and Marshall score were significant predictors (10). Biomarkers with differing cellular origins and temporal dynamics likely contribute differently to predicting recovery, and panels of several different biomarkers have been shown to improve outcome prediction following *severe* TBI compared to single biomarkers alone (44).

Plasma vs. Serum GFAP

The findings in this study for serum and plasma levels of GFAP were similar, but not identical. The levels were highly correlated, but not redundant, with most correlations ranging from 0.83 to 0.91 (**Table 2**). The reason for higher GFAP levels in plasma compared to serum is unclear. One possible explanation is that GFAP may become trapped in the fibrin-platelet matrix during clotting, which could account for lower levels of GFAP in serum compared to plasma (45, 46). GFAP levels in plasma and serum did not significantly differ for the subgroup that did not undergo head CT. It can be speculated that the trauma-induced coagulopathy was more severe in the patients who underwent CT scanning. Fibrin-platelet trapping is less likely in very mildly injured individuals, which could account for the lack of difference between serum and plasma GFAP levels in those who did not undergo head CT. In addition, the levels were measured using different assays, which could result in systematic bias of measurement, because the assays were not standardized to a common calibrator or a certified reference material. Different assays might also have different sensitivity to interferants that might be present in samples. The plasma samples in the present study were frozen and analyzed 1.5 years after the serum samples were analyzed, but the possible effect of this greater time in the freezer is thought to be negligible—especially because there was only one freeze-thaw cycle. There may also be matrix-related differences between plasma and serum of relevance to GFAP as a biomarker. Researchers examining a sample of adults with intracranial abnormalities following TBI (90% mild in severity) reported higher median GFAP levels in plasma than serum over 72 h after blood being drawn; however, the researchers did not statistically compare GFAP levels between fluids and the plasma and serum concentrations were nearly perfectly correlated (r = 0.994) (47).

Limitations

We ran the biomarker analyses in singlicates. This method does not allow us to account for variation within the assays. However, for both plasma and serum GFAP, two quality control samples were run in duplicates in the beginning and end of each run.

These quality control samples revealed low analytical variation for both assays and gave us reasonable confidence that running the samples in singlicates was appropriate.

Our study had small sample sizes in the abnormal head CT group and the group who did not undergo a head CT. When examining the two CT subgroups comparing plasma and serum levels, we had small sample sizes which reduces power. For the no head CT group ($n = 29$, $d = 0.001$), we had very low power (just 0.05 based on a *post-hoc* power analysis), but the low power is due to an extremely small effect size. As such, it is reasonable to accept the null hypothesis for this particular analysis. Considering the small effect, it is not likely that serum and plasma differ in this group. For the abnormal CT group ($n = 22$, $d = 0.31$), we observed a significant group difference, indicating that low power did not interfere with finding a significant effect for this analysis, despite a small sample size.

Given our small sample size of those with abnormal head CT scans, we were not able to further investigate why there was no significant correlation between GFAP and age in this subgroup. With a substantially larger sample of people with abnormal head CT scans, it would be possible to do a more careful analysis of the association between the specific types of abnormalities and lesion loads in relation to GFAP and age.

Conclusions

In conclusion, in a cohort of older adults following mTBI, GFAP levels were highly correlated in serum and plasma, and GFAP had similar ability to discriminate between individuals with and without intracranial abnormalities. Both plasma and serum GFAP levels had inadequate ability to discriminate between individuals with good and poor global functional outcome at 1-week following injury. Taken together, these findings suggest that the clinical impact of testing GFAP levels in plasma vs. serum is small. However, it is possible that the differences in fluid concentrations may have clinical significance in differential diagnosis between some patient groups in different clinical settings and are worthy of additional study. Plasma and serum GFAP were correlated with age in the total sample and all subgroups except the group with abnormal head CT scans. Additional research is needed to determine if neurotrauma resulting in intracranial lesions reduces or eliminates the correlation between GFAP and age.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (ethical code: R15045). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NH assisted with the literature review, the statistical analyses, writing of the manuscript, editing drafts of the manuscript, and he approved the final version for submission. TL was the principal investigator on the parent study, of which we used the data for secondary analyses. He contributed to the patient enrollment, literature search, reviewed drafts of the manuscript, and approved the final version for submission. JK assisted with the literature review and statistical analyses, edited drafts of the manuscript, and approved the final version for submission. KBe reviewed and coded the head CT scans, and approved the final version for submission. KBl and HZ co-supervised the laboratory in which the biomarker analyses were conducted and secured funding for the analyses, edited drafts of the manuscript, and approved the final version for submission. NA and JS assisted with running the biomarker analyses, edited the manuscript, and approved the final version for submission. JP and JG assisted with the literature review, edited drafts of the manuscript, and approved the final version for submission. GI conceptualized the study, conceptualized and ran the statistical analyses, wrote sections of the manuscript, and approved the final version for submission. All authors contributed to the article and approved the submitted version.

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Blunted Cardiac Parasympathetic Activation in Student Athletes With a Remote History of Concussion: A Pilot Study

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Introduction: Blunted cardiac autonomic nervous system (ANS) responses, quantified using heart rate variability (HRV), have been reported after sport-related concussion (SRC). Research suggests this persists beyond clinical recovery. This study compared cardiac parasympathetic responses in student athletes with a remote history of SRC (> 1-year ago, Concussion History: CH) with those who reported no lifetime history of SRC (Concussion Naïve: CN).

Design: Retrospective nested case-control.

Setting: University laboratory.

Patients or Other Participants: CH ($n = 9$, 18.3 ± 2 years, 44% male, median 2 years since injury) were student athletes with a remote history of concussion(s) from more than 1 year ago. CN ($n = 21$, 16.7 ± 3 years, 67% male) were student athletes with no lifetime history of concussion. Exclusion criteria included taking medications that could affect ANS function, history of concussion within the past year, persistent concussion symptoms, lifetime history of moderate to severe brain injury, and lifetime history of more than 3 concussions.

Material and Methods: Participants performed the Face Cooling (FC) test for 3-min after 10-min of supine rest while wearing a 3-lead electrocardiogram in a controlled environment.

Outcome Measures: Heart rate (HR), R-R interval (RRI), root mean square of the successive differences (RMSSD) of RRI, high frequency (HF) and low frequency to HF (LF:HF) ratios.

Results: At baseline, CH had a lower resting HR than CN (62.3 ± 11 bpm vs. 72.9 ± 12, $p = 0.034$). CH had a different HR response to FC than CN (+8.9% change from baseline in CH vs. −7.5% in CN, $p = 0.010$). CH also had a smaller RMSSD increase to FC than CN (+31.8% change from baseline in CH vs. +121.8% in CN, $p = 0.048$). There were no significant group differences over time in RRI ($p = 0.106$), HF ($p = 0.550$) or LF:HF ratio ($p = 0.053$).

Conclusion: Asymptomatic student athletes with a remote history of concussion had a blunted cardiac parasympathetic response to FC when compared with athletes with no lifetime history of concussion. These data suggest that an impaired autonomic response to a physiological stressor persists after clinical recovery from SRC for longer than previously reported.

Keywords: concussion, autonomic nervous system, sport, heart rate vaiability, face cooling

INTRODUCTION

Concussion, a subset of mild traumatic brain injury (mTBI), is a physiological, (1) metabolic, (2) and microstructural (3) insult to the brain resulting in non-specific somatic, cognitive, and emotional symptoms (4). There is no gold-standard method for diagnosing concussion, nor is there agreement on which measures need to normalize before beginning a return-to-play (RTP) strategy. The most recent International Concussion in Sport Group guidelines consider sport-related concussion (SRC) to be one of the most complex injuries in sports medicine to diagnose, assess, and manage; therefore, it is recommend that multi-modal clearance criteria be used for RTP decisions (5).

Emerging research indicates there is altered cardiovascular autonomic nervous system (ANS) function after concussion (6–9). Damage to the primary ANS control centers located in the brainstem following concussion has been confirmed by diffusion tensor imaging (10). Physiological research has shown that concussed subjects demonstrate reduced baroreflex sensitivity moving from supine to standing (11), and have reduced heart rate variability (HRV) at rest (12) and during exercise (12), which may reflect functional uncoupling of ANS control of cardiovascular function. Face Cooling (FC), i.e., cooling the forehead, eyes, and cheeks, stimulates the trigeminal nerve to evoke transient (∼1–2 min) increases in cardiac parasympathetic activity followed by sympathetically-mediated increases in blood pressure (13). In a prior study (14), concussed college-aged athletes within 10 days of injury demonstrated a blunted cardiac parasympathetic response to FC when compared with healthy controls (who did not report having a concussion within the past year). The concussed group also demonstrated lower sympathetically-mediated increases in blood pressure during FC. In this regard, we have also shown that recently concussed college athletes have blunted increases in heart rate (HR) and blood pressure during the cold pressor test, which is a sympathetic stressor (15). Collectively, these data indicate that both branches of the ANS do not respond properly to physiological stressors following a concussion.

Clinical recovery from a concussion tends to occur within ∼2 weeks in adults and by 3–4 weeks in adolescents (5). A recent systematic review, however, found that a myriad of physiological abnormalities are detectable for up to 1 month or more after the resolution of symptoms from concussion (16). It is not yet known when, or if, physiological function returns to baseline following a concussion, yet studies assessing the physiology of concussion recruit healthy controls with remote concussion histories. Additionally, a majority of studies have focused on sympathetic engagement (12, 17, 18). Hence, the purpose of this study was to determine the parasympathetic response to FC in asymptomatic student athletes with a remote history of concussion. For this, we retrospectively identified healthy student athletes who were initially recruited as healthy controls in previous studies (14, 19). The inclusion criteria for healthy controls in those studies included (1) not experiencing a concussion within the past year and (2) no more than 3 lifetime concussions. We separated these subjects into those who reported a remote history of concussion more than 1 year prior to testing (Concussion History: CH) and those who reported no lifetime history of concussion (Concussion Naïve: CN). We controlled for sex and age, which affect cardiac ANS tone (20). We hypothesized that CH participants would have a blunted cardiac parasympathetic response to FC vs. CN participants.

METHODS

This study was approved by the University at Buffalo IRB and conducted in accordance with the latest standards set forth by the Declaration of Helsinki. Athletic, healthy participants were recruited from local high school and college sport teams. The study was explained and consent was obtained. Parental consent/assent was obtained for all minors. On the day of the physiological assessment, participants completed a questionnaire that included demographics (including number of previous concussions), a Post-Concussion Symptom Scale (PCSS) (21), and current sport participation.

Participants

Participants in both groups had been recruited as healthy controls in previous studies (14, 19). CH were healthy male and female high school or college-aged athletes with a remote history of a concussion that occurred more than 1 year ago. CN were healthy male and female high school or college-aged athletes who reported never having experienced a concussion. Ages for both groups ranged from 13 to 24 years. Only physician (or relevant clinician)-diagnosed concussions were included. Participants were excluded if they (1) had a history of more than 3 lifetime concussions (because this is associated with persistent impairments) (22); (2) had a history of moderate or severe traumatic brain injury; (3) were currently on medications that would affect ANS function, e.g., mood disorder (tricyclic antidepressants) and/or learning disorder medications (methylphenidate, amphetamine), or beta-blockers; (4) did not participate in at least one organized sport; and (5) had a symptom severity score of more than 7/132 on the PCSS (23).

Experimental Approach

Participants were instructed to refrain from alcohol, caffeine, and exercise for 12 h and food for 2 h prior to their visit. Participants were instrumented with a 3-lead electrocardiogram (ECG) (DA100C, Biopac Systems, Goleta, CA) and assumed the supine position for 10 min in a quiet environment prior to FC. FC was performed by placing a pliable plastic bag filled with 2.5 L of ice water (∼0°C) on the forehead, eyes, and cheeks for 3 min. Room temperature was controlled and ranged from 20 to 23°C and humidity was controlled between 15 and 25%. Participants were allowed to end the test early if it became too uncomfortable. The FC test is based on mammalian diving reflex physiology (24), and the complete protocol and methods of data processing/analysis have been published previously (14). A variety of individual and environmental factors are known to affect HRV (25). These factors were assumed to affect each group equally and were not controlled for. This is discussed further in the limitations section.

Data and Statistical Analyses

ECG waveforms were analyzed using commercially available software (WinCPRS and Kubios HRV Software 5.0) with built-in tools for ECG clean-up, including a QRS detector, beat-to-beat analysis, and R-wave correction. ECG was visually inspected at the time of the experimental procedure and the first 5 min of supine rest was discarded. Baseline values were taken as the mean of minutes 6 and 7 of supine rest. HR, R-R interval (RRI), and root mean square of the successive differences (RMSSD) of RRI (26) were derived from the time domain while high frequency (HF) and the ratio of low frequency to HF (LF:HF) were derived from the frequency domain using Fast Fourier transformation (27). Mann-Whitney U-Test was used to test for group differences in age, height and weight. χ^2-test was used for group differences in sex. Mean values for HR, RRI, RMSSD, HF and LF:HF with 95% confidence intervals (CI) were calculated at baseline and during each minute of FC and compared using the Mann-Whitney U-Test. The HR, RRI, RMSSD, HF and LF:HF percent change from baseline with 95% CI were also calculated.

TABLE 1 | Participant demographics.

	Concussion History ($n = 9$)	Concussion Naïve ($n = 21$)	p-value
Age (years)	18.3 ± 2.4	16.7 ± 3.0	0.162
Sex	4 males (44%)	14 males (67%)	0.255
Height (cm)	171 ± 11	172 ± 8	0.774
Weight (kg)	68.8 ± 15	66.1 ± 18	0.702
Previous concussion			–
1	6	-	
2	2	-	
3	1	-	

Data are presented as mean ± standard deviations.

Mixed-models repeated measures ANCOVA with history of concussion as the grouping variable and sex (binary) and age (continuous) as covariates was used to assess for statistical differences in HR, RRI, RMSSD, HF and LF:HF change over time. Due to the pilot nature of this investigation, no *post-hoc* analysis for multiple comparisons was performed. A p-value of < 0.05 was considered significant. Statistical analyses were performed using SPSS Version 24 (Armonk, NY).

RESULTS

Thirty-four participants performed FC. Three participants did not complete all 3 min of FC and one participant's ECG had several artifacts on visual inspection and was discarded; hence, 30 participants were included in the analyses. Nine participants were categorized as CH and 21 participants as CN. CH's most recent concussion occurred a median of 2 years (interquartile range 1–3, range 1–8 years) prior to testing. Group demographics are presented in **Table 1**.

Absolute mean HR, RRI, RMSSD, HF and LF:HF at baseline and during each minute of FC are presented in **Table 2**. CH had lower HR at baseline and higher LF:HF ratio at minute 1 vs. CN. No other differences for absolute values were observed between groups.

HR percent change from baseline data are presented in **Figure 1**. There was a difference over time between groups ($p = 0.021$) that was not affected by sex ($p = 0.792$) or age ($p = 0.097$). At minute 1, CH had a mean change of +8.9% (−9.6, +27.4) while CN had a mean change of −7.5% (−13.3, −1.7). At minute 2, CH had a mean change of +15.0% (−8.0, +38.1) while CN had a mean change of −10.3% (−15.8, −4.7). At minute 3, CH had a mean change of +6.9% (−10.1, +24.4) while CN had a mean change of −8.3% (−12.6, −4.1).

RRI percent change from baseline data are presented in **Figure 2**. There was no difference over time between groups ($p = 0.161$) and there was no effect of sex ($p = 0.582$) or age ($p = 0.385$). At minute 1, CH had a mean change of +2.2% (−4.0, +8.4) while CN had a mean change of +11.4% (−10.5, +33.4). At minute 2, CH had a mean change of +7.7% (+2.0, +13.4) while CN had a mean change of +21.0% (−3.0, +44.9). At minute 3,

TABLE 2 | Absolute values for mean heart rate, RRI, RMSSD, HF, and LF:HF ratio during FC.

	Concussion History ($n = 9$)	Concussion Naïve ($n = 21$)	p-value
HR in beats per minute			
Baseline	62.3 ± 10.9	72.9 ± 12.2	**0.025**
Minute 1	66.6 ± 11.6	66.7 ± 10.9	0.689
Minute 2	70.1 ± 15.0	64.8 ± 10.9	0.209
Minute 3	65.2 ± 10.4	66.6 ± 12.4	0.859
RRI in milliseconds			
Baseline	908.9 ± 202	916.3 ± 71	0.926
Minute 1	916.5 ± 167	1016.1 ± 195	0.233
Minute 2	971.5 ± 198	1106.4 ± 234	0.168
Minute 3	940.4 ± 210	1050.0 ± 166	0.237
RMSSD in milliseconds			
Baseline	68.4 ± 36.4	73.9 ± 65.5	0.594
Minute 1	144.6 ± 108.0	151.7 ± 104.1	0.929
Minute 2	106.9 ± 78.1	171.8 ± 98.4	0.114
Minute 3	131.6 ± 50.4	134.6 ± 82.3	0.790
HF in milliseconds^2			
Baseline	1,491.1 ± 1,190	2,032.8 ± 2,446	0.910
Minute 1	12,417.6 ± 11,001	9,105.6 ± 10,814	0.213
Minute 2	5,633.5 ± 6,009	10,155.2 ± 9,184	0.263
Minute 3	6,056.9 ± 7,455	5,864.9 ± 5,612	0.965
LF:HF ratio			
Baseline	0.65 ± 0.40	0.99 ± 0.63	0.422
Minute 1	0.88 ± 0.41	0.62 ± 0.85	**0.050**
Minute 2	0.98 ± 0.84	0.51 ± 0.72	0.094
Minute 3	0.41 ± 0.21	0.58 ± 0.46	0.594

Data are presented as mean ± standard deviations. Bold values indicate statistical significance.

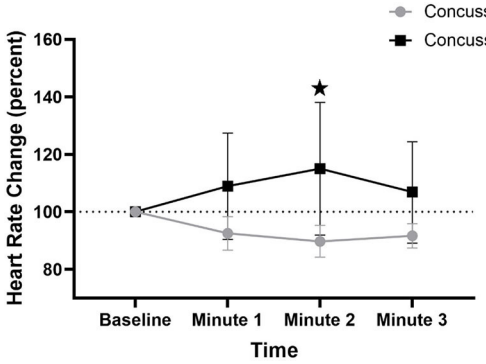

FIGURE 1 | HR percent change during FC with 95% CI. * indicating significant difference between groups on repeated measures.

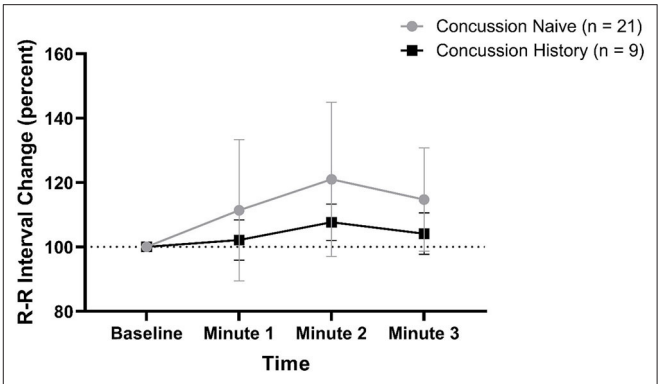

FIGURE 2 | RRI percent change during FC with 95% CI.

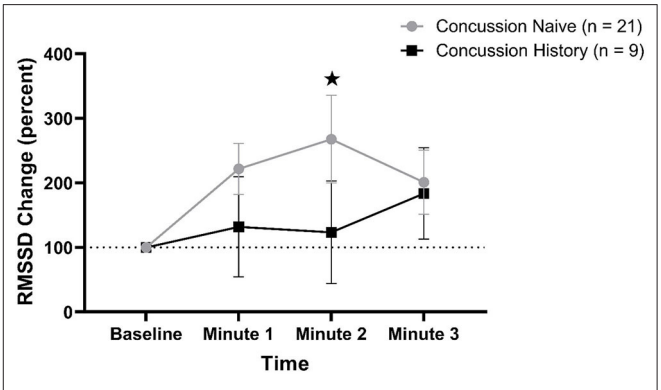

FIGURE 3 | RMSSD percent change during FC with 95% CI. * indicating significant difference between groups on repeated measures.

CH had a mean change of +4.2% (−2.3, +10.6) while CN had a mean change of +14.7% (−1.3, +30.8).

RMSSD percent change from baseline data are presented in **Figure 3**. There was a difference over time between groups ($p = 0.048$) that was not affected by sex ($p = 0.084$) or age ($p = 0.597$). At minute 1, CH had a mean change of +31.8% (−44.8, +109.4) while CN had a mean change of +121.8% (+82.1, +161.5). At minute 2, CH had a mean change of +23.4% (−56.1, +102.8) while CN had a mean change of +167.7% (+88.7, +235.5). At minute 3, CH had a mean change of +83.6% (+12.9, +154.2) while CN had a mean change of +100.9% (+51.2, +150.7).

HF percent change from baseline data are presented in **Figure 4**. There was no difference over time between groups ($p = 0.550$) and there was no effect of sex ($p = 0.275$) or age ($p = 0.660$). At minute 1, CH had a mean change of +1,177% (+221, +2,134) while CN had a mean change of +571% (+282, +861). At minute 2, CH had a mean change of +514% (+54, +975) while CN had a mean change of +1,217% (+286, +2,148). At minute 3, CH had a mean change of +1,019% (−500, +2,540) while CN had a mean change of +593% (+234, +952).

LF:HF percent change from baseline is presented in **Figure 5**. There was no difference over time between groups ($p = 0.062$) and there was no effect of sex ($p = 0.288$) or age ($p = 0.956$). At minute 1, CH had a mean change of +44.7% (−79.9, +169.3) while CN had a mean change of −18.5% (−75.2, +38.2). At minute 2, CH had a mean change of +72.0% (−116.9, +261.0) while CN had a mean change of −46.4%

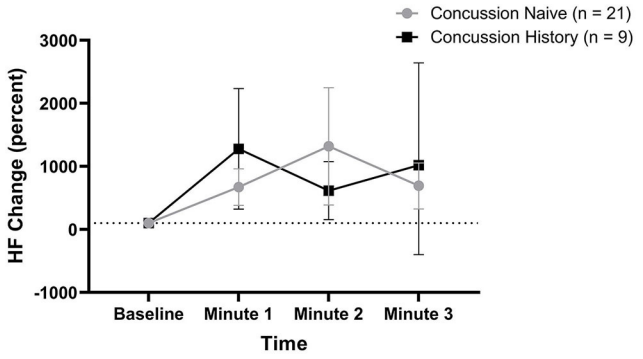

FIGURE 4 | HF percent change during FC with 95% CI.

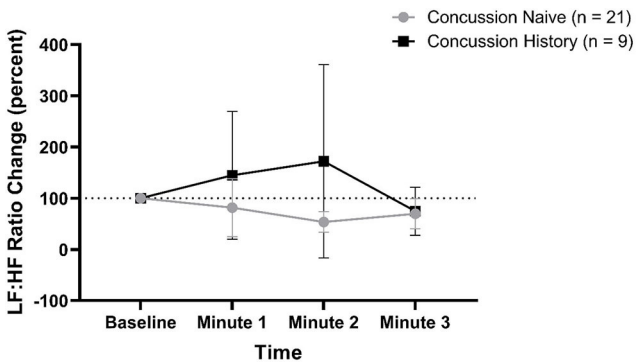

FIGURE 5 | LF:HF ratio percent change during FC with 95% CI.

(−66.5, −26.4). At minute 3, CH had a mean change of −25.4% (−73.3, +21.7) while CN had a mean change of −30.3% (−59.8, −0.8).

DISCUSSION

This pilot investigation shows that student athletes who reported experiencing a concussion more than a year ago had a blunted cardiac parasympathetic response to FC vs. student athletes who reported never having had a physician-diagnosed concussion. Participants without a history of concussion demonstrated the typical increase in RMSSD from baseline in response to stimulation of the trigeminal nerve with ice water, which is an indirect measure of cardiac parasympathetic activity (28). Participants who reported having a concussion more than a year ago, however, had a blunted response during the first 2 min of FC that was equivalent to the response we found in acutely (<10 days since injury) concussed college (14) and high school (19) athletes in previous studies. Heart rate in CH group increased from baseline to the end of minute 1 during FC, which is the opposite of the reduction in HR typically seen with cold stimulation of the trigeminal nerve. These data suggest that cardiac parasympathetic activity did not predominate during FC as it should in CH group, which supports the concept that athletes with prior concussions have difficulty "switching" to the appropriate branch of the ANS in response to environmental stimuli (29). CH had a significantly lower resting HR at baseline than CN yet had almost identical absolute HRs at minute 1 of FC; hence, the significant difference in change from baseline may reflect this difference in resting HR. Mean HR, however, continued to increase in CH from minutes 1 to 2 during FC whereas it declined in CN, which is consistent with normal parasympathetic function. HR change, LF:HF change and RMSSD change returned to normal by minute 3, which was expected due to engagement of the sympathetic response typically seen by minute 3 with continued FC (13). We did not identify significant changes in HF power between CN and CH. Although HF is thought to represent parasympathetic activity, it is commonly called the "respiratory frequency" because it corresponds to the HR variations related to the respiratory cycle (28). It is not considered, however, to provide additional information beyond time-domain measures (such as RMSSD) for vagal parasympathetic activity (27, 28). Since we did not collect respiratory data in this study, the HF data are difficult to interpret. Future studies in this realm should collect respiratory data to be able to interpret HF responses.

Other investigators have demonstrated persistent sympathetic ANS dysfunction in concussed athletes. Abaji et al. (17) reported that concussed patients in the post-acute to late phase after injury (mean 95 ± 63 days) had a blunted HRV response to isometric handgrip exercise (IHGE) vs. healthy controls. La Fountaine et al. (18) reported a blunted cardiac autonomic response during IHGE within 2 weeks after concussive head injury that was not present at rest. These studies may not, however, directly relate to our study since IHGE is a sympathetic stimulus that does not engage cardiac parasympathetic activity (30). Our data reveal that cardiac parasympathetic dysfunction persists for far longer beyond clinical recovery than previously shown in athletes after SRC.

Emerging research implicates ANS dysfunction as one cause of concussion signs and symptoms, including exercise intolerance (31), vision problems (32), and anxiety (33). Our data suggest, however, that the autonomic cardiac response to a stressor remains impaired in athletes with remote prior concussions who are not reporting any concussion-like symptoms at rest or during physical exertion. Our participants were doing well in school and playing organized sports without limitation. We must consider the possibility that the blunted autonomic response we measured over a year after recovering from a concussion is somehow mitigated by other mechanisms so that the body is able to function in this state without overt concussion-like symptoms or impairments. It is currently unclear if persistent autonomic dysfunction is a contributing factor to the increased susceptibility to repeat concussion in those who have had one or more concussions (34). Data from our study have relevance to future concussion research. Studies assessing physiological function after concussion may want to consider enrolling control participants with no lifetime history of concussion to reduce the possibility that previously concussed

(but currently asymptomatic) athletes have ongoing subclinical physiological dysfunction. This would aid researchers in developing diagnostic biomarkers with improved specificity for identifying acute concussion and for more objectively determining recovery.

LIMITATIONS

The major limitation of this study is the small sample size with unbalanced groups. This study was a retrospective analysis of participants who were recruited as healthy non-concussed controls in prospective case-control studies; hence, this convenience sample may not be representative of the general student-athlete population. We did not collect comprehensive details of previous concussions, such as loss of consciousness, recovery time or sport/activity. Future studies should obtain a detailed concussion history, including a history of concussion-like events that were never diagnosed by a physician/clinician, although it is recognized that self-reported concussion history may not be reliable (35). Future studies should also assess duration of participation and position played to explore a potential relationship between possible "sub-concussive" head impacts and ANS responses. Our study did not account for several variables that can affect HRV, including anxiety, variation in circadian rhythm patterns, sleep, endocrine factors and respiration (25). Future studies should attempt to control for these factors by either standardizing the time slept the night prior, performing the test soon after awakening or standardizing time since awakening to control for variation in circadian and endocrine cycles, measuring respiration and end-tidal CO_2 during the FC test, and performing more than one test to assess reliability. Finally, prospective longitudinal studies with multiple timepoints are needed to identify when, or if, impaired autonomic function returns to normal or baseline values after SRC.

CONCLUSION

Our data show that athletes with a remote history of concussion who are currently participating in sports and school without limitation have a blunted response to an effort-independent test of cardiac parasympathetic function. These results add to emerging data that physiological disturbances persist despite clinically-determined recovery from SRC. Our data suggest that cardiac autonomic dysfunction may persist for longer than expected. This has implications for the design of future concussion physiology studies, for susceptibility to repeat concussion, and potentially for finding a more objective determination of SRC recovery and readiness to return to sport.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University at Buffalo Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MH, BJ, JL, and BW contributed to study conception and design, interpretation of the results, statistical analysis, and manuscript writing. EH, CW, ER, MO'L, AB and LD contributed to participant enrollment, conducting experiments, data collection and preprocessing, and data quality assessment. All authors contributed to the article and approved the submitted version.

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Admission Levels of Interleukin 10 and Amyloid β 1–40 Improve the Outcome Prediction Performance of the Helsinki Computed Tomography Score in Traumatic Brain Injury

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Background: Blood biomarkers may enhance outcome prediction performance of head computed tomography scores in traumatic brain injury (TBI).

Objective: To investigate whether admission levels of eight different protein biomarkers can improve the outcome prediction performance of the Helsinki computed tomography score (HCTS) without clinical covariates in TBI.

Materials and methods: Eighty-two patients with computed tomography positive TBIs were included in this study. Plasma levels of β-amyloid isoforms 1–40 (Aβ40) and 1–42 (Aβ42), glial fibrillary acidic protein, heart fatty acid-binding protein, interleukin 10 (IL-10), neurofilament light, S100 calcium-binding protein B, and total tau were measured within 24 h from admission. The patients were divided into favorable (Glasgow Outcome Scale—Extended 5–8, $n = 49$) and unfavorable (Glasgow Outcome Scale—Extended 1–4, $n = 33$) groups. The outcome was assessed 6–12 months after injury. An optimal predictive panel was investigated with the sensitivity set at 90–100%.

Results: The HCTS alone yielded a sensitivity of 97.0% (95% CI: 90.9–100) and specificity of 22.4% (95% CI: 10.2–32.7) and partial area under the curve of the receiver operating characteristic of 2.5% (95% CI: 1.1–4.7), in discriminating patients with

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favorable and unfavorable outcomes. The threshold to detect a patient with unfavorable outcome was an HCTS > 1. The three best individually performing biomarkers in outcome prediction were Aβ40, Aβ42, and neurofilament light. The optimal panel included IL-10, Aβ40, and the HCTS reaching a partial area under the curve of the receiver operating characteristic of 3.4% (95% CI: 1.7–6.2) with a sensitivity of 90.9% (95% CI: 81.8–100) and specificity of 59.2% (95% CI: 40.8–69.4).

Conclusion: Admission plasma levels of IL-10 and Aβ40 significantly improve the prognostication ability of the HCTS after TBI.

Keywords: traumatic brain injury, biomarkers, outcome prediction, Helsinki CT score, interleukin 10 (IL10), beta amyloid 1–40, panel analysis

INTRODUCTION

Traumatic brain injury (TBI) is a highly heterogeneous disease (1) and a leading cause of long-term disability globally (2). It is clear that outcome after TBI solely does not depend only on the given care in the acute and late phases, but also on the injury type and severity, patient's clinical characteristics, and eventual brain tissue fate (3, 4). Improved outcome models may help better stratify patients for different treatment and monitoring strategies and provide information about expected gross outcomes to clinicians, patients, and their families.

TBI is classically divided into mild, moderate, and severe based on the initial assessment using the Glasgow Coma Scale (GCS) score upon admission (5). The GCS score is one of the strongest clinical outcome predictors (3) but does not consider the complex pathophysiological characteristics of TBI. Furthermore, GCS assessment may be confounded by subjective interrater variability and patient's intoxication or sedation (6, 7).

Early structural intracranial abnormalities detected on head computed tomography (CT) have been suggested as complementary or independent outcome predictors. The Marshall CT classification (8) was not originally designed to be an outcome measure tool, but its features have been successfully incorporated into the International Mission for Prognosis and Analysis of Clinical Trials in TBI (IMPACT) (9) and the Corticosteroid Randomization After Significant Head injury (10) prognostication models, which have been comprehensively validated (11). After the Marshall CT classification, outcome prediction-weighted CT classifications have emerged. Rotterdam CT score (12), Helsinki CT Score (HCTS), (13) and Stockholm CT score (14) have shown promise in prognostication of patients with CT-positive findings. The latter two reportedly provide more information on the structural pathology and more accurate outcome prediction than earlier models (15).

Several brain-enriched protein biomarkers have been studied in combination and isolation as tools for predicting TBIs of different severities (16–18). Biomarkers may offer incremental value in outcome prediction when used in combination with neuroimaging scores. We recently studied eight biomarkers [β-amyloid isoforms 1–40 [Aβ40] and 1–42 [Aβ42], glial fibrillary acidic protein [GFAP], heart fatty acid-binding protein [H-FABP], interleukin 10 [IL-10], neurofilament light chain [NF-L], S100 calcium-binding protein B [S100B], and total tau [t-tau]] and their ability to discriminate CT-negative and CT-positive patients with TBIs of different severities. We found that panels of biomarkers significantly outperformed individual biomarkers in this setting (19).

The overall aim of this study was to see whether the biomarkers listed earlier improved the prediction of outcome using an admission head CT score. As these biomarkers are of different cellular origins, we planned to investigate each separately as well as combined. The HCTS was chosen due to its ability to be reliably implemented, and it has an extensive validation background (15, 20–23). We hypothesized that the prognostic performance of the HCTS would improve after adding blood-based biomarkers.

METHODS

Study Population and Clinical Characteristics

This prospective study was part of the European Union-funded TBIcare (Evidence-Based Diagnostic and Treatment Planning Solution for Traumatic Brain Injuries) project, where we recruited patients with acute TBIs at the Turku University Hospital, Finland, from November 2011 to October 2013. All patients were treated according to the local protocols based on existing international guidelines and recommendations at that time (24).

The total available cohort of patients with head injury consisted of 620 patients. Of these, 203 patients met the following inclusion criteria: (i) age $\geq$ 18 years and (ii) clinical diagnosis of TBI and indications for acute head CT according to the National Institute for Health and Care Excellence criteria (25), and did not meet the following exclusion criteria: (i) blast-induced or penetrating injury, (ii) chronic subdural hematoma, (iii) inability to live independently due to a preexisting brain disease, (iv) TBI or suspected TBI not needing head CT, (v) more than 2 weeks from the injury, (vi) not living in the hospital district thereby preventing follow-up visits, (vii) not speaking the native language (Finnish), or (viii) no consent received.

In this study, we included those patients who had admission levels of plasma Aβ40, Aβ42, GFAP, H-FABP, IL-10, NF-L, S100B,

and t-tau obtained within 24 h after hospital admission available ($n = 160$). From these patients, we included those who had Glasgow Outcome Scale—Extended (GOSE) scores assessed 4–16 months after injury [assessed by an experienced neurologist [OT], $n = 137$, the average time between injury and GOSE was 7.82 months, ±3.33]. Outcomes were defined as favorable (GOSE 5–8), and unfavorable (GOSE 1–4), complete recovery (GOSE 8), and incomplete recovery (GOSE < 8) (17). Traditionally, the first categorization is used in terms of moderate to severe TBI and the latter in mild TBI. As the patients were not classified according to their initial GCS scores but according to their HCTS scores in the current study, we used both categorizations. The admission head CT scans were blindly evaluated by three senior neurotrauma researchers (neurosurgeons) as described later. The patients were divided into the main study cohort (CT-positive, $n = 82$, 60%) and comparison cohort (CT-negative, $n = 55$, 40%). Data on TBI-related deaths were collected up to 12 months after injury.

