Title: Time course and diagnostic utility of NfL, tau, GFAP, and UCH-L1 in subacute and chronic TBI

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Abstract

Objective To determine whether neurofilament light (NfL), glial fibrillary acidic protein (GFAP), tau, and ubiquitin C-terminal hydrolase-L1 (UCH-L1) measured in serum relate to traumatic brain injury (TBI) diagnosis, injury severity, brain volume, and diffusion tensor imaging (DTI) measures of traumatic axonal injury (TAI) in patients with TBI.

Methods Patients with TBI (n = 162) and controls (n = 68) were prospectively enrolled between 2011 and 2019. Patients with TBI also underwent serum, functional outcome, and imaging assessments at 30 (n = 30), 90 (n = 48), and 180 (n = 59) days, and 1 (n = 84), 2 (n = 57), 3 (n = 46), 4 (n = 38), and 5 (n = 29) years after injury.

Results At enrollment, patients with TBI had increased serum NfL compared to controls ($p < 0.0001$). Serum NfL decreased over the course of 5 years but remained significantly elevated compared to controls. Serum NfL at 30 days distinguished patients with mild, moderate, and severe TBI from controls with an area under the receiver-operating characteristic curve (AUROC) of 0.84, 0.92, and 0.92, respectively. At enrollment, serum GFAP was elevated in patients with TBI compared to controls ($p < 0.001$). GFAP showed a biphasic release in serum, with levels decreasing during the first 6 months of injury but increasing over the subsequent study visits. The highest AUROC for GFAP was measured at 30 days, distinguishing patients with moderate and severe TBI from controls (both 0.89). Serum tau and UCH-L1 showed weak associations with TBI severity and neuroimaging measures. Longitudinally, serum NfL was the only biomarker that was associated with the likely rate of MRI brain atrophy and DTI measures of progression of TAI.

Conclusions Serum NfL shows greater diagnostic and prognostic utility than GFAP, tau, and UCH-L1 for subacute and chronic TBI.

Classification of evidence This study provides Class III evidence that serum NfL distinguishes patients with mild TBI from healthy controls.

INTRODUCTION (< 3000 words)

Traumatic brain injury (TBI) is one of the leading causes of mortality and morbidity as well as high burden of disability.[1-3] TBI is a complex disorder where several pathophysiological processes may occur depending on the injury subtype including axonal injury, astrogliosis and neuronal injury or death.[4,5] TBI is also recognized as a risk factor for late-life neurodegeneration.[6-9] There is a great need to identify and measure these injury subtypes non-invasively and reliably in order to develop mechanistically appropriate therapies.

Traumatic axonal injury (TAI) is measured with fractional anisotropy (FA), radial diffusivity (RD), and mean diffusivity (MD) using diffusion tensor imaging (DTI).[4,5,10] However, DTI has several limitations, including limited availability, high cost, and cumbersome image postprocessing.[10] TAI can also be assessed using cerebrospinal fluid (CSF) tau or neurofilament light (NfL) protein, with the latter shown to be highly sensitive.[11-13] However, accessing CSF requires lumbar puncture (LP), which is invasive and not readily available. Recent developments in the immunoassay technology field have made it possible to reliably measure tau and NfL in blood samples.[14-16] This technique has been modified to quantify glial fibrillary acidic protein (GFAp), a marker of astrogliosis, and ubiquitin C-terminal hydrolase-L1 (UCH-L1), a cytosolic neuronal protein.[17] When measured acutely after TBI, these four candidate blood biomarkers have shown utility in distinguishing patients with intracranial hemorrhage on CT from those with negative CT findings.[17-19] Blood NfL and tau have also shown utility in athletes with concussion when measured within hours after injury.[20] Despite these recent studies, there are critical gaps in our knowledge related to these biomarkers, including the time course following injury, their relationship to injury severity or subtype, and the relationship to functional and neuroimaging outcomes.

