Serum-based GFAP, NFL, Tau, and UCH-L1
in assessing acute Traumatic Brain Injury

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Abstract

The purpose of this study was to compare the levels of Glial Fibrillary Acidic Protein (GFAP), Neurofilament Light Protein (NFL), Tau Protein, and Ubiquitin Carboxyl-Terminal Hydrolase Isozyme L1 (UCH-L1) in patients with varying TBI severities as determined by Glasgow Coma Scale Scores and CT imaging. Due to the complexity and intricate nature of each TBI case, we cannot depend solely on a single biomarker to interpret the entire body's pathophysiological state post-injury. While these proteins have been studied and researched individually in the past, using multiple biomarkers to assess a TBI event could create a much more precise understanding of the injury (9-10). Given this information, there is a need to understand the singular nature and behaviors of all potential biomarkers during the varying stages and severities of TBI before their use can be appropriately and effectively implemented within the field of medicine. As such, by analyzing the presence four different biomarkers within blood serum, this study tests each biomarkers' ability to classify TBI severity while providing further insight into how these biomarkers behave during the acute stages of TBI so that approximate cutoff values for the different severities of TBI could be created.

This study included a total of 240 patients with varying levels of TBI that were categorized using "Glasgow Coma Scale Scores". Patients enrolled in the study came from three different locations: Hospital Admissions, Emergency Department, and the Intensive Care Unit. The biomarker levels of these patients were compared to 30 commercially available control blood samples from patients without any incidence of TBI. Serum protein levels were measured using magnetic bead technology from the "Simoa Neuro-4-Plex A Advantage Kit." Within this study, results indicated that NFL was the single most useful biomarker in terms of its ability to distinguish between all severity categories of TBI; NFL was the only biomarker that received statistically significant results for all severity comparisons in the Kruskal-Wallis test. However, looking at each test individually, there were many instances where other biomarkers were better indicators in certain severity classes. For example, based on AUC values, GFAP, Tau, and UCH-L1 outperform Tau when differentiating between mild and moderate TBI. Thus, even though NFL may seem to be the single most useful biomarker in terms of its general application, the results of this study strongly suggest that even for severity classification, multiple biomarkers should be analyzed for accuracy purposes.

Introduction

According to the Centers for Disease Control and Prevention, an estimated 2.8 million Americans sustain some sort of Traumatic Brain Injury each year (1). However, despite our advancements in imaging technology, surgical expertise, and injury management methods in assessing and treating patients with TBI, we cannot ignore the lack of precise and efficient evidence-based diagnostic methods when dealing with TBI. Thus far, physicians have relied heavily on the use of Computed Tomography (CT) imaging and Glasgow Coma Scale (GCS) scores to assess the severity of brain injury. A derivative of the X-ray, a CT scan produces a 360-degree high definition image of the brain by utilizing computer software to stitch together 2.5 mm axial image slices (2). The GCS is a scale developed in 1974 that sums up the evaluation of eye-opening, verbal, and motor responses within TBI patients to gauge the severity of the injury (3). GCS scores range from 3 to 15, with 3 being the most severe and 15 being the least (3).

Both of these methods and their implementation in clinical practice has evolved over the course of many years, but there are still inherent flaws regarding the implementation of these modalities as diagnostic/prognostic tools. For example, even though CT imaging provides high-quality images of the brain's gross anatomy, it is still very difficult to differentiate mild TBI. A large part of this issue stems from the absence of a general consensus on what actually constitutes mTBI (4a-4c). Furthermore, while GCS provides a very useful classification tool for making quick clinical decisions, there are many factors that can prevent experts from obtaining an accurate score. Such factors include language/speech barriers, mental disabilities, and paralysis among others (3). In a Center-TBI study done on the variability of management policies for TBI patients, they found that 59% of the centers defined mTBI with GCS between 13 and 15 while 38% defined mTBI with a GCS of 14-15 (6). Without the use of a more concise indicator of TBI severity, it's very difficult for guidelines to be created and followed, especially during moments of emergency.

Combined with the lack of definitive indicators for mTBI, the complex nature of the mechanisms that occur during TBI has made it extremely difficult for physicians to agree on management/treatment methods (5). TBI changes the pathology and functioning of the brain; if these changes within the brain are not recognized and treated in a timely manner, acute TBI could subsequently induce a cascade of debilitating permanent vulnerabilities and disabilities within patients (7). Alterations in brain function include changes to the blood-brain barrier (BBB), cerebral blood flow (CBF), axonal/neuronal cell body damage, excitotoxicity, and inflammation amongst others (7-8). Since neurons/axons are easily susceptible to mechanical injury in the aftermath of any physical impact to the head (7, 9), this study includes biomarkers with the potential ability to assess neuronal/axonal injury. By comparing the relative levels of GFAP, NFL,

Tau, and UCH-L1 within the blood serum of patients of differing severity TBI levels determined using the GCS and CT abnormalities, a relationship between the level of protein present in the blood and the stage of TBI can be drawn.

Ultimately, the study's goal was to assess the value of a panel of biomarkers as a diagnostic tool for patients with TBI. Biomarkers will be assessed mainly on its ability to distinguish the severity based on the GCS. The biomarkers' ability to distinguish between CT abnormalities will also be tested for additional verification.

Methods

Study population

A sample of 240 patients was recruited at multiple European trauma centers to be included in this study for analysis. Patients were enrolled between September 2018 and January 2019. Inclusion criteria for enrollment in this study included patients who were admitted to the trauma center within 24 hours of the initial injury. Patients were excluded if they were unable to provide consent at the time they were presented within the hospital. After the initial triage from admissions, certain patients were moved to the Intensive Care Unit based on the initial analysis of their injury. Blood samples were collected from three different departments within the center: Admissions, Emergency Department, and Intensive Care Unit.

Sample Collection

CT Scans and blood samples were collected within 24 hours of the initial injury. Blood samples were left in a room temperature environment for at least 30 minutes to allow the blood to clot properly. On average, after 44 minutes had passed, samples were brought to be processed by means of centrifugation. After 15 minutes of centrifugation at 2500 RPM, blood serum was aliquoted into plastic collection tubes. Collection tubes were immediately frozen in -80 ℃ temperatures to prevent degradation of proteins within the serum. The samples were shipped inside styrofoam containers with adequate amounts of dry ice to prevent complete thawing of the samples. Samples were sent to the Wang Lab at the McKnight Brain Institute. Upon arrival, the samples were stored in -80 ℃ temperature until needed for testing.

Control Samples

Control samples were commercially available blood serum samples (Bioreclamation) from healthy humans without TBI.