The GCS scores were assessed by paramedics at the scene of the accident or during transport and/or by an emergency physician at the time of admission. The lowest recorded post-resuscitation GCS was used in the demographic data (16, 26). Hypoxia was defined as any event of oxygen saturation of <90% and hypotension as any period of systolic blood pressure level of <100 mmHg in patients aged 50–69 years and <110 mmHg in patients aged 18–49 years and ≥70 years (24). Anemia was defined as a hemoglobin concentration of <100 g/L. Hypoglycemia was defined as a glucose level of <4.4 mmol/L. These thresholds were based on the latest international recommendation (24). Injury Severity Score (ISS) (27) was used to evaluate the overall injury load.

The ethical review board of the Hospital District of Southwest Finland approved the study protocol (decision 68/180/2011). All patients or their next of kin were informed about the study in both oral and written forms. Written informed consent was obtained according to the World Medical Association's Declaration of Helsinki.

Biomarker Analyses

Blood samples for plasma Aβ40, Aβ42, GFAP, H-FABP, IL-10, NF-L, S100B, and t-tau were drawn within 24 h from admission. Plasma H-FABP and IL-10 were analyzed using the K151HTD and K151QUD kits, respectively, from Meso Scale (Meso Scale Diagnostics, Rockville, MD, USA), and S100B was measured using EZHS100B-33K kit from Millipore (Millipore, Billerica, MA, USA) according to the manufacturers' recommendations in a research laboratory in Geneva, Switzerland. The plasma levels of GFAP, NF-L, and t-tau were assessed using the Human Neurology 4-Plex A assay on an HD-1 Single molecule array (Simoa) instrument according to the instructions from the manufacturer (Quanterix, Billerica, MA, USA) in the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden. Plasma Aβ40 and Aβ42 concentrations were measured using a duplex Simoa immunoassay (Quanterix, Billerica, MA, USA) in a research laboratory in Bethesda, MD, USA.

The lower limits of detection, the lower limits of quantification, and the calibration ranges for the blood-based biomarkers are shown in **Supplementary Table 1**. One patient had an S100B level below the lower limit of detection range, and therefore, the concentration of 1 pg/ml was applied, permitting statistical analysis. This applied concentration did not affect the statistics results. All biomarker measurements were performed by board-certified laboratory technicians who were blinded to clinical data.

Computed Tomography Scan Grading

Three senior neurotrauma researchers (JP, RR, and TL) evaluated 137 head CT scans and classified them according to the HCTS (13). First, two researchers (JP and RR) independently and blindly analyzed the scans and coded the findings, and the third (TL) evaluated the results. Next, the third evaluated all the scans, emphasizing the cases with conflicting results provided by the two independent researchers. Last, the cases with disagreement were assessed in a joint meeting.

Statistical Analysis

The normality of distribution of the biomarker levels was assessed with the Kolmogorov–Smirnov test and by visually inspecting histograms. The demographic data on age, sex, pupil reactivity, extracerebral injuries, events of hypoxia, events of hypotension, events of hypoglycemia, anemia, hospital admission/discharge, and outcome were normally distributed and are presented as mean ± standard deviation. Differences between groups were analyzed with *t*-tests. There were patients with missing data on pupil reactivity, events of hypoxia, hypotension, and hypoglycemia, and these were excluded from the comparative analysis. Data on GCS, ISS, (27), and HCTS sum are presented in medians and ranges. Differences between groups are analyzed with the Mann–Whitney *U*-test. The levels of the biomarkers were not normally distributed and are presented as medians with interquartile ranges (IQRs). Differences in biomarker levels between the two outcome groups were analyzed with the Mann–Whitney *U*-test.

The partial area under the curve (pAUC) of the receiver operating characteristic (ROC) was used to compare only a portion of the biomarkers AUC curves, which here was set to the clinically relevant range of 90–100% sensitivity. Panels were developed by the iterative combination of biomarkers and thresholds method using the Panelomix toolbox (28). For each biomarker, several cutoffs were selected, and the best combination of markers and thresholds was selected to give the best panel performance. The size of the panels was set to a maximum of (i) first two and then (ii) three covariates (from the pool of the biomarkers and the HCTS) and was evaluated when sensitivity was set at 90–100%. Hence, an optimal predictive panel means combining covariates that yields a set of the best specificity, sensitivity, and pAUC. $P < 0.05$ were considered significant.

The first round of the head CT scan review included reviews by RR and JP. The inter-rater reliability was assessed with Cohen's kappa statistic. The overall inter-rater reliability between

the three reviewers was assessed with the intraclass correlation coefficient (two-way mixed-effects).

Excluding the Panelomix toolbox analysis, the statistical analysis was carried out using the IBM SPSS Statistics version 25 (IBM Corp, New York).

RESULTS

Demographics, Computed Tomography Findings, Outcomes, and Blood Samples

The number of eligible patients was 137. Out of these, 82 patients (60%) were CT-positive, and 55 patients (40%) were CT-negative (**Figure 1**). The CT-positive patients constituted the main study group. Differences in baseline characteristics between CT-positive (main study group) and CT-negative patients (comparison study group) are shown in **Supplementary Material**. Briefly, patients in the CT-positive group were older (mean age 50 vs. 44 years), more often male (78 vs. 62%), had lower GCS scores (median 14 vs. 15), more often abnormal pupillary light reactions (15 vs. 4%), higher ISSs (median 18 vs. 6), and less frequently had a favorable outcome (60 vs. 93%) compared with patients in the CT-negative group. The main study group differed from the total potential head injury population ($n = 620$) only in terms of sex: in the main study group, 78% were males and in the total available cohort 71%.

In the CT-positive group, the mean age was 50.5 years (SD ±20.4), 78% were male, the median GCS score was 14 and 60% had a favorable outcome. The CT-positive patients with a favorable outcome were younger, had higher GCS scores, lower ISSs, and underwent less mass lesion evacuations compared with the CT-positive patients with an unfavorable outcome (**Tables 1**, **2**). In the CT-positive group, there were no differences in time elapsed between injury date and outcome assessment date when a patient had favorable and unfavorable outcomes ($p = 0.584$) and when complete and incomplete recovery ($p = 0.320$) were compared.

Utilizing the HCTS classification, the first two head CT scan reviewers reached a substantial agreement in terms of subdural hematoma, intracerebral hematoma, mass lesions (size > 25 cm^3), and intraventricular hemorrhage, whereas the agreement was moderate in terms of epidural hematoma and suprasellar cistern features as assessed according to Cohen (29) (**Supplementary Table 2**). The overall agreement reliability between the reviewers RR, JP, and TL was excellent in terms of subdural hematoma, intracerebral hematoma, mass lesions (size > 25 cm^3), and intraventricular hemorrhage, whereas the agreement reliability was good in terms of epidural hematoma and suprasellar cistern features as assessed according to Koo and Li (30) (**Supplementary Table 3**).

The blood samples of all the patients were obtained within 24 h from admission. In those patients for whom the exact time of injury was available, the time elapse from injury to blood sampling was 13.1 ± 10.4 h ($n = 62$). Among those patients in whom the exact injury time was unavailable, the time of injury was estimated based on the best available information. Among these patients, 26 patients were sampled within 24 h, and 49 patients were sampled after 24 h from the injury.

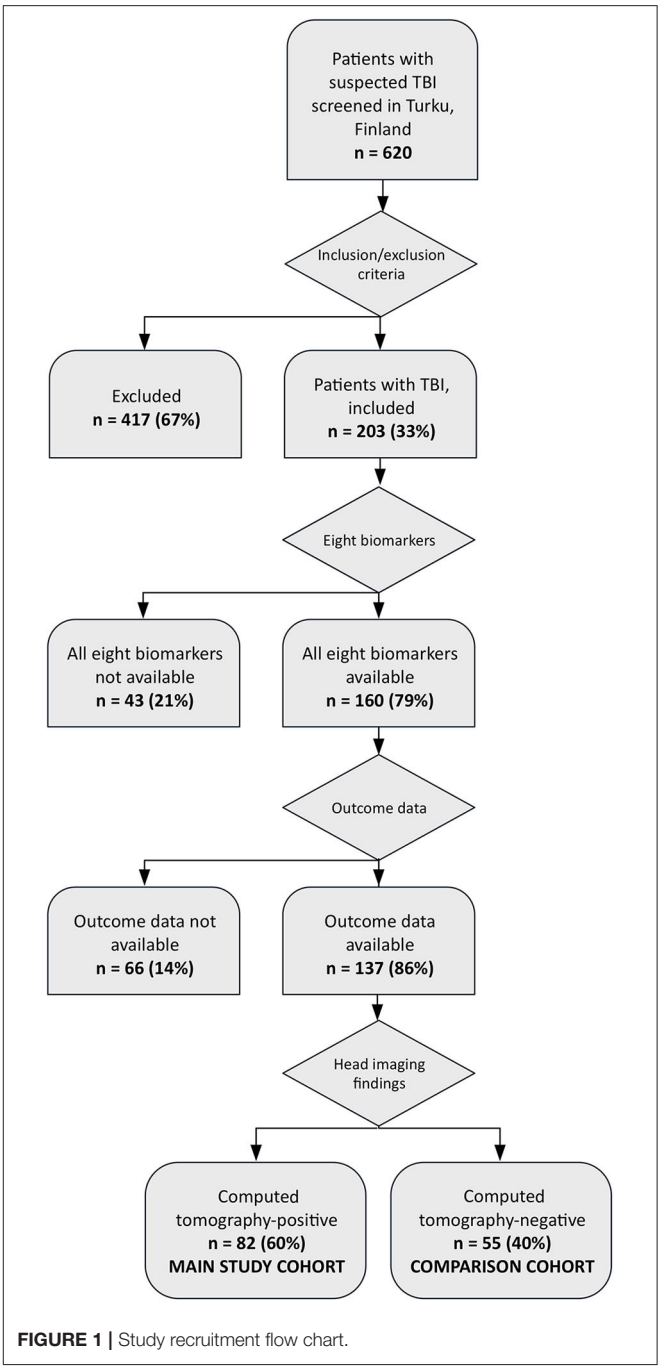

FIGURE 1 | Study recruitment flow chart.

The biomarker levels in different outcome groups are presented in **Supplementary Tables 4**, **5**.

Helsinki Computed Tomography Scale Alone in Outcome Prediction

The HCTS alone yielded a pAUC of the ROC of 2.5% (1.1–4.7) with a sensitivity of 97.0% (95% CI 90.9–100) and a specificity of 22.4% (95% CI 10.2–32.7) in detecting patients with unfavorable

TABLE 1 | Demographics of the whole study cohort—all patients.

Variable type	Variable		Main study cohort, CT-positive ($n = 82$)	Comparison study cohort, CT-negative ($n = 55$)	p-value
Demographic	Age (years, mean ± SD)		50.46 ± 20.35	43.67 ± 18.21	**0.048**
	Sex (male/female)		64 (78%)/18 (22%)	34 (62%)/21 (38%)	**0.039**
	GCS (median [range])		14 (3–15)	15 (3–15)	**0.043**
	Pupil reactivity	Unreactive/sluggish/reactive	9 (11%)/3 (4%)/61 (74%)[a]	1 (2%)/1 (2%)/52 (95%)[b]	**0.020**
	ISS (median [range])		18 (1–50)	6 (1–57)	**0.001**
	Isolated TBI		49 (60%)	32 (59%)	0.856
	Evacuated mass lesion		24 (29%)	0 (0%)	**<0.001**
	Hypoxia		6 (7%)[c]	1 (2%)[d]	0.203
	Hypotension		3 (4%)[e]	0 (0%)[f]	0.182
	Hypoglycemia		0 (0%)[g]	0 (0%)[f]	–
	Anemia		3 (4%)	0 (0%)	0.175
	Admitted to hospital		76 (93%)	33 (60%)	**<0.001**
	Outcome	Favorable (GOSE 5–8)	49 (60%)	51 (93%)	**<0.001**
		Unfavorable (GOSE 1–4)	33 (40%)	4 (7%)	**<0.001**
		Complete (GOSE 8)	10 (12%)	23 (42%)	**<0.001**
		Incomplete (GOSE 1–7)	72 (88%)	32 (58%)	**<0.001**
	TBI-related deaths		11 (12%)	1 (2%)	**<0.001**
HCTS	Mass lesion types	Subdural hematoma	53 (65%)	–	–
		Intracerebral hematoma	53 (65%)	–	–
		Epidural hematoma	11 (13%)	–	–
	Mass lesion size >25 cm^3		26 (32%)	–	–
	Intraventricular hemorrhage		21 (26%)	–	–
	Suprasellar cisterns	Normal	47 (57)	–	–
		Compressed	31 (38%)	–	–
		Obliterated	4 (5%)	–	–
	Sum (median [range])		4 (−3 to 14)	0	**<0.001**

Statistically significant p-values are in bold. SD, standard deviation; GCS, Glasgow Coma Scale; ISS, Injury Severity Score; Isolated TBI, traumatic brain injury without concomitant extracerebral injuries; Hypoxia, event of hypoxia after injury; Hypotension, event of hypotension after injury; Anemia, anemia after injury; TBI, traumatic brain injury; GOSE, Glasgow Outcome Scale—Extended; HCTS, Helsinki Computed Tomography Score; CT-positive, Computed tomography-positive; CT-negative, Computed tomography-negative.

[a] *Data missing on nine patients.*

[b] *Data missing on one patient.*

[c] *Data missing on seven patients.*

[d] *Data missing on 11 patients.*

[e] *Data missing on two patients.*

[f] *Data missing on eight patients.*

[g] *Data missing on three patients.*

outcome. The threshold to detect a patient with unfavorable outcome was an HCTS sum of >1 (**Table 3**). In terms of discriminating patients with complete recovery and incomplete recovery, the HCTS did not reach clinically relevant sensitivity and specificity (**Table 4**).

Biomarkers Alone in Outcome Prediction

In discriminating patients with favorable and unfavorable outcomes, the three best individually performing biomarkers in outcome prediction were Aβ40, Aβ42, and NF-L (**Table 3**). Patients with unfavorable outcome had significantly higher levels of Aβ42 (unfavorable outcome: median 21.9 pg/ml, IQR 40.6 pg/ml; favorable outcome: median 16.9 pg/mL, IQR 16.4 pg/ml; $p = 0.040$) and NF-L (unfavorable outcome: median 99.9 pg/ml, IQR 120.0 pg/ml; favorable outcome: median 36.9 pg/ml, IQR 57.6 pg/ml; $p = 0.001$) compared with those with favorable outcome, whereas levels of Aβ40 were not different between the groups ($p = 0.490$).

In terms of discriminating patients with complete and incomplete recovery, the three best individually performing biomarkers in outcome prediction were Aβ40, NF-L, and Aβ42 (**Table 4**).

Patients with incomplete recovery had significantly higher levels of NF-L (incomplete recovery: median 66.9 pg/ml, IQR 87.0 pg/ml; complete recovery: median 9.2 pg/ml, IQR 13.5 pg/ml; $p = 0.001$) compared with those with complete recovery, whereas levels of Aβ40 and Aβ42 were not different between the groups ($p = 0.436$ and $p = 0.257$, respectively).

TABLE 2 | Demographics of the main study cohort—Computed tomography-positive patients divided into patients with favorable outcome (Glasgow Outcome Scale—Extended 5–8) and unfavorable outcome (Glasgow Outcome Scale—Extended 1–4).

Variable type	Variable		Favorable outcome ($n = 49$)	Unfavorable outcome ($n = 33$)	*p*-value
Demographic	Age (years, mean ± SD)		44.69 ± 19.55	59.03 ± 18.67	**0.001**
	Sex (male/female)		37 (76%)/12 (24%)	27 (82%)/6 (18%)	0.505
	GCS (median [range])		14 (3–15)	9 (3–15)	**0.001**
	Pupil reactivity	Unreactive/sluggish/reactive	3 (6%)/1 (2%)/38 (78%)[a]	6 (18%)/2 (6%)/52 (70%)[b]	0.075
	ISS (median [range])		17 (1–41)	24 (6–50)	**0.001**
	Isolated TBI		29 (60%)	20 (61%)	0.889
	Evacuated mass lesion		12 (25%)	12 (36%)	**<0.001**
	Hypoxia		3 (6%)[c]	3 (9%)[d]	0.608
	Hypotension		2 (4%)	1 (3%)	0.813
	Hypoglycemia		0 (0%)[e]	0 (0%)[f]	–
	Anemia		2 (4%)	1 (3%)	0.800
	Admitted to hospital		44 (90%)	32 (98%)	0.226
	Outcome	Complete recovery (GOSE 8)	10 (20%)	0 (0%)	**0.005**
		Incomplete recovery (GOSE 1–7)	39 (80%)	33 (100%)	**0.005**
	TBI-related deaths		0 (0%)	11 (33%)	**0.001**
HCTS	Mass lesion types	Subdural hematoma	27 (55%)	26 (79%)	**0.028**
		Intracerebral hematoma	27 (55%)	26 (79%)	**0.028**
		Epidural hematoma	7 (14%)	4 (12%)	0.781
	Mass lesion size >25 cm^3		10 (20%)	16 (49%)	**0.007**
	Intraventricular hemorrhage		9 (18%)	12 (36%)	0.069
	Suprasellar cisterns	Normal	33 (67%)	14 (42%)	**<0.001**
		Compressed	13 (27%)	18 (54%)	**0.010**
		Obliterated	3 (6%)	1 (3%)	**0.031**
	Sum (median [range])		3 (−3 to 14)	5 (0–10)	**0.004**

Statistically significant p-values are in bold. SD, standard deviation; GCS, Glasgow Coma Scale; ISS, Injury Severity Score; Isolated TBI, traumatic brain injury without concomitant extracerebral injuries; Hypoxia, event of hypoxia after injury; Hypotension, event of hypotension after injury; Anemia, anemia after injury; TBI, traumatic brain injury; GOSE, Glasgow Outcome Scale—Extended; HCTS, Helsinki Computed Tomography Score.

[a] *Data missing on seven patients.*

[b] *Data missing on two patients.*

[c] *Data missing on four patients.*

[d] *Data missing on three patients.*

[e] *Data missing on two patients.*

[f] *Data missing on one patient.*

Biomarkers Improve the Outcome Predictive Performance of the Helsinki Computed Tomography Scale

We studied if combinations of biomarkers could improve the predictive performance of the HCTS in distinguishing patients with unfavorable outcome from patients with a favorable outcome. The best panel consisting of HCTS and a single biomarker included IL-10, and it yielded a pAUC of 3.0% (95% CI 1.3–6.0) with a sensitivity of 90.9% (95% CI 78.8–100) and a specificity of 55.1% (95% CI 40.8–69.4). In this panel, the threshold for the HCTS was >4 and for IL-10 <0.48 mg/ml (**Table 5A**, **Figure 2**). A corresponding analysis was conducted with HCTS and a combination of two biomarkers. The optimal panel included IL-10 and Aβ40, and it reached a pAUC of 3.4% (95% CI 1.7–6.2) with a sensitivity of 90.9% (95% CI 81.8–100) and a specificity of 59.2% (95% CI 40.8–69.4). In this panel, the threshold for the HCTS was >4, for Aβ40 >7.38 pg/ml, and for IL-10 <0.48 pg/ml (**Table 5B**, **Figure 3**).

Panel analysis for outcome prediction of incomplete recovery was not conducted because the HCTS did not have a clinically meaningful outcome prediction performance in this setting (**Table 4**).

Biomarkers in Outcome Prediction in Patients With Normal Head Computed Tomography Findings

To further elucidate the outcome prediction performance of the biomarkers, we also studied patients with CT-negative TBIs (comparison study cohort). The three best individually performing biomarkers in discriminating patients with a favorable outcome and an unfavorable outcome were Aβ40, GFAP, and NF-L (**Table 6**). The three best individually

TABLE 3 | Individual abilities of the Helsinki Computed Tomography Score and eight different biomarkers in discriminating patients with favorable and unfavorable outcomes sorted by partial area under the curve of the receiver operating characteristic (all, $n = 82$; favorable outcome, $n = 49$; unfavorable outcome, $n = 33$).

Biomarker	Threshold, pg/ml	% pAUC (95% CI)	% Specificity (95% CI)	% Sensitivity (95% CI)
HCTS	1	2.5 (1.1–4.7)	22.4 (10.2–32.7)	97.0 (90.9–100)
Aβ40	15.1	2.2 (0.9–4.1)	32.7 (18.4–46.9)	90.9 (81.8–100)
Aβ42	7.9	1.0 (0.1–2.7)	18.4 (8.2–30.6)	90.9 (78.8–100)
NF-L	179.6	0.6 (0.0–3.2)	22.4 (12.2–34.7)	90.9 (78.8–100)
H-FABP	56.3	0.6 (0.0–1.5)	6.1 (0.0–14.3)	100 (100–100)
t-tau	56.5	0.5 (0.0–3.0)	24.5 (12.2–36.7)	90.9 (78.8–100)
IL-10	13.9	0.3 (0.0–1.5)	8.2 (2.0–6.1)	93.9 (84.8–100)
S100B	2300.8	0.2 (0.0–1.5)	2.0 (0.0–6.1)	100 (100–100)
GFAP	94.7	0.1 (0.0–2.6)	12.2 (4.1–22.4)	90.9 (81.8–100)

Threshold indicates a value or level that needs to be exceeded to detect unfavorable outcome. pAUC, partial area under the curve of the receiver operating characteristic; HCTS, Helsinki Computed Tomography Score; Aβ40, β-Amyloid isoform 1–40; Aβ42, β-Amyloid isoform 1–42; GFAP, glial fibrillary acidic protein; H-FABP, heart fatty acid-binding protein; IL-10, interleukin 10; NF-L, neurofilament light; S100B, S100 calcium-binding protein B; t-tau, total tau.

TABLE 4 | Individual abilities of the Helsinki Computerized Tomography Score and eight different biomarkers in discriminating patients with complete and incomplete recovery sorted by partial area under the curve of the receiver operating characteristic (all, $n = 82$; complete recovery, $n = 10$; incomplete recovery, $n = 72$).

Biomarker	Threshold, pg/ml	% pAUC (95% CI)	% Specificity (95% CI)	% Sensitivity (95% CI)
Aβ40	35.0	2.3 (0.0–5.3)	40.0 (10.0–70.0)	90.3 (83.3–95.8)
NF-L	245.1	1.2 (0.0–4.1)	20.0 (0.0–50.0)	93.1 (86.1–98.6)
Aβ42	32.9	1.2 (0.0–4.1)	20.0 (0.0–50.0)	91.7 (84.7–97.2)
GFAP	113.9	1.0 (0.0–3.2)	22.4 (12.2–34.7)	94.4 (88.9–98.6)
H-FABP	56.4	0.7 (0.0–2.6)	10.0 (0.0–30.0)	97.2 (93.1–100)
HCTS	–	0.4 (0.0–2.3)	0.0 (0.0–0.0)	100 (100–100)
t-tau	–	0.3 (0.0–3.1)	20.0 (0.0–50.0)	90.3 (83.3–95.8)
IL-10	–	0.0 (0.0–0.0)	0.0 (0.0–0.0)	100 (100–100)
S100B	–	0.0 (0.0–1.4)	0.0 (0.0–0.0)	100 (100–100)

Threshold indicates a level that needs to be exceeded to detect incomplete recovery. pAUC, partial area under the curve of the receiver operating characteristic; HCTS, Helsinki Computerized Tomography Score; Aβ40, β-Amyloid isoform 1–40; Aβ42, β-Amyloid isoform 1–42; GFAP, glial fibrillary acidic protein; H-FABP, heart fatty acid-binding protein; IL-10, interleukin 10; NF-L, neurofilament light; S100B, S100 calcium-binding protein B; t-tau, total tau.

performing biomarkers in discriminating patients with complete and incomplete recovery were NF-L, Aβ40, and IL-10 (**Table 7**).

TABLE 5A | Ability of the Helsinki Computed Tomography Score alone and a panel consisting of the Helsinki Computed Tomography and interleukin 10 in distinguishing patients with unfavorable outcome from patients with favorable outcome.

	Markers (threshold to be classified as positive)	% pAUC (95% CI)	% Specificity (95% CI)	% Sensitivity (95% CI)
HCTS	HCTS (>1)	2.5 (1.2–4.6)	22.4 (12.2–34.7)	97.0 (90.9–100)
Panel	HCTS (>4) + IL-10 (<0.48 pg/ml)	3.0 (1.3–6.0)	55.1 (40.8–69.4)	90.9 (78.8–100)

Marker thresholds to detect patients with unfavorable outcome are presented in the second column. At least one marker needs to exceed the threshold in order for the panel to be positive. In the figure, a value before the parenthesis indicates that at least one marker needs to be positive (exceed the threshold) in the panel. Values in the parenthesis are the specificity and sensitivity of the panel.
HCTS, Helsinki Computerized Tomography Score; IL-10, interleukin 10.

DISCUSSION

This prospective, observational study of patients with acute TBI investigated whether admission levels of eight different plasma protein biomarkers obtained from CT-positive patients can improve the outcome prediction ability of the HCTS without clinical covariates in a well-characterized cohort. We also studied the prognostic ability of the biomarkers without the HCTS in discriminating complete recovery and incomplete recovery in CT-positive patients and CT-negative patients. The main finding of the study is that the admission levels of IL-10 and Aβ40 improve the ability of the HCTS in discriminating patients with unfavorable and favorable outcomes with increasing the specificity by 27% points (from 22 to 59%) while maintaining a sensitivity above 90%. In other words, when using only the HCTS, 11 patients out of the 49 with favorable outcomes were correctly detected, and when using the HCTS together with biomarkers, 29 patients with favorable outcomes were correctly detected. When studied alone, the HCTS had the highest pAUCs of the tested covariates, followed by Aβ40 and Aβ42. The individual specificities of the HCTS and biomarkers remained low (2–33%) in isolation, but the optimal combination panel yielded a specificity of 59% when the sensitivity was set above 90%.

Most modern TBI biomarker studies have investigated the individual prediction abilities of different molecules. The studies

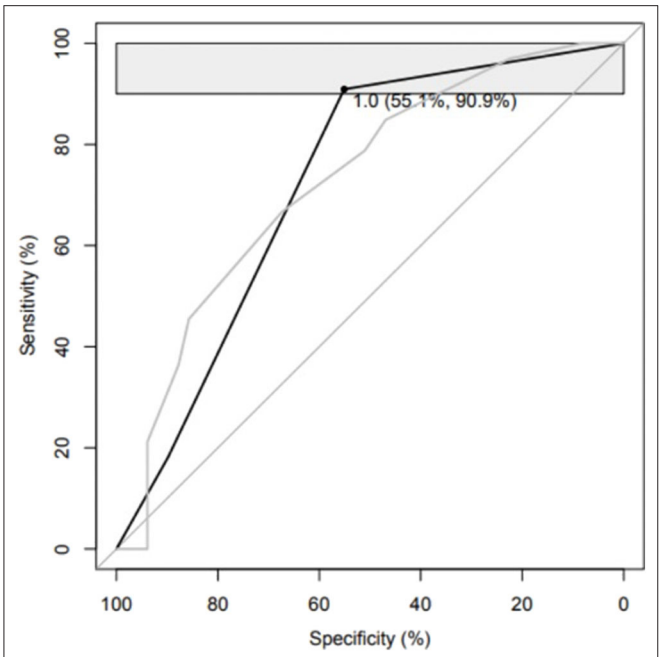

FIGURE 2 | Please see **Table 5A**.

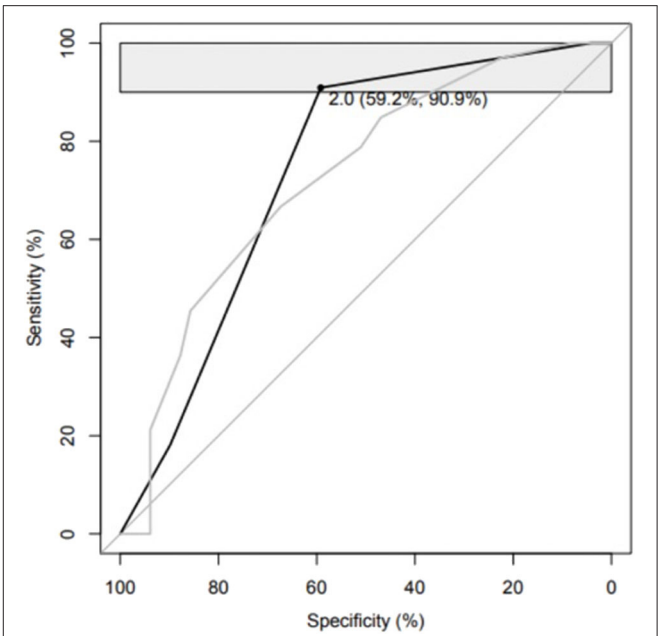

FIGURE 3 | Please see **Table 5B**.

TABLE 5B | Abilities of the Helsinki Computed Tomography Score alone and a panel consisting of the Helsinki Computed Tomography, interleukin 10, and β-Amyloid isoform 1–40 in distinguishing patients with unfavorable outcome from patients with favorable outcome.

	Markers (threshold to be classified as positive)	% pAUC (95% CI)	% Specificity (95% CI)	% Sensitivity (95% CI)
HCTS	HCTS (>1)	2.5 (1.2–4.6)	22.4 (12.2–34.7)	97.0 (90.9–100)
Panel	HCTS (>4) + IL-10 (<0.48 pg/ml) + Aβ40 (>7.38 pg/ml)	3.4 (1.7–6.2)	59.2 (44.9–71.4)	90.9 (78.8–100)

Marker thresholds to detect patients with unfavorable outcome are presented in the second column. At least two markers need to exceed the threshold in order for the panel to be positive. In the figure, a value before the parenthesis indicates that at least two markers need to be positive (exceed the threshold) in the panel. Values in the parenthesis are the specificity and sensitivity of the panel.
HCTS, Helsinki Computerized Tomography Score; IL-10, interleukin 10; Aβ40, β-Amyloid isoform 1–40.

TABLE 6 | Individual abilities of eight different biomarkers in discriminating patients with favorable and unfavorable outcomes without head imaging abnormalities sorted by partial area under the curve of the receiver operating characteristic (all, $n = 55$; favorable outcome, $n = 51$; unfavorable outcome, $n = 4$).

Biomarker	Threshold, pg/ml	% pAUC (95% CI)	% Specificity (95% CI)	% Sensitivity (95% CI)
Aβ40	16.7	5.1 (3.7–7.3)	51.0 (37.3–64.7)	100 (100–100)
GFAP	0.4	4.5 (3.3–8.0)	45.1 (31.4–58.8)	100 (100–100)
NF-L	8.3	4.1 (2.7–10.0)	41.2 (27.5–54.9)	100 (100–100)
t-tau	1.6	3.6 (2.4–7.5)	35.3 (21.6–49.0)	100 (100–100)
H-FABP	3.8	3.3 (2.2–8.0)	33.3 (19.6–47.1)	100 (100–100)
IL-10	0.2	2.4 (1.4–9.4)	23.5 (13.7–35.3)	100 (100–100)
S100B	45.3	1.4 (0.6–6.7)	13.7 (5.9–23.5)	100 (100–100)
Aβ42	–	0.0 (0.0–6.5)	0.0 (0.0–0.0)	100 (100–100)

Threshold indicates a level that needs to be exceeded to detect unfavorable recovery. pAUC, partial area under the curve of the receiver operating characteristic; Aβ40, β-Amyloid isoform 1–40; Aβ42, β-Amyloid isoform 1–42; GFAP, glial fibrillary acidic protein; H-FABP, heart fatty acid-binding protein; IL-10, interleukin 10; NF-, neurofilament light; S100B, S100 calcium-binding protein B; t-tau, total tau.

show that single biomarkers tend to have low specificities when sensitivity is set above 90%. Therefore, individual blood-based biomarkers may not be applicable for clinical practice as stand-alone tools (19, 28, 31), which is expected due to the complexity of TBI. Combining several biomarkers or combining biomarkers with clinical characteristics have been suggested to improve diagnostic and predictive abilities (31, 32). Thus, biomarkers may provide additional value in outcome prediction of TBI when used in combination with predictive neuroimaging scores. However, studies on blood-based biomarkers complementing head imaging scores are scarce. The results presented here suggest that protein biomarkers IL-10 and Aβ40 provide incremental value in outcome prediction when used in

TABLE 7 | Individual abilities of the eight different biomarkers in discriminating patients with complete and incomplete recovery without head imaging abnormalities sorted by partial area under the curve of the receiver operating characteristic (all, $n = 55$; complete recovery, $n = 32$; incomplete recovery, $n = 23$).

Biomarker	Threshold, pg/ml	% pAUC (95% CI)	% Specificity (95% CI)	% Sensitivity (95% CI)
NF-L	4.9	0.9 (0.0–2.9)	17.4 (4.3–34.8)	93.8 (84.4–100)
Aβ40	4.3	0.5 (0.0–2.6)	4.3 (0.0–13.0)	100 (100–100)
IL-10	8.0	0.4 (0.0–1.7)	4.3 (0.0–13.0)	100 (100–100)
GFAP	–	0.2 (0.0–1.3)	0.0 (0.0–0.0)	100 (100–100)
H-FABP	–	0.2 (0.0–2.8)	0.0 (0.0–0.0)	100 (100–100)
Aβ42	–	0.0 (0.0–2.4)	0.0 (0.0–0.0)	100 (100–100)
S100B	–	0.0 (0.0–1.1)	0.0 (0.0–0.0)	100 (100–100)
t-tau	–	0.0 (0.0–3.9)	0.0 (0.0–0.0)	100 (100–100)

Threshold indicates a level that needs to be exceeded to detect incomplete recovery. Statistically significant p-values are in bold. Mann U, Mann–Whitney U-test; pAUC, partial area under the curve of the receiver operating characteristic; Aβ40, β-Amyloid isoform 1–40; Aβ42, β-Amyloid isoform 1–42; GFAP, glial fibrillary acidic protein; H-FABP, heart fatty acid-binding protein; IL-10, interleukin 10; NF-L, neurofilament light; S100B, S100 calcium-binding protein B; t-tau, total tau.

combination with the HCTS. Intriguingly, in both panels in the panel analysis, the thresholds for IL-10 (many patients with lower GCS scores—indicating a more severe TBI—have relatively low levels of IL-10) and Aβ40 are considerably lower and for the HCTS higher compared to analyses where the parameters are studied in isolation. In line with this finding, it has been previously reported that most of the clinical studies have not identified a correlation between blood IL-10 levels and GCS scores (33). These results suggest that the best diagnostic value in discriminating patient outcomes after TBI is achieved by utilizing biomarkers in combination, which echoes our other recent findings in the acute diagnostics of TBI (19) and outcome prediction (34). A possible explanation for the higher HCTS threshold in the panel analysis is that biomarkers provide additional accuracy to the predictive power of the HCTS permitting patients with a favorable outcome to have some traumatic intracranial findings. We have recently reported IL-10 thresholds of 0.38 and 0.44 pg/ml depending on other markers included in the panels for predicting unfavorable outcomes. Correspondingly, when the HCTS is included in the panels, IL-10 thresholds need to be lower to capture patients with low IL-10 levels, low GCS scores, and unfavorable outcomes.