In this study, we examine NfL, GFAp, tau and UCH-L1 in clinic-based patients with mild, moderate and severe TBI for up to five years following injury. We hypothesized that: (*1*) patients with TBI would have increased serum concentrations of axonal and glial proteins compared with controls, with higher concentrations in moderate or severe cases, and (*2*) the concentrations of axonal and glial proteins would correlate with brain white matter (WM) volumes and DTI measures of WM integrity.

METHODS

Standard protocol approvals, registrations, and patient consents

The institutional review board at the National Institutes of Health (NIH), Bethesda, MD, USA approved the study. Written and informed consent was obtained from all participants.

Study population

A prospective study of clinic-based patients with subacute and chronic mild, moderate, and severe TBI enrolled between January 2009 to July 2018 at the NIH Clinical Center, Bethesda, MD, USA. Severity of TBI was classified according to VA/DoD guidelines.[21] The inclusion criteria and the study population are described in **Supplement 1**.

Outcome measures

The primary outcome measures were changes in serum levels of NfL, GFAp, tau, and UCH-L1 from 30 days to 5 years after injury in relation to severity of TBI, functional outcome, brain MRI volumes, and DTI. Functional outcome was assessed with the Glasgow Outcome Scale-Extended (GOS-E).[22] Associations were also tested between blood biomarkers and gray matter (GM), WM, and corpus callosum (CC) volumes. We also conducted a detailed DTI characterization of CC. CC is a central WM structure and has been shown to be highly vulnerable to trauma to the brain.[23]

Biochemical assessment

Serum NfL, GFAp, tau, and UCH-L1 concentrations were measured simultaneously using the Neurology 4-plex assay kit (Quanterix Corporation, Lexington, MA, USA) on a Single molecule array (Simoa) HD-1 Analyzer (Quanterix Corporation, Lexington, MA, USA). The average coefficient of variation (CV) of measurement of NfL, GFAp, tau, and UCH-L1 were 4%, 3%, 33%, and 30%, respectively. The sample processing procedure is described in detail in **Supplement 1**.

Imaging acquisition and processing

MR images were acquired on a 3 tesla MR scanner (Siemens Biograph) with a 16-channel head coil in Radiology and Imaging Sciences at the Clinical Center, NIH, Bethesda, MD, USA. The image acquisition protocol and post-processing are detailed in **Supplement 1**.

Statistical analyses

The diagnostic utility of serum biomarkers was determined by calculating the Area Under the Receiver Operating Characteristic Curve (AUROC). The association between serum biomarkers and functional and imaging outcomes were tested using linear models, adjusted for covariates. All tests were two-sided and statistical significance was determined at $P < .05$. All statistical calculations were performed using R (v. 3.0.3, The R Foundation for Statistical Computing). The statistical tests are detailed in **Supplement 1**.

Data availability

The data supporting the findings are available upon request to the corresponding author.

RESULTS

Demographics and clinical characteristics

A total of 613 individuals were screened between 2009 and 2018 of whom 243 participants (175 with TBI [median, 7 months after recent TBI], and 68 healthy controls) were enrolled. Thirteen patients with TBI were excluded due to screening failure. Of 162 patients with TBI, 106 underwent repeated blood, MRI, and outcome assessment at 30 ($N = 30$), 90 ($N = 48$), and 180 ($N = 59$) days, and 1 ($N = 82$), 2 ($N = 57$), 3 ($N = 46$), 4 ($N = 38$), and 5 ($N = 29$) years after injury. Of 162 patients with TBI, 89 were classified as mild TBI (mTBI) and had no abnormalities on conventional MRI, 48 were moderate, and 25 were severe. The demographic and clinical characteristics of the participants at enrollment are shown in **eTable 1**.

Biomarker concentrations at enrollment

At enrollment, patients with TBI (all severities) had increased concentrations of NfL and GFAp compared with controls ($P = .0001$ and $P < .001$, respectively; **eTable 1**). There were no

differences in the concentrations of tau and UCH-L1 between TBI and controls (P = .17, and P = .11, respectively; **eTable 1**).