Analysis of GFAP, NFL, Tau, and UCH-L1

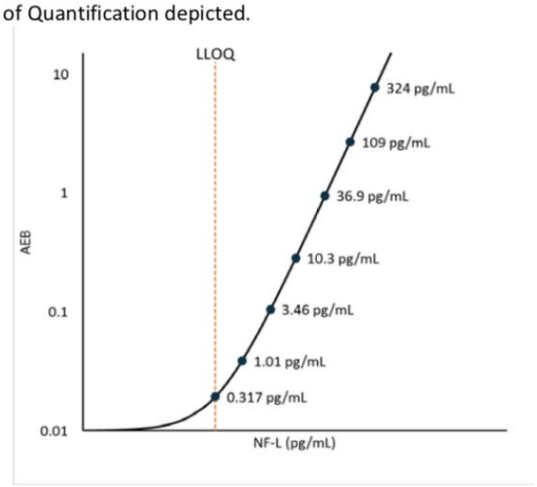
Serum GFAP, NFL, Tau, and UCH-L1 were measured using the Human Neurology 4-Plex A Advantage Kit (N4PA). This kit is used in conjunction with the provided SR-X machine, Microplate Washer, and Microplate Shaker (Simoa) according to the instructions from the manufacturer (Quanterix, Lexington, MA). The magnetic bead technology utilized in the N4PA not only allows multiple biomarkers to

be analyzed simultaneously but also increases the sensitivity to each biomarker. For blood serum/plasma, only 38µL of the sample is required for analysis. The SR-X machine analyzes the sample to provide an Average Enzyme per Bead (AEB) value which can then be converted to a concentration value (pg/mL) based on the dilution factor used.

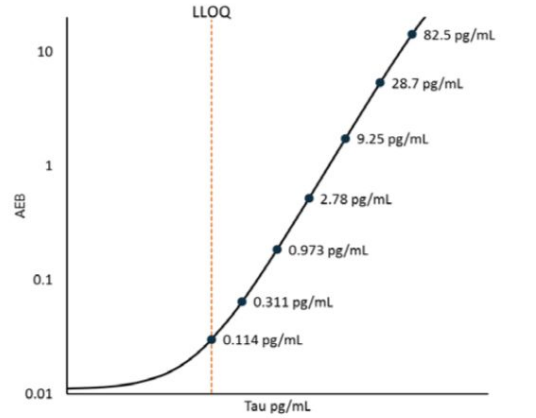
Samples were frozen in -80℃ and only thawed when necessary to preserve the integrity of the proteins within the serum.

Occasionally, the machine would produce errors dealing with the limits of quantification of the sample. Upon receiving these errors, the sample label and specific error was noted and then retested in later runs with a higher dilution factor until the sample was quantifiable.

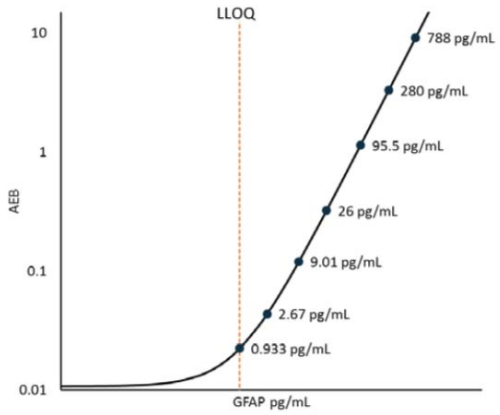
NF-L Curve: Calibrator concentrations and Lower Limit of Quantification depicted.



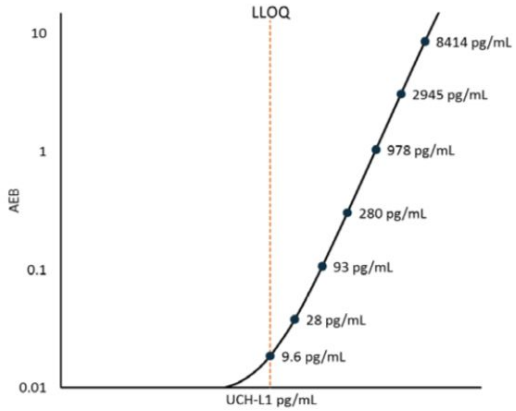
Tau Curve: Calibrator concentrations and Lower Limit of Quantification depicted.



GFAP Curve: Calibrator concentrations and Lower Limit of Quantification depicted.



UCH-L1 Curve: Calibrator concentrations and Lower Limit of Quantification depicted.



*Graphs adopted from [N4PA SR-X Data Sheet].

Statistical analysis

Using Kolmogorov-Smirnov tests and visual analysis of the data histograms/residual plots to assess normality, I found that the levels of GFAP, NFL, Tau, and UCH-L1 were not normally distributed. However, with a sample size of 240 individuals, according to the Central Limit Theorem, the violation of the normality assumption should not cause major issues when using parametric tests (11-12). Furthermore, using the ROUT method (Q = 1%), several outliers were excluded for accuracy. However, as a safety measure, I analyzed the data using nonparametric tests to maintain statistical integrity.

Spearman rank correlation coefficient was used to compare the relative levels of biomarkers to the severity of the injury as determined by GCS. Mann-Whitney U test was used to compare the differing levels of biomarkers between the varying severity groups for individual groups of severity classification. The Kruskal-Wallis test was also executed to corroborate these findings. Furthermore, the area under (AUC) the receiver operating characteristic (ROC) curve was used to assess the diagnostic ability of biomarkers in predicting injury severity. For all significance tests, p-values < 0.05 were considered statistically significant. For data analyses, GraphPad PRISM Version 8.0 (GraphPad Inc., San Diego, CA) Software was utilized to obtain results.

Results

Patient demographics, injury severity/GCS, and CT Scans

Protein analysis was executed on a total of 240 patients within this study. (**Table 1**) Neither gender nor age played a part in the inclusion criteria. As long as TBI patients were brought into the trauma center within 24 hours of their injury and gave consent to have their blood samples taken, they were included in this pilot study. Of the patients admitted to the participating trauma centers, 36 patients (15%) were classified with severe TBI (GCS 3-8), 24 patients (10%) with moderate TBI (GCS 9-12), and 175 (72.9%) with mild TBI (GCS 13-15). GCS scores were unavailable for 5 patients; these patients' biomarker levels were excluded from statistical analysis. Additionally, intracranial CT findings were described with an array of possible abnormalities: midline shifting, acute subdural hematoma, subarachnoid hemorrhage, and contusions.

Table 1. Patient Characteristics

Total Patients:	240
Glasgow Coma Scale (GCS)	
3-8:	36 (15%)
9-12:	24 (10%)
13-15:	175 (72.9%)
Missing:	5 (2.08%)
CT	
Midline Shift:	34 (14%)
Subd. Hematoma:	66 (27.5%)
Suba. Hemorrhage:	89 (37.1%)
CT Contusion:	85 (35.4%)

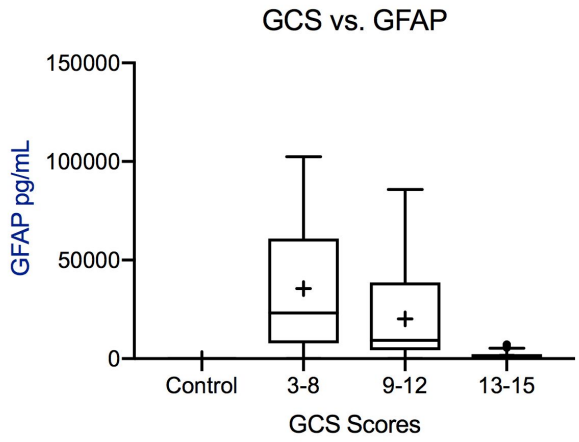
GFAP & results

The levels of GFAP of patients (median, 1609 pg/mL; IQR, 4184.9 pg/mL) of all GCS scores/TBI severities was significantly different (p-value < 0.0001) from control subjects (median, 3.316 pg/mL; IQR, 5.19 pg/mL) without any incidence of TBI. For all the intracranial abnormalities that were observed using CT imaging, GFAP levels significantly differed (p-value < 0.0001) between patients who did and didn't show abnormalities.