To better illuminate the predictive power of biomarkers in patients with CT-positive findings, we also investigated their abilities in distinguishing between patients with complete and incomplete recovery. The best-performing biomarkers were the same as in discrimination of patients with favorable and unfavorable outcomes, but the predictive performance of the HCTS was low. The HCTS was designed to predict functional outcome according to the GOS (13). Thus, unsurprisingly, the HCTS does not provide enough information to clinically meaningfully discriminate between patients with complete and incomplete recovery.

We also conducted a comparative analysis of CT-negative patients. In discriminating CT-negative patients with favorable and unfavorable outcomes, the best performing biomarkers were Aβ40, GFAP, and NF-L. However, these results should be interpreted with caution due to the small number of patients with unfavorable outcomes among CT-negative patients. In predicting a full recovery in CT-negative patients, only NF-L, Aβ40, and IL-10 showed a modest predictive power, whereas the other proteins did not have any prognostic value.

We utilized the pAUC instead of the conventional AUC test. The AUC indexes diagnostic performance summarizing the entire ROC curve, including regions that might not be relevant to a certain clinical application (e.g., regions with low levels of sensitivity or specificity). To overcome this disadvantage, we used the pAUC that summarizes a portion of the ROC curve over the prespecified range of interest (35)—in the context of the current study, sensitivity >90%. Thus, the pAUC yields more information regarding the predictive information provided by the HCTS and biomarkers than, for example, overall median value comparison using the Mann–Whitney *U*-test. This explains the finding that median levels of Aβ40 were not different between the favorable and unfavorable outcome groups, but the biomarkers still yield a good pAUC and specificity when studied in panels within a fixed sensitivity area. This also applies to the finding why Aβ40 and Aβ42 are not different between the complete and incomplete recovery groups.

Clinical features are known to contribute to explaining outcome variance (3). However, given the primary purpose of the current analysis was to explore the prognostic and diagnostic performance of the biomarker studied as an adjunct to CT imaging, they were not integrated into the overall prognostic models. In the main study cohort, there were no differences in sex distribution, pupil reactivity, events of hypoxia, events of hypotension, hypoglycemia, anemia, and the proportion of hospital admissions. Extracranial injuries may affect the levels of GFAP, H-FABP, IL-10, NF-L, S100B, and t-tau (18, 19, 26), but in terms of patient group comparisons in the main study cohort, this effect can be considered negligible because the proportion of patients with concomitant extracranial injuries was similar. Moreover, we have previously demonstrated that the levels of IL-10 and Aβ40—the proteins included in the outcome prediction panels in the current study—are not affected by the presence of extracranial injuries in patients with TBIs of all severities and CT-positive findings (19). The differences in the HCTS features reflect more serious lesion load in patients with unfavorable outcomes. The patients were also older in the unfavorable outcome group.

We studied several biomarkers that are known to be correlated with TBI prognosis, but we also selected biomarkers less

investigated in the literature due to their recent promising results in acute TBI diagnostics (19, 36, 37). Astroglial marker S100B is the most studied TBI biomarker to date (38–40). Acutely (12–36 h) measured blood S100B levels are associated with outcome (41). An earlier study reported that levels of S100B and GFAP in combination are correlated with unfavorable outcome in patients with severe TBI (42). S100B is expressed in many bodily tissues outside the central nervous system, and its levels increase, e.g., after extracranial injuries (43) and physical exercise (44), which may complicate interpretation of the results if the patient has significant extracranial injuries and if the levels are assessed in polytrauma patients immediately after injury (45, 46). After S100B, the astroglial marker GFAP, which is expressed in the cytoskeleton of glial cells (47), is probably the most studied TBI biomarker. Many studies have shown a significant association between increased GFAP levels and unfavorable outcome (16, 17, 42, 48). NF-L and tau have been mostly studied in the subacute after TBI. NF-L is abundantly expressed in the long myelinated subcortical axons (49). NF-L has been reported to be significantly correlated with late outcome after TBI by three studies (17, 50, 51). Tau is a microtubule-associated protein expressed in the axonal cytoskeleton (52, 53). Significant increases in tau levels have been reported in concussed professional ice hockey players (54), and tau levels have been correlated with outcome after severe TBI (55). Aβ40 and Aβ42 (52, 56) are associated with amyloidogenic amyloid precursor protein metabolism and have been suggested as potential biomarkers of axonal damage in TBI (57). However, it has been reported that especially in the case of mild TBI, Aβ40 and Aβ42 do not exhibit prognostic value (58–60). Cytosolic trafficking protein H-FABP and anti-inflammatory mediator protein IL-10 are related to traumatic intracranial findings (19, 36, 37). The outcome prediction ability of IL-10 after TBI has been controversial, although it has shown some potential in predicting mortality (33). However, a recent study utilizing partially same cohort as in this study demonstrated that both IL-10 and H-FABP improved outcome prediction abilities of panels consisting of more studied biomarkers and clinical covariates in both mild TBI and TBIs of all severities (34).

Previous studies suggest that biomarkers may perform in the outcome prediction of TBI better in combination than in isolation (50, 61, 62). Czeiter et al. (63) have reported that GFAP has an added value when combined with a modified IMPACT model consisting of age, GCS motor score, and pupil status. Both Gradisek and Vos have reported that GFAP and S100B improve the performance of clinical parameters in outcome prediction (61, 62). These findings are consistent with a recent study by Thelin et al. (18), where they reported that GFAP and NF-L enhanced the predictive ability of the IMPACT model combined with the Stockholm CT findings. With regard to current results, there was no benefit to combining HCTS, GFAP, and S100B with HCTS.

Currently, the most widely used CT scores are the Marshall CT classification and Rotterdam CT score. The Marshall CT classification grades injuries—in non-ordinal fashion—as different levels of diffuse injuries or mass lesions in case hematoma volume exceeds 25 cm^3 (8). Although the Marshall CT classification was not designed to be used as an outcome prediction tool, the Rotterdam CT score was developed based on the Marshall CT classification features adding traumatic subarachnoid and intraventricular hemorrhage (12). The most recent additions to the outcome prediction-weighted CT classifications are the HCTS and Stockholm CT score. The Stockholm CT score includes a separate traumatic subarachnoid hemorrhage score and a tally comprising midline shift as a continuous variable, epidural hematoma, dual-sided subdural hematoma, diffuse axonal injury, and the traumatic subarachnoid hemorrhage score (14). The HCTS focuses on the types of intracranial gross pathologies (13). It has been reported that the Stockholm CT score and HCTS outperform the older scores in outcome prediction (15). We chose the HCTS because its implementation is reliable, it is widely validated, and it takes into account different types of intracranial injuries that may be associated with differently elevated biomarker levels.

The strengths of this study are the use of several biomarkers of different cellular origins in the same cohort, the use of sensitive advanced analytics, and a prospectively recruited well-characterized study population. Although a minority of the screened patients were included in the current analyses, the patient selection did not introduce a significant bias, as the only difference was sex distribution.

The main limitation of the study is the variable delays between injuries and blood sampling. This may have affected biomarkers with a short half-life in blood, such as H-FABP, IL-10, and S100B. Furthermore, for NF-L, the sampling time-points might have been too close to the injury (64). Earlier mean sampling time would probably have resulted in different sensitivities and specificities for the panels. In addition, we could not use the levels of UCH-L1 from the Human Neurology 4-Plex assay in the current analyses because the coefficients of variation were at a level where the results are not reliable. We also used the National Institute for Health and Care Excellence criteria for head CT imaging, and the results might not be applicable for other head CT rules due to differences in case selection. The fairly small study cohort also increases the risk of over-fitting bias, and therefore, the results should be verified and validated in a larger cohort. Moreover, the assays utilized in this study are developed for research purposes, limiting the generalizability of the results. However, this limitation also concerns most of the current TBI biomarker studies because there is a paucity of commercialized assays for clinical TBI diagnostics. The possibility of some degree of selection bias should be noted, as only a third of the patients treated at the recruiting hospital were eventually enrolled in the study. The current cohort is somewhat less severely injured than those in which the HCTS has been earlier validated. The HCTS was originally designed using a neurocritical care cohort. Finally, these results specifically speak to the additional ability of the biomarkers studied to improve on the ability of the Helsinki CT Score to explain outcome variance. Integration

into well-established TBI outcome prediction schemes such as IMPACT (9) and Corticosteroid Randomization After Significant Head injury (10) will require further study. The authors acknowledge the limitations of the GOSE in detecting subtle functional and cognitive deficits, especially in patients with higher GOSE scores. However, the main aim of the study was slightly grosser in terms of prognostication, as we studied whether different protein biomarkers can improve the outcome prediction performance of the HCTS in discriminating patients with favorable and unfavorable outcomes. The variability in the time interval between injury and GOSE assessment may have affected the results.

CONCLUSION

Admission levels of IL-10 and Aβ40 improve the prognostic performance of the HCTS in discriminating patients with unfavorable and favorable outcomes. When studied alone, HCTS had the highest pAUCs of the tested covariates, followed by Aβ40 and Aβ42. Although the individual specificities of the HCTS and biomarkers remained low (2–33%) in isolation, the optimal combination panel yielded a specificity of 59% when the sensitivity was set above 90%. The current results suggest that outcome prediction ability of the HCTS could be significantly enhanced with rapid point-of-care measurement of plasma levels of IL-10 and Aβ40. This may allow the identification of initially neurologically stable patients who, however, are developing severe secondary brain injury that significantly impairs their recovery.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical review board of the Hospital District of Southwest Finland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JP, RT, RR, and TL conceived and designed the current study. JP, RT, AK, H-RM, JT, and OT recruited the patients. JP, RT, AK, MM, IH, H-RM, JT, PK, and OT collected and curated the data. LA, LL, J-CS, and JP conducted the statistical analyses. JG, HZ, KB, and J-CS supervised the biomarker analyses. MG, PH, DM, VN, and OT supervised the TBIcare study. JP drafted the manuscript with critical contributions from RT, RR, and TL. JP takes the responsibility for the paper as a whole. All authors substantially contributed to the revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.549527/full#supplementary-material

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Association Between Proteomic Blood Biomarkers and DTI/NODDI Metrics in Adolescent Football Players: A Pilot Study

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While neuroimaging and blood biomarker have been two of the most active areas of research in the neurotrauma community, these fields rarely intersect to delineate subconcussive brain injury. The aim of the study was to examine the association between diffusion MRI techniques [diffusion tensor imaging (DTI) and neurite orientation/dispersion density imaging (NODDI)] and brain-injury blood biomarker levels [tau, neurofilament-light (NfL), glial-fibrillary-acidic-protein (GFAP)] in high-school football players at their baseline, aiming to detect cumulative neuronal damage from prior seasons. Twenty-five football players were enrolled in the study. MRI measures and blood samples were obtained during preseason data collection. The whole-brain, tract-based spatial statistics was conducted for six diffusion metrics: fractional anisotropy (FA), mean diffusivity (MD), axial/radial diffusivity (AD, RD), neurite density index (NDI), and orientation dispersion index (ODI). Five players were ineligible for MRIs, and three serum samples were excluded due to hemolysis, resulting in 17 completed set of diffusion metrics and blood biomarker levels for association analysis. Our permutation-based regression model revealed that serum tau levels were significantly associated with MD and NDI in various axonal tracts; specifically, elevated serum tau levels correlated to elevated MD ($p = 0.0044$) and reduced NDI ($p = 0.016$) in the corpus callosum and surrounding white matter tracts (e.g., longitudinal fasciculus). Additionally, there was a negative association between NfL and ODI in the focal area of the longitudinal fasciculus. Our data suggest that high school football players may develop axonal microstructural abnormality in the corpus callosum and surrounding white matter tracts, such as longitudinal fasciculus. A future study is warranted to determine the longitudinal multimodal relationship in response to repetitive exposure to sports-related head impacts.

Keywords: brain injury, diffusion tensor imaging, neurite orientation dispersion and density imaging, blood biomarker, football, youth, concussion, subconcussion

INTRODUCTION

Concussive and subconcussive brain injury in sports have emerged as a complex public health issue. Policy and rule changes, as well as societal awareness, have played a catalytic role in decreasing concussion incidence in sports (1). However, despite decades of investigation, there is no concrete evidence on gold-standard diagnostic biomarkers for concussion, preventive tools that can increase neural resiliency to trauma, or factors contributing to the potential long-term consequence of subconcussive head-impact exposure. This knowledge gap is partly due to the unimodal approach, in which many papers report data derived from a single modality (e.g., neuroimaging, blood biomarker, behavioral measures). This precludes validation of study findings. For example, elevated tau protein in blood theoretically indicates axonal damage or degeneration, but without cross-referencing against neuroimaging data, the usefulness of the tau protein as a surrogate for brain damage remains speculative at best.

Several interdisciplinary groups have begun testing two- and three-way multimodal relationships that reflect subconcussive neuronal stress. In 2014, initial studies by Talavage et al. (2) and Bazarian et al. (3) revealed head impact-dependent declines in neural activation patterns and axonal microstructural integrity after a single high school and college football season, respectively. These neuroimaging findings were correlated with declined cognitive function (2, 4), and the development of autoimmune response to brain-derived blood biomarkers (e.g., ApoA1, S100B) (3, 5). Despite the unequivocal importance of the multimodal approach, association studies in subconcussion research are limited (6, 7).

Neuroimaging techniques, especially diffusion MRI, and brain-derived blood biomarkers are the fastest growing areas of neurotrauma research. Diffusion tensor imaging (DTI) is the most extensively used technique worldwide to examine the white matter microstructural properties in humans. However, DTI metrics such as mean diffusivity (MD) and fractional anisotropy (FA) represent basic statistical descriptions of diffusion that do not directly correspond to biophysical properties of neuronal axons (8). In 2012, Zhang et al. (9) introduced the neurite orientation and dispersion density imaging (NODDI) technique that can measure axonal density within white matter, dispersion of axonal orientation, and free water diffusion (see **Table 1** for descriptions of these metrics). The combined use of DTI and NODDI has been shown to detect progressive axonal degeneration even 6 months after a concussion (13). Similar to neuroimaging techniques, blood biomarker technology has evolved to be able to detect neural factors at a femtomolar concentration. Among the many potential biomarkers for brain injury, tau, neurofilament-light (NfL), and glial fibrillary acidic protein (GFAP) have shown their superior ability to predict concussion recovery time (30, 31), cumulative subconcussive axonal damage (26, 32, 33), and absence of intracranial bleeding (34–36). However, the relationships between DTI/NODDI metrics and blood biomarkers in reflecting cumulative neural stress from football head impacts have never been reported in the literature.

Therefore, we conducted a pilot, cross-sectional study in high school football players to examine the relationship between diffusion neuroimaging metrics and blood biomarkers at their preseason baseline, aiming to detect potential residual neuronal damage from prior football seasons. We hypothesized that there would be significant associations between neuroimaging and blood biomarkers to reflect axonal microstructural damage in some areas of the brains of football players. Specifically, tau and NfL levels will correlate with higher (worse) levels in DTI and NODDI metrics (e.g., FA, MD, NDI), whereas GFAP will not show a notable correlation with DTI and NODDI metrics.

MATERIALS AND METHODS

Participants

This single-site, cross-sectional study enrolled 25 male high school football athletes. None of the 25 participants was diagnosed with a concussion or traumatic brain injury in the 12 months prior to the enrollment. Inclusion criterion was being an active high school football team member. Exclusion criteria included a history of head and neck injury, including concussion within 12 months prior to the study or history of neurological disorders. However, participants were allowed to have a history of concussion if it was beyond 12 months prior to the study. Conditional exclusion criteria for the neuroimaging data collection were metal implants in the body or implanted electro/magnetic devices (e.g., orthodontic braces, pacemakers, aneurysm clips). The Indiana University Institutional Review Board approved the study, and all participants and their legal guardians gave written informed consent. The data were collected during the preseason baseline assessment in July 2019 and included self-reported demographic information (age, race/ethnicity, height, weight, number of previously diagnosed concussions, and years of tackle in American football experience), 7 mL of blood samples, and MRI scans.

Blood Biomarker Assessments

Seven-milliliter samples of venous blood were collected into red-cap serum vacutainer sterile tubes (BD Bioscience). Blood samples were allowed to clot at room temperature for a minimum of 30 min. Serum was separated by centrifugation (1,500 × g, 15 min) and stored at −80°C until analysis. Serum levels of tau, NfL, and GFAP were measured using the Simoa^{TM} Platform (Quanterix), a magnetic bead-based, digital enzyme-linked immunosorbent assay (ELISA) that allows detection of proteins at femtomolar concentrations (37). An analytical protocol was previously described in detail (38). The analyses were performed by a board-certified laboratory technician blinded to the study design and subject characteristics. Limit of detection was 0.024 pg/mL for tau, 0.104 pg/mL for NfL, and 0.221 pg/mL for GFAP. The average intra-assay coefficients of variation for the samples were 6.7 ± 5.2% for tau, 8.3 ± 6.0% for NfL, and 3.7 ± 2.7% for GFAP.

TABLE 1 | Summary of DTI/NODDI and blood biomarker characteristics.

Outcome metrics	Definition	Cause of increase	Cause of decrease
Fractional anisotropy (FA)	A precise assessment of white matter microstructure properties like myelination, packing density, axonal coherence, axonal size, and microstructural connectivity, which is characterized by the directionality of constrained water diffusion in the brain tissue (10–12).	- Increase found following repetitive subconcussive hits over long periods of time, conflating injury, and recovery effects (13). - Athletes found to have an elevation with a history of concussions, noting hindered water diffusion within white matter tracts (14).	- A lower level of FA observed 2 weeks post-injury may be due to an influx of water content as a response to neuroinflammation (13). - In cases of more severe TBI, a decrease of FA is generally noted as individuals display more clinical symptoms. The persistent microstructural changes noted with concussions may be more distinguishable from severe TBI (14).
Mean diffusivity (MD)	The average rate of molecular diffusion measured from all directions with the assumption cellular size and integrity play a role (15, 16).	- An increase following 2 weeks post-injury may be due to an influx of water content as a response to neuroinflammation (13). - Increase is observed in individuals who experience a more severe form of TBI (14).	- A decrease was noted in athletes with a history of concussion, due to a hampering of water diffusion within white matter tracts (14). - Following a concussion, during the post-injury phase, MD was noted to be decreased (17).
Axial diffusivity (AD)	The rate at which water molecules diffuse parallel to the tract within the voxel of interest (18).	- Following repetitive hit hockey season an increase in AD was noted (19).	- Decrease noted 24 h post-concussion (17).
Radial diffusivity (RD)	The immensity of water molecule diffusion occurring perpendicular to the tract within the voxel of interest (18).	- Following repetitive hit hockey season an increase in RD was noted (19).	- Decrease noted 24 h post-concussion (17).
Neurite density index (NDI)	The volume and density of neurites within intra-neurite space (20, 21).	- An increase is found after repetitive subconcussive hits over an extended period of time, prolonging injury and recovery effects (13).	- Decrease is observed over time in both initial and replication of mTBI suggesting progressive axonal degeneration (13). - A decrease is noted in NDI following a mTBI compared to orthopedic trauma controls and friend controls (13).
Orientation dispersion index (ODI)	Assess the characteristics of neurite angular variation of extra-neurite space as well as cell membranes, somas, glial cells, and fiber orientations in white matter (20, 21).	- Following mTBI, ODI was noted to be higher when observed in lower functioning individuals (13).	- ODI was noted to be lower in patients who did not show symptomatic or cognitive improvement following mTBI (13). - Concussed athletes displayed a decrease of ODI (14).
Tau	A microtubule binding protein in which promotes polymerization and plays a role in maintaining azonal transport and neuronal integrity (22).	- Noted to be elevated following high head impact pregame vs. post-game and preseason vs. post-season (23).	N/A
Neurofilament-light (NfL)	Protein associated with neuronal cytoskeletal element, which is involved in normal axonal and dendritic structure, growth, and function (24).	- Exposure to repetitive subconcussive head trauma results in an increase of NfL and remains elevated throughout the season (25). - Higher frequency and magnitude of head impacts results in an increase of NfL (24). - Following a bout of 10 subconcussive soccer heading impacts, NfL gradual increased, which was detected 2 h post-impact (26).	N/A
Glial fibrillary acidic protein (GFAP)	The most abundant cell type in the brain responsible for supporting structural integrity of the astrocytic cytoskeleton following disruptive forces (27).	- The severity of the TBI is dependent upon the rise in GFAP (28). - Within 1 h of a concussion, GFAP levels were noted to be increased, and reaching a peak level at 20 h post-injury (29).	N/A

MRI Acquisition

The MRI data were acquired on a 3T Siemens Prisma MRI scanner (Siemens, Erlangen, Germany) equipped with a 64-channel head/neck coil. High-resolution anatomical images (T1 weighted) were acquired using 3D MPRAGE pulse sequence with the following parameters: repetition time/echo time (TR/TE) = 2,400/2.3 ms, inversion time (TI) = 1,060 ms, flip angle = 8, matrix = 320 × 320, bandwidth = 210 Hz/pixel, iPAT = 2, resulting in 0.8 mm isotropic resolution. For diffusion analysis, two consecutive diffusion weighted imaging (DWI) sessions with opposite phase encoding directions were performed with a simultaneous multi-slice single-shot spin-echo echo-planar pulse sequence with the following parameters: TE = 89.4 ms; TR = 3,590 s, flip angle = 90, 1.5 mm isotropic resolution. Each session had 103 images with different diffusion weightings and gradient directions summarized as following: 7 b = 0 s/mm^2, 6 directions with b = 500 s/mm^2, 15 directions with b = 1,000 s/mm^2, 15 directions with b = 2,000 s/mm^2, and 60 directions b = 3,000 s/mm^2.

Imaging Processing

First, the DWI images were denoised using the principal component analysis (PCA)-based denoising tool in Mrtrix (https://www.mrtrix.org/) (39), and then magnetic field map information for susceptibility artifacts correction was derived from the b0 ($b = 0$ s/mm^2) images with opposite phase encoding directions using "topup" tool in FSL (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki) (40). The images were then corrected for susceptibility artifact, eddy current distortions, and motion artifacts simultaneously using the "eddy" command of FSL and the average of the b0 volumes as a reference. DTI analysis was performed using the FSL Diffusion Toolbox. The diffusion metrics of FA, MD, axial diffusivity (AD), and radial diffusivity (RD) maps were calculated.

Meanwhile, the NODDI metrics including neurite density index (NDI) and orientation dispersion index (ODI) were derived using the NODDI Matlab toolbox v1.01 (http://mig.cs.ucl.ac.uk/index.php?n=Tutorial.NODDImatlab) using the default settings. NDI primarily represents axonal density within white matter, and ODI represents organization of white matter tracts (13).

Statistical Analysis

The whole-brain, tract-based spatial statistics was conducted for six diffusion metrics: FA, MD, AD, RD, NDI, and ODI in FSL (41). The FA maps were co-registered to a template *via* non-linear transformation. A skeleton of mean white matter tracts was obtained, and FA values of nearby voxels were projected to the template to obtain skeletonized FA maps. The non-linear warps and skeleton projection derived from FA maps were applied to all other diffusion metrics to obtain skeletonized maps of MD, AD, RD, NDI, and ODI as well.

The analysis used complete samples to test the relationship between blood biomarker and MRI data. Univariate regression analyses were conducted for each diffusion metric against blood biomarker levels *via* randomized permutation. The model included years of tackle football experience and number of concussion occurrences as covariates. The Threshold-Free Cluster Enhancement (TFCE) option was used in the permutation test, which gives cluster-based thresholding for familywise error correction (42). As a result, the TFCE p-value images obtained were fully corrected for multiple comparisons across space. When there was a significant association, *post-hoc* analysis using a Pearson correlation coefficient was computed between the blood biomarker level and an average value of the imaging voxels that showed a significant effect in the regression analysis.

RESULTS

Demographics

Five of 25 participants in the football group were excluded from MRI due to a metal implant in the body ($n = 4$) and orthodontic braces ($n = 1$). Three serum samples in the football group were not assessed for biomarkers due to severe hemolysis, which has shown to influence the detection of biomarkers, especially NfL (43). As a result, 17 completed sets of the neuroimaging-blood biomarker data for the analysis. Demographic information is detailed in **Table 2**.

TABLE 2 | Demographics and biomarker levels.

Variables	Football
n	17
Sex (%)	17M (100)
Age, y	16 (16–17)
BMI, kg/m^2	26.5 (23.9–28.7)
No. of previous concussion	
0, *n* (%)	11 (65.0)
1, *n* (%)	5 (29.0)
2, *n* (%)	1 (6.0)
Tackle football experience, y	7 (3–8)
Race, *n* (%)	
White	14 (82)
Black/African American	0 (0)
Asian	0 (0)
African Indiana/Alaska	0 (0)
Multiracial	3 (18)
Ethnicity, *n* (%)	
Not Latino/Hispanic	14 (82)
Latino/Hispanic	3 (18)
Psychiatric condition	
ADHD	0 (0)
Learning disability	0 (0)
Major depressive disorder	0 (0)
Blood biomarker levels, mean±SD, pg/mL	
Tau	2.26 ± 1.10
Neurofilament light	4.27 ± 1.89
Glial fibrillary acidic protein	60.00 ± 22.04

Data for age, BMI, and football experience are expressed as median (interquartile range), as these data were not normally distributed. Blood biomarker data were normally distributed; hence, it is presented as mean ± standard deviation. BMI, body mass index. ADHD, attention-deficit/hyperactivity disorder.

Associations Between Diffusion Metrics and Blood Biomarker Levels

Examples of the six different diffusion metrics are shown in **Figure 1A** on one slice of a representative subject that is mapped on FMRIB58_FA standard space. These parameter maps are distinct from one another, with each metric (FA, MD, AD, RD, NDI, and ODI) characterizing different diffusion features arising from underlying tissue microstructure.

Tract-based regression analyses revealed significant associations between several diffusion metrics and blood biomarker levels in football players. Specifically, serum tau level was positively associated with MD (**Figure 1B**) and negatively associated with NDI (**Figure 1D**) mainly in the corpus callosum. The tau-MD association was widespread over the corpus callosum ($p = 0.027$) and longitudinal fasciculus, whereas the tau-NDI association was focal on the anterior body of the corpus callosum ($p = 0.048$). In our *post-hoc* analysis using a Pearson correlation coefficient, we found a significant positive correlation

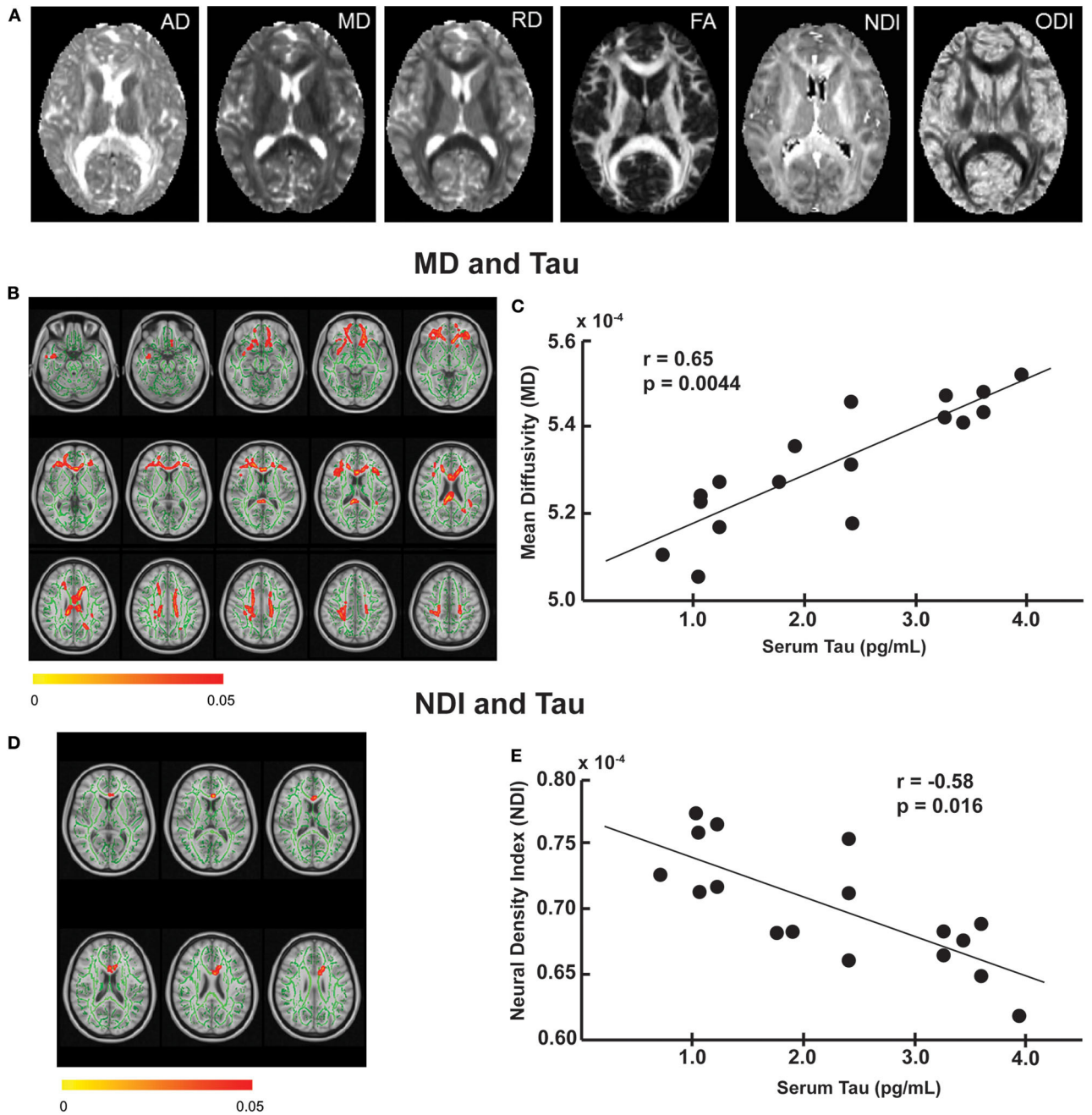

FIGURE 1 | The relationship between imaging and serum tau levels. **(A)** Example maps of DTI [axial diffusivity (AD), mean diffusivity (MD), radial diffusivity (RD), fractional anisotropy (FA)] and NODDI [neurite density index (NDI), and neurite orientation dispersion index (ODI)] from a single subject in FMRIB58_FA template. Tract-based regression analysis showed that tau was positively associated with mean diffusivity **(B)** and negatively associated with neurite density index **(D)** in several white matter tracts (red-yellow, corresponding to *p*-value 0.05 to 0). The regression analyses were performed on the skeletonized white matter tracts (green) derived from the mean FA maps of the study subjects, to which diffusion metrics of nearby voxels were projected. All results were corrected for multiple comparisons using threshold-free cluster enhancement (TFCE) at $p \leq 0.05$. The results are overlaid on the 1-mm resolution MNI (Montreal Neurological Institute) template. *Post hoc* correlation analysis revealed a positive correlation between tau and mean MD of the voxels that showed a significant effect in the regression analysis **(C)** and a negative correlation between tau and mean NDI of the voxels that showed a significant effect in the regression analysis **(E)**.

between an average value of MD voxels that showed significant associations in **Figure 1B** and serum tau levels ($r = 0.65$, $p = 0.0044$: **Figure 1C**). Similarly, there was a significant negative correlation between an average value of NDI voxels that showed significant associations in **Figure 1D** and serum tau levels ($r = -0.58$, $p = 0.016$: **Figure 1E**). All *post-hoc* comparisons are

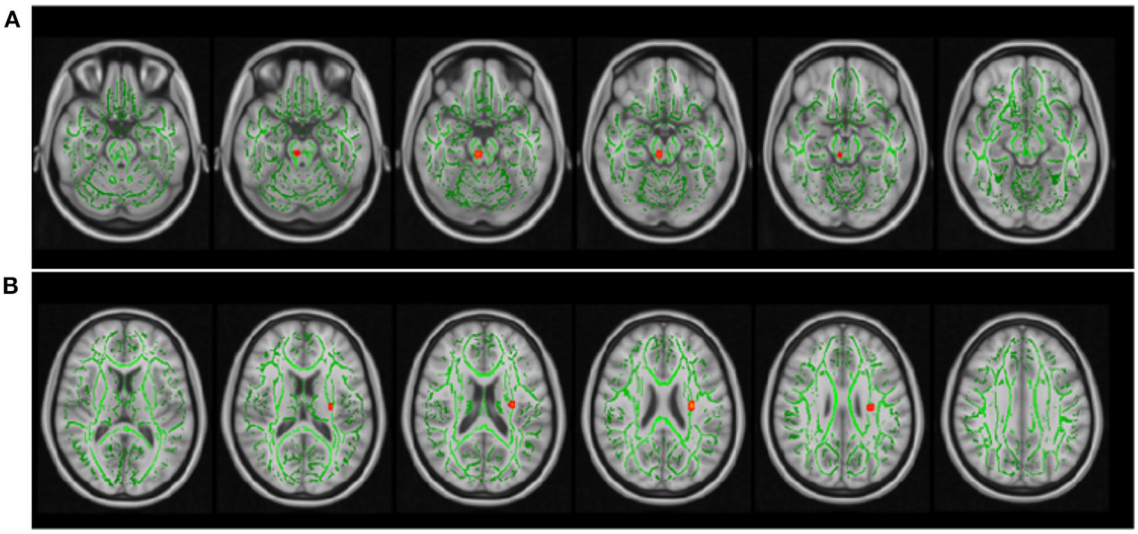

FIGURE 2 | The relationship between imaging and serum GFAP and NfL levels. The same tract-based regression analysis as used in **Figure 1** showed that GFAP was positively associated with axial diffusivity in the brain stem **(A)**, and NfL was negatively associated with neurite orientation dispersion index **(B)** in a small portion of the longitudinal fasciculus. The skeletonized white matter tracts (green) are the same as that in **Figure 1**. The TFCE p-value was set to $p \leq 0.05$. The results are overlaid on the 1-mm resolution MNI template.

shown in **Supplementary Table 1**. It is worth noting that both covariates, years of tackle football experience, and number of previous concussions had a non-significant influence on the imaging-blood biomarker association.

Additionally, a small number of voxels showed positive association between GFAP and AD in the brain stem ($p = 0.052$: **Figure 2A**) and negative association between NfL and ODI in the longitudinal fasciculus ($p = 0.046$: **Figure 2B**).

DISCUSSION

The novelty of the current study was the multimodal association of the neural injury blood biomarkers (tau, NfL, and GFAP) and diffusion imaging metrics derived from DTI and NODDI to gauge the potential cumulative stress in the brains of high school football players. Primary findings from this pilot study are that elevated serum tau levels at preseason baseline were reflective of increased MD in various white matter tracts and decreased neurite density in the corpus callosum. However, these associations were independent from a self-reported number of previous concussions and years of tackle football experiences.