Serum NfL was elevated in patients with mTBI versus controls, moderate versus mild, and severe versus moderate ($P_{adjusted}$ = .0001, $P_{adjusted}$ = .0001, and $P_{adjusted}$ = .05, respectively; **Figure 1A**). Serum GFAp was elevated in mTBI versus controls and severe versus moderate cases but not moderate versus mild ($P_{adjusted}$ < .0001, $P_{adjusted}$ =.005, and $P_{adjusted}$ =.17, respectively; **Figure 1B**). Serum concentrations of tau and UCHL-1 were significantly higher in patients with severe TBI versus moderate but did not significantly distinguish mild, moderate, and controls (**Figure 1C** and **D**).

Time course of blood-based biomarkers

Figure 2A and **eFigure 1A** show the time course of serum NfL at group and individual level. Serum NfL was increased at the 30-day time point, with levels decreasing over 5 years (ß = – 0.09, P <.0001). Next, we conducted pairwise group comparisons across TBI severity and controls at different time points. The significant results adjusted for multiple group comparisons are summarized in **eTable 2**. In summary, serum NfL was increased in patients with mild, moderate, and severe TBI compared with controls even at 5 years after injury (**eTable 2**).

Figure 2B and **eFigure 1B** show the time course of serum GFAp at group and individual level. The longitudinal changes in GFAp concentrations from 30 days to 5 years were not statistically significant (ß = 0.003, P =.60). There was no difference in serum GFAp between mTBI and controls at 30, 90, and 180-day sampling time points after correcting for multiple comparisons; however, those with moderate or severe TBI had higher serum GFAp concentrations at all measured time points compared with controls (**eTable 2**).

Figure 2C and **eFigure 1C** show the time course of serum tau at group and individual level. Serum tau concentrations were variable over the course of the 5-year period, and there was no difference in tau concentrations over time (ß = – 0.02, P =.23). Serum tau concentrations were elevated in severe cases compared with controls at the 90-day, 1, and 2-year time points (**eTable 2**).

Figure 2D and **eFigure 1D** show the time course of serum UCH-L1 at group and individual level. The time course for serum UCH-L1 over the 5-year period was variable, and there was no effect of time on UCH-L1 concentrations (ß = – 0.025, *P* =.08). Also, there were no significant differences in UCH-L1 concentrations either across TBI severity or compared with controls at any measured time point except for 30-day time point, where UCH-L1 was elevated in moderate cases versus controls ($P_{adjusted}$ =.032; **eTable 2**).

Diagnostic utility of the biomarkers

Serum NfL distinguished mTBIs from controls at the 30-day time point with an AUROC of 0.84 (**Figure 3**). The AUROC for serum NfL at the following time points decreased (0.72-0.81; **Figure 3)**. The highest AUROCs for serum NfL were measured for moderate to severe cases at the 30, 90 and 180-day time points (ARUOCs, 0.84-0.98; **Figure 3**).

The AUROCs for serum GFAp over the course of five years ranged from 0.60-0.89, across TBI severity (**Figure 3**). Serum GFAp distinguished mTBIs from controls at the 30-day time point with an ARUOC of 0.71, while for the moderate and severe cases the AUROCs were 0.89, and 0.89, respectively (**Figure 3**).

The AUROC for serum tau distinguishing TBI cases from controls over the course of 5 years ranged from 0.50-0.74, with the highest AUROC measured at 2 years, distinguishing moderate and severe TBIs from controls (0.74, and 0.74, respectively; **Figure 3**).

Overall, the AUROCs for UCH-L1 decreased over the 5-year period, ranging from 0.50-0.77. The highest AUROCs for distinguishing mTBI from controls were measured at the 30-day time point, 0.70, while the AUROC for moderate and severe were 0.77 and 0.77, respectively (**Figure 3**).