The levels of GFAP were also compared between patients of varying GCS Scores using various significance tests. From the Mann-Whitney U tests, the difference of GFAP levels was statistically significant (p-value < 0.0001) when comparing each severity group separately. However, when comparing the means between the severity classes using Kruskal-Wallis, the results showed that the GFAP levels were significantly different when comparing mild-severe and mild-moderate but not for moderate-severe (p-value = 0.6138). AUC scores obtained from the ROC Curve further confirmed the findings of the significance tests but also gave insight into approximate cut-off values between each severity class.

Although the relationship between the levels of GFAP and GCS score wasn't exactly linear due to the varying spectrum of TBI, they were found to be significantly negatively correlated (p-value < 0.0001) with a Spearman correlation coefficient of -0.8418.

Figure 1*



*Refer to **Table 2B** for further information
+ Symbol refers to the mean

Figure 2

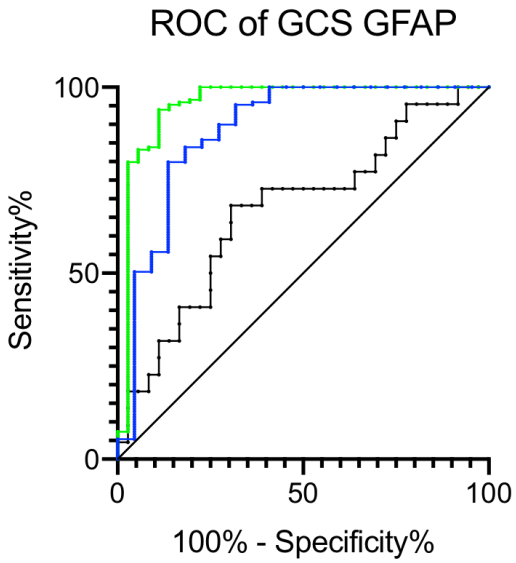


Table 2A. ROC GFAP Curve

Black:	GCS 3-8 vs. 9-12	**AUC: 0.6705**
Blue:	GCS 9-12 vs. 13-15	**AUC: 0.8829**
Green:	GCS 3-8 vs. 13-15	**AUC: 0.9545**

Table 2B GFAP Significance Tests

Mann-Whitney U Test

GCS

3-8 vs. 9-12:	*p*-value = 0.0303	Median(3-8, 9-12): 23150, 9469
3-8 vs. 13-15:	*p*-value < 0.0001	Median(3-8, 13-15): 23150, 1422
9-12 vs. 13-15:	*p*-value < 0.0001	Median(9-12, 13-15): 9469, 1422

Kruskal-Wallis Test

Dunn's multiple comparisons	Mean rank diff.	Significant?	Summary	Adjusted P Value
3-8 vs. 9-12	20.56	No	ns	0.6138
3-8 vs. 13-15	96.26	Yes	****	<0.0001
9-12 vs. 13-15	75.69	Yes	****	<0.0001

Spearman Correlation

GCS vs. GFAP (pg/mL)

Spearman r = -0.4424 95% Confidence Interval: (-0.5539, -0.3153) *p*-value < 0.0001

ROC (AUC)

3-8 vs. 9-12:	*p*-value = 0.0305	AUC = 0.6705	*Cutoff < 58729 pg/mL
9-12 vs. 13-15:	*p*-value < 0.0001	AUC = 0.8829	*Cutoff < 4930 pg/mL
3-8 vs. 13-15:	*p*-value < 0.0001	AUC = 0.9545	*Cutoff < 4930 pg/mL

*Cutoff values determined with a minimum of 90% Sensitivity.

Table 2C. ROC GFAP Curve

GCS 3-8 vs. 9-12	Sens, Spec: 90.91, 25
GCS 9-12 vs. 13-15	Sens, Spec: 90.6, 68.2
GCS 3-8 vs. 13-15	Sens, Spec: 90.6, 88.9

Cutoff values for GFAP

The cutoff value between severe and moderate TBI is 58729 pg/mL (90% Sensitivity, 25% Specificity). Levels greater than this value should be classified as severe TBI while anything lower should be moderate TBI. The AUC value shows that on average, a severe TBI patient (GCS 3-8) will show higher levels of GFAP than 67.05% of moderate TBI patients (GCS 9-12). For consistency, the cutoff values between severe and mild TBI were also included.

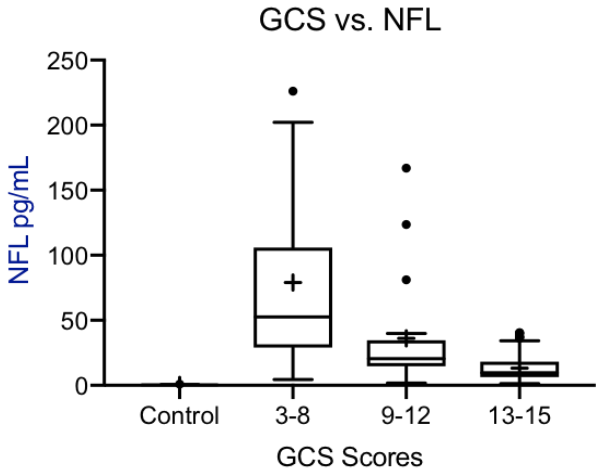
NFL and results

The levels of NFL in patients (median, 13.95 pg/mL; IQR, 15.96 pg/mL) of all TBI severities/GCS was significantly different (p-value < 0.0001) from control subjects (median, 0.354 pg/mL; IQR, 0.328 pg/mL) without any incidence of TBI. For all the intracranial abnormalities that were observed using CT imaging, NFL levels significantly differed (p-value < 0.0004) between patients who did and didn't show abnormalities.

The levels of NFL were also compared between patients of varying GCS Scores using various significance tests. From the Mann-Whitney U tests, the difference of NFL levels was statistically significant (p-value ≤ 0.0009) when comparing each severity group separately. Comparing the means between the severity classes using Kruskal-Wallis also showed that the NFL levels were significantly different between varying TBI severities as well. AUC scores obtained from the ROC Curve further confirmed the findings of the previous significance tests but also gave insight into approximate cut-off values for each severity class.

Although the relationship between the levels of NFL and GCS score could not be analyzed with regression analysis, they were found to be significantly negatively correlated (p-value < 0.0001) with a Spearman correlation coefficient of -0.2974.