The corpus callosum is comprised of nearly 200 million myelinated axonal tracts that enable interhemispheric neuronal communications (44). The corpus callosum has been shown to be one of the most vulnerable areas of the brain to concussive and subconcussive mechanical forces (e.g., shear, stretch, shortening) (45–47), and significant atrophy has been found in brains with chronic traumatic encephalopathy (48, 49). Furthermore, the immature (teenage) brain has shown to exhibit more pronounced axonal diffusion in the corpus callosum and longitudinal fasciculus (50, 51) following repetitive subconcussive head impacts compared to the mature brain (52), given that neural networks in the frontal cortex proliferate throughout adolescence and reinforce executive functions (53).

Our data on the imaging-blood biomarker associations in the corpus callosum and longitudinal fasciculus are intriguing, in that even at preseason baseline, increased MD and reduced NDI in these tracts were highly correlated to serum levels of tau in high school football players. The interpretation of DTI imaging data on subconcussive neurotrauma is challenging, as a recent systematic review (54) concluded that there are divergent findings in DTI measures. As for MD, it is thought that increased MD often attributes to more severe form of traumatic brain injury (TBI), whereas decreased MD is frequently reported due to repetitive subconcussive head impacts, as revealed in a recent systematic review (54). Given that our data is cross-sectional and we do not possess head impact kinematic data from previous seasons, it remains unclear of the cumulative head impact effects on the MD values. However, we identified that elevated MD in various axonal tracts was correlated strongly to elevated serum tau, which has shown to gauge the severity of axonal damage and neurodegenerative progression, including diagnosis of Alzheimer's disease (55), prediction of concussion recovery duration (30, 31), and association with short- (23) and long-term subconcussive neural stress (56).

This MD-tau finding is further substantiated by NDI data derived from NODDI analysis. NDI is a measurement of the intracellular volume fraction; in other words, NDI primarily represents axonal density within white matter. Palacios et al. (13) previously reported that concussion can significantly and acutely reduce NDI, and NDI values continues to decrease over time in these concussion patients, suggesting progressive axonal degeneration. We found a significant negative correlation

between serum tau and NDI particularly in the corpus callosum. It is possible that football-related head impacts can trigger microstructural disruption and progressive degeneration in axons, as represented in elevated MD and reduced NDI, and concurrently induce tau dissociation from microtubules. Dissociated tau can reach peripheral circuitry through either blood–brain barrier leakage or glymphatic pathway (57). These associations are physiologically reasonable and indicate that playing American football may relate to chronic microstructural abnormality in axonal tracts. Previous studies support this hypothesis in such a way that astrocyte-enriched protein, S100B, is released into the peripheral circuitry in a subconcussive impact-dependent manner in college (58) and high school football players (5, 59). Recurring spikes of plasma S100B levels due to head impacts from practices and games can develop autoimmune reaction to S100B within neurons and astrocytes (5). Furthermore, abundant S100B in the brain parenchymal space can act as a ligand for the advanced glycation end products receptors in the neuronal plasma membrane (60), which then trigger a cascade of events including c-Jun N-terminal kinase, Dickkopf-1, and glycogen synthase kinase 3β, which collectively induce hyperphosphorylation of the tau protein and contribute to tau tangle formation (57). While it is unlikely to observe distinct neurodegenerative features in adolescent football players, a recent study suggests that subconcussive head impact exposure may blunt positive neurologic effects (i.e., increased axonal integrity, better cognitive performance) from participating in sports. Strauss et al. (61) demonstrated that these beneficial effects were absent in soccer players who experienced high exposure to soccer headings, pointing to the possibility that axonal microstructural damage from head impacts may attenuate neurologic well-being as part of healthy development in adolescents.

Similar to tau protein, NfL functions as a scaffolding structural protein in axonal and dendritic branching and growth, and NfL undergoes post-translational modification by a series of phosphorylation events, which can make it vulnerable to mechanical stretch and shear stress (62). Growing evidence supports that NfL levels in blood have shown to reflect the progression of neurodegenerative condition (e.g., Alzheimer's disease, multiple sclerosis) (63–65), differentiate severities of TBI (66), predict clinical outcome (e.g., functional recovery, return-to-play) after severe TBI and concussion (67, 68), and correlate with subconcussive head impact exposure (24, 26, 32, 33). Unlike tau, however, we failed to observe significant correlations between NfL and diffusion metrics, which opposes the data by Ljungqvist et al. (69) that showed an association between serum NfL and FA ($R^2 = 0.83$) 12 months after severe TBI. This discrepancy may attribute to the severity of injury, whereby despite repeated exposure to subconcussive head impacts in previous seasons, these stimuli may not be sufficient to chronically elevate NfL levels. In fact, Joseph et al. (23) reported that serum NfL was unchanged after a high school football season, while tau protein elevated up to five-fold post-season, especially in players with frequent head impacts. A multimodal longitudinal study is needed to address whether serum levels of tau and NfL elevate due to subconcussive head impacts over time and associate with progressive axonal microstructural damage, as assessed *via* DTI/NODDI.

Limitations

While the current study used state-of-the-art technologies to examine the brain microstructural integrity of adolescent athletes, there were limitations to be noted. A relatively small sample size from a single site, lack of female sports, and non-collision control group limit generalizability of the results. Increasing data consistently suggest sex-related differential response to concussion, with females experiencing greater severity of symptoms and longer recovery time than their male counterparts. Therefore, the data from the current study in male football players unlikely translate into female athletes with subconcussive exposure (e.g., soccer, ice hockey, rugby). Additionally, our findings in tau and DTI/NODDI cannot fully attribute to football-related neural burden, given that we were unable to account for developmental factors and positive effects from exercise due to lack of control group.

We are also aware that the true novelty lies with a longitudinal multimodal relationship, by testing if parameters of neuroimaging and blood biomarkers change over time in relation to head impact exposure. Hirad et al. (70) recently showed longitudinal agreement between DTI and tau, but other biomarkers and NODDI were not included. Therefore, this study is an excellent step to encourage interdisciplinary collaborations between neuroimaging and blood biomarker scientists since these fields rarely intersect to delineate subconcussive brain injury. The potential residual neural burden was accounted for by the number of previous concussions and years of tackle football experience. Although these are commonly used variables, there might be an unquantifiable recall bias in self-reporting. A more rigorous approach would be to use head-impact data from previous seasons and conduct a medical chart review to validate prior concussion history.

CONCLUSION

Evidence is beginning to uncover the effects of cumulative concussive and subconcussive head impacts in sports. Neuroimaging and blood biomarkers have been two of the most active areas of research in the neurotrauma community. Our data from DTI/NODDI and blood biomarkers suggest that football players may develop axonal microstructural abnormality particularly in the corpus callosum and surrounding white matter tracts, such as longitudinal fasciculus. Future study is warranted to determine the longitudinal multimodal relationship in response to repetitive exposure to sport-related head impacts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Indiana University Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KKa conceptualized and designed the study, obtained funding, collected data, conducted analysis, drafted the initial manuscript, and reviewed and revised the manuscript. JS and JM designed the study, recruited subjects, collected data, and reviewed and revised the manuscript. MH, MN, and KKe recruited subjects, collected data, reviewed and revised the manuscript. DR conducted follow-up analysis and revised the manuscript. SN and AS contributed to conceptualize the study, obtained funding, reviewed and finalized study protocol, and provided critical review of the manuscript. HC, ZC, and KE designed the study, conducted initial and final analyses, helped draft the manuscript, and reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Insights Into the Proteomic Profiling of Extracellular Vesicles for the Identification of Early Biomarkers of Neurodegeneration

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Extracellular vesicles (EVs) are involved in the development and progression of neurodegenerative diseases, including Alzheimer's and Parkinson's disease. Moreover, EVs have the capacity to modify the physiology of neuronal circuits by transferring proteins, RNA, lipids, and metabolites. The proteomic characterization of EVs (exosomes and microvesicles) from preclinical models and patient samples has the potential to reveal new proteins and molecular networks that affect the normal physiology prior to the appearance of traditional biomarkers of neurodegeneration. Noteworthy, many of the genetic risks associated to the development of Alzheimer's and Parkinson's disease affect the crosstalk between mitochondria, endosomes, and lysosomes. Recent research has focused on determining the role of endolysosomal trafficking in the onset of neurodegenerative diseases. Proteomic studies indicate an alteration of biogenesis and molecular content of EVs as a result of endolysosomal and autophagic dysfunction. In this review, we discuss the status of EV proteomic characterization and their usefulness in discovering new biomarkers for the differential diagnosis of neurodegenerative diseases. Despite the challenges related to the failure to follow a standard isolation protocol and their implementation for a clinical setting, the analysis of EV proteomes has revealed the presence of key proteins with post-translational modifications that can be measured in peripheral fluids.

Keywords: extracellular vesciles, central nervous system, neurodegenenerative diseases, biomarkers, proteomic analyses, Parkinson' s disease, Alzheimer's disease, endolysosomal dysfunction

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INTRODUCTION

Neurodegenerative diseases (NDs) are complex disorders with devastating consequences for the patient and their immediate social environment. In Alzheimer's disease (AD) and Parkinson's disease (PD), the neuronal dysfunction and secondary effects progress gradually with heterogeneous clinical outcomes. Despite the advances in management of AD and PD, these diseases are among the leading causes of disabilities and showed an increased burden especially in low- and middle-income countries (1). One of the major challenges in the field of neuroscience is to achieve an early and precise detection of NDs to procure an adequate treatment and delay their

progression. Most of the patients are diagnosed when overt symptoms of neurodegeneration are displayed. Furthermore, it has been documented that the misdiagnosis rate of AD and PD could be as high as 17 and 25%, respectively (2–5). Thus, there is a need to develop new diagnostic strategies to aid in the identification of people developing AD and PD.

The specific cause of neural cell death has not been resolved, but it has become clearer that in AD and PD, both genetic and environmental factors operate. In the last two decades, several chromosomal regions have been identified containing genes that are associated with the risk of developing these NDs. In the early-onset variety of AD and PD, a point mutation could be identified as a causal agent due to the impairment of the function of a specific protein. Among the most relevant genes that contain genetic variants for AD development are the *APP*, *PSEN1*, *PSEN2*, *APOE*, *ADAM10*, and *ACE* genes (6). In the case of PD, the development of the disease has been linked to the following genes: *SNCA*, *PRKN*, *PINK1*, *DJ-1*, *LRRK2*, *ATP13A2*, *PLA2G6*, *FBX07*, and *VPS35* (7, 8). Despite early-onset cases representing <10% of the total AD and PD cases, these studies have helped to determine the main processes that underlie neurodegeneration processes. In general, these two diseases are characterized by alterations in mitochondrial function, in management of oxidative stress, in protein folding and aggregation, and in immune function (7–9).

The diversity and severity of symptoms and the differences in the timing of neurodegenerative progression have made it difficult to create clinically applicable tests for the diagnosis of AD and PD. In many cases, the confirmation of a ND is performed post-mortem. For this reason, the definition of a set of biomarkers is essential for diagnosis, stratification of patients and therapeutic monitoring. In recent years, the fact that altered protein homeostasis is a common event in the development of NDs has guided the development of diagnostic methods. For instance, several studies have found that decreased levels of amyloid β peptide of 42 amino acids length (Aβ42) in AD and of α-synuclein (α-syn) in PD in cerebrospinal fluid (CSF) indicate an overt development of the disease, but low specificity and contradictory results have been reported (10, 11). Nevertheless, these findings set the precedent for the identification of circulant biomarkers indicating the progress of NDs. A relevant aspect in the development of AD and PD is the dysfunction of the interaction between endosomes, lysosomes, and mitochondria at various levels. These alterations modify the secretory capacities of the cells in the brain, which is reflected in the molecular composition of organelles released into the extracellular space, which are known as extracellular vesicles (EVs).

The generic term of EVs refers to the lipidic vesicles that are released from the endosomal system (exosomes) and from the plasma membrane (microvesicles or ectosomes) (**Figure 1A**). EVs are considered a novel system of intercellular communication that involves the transfer of proteins, RNA, lipids, and metabolites. An alteration of the molecular cargo of EVs has been associated with the development of several diseases. In the case of NDs, it has been proposed that these organelles are involved in the propagation of mis-folded proteins in the brain (12). Moreover, it has also been documented that part of the communication between glia and neurons is mediated by EVs and their alteration has implications for the development of NDs. The advantage of studying EVs is that exosomes and microvesicles can be isolated from fluids such as CSF, blood, saliva, or urine. Thus, the molecular characterization

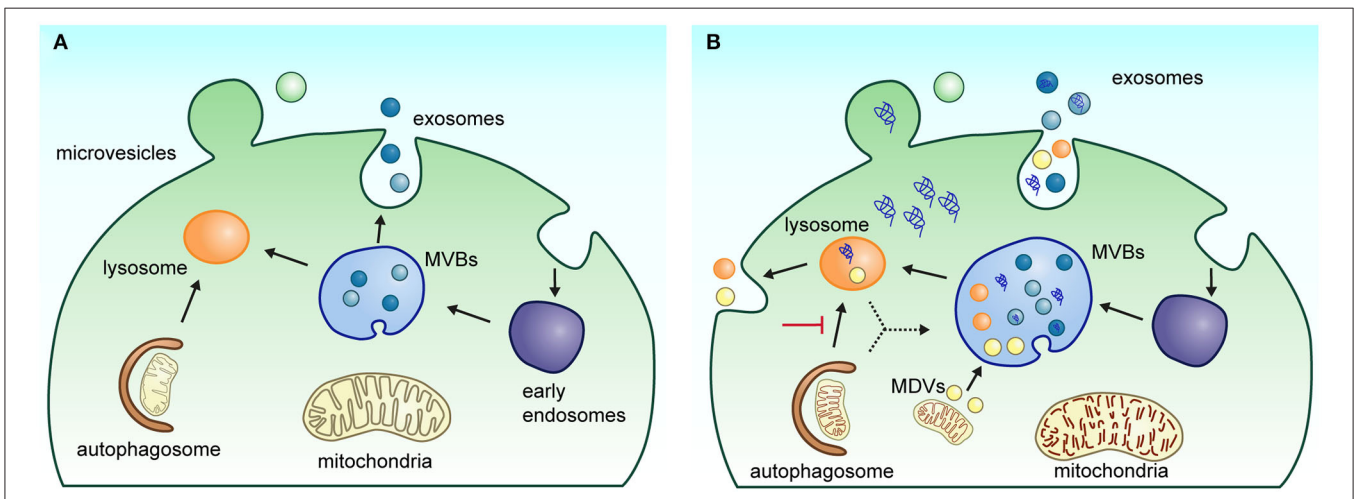

FIGURE 1 | Biogenesis and secretion pathway of EVs. **(A)** Under physiological conditions, extracellular vesicle biogenesis and secretion process occur in two main ways: (1) exosomes are generated in multivesicular bodies (MVBs) following the endolysosomal pathway; when MVBs fuse with the plasma membrane, exosomes are released into the extracellular space; or (2) microvesicles are released directly by budding from the plasma membrane. **(B)** Under neuropathological conditions such as Parkinson's and Alzheimer's disease, alterations in the endolysosomal system can affect biogenesis and release of EVs in the brain cells. The impairment of the autophagic–lysosomal pathway and alteration in protein sorting contribute to the increased accumulation of pathological markers in EVs. Several neurological diseases show an enlargement of MVBs with an increased number of intraluminal vesicles. The deficient mitophagy process also induces dysfunctional mitochondria accumulation, favoring the generation and release of mitochondrial-derived vesicles (MDVs) by cells.

of EVs is promising because the analysis in both patient samples and disease models may reflect the type of molecules that are expressed at different stages of the development of NDs. Interestingly, it has been described that brain-derived exosomes can be found in peripheral circulation. In this review, we will focus on discussing the scope and limitations of the proteomic characterization of EVs and their potential to identify molecules that guide the development of non-invasive tests and to help unravel neurodegenerative pathophysiology.

ROLE OF EVs IN THE CENTRAL NERVOUS SYSTEM

The intercellular communication mediated by EVs has been linked to various processes of normal brain function both in development and in adulthood. During the synaptogenesis process and proliferation of neural progenitors, the gradients of hydrophobic molecules such as Wnt and Sonic the Hedgehog are generated by their release and transport in EVs (13–15). For neural circuit formation, neuronal exosomes promote neuronal precursor proliferation and cell differentiation both *in vivo* and *in vitro*. The EVs derived from human induced pluripotent stem cells (iPSC) are enriched in proteins related to neurodevelopmental functions including neuritogenesis and morphology of the nervous system. These functions are impaired due to the loss of MECP2, which also alters synaptic density and neuronal firing capabilities (16). In the subventricular zone, the neural stem cells release vesicles that function as morphogens by decreasing the number of microglial processes to induce its traditional stellate morphology. In response, microglia can reduce the proliferation of neural precursors through cytokine release (17). It appears that EV-mediated communication is an active system that continues into adulthood. In ultrastructural studies, the presence of vesicles in the intercellular space in various brain structures is notorious, which suggests the existence of communication axes between the different cell types in the brain: neurons, astrocytes, microglia, and endothelial and epithelial cells (18, 19).

At a neuronal level, synaptic maturation and remodeling are influenced by the molecular cargo of EVs. For example, the degradation of the post-synaptic density protein-95 is promoted by the delivery of Proline-Rich 7, which results in the elimination of excitatory synapses in rat hippocampal neurons (15). The release of EVs from the presynaptic terminal is activity dependent and promotes the delivery of molecules to the post-synapsis such as Synaptotagmin-4, which, by retrograde signaling, promotes the maturation of the presynaptic terminal at the neuromuscular junction of Drosophila (20, 21). Likewise, synaptic plasticity is influenced by EV-mediated transfer of Arc1 mRNA to the post-synaptic region (22, 23). While epileptic status selectively modifies the miRNA content of hippocampus-derived EVs, it does not modify the process of miRNA editing that occurs inside the vesicle, which modulates target recognition (24). These findings suggest that the information contained in the EVs add an extra layer of modulation for normal synaptic functioning, which can be bidirectional among neurons.

Regarding the communication between different brain cell types, there is evidence that EVs are targeted to specific cell types. Selective expression of CD63 coupled to GFP in neurons has been achieved in rodent models. Neuron-derived EVs containing GFP are incorporated preferentially by astrocytes and also in a minor proportion by certain neurons, both *in vivo* and *in vitro* experiments (25, 26). These vesicles are mainly secreted at the neuronal soma and dendrites, and they transfer the miR-124-3p, which in turn increases protein levels of the glutamate transporter GLT1 in astrocytes (26). Astrocyte-derived EVs seem to be directed mainly toward neurons to modulate a variety of functions (27). The effect of astrocyte-derived EVs depends on the brain microenvironment, being beneficial when there are trophic stimuli or neurotoxic when there are pro-inflammatory conditions. After incubating astrocytes with ATP, the EVs are enriched with proteins related to synaptogenesis (NETO1) and neurite growth (RPL10). In contrast, astrocyte stimulation with IL-1β or TNF-α enriches proteins and miRNAs associated with reduced growth and network neuronal activity (28, 29). In addition, the activation of astrocytes by IL-1β is associated with the production of vesicles that are released into peripheral circulation and that promote migration of leukocytes into the brain (30). In the case of microglia, an interaction with neurons through EVs has also been documented. The incubation of the MG6 microglial cell line with EVs from depolarized neurons promotes the degeneration of neurites from PC12 cells (31). A deleterious effect is also observed with the EVs derived from microglia BV2 cells after the activation of the metabotropic glutamate receptor 5, which is involved in neuroinflammation (32). LPS increases the levels of TNF-α and IL-6 in microglia-derived EVs and promotes the loading of proteins related to transcription and protein translation (33). Further research is needed to determine the role of microglia-derived EVs in normal conditions and after traumatic brain injury, which induces changes in miRNA cargo that have been related to anti-inflammatory effect on neurons (34).

Endothelial cells constitute another important participant of the intercellular communication in the brain. The survival and proliferation of oligodendrocyte precursor cells are promoted by EVs derived from microvascular endothelial cells (35). Likewise, endothelial cell-derived EVs inhibit apoptosis of neurons deprived of oxygen and glucose through the transfer of miR-1290 (36). Endothelial cells can also be influenced by astrocyte-derived vesicles that regulate the expression of tight-junction proteins (claudin-5, occluding, ZO-1) to maintain the integrity of the blood–brain barrier (37). After TNF-α exposure, the protein related to TNF signaling, immune response, and mitochondrial proteins are enriched in the EVs derived from endothelial cells (38). These findings indicate that EV secretion by endothelial cells has a role in the activation of an inflammatory systemic response. Indeed, a focal brain lesion induced by a microinjection of IL-1β in the brain parenchyma increases the circulating EVs from endothelial cells and induces the release of acute-phase response proteins by the liver (39). Conversely, the brain can be exposed to signals from the periphery. Breast cancer-derived EVs can cross the blood–brain barrier through a transcellular mechanism that involves the downregulation of

Rab7 to switch the endocytic pathway to a recycling mode (40). The choroid plexus epithelium is another sensor of peripheral conditions that can modify brain cell physiology. After LPS injection in mice, choroid plexus epithelial cells release EVs into the CSF containing inflammatory miRNAs (miR-1a, miR-9, miR-146a, and miR155) that eventually activate an inflammatory response in astrocytes and microglia (19).

Because CSF contains a mixture of molecules originating from different cells in the brain, it has been used to monitor the physiological status of the CNS. In relation to EVs, there is evidence showing that the isolation of vesicles from CSF could be informative about the progression of neurological diseases. Recently, the analysis of patient-derived glioblastoma cells revealed that a subpopulation of EVs is mainly enriched in mRNA and non-vesicular ribonucleoprotein complexes are enriched in tRNAs and Y RNA fragments (41, 42). These changes can be detected by the analysis of EVs in CSF samples and help to provide a molecular diagnosis (43). The principle of monitoring pathological events in the brain is reinforced by the fact that EVs derived from peripheral blood reflect the presence of biomarkers of the developing tumor in the brain and its levels correlate with cell invasiveness (41, 42, 44). Similarly, the protein cargo of EVs from blood and CSF samples reflects early events of neurodegeneration related to mild traumatic brain injury and cognitive impairment (45–47). These proteomic profiling studies indicate alterations in the endosomal and lysosomal functions which are key processes underlying NDs. A possible role of EVs in the brain is to discard an overload of mis-folded proteins to maintain synaptic homeostasis (48, 49).

OVERVIEW OF THE ROLE OF EVs IN DEVELOPMENT AND PROPAGATION OF NDS

Recently, it has been suggested that the classification of EVs in exosomes and microvesicles is an oversimplification of the heterogenous mixture of EVs that originate from different intracellular pathways (50, 51). While the exact composition and function of each EV subtype is still not well-defined, the current available methods for its molecular characterization indicate that a set of molecules associated with the development of NDs can be enriched or depleted under certain conditions. These changes may result from an alteration in the biogenesis of EVs or from a neuronal response to eliminate protein aggregates to counteract impairments in lysosomal and autophagy functions. In agreement with this notion, it has been observed that in several models of neurodegeneration, there are morphological alterations in the endolysosomal system, including larger multivesicular bodies (MVBs) with more intraluminal vesicles (52–54). In addition, neurodegeneration is related to changes in the expression of endolysosomal markers (EEA1, Rab5B, Rab7, CD63, TSG101, Rab35, LAMP1, and CTSD) and lipid metabolism (sphingolipids and lysobisphosphatidic acid) (49, 54, 55). Neurons with a reduced capacity of protein and organelle degradation are prone to accumulate protein aggregates, which apparently are propagated through EV release (**Figure 1B**).

Histological, biochemical, and proteomic analysis of EVs in different research models and patient samples have shown that Aβ and Tau in AD, and α-syn in PD are associated with EVs (56). In human brain sections from AD patients, the exosome marker flotillin-1 colocalizes with oligomers of Aβ (57). The injection of EVs isolated from familial AD patient-derived iPSCs in mouse hippocampus was sufficient to induce Tau phosphorylation in different regions of the hippocampus after 5 weeks (58). The Swedish mutation in the amyloid precursor protein (APP), which increases abnormal cleavage of cellular APP, favors the loading of Aβ40, Aβ42, and APP C-terminal fragments (CTFs) into EVs that are incorporated mainly by neurons (59). In an elegant study, it was shown that the disruption of the PI3K/Vps34 signaling pathway is involved in the secretion of APP-CTFs by causing a dysregulation of lipid metabolism that induces damage to endolysosomal membranes (49). A reduced EV release has been observed in a humanized APOE4 mouse model. The presence of the risk allele of apolipoprotein E is associated with decreased levels of Tsg101 and Rab35 (55). These findings are of great relevance considering that genetic risk factors of NDs usually involve proteins of endosomal and lysosomal trafficking. A compromised ability to handle cellular waste by the disturbance of endolysosomal trafficking can be reflected in the molecular content of EVs.

Similar evidence has been described for PD in which the dysfunction of key elements of lysosomal and endosomal pathways has been associated with the development of the disease (7). An analysis of PD human brain extracts showed that α-syn accumulation is associated with decreased levels of the lysosomal protein LAMP2 (60). For instance, the loss of the mitochondrial protein Parkin (PARK2) decreased the endosomal tubulation and increased the expression of late endosome markers such as CD63 and the number of intraluminal vesicles in MVBs (61). The overexpression of the lysosomal protein ATP13A2 (PARK9) increases its presence in MVBs and promotes the secretion of α-syn in EVs (62). Interestingly, the inhibition of the enzyme neutral sphingomyelinase (nSMase2), involved in the biogenesis of intraluminal vesicles, decreased the transfer of oligomeric aggregates of α-syn in co-culture experiments (63). The leucine-rich repeat kinase 2 (LRRK2) is naturally found in EVs from kidney, immune, and brain cells, and it is proposed to have a key role in autophagocytic pathway (64, 65). Levels of phosphorylated LRRK2 at residue S1292 in CSF and urine correlate with severity of the disease and help to predict mutation carriers (66). Overall, an early event in protein aggregation seems to involve components of the endosome and lysosome compartments.

EV PROTEINS AS BIOMARKERS IN PD

PD is characterized by non-motor and motor alterations that are associated with the progressive loss of dopaminergic neurons in the substantia nigra pars compacta. A well-established histological feature is the presence of cytoplasmic inclusions known as Lewy bodies (LBs) that are composed of α-syn aggregates and sequestered organelles including mitochondria, endosomes, and lysosomes (67). Growing evidence indicates that

α-syn pathology initiates in the periphery even earlier than in the central nervous system (68, 69). The mechanisms considered for α-syn pathology spreading include axonal transport, fluid phase, prion-like process, and EV-mediated transmission (70). Considering that EVs reflect cellular changes that occur in response to pathological conditions, it is conceivable that they are an effective source of biomarkers for both peripheral and central events of neurodegeneration.

Among the intracellular mechanisms proposed to explain PD pathogenesis, included is the disturbance of the interactions between mitochondria, lysosomes, and endoplasmic reticulum, which results in the accumulation of protein aggregates and cellular toxicity. The deletion of mitochondrial proteins (AIF, OPA, and PINK1) or inhibition of mitochondrial complex I with rotenone cause the appearance of lysosomal vacuoles, increase lysosomal permeability, and alter protein degradation (71, 72). The absence of PINK1 and Parkin induces the formation of mitochondrial-derived vesicles that can be shuttled to late endosomes and secreted to extracellular space (73). Similarly, the inhibition of the maturation of autophagosomes with bafilomycin promotes their fusion with multivesicular bodies and favors the release of α-syn through EVs (74). Altogether, these data indicate that dysfunction of organelle crosstalk not only creates a microenvironment for protein aggregation but also alters the dynamics of protein cargo that is packaged into EVs.

The protein α-syn is expressed in the brain and is present in an oligomeric or aggregated state in body fluids, CSF, and plasma (75). The levels of α-syn in EVs from CSF allow the discrimination of patients with PD and dementia with LBs from other neurological conditions and healthy controls (76). To achieve a better resolution, EVs from the central nervous system have been isolated by immunocapture using an anti-L1CAM antibody, which recognizes a cell adhesion marker enriched in neurons (77–79). The amount of neuron-derived EVs and α-syn levels in these EVs is higher in plasma of PD patients than in plasma of healthy controls (77, 79). A relevant point to mention is that measurement of α-syn in EVs from a specific cell population could provide a more consistent assessment of the quantity of a biomarker at different stages and types of NDs than measurements directly from total CSF or plasma. A cross-sectional study showed that α-syn in neuronal EVs is increased by two-fold in prodromal and clinical PD in comparison to multiple system atrophy, controls, or other NDs (80). Combined evaluation of α-syn and clusterin in neuron-derived EVs improves the differential diagnosis to predict PD from non-α-syn proteinopathies (80). Furthermore, an increased proportion of oligodendrocyte-derived EVs and astrocyte-derived EVs in relation to neuron-derived EVs in plasma shows a significant correlation with the Unified Parkinson's Disease Rating (UPDRS) part III scores in the patients with PD (79). Taken together, these data suggest that EVs derived from the central nervous system would be a helpful strategy with elevated specificity and sensitivity to differentiate and monitor the progression of PD.

The presence of relevant biomarkers in EVs has also been studied in other biofluids. In a small sample of Korean PD patients, α-syn was not detected in urine EVs (81). In contrast, the levels of α-syn oligomers and the ratio of α-syn oligomers to total α-syn is increased in salivary EVs isolated from PD patients (82). Additionally, the concentration of salivary EVs of neuronal origin is higher in PD patients than in control individuals, as well as the levels of phosphorylated α-syn (83). Tear fluid is also accessible and mirrors the pathophysiological changes in systemic and ocular diseases. A mass spectrometry analysis of tear samples in a small cohort of PD patients revealed that almost half of the deregulated proteins in PD are related to neuronal functions. Remarkably, DJ-1 levels are increased in PD tear samples vs. control tears (84). Dysregulation of core networks of proteins involved in lipid metabolism, oxidative stress, vesicle secretion, and immune response supports the participation of these mechanisms in PD pathogenesis (84). Further studies, including proteomic studies of tear fluid EVs in PD, are needed to deeper evaluate them as a feasible biomarker source for PD.

Regarding the observation of an altered EV content and secretion in PD patients, it can be correlated to impaired lysosomal function. Decreased glucocerebrosidase 1 (GBA-1) enzymatic activity in the brain is a common trait in PD. Dysfunction of the endocytic pathway through the inhibition of GBA-1 results in increased release of brain EVs containing α-syn oligomers (85, 86). The increased production of EV associated with α-syn contributes to accelerate protein aggregation in receptor cells *in vivo* (85). GBA1-associated neurodegeneration in PD is associated with impairment of the autophagic–lysosomal pathway and mitochondrial dysfunction, possibly attributable to the accumulation of dysfunctional mitochondria as a consequence of defective mitophagy (87). Moreover, α-syn aggregation can be involved in the dysregulation of autophagic and endolysosomal pathways and is also associated with mitochondrial dysfunction (88).

Mutations in the genes encoding for LRRK2 (PARK8) and DJ-1 have been associated with PD. The mutations in the kinase LRRK2 account for 40% of the familial cases and its loss of function alters the endolysosomal pathway. The most prevalent mutation LRRK2-G2019S interferes with the mitochondrial fission through increasing the expression of the factor DLP1 (89). Regarding the possible role of these proteins as biomarkers, it is known that the levels of DJ-1 and LRRK2 in urine-derived EVs differ between sexes (81). Notably, DJ-1 levels increase with age in PD male patients (81). A high ratio of Ser(P)-1292 LRRK2 to total LRRK2 in urine EVs seems to be useful in discriminating between LRRK2 mutation carriers and non-carriers with or without PD. Moreover, LRRK2 mutation carriers with PD have a higher ratio than control individuals (66). Also, Ser(P)-1292 LRRK2 levels correlate with the severity of cognitive impairment and difficulty in accomplishing activities of daily living (90). While its exact function in PD pathogenesis is not fully understood, DJ-1 may act as an antioxidant and chaperone and play a role in mitochondrial homeostasis, possibly involving the PINK1/Parkin pathway. The levels of DJ-1 in EVs derived from CNS and the ratio of EV DJ-1 to total DJ-1 are substantially higher in the plasma of PD patients compared to healthy controls (91). These data show promising results regarding the quantification of LRRK2 and DJ-1 in EVs and considering post-translational modifications could increase the accuracy for PD diagnosis and prognosis.

Biomarker discovery can be accelerated by the characterization of EVs using mass spectrometric analysis for the high-throughput identification of dysregulated proteins in NDs. Despite the challenges of EV isolation from a reduced sample volume and the purity of EV preparations, shotgun proteomic profiling experiments have started to reveal the families of molecules that are associated with EVs. A total of 1,033 proteins were identified in a proteomic analysis of EVs isolated from sera of healthy controls and PD patients in early stages of the disease. Of these, 21 proteins were upregulated and 2 were downregulated in PD samples, while the quantity of vesicles and the amount of classical exosome markers such as flotillin were similar (92). Among the most abundant proteins are the vacuolar protein sorting-associated protein 13D, peroxiredoxin-2, S100A8, cytochrome b-245 heavy chain, and syntenin 1 (92). A more refined analysis using neural immunocaptured EVs from serum identified 429 proteins samples from controls and patients with mild (Hoehn and Yahr score < 3) and severe PD (Hoehn and Yahr score > 3) (93). The upregulated proteins in mild and severe cases include the clusterin, complement C1r subcomponent, afamin, apolipoprotein D, gelsolin, and pigmented epithelium-derived factor (PEDF). The down-regulated proteins in serum-derived EVs comprise human neuroblastoma cDNA clone CS0DD006YL02, complement C1q subcomponent, myosin-reactive immunoglobulin, Ig kappa chain, and Ig mu chain (93). In contrast, the proteomic characterization of plasma-derived EVs isolated by size exclusion chromatography showed that the levels of clusterin and C1r subcomponent were decreased in PD patients compared to control individuals (94). These disparate results highlight the importance of the isolation method and the need to determine whether differentially abundant proteins correspond to vesicular or extravesicular material.

Recently, a strategy to define the origin of EV subpopulations in blood was generated through the phenotyping of EVs by flow cytometry. The mean number of erythrocyte-derived EVs ($CD235a^+$) correlated with the UPDRS scores for different disease stages. The proteomic analysis of EVs from erythrocytes revealed that 8 proteins of a total of 818 identified proteins showed significantly different levels as a function of PD stage (95). The protein markers that were highly expressed in controls include axin interactor dorsalization-associated protein (AIDA), alpha/beta hydrolase domain-containing protein 14Bv (ABHD14B), and glutamine-dependent NAD^+ synthetase (NADSYN1), while proteins highly expressed in mild PD were quinoid dihydropteridine reductase (QDPR), alcohol dehydrogenase $NADP^+$ (AKR1A1), and cannabinoid receptor-interacting protein 1 (CNRIP1). In contrast, the ubiquitin carboxyl-terminal hydrolase 24 (USP24) and ATP synthase subunit alpha mitochondrial (ATP5A1) were increased in moderate PD cases (Hoehn and Yahr score 2–2.5) (95). Future studies should explore differential protein signatures in other blood cell types such as immune cells, considering the relevance of persistent inflammation as a risk factor for developing PD.

The isolation of EVs from plasma, serum, and CSF samples represents several limitations including the limited volume obtained per patient and the technical procedures for its obtention. Conversely, urine samples can be used to maximize the yield of EVs and still capture the pathological processes occurring in different parts of the body. Consistent with this notion, a quantitative analysis of urinary EVs from a cohort of PD patients revealed that enriched proteins are related to neurological disorders including Parkinson's, Huntington's, and Alzheimer's diseases (96). Among the enriched endolysosomal proteins related to NDs, synaptosomal-associated protein 23 (SNAP23) and calbindin were particularly prominent in PD cases. The measurement of these proteins have a prediction success in a range of 76–86% for disease diagnosis in two independent cohorts (96). Moreover, this study demonstrated that EV-related proteins show low interindividual variability and can be tracked over time. Overall, these findings open the possibility of monitoring long term the abundance of proteins associated with neurological disorders and how their levels are modified in response to treatments.

