

## Performance Evaluation of a Multiplex Assay for Simultaneous Detection of Four Clinically Relevant Traumatic Brain Injury Biomarkers

Frederick K. Korley,<sup>1,\*</sup> John K. Yue,<sup>2,\*</sup> David H. Wilson,<sup>3</sup> Kevin Hrusovsky,<sup>3</sup> Ramon Diaz-Arrastia,<sup>4</sup> Adam R. Ferguson,<sup>2</sup> Esther L. Yuh,<sup>5</sup> Pratik Mukherjee,<sup>5</sup> Kevin K. W. Wang,<sup>6</sup> Alex B. Valadka,<sup>7</sup> Ava M. Puccio,<sup>8</sup> David O. Okonkwo,<sup>8</sup> and Geoffrey T. Manley<sup>2</sup>

### Abstract

Traumatic brain injury (TBI) results in heterogeneous pathology affecting multiple cells and tissue types in the brain. It is likely that assessment of such complexity will require simultaneous measurement of multiple molecular biomarkers in a single sample of biological fluid. We measured glial fibrillary acidic protein (GFAP), ubiquitin c-terminal hydrolase L1 (UCH-L1), neurofilament light chain (NF-L) and total tau in plasma samples obtained from 107 subjects enrolled in the Transforming Research and Clinical Knowledge in Traumatic Brain Injury Pilot (TRACK-TBI Pilot) Study using the Quanterix Simoa 4-Plex assay. We also measured NF-L using the Simoa singleplex assay. We computed the correlation between the different biomarkers and calculated the discriminative value of each biomarker for distinguishing between subjects with abnormal versus normal head computed tomography (CT). We found a strong correlation between NF-L values derived from the multiplex and singleplex assays (correlation coefficient = 0.997). Among biomarker values derived from the multiplex assay, the strongest correlation was between the axonal and neuronal markers, NF-L and UCH-L1 (coefficient = 0.71). The weakest correlation was between the glial marker GFAP and the axonal marker tau (coefficient = 0.06). The areas under the curves for distinguishing between subjects with/without abnormal head CT for multiplex GFAP, UCH-L1, NF-L, and total tau were: 0.88 (95% confidence interval 0.81–0.95), 0.86 (0.79–0.93), 0.84 (0.77–0.92), and 0.77 (0.67–0.86), respectively. We conclude that the multiplex assay provides simultaneous quantification of GFAP, UCH-L1, NF-L, and tau, and may be clinically useful in the diagnosis of TBI as well as identifying different types of cellular injury.

**Keywords:** biomarkers; glial fibrillary acidic protein; multiplex immunoassay; neurofilament light chain; total tau; traumatic brain injury; ubiquitin c-terminal hydrolase L1

**O**BJECTIVE DIAGNOSIS of traumatic brain injury (TBI) and accurate risk-stratification of patients with TBI for clinical trials of promising treatments remain unique challenges with important public health implications. Blood-based biomarkers have shown promise for aiding TBI diagnosis, with the goals of identifying and stratifying TBI for rapid and tailored diagnoses and/or treatment.

Because the brain is made up of different cell types, it is unlikely that one biomarker will be sufficient for quantifying the full extent of the heterogeneous pathobiology underlying TBI.

Over the past two decades, several circulating brain-enriched proteins have emerged with support from the literature as candidate biomarkers of TBI. These include glial fibrillary acidic protein

<sup>1</sup>Department of Emergency Medicine, University of Michigan, Ann Arbor, Michigan.

<sup>2</sup>Department of Neurological Surgery, University of California, San Francisco, San Francisco, California.

<sup>3</sup>Quanterix Corporation, Lexington, Massachusetts.

<sup>4</sup>Department of Neurology, University of Pennsylvania, Traumatic Brain Injury Clinical Research Center, Penn Presbyterian Medical Center, Philadelphia, Pennsylvania.

<sup>5</sup>Department of Radiology, University of California, San Francisco, San Francisco, California.