Figure 3*



*Refer to **Table 4B** for further information
+ symbol refers to the mean

Figure 4

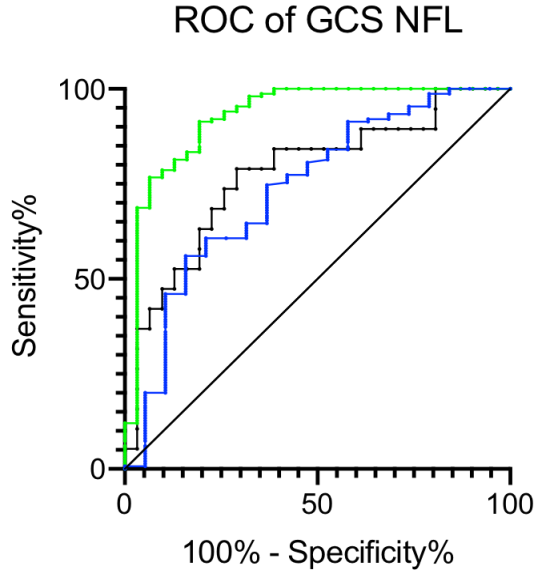


Table 4A. ROC NFL Curve

Black:	GCS 3-8 vs. 9-12	**AUC: 0.7759**
Blue:	GCS 9-12 vs. 13-15	**AUC: 0.7351**
Green:	GCS 3-8 vs. 13-15	**AUC: 0.9258**

Table 4B NFL Significance Tests

Mann-Whitney U Test

GCS

3-8 vs. 9-12:	*p*-value = 0.0009	Median(3-8, 9-12): 52.62, 20.48
3-8 vs. 13-15:	*p*-value < 0.0001	Median(3-8, 13-15): 52.62, 9.725
9-12 vs. 13-15:	*p*-value = 0.0006	Median(9-12, 13-15): 20.48, 9.725

Kruskal-Wallis Test

Dunn's multiple comparisons	Mean rank diff.	Significant?	Summary	Adjusted P Value
3-8 vs. 9-12	42.40	Yes	*	0.0358
3-8 vs. 13-15	86.78	Yes	****	<0.0001
9-12 vs. 13-15	44.38	Yes	**	0.0049

Spearman Correlation

GCS vs. NFL (pg/mL)

Spearman r = -0.2974 95% Confidence Interval: (-0.4241, -0.1593) *p*-value < 0.0001

ROC (AUC)

3-8 vs. 9-12:	*p*-value = 0.0012	AUC = 0.7759	*Cutoff < 145.3 pg/mL
9-12 vs. 13-15:	*p*-value = 0.0009	AUC = 0.7351	*Cutoff < 25.96 pg/mL
3-8 vs. 13-15:	*p*-value < 0.0001	AUC = 0.9258	*Cutoff < 25.96 pg/mL

*Cutoff values determined with a minimum of 90% Sensitivity.

Table 4C. ROC NFL Curve

GCS 3-8 vs. 9-12	Sens, Spec: 94.7, 19.4
GCS 9-12 vs. 13-15	Sens, Spec: 90.0, 42.1
GCS 3-8 vs. 13-15	Sens, Spec: 90.0. 80.7

Cutoff values for NFL

Please refer to "*Cutoff levels for GFAP"* for detailed interpretation of cutoff values.

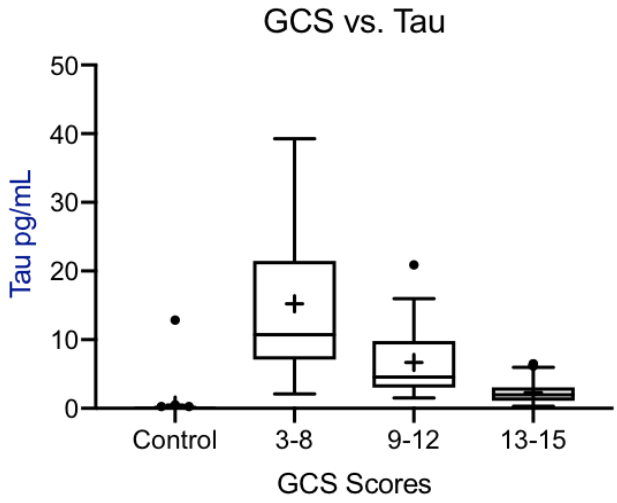
Tau and results

The levels of Tau in patients (median, 2.25 pg/mL; IQR, 2.675 pg/mL) of all TBI severities/GCS were significantly different (p-value < 0.0001) from control subjects (median, 0.0129 pg/mL; IQR, 0.082 pg/mL) without any incidence of TBI. For all the intracranial abnormalities that were observed using CT imaging, Tau levels significantly differed (p-value < 0.0001) between patients who did and didn't show abnormalities.

The levels of Tau were also compared between patients of varying GCS Scores using various significance tests. From the Mann-Whitney U tests, the difference in Tau levels were all statistically significant (p-value ≤ 0.0011) when comparing each severity group separately. However, when comparing the means between the severity classes using Kruskal-Wallis, the results showed that the Tau levels were significantly different when comparing mild-severe and mild-moderate but not for moderate-severe (p-value = 0.1760). AUC scores obtained from the ROC Curve further confirmed the findings of the previous significance tests but also gave insight into approximate cut-off values for each severity class.

Although the relationship between the levels of Tau and GCS could not be analyzed using regression analysis, they were found to be significantly negatively correlated (p-value < 0.0001) with a Spearman correlation coefficient of -0.6151.

Figure 5*



*Refer to **Table 6B** for further information
+ symbol refers to the mean

Figure 6

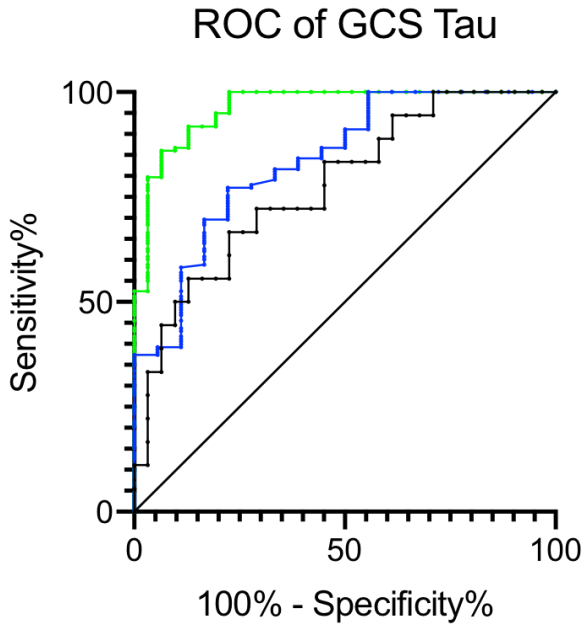


Table 6A. ROC Tau Curve

Black:	GCS 3-8 vs. 9-12	**AUC: 0.7760**
Blue:	GCS 9-12 vs. 13-15	**AUC: 0.8356**
Green:	GCS 3-8 vs. 13-15	**AUC: 0.9624**

Table 6B Tau Significance Tests

Mann-Whitney U Test

GCS		
3-8 vs. 9-12:	*p*-value = 0.0011	Median(3-8, 9-12): 10.71, 4.57
3-8 vs. 13-15:	*p*-value < 0.0001	Median(3-8, 13-15): 10.71, 1.97
9-12 vs. 13-15:	*p*-value < 0.0001	Median(9-12, 13-15): 4.57, 1.97

Kruskal-Wallis Test

Dunn's multiple comparisons	Mean rank diff.	Significant?	Summary	Adjusted P Value
3-8 vs. 9-12	33.56	No	ns	0.1760
3-8 vs. 13-15	98.41	Yes	****	<0.0001
9-12 vs. 13-15	64.85	Yes	****	<0.0001

Spearman Correlation

GCS vs. Tau (pg/mL)

Spearman r = -0.6151 95% Confidence Interval: (-0.6908, -0.5260) *p*-value < 0.0001

ROC (AUC)

3-8 vs. 9-12:	*p*-value = 0.0014	AUC = 0.7760	*Cutoff < 16.22 pg/mL
9-12 vs. 13-15:	*p*-value < 0.0001	AUC = 0.8356	*Cutoff < 4.895 pg/mL
3-8 vs. 13-15:	*p*-value < 0.0001	AUC = 0.9624	*Cutoff < 4.895 pg/mL

*Cutoff values determined with a minimum 90% Sensitivity.