Mitochondrial dysfunction causes oxidative stress favoring aberrant protein folding and protein accumulation (i.e., Aβ, huntingtin, Tau, and α-syn). Furthermore, mitochondrial damage and impairment of the removal of damaged mitochondria (mitophagy) have been proposed as a central event in the pathogenesis of PD. The analysis of circulant mitochondrial-derived vesicles can be promising for the identification of early biomarkers of PD, considering that it is a cellular mechanism of antigen presentation in inflammatory conditions (73). A recent characterization of small EVs from the serum of PD patients identified a reduction in the levels of mitochondrial markers such as adenosine triphosphate 5A (ATP5A), NADH: ubiquinone oxidoreductase subunit S3 (NDUFS3), and succinate dehydrogenase complex iron sulfur subunit B (SDHB), and of the levels of the tetraspanins CD9 and CD63 (97). A more precise protein signature of mitochondrial-derived vesicles should be addressed in future studies including genetic models of PD and patient samples. A better characterization of the temporal dynamics of mitochondrial markers in circulation would help to understand the interconnection with the endolysosomal system.

The studies described above account for the most recent advances in the identification of prospective protein biomarkers contained in EVs. However, some points need to be resolved before a valid biomarker profile based on EV characterization can be efficiently used for the diagnosis and prognosis of PD. Ideally, an exhaustive phenotyping of EV subtypes could improve the detection of altered proteins related to neurodegeneration. Moreover, a multicenter study that guarantees the standardization of the protocols used for EV isolation and proteomic analysis would benefit the phase of validation of biomarkers in large size cohorts. Finally, as the familial forms of PD are associated with endolysosomal or mitochondrial defects, a targeted analysis of proteins associated with these cellular pathways in the EVs derived from body fluids of PD patients would be of great value in understanding the

evolution of the disease and determining its relation to pharmacological treatments.

EVs PROTEINS AS BIOMARKERS IN AD

The histopathological hallmarks of AD are the presence of extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated Tau protein. The exact mechanism that provokes this malignant protein aggregation is not fully understood. In the development of AD, EVs show a multifaceted role. Several studies have demonstrated a deleterious effect of EVs as carriers of pathogenic proteins that contribute to their spreading across the brain. However, others state that it is possible that EVs have a neuroprotective role by buffering the formation of toxic protein aggregates. The specific function of brain EVs seems to depend on their cellular origin and on the inflammatory status of the brain.

Neuroblastoma N2a-derived EVs act as scavengers of neurotoxic forms of Aβ by trapping the peptides on its surface through glycosphingolipids. These Aβ-EV complexes can be internalized by microglia for degradation (98). Moreover, the intracerebral infusion of EVs isolated from neuroblastoma cells diminishes Aβ pathology and amyloid deposition in APP transgenic mice (99). These findings suggest the use of EVs as a novel therapeutic approach to preventing plaque deposition in AD. In support of this notion, it has been proposed that the administration of EVs from hypoxic mesenchymal stem cells (MSCs) improves the learning and memory capabilities of the APP/PS1 mice by restoring the synaptic function. The EVs from hypoxic MSCs reduce the glial activation through the increase of anti-inflammatory cytokines (IL-10, IL-4, IL-6, and VEGF) and the decrease of proinflammatory cytokines (TNF-α and IL-1β) concomitantly *via* a reduction in the activation of the STAT3 and NF-κB pathway (100, 101). The administration of MSC-EVs also influences presynaptic functions by the induction of long-term potentiation (cellular correlate of learning and memory) at Schaffer collateral to CA1 synapses, which improves cognitive behavior (102). MSC-EVs also protect neurons by reducing oxidative stress through the transfer of catalase and by promoting synapse integrity (101).

An additional beneficial effect of EVs has been postulated through the transport of the cellular prion protein (PrPC), which is a glycoprotein with high affinity for the oligomeric form of the Aβ42. The EVs purified from N2a, SHSY-5Y, and cortical neurons, highly enriched in PrPC, show a higher binding affinity for dimeric, pentameric, and oligomeric Aβ species (103, 104). The EV–PrPC complex plays a protective role in sequestering soluble oligomeric forms of Aβ, reducing its neurotoxic effects by accelerating its fibrillization (103). A similar mechanism could also occur *in vivo* as the intracerebroventricular injection of human neural EVs in the brain rat prevents the inhibition of long-term potentiation caused by Aβ oligomers (105).

The notion of a neurotoxic role of EVs has been based on data showing the release of pathological forms of Aβ in the extracellular space by microglial and neuronal cells. As suggested by studies in AD animal models, EVs can diffuse throughout the brain and play a role in the dynamics of amyloid deposition (106). After Aβ internalization into microglia, fibrils can be converted into an oligomeric toxic form and reintroduced into endosomal secretion pathway (107, 108). In neurons, the loss of endosomal sorting complexes required for transport (ESCRT) components or the ubiquitination factor E4B (UBE4B) increases the levels of Aβ42 in late endosomes and its secretion through EVs (109). Both *in vivo* and cell culture assays have demonstrated that the dysfunction of the enzymes ECE-1 and−2, located in MVBs, could lead to the accumulation of intraneuronal Aβ aggregates and their subsequent release through EVs (110). Thus, mutations or alterations in the molecular machinery involved in APP trafficking can favor the loading and secretion of EVs with toxic aggregates into the circulation. Interestingly, the measurement of Aβ peptides and Tau levels in plasma-derived EVs has shown promising results in designing a score to follow AD progression and to discriminate from other types of dementias. With a sensitivity of 96%, the levels of phospho-Tau (pTau)-T181, pTau-S396, and Aβ42 levels in neuronal EVs discriminate AD patients from match-case controls. Moreover, EV levels of Aβ42 are an indicator of AD progression in preclinical cases (111).

The molecular characterization of EVs indicates that there is a basic set of proteins with a complex dynamic overtime, consisting of subtle changes in protein concentration and post-translational modifications. Mild cognitive impairment has been associated with lower levels of total Tau and APP and a higher ratio of pTau-T181/total Tau in comparison to controls. AD can be discriminated from healthy individuals considering the ratio of pTau-T181/total Tau, and from mild cognitive impairment considering the pTau-T181/total Tau ratio and the APP levels in plasma-derived EVs. In comparison to controls, the APP levels are lower in mild cognitive impairment and in mild and moderate AD, while high levels of APP characterize severe AD (112). These data indicate that the protein signature of EVs changes gradually during the development of AD. Other research groups have explored neuronal-derived EVs from plasma, the expression of transcriptional factors involved in neuronal defense against diverse stresses on AD patients. EVs of AD patients contain low levels of the low-density lipoprotein receptor-related protein 6 (LRP6), heat-shock factor-1 (HSF1), and repressor element 1-silencing transcription factor (REST). These transcription factors are diminished 2–10 years before the clinical diagnosis of AD (113).

In AD, the increased production of EVs is associated with the progression of Tau pathology (111, 114). The inhibition of exosome synthesis in microglia significantly reduces Tau propagation *in vitro* and *in vivo* (115). It is possible that EVs can contain different Tau species, including monomers, oligomers, and aggregates. However, only the aggregated forms can promote the aggregation of new Tau molecules. The release of EVs containing Tau is promoted by neuronal activity and thus may contribute to the spreading of Tau pathology through trans-synaptic transmission (116). EVs from the Tg4510 mouse (carrying the P301L Tau mutation) contain high levels of Tau with an altered pattern of Tau-phosphorylation (AT8, AT100, and AT180) that promote the formation of Tau inclusions. The P301L Tau-containing EVs transport Tau seeds, which are able

to induce the aggregation of endogenous Tau in recipient cells, supporting the active role of exogenous seeds in nucleating nascent Tau inclusion through vesicle transport (117). Genome-wide association studies suggest a strong connection of late-onset AD with Bridging INtegrator 1 (BIN1), which is involved in endosomal trafficking (118). In animal models and CSF of AD, BIN1 is related to Tau secretion *via* EVs and could contribute to Tau pathology by altering Tau clearance and promoting the release of Tau enriched EVs by microglia (119). The characterization of EVs from human iPSCs, CSF, and plasma shows that the main type of Tau inside of EVs is the full length form, suggesting a dysfunction of autophagy (120). The presence of P301L and V337M Tau mutations in iPSC dysregulates the EV proteome. Some of the proteins exclusively present in mutant Tau-EVs have been reported to participate in cellular processes related to synaptic dysfunction, memory loss, and neuropathology. Remarkably, mutant Tau-EVs contain ANP32A, which is an endogenous inhibitor of protein phosphatase-2A (PP2A) that in turn regulates the dephosphorylation of Tau (121). Therefore, common mutations related to AD pathogenesis that alter endolysosomal trafficking may result in a noxious molecular cargo in EVs that contributes to the spread of toxic protein aggregates.

One of the major genetic risks associated with the development of late-onset AD is the allele ε4 of the apolipoprotein E (APOE4). The expression of APOE4 is associated with decreased levels of EVs in the human brain and in APOE4 transgenic mice. The exosomal biogenesis pathway seems to be compromised by the presence of APOE4, as indicated by the downregulation of the transcription and translation of Tsg101 and Rab35 in the humanized APOE mice (55). Additional effects related to APOE4 include enlarged endosomes, cholesterol accumulation, and increased secretion of Aβ42 (55, 122). The APOE4 genotype may contribute to the disruption of endosomal–lysosomal system function, probably due to disturbances in lipid metabolism that increase neuronal vulnerability over time. The exact mechanism has not been resolved, but it could involve a vicious cycle between astrocytes and neurons that ends in an inability of Aβ42 clearance and altered EV molecular cargo. A mass spectrometry analysis of the EV content from a mixed co-culture of mouse primary astrocytes, neurons, and oligodendrocytes found that the exposure to Aβ42 protofibrils altered the levels of ApoE, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase), heavy chain 1, 60S ribosomal protein L4, and cytoplasmic dynein 1 heavy chain 1 proteins (123). Previously, the same group showed that the inability of astrocytes to clear Aβ leads to the increased release of truncated forms of Aβ in EVs, thus inducing neural apoptosis (124). In line with these studies, an altered EV generation has been proposed from mass spectrometry analysis of neocortical brain tissue samples from AD patients. A pathway enrichment analysis revealed changes in proteins related to exocytic and endocytic pathways. Interestingly, among the proteins with higher levels in AD compared to control include EV-related markers such as CD9, HSP72, PI42A, TALDO, and VAMP2 (125). EVs from CSF and plasma of AD patients and AD mouse models expressing a presenilin-1 mutation show an increased Aβ42/Aβ40 ratio and impair Ca2+ handling and mitochondrial function (126). These findings suggest that distorted intercellular communication may contribute to neuronal dysfunction and highlights the role of glia in maintaining a healthy neuronal state.

Recent evidence also indicates the potential role of EVs in the transport of APP and its catabolites (CTFs). Mouse N2a cells expressing the Swedish mutation of human APP show a differential loading of CTFs into EVs. The Swedish mutation enriches CTF-α and CTF-β, but not CTF-η, into a subset of EVs that lack CD63. This subpopulation of EVs carrying CTFs only bind to dendrites of neurons, while $CD63^{+}$ EVs are targeted to both neurons and glial cells (59). These changes in composition and cellular selectivity may result from an impairment of the endolysosomal function that modifies lipid composition, membrane proteins type, and protein glycosylation. The chemical inhibition or genetic ablation of the kinase Vps34 results in endosomal abnormalities and autophagic blockage in primary cortical neurons and N2a cells (49). The EVs collected from these cultures are enriched in CTFs, in cholesterol and sphingolipid subclasses (dihydrosphingomyelin, monohexosylceramide, and lactosylceramide), and in the phospholipid bis(monoacylglycerol)phosphate. Furthermore, the global protein glycosylation is modified in specific brain regions of AD patients, and some of these changes are reflected in serum samples (127). An abnormal protein glycosylation pattern has been associated not only with AD development, but also with a differential cellular uptake of EVs (128–130). The fact that APP-CTFs and Aβ are selectively sorted into a subpopulation of EVs and are specifically endocytosed by neurons suggests the mechanism in which EVs contribute to the spread of pathological fragments throughout the brain. In agreement with this notion, EVs isolated from human iPSC-derived neuronal cultures harboring the A246E presenilin-1 mutation can induce Tau hyperphosphorylation. Although the mechanism remains unknown, the injection of these EVs into the CA1 region of the mouse hippocampus generates Tau aggregation in the hippocampus after 5 weeks (58, 131). Likewise, EVs from mice containing Tau with the familial dementia mutation P301L accelerate Tau phosphorylation and soluble aggregate formation *in vivo* (132). These findings support the role of EVs in the spreading of Tau pathology in AD, but long-term studies are required to determine the intracellular mechanism by which EVs induce Tau phosphorylation and whether there is a direct participation in neurofibrillary tangle formation.

A dysfunctional endosomal system is an early feature of neurons of individuals with Down syndrome (DS), which includes larger endosomes and an accumulation of APP metabolites in endosomal vesicles. EVs isolated from the brains of DS patients, from the Ts2Cje mouse model of DS (Ts[Rb(12.17(16))]2Cje), and from human DS fibroblasts are enriched in APP-CTFs. The Ts2Cje model revealed that APP-CTFs levels in brain EVs increase in an age-dependent manner considering a period of up to 24 months of age (133). An electron microscopy study of the neuronal endosomal system of Ts2Cje mice shows that MVBs are larger, more abundant,

and contain a higher number of intraluminal vesicles (ILVs) compared to controls. The biochemical analysis of Ts2Cje brain endosomes revealed an enhanced content of Rab5b, Rab7, CD63, Tsg101, and Rab35, suggesting an alteration in the biogenesis and secretion of EVs (54, 134). The up-regulation of the endosomal–EV pathway could be a homeostatic mechanism to improve endosomal dysregulation in NDs. In this regard, the brain-derived EVs from 3xTgAD mouse (APP Swedish mutation, Tau P301L, and Presenilin 1 M146V) or from neuroblastoma cells show an accumulation of APP-CTFs induced by the inhibition of the γ-secretase, a molecular complex involved in Aβ peptide generation. This seems to be a consequence of a defective retrograde transport of APP-CTFs from the endosomal compartments to the trans-Golgi network. The monomeric forms of APP-CTFs were mainly localized in the trans-Golgi network, whereas oligomeric forms were confined to endosomes and lysosomes, explaining the selective recovery of APP-CTFs in EVs (135).

The role of brain insulin resistance in the development of AD has been widely documented. Thus, the level of phosphorylation of the adaptor insulin receptor substrate (IRS)-1, a protein associated with the insulin signaling status, has been considered as a biomarker of cognitive changes. In a cohort of AD and diabetes mellitus, the ratio of phospho-serine 312-IRS-1 to pan-phospho-tyrosine-IRS-1 in neuronal EVs from plasma was found to discriminate AD cases from diabetic and control individuals. This insulin resistance index is higher in AD than in diabetic patients (136). Interestingly, the abnormal phosphorylation of IRS-1 could predict the development of AD up to 10 years before clinical manifestations and correlates with cognitive performance especially in APOE4 non-carriers (137). Moreover, the pS312-IRS-1/p-tyrosine-IRS-1 ratio in neuronal EVs has also been associated with the degree of brain atrophy in AD patients due to the volume of brain regions showing the presence of pS312-IRS-1, as assessed by magnetic resonance image (138).

Vascular pathology is considered an early event of AD pathogenesis. The reduction of blood flow affects the content of brain-derived EVs from mice subjected to bilateral common carotid stenosis. A mass spectrometry analysis of these EVs revealed the appearance of proteins implicated in neuroprotection (glutathione peroxidase and serine protease inhibitor A3), hypoxia (agrin and myosin light chain kinase, and EGF-containing fibulin-like protein), angiogenesis (angiomotin), and AD pathogenesis (sortilin-related receptor and PACS2). Similar groups of proteins are upregulated in EVs derived from brain tissue of preclinical AD and AD with cerebrovascular disease, and in EVs derived from the serum of mice subjected to blood flow reduction to the brain (139). These findings reinforce the notion that early brain damage could be monitored in the peripheral fluids, as has been recently documented in an analysis for the identification of markers for neurocognitive impairment. The proteome of CSF -derived EVs from HIV-positive individuals with neurocognitive disorders showed an enrichment of exosomal markers like Alix, Syntenin, tetraspanins, ARF, Rab proteins, and heat-shock proteins, as well as proteins related to synapses (NPTN, NRXNs, NPTXs, and SYN1), immune/inflammatory response (ANXA, CRP, DPYSL2, ENO1, EZR, and TIMP), stress response (GST, HSPs, PARK7, PRDX, SNCA, and SNCB), mitochondrial functions (ACOT, DNM1L, DNPEP, GLUD1, RAN, and VDAC), and the blood–brain barrier (GFAP, GLUL, AGRN, AQP1, AQP4, DAG1, FBLNs, and NIDs) (140). A chronic inflammatory environment has been considered a factor underlying early events of neurodegeneration associated with the progression of dementia. Advanced glycation end products such as N-(1-carboxymethyl)-L-lysine increase their formation during chronic inflammation, and their levels in serum-derived EVs can differentiate early to moderate AD (141).

The development of new and improved methodologies has enabled large-scale proteomics studies to characterize EVs obtained directly from brain tissue of animal models and AD patients (142, 143). This kind of study allows the analysis of the EV composition while preserving the tissue microenvironment and without physiological alterations associated with cell cultures. The proteomic analysis of the EVs isolated from frozen brains of mouse AD models have shown that their contents change along with the development of histopathological and behavioral hallmarks of AD. In a mouse containing mutations in the APP and PSEN1 genes (5xFAD), the EV proteome reflects complex changes in protein abundance that involve both the increase and the decrease of various components differentially at 2 and 6 months of age. For instance, at 2 months, the proteins neuromodulin (Gap43), microtubule-associated protein 2 (Map2), glia maturation factor beta (Gmfb), and oxidation resistance protein 1 (Oxr1) are enriched in the EVs of AD mice. However, at 6 months, these proteins are markedly decreased in the EVs of AD mice (143). A proteomic profiling of human brain-derived EVs suggested that EV biogenesis might be altered in preclinical AD as indicated by an increment of MHC class I levels. In addition, EVs of preclinical cases are enriched in proteins that indicate the activation of immune response (SSBP1, PAF, and CD90), a modulation of synaptic structure (NLGN3), and a dysregulation of mitophagy (GABARAP). The progression of AD is characterized by an alteration in lysosome dynamics (LAMP1), an upregulation of amyloid associated proteins (APP, PrP, and ENPP2), and an induction of neurite regeneration (GAP43) (144). A similar analysis of brain-derived EVs from diagnosed AD cases revealed an increase of Aβ42 and pS396-Tau levels in AD samples as determined by ELISA. The label-free proteomic comparison of these EVs identified a set of differentially expressed proteins in the samples from the brain of AD patients, including APOE, SNCA, ANXA5, MITCH2, GPM6A, VGF, and ACTZ. Interestingly, ANXA5 shows a positive correlation with Braak stages (145). As can be appreciated from these studies, there is not an exact match of the modifications of the EV cargo. Moreover, the central core of proteins remains constant between healthy individuals and patients. This highlights the importance of the protocols employed to isolate different subpopulations of EVs and the inclusion of quantitative

approaches to detect subtle changes in the abundance of key proteins.

CURRENT ADVANCES AND CHALLENGES IN PROTEOMIC CHARACTERIZATION OF EVs

During the last two decades there have been numerous efforts directed to determine the protein composition of several biofluids in health and disease. One of the main challenges in the molecular characterization of any biofluid is that the difference of concentrations between the most and the least abundant proteins can cover more than 10 orders of magnitude (146, 147). Currently, the most common and efficient method for studying complex protein mixtures is mass spectrometry; however, the limit of detection of modern instrumentation is up to five orders of the proteome dynamic range (148). This introduces a bias where only abundant proteins are confidently detected, while low-abundant proteins escape identification. Over the past few years, several strategies including labeling techniques, fractionation protocols, acquisition methods, and bioinformatic pipelines have been implemented to increase the resolution of proteomic analysis and improve quantification of proteins in clinical samples. As a result of this refinement of the mass spectrometry analysis, the compendium of plasma proteins went from 280 identified proteins in 2002 to 3,509 proteins in 2017 (147, 149). Nevertheless, there are still limitations in finding consistent biomarkers, especially in early phases, for AD and PD. In this regard, EVs proteomics, especially from plasma, represents a promising strategy to enrich relevant molecules based on the fact that its cargo reflects the physiological state of a cell population. To further advance the discovery of biomarkers for NDs, there is a need for the standardization of protocols for EV isolation to assure reproducibility in the measurement of altered proteins.

Recent advances in the EV field have shown that there are diverse routes involved in their biogenesis, and potentially each vesicle subtype can indicate the dysfunction of a cellular process. A challenge remains in designing strategies to efficiently separate EV subtypes to identify pathogenic cargo. Most of the protocols available to isolate EVs rely on the size and density of vesicles. The proteomes of EVs from different cell sources and biofluids obtained by ultracentrifugation, precipitation, size exclusion chromatography, or ultrafiltration display components that are not necessarily EV components. The use of bottom-loaded density gradients has served to separate soluble components previously thought to be part of EV cargo such as histones, ribonucleoproteins, Argonautes, and major vault protein (51). Furthermore, the identification of proteins sensitive to trypsin digestion combined with a systems biology approach has helped to gain an insight into the real components of EVs, suggesting the presence of contaminants even after the combination of several methods of isolation (150). Particularly for CSF- and plasma-derived EVs, the elimination of non-vesicular components should be addressed in the future as a great proportion of the proteins identified in mass spectrometry studies include IgGs, coagulation factors, histones, and complement factors. The successful recovery of brain-derived EVs is one step forward in favor for the detection of low abundant proteins that could be relevant for biomarker discovery (77–79).

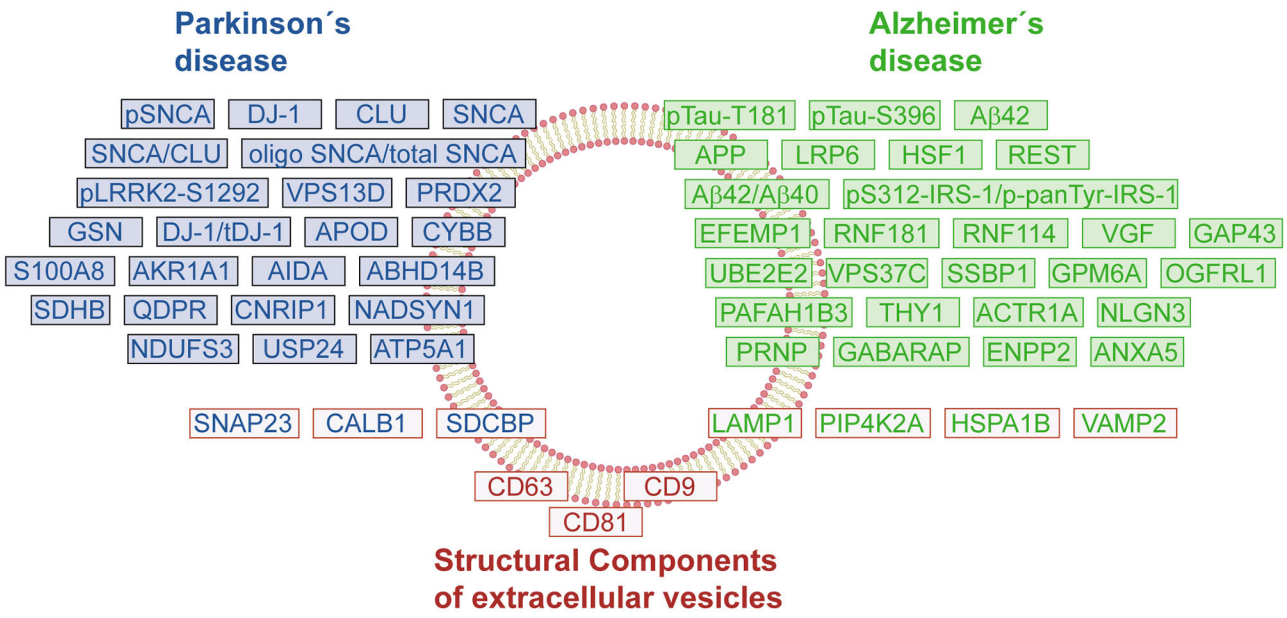

FIGURE 2 | Protein signature of EVs in Parkinson's and Alzheimer's disease. The schematic representation shows a compilation of changes in the protein content of EVs under pathological conditions. The proteins/genes were classified by groups: Parkinson's disease (PD, blue boxes), Alzheimer's disease (AD, green boxes), and endolysosomal pathway-related proteins or structural components of EVs (red boxes). The color-coding of protein names follows the same classification criteria.

A general conclusion of the studies involving the proteomic analysis of EV samples from AD and PD patients is that there is a big overlap of proteins between healthy individuals and patients. Only a few dozens of proteins are exclusively found in the disease state, at least with the resolution of current methods. This highlights the importance in developing quantitative proteomic approaches with enough sensitivity that will not only register an alteration in the concentration of certain components but also allow for patient stratification (151, 152). A simple but powerful modification in the protocol of EV isolation accompanied by an exhaustive fractionation of the samples with nano-ultra-high-performance liquid chromatography has proved to impact the resolution of mass spectrometry analysis. Over 5,000 proteins related to the biogenesis and function of small vesicles were identified in an analysis of EV-derived from plasma obtained at low-speed centrifugation (20,000 × *g*) (153). These findings open the possibility to analyze other clinical samples with the same strategy and to focus on determining the tissue origin of EVs. As we detailed in this review, alterations in the phosphorylation and glycosylation of certain proteins is an aspect to be included in future mass spectrometry analysis due to its relevance in development of neurodegeneration. We envision that the study of post-translational modifications would accelerate the discovery of cellular pathways altered in the development of PD and AD.

CONCLUSIONS AND PERSPECTIVES

There is a growing interest in the proteomic analysis of EVs due to their potential to transport biomarkers indicating the development of various chronic and degenerative diseases. The recent technical advances in proteomics have accelerated the characterization of EVs, revealing that their cargo reflect the cellular changes that occur in physiological and pathological conditions. Recently, several research groups have begun to elucidate the role of EVs in the progression of AD and PD. At a cellular level, both diseases present alterations in the interaction between endosomes, lysosomes, and mitochondria, which are considered as early events of neurodegenerative processes. The dysfunction of these organelles affects the biogenesis and release of EVs by the cells in the brain. Interestingly, several genetic risk factors of NDs involve proteins implicated in the endosomal and lysosomal trafficking. The disturbance of the endolysosomal system alters normal intracellular trafficking and organelle turnover. These events are reflected in impared degradation of proteins and alterations in protein sorting (**Figure 1B**). Overall, the cell ability to handle cellular components and waste is compromised, altering the molecular content of EVs.

To further promote the use of EVs as biomarkers of NDs, it is necessary to develop standardized isolation protocols that ensure the quality and purity of the EV preparations. Moreover, considering that a central core of proteins remains constant between healthy and diseased individuals, the characterization of the different subpopulations of EVs is essential to identify those vesicles relevant for diagnosis purposes, and to follow the progression of NDs. During the discovery and verification phases, it will be key to correlate the EV cargo (protein, RNA, or metabolites) with relevant clinical data. The current discoveries concerning EV proteomes have already generated a list of interesting protein markers and post-translational modifications that should be corroborated in large cohorts (**Figure 2**). It is expected that in the following years, more candidates will be available that allow a differential diagnosis between different NDs. The EV proteins can also be useful biomarkers to follow the therapeutic response in drug trials (154). In the future, the development of new technology that provides enough sensitivity, wide dynamic range, and reliable quantification without an enrichment step of EVs would be desirable (155).

From a mechanistic point of view, a remaining challenge is to determine whether the reported changes in the content of EVs in AD and PD are a functional mechanism of communication or an indirect effect of the impairment of the management of cellular waste. This will help in the understanding of how the process of neurodegeneration starts and will give an insight into possible alternatives to halt the development of NDs. In this regard, it will be useful to perform cross-sectional studies to determine the molecular content of EVs at different ages and consider peripheral sources of EVs to evaluate its influence on the function of the nervous system. An omic approach considering the association of EV cargo with blood-relevant analytes (e.g., inflammatory, metabolic) combined with brain imaging will help to build a strong diagnostic strategy.

AUTHOR CONTRIBUTIONS

RQ-B and KH-O wrote the manuscript and EM-M conceived and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Delayed Neurosurgical Intervention in Traumatic Brain Injury Patients Referred From Primary Hospitals Is Not Associated With an Unfavorable Outcome

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Background: Secondary transports of patients suffering from traumatic brain injury (TBI) may result in a delayed management and neurosurgical intervention, which is potentially detrimental. The aim of this study was to study the effect of triaging and delayed transfers on outcome, specifically studying time to diagnostics and neurosurgical management.

Methods: This was a retrospective observational cohort study of TBI patients in need of neurosurgical care, 15 years and older, in the Stockholm Region, Sweden, from 2008 throughout 2014. Data were collected from pre-hospital and in-hospital charts. Known TBI outcome predictors, including the protein biomarker of brain injury S100B, were used to assess injury severity. Characteristics and outcomes of direct trauma center (TC) and those of secondary transfers were evaluated and compared. Functional outcome, using the Glasgow Outcome Scale, was assessed in survivors at 6–12 months after trauma. Regression models, including propensity score balanced models, were used for endpoint assessment.

Results: A total of $n = 457$ TBI patients were included; $n = 320$ (70%) patients were direct TC transfers, whereas $n = 137$ (30%) were secondary referrals. In all, $n = 295$ required neurosurgery for the first 24 h after trauma (about 75% of each subgroup). Direct TC transfers were more severely injured (median Glasgow Coma Scale 8 vs. 13) and more often suffered a high energy trauma (31 vs. 2.9%) than secondary referrals. Admission S100B was higher in the TC transfer group, though S100B levels 12–36 h after trauma were similar between cohorts. Direct or indirect TC transfer could be predicted using propensity scoring. The secondary referrals had a shorter distance to the primary hospital, but had later radiology and surgery than the TC group (all $p < 0.001$). In adjusted multivariable analyses with and without propensity matching, direct

or secondary transfers were not found to be significantly related to outcome. Time from trauma to surgery did not affect outcome.

Conclusions: TBI patients secondary transported to a TC had surgical intervention performed hours later, though this did not affect outcome, presumably demonstrating that accurate pre-hospital triaging was performed. This indicates that for selected patients, a wait-and-see approach with delayed neurosurgical intervention is not necessarily detrimental, but warrants further research.

Keywords: traumatic brain injury, secondary referral hospital, pre-hospital management, human, neurosurgery

INTRODUCTION

Traumatic brain injury (TBI) is a considerable public health problem globally, and approximately 5.5 million people suffer a severe TBI annually (1). The estimated incidence in Europe is 262/100,000 of patients admitted to hospital, with an average-related mortality of 11/100,000 (2, 3) and many survivors living with life-long disabilities, resulting in substantial costs for society (4).

Actions taken in the pre-hospital setting focus primarily on preventing secondary insults, such as hypotension, hypothermia, and hypoxia, which have been shown to be detrimental to TBI patients, as they may aggravate secondary brain injury development (5–7). While it is possible to initiate resuscitation at the scene of accident (SoA), it is of key importance to reach a hospital for diagnosis and intervention (8). Furthermore, prolonged pre-hospital time, as well as long distances to the hospital, has been shown to be associated with unfavorable outcome (9–11). Transport destination is often based on local guidelines, where unconscious patients with deranged physiological parameters generally will be transferred directly to level 1 trauma centers (TCs), whereas patients with less severe injuries are taken to tertiary hospitals for diagnostic work-up (12, 13).

Many patients suffering from milder TBI will not require neurosurgical interventions and/or monitoring in neurosurgical departments (14) and, thus, do not necessarily need direct referral to a level 1 TC. However, in severe TBI patients (unconscious at the SoA), increased intracranial pressure (ICP) is present in up to 70% of patients (15), a central metric in the management of moderate-to-severe TBI patients requiring either monitoring or evacuation surgery (16). Studies have shown that TBI patients with delayed surgery have worse outcome as compared with those with early interventions (17, 18). Further, in patients where decompressive hemicraniectomy is warranted, the time to surgery might be of importance (19). Compared with patients who have rapid surgery, an un-operated patient could have an ongoing intracranial mass-effect and, thus, a longer burden of increased ICP and lower cerebral perfusion pressure (CPP), which have been shown to affect outcome (20). Thus, affected patients suffering from mass occupying traumatic lesions should benefit from transportation directly to a designated TC where rapid diagnostics and potential neurosurgical intervention may take place (21, 22).

In the literature, studies comparing direct vs. secondary transfers of TBI patients to appropriate TCs in large conclude that for secondary TC referrals, there is a delay in transfer times and longer dwell times in the emergency rooms (ERs) before patients are adequately managed (21–23), with some studies demonstrating that these delays result in worse outcome (24, 25). However, a systematic review showed no difference in mortality between patients who were directly transferred to TCs or to non-TCs (26). Furthermore, sending too many patients with suspected TBIs directly to TCs resulted in substantial over-triaging (27), with erroneous allocations of resources. This stresses the need for primary hospitals to perform diagnostic work-ups on the majority of TBI patients who are not in the severe category and might require neurosurgical care. However, in major trauma, not specifically TBI, potentially dangerous under-triaging occurs in about 11–22% (28, 29) of cases; thus, numerous patients are at risk of not getting the level of care they require expeditiously. While different delays have been shown between direct and secondary transfers, we have not been able to identify any studies that look specifically at delayed neurosurgical intervention (time from trauma to surgery) and its effect on functional outcome in the emergency setting.

Therefore, this study aims to primarily investigate the long-term outcomes between TBI patients who were primarily or secondarily transported to a neurosurgical unit, specifically looking at the time from trauma-to-surgery in the two groups.

MATERIALS AND METHODS

Ethics

The study received ethical approval from the Regional Ethical Review Board in Stockholm with reference numbers 2007/1113-31 (with follow-up amendments 2010/1979-32, 2013/1718-32, and 2014/691-32), as well as 2015/1675-31/1. The ethical review board waived the need for informed consent.

Study Design and Population

This was a retrospective observational cohort study. Inclusion criteria were: adults and late adolescent trauma patients (>14 years old) with existing pre-hospital charts, documented TBI on a head/brain computed tomography (CT) scan [International Classification of Disease (ICD)-10 S06.2–S06.9], and treated at the Department of Neurosurgery at the Karolinska University Hospital (KUH). Exclusion criteria were: patients admitted to the reporting hospital [TC or non-trauma center (NTC)] >6 h

after the trauma, cases where the reported time of the trauma was unclear or unknown, patients transferred to the KUH >24 h after admission to any other hospitals, or patients transported from another county to the KUH.

Patients were included during the period of 1st of January 2008 to the 31st of December 2014 in Region Stockholm, Sweden. This data set has previously been used to study the clinical efficacy of pre-hospital intubation (30).