<sup>6</sup>Center for Neuroproteomics and Biomarkers Research, Department of Psychiatry, McKnight Brain Institute, University of Florida, Gainesville, Florida.

<sup>7</sup>Department of Neurological Surgery, Virginia Commonwealth University, Richmond, Virginia.

<sup>8</sup>Department of Neurological Surgery, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania.

\*These authors contributed to this work equally.

(GFAP), an astrocytic intermediate filament protein,<sup>1,2</sup> ubiquitin c-terminal hydrolase L1 (UCH-L1), an enzyme expressed in the cytoplasm of neurons,<sup>3,4</sup> tau, a microtubule associated protein found predominantly in cortical nonmyelinated axons,<sup>5,6</sup> and neurofilament light (NF-L), which is predominantly expressed in subcortical myelinated axons.<sup>7,8</sup>

It has been suggested that multiple TBI biomarker measurements may provide additional diagnostic and prognostic information over single biomarker measurements.<sup>9</sup> Recent introduction of the Quanterix Simoa Human Neurology 4-Plex A assay (Quanterix, MA) provides the ability to simultaneously measure multiple clinically relevant TBI biomarkers using one assay. The development of multiplex immunoassays for measuring TBI biomarkers could be an important step in advancing research on TBI biomarkers because the assays would allow faster and lower cost measurement of biomarkers and utilize smaller sample volumes. They may also provide information about the types of cells that are injured in TBI and give insight into the pathobiology of the injury. There are concerns, however, regarding the analytic performance of the multiplex assays compared with singleplex assays. The purpose of this study was to evaluate the multiplex assay for simultaneous measurement of GFAP, NF-L, UCH-L1, and tau in a cohort of subjects from the Transforming Research and Clinical Knowledge in Traumatic Brain Injury Pilot (TRACK-TBI Pilot) study.<sup>9–12</sup>

We measured plasma levels of GFAP, UCH-L1, NF-L, and total tau in stored biospecimens collected from subjects enrolled in the TRACK-TBI Pilot study, a multi-center prospective observational study conducted at three Level I trauma centers in the United States, including San Francisco General Hospital (San Francisco, CA), University of Pittsburgh Medical Center (Pittsburgh, PA), and University Medical Center Brackenridge (Austin, TX).<sup>10</sup> TRACK-TBI Pilot was the first study to utilize the National Institutes of Health (NIH) and National Institute of Neurological Disorders and Stroke (NINDS) Common Data Elements (CDEs).<sup>13–15</sup> Inclusion criteria were: external force trauma to the head, presentation to the emergency department (ED) of the three participating sites, and a clinically indicated brain computed tomography (CT) scan performed within 24 h of injury. Exclusion criteria were: pregnancy, comorbid life-threatening disease, incarceration, on psychiatric hold, and non-English speakers because of limitations in participation with outcome assessments.

Eligible subjects were enrolled through convenience sampling from 2010 to 2012. The current analysis included only TRACK-TBI Pilot subjects with sufficient quantities of plasma samples remaining to run the assay. Institutional Review Board approval was obtained at all sites. Informed consent was obtained from all subjects before enrollment. For patients unable to provide consent because of their injury, consent was obtained from their legally authorized representative. Patients were then re-consented, if cognitively able at later inpatient and/or outpatient follow-up assessments for continued participation in the study. Demographic (age, gender, race, and ethnicity) and clinical (history of previous episode of TBI, injury mechanism, admission Glasgow Coma Scale score, initial ED vital signs, ED disposition) data were collected through patient interviews and abstraction of medical records in accordance to the NINDS CDE definitions.<sup>16</sup>

Blood samples were collected from consenting subjects within 24 h of injury. Samples were centrifuged and plasma aliquots were stored at  $-80^{\circ}\text{C}$  per the NINDS CDE Biospecimens and Biomarkers Working Group Guidelines.<sup>13</sup> All samples were de-identified and assigned a unique study number specific to site and

subject. Blinded sample analysis occurred in a laboratory using a digital immunoassay based on a single molecule array technology (Simoa) developed by Quanterix Corporation. We utilized the Simoa neurology 4-plex A assay, which measures GFAP, UCH-L1, total tau, and NF-L on the same blood sample and at the same time. The lower limits of detection of the assay for GFAP, UCH-L1, total tau, and NF-L are 0.221 pg/mL, 1.74 pg/mL, 0.024 pg/mL, and 0.104 pg/mL, respectively. The inter lot and inter instrument coefficient of variation (CV) for each of the proteins were  $<5\%$ .