Table 6C. ROC Tau Curve

GCS 3-8 vs. 9-12	Sens, Spec: 94.4, 38.7
GCS 9-12 vs. 13-15	Sens, Spec: 90.5, 50.0
GCS 3-8 vs. 13-15	Sens, Spec: 90.5. 87.1

Cutoff values for Tau

Please refer to "*Cutoff values for GFAP*" for detailed interpretation of cutoff values.

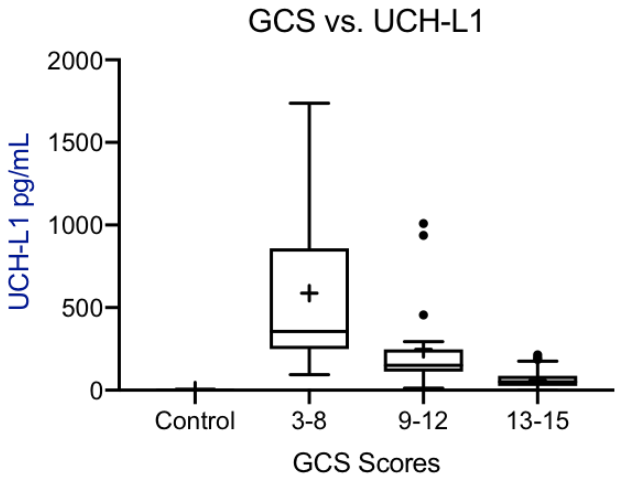
UCH-L1 and results

The levels of UCH-L1 in patients (median, 66.0 pg/mL; IQR, 97.64 pg/mL) of all TBI severities/GCS were significantly different (p-value < 0.0001) from control subjects (median, 0.909 pg/mL; IQR, 2.352 pg/mL) without any incidence of TBI. For all the intracranial abnormalities that were observed using CT imaging, UCH-L1 levels significantly differed (p-value < 0.0001) between patients who did and didn't show abnormalities.

The levels of UCH-L1 were also compared between patients of varying GCS Scores using multiple significance tests. From the Mann-Whitney U tests, the difference in UCH-L1 levels were all statistically significant (p-value ≤ 0.0003) when comparing each severity group separately. However, when comparing the means between the severity classes using Kruskal-Wallis, the results showed that the UCH-L1 levels were significantly different when comparing mild-severe and mild-moderate but not for moderate-severe (p-value = 0.1283). AUC scores obtained from the ROC Curve further confirmed the findings of the previous significance tests but also gave insight into approximate cut-off values for each severity class.

Although the relationship between the levels of UCH-L1 and GCS could not be analyzed using regression analysis, they were found to be significantly negatively correlated (p-value < 0.0001) with a Spearman correlation coefficient of -0.4503.

Figure 7*



*Refer to **Table 8B** for further information
+ symbol refers to the mean

Figure 8

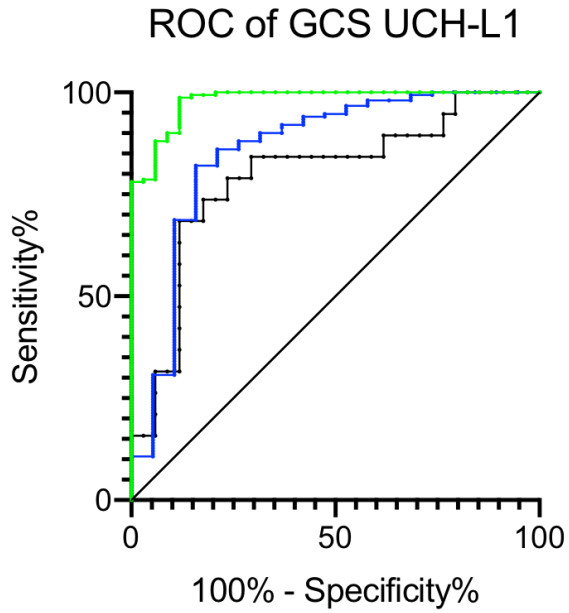


Table 8A. ROC UCH-L1 Curve

Black:	GCS 3-8 vs. 9-12	**AUC: 0.7957**
Blue:	GCS 9-12 vs. 13-15	**AUC: 0.8572**
Green:	GCS 3-8 vs. 13-15	**AUC: 0.9800**

Table 8B UCH-L1 Significance Tests

Mann-Whitney U Test

GCS

3-8 vs. 9-12:	*p*-value = 0.0003	Median(3-8, 9-12): 356.6, 151.3
3-8 vs. 13-15:	*p*-value < 0.0001	Median(3-8, 13-15): 356.5, 51.5
9-12 vs. 13-15:	*p*-value < 0.0001	Median(9-12, 13-15): 151.3, 51.5

Kruskal-Wallis Test

Dunn's multiple comparisons	Mean rank diff.	Significant?	Summary	Adjusted P Value
3-8 vs. 9-12	34.09	No	ns	0.1283
3-8 vs. 13-15	100.7	Yes	****	<0.0001
9-12 vs. 13-15	66.63	Yes	****	<0.0001

Spearman Correlation

GCS vs. UCH-L1 (pg/mL)

Spearman r = -0.4503 95% Confidence Interval: (-0.5575, -0.3283) *p*-value < 0.0001

ROC (AUC)

3-8 vs. 9-12:	*p*-value = 0.0004	AUC = 0.7957	*Cutoff < 946 pg/mL
9-12 vs. 13-15:	*p*-value < 0.0001	AUC = 0.8572	*Cutoff < 125.3 pg/mL
3-8 vs. 13-15:	*p*-value < 0.0001	AUC = 0.9800	*Cutoff < 125.0 pg/mL

*Cutoff values determined with a minimum of 90% Sensitivity.