Trauma Organization and Pre-hospital Data Collection

The structure of hospital trauma care in Scandinavia is similar to the American College of Surgeons trauma level system (31), a classification system for hospitals receiving trauma patients. This is based on the level of care that the hospital can provide where, e.g., a level 1 (highest care) hospital can provide neurosurgical and neurointensive care at all times. Region Stockholm consists of seven emergency departments and has a population of about 2.3 million. KUH is the only hospital in the region serving functionally as a level 1 trauma hospital.

In 2008, new pre-hospital guidelines were implemented regionally in order to better accurately triage of more severely injured patients directly to the TC (13, 32). The algorithms therein base TBI triage on known TBI outcome predictors that have been identified primarily on direct TC cohorts, including low Glasgow Coma Scale (GCS) (3–13, severe-to-moderate TBI), unresponsive pupils, hypotension (<90 mmHg), hypoxia (<90% saturation), or respiratory rate <10 or >29 per min, and penetrating injuries or presence of extracranial multi-trauma (13).

The studied region consists of approximately 6,500 km^2, including an archipelago of over 30,000 islands. At the time of the study, the organization of the emergency medical services (EMSs) in Region Stockholm included one publicly owned company and two private contractors coordinated by one Emergency Medical Communications Center. Between 07:00 and 20:00 (i.e., daytime), there were 55–61 ground ambulance and three rapid-response vehicles operating in the area (33). The ground-based ambulance crew consisted of an emergency medical technician (EMT) and a registered nurse. One of the rapid-response vehicles was physician-manned and the two others by a nurse anesthetist and an EMT. During nights, there was no physician on duty, and there were about 38 functioning ambulances in the area. Furthermore, the region also had one helicopter (and one additional helicopter during summer) manned by a nurse anesthetist and one mobile intensive care unit (ICU) for transfers between critical care units.

Clinical Variables

Data were extracted from the neuro-trauma registry at KUH. The pre-hospital network (CAK-net) used by all pre-hospital staff during the study period was used to extract the pre-hospital data. The ambulances are rigged with a global positioning satellite system (GPS) that supplies a GPS coordinate according to the SWEREF 99 (Swedish reference frame 1999) system (34).

Gender and age were extracted from hospital charts. Time from arrival at the scene to hospital arrival was extracted from the pre-hospital charts, as well as systolic blood pressure (SBP), respiratory rate, and GCS on the scene and during transport. The distances and time periods were defined as follows: distance from the SoA to hospital was defined in kilometers, and the time on the scene and the departure from the scene until hospital arrival were defined in minutes and seconds. The presence of multi-trauma was noted, defined as significant injury to any other major organ systems except the spine and head as per previous definitions (35). If available, it was noted if it was a high energy trauma as defined by the Advance Trauma and Life Support (ATLS) guidelines (36). At the SoA, GCS was recorded, and if not specified, "unconscious" patients were defined as GCS 3–8 (37). "Pupil unresponsiveness" was categorized as one or two pupils presenting without a light reflex. To evaluate the neuro-radiological damage, we assessed the primary CT scans according to Rotterdam CT scores (38), Marshall classification (39), and Stockholm CT scores (40). The Stockholm CT scores have been shown to exhibit best correlation to outcome; therefore, we used it in our analysis (41). Furthermore, head abbreviated injury score (AIS) [version 2005 update 2008 (42)] was noted, together with injury severity score (ISS) and new injury severity score (NISS). S100B, a protein biomarker of brain tissue fate (43), was sampled at admission and every 12 h at KUH. Samples from admission and after 12 h following TBI (as these samples have been shown to be less affected by external trauma) were registered (44, 45). Survival status and 30-day mortality were noted, as well as the length of stay in the critical care unit. Surgical intervention was included and defined as the primary surgical intervention ICD code. Long-term outcome was assessed by clinic visits and questionnaires concerning health-related quality of life and was used to extract functional outcome at 6–12 months using the five stages of the Glasgow Outcome Score (GOS) (37).

Statistical Analysis

In describing patient demographics, categorical data are presented as count (percentage) and continuous data as medians (interquartile range). Group-wise comparisons were conducted using χ^2/Fisher's exact test and Mann–Whitney *U*-test for categorical and continuous variables, respectively. For outcome predictions, multivariable logistic or else proportional odds regression was used with either dichotomized or the full-scale GOS (proportional odds) (using the "rms" package in R) (46) as a dependent variable. The proportional odds analyses are available as **Data Sheet 1**. The International Mission for Prognosis and Analysis of Clinical Trials in TBI (IMPACT) study group has previously shown that the proportional odds analyses are adequate to use in TBI cohorts (47), so even if the proportional odds assumption does not necessarily apply in our cohort, it could be considered an analysis of a larger group where these assumptions are true.

An aim of the study is to focus on signs and symptoms available first during the initial triage at the SoA in a cohort that is later deemed in need of neurosurgical treatment or surveillance and evaluate the initial decision-making and triage in relation to choice of hospital and later outcomes, also comparing this with

later available information at admission. Inherently, the patient cohort immediately transferred to the TC was likely to differ from patients secondarily transferred. We approached this expected confounding *via* two different analytical approaches. Firstly, we performed multivariable analysis adjusting for known predictors and potential confounders using variables in the IMPACT TBI model by Steyerberg et al. (48). This approach uses late available information, such as data from CT scans, to investigate if "secondary referral" was a significant predictor of outcome. Secondly, we employed propensity score estimation modeling where "secondary referral" was used as dependent variable in a logistic regression model. Here, we did *not* include metrics that could not be assessed at the SoA (such as radiological variables) or variables relating to hospital proximity, as those do not form part of the guidelines (48). This approach will inherently focus the analysis in the matched sample on an intermediate group that has similar presentation at the SoA but is triaged differently. For this, we utilized the pre-hospital guidelines available but extended our model somewhat (13). Independent variables comprised all pre-hospital vital parameters, neurological features that can be determined and were recorded at the SoA, and trauma characteristics. In the pre-hospital setting, GCS motor score at the SoA was frequently missing. Whenever we had a SoA GCS of four, we assumed the motor score to be two, representing the "best possible" motor score. Equivalently, when SoA GCS was 14, we assumed that GCS motor score was six. There was also limited documentation of pupillary reactions, prior to the arrival at the TC. For the final propensity score estimation, variables included were: respiratory rate, pulse, oxygen saturation, GCS, multi-trauma, low-energy trauma [according to the Utstein definition (49)], compromised airway, and age (where all variables except accurate age were possible to determine at the SoA, though the EMS would know if the patient was young or old). Using propensity scores, we created a balanced patient subset ($n = 128$ out of 275) using the MatchIt package in R (50), using a nearest neighbor matching algorithm. Following this, group balance was still not perfect when we added a maximum propensity score distance on the observations (51). Thereby, we obtained balanced groups, as deemed through inferential testing and graphical observation. The propensity scores are available as **Data Sheet 1**.

Due to missing data in several pre-hospital variables, we employed multiple imputation ($n = 7$) of our data using the "mice" package in R (52). For each imputation, we recalculated the inferential operations stated above, before pooling results estimate. Our propensity score estimation approach on the imputed data has been deemed superior in the context of inverse probability of treatment weighting as compared with other methods (53, 54). We employed this approach as it adhered with the idea of multiple imputation, but some caution is warranted when interpreting these results as it risks interfering bias. Finally, for each imputation, outcome analysis was calculated using proportional odds regression. For these analyses, we employed the MASS package in R (55), as it allows for pooling regression outputs of all imputed data. All statistical calculations were performed in the program R with the interface program R-studio (56).

RESULTS

Included Patients

During the inclusion period, a total number of $n = 738$ TBI patients were admitted to the KUH in need for neurosurgical management. A total of $n = 457$ patients met the inclusion criteria (see flowchart in **Figure 1**). Of those 457 patients, $n = 320$ (70%) patients were directly transported to the TC, whereas $n = 137$ (30%) patients were initially transported to another primary hospital before undergoing a secondary transfer to the TC.

Comparison Between Direct and Secondary Transfers

The descriptive analysis of the included patients is described in **Table 1**. The pre-hospital data suggest a tendency toward older patients more frequently being transferred to the non-TCs and the TC receiving younger, more unstable, and more highly injured patients (e.g., higher frequency of pre-hospital hypotension, unconsciousness, high respiratory rate, high trauma energy, multi-trauma, and low GCS). Hospital data showed that patients transported to the TC had a higher incidence of unresponsive pupils and higher ISS/NISS. While S100B levels at admission were higher in the TC, Stockholm CT score and peak levels of S100B sampled after the initial phase were similar between the two cohorts.

There was no difference in the hospital length of stay, whereas the ICU length of stay was longer in the group initially transported to the TC. Approximately 75% of the patients in both direct referrals and secondary transports had surgery performed. The primary surgical intervention differed somewhat between the two groups (**Table 2**), but neurosurgical hematoma evacuation surgeries and monitoring surgery dominated in both. The incidence of evacuated acute subdural and epidural hematomas (EDHs) was higher in the secondary transfer group. Insertion of intracranial monitoring surgery (ICD AAA99) was the primary initial surgery

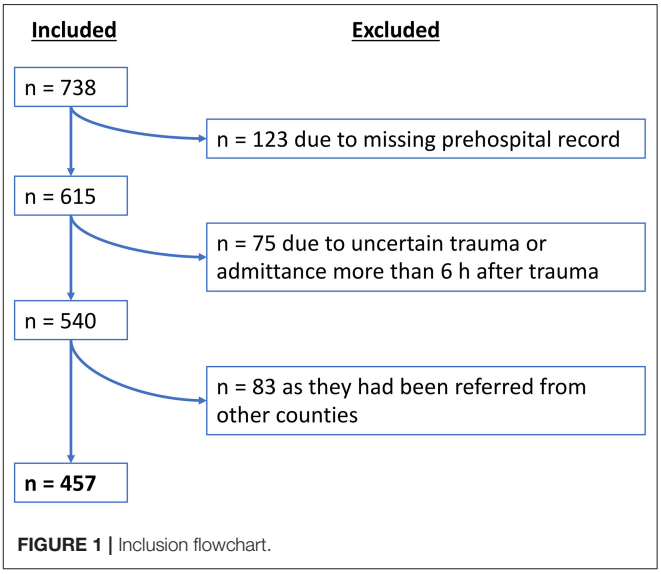

FIGURE 1 | Inclusion flowchart.

(**Supplementary Figure 1**), though often in conjecture with acute subdural hematoma (SDH) evacuation, but generating two separate sub-ICD codes. No unadjusted differences between the groups were seen in-hospital mortality or long-term GOS (**Table 1**).

Functional Outcome Between Transfer Groups

Multivariable logistic regression showed no significant difference in dichotomized long-term functional outcome (GOS) between the group that was transported directly to the TC compared with the group that was secondarily transported to the TC ($p = 0.140$ unadjusted, $p = 0.297$ adjusted for age, pupil responsiveness, admission GCS, and Stockholm CT score). Similarly, a proportional odds analysis, using all levels of GOS, showed a non-significant trend toward more favorable outcome in patients who were secondarily transferred ($p = 0.062$ unadjusted, $p = 0.32$ adjusted for the same parameters above) (**Supplementary Data 1**).

Transportation and GCS Dynamics

Patients transported directly to the TC had a similar total pre-hospital time (time from reported trauma to arrival at the hospital) as compared with patients not transported directly to the TC, 45 vs. 44 min, respectively. However, the on-scene time (29 vs. 26 min) and the distance (at average almost 4 km longer) were longer for the direct-to-TC transfers (**Table 3**). The time from trauma to initial CT scan (performed either at the TC or at the primary hospital) was at average approximately an hour longer for patients with non-direct TC transfers (**Table 3**).

GCS was already low in a majority of patients directly transferred to the TC (52%) (**Table 4**). The share of patients who were unconscious increased dramatically in the non-direct TC transfers from the primary hospital to admission at the TC (11–33%) (**Table 4**). While some represent "true" deteriorations, some may include intubations (or probably both).

Outcome Related to Delayed Surgeries

The direct transports had a median time from trauma to surgery of 3 h 39 min, whereas, patients with secondary transports

TABLE 1 | Patient demographics.

Variable	Direct transfer to TC *N* = 320		Secondary transfer *N* = 137		*p*-value	Adjusted *p*-value
	Data	Missing	Data	Missing		
Patient demographics						
Demographics						
Age (years)	47 (26–63)	0 (0)	56 (39–64)	0 (0)	0.010	0.20
Male	231 (72)	0 (0)	103 (75)	0 (0)	0.57	1
Pre-hospital data						
GCS SoA	8 (4–13)	0 (0)	13 (11–14)	3 (2.2)	<0.001	**<0.001**
Unconscious SoA	164 (51)	0 (0)	14 (10)	0 (0)	<0.001	**<0.001**
Hypotension SoA	10 (3.1)	51 (16)	0 (0)	45 (33)	0.070	1
Hypoxia SoA	34 (11)	43 (13)	3 (2.2)	47 (34)	0.014	0.28
High-energy trauma	98 (31)	144 (45)	4 (2.9)	106 (77)	<0.001	**<0.001**
Multi-trauma	117 (37)	0 (0)	14 (10)	0 (0)	<0.001	**<0.001**
Hospital data						
Pupil unresponsiveness	63 (20)	6 (1.9)	15 (11)	3 (2.2)	0.03	0.58
Stockholm CT score	2.1 (1.5–3.1)	0 (0)	2 (1–3)	1 (0.73)	0.015	0.30
S100B peak 12–36 h (μg/L)	0.3 (0.19–0.59)	69 (22)	0.2 (0.1–0.57)	52 (38)	0.017	0.35
S100B admission (μg/L)	2.1 (0.87–5.0)	136 (43)	0.34 (0.21–1.45)	114 (83)	<0.001	**<0.001**
AIS > 3	249 (78)	3 (0.93)	99 (72)	4 (2.9)	0.38	1
ISS	25 (17–30)	3 (0.93)	19 (16–25)	4 (2.9)	<0.001	**<0.001**
NISS	41 (27–50)	3 (0.93)	29 (24–38)	4 (2.9)	<0.001	**<0.001**
Surgery	251 (78)	0 (0)	103 (75)	0 (0)	0.46	1
Outcome data						
In-hospital mortality	37 (12)	0 (0)	13 (9.5)	0 (0)	0.62	1
NCCU LoS	6.0 (1.1–16)	0 (0)	1.4 (0–6.7)	0 (0)	<0.001	**<0.001**
TC LoS	16 (7–30)	0 (0)	8 (4–16)	0 (0)	<0.001	**<0.001**
Unfavorable GOS	131 (41)	0 (0)	46 (34)	0 (0)	0.14	1

Data are presented as median (IQR) or count (percentage) for continuous and categorical data, respectively. p-values were calculated using either Fisher's exact test (categorical data) or Mann–Whitney U-test (continuous data). Bonferroni correction was applied for multiple testing and is presented as adjusted p-values. Significant adjusted p-values in bold.
AIS, abbreviated injury score; CT, computed tomography; GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Scale; IQR, interquartile range; ISS, injury severity score; LoS, length of stay; NCCU, neuro-critical care unit; NISS, new injury severity score; SoA, site of accident; TC, trauma center.

TABLE 2 | Type of surgery performed.

Type of surgery	Direct to TC, 251 procedures	Secondary referrals, 103 procedures
Type of primary surgical intervention performed, divided by transportation type		
Evacuation of acute subdural hematoma	71 (28%)	47 (46%)
Ventriculostomy (external ventricular drain)	51 (20%)	8 (8%)
Placement of intracranial pressure device	46 (18%)	3 (3%)
Evacuation of epidural hematoma	31 (12%)	23 (22%)
Evacuation of traumatic intracerebral hematoma (contusions)	7 (3%)	6 (6%)
Revision of skull fracture	6 (2%)	5 (5%)
Revision of penetrating or perforating head injury	5 (2%)	4 (4%)
Microsurgical discectomy of the cervical spine	3 (1%)	0
Other neurosurgical interventions	14 (6%)	4 (4%)
Other non-neurosurgical interventions	17 (7%)	3 (3%)

commenced surgery at 8 h 47 min following the reported trauma (**Table 3**). A univariate analysis looking at a subgroup of patients with surgeries within the first 24 h of trauma ($n = 295$) actually suggests a significant positive correlation between better outcome and a more extended time between trauma and surgery ($p = 0.023$) (**Figure 2A**; **Supplementary Data 1**). This can be assumed due to confounding by indication. However, and more importantly, there was no increase in unfavorable outcomes as time-from-trauma to surgery progressed up until 24 h after injury. The early negative association of early surgeries toward outcome was maintained even when adjusting for the group that was secondarily transferred ($p = 0.043$). However, if adjusted for known TBI predictors (age, pupil responsiveness, admission GCS, and Stockholm CT score) and secondary transfer, time between trauma and surgery was no longer significant ($p = 0.431$). In an exploratory approach, we isolated subdural and EDH evacuation surgeries as these could be considered the most relevant to perform as early as possible after trauma, but the results remained similar (data not shown).

We executed propensity score analysis followed by multiple imputation and then re-did these analyses on imputed, matched samples. The multiple imputation data sets showed adequate distributions in comparison with available data, which were also supported by the imputation index sample and imputation quality plots (**Supplementary Figures 2–4**). Univariate proportional odds regression, pooled for all imputations, once again demonstrated that outcome assessed by GOS among the subset of patients operated on within 24 h did not depend on time to surgery (estimate = 0.0007, $p = 0.204$). Hence, this highlights inherent differences between the two groups that when accounted for using propensity score estimation did not translate to differences in outcome. **Figure 2B** shows the distribution of surgery initiation depending on if the patient was directly transferred to the TC or admitted as a secondary referral.

DISCUSSION

Despite differences ranging several hours between trauma and neurosurgery in patients directly transferred to TC and secondary transfers, we found that neither long-term GOS nor mortality was significantly different between the two groups. These results remained, before and after adjusting for known severity predictors, or propensity matching cohorts. Late deterioration and surgery appear, thus, to trigger adequate responses in this setting, and the longer time from trauma to surgery in the transfer group does not appear crucial in this study. A possible relevant metric to explore in future studies would be time from clinical deterioration to surgery, a potential outcome metric that could be affected by transfer times. Interestingly, the transferred group is a selected group that deteriorates as to require transfer and would be expected to do worse than variable adjustment from the initial (prior deteriorating) variables would predict. This does, however, not seem to be the case, again suggesting that late deterioration may be a slower process that can be handled adequately. In aggregate, we cannot identify secondary transports or delayed surgery to be associated with increased risk. However, this observational retrospective data set requires specific discussion.

Triage

Triage at the SoA will be responsible for major differences in the TC and transfer cohort. The patients who were directly transferred to the TC were younger, more often unconscious, hypotensive, and had abnormal pupillary responses. That these TBI patients are prioritized for direct transfers are in line with several other studies (21–23, 57) and follow established triaging guidelines implemented in the region (13). This is supported by an earlier analysis of this cohort (30), where triage concerning on site intubation closely followed local guidelines. Similarly, propensity scoring indicated that pre-hospital clinical variables were different between patients at the SoA. Our pre-hospital trauma guidelines stipulate that blood pressure, respiratory rate, mechanism of injury, and GCS should direct pre-hospital triaging (13, 32). In addition, we found that low-energy trauma, multi-trauma, and heart rate were important parameters. Even though this study does not allow for causation, it might be interpreted as an indication that additional factors may be usable in the pre-hospital setting. Aside from this, the triaging was performed in accordance with established guidelines, so while the amount of secondary transports was high, it is difficult to say if it is due to actual under-triaging or not. It should be acknowledged that

TABLE 3 | Pre-hospital transportation distance and times.

Variable	Direct transfer to TC N = 320		Secondary transfer N = 137		*p*-value	Adjusted *p*-value
	Data	Missing	Data	Missing		
Pre-hospital and hospital Δtimes and Δdistances						
Distance SoA to PH (km)	13 (6.5–25)	0 (0)	9.1 (3.5–15)	0 (0)	<0.001	<0.001
Time at SoA (min)	29 (23–42)	4 (1.3)	26 (20–37)	1 (0.73)	0.012	0.14
Time from trauma to arrival PH (min)	45 (35–61)	1 (0.31)	44 (36–60)	10 (7.3)	0.77	1
Time from trauma to CT (h, min)	1 h 22 min (1 8–1 h 43 min)	1 (0.31)	2 h 29 min (1 36–4 h 5 min)	1 (0.73)	<0.001	<0.001
Time to surgery (h, min)	3 h 39 min (2 31–7 h 30 min)	72 (23) (no surgeries performed)	8 h 47 min (5 25–16 h 10 min)	36 (26) (no surgeries performed)	<0.001	<0.001

Time and distance calculations are presented as median (interquartile range), unless otherwise stated. p-values were computed using the Mann–Whitney U-test, and subsequent Bonferroni correction was applied (yielding adjusted p-values).
CT, computed tomography; PH, primary hospital; SoA, site of accident.

TABLE 4 | Dynamics of Glasgow Coma Scale over time.

	Scene of accident	Primary hospital	Admission trauma center
Secondary referrals			
Median GCS (IQR)	13 (12–14)	13 (12–14)	13 (4–14)
Percentage GCS 3–8	12%	11%	33%
Missing (*n*, %)	14 (10%)	9 (7%)	6 (4%)
Direct referrals			
Median GCS (IQR)	8 (4–13)	NA	7 (3–13)
Percentage GCS 3–8	52%	NA	54%
Missing (*n*, %)	20 (6%)	NA	12 (4%)

GCS dynamics from scene of accident, primary hospital (for secondary referrals), and at admission to trauma center.
GCS, Glasgow Coma Scale; IQR, interquartile range.

the pre-hospital care of TBI in Europe is very heterogeneous, as is highlighted by data and results from the CENTER-TBI consortium (58, 59). Novel markers that could improve accurate triage are, thus, needed and could help design better triaging protocols in the future.

Despite generally correct triage, the AIS, as well as Stockholm CT score, did not differ between the two groups. As Head-AIS is assessed during the treatment period (and Stockholm CT score following the admission CT scan), and not on site, this is in line with that the arriving EMS observes and acts on clinical signs and measurements at the SoA that are incomplete as to distinguish TBI severity. This is also reflected in the propensity matching. Furthermore, both the AIS-derived indices NISS and ISS were higher in the direct transfer cohort, likely due to the higher degree of multi-trauma in the TC group. The S100B levels reflected an interesting pattern with early levels being markedly higher in the direct TC group. While this is presumably due to a higher injury burden from both intracranial and extracranial sources (44, 60), the group with secondary referrals had their samples acquired on average hours later, which also has been shown to result in lower levels due to rapid serum clearance in the absence of ongoing injury (61, 62). However, 12–36 h samples, better corresponding to the brain injury (44), were relatively similar between the two groups, suggesting again that the cerebral injury burden was perhaps more similar for the patients in need of neurosurgical management for the first 24 h after injury, but not identifiable during early triage. Additionally, GCS decreased among some patients in the secondary referral cohort while at the primary hospital. It is possible that sedation was initiated, and that intubation was performed, in order to prepare for surgery, hence resulting in a GCS of three when arriving at the TC.

Our interpretation is that while the group directly transferred to the TC had more severe symptoms at the SoA, the fact that AIS, Stockholm CT score, and S100B were not significantly different between the groups shows that the radiological and biochemical aspects of the injury were similar when the admission CT was performed. Another possibility is that the older cohort that later needed secondary transfer to the TC had similar injuries in terms of mass lesions size and severity, but they were less affected neurologically by them at the SoA. Though, the delay at the primary hospital from trauma to CT scan could also play a role as lesions may have significantly progressed.

This study illustrates a good example of using a CT scoring system and brain tissue damage markers to establish a more objective baseline stratification between two cohorts, showing that while different in symptomatology, the cohorts shared similarities related to their intracranial pathology. It also demonstrates that serial measurements of S100B and other markers could be used to monitor cerebral deterioration (62), although currently, this is only performed at the TC in our region.

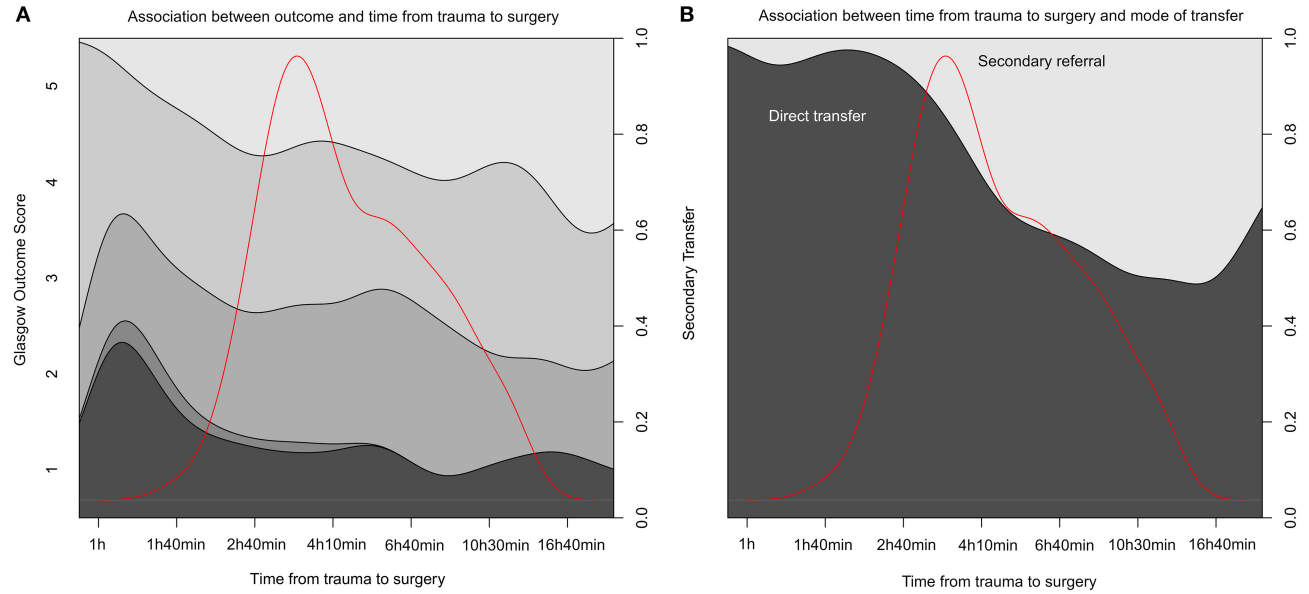

FIGURE 2 | Time from trauma to surgery and association to outcome and transportation mode. **(A)** A conditional density (CD) plot of the different stages of Glasgow Outcome Score (y-axis left) over time between trauma and surgery (x-axis) for the first 24 h after injury. y-Axis right summing the proportion to one. **(B)** A similar CD plot of the cohort divided by transportation mode (direct or secondary referrals) on y-axis left. The red line in both graphs illustrates data distribution. Data is logged to visualize it more clearly.

In summary, direct transfers were performed on patients who were deemed to have more severe injuries at the SoA, but patients in need of neurosurgical intervention deteriorated and had similar intracerebral injuries during the first 24 h. In aggregate, the cohorts are intrinsically different as expected by triage, and as such, multivariable adjustment and propensity matching are required to match and compare groups.

Secondary Transfers

Secondary transfers appear relevant as nearly 75% of the patients, in both groups, eventually needed surgical interventions of some kind. While ultimately suffering from brain hemorrhages requiring surgery, many of the secondary referrals did not present with signs of severe injuries at the SoA and, thus, had progressive injuries that were later detected at the non-TCs. An appealing hypothesis is that among a small subset of low-energy trauma cases, injury progression occurs slowly throughout the course of stay at the non-TC. This emphasizes the need to closely monitor initially conservatively treated TBIs, as many will be eligible for delayed surgery.

We could not see that secondary transfers had worse outcome, contrasting some of the literature. In a systematic review of pre-hospital time's influence on outcome in trauma patients by Harmsen et al. (10), two studies from Dinh et al. (63) and Tien et al. (64) are highlighted. The former notice a survival effect in patients arriving >1 h 30 min, whereas the latter see that patients referred within 1 h (previously referred to as the "golden hour") have a better outcome than patients arriving later. An additional study by Hartl et al. showed that severe (GCS 3–8) TBI patients, not transferred to a TC, had 50% higher mortality than direct TC transfers (24). Prabhakaran et al. noted similar dangers with delayed transfers, highlighting risks with prolonged pre-hospital times and subsequent dwell times in non-TC emergency departments (25).

However, similar to the systematic review on pre-hospital strategies in TBI by Pickering et al. outcome in our study was not affected if patients were transported direct to a TC or not (26), which we believe could be due to a number of factors. First, the time duration from reported trauma to hospital arrival was relatively short for both the direct and secondary referrals (45 and 44 min, respectively), with no patients even in the interquartile range falling outside 1 h. In previous studies from this cohort and northern Sweden, secondary insults during these short periods do not seem to affect outcome to the degree earlier thought (30, 65). Swedish pre-hospital data are also unique in that we report time from the reported trauma, whereas other studies most commonly report time from when EMS arrives at the scene to hospital arrival, underestimating the time since the trauma occurred. Second, almost exclusively, all "severe" traumas were directly transferred to the TC (looking at trauma energy level, presence of multi-trauma, and GCS); thus in comparison with Hartl and Prabhakaran (24, 25), we had very few patients who were severely injured that were not directly transferred. In the review by Harmsen, it is also difficult to establish exactly what non-TCs could and could not provide for the patients. While referring emergency hospitals in the Stockholm region do not have neurosurgery departments, all have a general surgeon and an anesthesiologist present in the ER when the ambulance arrives. Thus, immediate resuscitation measures may be undertaken and intubation performed, if necessary, prior to the CT scan. The

study by Prabhakaran et al. also indicated that prolonged time on scene seems to be associated with an unfavorable outcome (25). In contrast, in a study by Kim et al. studying general trauma (a majority were TBI patients) from South Korea, they found that longer on-scene time significantly decreased mortality (66). This highlights that it is difficult in retrospect to analyze this, as the EMS on scene will do what is necessary in order to stabilize the patient for transportation in varying situations and settings. Age is another important aspect of this study. In TBI, older age is a key independent predictor of poor functional outcome (67, 68). The secondary transferred patients in this study showed a higher median age of 56 (vs. 47 in the TC cohort). This might contribute to the non-significant difference of poor outcome in our material, as we are comparing more severely injured, younger multi-trauma patients with older, isolated brain injury patients. However, we adjusted for this in our propensity score estimation model, why we do not believe this to cause any major residual confounding.

In aggregate, the analyses showed no outcome difference between the TC and non-TC cohorts, hence indicating correct triaging but could also be due to rapid transfer times in general and escalated therapy measures even in the non-TC cohort.

Timing of Surgery

In addition to secondary transfers, we did not see that delayed neurosurgical interventions affected functional outcome in our TBI cohort. The relevance of time from trauma to surgery is difficult to explore in a retrospective study and presumably unethical in a prospective randomized study. However, historical TBI cohorts from the 1970s and 1980s showed that mortality increases from 30–50% to 80–90% after 4 h for acute SDH and from 17 to 65% after 2 h for EDH (69, 70). Clearly, advances in trauma management have been done since, including improved pre-hospital resuscitation, logistics, neurosurgery, and rapid diagnostics. Still, due to underdeveloped pre-hospital systems, studies from Tanzania and Uganda in recent years report high mortality (around 50%) where time from trauma to surgery can take days (17, 71). This emphasizes that delayed neurosurgical intervention for patients in need of rapid surgery is still a global problem that needs to be acknowledged. The more intricate infrastructures of modern trauma regions may make it difficult to evaluate and compare strategies. Our data seem to suggest that critical patients with more severe injuries are operated on rapidly, whereas patients less affected by their injuries are not rushed to the same extent.

That delayed surgery does not seem to increase unfavorable outcome appears in line with Joosse et al. (21). The time 2 h 30 min was also the average time from ED arrival to surgery for emergency craniectomies in a Canadian study from 2016 in patients directly transferred to the TC (72). This highlights that our median time from trauma to surgery of 3 h 39 min (ours including pre-hospital time) is within what could be considered normal for similar trauma cohorts. That time from trauma to surgery did not affect outcome is also in line with the results from Fountain et al. who noticed no difference in outcome following acute SDH surgery vs. time from trauma (>1 h 30 min or not) over a period of 20 years (73). It should also be noted that in comparison, our cohort consists not only of critical, unconscious TBI patients, in which case transportation and management is presumably performed in greater haste. In fact, the secondary transferred group had a median GCS more equivalent with mild TBI (mTBI) cohorts, and there is still scarce evidence about the long-term outcome for mTBI requiring neurosurgical interventions at a later stage. One study from Tierney et al. (18) found unfavorable neurological outcome (GOS score <4) in 44% of patients with mTBI, especially in mild TBI patients with delayed surgery. This is markedly higher than previously reported poor outcome rates (of 25%) in TBI patients (67) and is suggested to be due to anti-coagulant use and high GCS scores despite high Head-AIS/ISS in that specific study (18). In our study, the median time from trauma to surgery for the secondary transfers was more than 5 h longer (8 h 47 min), and as no difference in outcome was seen, this indicates that for selected patients, delayed surgeries with a wait-and-see approach are not necessarily detrimental. Presumably, adequate monitoring, including vigilant staff in referring hospitals providing optimized medical treatment to the patient, and an on-going consultation with the neurosurgical departments will appropriately triage correct patients for transport and surgery.

Altogether, we found that the delayed surgeries in secondary transports to the TC did not result in more unfavorable outcomes, presumably as appropriate pre-hospital management and triage systems were applied adequately, sorting correct patients to the correct level of care and identify deterioration and need of transfer in a timely manner.

Limitations

The retrospective design is a natural limitation. Some data were missing, which were imputed in the multivariable analyses. While this could result in less robust analyses, we only draw conclusions from findings that we believe are highly significant from a statistical standpoint. Additionally, for the propensity scoring, the imputed data showed adequate distribution in the imputation index sample and imputation quality plots (**Supplementary Figures 2–4**), supporting the imputations. A majority of the missing data were centered around pre-hospital ambulance charts concerning blood pressure, respiratory rate, and oxygen saturation at the SoA (**Data Sheet 2** and **Supplementary Figure 5**). It is difficult to assess if these are missing at random. Presumably, milder traumas with higher GCS are more likely to have this data missing as these are not as important to monitor, whereas in more critical patients being actively resuscitated, these will be targeted metrics and, thus, monitored more frequently.

Another key limitation by study design is that our database used for this study only includes patients who were managed by the neurosurgical department, thus patients where a neurosurgical intervention was deemed to be futile (either a too severe injury not associated with survival or an injury too mild to benefit from patient transport to the neurosurgical unit) were not included. The finding that very early surgery was associated with more unfavorable outcome (as seen in **Figure 2**) could be due to that some very severe cases (e.g., in young patients) were transported to the TC directly and everything was attempted,

including surgery, to save them despite knowledge of a probable poor outcome. The database we used also lacks the TBI patients who never needed neurosurgical management or monitoring, i.e., with lesions that could be monitored conservatively at non-TCs. While this would constitute quite an extensive group in terms of sample size, we believe that it is more important to study patients who truly did deteriorate to the extent where neurosurgical intervention was needed.

Moreover, by definition, patients triaged to a non-TC should be less severely injured than those immediately triaged to the TC. This hampers the statistical approach, since the two groups for comparison are inherently different. In order to compensate for this, we employed propensity score modeling. This would allow us to better validate pre-hospital triaging routines, as well as our outcome analysis results. This method has been suggested to be able to compensate for approximately 90% of confounding in similar studies (74). In total, we believe this to be the most solid approach to this type of research, since it is ethically inappropriate to design a prospective study where surgery is delayed in a randomized fashion in severe TBI patients.