Serum NfL shows associations to functional outcome

At enrollment, increased serum NfL concentrations were associated with worsened GOS-E scores (ß = – 0.28, *P* = .0019; **eFigure 2A**). There were no associations between GOS-E scores and other biomarkers (**eFigure 2B-D**).

Serum NfL and GFAp measured at 30 days were associated with an improvement in GOS-E at 90-day (ß = 0.64, *P* = .0002, ß = 1.14, *P* = .032; **eFigure 3** ***row A*** and ***B***). No changes were observed beyond the 30-day time point (**eFigure 3** ***row A and B***). Changes in GOS-E scores were not associated with tau and UCH-L1 at any of the time points (**eFigure 3** ***row C*** and ***D***).

Associations between blood biomarkers and brain volumes

At enrollment, increased serum NfL was associated with decreased GM, WM, mid anterior, central, and mid posterior CC volumes (**Figure 4A, E** and **eFigure 4**). Increased serum GFAp was associated with decreased GM, WM, anterior, mid anterior, central, and posterior CC volumes (**Figure 4B, F** and **eFigure 4**). Increased serum tau was associated with decreased GM volume, but WM volume or CC volumes (**Figure 4C** and **eFigure 4**). Also, increased serum UCH-L1 was associated with decreased GM volumes but not CC volumes. (**Figure 4D** and **eFigure 4**).

The summary of serum biomarkers predicting *future* change in brain volumes are presented in **eTable 3**. Serum NfL measured at the 180-day time point predicted WM volume loss at 1-year (ß = – 3881, *P* = .001; **eTable 3**). Serum NfL at 1-year predicted a loss in mid-anterior and central CC volumes at the 2-year time point (**eTable 3**). Also, serum NfL at the 3-year time point was associated with loss in central CC volume at the 4-year time point (**eTable 3**).

There were no significant relationships between GFAp, tau or UCH-L1 and changes in brain volumes over time after correcting for multiple comparisons (**eTable 3)**.

Associations between blood biomarkers and DTI measures of WM integrity

At enrollment, increased NfL was associated with decreased DTI FA for all segments of CC including genu, body, and splenium (ß = – 0.0075, *P* < .0001, ß = – 0.0071, *P* = .004, and ß = – 0.0075, *P* < .0001, respectively; **Figure 5A-C**). Similarly, increased GFAp was associated with DTI FA for all segments of CC (ß = – 0.0075, *P* < .0001, ß = – 0.0071, *P* = .004, and ß = – 0.0075, *P* < .0001, respectively; **Figure 5D-F**). Similarly, increased serum concentrations of NfL

and GFAp were associated with increases in DTI RD and MD for all segments of CC (**eFigure 5** and **eFigure 6**). Increased serum GFAp was also associated with DTI AD for all segments of CC (**eFigure 7**). There were no relationships between tau and UCH-L1 and DTI FA, AD, RD, and MD, except for UCH-L1 showing an association with DTI FA and CC splenium (ß = – 0.005, *P* = .017; **eFigure 5-8**).

The results testing whether serum NfL, GFAp, tau, and UCH-L1 could predict *future* DTI CC changes are summarized in **eTable 4A-D**. Serum NfL at the 3-year time point predicted a change in DTI FA for genu CC at the 4-year time point (ß = – 0.010, *P* < .0001, **eTable 4A**). Similarly, serum NfL at the 3-year time point predicted a change in DTI RD for genu CC from the 3 to 4-years (ß = 16.4, *P* = .006, **eTable 4A**). Serum GFAp at 180-day time point predicted a change in DTI FA for splenium CC from 180-day to 1-year (ß = 0.009, *P* = .0013; **eTable 4B**). Also, GFAp measured at 3-year predicted a change in DTI FA for genu CC at 4-years (ß = 0.011, *P* = .0003; **eTable 4B**). Serum tau measured at the 4-year time point predicted a change in DTI FA for splenium CC from 4-year to 5-year (ß = 0.003, *P* = .007; **eTable 4C**). Serum tau at 1-year predicted DTI MD changes for body of CC from 1 to 2 years (ß = 8.4, *P* = .009; **eTable 4C**). There was no relationship between serum UCH-L1 and changes in DTI measures for CC integrity after correcting for multiple comparisons (**eTable 4D**).