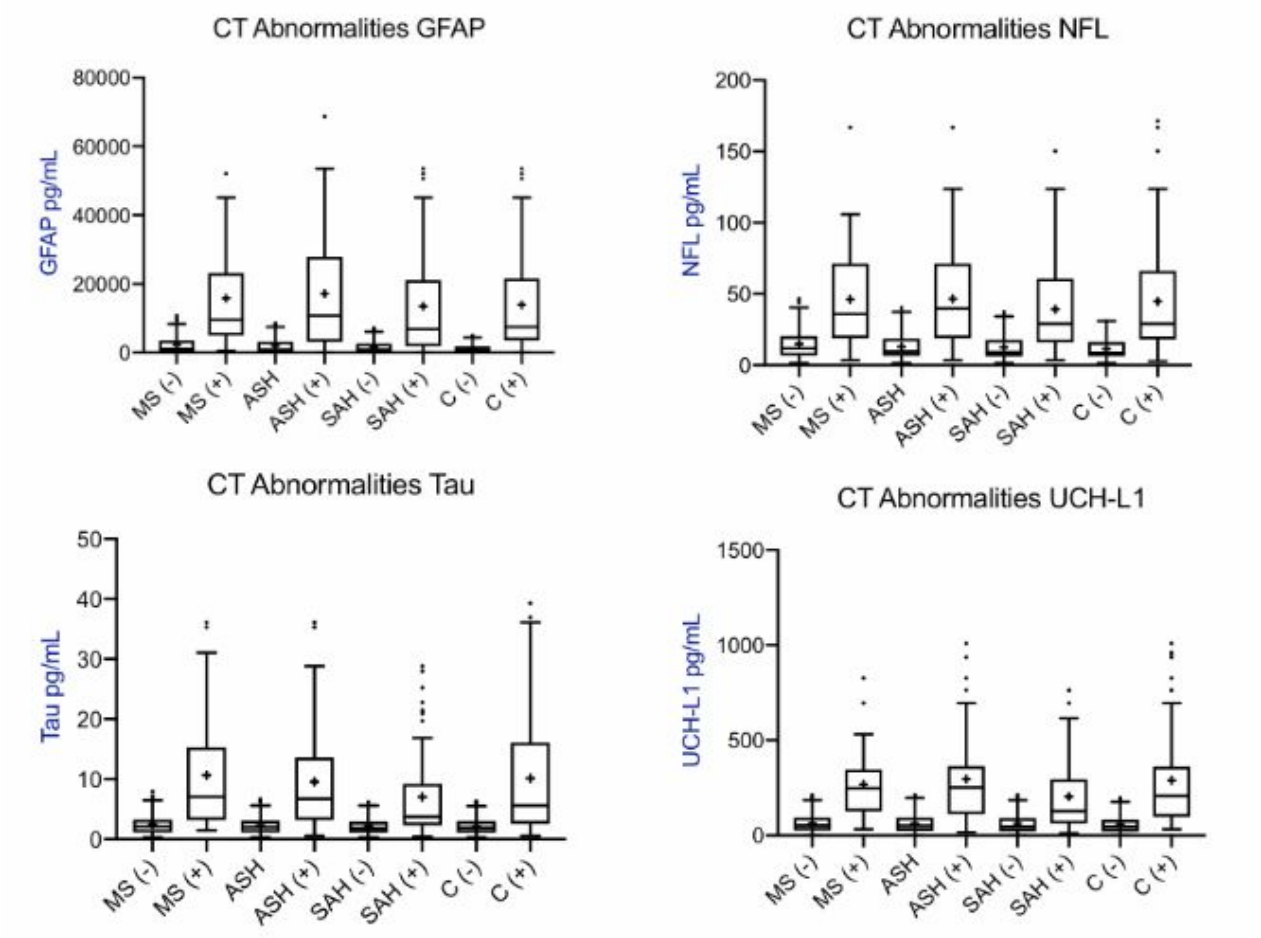
Table 8C. ROC UCH-L1 Curve

GCS 3-8 vs. 9-12	Sens, Spec: 94.7, 23.5
GCS 9-12 vs. 13-15	Sens, Spec: 90.0, 68.4
GCS 3-8 vs. 13-15	Sens, Spec: 90.0. 91.2

Cutoff values for UCH-L1

Please refer to "*Cutoff values for GFAP*" for detailed interpretation of cutoff values.

CT Abnormalities results



* **MS**: Midline Shift; **ASH**: Acute Subdural Hematoma; **SAH**: Subarachnoid Hemorrhage; **C**: Contusion

Table 9 ROC CT Abnormalities

GFAP:	0.8903; 0.8606; 0.8221; 0.9094
NFL:	0.8147; 0.8511; 0.8178; 0.8503
Tau:	0.8618; 0.8418; 0.7924; 0.8345
UCHL1:	0.9139; 0.8690; 0.7930; 0.8799

** Please note that AUC values are in the order as presented on the graphs: MS; ASH; SAH; C

Discussion

Traumatic brain injury plays a major role in death and disability following most physical traumas. According to Johns Hopkins University, TBI is also the leading cause of death from a sports-related injury (13). Even though current imaging methods and neurological examinations used to quickly assess injury severity have improved over time, there is still a lack of a general definitive understanding of what truly defines the severity of TBI, an important distinction to make to produce efficacious treatment plans. Furthermore, CT Scanning/MRI and GCS scores are only useful to a certain extent. CT scanning has low sensitivity to diffuse brain damage and the use of MRI is costly and inefficient for unstable patients (14). The Glasgow Coma Scale Scores, while representing a wide spectrum of TBI, can misrepresent many patients.

In contrast, the use of diagnostic biomarkers provides many advantages that are not present within other modalities. The mere fact that the concentration of biomarkers can be quantified within serum, plasma, or even CSF provides much less room for subjective questioning of what constitutes the varying severity of TBI. This provides a more objective way to define TBI while complementing the information gathered from other diagnostic methods. However, in order to implement theranostic biomarkers within a clinical setting, we must understand the overall mechanism, behavior, function, and lifespan of the biomarkers under different circumstances.

Biomarker presence within blood

Upon sustaining a traumatic brain injury, physiological changes occur within the CNS that can disrupt the normal functioning of certain parts of the CNS. One of these issues is the disruption of the blood-brain barrier (BBB). During the primary injury event, the BBB is susceptible to mechanical deformations that lead to tight junction instability, resulting in axonal strain (15-17). Along with BBB disruption, this axonal strain induces production and release of stress factors/proteins that are able to enter the peripheral bloodstream through the compromised sections of the BBB (15-17). This explains the presence and detectability of biomarkers in the bloodstream.

Cutoff Values/ROC Curve

Within this study, I used Area under the ROC Curve to approximate cutoff values for mild, moderate, and severe TBI as determined by GCS Scores. ROC Curves are normally used to determine the diagnostic ability to distinguish between a control with no symptoms and a patient that is showing symptoms. However, I believe that its use can be extrapolated to different situations if the perspective of what constitutes the control and patient group is changed. For example, when setting the control group as GCS 3-8 (severe TBI) and the patient group as GCS 9-12 (moderate TBI), since all of the biomarker levels were negatively correlated with (p-value < 0.0001) to the severity level, it is safe to assume that patients with moderate TBI should have lower levels of

biomarkers present in their blood. Therefore, by setting the patients with expectedly lower levels of biomarker as the control, I used the ROC curve to construct a rough outline of mild, moderate, and severe TBI for each biomarker based on the severity classifications from GCS scores provided.

Control values & CT Abnormalities

This study provides further evidence that all four of the tested biomarkers tested were able to correctly distinguish the incidence of TBI with (p-value < 0.0001, AUC > 0.967) when compared to a control subject. While many studies have already proved their ability to determine the occurrence of TBI (6, 14) it is important to prove this point in all studies as a minimum requirement prior to moving forward with any type of biochemical analysis.

Regarding CT Abnormalities, it's clear that biomarker levels are markedly higher in patients that are found to have some sort of intracranial abnormality (p-value < 0.0001, AUC > 0.798). This further proves that the difference in serum biomarker levels is large enough to portray the presence of any TBI, especially when detected by CT Imaging. However, while the levels of all biomarkers are significantly different when comparing between the absence and presence of CT abnormalities, the primary focus of this study is to find a diagnostic tool to detect, classify, and assess TBI when no abnormalities are observed using the classical CT imaging techniques (mild TBI).