From the data available, we could see that while 12% reaching the non-TC primary hospital were unconscious, about 33% of this cohort was unconscious upon arrival at the TC, but more accurate details of how and when deterioration occurred were not included in the data. Deterioration of intracranial pathology has been reported to occur in up to 50% of cases (75). It is probable that both injury and clinical status deteriorated over time so that a new more accurate decision based on the current state of the patient could be made. For future studies, as previously mentioned, it would be of key interest to obtain data on all secondarily transferred TBI patients who had CT-verified intracranial injuries, in order to find if any early clinical variables are predictive of subsequent/delayed injury progression, in order to find "high-risk" patients who initially are conservatively managed. This information should include information such as comorbidities and anti-coagulants, unfortunately not available in this study, as they may play an important role in the pathophysiology of neurological deterioration (76). We hypothesize that serial protein biomarker monitoring, in addition to clinical signs and radiology, may play an important role here, as the TBI field currently is developing a panel of protein biomarkers, suitable at different stages following the trauma (62, 77). Additionally, due to the retrospective nature of the study, it is unknown if any delay was due to the patient not needing immediate surgery in contrast to if there were logistical difficulties or iatrogenic delays in patients reaching the TC in time.

We did not include the use of helicopter transport in our study, which of course would have facilitated the transportation and hence decreased the pre-hospital time. Our experience of helicopter use in this cohort is that transport will be escalated to helicopter transport if deemed necessary due to long distances (30). Further, while an ambulance might have been used for transport to the primary hospital, helicopters were sometimes used for the secondary transfers, making it difficult to study the direct benefit of them in the current study setting.

CONCLUSIONS

Despite significant delays until neurosurgery could be initiated, this study shows no difference with regard to long-term functional outcome between TBI patients directly or secondarily transferred to the TC in a large urban area. While patients were more rapidly admitted to the nearest primary hospital, there were significant delays in trauma to CT and surgery times. The pre-hospital decision-making regarding transport destination was presumably correct in large, as more severely injured patients were transported directly to the TC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Ethical Review Board in Stockholm. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ET, MS, DN, and RR contributed to the conception and design of the study. ET, RR, NG, and CL organized the database. NG, DN, and CL performed the statistical analysis. NG wrote the first draft of the manuscript. CL, DN, ET, and RR wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.610192/full#supplementary-material

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Peripheral Blood and Salivary Biomarkers of Blood–Brain Barrier Permeability and Neuronal Damage: Clinical and Applied Concepts

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Within the neurovascular unit (NVU), the blood–brain barrier (BBB) operates as a key cerebrovascular interface, dynamically insulating the brain parenchyma from peripheral blood and compartments. Increased BBB permeability is clinically relevant for at least two reasons: it actively participates to the etiology of central nervous system (CNS) diseases, and it enables the diagnosis of neurological disorders based on the detection of CNS molecules in peripheral body fluids. In pathological conditions, a suite of glial, neuronal, and pericyte biomarkers can exit the brain reaching the peripheral blood and, after a process of filtration, may also appear in saliva or urine according to varying temporal trajectories. Here, we specifically examine the evidence in favor of or against the use of protein biomarkers of NVU damage and BBB permeability in traumatic head injury, including sport (sub)concussive impacts, seizure disorders, and neurodegenerative processes such as Alzheimer's disease. We further extend this analysis by focusing on the correlates of human extreme physiology applied to the NVU and its biomarkers. To this end, we report NVU changes after prolonged exercise, freediving, and gravitational stress, focusing on the presence of peripheral biomarkers in these conditions. The development of a biomarker toolkit will enable minimally invasive routines for the assessment of brain health in a broad spectrum of clinical, emergency, and sport settings.

Keywords: neurovascular unit, blood biomarkers, saliva, concussion, epilepsy, neurodegeneration, traumatic brain injury, extreme sports

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INTRODUCTION: FROM BLOOD–BRAIN BARRIER TO BLOOD–BRAIN DYNAMIC INTERFACE

The blood–brain barrier (BBB) is the complex and finely tuned network of brain capillaries governing the homeostatic exchange of ions, molecules, and cells between the brain and the peripheral blood (1–3). The importance of the BBB in the understanding and diagnosis of neurological disorders and brain health is recognized (4). The notion of BBB has evolved from

that of a static brain shield to that of a dynamic blood–brain interface where endothelial cells continuously communicate with mural cells (pericytes and smooth muscle) and glia (astrocytes and microglia), located near neurons and spatially assembled to constitute the neurovascular unit (NVU) (**Figure 1**) (2). A precise layering of cells and extracellular matrixes forms an impermeable wall (**Figure 1B**). BBB dysfunction has etiologic and diagnostic significance (4), and BBB permeability is a key element of perivascular and neuroinflammation (**Figure 1B1**) (5, 6). Increased BBB permeability provokes an immediate loss of homeostatic control of ions, ATP, and neurotransmitters levels in the brain, promoting abnormal synaptic transmission or neuronal firing, possibly leading to neurological sequelae (6–13). On the other hand, neuronal activity significantly influences cerebrovascular functions in health and disease conditions (14, 15). Diagnostically and because of increased BBB permeability, peripherally injected imaging contrast agents can access the brain parenchyma while a suite of central nervous system (CNS) proteins (see **Table 1**) or nucleic acids [circulating free DNA and microRNA; for a review see (44, 45)] can exit into the peripheral blood (**Figures 2A–C**, **3A–C**). Contrast MRI and CT scans are common clinical tools, while monitoring the levels of CNS proteins in peripheral body fluids represents a novel strategy for identifying BBB and neuronal damage (46). Importantly, the NVU connects with specialized brain acellular spaces through which the cerebrospinal and interstitial fluids carry ions, molecules, and proteins across the parenchyma or toward waste clearance pathways (**Figure 2B**) (47–50). This spatial perivascular and interstitial connectivity is important in the context of contrast-based brain imaging, possibly influencing the availability of biomarkers and their exit trajectories from the CNS [**Figure 2**; see (46, 51, 52) for a review]. Starting from these fundamental concepts, we here examine the evidence supporting the development and the use of specific peripheral biomarker proteins to detect glioneuronal damage and BBB permeability in a plethora of clinical, emergency and sport-related settings.

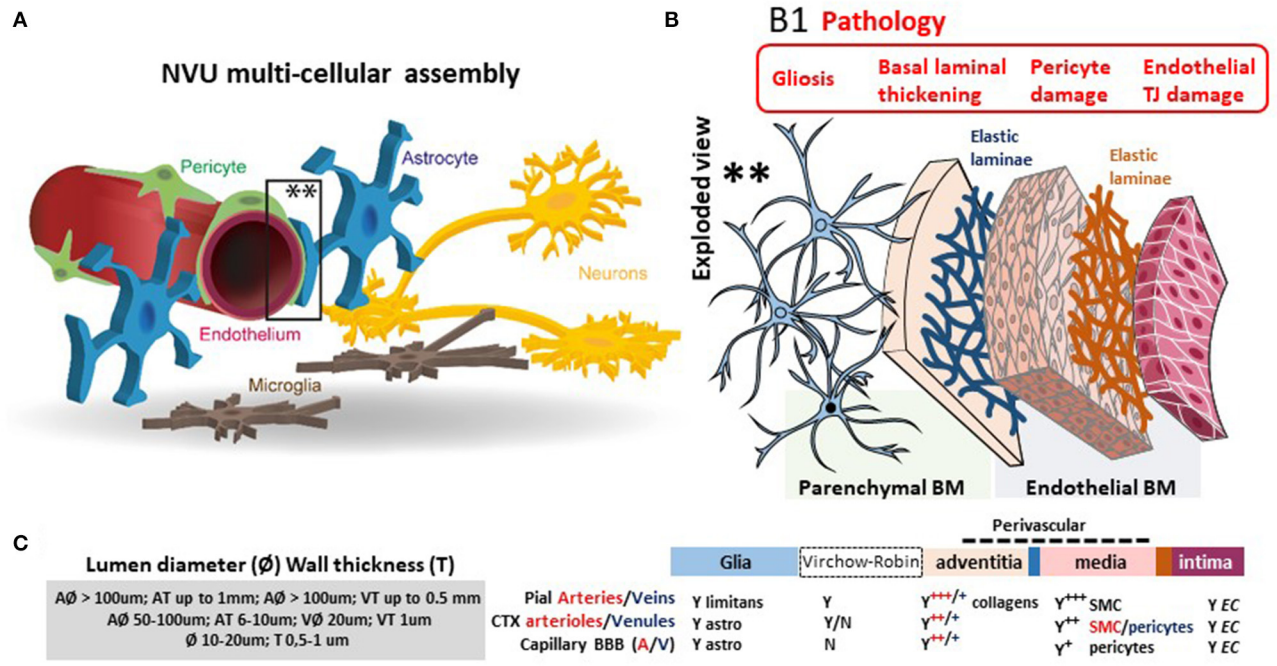

FIGURE 1 | The dynamic NVU multicellular layout. (**A**) Within the NVU, the BBB encapsulates a set of unique properties of the microvascular capillary and post-capillary venules. The BBB endothelium lacks fenestrations, is assembled by structured tight junctions (TJs), and expresses luminal or abluminal transporters, altogether finely regulating brain homeostasis for proper neuronal physiology. These endothelial specializations are generated and controlled by precise interactions with pericytes, astrocyte end-feet, and microglial cells, all participating to the NVU. **(B)** Exploded view to illustrate the varying cellular composition and wall thickness of the intima, media, and adventitia layers. The endothelial basement membrane (BM) embeds pericytes. A second basement membrane is deposited by astrocytes and surrounds the end-feet. At the capillary level, the endothelial and parenchymal basement membranes merge. At the post-capillary venules, the two basement membranes separate to provide a perivascular space that allows for immune cells homing. (**B1**) Commonly reported pathological modifications leading to BBB permeability or NVU damage. **(C)** The cerebrovasculature in numbers (A, arteries; V, veins; CTX, cortex; SMC, smooth muscles cells). Proper BBB commences as the deepening cortical arteries (diameter > 100 μm in mice) branch into arterioles (diameter 15–50 μm; wall thickness 5–10 μm) and capillaries (diameter < 10 μm; wall thickness a few μm). Pial vessels have glia *limitans* and an anatomically distinguishable Virchow-Robin space **(B)**. Blood flow velocity rates in cortical mouse arterioles and capillaries are 3 and <0.5 mm/s, respectively. Capillary blood flow decreases with cortical depth (down to 0.1 mm/s). Diameter ranges (rodent) and anatomical abundance of glia, Virchow-Robin space, collagens, and mural cells (smooth muscles or pericytes) is provided. The multicellular layering within the tunica media (sign + + + indicates more than three smooth muscle cells at the arteries; + indicates one layer of pericytes at the BBB) is the major determinant of vascular thickness and elasticity. Original images by NM and IGF graphical service.

TABLE 1 | Peripheral biomarker proteins of glio-neuronal damage and BBB permeability.

Proteins	MW (kDa)	Role as biomarker	Estimated half-life in blood	Usage temporal trajectories	Source	Sampling methods	CNS disease	Reported (and varying) blood baselines	References
GFAP	50	Astrocyte damage or astrogliosis	48 h (16)	Acute and Subacute (hours–days) (17)	Astrocyte cytoskeleton No clearly reported extra-cranial sources (18)	Venipuncture CSF	TBI Multiple sclerosis AD	Baseline 0.01 ng/ml TBI with negative CT 0.21 ng/ml TBI with positive CT 0.73 ng/ml	(17–21)
S100B	11	BBB and astrocyte damage, astrogliosis	2–6 h (22)	Acute (min, hours) (17)	Astrocyte calcium binding protein CNS development Extra-cranial sources [adipocytes (18)]	Venipuncture CSF Urine Saliva	TBI Epilepsy Multiple sclerosis	Pediatric 0.11 ng/ml (23) Adults 0.045 ng/ml (24) (sub)concussion, mTBI: 0.1 ng/ml (25, 26)	(17, 23, 25, 27–29)
UCH-L1	24	Neuronal cell damage (30)	7–9 h (31)	Acute (min, hours) Subacute (days) (17)	Axonal integrity Extra-cranial sources [neuromuscular junction (18)]	Venipuncture CSF	TBI Neurodegeneration	Pediatric 0.09 ng/ml (19) TBI with negative CT: 0.14 ng/ml TBI with positive CT: 0.44 ng/ml TBI with negative CT 261 pg/ml (21)	(19, 21)
NSE	47	Neuronal cell damage	30 h (32)	Acute (min, hours) Subacute (days) (17)	Neuron cytoplasmic enolase Detected in blood erythrocytes	Venipuncture CSF	TBI Epilepsy	Adults 6.1 μg/ml (24)	(33, 34)
NFL	68	Axonal injury, neuronal death	3 weeks (35)	Subacute days to weeks (17) and chronic	Neuron class IV intermediate filaments of cytoskeleton	Venipuncture CSF	TBI Neurodegeneration Multiple sclerosis	Threshold CSF 386 ng/ml (36)	(37, 38)
PDGFRβ	123	Pericyte reactivity or damage	na	Subacute (39)	Pericytes–endothelial interface	CSF	Neurodegeneration	See (40) for graphic baseline (115 % increase in CSF between no and mild cognitive impairment)	(39)
Tau, pTau	50–80	Neuronal or axonal damages, neurodegeneration	10 h (41)	S majuscle, subacute, and chronic (17)	Neuronal microtubule-associated proteins Aggregates into neurofibrillary tangles	Venipuncture CSF	Neurodegeneration AD	Blood total and phosphorylated tau, (42) T-tau control = 65.59 fg/ml P-tau control = 20.85 fg/ml P-tau/T-tau ratio control = 30.94 Total-tau in serum 4.4 pg/ml (24) Threshold CSF P-tau 78 pg/ml (39)	(17, 24, 43)

PERIPHERAL BIOMARKERS: BASIC CONCEPTS AND FOCUS ON TRAUMATIC BRAIN INJURY

Elevated BBB permeability, or dysfunction, occurs in response to an acute injury (e.g., head trauma, stroke, and status epilepticus) and may be present throughout CNS disease progression (e.g., neurodegeneration, epileptogenesis, and multiple sclerosis), often due to inflammation (5–7, 13). Peripheral biological fluids represent suitable matrices to detect and quantify brain-derived proteins reporting BBB permeability and susceptibility to glio-neuronal damage (27–29, 53). **Table 1** provides a list of protein biomarkers and their characteristics, properties, and proposed use in diagnostics. In general, peripheral biomarker proteins must (i) be present in brain interstitial fluids or be released by neurovascular cells into the interstitial or perivascular spaces, reaching the peripheral blood across a leaky BBB or by cerebrospinal fluid (CSF)–blood exchange (**Figures 2A**, **3A,B**); (ii) have a concentration gradient driving passive diffusion [**Figure 2C**; see (29)]; (iii) have a known and appropriate half-life to allow diagnostic interpretation (29) (biomarker half-life in peripheral fluids may impact usefulness in acute vs. long-term settings; see **Table 1**); and (iv) have a low molecular weight to allow a rapid egress across the damaged barriers or interfaces (19, 25, 27).

The bulk of neurological clinical biomarker literature has often focused on traumatic brain injury (TBI), with a recent emphasis on mild TBI (mTBI) (18, 54). Within this framework, the astrocytic protein S100B (55) has been examined as a peripheral biomarker of BBB permeability and gliosis (**Table 1** and **Figures 3A,B**). Early proof-of-principle studies showed serum S100B levels to rapidly increase in response to a sudden BBB permeability, supporting the hypothesis that perivascular S100B can readily exit the brain (27, 28, 56). S100B was reported to rule out mTBI sequelae in emergency room settings (57), and measurement of blood S100B levels displayed a 99.7% negative predictive value (NPV) (57–60). Further evidence indicated that monitoring S100B after a mTBI could override the need for a CT scan for the identification of intracranial injury, with an excellent NPV (61). However, another study reported no relationship between serum S100B concentration and mTBI severity (62). In sports, S100B blood levels increased immediately after football games as compared to pregame baselines in players experiencing repeated head hits (25, 63). The evidence of a rapid S100B surge in blood after sub concussive hits was confirmed in follow-up studies (63–65). Importantly, extra-CNS sources of S100B were reported, representing a potential confounding factor if timing of blood draws in relation to injury is not adequately controlled and standardized (18, 66). These concerns have been discussed in (23, 67, 68).

The astrocytic glial fibrillary actin protein (GFAP) and the neuronal ubiquitin carboxyl-terminal hydrolase isoenzyme L1 (UCH-L1) are important biomarker candidates for glioneuronal damage (**Table 1** and **Figures 3A,B**). UCH-L1 is also expressed at the neuromuscular junction (69, 70) while the contribution of extracranial sources of GFAP is debated (20, 71, 72). Monitoring

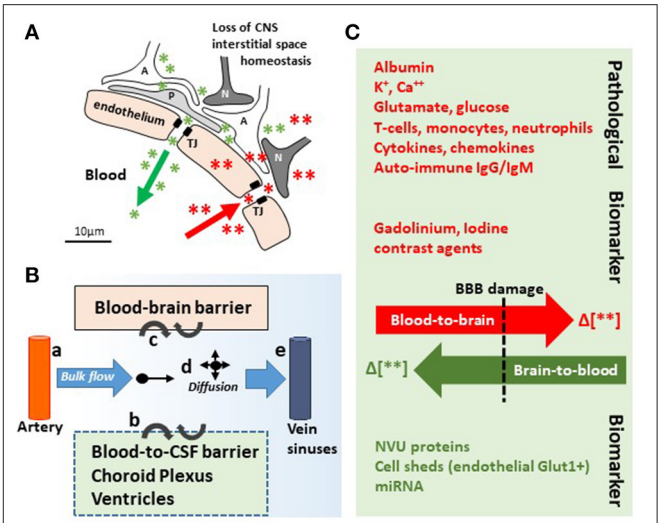

FIGURE 2 | In and out the brain: plausible biomarker exit routes and transport. **(A)** NVU cell disassembly causes disruption of brain homeostasis, allowing blood (red asterisks and arrow) and brain (green asterisks and arrow) components directly crossing the permeable BBB (TJ, tight junctions; A, astrocytes; P, pericytes; N, neurons). **(B)** Brain fluid paths regulate the movement of molecules (e.g., biomarkers) within the brain parenchyma into the blood and the CSF: (a) the CSF is produced by subarachnoid arteries and (b) the choroid plexus (blood-to-CSF barrier). By bulk flow mechanisms, the CSF diffuses at the cortical levels and in periventricular organs, constituting a possible vehicle for biomarker transport. (c) BBB damage allows biomarkers exiting (or entering) the brain. (d,e) Parenchymal bulk flow and CSF reabsorption occurs at larger veins, dural venous sinuses, and dural lymphatic vessels, sites where biomarkers could accumulate. **(C)** Concentration gradients (green/red Δ or arrows) between the peripheral blood and the brain parenchyma are the underpinning for pathological modifications and the driving force for biomarkers (e.g., MR contrast agent brain entry, red; protein biomarkers brain exit, green). Original images by NM.

of blood GFAP and UCH-L1 levels was used to grade brain injury after TBI. GFAP and UCH-L1 levels were increased in non-concussive and concussive head trauma as compared to body trauma (73, 74). The analysis of blood GFAP (or S100B) levels within 24 h from the head injury was proposed as a means to improve the detection of TBI and to identify patients in need of a subsequent MRI, in addition to routine CT surveillance (75, 76). GFAP and UCH-L1 blood levels were used to rule out intracranial injuries and the need for CT scans, showing high test sensitivity and NPV (21). One study reported no significant difference in blood UCH-L1 between control and players who sustained repetitive head hits (77). Collectively, this evidence points to GFAP as a diagnostic candidate to be used in TBI (33, 54, 71, 72). In two studies (78, 79), however, GFAP and UCH-L1 levels were below the lower limits of quantification or detection (LLOQ or LLOD, respectively) in a percentage of both TBI and trauma control groups, representing a possible concern for estimating NPV (20, 80).

Important biomarkers detecting neuronal damage are myelin basic protein (MBP), neuron-specific enolase (NSE), tau, and neurofilament light chain [NfL; **Table 1** and **Figures 3A,B**; see

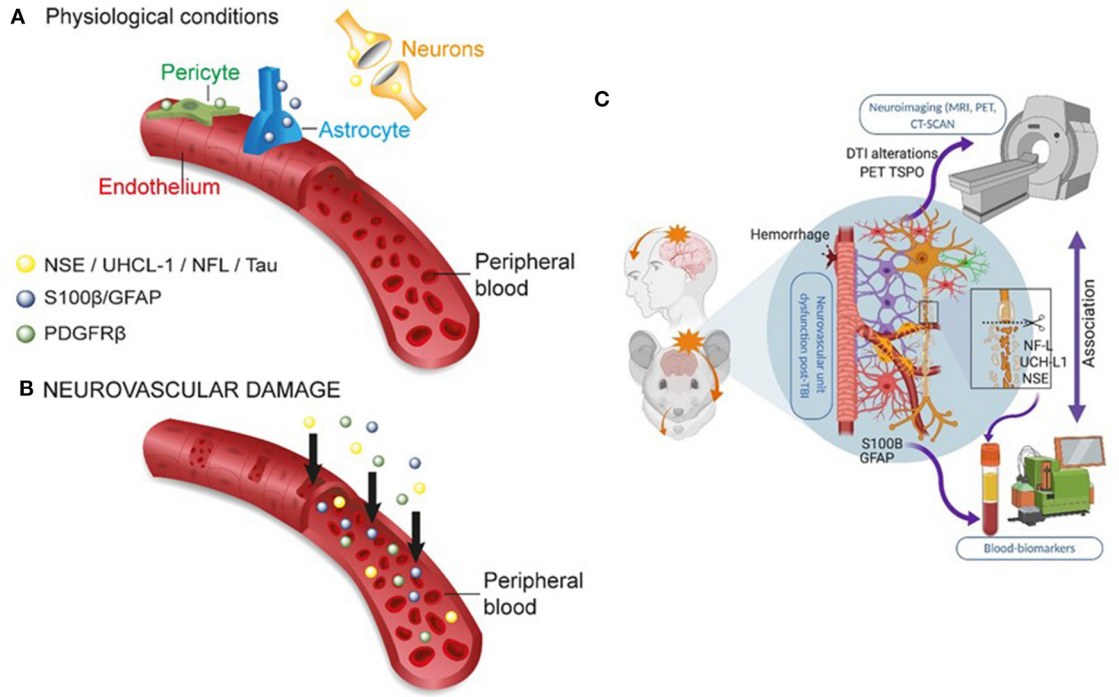

FIGURE 3 | Peripheral biomarkers of BBB permeability and brain damage. **(A)** Under physiological conditions, BBB tightness within a healthy NVU limits proteins from exiting the brain into the peripheral blood. **(B)** In conditions of brain damage, each neurovascular cell acts as a source of specific biomarker(s) (color coded), accessing the peripheral blood across a leaky BBB. The production or secretion of biomarkers at each NVU cell type depends on the severity, time, and the progression of disease states (see **Table 1**). **(C)** Integrating the use of brain imaging and peripheral biomarkers is a developing strategy to detect brain damage and to validate the usefulness of specific biomarker proteins in peripheral fluids. Original images by NM, JB, and IGF graphical service.

(43)]. Blood MBP levels were unchanged in a pediatric mTBI population as compared to controls. Interestingly, MBP levels remained elevated for up to 2 weeks in case of intracranial hemorrhage (81). NfLs are found in axons and have been proposed as biomarkers of axonal damage triggered by mTBI, for example, after an amateur boxing bout (82–84). S100B levels were also increased following amateur boxing (85). Further evidence indicated neurofilament heavy chain increase after mTBI (82). Finally, NSE levels in CSF were shown to be proportional to TBI severity, in the setting of moderate or severe TBI (86–88). NSE in the blood is less investigated due to its presence in erythrocytes (89, 90). Collectively, these data support the further development of blood biomarker toolkits of TBI, with a special relevance to mild head injury and sport-related (sub)concussions, when emergency and sideline diagnostic solutions need to be readily accessible.

PHOSPHORYLATED TAU AS AN EMERGING BLOOD BIOMARKER OF ALZHEIMER'S AND NEURODEGENERATIVE DISEASES

Accumulating evidence points to blood phosphorylated tau as a promising biomarker to improve the diagnosis and staging of and to enable trials in Alzheimer's disease (AD) subjects. In a cross-sectional study performed in AD patients, phosphorylated tau isoforms were used as diagnostic biomarkers to track disease progression (91). A method measuring attomolar concentrations of tau isoforms in plasma was implemented using stable isotope labeling kinetics and mass spectroscopy. Changes in plasma p-tau, particularly p-tau217, mirrored specific changes in CSF to detect phosphorylation of soluble tau and amyloidosis. No correlation was found between CSF and plasma p-tau202 levels. Plasma p-tau217 level distinguished amyloid-negative from amyloid-positive groups regardless of the cognitive status, indicating that p-tau217 in plasma may be an accurate biomarker of abnormal brain tau metabolism. Furthermore, a longitudinal study of familial AD (presenting pathogenic mutations in PSEN1 or APP genes) included 19 symptomatic and 51 asymptomatic participants where plasma p-tau181 levels were quantified by using a single-molecule array (Simoa) method (92). Elevated plasma p-tau181 concentrations segregated symptomatic mutation carriers from non-carriers. In another cross-sectional study including the Arizona-based neuropathology cohort (37 AD and 47 without AD), the Swedish BioFINDER 2 cohort [121 AD, 178 mild cognitive impairment [MCI], 301 without AD, and 99 other neurological disorders], and a Columbian autosomal-dominant AD kindred (365 PSEN1 E280A mutation carriers and 257 mutation non-carriers), plasma tau phosphorylated at the threonine 217 (p-tau217) was quantified by the Meso Scale Discovery (MSD) assay as a

diagnostic AD biomarker (93). Among 1,402 participants from the three cohorts, plasma p-tau217 discriminated AD from other neurological disorders with higher accuracy compared with plasma p-tau181, plasma Nfl, CSF p-tau181, and CSF Aβ42:Aβ40 ratio. A positive correlation between CSF and plasma p-tau217 was found in the Swedish BioFINDER 2 cohort. Finally, a high-sensitivity immunoassay measuring p-tau181 in plasma and serum was developed (94). A positive correlation was reported between plasma and CSF p-tau181 levels, distinguishing Aβ-negative cognitively unimpaired older adults from Aβ-positive older adults and Aβ-positive individuals with MCI.

Furthermore, at the BBB, the low-density receptor-related protein 1 (LRP1) plays an important role in regulating cerebrovascular permeability (95). sLRP1, a truncated soluble form of LRP1, freely circulates in plasma, and it sequesters unbound Aβ in the peripheral circulation (96). Plasma sLRP1 levels are significantly reduced in AD patients, and sLRP1 binding to Aβ is disrupted by oxidation (96, 97). Impaired sLRP1-mediated binding of plasma Aβ was suggested as an early biomarker for MCI preceding AD-type dementia (97). In summary, this evidence supports the further development of tau-based blood biomarkers as an accessible test for the screening and diagnosis of AD within the spectrum of cognitive impairments and dementia.

PERIPHERAL BIOMARKERS OF BBB PERMEABILITY AND SEIZURE CONDITIONS

The use of blood biomarkers extends to epilepsies, a cluster of diseases where BBB damage represents an etiological or a contributing pathophysiological player (98–100). A first study (67) demonstrated that blood S100B is elevated at seizure onset and after seizures, in support of the hypothesis that BBB damage may trigger a seizure (7, 101–103). A systematic review analyzed 18 studies and a total of 1,057 subjects, indicating that epileptic patients displayed elevated S100B blood levels as compared to controls (104). Meta-regression analyses showed that gender and mean age can impact serum S100B levels (104). Another study correlated MRI T1 peri-ictal imaging to blood S100B in drug-resistant epileptic patients, confirming the increase in BBB permeability during a seizure (105). Increased S100B blood levels were reported in pediatric temporal lobe epilepsy, with blood samples obtained 30 min after a complex partial seizure (106).

Children suffering from intractable focal epilepsy displayed elevated blood S100B levels as compared to controls (107). One study included 39 patients suffering from simple febrile seizures and age- and sex-matched controls, showing no S100B differences between groups when assessed immediately after seizures (108). These findings were corroborated in a follow-up study, (109) with the conclusion that febrile seizures are relatively harmless to the developing brain. Currently, a clinical trial is investigating whether S100B, as well as other protein biomarkers, increase in blood after a first generalized seizure could be used to predict first-to-chronic seizure conversion in adult subjects (https://clinicaltrials.gov/ct2/show/NCT02424123). Moreover, NSE elevations were reported in blood over time in patients affected by temporal lobe and extratemporal lobe epilepsies (110). Finally, recent evidence indicates miRNA in blood, or body fluids, as potential biomarkers indicating neurovascular and neuroinflammatory modifications occurring in specific forms of epilepsies [see (111–113) for comprehensive topic reviews]. In summary, blood biomarkers could represent a surrogate method of clinical electroencephalographic explorations to examine damage and brain neurophysiology in epileptic patients.

IMAGING BBB PERMEABILITY AND BRAIN DAMAGE: IS THE INTEGRATION WITH BLOOD BIOMARKERS POSSIBLE?

Available evidence supports the prospective use of blood biomarkers to detect NVU damage in acute and chronic neurological conditions. In this context, can peripheral biomarkers replace brain imaging? This is an important question especially if one considers the logistics (scarce imaging availability in rural areas and emergency, sport, and combat settings) and economic advantages that come with peripheral biomarkers, notwithstanding the complications associated with radiation exposure (e.g., CT scan). As a result, the diagnostic equivalence of blood biomarkers and enhanced MRI or CT scans (114–119) is being investigated. Accumulating evidence has shown that mTBI represents an optimal clinical arena to study the usefulness of imaging and peripheral biomarkers, also fulfilling an urgent clinical need (120–122). Neuroimaging techniques [CT scan (61)] show limitations for the diagnosis of mTBI patients (122, 123). Importantly, blood levels of GFAP, tau, and NfL were higher in patients with TBI-related findings on CT as compared to subjects presenting with normal CT, where the only significant predictor of damage was GFAP (124). Combining the biomarkers tau, NfL, and GFAP showed a good discriminatory power for detecting MRI abnormalities, even in mTBI patients with a normal CT (124). Furthermore, peak serum S100B levels negatively correlated with resting-state brain connectivity and behavioral outcomes in mTBI to severe TBI cases (125). S100B has proven its high NPV to rule out intracranial bleeding in patients after mTBI. However, its specificity for brain parenchyma structural lesions remains debated, and MRI is required for a specific explanation of clinical symptoms (76, 126, 127). Positron emission tomography (PET) and radiolabeled biomarkers were tested along with blood biomarkers. The [^{18}F]AV1451 (flortaucipir) tau ligand was detected at the white/gray matter junction in frontal, parietal, and temporal brain regions, a typical localization of chronic traumatic encephalopathy (CTE) and tauopathy in veterans. Elevated levels of Nfl were also reported in plasma (43). Finally, TBI is associated with inflammation as blood levels of IL6, TNFα, and VEGF were increased in CT- and MRI-positive patients as compared to controls (126).

Importantly, newer brain imaging approaches are being tested. Proton magnetic resonance spectroscopy (^{1}H-MRS) represents an emerging neuroimaging modality to track the

metabolic changes occurring after TBI (128, 129). Spectroscopy can predict changes of key metabolites such *N*-acetylaspartate (NAA), a marker of neuronal loss (130), and its early decrease associates with long-term poor outcomes in clinical pediatric mTBI and moderate TBI (130). Experimentally, spectroscopy modifications post injury were linked to altered astrocyte metabolism (131). Brain structural changes observed using diffusion tensor imaging were correlated to astrocyte dysfunction and astrogliosis at early (1–7 days) and late (60 days) time points after injury (132, 133). Tractography provides an opportunity for measuring structural alterations in the white matter that are not detected by conventional structural MRI (134). Magnetic encephalography has also been proposed to study mTBI damage, in addition to being used for post-traumatic stress disorders (135, 136). Collectively, these data underscore the need for integrating the temporal and quantitative profiles of emerging imaging read-outs with the dynamics of peripheral biomarker of NVU damage. These studies will allow us to fully understand whether blood biomarkers can reliably act as surrogates for brain imaging.

SALIVA AS A BIOMARKER MATRIX: GENERAL CONCEPTS

Another key cellular "barrier" can be exploited for diagnostic purposes, namely, the salivary glands and gingival vessels, both interfacing with the peripheral blood (**Figures 4A,B**) (53, 137–144). While plasma and serum are considered as classic biofluids for assessment of systemic biomarkers, saliva is being increasingly viewed as a matrix with a high diagnostic value (141, 145). Saliva collection is economical, safe and can be performed without the assistance of specialized health care personnel, allowing for point-of-injury (POI) sampling. Saliva lacks cellular and soluble components (e.g., coagulation cascade). As the leakage of brain-derived biomarkers in saliva undergoes a process of biological filtration (53, 137, 146), the use of saliva does not require separation steps that are an obstacle to the development of POI blood tests (138, 139). Human saliva is a clear, slightly acidic (pH 6.0–7.0) heterogeneous biofluid composed of water (99%), proteins (0.3%), and inorganic substances (0.2%) (147). Saliva contains enzymes, hormones, antibodies, nucleic acids, antimicrobial constituents, and cytokines (148), which accumulate in salivary glands and are secreted into the oral cavity through acinar cell ducts (149). Available protocols indicate that saliva samples can be stored short term at room temperature and long term at −20°C or −80°C without significant protein degradation, similar to serum or plasma samples (150, 151). Relevant information inherent to the preparation and the technical handling of saliva samples can be found in (150, 152–154).