DISCUSSION

The main findings of this study are: (*1*) at median time of 7 months after injury, patients with mild, moderate, and severe TBI had increased concentrations of NfL and GFAp compared to controls, while serum tau and UCHL-1 were increased in the moderate to severe cases only; (*2*) serum NfL distinguished patients with TBI from controls at 30, 90, and 180-days with good to excellent accuracy; the diagnostic accuracy of GFAp, tau, and UCH-L1 were lower; (*3*) serum NfL was the only biomarker that showed relationships with functional outcome, cerebral WM, and CC volume changes; both serum NfL and GFAp showed relationships with DTI measures of WM axonal integrity.

NfL is a component of the axonal cytoskeleton and is primarily expressed in large-caliber myelinated axons that extend subcortically.[24] In the context of TBI, serum NfL measured within

48 hours of injury has been shown to distinguish patients with CT findings from those without CT findings.[17,25,26] In contrast to the existing studies, we herein found that serum NfL can distinguish patients with mild, moderate, and severe TBI from each other as well compared to controls months to years after injury. The AUROCs at 30 days for serum NfL distinguishing mTBI from controls was 0.84, while for moderate and severe cases were 0.94. Although the AUROCs decreased over five years, it remained significant in the mild and moderate cases. Serum NfL also showed modest associations with GOS-E score and was the only biomarker in this study associated with functional outcome at enrollment. In direct comparison to these findings, we previously observed increased concentrations of serum NfL up to 1-year after injury in patients with severe TBI, with initial levels associated with GOS-E score.[16] Together, these findings suggest that a single TBI may cause long-term axonal degeneration which could be detectable in serum months to years after injury using NfL as the biomarker.

GFAp is an intermediate filament protein that is predominantly expressed by astrocytes.[27] In the context of TBI, serum GFAp measured acutely after injury distinguish patients with intracranial hemorrhage on CTI from those with normal CT finding.[17-19] Herein, serum GFAp measured median of 7 months did not relate to injury severity, but could distinguish mild, moderate and severe TBI from controls. Additionally, in the previous study, the concentration of serum GFAp decreased in the days following injury.[18] In contrast, we found increased concentrations of serum GFAp at 30 days after TBI, with lower levels over the following time points. The highest ARUOC for GFAp was measured at 30-day time point for moderate to severe TBI cases (AUCROC, 0.89), however, beyond 30-day time point the AUROCs were limited. Also, there was no association between GFAp and outcome. Therefore, unlike previous studies in acute TBI,[11,28] these results indicate that serum GFAp may not perform well as a diagnostic biomarker in subacute and chronic TBI.

Tau is a microtubule-associated protein predominantly expressed in short cortical unmyelinated axons.[29] In the context of TBI, increased concentrations of CSF tau have previously been found in acute samples from patients with moderate to severe TBI.[13,28] Also, plasma tau increased within hours after concussion in athletes compared with preseason baseline.[15,20,30] In the present study, serum tau concentrations were elevated in patients with TBI

at enrollment, with higher concentrations in moderate to severe cases. Longitudinally, the levels of serum tau were variable and did not relate to injury severity (AUROCs ranged from 0.50-0.74) or functional outcome. Similar findings have also been observed in CSF of athletes with a history of repetitive concussions, where there was no association between CSF tau and outcome.[31,32] These findings are consistent with previous reports showing elevated concentrations of tau following TBI, however, with limited prognostic utility.[31,32]