All patients underwent CT imaging of the brain at the time of ED presentation. Brain CTs were characterized according to NINDS CDE Neuroimaging Working Group recommendations.<sup>10,14</sup> Each CT was de-identified, electronically uploaded to a central imaging database, and reviewed by a blinded central reader who was a board-certified neuroradiologist. Imaging features were extracted and entered into the TRACK-TBI Pilot database. The primary outcome of this analysis is traumatic intracranial abnormality on head CT scan, hereafter referred to as abnormal head CT. In addition, head CTs were further categorized into: contusion only; diffuse axonal injury (DAI) only; multiple traumatic intracranial lesions (defined as any traumatic intracranial abnormality that could not be classified as contusion only or DAI only); or no intracranial abnormality (normal CT).

Descriptive statistics with medians, interquartile range, and proportions were used to describe continuous and categorical variables, respectively. Biomarker levels were treated as continuous data. Intracranial lesions on initial brain CT were scored and analyzed as categorical variables (present/absent), with biomarker levels as the dependent variables. Biomarker data were examined for normality with distributional plots and the Shapiro-Wilk test. The Mann-Whitney *U* test was used to assess for between-group differences for continuous variables. The Pearson chi-square test was used to assess for between-group differences for categorical variables, except in comparisons with individual cell counts  $\leq 5$  where the Fisher Exact Test was used. Correlations between biomarkers were computed using the Pearson correlation coefficient.

We examined differences in NF-L values derived from the two assays visually, using a Bland-Altman Plot. We also determined the correlation between NF-L values derived from the singleplex assay (reference standard) and multiplex assay using the Pearson correlation coefficient. The discriminative ability of each biomarker for distinguishing between subjects with abnormal and those with normal head CTs were quantified using the area under the receiver-operating characteristic curve (AUC; ROC). In line with current statistical consensus, AUC of 0.8–0.9 is considered very good, 0.7–0.8 is considered adequate, and  $<0.7$  is considered poor. In a proof-of-concept analysis, we examined the discriminative ability of all four biomarkers combined, using a multi-variable logistic regression model and calculated the corresponding AUC, acknowledging that with our modest sample size, we will not have sufficient power to detect a statistically significant increase in AUC values. Data analysis was performed using STATA/MP 11.1 and RStudio 1.0.143.

We studied 107 subjects enrolled in TRACK-TBI Pilot. Our study population was predominantly male (72.9%) and Caucasian (83.2%) with median age of 42 years (interquartile range: 25–55 years). Fall was the most common injury mechanism (29.2%). A total of 43 (40.2%) subjects had abnormal head CT scans. Among those with abnormal head CTs, five had contusion only; nine had DAI only, and 29 were classified as multiple traumatic intracranial lesions (21 with at least both a subarachnoid hemorrhage (SAH)

and a subdural hemorrhage, five with epidural hemorrhage with/without contusion, one with both contusion and DAI, one with SAH and contusion among others, and one with SAH only). A detailed description of the demographics and clinical characteristics of the study population is presented in Table 1.

There was very good correlation between NF-L values derived from the multiplex and singleplex assays (correlation coefficient=0.997). Overall, NF-L values from the multiplex assay were 10% higher than NF-L values from the singleplex assay (mean: 22.39 ng/mL vs. 20.52 ng/mL,  $p < 0.001$ ). Higher multiplex NF-L values seem to be because in subjects with the highest singleplex NF-L values, while for lower concentrations, the NF-L values are more similar (Supplementary Fig. 1; see online supplementary material at [ftp.liebertpub.com](http://ftp.liebertpub.com)).