The whole saliva (WS) proteome, when compared with the plasma proteome, displays a larger proportion (14.5%) of low-molecular-weight proteins (<20 kDa), in contrast to only 7% for the plasma proteome (154). The highest fraction of proteins found in WS ranges from 20 to 40 kDa, whereas the 40–60 kDa range is the largest fraction for plasma. This is consistent with selective permeability between blood and saliva for low-molecular-weight proteins. Five diagnostic alphabets are outlined in saliva, including proteome (153, 155), transcriptome

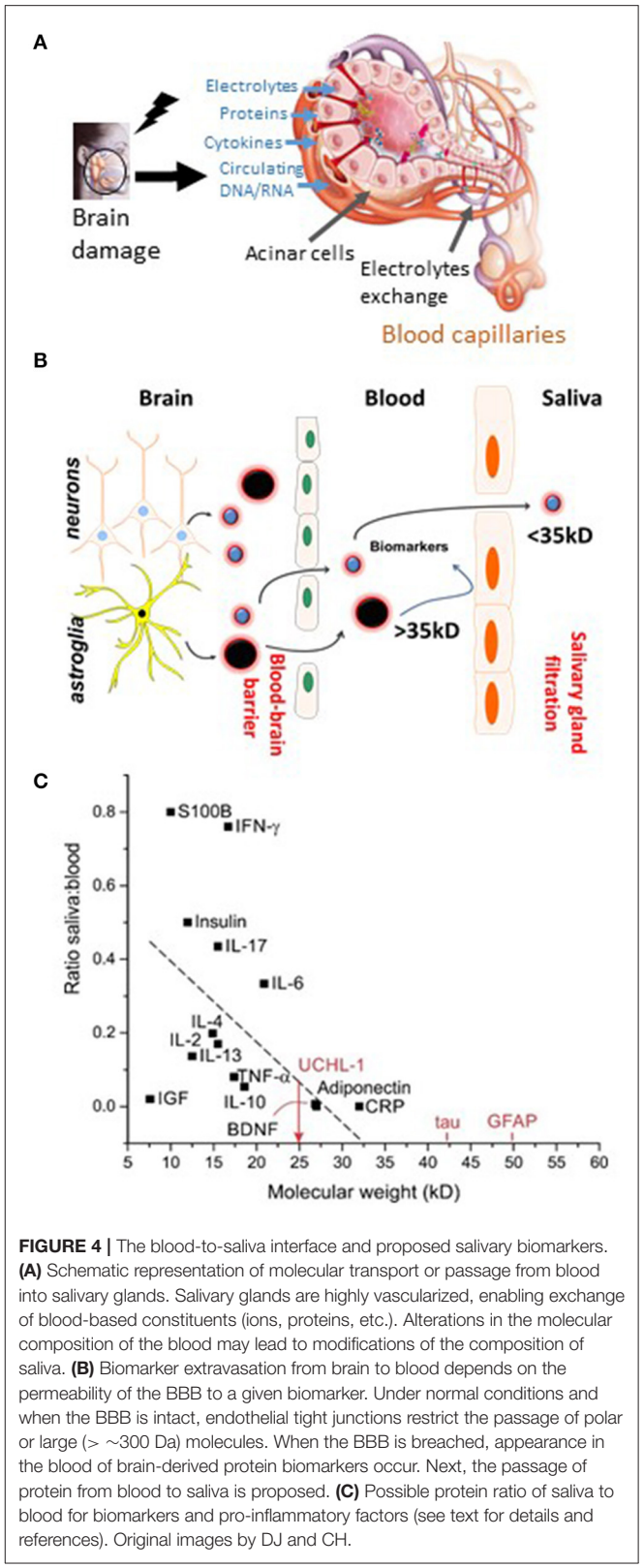

FIGURE 4 | The blood-to-saliva interface and proposed salivary biomarkers. **(A)** Schematic representation of molecular transport or passage from blood into salivary glands. Salivary glands are highly vascularized, enabling exchange of blood-based constituents (ions, proteins, etc.). Alterations in the molecular composition of the blood may lead to modifications of the composition of saliva. **(B)** Biomarker extravasation from brain to blood depends on the permeability of the BBB to a given biomarker. Under normal conditions and when the BBB is intact, endothelial tight junctions restrict the passage of polar or large (> ~300 Da) molecules. When the BBB is breached, appearance in the blood of brain-derived protein biomarkers occur. Next, the passage of protein from blood to saliva is proposed. **(C)** Possible protein ratio of saliva to blood for biomarkers and pro-inflammatory factors (see text for details and references). Original images by DJ and CH.

(156, 157), microRNA (158), metabolome (159), and microbiome (160). Saliva is used by clinical laboratories for the detection of secretory IgA antibodies, for the analysis of salivary cortisol and hormones, and for genetic purposes (161–163).

SALIVARY BIOMARKERS OF NVU DAMAGE: A NEW DIAGNOSTIC OPPORTUNITY?

The salivary proteome has been characterized in CNS disease conditions, such as schizophrenia, bipolar disorders, and genetic disorders including Down's syndrome and Wilson disease (164). An overview of biomarkers identified in saliva for the diagnosis of neurodegenerative diseases such as AD, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis is provided in (165). Inflammatory biomarkers (e.g., IL-1β, TNF-α, and IL-6) have been quantified in saliva (166).

The deployment of POI salivary tests represents an opportunity for the detection of time-sensitive brain injuries (139–141, 167, 168). NSE was shown as a possible diagnostic salivary biomarker for neuronal damage in patients post stroke (169). Saliva samples have been analyzed for S100B levels, pro-inflammatory factors, and microRNAs in the settings of TBI (168, 170, 171). In particular, S100B levels in saliva were elevated in children post TBI (171). In another pilot study, 15 adult patients with suspected TBI and 15 control subjects were studied. Average salivary S100B level was 3.9-fold higher than blood S100B level, regardless of the presence of pathology $[S100B]_{saliva}$ correlated positively with $[S100B]_{serum}$, and salivary S100B levels were as effective in differentiating TBI patients from control subjects as serum levels (172).

In an attempt to further accentuate the diagnostic significance of salivary testing, we reviewed the literature to obtain potential blood-to-saliva ratios for a number of proteins (**Figure 4C**). This search was directed to proteins that are not secreted by salivary glands. These proteins can access the salivary fluid by pericellular capillary leak, primarily the crevicular fluid. Importantly, it is currently unknown whether the steady-state permeability of the blood-to-saliva protein diffusion is preserved even at times when the BBB is breached due to brain insults. Literature references were used to examine insulin (173, 174), EGF (175), HGH (19, 176), S100B (18, 54–56, 177–180), adiponectin (181), prostate-specific antigen (PSA) (182), and cytokines (183). To our knowledge, there are no reports of salivary BDNF or NFL levels. All retrieved values were plotted to outline the theoretical cutoff properties of salivary filtration (**Figure 4C**). Large molecules (e.g., IgG) can be present in saliva owing to active secretion or local production.

Finally, we examined whether blood-to-saliva biomarkers' passage could be empirically predicted or modeled (153). Available data indicate that saliva is not a diluted substitute for the determination of plasma protein levels, as indicated by the incoherent plasma and saliva proteomes (152). Therefore, understanding the kinetic protein passage from blood to saliva is difficult. In the past, a model describing the passage of biomarkers from the brain into the peripheral blood was proposed (27–29). A physiologically based pharmacokinetic model can be used to describe the distribution of drugs and small molecules in body fluids (184). This computational approach can estimate the extent and time course of salivary biomarkers originating from the brain, offering the likelihood of a protein in saliva to be blood-borne (185). The physiologically based pharmacokinetic model used to describe the distribution of brain-derived biomarkers in blood was expanded to include an idealized salivary gland receiving its vascular supply from the external carotid. The venous output was mimicked according to the properties of jugular vein branches. To approximate the combined contribution of transcellular and paracellular pathways of protein extravasation across capillary endothelial cells and salivary gland epithelia, the following equation was used to calculate Js, the transfer of protein from blood to saliva:

$$Js = Jv^{*}(1 - R)*Cp + (Cp - Ci)*PS \quad (1)$$

where Js (mol/min) is the mass transfer from blood to saliva, Jv (ml/min) is the blood flow to the salivary gland, R is the reflectance of the vascular wall, Cp (mol/L) is the concentration of biomarker in the serum, Ci (mol/L) is the concentration of biomarker in the saliva, and P and S refer to permeability (cm/s) and surface of exchange (cm^2), respectively. The value of reflectance has no dimension and has a range from one (no passage of protein) to 0 (protein passage dictated by diffusion alone). The value of reflectance is derived from pore radius and molecular radius. To estimate PS, we used PS = Jv * Ci/(Cp – Ci) with salivary flow at 1 ml/min and Ci and Cp at 2.5 and 61.5 mg/ml, respectively. These values were derived by measurements and transfer of albumin levels from blood and saliva. The equation can be greatly simplified by fitting experimental data to confirm their accuracy. Once this is done, the predictors of passage of a given protein are primarily related to its molecular size (vascular wall reflectance) and the presence of a gradient for passage from blood to crevicular fluid. For (1), note that if the reflectance tends toward 1 (large molecular weight), the first term equals zero, thus leaving only the permeability of the capillary wall and the osmotic gradient as variables. Considering that permeability also depends on molecular size, a cutoff for extravasation seems to be mostly related to the size of the permeating protein. By using other computational models, it was shown that the physicochemical properties of proteins were the main predictors of presence in saliva. Among several properties, molecular size was the most relevant (185, 186).

It is important to underscore that the use of saliva samples comes with confounding factors. For instance, gingivitis or periodontal disease can affect the identification and quantification of proteins. It has been shown that submandibular saliva flow rates are lower in AD patients as compared to controls (187), possibly impacting the proportion of proteins detectable (188). In summary, fully defining the qualitative and quantitative characteristics of salivary biomarkers in physiological and neuropathological conditions is important to develop non-invasive point of care applicable to NVU screening.

PUSHING THE BBB LIMITS: RELEVANCE OF PERIPHERAL BLOOD BIOMARKERS IN HUMAN MODELS OF EXTREME BRAIN PHYSIOLOGY

Here, we focus on extreme sport settings that can be exploited as 'human' models to study BBB permeability, neuronal damage, and hemodynamic modifications in a controlled spatiotemporal manner. We review the evidence supporting the use of blood biomarkers to detect neurovascular modifications associated with extremes of cerebral blood flow (**Figure 5A**). These models share similar pathophysiological features unified by the cerebral formation of free radicals, associated reactive oxygen/nitrogen species (ROS/RNS), and impaired cerebral autoregulation (CA).

EXERCISE, CEREBROVASCULAR REGULATION, AND BLOOD BIOMARKERS

Evidence indicates that moderate-intensity continuous training (MICT) and corresponding improvements in cardiorespiratory fitness (CRF) can increase cerebral perfusion and vasoreactivity across the human life span (192, 193), translating into a lower risk of stroke mortality and dementia (194, 195). The primary mechanisms include accelerated neurogenesis, in particular of the hippocampal dentate gyrus (196); reduction in β-amyloid (197); neuro-oxidative inflammatory nitrosative stress (198); proprioceptive adaptations incurred by movements that require sustained mental effort (199); increased brain-derived neurotrophic factor that modulates brain plasticity by promoting neuritic outgrowth and synaptic function (200); and improved BBB integrity and bolstering of tight junctions (201). More recently, high-intensity interval training (HIIT) has emerged as a more time-efficient model of exercise that can potentially promote superior improvements in CRF and cerebrovascular adaptation (191). However, this type of exercise characterized by high-flow/high-arterial-pressure transmission poses unique challenges for the brain with emerging evidence suggesting that an acute bout of HIIT could increase BBB permeability in the absence of neuronal injury (e.g., increased blood S100B and no NSE changes), subsequent to a free radical-mediated impairment in dynamic CA that persists into the recovery period (202) (**Figure 5B**).

HIGH-ALTITUDE MOUNTAINEERING, FREEDIVING, NVU DYNAMICS, AND BLOOD BIOMARKERS

High-altitude (HA) mountaineering (**Figure 6A**) and freediving (**Figure 6B**) represent unique physiological models to study severe arterial hypoxemia (O_2 lack) and hypocapnia/hypercapnia (CO_2 lack/excess) in 'extreme' athletes who consistently operate at, or very close to, the limits of human consciousness (189, 212). Diffusion-weighted magnetic resonance imaging has identified increases in brain volume, T_2 relaxation time (T_2-rt), and apparent diffusion coefficients (ADCs) in healthy participants acutely exposed to hypoxia, taken to reflect extracellular vasogenic edematous brain swelling (205, 206). These changes were pronounced in the splenium and genu of the corpus callosum, the likely consequence of a unique vascular constitution. Densely packed horizontal fibers characterized by short arterioles that lack adrenergic tone likely render it more susceptible to hyperperfusion edema in the setting of hypoxic cerebral vasodilatation and/or autoregulatory impairment (205, 206). Local sampling of CSF and arterial–jugular venous blood concentration gradients of biomarkers including S100B indicated that BBB disruption is likely minor and linked to increased free radical formation (207, 216).

Some mountaineers, notably those who ascend (too) rapidly to altitudes above 2,500 m and thus not adequately acclimatized, can develop acute mountain sickness (AMS), a primary disorder of the CNS characterized by headache that is associated with, if not the primary trigger for, other vegetative symptoms (217). Traditionally, AMS has been considered a mild form of HA cerebral edema (HACE, the most malignant of all HA illnesses, oftentimes proving fatal) with a common pathophysiology of intracranial hypertension subsequent to vasogenic edematous brain swelling at opposing ends of a clinical continuum. An increase in intracranial pressure (ICP) could potentially result in the mechanical stimulation of pain-sensitive unmyelinated fibers that reside within the trigeminal–vascular system, triggering the symptoms of a headache (218). This makes intuitive sense in light of an early study that identified an increased T_2 signal in the white matter of mountaineers with moderate to severe AMS in whom clinical HACE had not yet developed (no ataxia or altered consciousness) (219). However, follow-up MRI studies consistently failed to support this concept, with no clear relationships observed between hypoxia-induced increases in brain volume or T_2-rt and cerebral AMS scores (206, 220). Indeed, the only defining morphological feature that distinguishes the AMS brain from its healthy counterpart is a selective attenuation in the ADC signal taken to reflect intracellular (cytotoxic) edema that likely coexists with extracellular vasogenic edema (206, 220). Attenuation of the ADC signal likely reflects fluid redistribution from within the extracellular space, as intracellular (astrocytic) swelling proceeds without any additional increment in brain volume, edema, or ICP (221). The underlying causes and temporal sequence are unknown, perhaps a reflection of ion pump suppression subsequent to (free radical-mediated) downregulation of Na^+/K^+-ATPase activity (211). More recent evidence suggests that a functional impairment in cerebral 'venous outflow' at the level of the transverse venous sinus may prove the unifying risk factor for AMS (222).

Freediving (**Figure 6B**) offers yet another remarkable model of severe arterial hypoxemia (189, 212). The static apnea world record currently stands at an impressive 11 min 35 s held by Stéphane Mifsud. However, unlike mountaineers, apnea results in severe hypercapnia, further compounding the cerebral hyperemic stimulus (**Figure 6B**), with freedivers also having to contend with the additional challenge of

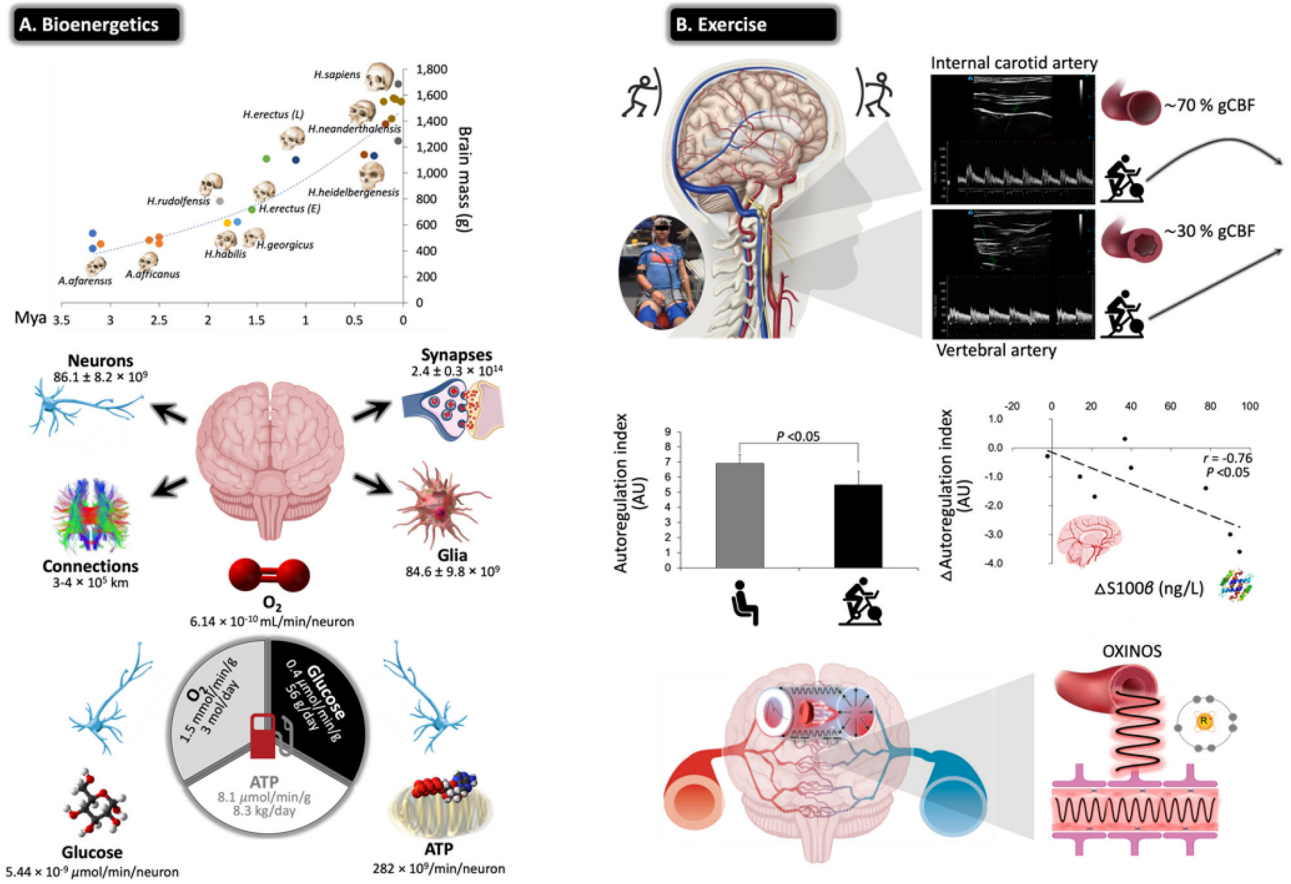

FIGURE 5 | Challenges for the exercising human brain: applicability of NVU biomarkers. **(A)** Evolutionary 'drive-for-size' with exponential increases in estimated brain mass observed in fossil hominids. Note the structural complexities and corresponding bioenergetic demands that define the 'modern' human brain, highlighting its limited energy reserves in the form of oxygen (O_2), glucose, and adenosine triphosphate (ATP) in the face of extraordinarily high rates of neuronal metabolism. This renders the human brain exquisitely sensitive to anoxia and ischemia, and thus, it has developed a sophisticated armory of mechanisms that collectively defend O_2 homeostasis. Calculations cited and figures modified from (189). **(B)** Physical exercise poses unique challenges for the human brain with perfusion typically characterized by preferential redistribution to the phylogenetically 'older' regions subserved by the posterior circulation (typical B-mode Doppler images illustrated). This makes teleological sense given that it is one of the most primitive neuroanatomical regions of the human brain, which has remained highly conserved across vertebrate evolution housing (almost exclusively) all the major cardiovascular and respiratory control centers essential for the integrated regulation of autonomic nervous control (190). However, this can come at a cost, with emerging evidence indicating that high flow/pressure and systemic/cerebral formation of free radicals and oxidative inactivation of nitric oxide [oxidative–nitrosative (OXINOS) stress] contribute to impaired cerebral autoregulation and BBB disruption. The latter is confirmed through proportional extravasation of brain-specific proteins, including S100B, in the absence of structural tissue damage. BBB permeability can cause extracellular vasogenic edema resulting in a regional O_2 diffusion limitation, with the potential to adversely affect cerebral bioenergetics and cognition. This is relevant to patients already suffering from impaired cerebral autoregulation/autonomic dysfunction, including older adults, notwithstanding patients diagnosed with diabetes, hypertension, stroke, and AD (191) (original images by DMB).

elevated hydrostatic pressure when competing 'at depth' in select disciplines and complications associated with pulmonary barotrauma, nitrogen narcosis, decompression sickness, and high-pressure neurologic syndrome (212). Competitive freedivers oftentimes experience shallow-water blackout due to severe cerebral hypoxia and loss of motor control, clinical signs that are the frustrating cause for disqualification from competition, notwithstanding immunochemical evidence for structural NVU damage, e.g., increased peripheral blood S100B and NSE after a maximal apnea, with potential long-term neuropsychological consequences (223, 224).

More recent, direct approaches have taken advantage of sampling arterial–jugular venous blood and combining regional measurements of CBF during the course of an apnea in champion freedivers (213, 214). Despite no detectable O_2 gradient across the brain, a truly remarkable observation, CDO_2 subsequent to increased perfusion was well maintained even at PaO_2s as low as 23 mmHg (**Figure 6B**). Similar to the aforementioned acute hypoxia study (207), apnea was associated with a net trans-cerebral outflow of free radicals and S100B (in the absence of any local gradients in NSE or MBP) that may reflect minor BBB permeability due to the combination of hemodynamic (increased intracranial

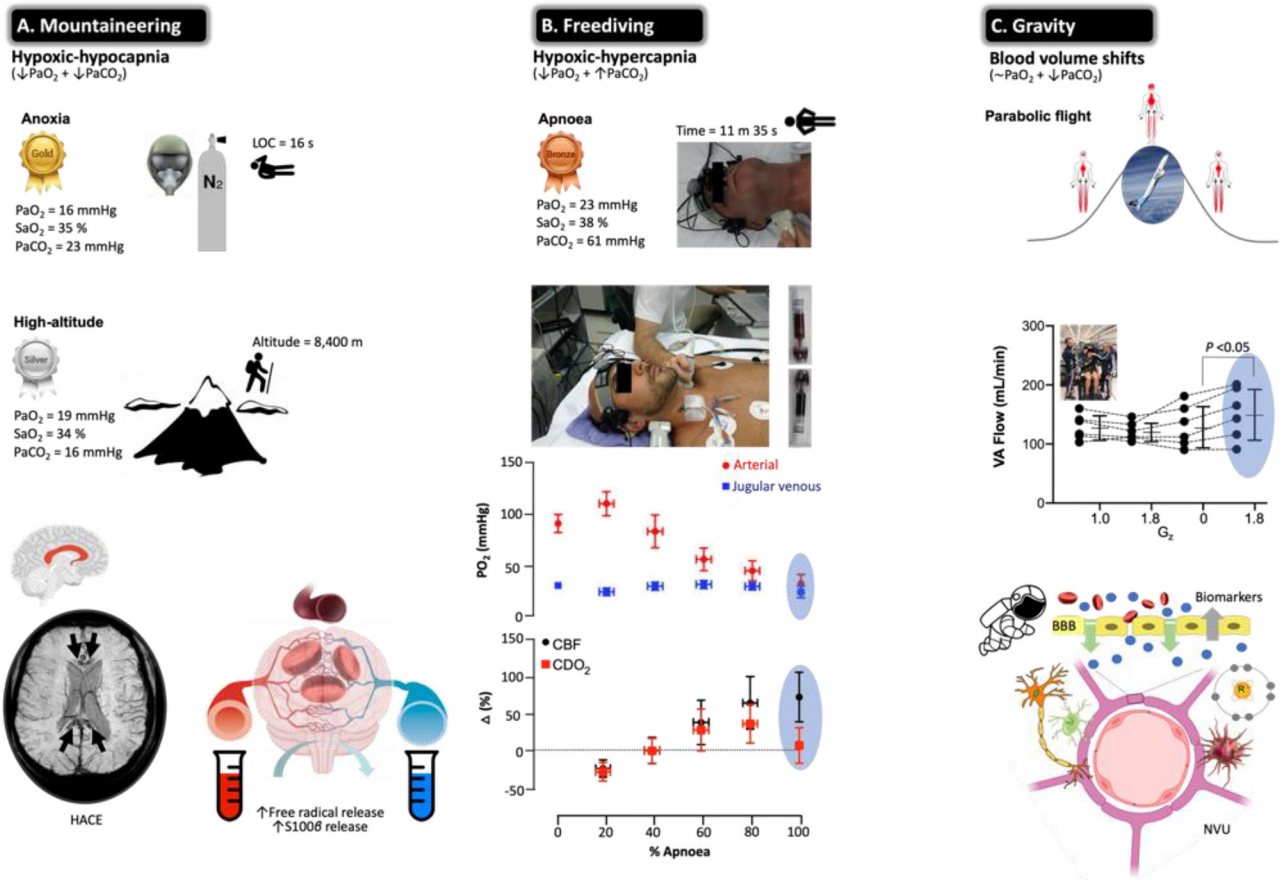

FIGURE 6 | Beyond barriers: the brain under pressure and NVU blood biomarkers. Extreme examples highlighting how the human brain adapts to the most severe swings in circulating oxygen, carbon dioxide, and blood volume recorded in the published literature with data obtained from apparently healthy participants. All examples are unified by a general increase in CBF to ensure preservation of O_2 and glucose substrate supply. Note the most hypoxemic (notwithstanding hypocapnic/hypercapnic) measurements documented in humans (gold, silver, and bronze awarded according to severity of hypoxemia) who regularly operate at the 'cusp' of consciousness or indeed beyond. **(A)** Acute exposure to anoxia (pure nitrogen) resulted in an arterial partial pressure of O_2 (PaO_2) of 16 mmHg (lowest ever recorded) that resulted in unconsciousness within 16 s (203). The highest recorded femoral arterial puncture performed on an acclimatized mountaineer at 8,400 m on Mt. Everest revealed the second lowest (silver award) PaO_2 (and lowest $PaCO_2$) of 19 mmHg (204). Prolonged exposure to the severe hypoxia of terrestrial HA can result in HACE, a rare albeit deadly syndrome characterized by ataxia resulting in rapid progression to coma. Susceptibility-weighted MRI has revealed hemosiderin deposits (insoluble iron(III) oxide-hydroxide, reflecting micro-hemorrhages) in nonlethal HACE confined to the genu and splenium of the corpus callosum. In combination with vasogenic edematous brain swelling previously documented by MRI studies in healthy participants exposed to acute hypoxia (205, 206) and net trans-cerebral (arterio-jugular venous) outflow of free radicals and S100B (207–209) in the face of impaired dynamic cerebral autoregulation (210) has led to the suggestion that severe hypoxia results in permeability of the BBB subsequent to molecular (free radical-mediated increase in permeability) and hemodynamic (cerebral vasodilatation) stress (211). (**B**) The third lowest PaO_2 (and highest $PaCO_2$) recorded in a single freediver during the course of a static breath-hold (apnea) (212, 213). Note that the level of hypoxemia was so marked there was no observable arterio-jugular venous O_2 gradient across the brain (marked cyanosis and 'black blood' visible toward the end of apnea). Hypoxic-hypercapnic increase in CBF was sufficient to offset arterial hypoxemia and preserve the cerebral delivery of O_2 (CDO_2) (213), though a mild net trans-cerebral outflow in S100B was observed in a follow-up study taken to reflect mild, diffuse BBB disruption (214). **(C)** Gravitational stress and reciprocal changes in central blood volume were recently shown to increase blood flow to the posterior region of the brain (VA, vertebral arteries) arguably more prone to hyperperfusion injury. This was associated with a free radical-mediated reduction in nitric oxide (NO) bioavailability and mild damage of the NVU reflected by the extravasation of S100B and GFAP (215). Image credits: mountaineering (T*MRI of a 65-year-old woman 7 weeks after having developed HACE at 3,580 m, courtesy of Professor M Knauth, University Hospital Gottingen, Germany); freediver [photographs courtesy of Prof. PN Ainslie and the late Dr. CK Willie, University of British Columbia, Canada (213) with permission]; Gravity (Novespace's ZERO-G Airbus A300 credit: Novespace/CNES/DLR/ESA).

pressure) and molecular (increased free radical formation) stress in the absence of neuronal damage (214). Rather than consider this simply as a damaging maladaptive response, vasogenic edematous brain swelling may prove the adaptive phenotypical response in the hypoxia-tolerant human brain (211, 225).

GRAVITATIONAL STRESS, CEREBROVASCULAR REGULATION, AND BLOOD BIOMARKERS

Alterations in gravitational fluid pressure gradients caused by the microgravity of orbital spaceflight and hypergravity

associated with takeoff and landing pose unique physiological challenges for the astronaut brain. Recent interest has focused on the complex pathophysiology underlying a constellation of debilitating neurological, ophthalmological, and neurovestibular symptoms, known collectively as spaceflight-associated neuro-ocular syndrome (SANS) (226). At the cellular level, microgravity has been associated with a loss of cytoskeletal integrity through dissociation of actin and tubulin bundles (227), and evidence obtained using animal models suggests that BBB disruption may occur during the early phases of unloading induced by suspension or microgravity (228) and during hypergravity induced by prolonged centrifugation (229, 230). In a recent study (215), parabolic flight (PF), a ground-based spaceflight analog, was used as a human model to induce rapidly alternating shifts in central blood volume during repeated exposures to microgravity (0 G_z) interspersed with hypergravity (1.8 G_z) (231) to explore how altered CBF impacts the NVU (232) (**Figure 6C**). Blood flow to the posterior cerebral circulation (vertebral arteries) was selectively elevated during the most marked gravitational differential from microgravity to hypergravity. Posterior hyperperfusion was associated with a free radical-mediated reduction in nitric oxide bioavailability (oxidative–nitrosative stress) and selective increases in blood S100B and GFAP that persisted following return to microgravity, whereas blood biomarkers of neuronal–axonal damage (NSE, NFL, UCH-L1, and tau) remained stable (215). These findings suggest that the cumulative effects of repeated gravitational transitions may promote minor BBB damage due to the combined effects of hemodynamic-molecular stress. While we appreciate that PF is an entirely different stimulus dominated by hypergravity, these findings provide important mechanistic insight to help understand the neurological risks associated with prolonged microgravity during spaceflight, given that increased BBB permeability directly impacts neuronal function, predisposing to neurological sequelae and brain disease (6).

BLOOD BIOMARKERS OF NVU DAMAGE: AVAILABLE ANALYTICAL TOOLS, LIMITATIONS, AND CONTROVERSIES

No single ideal peripheral biomarker exists; rather, a suite of biomarkers could have a significant diagnostic impact. In recent years, innovative methods for biomarker detection have been implemented (**Table 2**). Reaching high sensitivity has several advantages, particularly in the context of neurological settings. Foremost is the ability to detect biomarkers such as NfL, Tau, or GFAP that are readily present in the CSF and in low concentrations in the blood. New technology has enabled the quantification of brain-derived protein biomarkers in blood, getting one step closer to a minimally invasive diagnosis of brain damage and neurodegenerative processes. Furthermore, high-sensitivity methods use microliter quantities of biofluid, allowing the quantification of several analytes and multiplex measurement. As an example, we here provide NfL, GFAP, and tau serum baseline levels as measured in our laboratory using Simoa (**Table 2**). We include specific LLOD and LLOQ values relative to our particular experience. Obviously, this new technology presents limitations. A shortcoming of high-sensitivity assay resides in the fact that Research Use Only (RUO) kits are not able to provide, yet, a level of robustness and precision that one would expect for a clinical *in vitro* diagnostics (IVD) use. To date, the impact of analytic interference is not sufficiently investigated. Therefore, the expectations formulated following cohort-based studies need confirmations in large preclinical studies and multicentric clinical trials.

TABLE 2A | Available biomarker detection tools.

Novel diagnostic technology	Providers
Electrochemiluminescent immunoassay	Meso scale discovery
Single-molecule array immunoassay	Simoa® (Quanterix)
Immunomagnetic reduction	MagQu Co
Proximity extension assay	Olink
Immunocapture mass spectrometry	Thermo Fisher, Shimadzu, Agilent, AB Sciex, Waters

TABLE 2B | Examples of analytical parameters.

	NFL	GFAP	Tau
Control baseline levels	8 pg/ml	54 pg/ml	0.35 pg/ml
Limit of detection (LOD)	0.10 pg/ml	0.22 pg/ml	0.02 pg/ml
Limit of quantification (LOQ)	0.24 pg/ml	0.47 pg/ml	0.05 pg/ml

Although the use of blood biomarkers of BBB or neuronal damage is appealing, a number of clinical stumbling blocks currently limit full applicability. The usefulness of blood biomarkers in a given human depends on the availability of reference values, correcting for age, ethnicity, kidney function, and body mass index (29). Adequateness of the blood sampling schedule and availability of baseline controls are crucial for a reliable biomarker outcome. Sample readiness before and after pathological events (e.g., inpatient seizure monitoring and head trauma as in contact sports) provides the optimal framework to calculate biomarker differential in the same individual and within a controlled time frame (24, 25). Availability of *ad hoc* baseline samples (e.g., specific enrollments for sport events and military personnel) represents a robust method enabling personalized medicine.

As examined so far, the appearance of NVU proteins in blood is reported for neurodegenerative diseases (91, 94), brain tumors (115, 117), TBI (25, 233), neurologic manifestations of systemic disease (234), psychiatric diseases, and seizures (21, 53, 235). Peripheral biomarkers have an excellent NPV to rule out disease(s) but have a poor positive predictive value (PPV) to identify a specific pathological condition (27, 28, 46, 53, 236–238). Another concern is the potential contamination related to extra-CNS sources of protein biomarkers. For example, S100B could be derived from adipose tissue with levels directly depending on body mass index (239). A study excluded the impact of adipose tissue on S100B serum levels (23).

Elevated serum S100B was reported in patients presenting with extracranial pathology (240), such as polytrauma and burns (66).

Another important question is whether peripheral biomarkers have a prognostic value for the development of long-term brain pathology. Currently, there is no collective agreement on whether an unhealthy BBB may already exist, and could be diagnosed, in an otherwise apparently healthy brain (241, 242). However, recent evidence indicates that subjects presenting early cognitive impairment had preexisting BBB damage. The platelet-derived growth factor receptor beta (PDGFRβ; **Table 1**) (243, 244) shedding from perivascular pericytes was proposed as a biomarker of BBB integrity anticipating and predicting neurodegeneration (39, 243). A high-sensitivity method for detecting pericyte injury quantifying PDGFRβ in CSF was recently proposed (245). This method could be extended to study brain pericyte–endothelial damage in neurodegenerative disorders. Moreover, repetitive head hits during contact sports [American football (25)] were shown to associate with recurrent BBB permeability and S100B increases in blood. Players experiencing recurrent BBB permeability presented higher serum reactive autoantibodies, with a possible correlation with cognitive defects (25). The clinical significance of repeated BBB damage in sports is currently debated, with evidence pointing to a role in accelerated neurodegeneration (73). Moreover, total tau in blood was reported as a biomarker of axonal damage in hockey (24). Tau and amyloid monitoring in CSF is undergoing validation processes for dementia and AD (246, 247).

OUTLOOK AND FINAL REMARKS

Using peripheral biomarkers to monitor BBB permeability could extend to clinical cases where opening of the BBB is necessary to enhance drug penetration into the brain (13) or when re-establishment of physiological BBB tightness is justified to treat brain diseases (248, 249). Emerging evidence supports a holistic approach to tackle CNS diseases, where neuronal and cerebrovascular contributors of diseases are synchronously targeted. An increasing number of BBB-repairing molecules are currently being tested [for review, see (5, 6)], targeting NVU cells and neuroinflammation. Importantly, BBB biomarker and repairing strategies could become important in the settings of acute or chronic peripheral diseases (infections, metabolic or inflammatory) where immunity and inflammation negatively impact BBB permeability and, consequentially, synaptic transmission (5, 7, 250).

In conclusion, the NVU represents a modern and integrated entry point for the investigations of brain functions, and a continuous technological advancement will be instrumental to improve our ability to link NVU damage with diagnostics. The field of biomarkers of NVU damage, or dysfunction, is expanding together with the use of omic techniques and machine-learning routines for the discovery of signatures of acute or chronic disease conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

NM coordinated this effort, generated most of the figures, table, and contributed or wrote all parts. DJ focused on salivary biomarkers and provided parts of figures. DB focused on extreme conditions and biomarkers and providing relevant figures. JB and NM focused on imaging. SL, RO'F, and CH focused on salivary biomarkers and revised applicability of biomarkers. All authors contributed to the article and approved the submitted version.

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