Neuro 4- Plex A Advantage Kit

The protein assay (N4PA) that was executed as the main data collector for this study provides a solution to some of the challenges attributed with creating blood-based tests for detection of biomarkers within the peripheral blood circulation (18). Serum levels of biomarkers allow for detection/quantification at subfemtomolar concentrations. The magnetic bead-based technology that is utilized within the Simoa platform (Quanterix, Lexington, MA) effectively increases the sensitivity of these magnetic beads for the specific biomarkers in blood, thereby lowering the limit required for detection and quantification.

Significance Tests

In this study, I decided to use nonparametric tests for all of the significance tests that were conducted. While I still had well over 30 individuals after removing outliers and could technically assume normality for the entire sample through the Central Limit Theorem, it was best to practice caution given the circumstances. Upon splitting up the patients into mild, moderate, and severe (GCS) there seemed to be a lack of moderate TBI patients (24 members). Furthermore, since the mean was always much greater than the median (indicated on boxplots), it proved that the samples still did not follow a normal sampling distribution. Therefore, in order to preserve the statistical integrity of this study, I opted to use nonparametric significance tests that aren't as influenced by outliers and skewed distributions.

GFAP

Glial Fibrillary Acidic Protein (GFAP) is an intermediate filament found mainly in astrocytes located in the central nervous system (19). Upon brain trauma, GFAP is strongly upregulated (14) and released from injured cells into the surrounding environment. Due to the compromise of the blood-brain barrier, GFAP then flows into the peripheral blood circulation (20, 21). With the N4PA, the lower limit of quantification is 0.933 pg/mL and had no issues with detection.

Within this study, the results indicate that serum GFAP levels were the most extreme in the acute stages (< 24h) of TBI in comparison to the others. Even after accounting for outliers, GFAP levels seem to be the most reactive towards the injury. The Mann-Whitney significance tests seem to show that GFAP serum levels are different enough to distinguish between all three levels of TBI severity. Surprisingly, the Kruskal-Wallis test seems to contradict the findings of the Mann-Whitney test in relation to severe and moderate TBI. However, with the ANOVA significance test, I received a significant difference (p-value < 0.0001) in GFAP levels between all the severity groups. Nonparametric tests tend to have less power (ability to detect true differences) compared to parametric tests. Even with the removal of outliers and the large sample size, it is obvious that the sampling distribution is skewed because the mean is greater than the median by a large amount. Furthermore, the moderate TBI group did not meet the CLT (24 patients). As such, any cutoff-value that is determined must be interpreted with caution and not be used as a strict guideline.

The AUC for the ROC curve confirmed my suspicions when I saw that the AUC for distinguishing moderate and severe TBI were significantly lower (AUC = 0.6705) than the other two. There seems to be more overlap between the moderate and severe TBI groups than any others. Even though this value indicates that the difference between the severity groups may not be fully attributed to chance, the cut-off value that was interpreted from this test should definitely be utilized as a complement to other current methods or in conjunction with other biomarkers.

Serum GFAP's extreme upregulation during brain injury makes it easy to determine the presence of TBI. While GFAP is not the best biomarker to distinguish severe TBI, GFAP is able to distinguish between mild and moderate TBI which is more important within the clinical setting. As stated in many studies (4), mTBI is hardest to define because the mechanism of injury is not understood and patients may not be exhibiting major symptoms. Severe TBI should be fairly obvious within the clinical setting because the patient will likely show more major physical symptoms that require immediate medical attention. As such, though GFAP may not be the best for differentiating between moderate and severe TBI, GFAP definitely seems like a viable option for diagnosing acute mild TBI, which is the most important.

NFL

Neurofilaments are intermediate filament heteropolymers that consist of light, medium, and heavy chains. NFL is a protein populated within the CNS, more specifically the myelinated subcortical white matter axons; it functions as part of a scaffolding protein in the neurocytoskeleton and is important for growth and management of the axons (22-23). Even though the heavy chain has shown some promising results as a biomarker within CSF (24), this study focuses on serum levels of NFL (light chain) instead. Upon any type of brain injury, there are often neuronal/axonal mechanical injuries (DAI) that follow due to the axon's viscoelastic properties. Additionally, calcium entry into damaged axons is hypothesized to extenuate the mechanical injury through protease activation (25).

While studies have shown the dramatic increase in NFL within CSF post-injury (26,27), we are in need of a diagnostic tool that is efficient at diagnosing mTBI; since blood is more accessible than CSF, not only is it inherently more efficient but also less invasive for the patient as well. However, creating an assay to quantify NFL posed an obstacle for many because NFL is only present at very low concentrations in peripheral blood. Fortunately, with the development of the N4PA, the LLOQ was lowered to 0.317 pg/mL which is much more sensitive than the standard ELISA (28); even with the fourfold dilution factor, the SR-X machine had no issues detecting serum NFL levels.

Serum NFL was the only biomarker to receive statistically significant results on all of the tests without contradiction. However, while the AUC value for NFL was higher when comparing between severe and moderate TBI (AUC = 0.7759), GFAP levels had a higher AUC value for mild and moderate (AUC = 0.8829). The results support a study done on NF-L by Shahim, stating that NFL is a highly sensitive biomarker for diagnosing and assessing severe TBI (18). However, in this study, the results show that GFAP, Tau, and UCH-L1 may be better than Tau at distinguishing between mild and moderate TBI.

Overall, NFL proves to be a potentially useful biomarker within the clinical setting as it can be applied in a variety of ways. In this study, I only discussed the ability of NFL to distinguish the severity of TBI. However, NFL has shown to be a strong potential candidate as an indicator of DAI (18). Currently, with our lack of technology and knowledge on DAI, it is extremely difficult to detect DAI noninvasively (25). The presence of DAI already presents an unfavorable prognosis for the patient, but if proper supportive care and prevention of secondary injuries are not pursued, the patient will likely face many challenges in the near future (29). Currently, MRI technology is the best way to detect DAI and even though there are many differential diagnoses that share similar symptoms of DAI (29), we should work towards understanding the relationship between serum NFL levels and DAI presence and severity for increased efficiency and efficacy.

Tau

Tau is an intracellular, microtubule-associated protein that is found in high concentrations within axons. It functions as part of an assembly unit for axonal microtubule bundles and also participates in anterograde axoplasmic transport (14). Since Tau protein is highly expressed in nonmyelinated axons of cortical interneurons, it's presence within CSF or blood is thought to be an indicator for axonal damage in gray matter neurons (30). Upon injury, tau is proteolytically cleaved before moving into CSF and serum (14).