Among the four biomarkers studied, the strongest correlation was between NF-L and UCH-L1 (coefficient=0.71) and the weakest correlation was between GFAP and total tau (coefficient=0.06). The correlation matrix of all four biomarkers is presented in Supplementary Figure 2; see online supplementary material at [ftp.liebertpub.com](http://ftp.liebertpub.com).

Serum levels of all four biomarkers were higher in subjects with abnormal head CT than those with normal head CT (Table 1 and Figure 1). The discriminative ability of GFAP ( $AUC_{GFAP}$ ) for distinguishing between subjects with and without abnormal head CT was 0.88 (95% CI: 0.81–0.95). The  $AUC_{GFAP}$  was not statistically significantly different from the  $AUC_{UCH-L1}$  (0.86 [95% CI:

0.79 – 0.93],  $p = 0.62$ ) or the  $AUC_{NF-L}$  (0.84 [95% CI: 0.77–0.92],  $p = 0.44$ ); however, it was higher than the  $AUC_{Tau}$  (0.77 [95% CI: 0.67–0.86],  $p = 0.04$ ), see Figure 2.

Compared with subjects with normal head CTs ( $n = 63$ ), those with isolated contusion only ( $n = 5$ ) on head CT had higher values of GFAP, UCH-L1, and NFL but not tau. Median values of the biomarkers were as follows: 7351 (IQR: 3445–14310) pg/mL versus 540 (IQR: 104–1265) pg/mL,  $p < 0.001$  for GFAP; 157 (71–378) pg/mL versus 39 (IQR: 22–62) pg/mL,  $p = 0.002$  for UCH-L1; 20.4 (IQR: 14.8–70.9) pg/mL versus 8.8 (5.6–13.8) pg/mL,  $p = 0.004$  for NF-L; and 3.9 (IQR: 3.5–604) pg/mL versus 3.5 (IQR: 2.2–6.5) pg/mL,  $p = 0.17$  for tau.

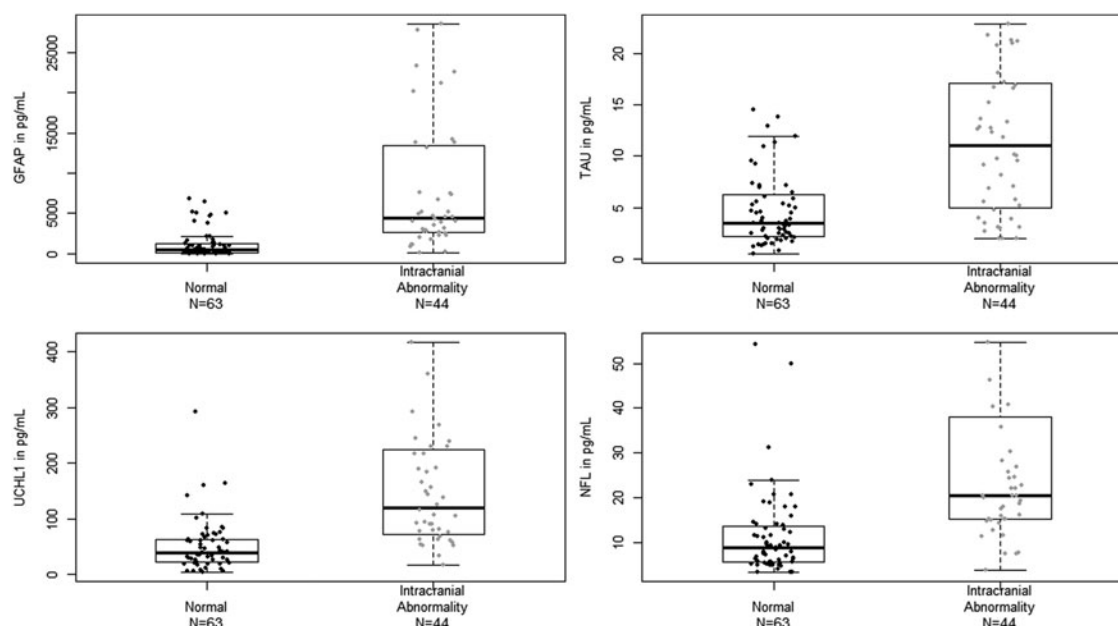
Compared with subjects with normal head CTs ( $n = 63$ ), those with isolated DAI only on head CT ( $n = 9$ ) had higher values of GFAP, UCH-L1, and NFL but not tau. Median values of the biomarkers were as follows: 2843 (IQR: 1896–4172) pg/mL versus 540 (IQR: 104–1265) pg/mL,  $p = 0.001$  for GFAP; 105 (57–217) pg/mL versus 39 (IQR: 22 – 62) pg/mL,  $p = 0.001$  for UCH-L1; 17.5 (IQR: 11.6–39.6) pg/mL versus 8.8 (5.6–13.8) pg/mL,  $p = 0.015$  for NF-L; and 13.3 (IQR: 2.5–19.4) pg/mL versus 3.5 (IQR: 2.2–6.5) pg/mL,  $p = 0.07$  for tau.