UCH-L1 is abundantly found in neurons.[33] Similar to serum GFAp, previous studies have reported that serum UCH-L1 measured within 48 hours after injury distinguish patients with intracranial hemorrhage on CT from those with normal CT finding.[17-19] In contrast to the previous studies, serum UCH-L1 measured at median of 7 months after injury was elevated in patients with severe TBI compared with controls but not mild or moderate TBI. Longitudinally, the levels of serum UCH-L1 was variable but did not relate to injury severity or outcome. Together, these findings indicate that serum UCH-L1 may not perform as well when measured in the subacute and chronic phase of TBI. This is further supported by the higher analytical variability seen for lower concentrations of serum UCH-L1 herein as well as previously.[17]

In the last part of this study, we attempted to cross-validate NfL, GFAp, tau, and UCH-L1 with brain MRI volumetric analysis and DTI. Increased serum NfL was related to decreased GM, WM, and CC volumes. Decreases in FA, along with increased RD and MD were also observed for NfL. These findings are in direct comparison to a recent prospective study of nine patients that revealed a strong relationship between serum NfL measured six days after injury with TAI assessed with DTI 12 months later.[34] Similar to NfL, serum GFAp showed a relationship with both brain volumes and DTI metrics at enrollment, however, with limited predictive utility. As expected, the association of tau and UCH-L1 with brain volumes and DTI measures were weak, further indicating that tau and UCH-L1 measured in the subacute and chronic phase of TBI may not be as informative as when measured acutely. Importantly, the convergent findings provided by the methodologies used herein (*i.e.*, serum biomarker levels, MRI volumetric analysis, and DTI) is a principal proof of independent cross-validation of these methods. From the scientific perspective, the data provided by these three methods greatly increases the confidence of a relationship between serum biomarker levels and underlying

neuropathology. From a clinical perspective, the complementary strengths of these methods and their relationship with clinical outcome provide multiple diagnostic and prognostic options to the clinician. For instance, MRI volumetric analysis provides no information regarding WM axonal microstructure yet is readily available and not technically challenging, while DTI provides more detailed and region-specific information of WM microstructure but has limited availability and high cost. On the other hand, serum NfL concentrations are related to both imaging outcomes as well as clinical outcome and TBI severity, and they are readily measured using standard laboratory techniques.

Comparing the four serum neuronal injury biomarkers measured herein, the performance of NfL was robust in distinguishing patients with different TBI severities at enrollment and over time as well as showing stronger associations with functional outcome, brain volumes, and DTI measures of TAI compared to the other biomarkers. Additionally, serum NfL was elevated in mild and moderate cases compared with controls up to 5 years after injury. Serum GFAp showed a biphasic releasing pattern, with levels decreasing during the first 6 months of injury but increased over the following time points. These findings indicate that axonal injury and astrogliosis may persist for years after TBI and are more evident in moderate to severe cases, which is consistent with existing animal model and human histopathological studies.[35,36] From a clinical stand point, although serum GFAp was increased in the subacute and chronic TBI patients, it did not relate to injury severity or outcome. Finally, the level of tau and UCH-L1 were variable and showed weak or no relationships to injury severity and function and neuroimaging outcomes, suggesting that tau, and UCH-L1 may not be sensitive biomarkers for subacute and chronic TBI.

Limitations

We did not have longitudinal blood samples and MRI assessments at all measured time points, which is an inherent issue of many long-term longitudinal studies. Second, the present study was designed to include participants at 30 days and onwards after injury, precluding comparison to their acute biomarker concentrations as well as direct comparison to the existing studies of these biomarkers. Third, serum may not be the optimal source for measurement of tau, as tau concentrations in serum are lower than in plasma, possibly explaining the higher analytical

variation for this biomarker. Lastly, we also observed higher analytical variations for UCH-L1, especially for the lower concentrations, limiting the utility of serum UCH-L1 as a biomarker for subacute or chronic mTBI.

Conclusion and clinical relevance

These findings suggest that serum NfL and GFAP, which are reflective of axonal injury and astroglial injury/activation, respectively, can be detected months to years after injury, with serum NfL showing greater diagnostic utility and greater associations with functional outcome, brain MRI volumes, and DTI measures of WM integrity. In order to implement these findings into clinical practice, future directions include standardization of methods of quantification across analytical platforms and determining cutoffs across age, severity, and for different populations such as athletes and military personnel.