Looking at the significance tests, Tau faces the same contradiction that GFAP did, but to a lesser extent. The Mann-Whitney tests suggest that Tau levels are significantly different in all three severity groups. However, with a *p*-value of 0.1760, the levels of Tau were not different enough in patients with severe/moderate TBI for the Kruskal-Wallis test to deem it significant. However, upon using the ANOVA test instead, I received significant results. While I discussed the discrepancy in the GFAP discussion section, it is still unclear as to why these results contradict each other. As such, I believe that the results of the Mann-Whitney test and ROC curve should be interpreted with caution. Interestingly, the AUC value for the ROC curve of severe and moderate TBI was 0.7760. Compared to the 0.7759 for NFL, the similarity in the results leads me to believe that there should be additional research done on the correlation of serum Tau and NFL. Since they are both indicators for axonal injury, there may be some significance in the similarity of these values that warrant further research. Additionally, the AUC value for Tau was higher than NFL when comparing between mild and moderate TBI. This suggests that Tau might be a better classification tool when distinguishing mild from moderate TBI. As mentioned in previous sections, this provides more practical advantage within the clinical setting because severe TBI is usually identifiable due to physical symptoms.

Compared to the other biomarkers tested in this study, Tau seems to be stronger than GFAP but weaker than UCH-L1 at distinguishing severe from moderate TBI based on the AUC values. Many studies also suggest that serum Tau could also potentially predict outcome in TBI patients (31-32).

Overall, Tau seems to hold a lot of potential as a diagnostic biomarker. Given the results, Tau seems to be an important biomarker that can work with NFL to better assess DAI. It's increased ability to distinguish mild versus moderate TBI also makes it an attractive biomarker within the clinical setting. The Simoa platform and its increased sensitivity to biomarkers (Tau LLOQ = 0.114 pg/mL) provide a great advantage for detecting Tau levels within the blood. Comparing the cutoff values, it is clear that Tau is present in the blood at the lowest concentrations, proving difficult to detect and quantify in other studies (32). While further research must still be conducted to understand the detailed relationship of Tau within the lifespan of TBI, this study proves that Simoa could be a very valuable platform in the clinical setting.

UCH-L1

This protein is essential for maintaining axonal integrity by adding and removing ubiquitin from other proteins (14). The ubiquitin system functions to regulate cellular processes including protein degradation, protein-membrane trafficking, endocytosis, and even DNA repair (34). UCH-L1 is abundantly expressed in neurons and so its presence within blood serum is indicative of neuronal injury (14). UCH-L1 is a very abundant protein within neurons, representing around 1-5% of all soluble proteins in the brain (35-37). It is believed that UCH-L1 reaches peripheral blood due to a compromised BBB, an may have some utility in monitoring this disruption following TBI (38).

Looking at the significance tests, it appears that UCH-L1 also faces the same contradiction as Tau and GFAP whereby the Kruskal-Wallis test between severe and moderate TBI received insignificant results (p-value = 0.1283) while the Mann-Whitney test showed that the levels were significantly different (p-value = 0.0003). While a definite explanation as to why this occurred is unavailable, I suspected that it was due to the skewed distribution. Especially since the moderate TBI group only had 24 patients, I could not safely assume normality for this specific distribution. However, looking at the AUC scores, UCH-L1 is the strongest candidate for detecting severe/moderate TBI and second strongest for moderate/mild TBI as indicated by AUC scores 0.7957 and 0.8572 respectively. With an LLOQ of 9.6 pg/mL, the Simoa SR-X machine had no issues quantifying this protein in blood serum.

Overall, UCH-L1 shows a lot of potential as a biomarker. The difference in UCH-L1 is able to detect controls from TBI patients (GCS 3-15) with p-value < 0.0001 and AUC = 0.9876. While our patient inclusion factors only required patients to be admitted within 24 hours of their injury, many studies have shown that UCH-L1 is detectable within blood within an hour after TBI (39-40). The fact that UCH-L1 is present in peripheral blood at such acute stages of TBI provides many practical advantages within the clinical setting. In this study, I chose to use the N4PA kit because of the magnetic bead technology and its ability to detect and quantify lower amounts of protein within the blood. However, looking at the cutoff values provided in this study, it's clear that the UCH-L1 levels required to make a classification were well above the LLOQ. As such, an assay with lower sensitivity (ELISA) could probably be used in the clinical setting for quicker results. During drastic situations, emergency responders could prepare blood serum samples for testing en route the hospital so that proper and accurate treatment methods could be devised as soon as blood is tested. If GCS scores are unavailable to the physician or medical staff for any number of complications, the quantification of UCH-L1 could help set-up preliminary injury management methods in a short period of time. However, quantification of UCH-L1 within an hour of TBI needs to be thoroughly tested in future studies to understand and create baseline levels in patients.

Limitations/Future Research

The greatest limitation of this study was that the sample size for moderate TBI patients was too small for the Central Limit Theorem to be used. While it is clear that the sampling distributions are not normal if there are at least 30 members we can safely assume that the sampling distributions are approximately normal and would not cause any major issues upon the use of parametric tests. Parametric tests tend to have higher power and will produce more accurate results.

Even though the data shows promising results, the results should be interpreted with caution and future research needs to be done to corroborate the findings. In this study, there were 36 patients with severe TBI, 24 with moderate TBI, and 175 patients with mild TBI. This vast difference in sample size may have possibly affected the results. In future studies, while samples should be collected at random, there should be an effort to create similar sample sizes for the differing severities.

This study also included a limited number of trauma control patients; the control serum samples came from commercially available patients that do not have TBI. Even though the study proves that the biomarker levels are significantly different than controls for all severities, future studies should include real patients collected within the same time frame as other samples to lessen any confounding variables present.

The study is also limited in its scope. Since the inclusion criteria for enrollment was very lenient, the results are general and cannot be applied in specific instances. Future studies need to have more specific boundaries set so that the parameters set could control for any confounding variables. Variables like age, gender, previous TBI history, drugs, alcohol and etc. need to be accounted for to establish more precise baseline levels in different situations.

Although these biomarkers have been suggested to have predictive qualities for the outcome, this study only assessed patients within the acute stages of TBI. Future studies should include sample collection at multiple time points to better understand the relationship between biomarker level and relative stage of injury and recovery.

Lastly, while many of these proteins are abundantly expressed within the brain. It is important that the proteins that are quantified within the blood only came from the brain and not other cells within the body. The best way to control for this is to increase the sample size to limit the variability within data.

Conclusion

In this study, I assessed serum GFAP, NFL, Tau, and UCH-L1's ability to distinguish between control groups/TBI patients, varying TBI severity groups as determined by GCS, and presence/absence of CT abnormalities. Furthermore, using ROC analysis, general cutoff values were created for each biomarker to provide a numerical description for mild, moderate, and severe TBI.

With the use of the Simoa SR-X machine and the commercially available N4PA kit, the results indicated that serum levels of biomarkers were significantly higher in TBI patients and CT positive vs. controls and CT negative patients respectively. The AUC values also suggest that multiple biomarkers should be used in determining the severity of the injury. While the correlation factors are low, serum biomarker levels are significantly negatively correlated with GCS scores. Based on the negative correlation of biomarkers with GCS scores, cutoff values were created with a minimum of 90%. These cutoff values could further help physicians determine the severity of TBI, especially when GCS scores are unavailable

This study provides ample evidence that serum blood biomarkers could be a very advantageous diagnostic tool within the clinical setting due to its high efficiency, accuracy, and noninvasive nature. The use of biomarkers for TBI could authenticate and verify any findings from GCS and CT Imaging while gaining a more in-depth understanding of the pathophysiological changes occurring within the patient's body post-injury.

Nonetheless, results should be validated with further research prior to any clinical application.

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