FIGURE LEGENDS

Figure 1. Biomarker concentrations across TBI severities at enrollment

Plots (**A-D)** show serum concentrations of NfL, GFAp, tau, and UCH-L1 across TBI severities at median 7 months after TBI. The boxplots show the median and interquartile range. The *P* values are adjusted for multiple comparisons using Holm-Bonferroni method. *Abbreviations*: TBI, traumatic brain injury; NfL, neurofilament light; UCH-L1, ubiquitin C-terminal hydrolase-L1; GFAp, glial fibrillary acidic protein.

Figure 2. Time course of the blood biomarkers

Plots (**A-D**) show the time course of serum NfL, GFAp, tau, and UCH-L1 across TBI severities. The error bars indicate the standard error of mean. The y-axes are long-transformed (log 10) for better visual clarity. The x-axes show the sampling time points after TBI. *Abbreviations*: TBI, traumatic brain injury; NfL, neurofilament light; GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1.

Figure 3. Diagnostic utility of blood biomarkers over time

The plots show the diagnostic utility (AUROC) of serum NfL, GFAp, tau, and UCH-L1 in distinguishing TBI patients from controls. The error bars indicate the 95 % confidence interval. *Abbreviations*: TBI, traumatic brain injury; AUROC, area under the receiver operating characteristics curve; NfL, neurofilament light; GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1.

Figure 4. Association between blood biomarkers and GM, WM volumes at enrollment

Plots (**A-H**) show the association between serum concentrations of NfL, GFAp, tau, and UCH-L1, and GM and WM volumes. The ß estimates and *P* values are from regression models, covaried for age, education, and gender. The fitted lines including the confidence interval are from the adjusted regression models. *Abbreviations*: GM, grey matter; WM, white matter; NfL, neurofilament light; GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1.

Figure 5. Serum NfL and GFAp concentrations at enrollment in relation to DTI fractional anisotropy

Plots (**A**-**F**) show the relationship between serum NfL and GFAp measured at enrollment and DTI FA for CC integrity measured at enrollment. The ß estimates and *P* values are from regression models, covaried for age, education, and gender. The fitted lines including the confidence interval are from the adjusted regression models. The ρ denotes the Spearman's rank correlation coefficient (univariate analysis). *Abbreviations*: NfL; neurofilament light; GFAp, glial fibrillary acidic protein; CC, corpus callosum; diffusion tensor imaging; FA, fractional anisotropy.

eFigure 1. Time course of the blood biomarkers

Rows (**A**-**D**) show the time course of serum NfL, GFAp, tau, and UCHL-1 for individual patients across TBI severities. *Abbreviations*: TBI, traumatic brain injury; NfL, neurofilament light; GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1.

eFigure 2. Association between blood biomarkers and functional outcome at enrollment

Plots (**A**-**D**) show the association of GOS-E with serum concentrations of NfL, GFAp, tau, and UCH-L1. The *x*-axes show the log-transformed values for visual clarity. The ß estimates and *P* values are from linear regression models, covaried for age, education, and gender. The fitted lines including the standard error are from the regression models. *Abbreviations*: GOS-E, Glasgow Outcome Scale Extended; NfL, neurofilament light; GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1.

eFigure 3. **Serum NfL shows association with clinical outcome**

Rows (**A**-**D)** show serum NfL, GFAp, tau, and UCH-L1 measured at different sampling time point in relation to change in GOS-E score from the previous time point. The *x* axes show the log-transformed values for visual clarity. The ß estimates and *P* values are from linear regression model, covaried for age, education, and gender. The fitted are from the regression models. The models were also tested with Spearman's rank correlation, however, none of the models were significant. *Abbreviations*: GOS-E, Glasgow Outcome Scale Extended; NfL, neurofilament light;

GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; N.S., non-significant.

eFigure 4. Associations between blood biomarkers and GM, WM, and CC volumes at enrollment

Rows (**A**-**E**) show the association between serum concentrations of NfL, GFAp, tau, and UCH-L1, and GM, WM, and CC volumes. The ß estimates and *P* values are from regression models, covaried for age, education, and gender. The fitted lines including the confidence interval are from the adjusted regression models. *Abbreviations*: GM, grey matter; WM, white matter, CC, corpus callosum; NfL, neurofilament light; GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1.

eFigure 5. Serum NfL, GFAp, tau, and UCH-L1 concentrations at enrollment in relation to DTI radial diffusivity at enrollment

Rows (**A**-**D**) show the association between serum NfL, GFAp tau, and UCH-L1 measured at enrollment and DTI AD for CC integrity measured at enrollment. The ß estimates and *P* values are from regression models, covaried for age, education, and gender. The fitted lines including the confidence interval are from the adjusted regression models. The ρ denotes the Spearman's rank correlation coefficient (univariate analysis). *Abbreviations*: NfL; neurofilament light; GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; CC, corpus callosum; DTI, diffusion tensor imaging; RD, radial diffusivity.

eFigure 6. Serum NfL, GFAp, tau, and UCH-L1 concentrations at enrollment in relation to DTI mean diffusivity at enrollment

Rows (**A**-**D**) show the association between serum NfL, GFAp tau, and UCH-L1 measured at enrollment and DTI AD for CC integrity measured at enrollment. The ß estimates and *P* values are from regression models, covaried for age, education, and gender. The fitted lines including the confidence interval are from the adjusted regression models. The ρ denotes the Spearman's rank correlation coefficient (univariate analysis). *Abbreviations*: NfL; neurofilament light; GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; CC, corpus callosum; DTI, diffusion tensor imaging; MD, mean diffusivity.

eFigure 7. Serum NfL, GFAp, tau, and UCH-L1 concentrations at enrollment in relation to DTI axial diffusivity at enrollment

Rows (**A-D**) show the association between serum NfL, GFAp tau, and UCH-L1 measured at enrollment and DTI AD for CC integrity measured at enrollment. The ß estimates and *P* values are from regression models, covaried for age, education, and gender. The fitted lines including the confidence interval are from the adjusted regression models. The ρ denotes the Spearman's rank correlation coefficient (univariate analysis). *Abbreviations*: NfL; neurofilament light; GFAp, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; CC, corpus callosum; DTI, diffusion tensor imaging; AD, axial diffusivity.

eFigure 8. Serum tau and UCH-L1 concentrations at enrollment in relation to DTI axial diffusivity at enrollment

Rows (**A-D**) show the association between serum NfL, GFAp tau, and UCH-L1 measured at enrollment and DTI AD for CC integrity measured at enrollment. The ß estimates and *P* values are from regression models, covaried for age, education, and gender. The fitted lines including the confidence interval are from the adjusted regression models. The ρ denotes the Spearman's rank correlation coefficient (univariate analysis). *Abbreviations*: UCH-L1, ubiquitin C-terminal hydrolase-L1; CC, corpus callosum; DTI, diffusion tensor imaging; AD, axial diffusivity.

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CONFLICTS OF INTEREST

Dr. Shahim reports no conflicts of interest. Dr. Diaz-Arrastia serves in the Scientific Advisory Board or BrainBox, Inc, and Neural Analytics (all unrelated to the work presented in this paper). Dr. Zetterberg has served at scientific advisory boards for CogRx, Samumed, Roche Diagnostics and Wave, has given lectures as symposia sponsored by Biogen and Alzecure, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg (all unrelated to the work presented in this paper). Dr. Blennow has served as a consultant or at advisory boards for Alector, Alzheon, CogRx, Biogen, Lilly, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg (all

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AUTHOR CONTRIBUTION

Dr. Shahim had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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