In a proof-of-concept analysis, the combination of all four biomarker values increased the AUC to 0.90 (95% CI: 0.84–0.96); however, compared with the AUC of GFAP only (0.88), this increase did not reach statistical significance ( $p = 0.34$ ) given the modest sample size of our study population.

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF STUDY SUBJECTS

Demographic characteristics ( $N = 107$ )	No intracranial abnormality on head CT scan $n = 63$	Intracranial abnormality on head CT scan $n = 44$	Sig. (p)
Median age in years (IQR)	39.0 (24.0–52.0)	44.0 (28.8–61.0)	0.037
Gender (%)			0.009
• Male	40 (63.5%)	38 (86.4%)	
• Female	23 (36.5%)	6 (13.6%)	
Race (%)			0.056
• Caucasian	48 (76.2%)	41 (93.2%)	
• African American	6 (9.5%)	2 (4.5%)	
• Other	9 (14.3%)	1 (2.3%)	
Mechanism			0.440
• MVA/MCC	17 (27.0%)	17 (39.5%)	
• PVA	4 (6.3%)	1 (2.3%)	
• Fall	31 (49.2%)	19 (44.2%)	
• Assault	8 (12.7%)	6 (14.0%)	
• Struck by	3 (4.8%)	0 (0.0%)	
GCS			<0.001
• 3–8	0 (0%)	11 (25.0%)	
• 9–12	1 (1.6%)	4 (9.1%)	
• 13–15	62 (98.4%)	29 (65.9%)	
Post-traumatic amnesia			0.003
• Yes	35 (55.6%)	19 (43.2%)	
• Suspected	1 (1.6%)	8 (18.2%)	
• No	21 (33.3%)	8 (18.2%)	
• Unknown	6 (9.5%)	9 (20.5%)	
ED drug screen positive	1 (1.6%)	5 (11.4%)	0.079
Median GFAP value in pg/mL (IQR)	540.0 (104.5–1265.9)	4406.3 (2677.5–13630.7)	<0.001
Median UCH-L1 value in pg/mL (IQR)	38.8 (22.4–62.5)	120.6 (70.7–226.4)	<0.001
Median NF-L value in pg/mL (IQR)	8.8 (5.6–13.8)	20.4 (15.2–39.1)	<0.001
Median tau value in pg/mL (IQR)	3.5 (2.2–6.5)	11.0 (4.9–17.2)	<0.001

CT, computed tomography; IQR, interquartile range; MVA, motor vehicle accident; MCC, motorcycle crash; PVA, pedestrian vehicle accident; GCS, Glasgow Coma Scale score; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin c-terminal hydrolase L1; NF-L, neurofilament light chain.

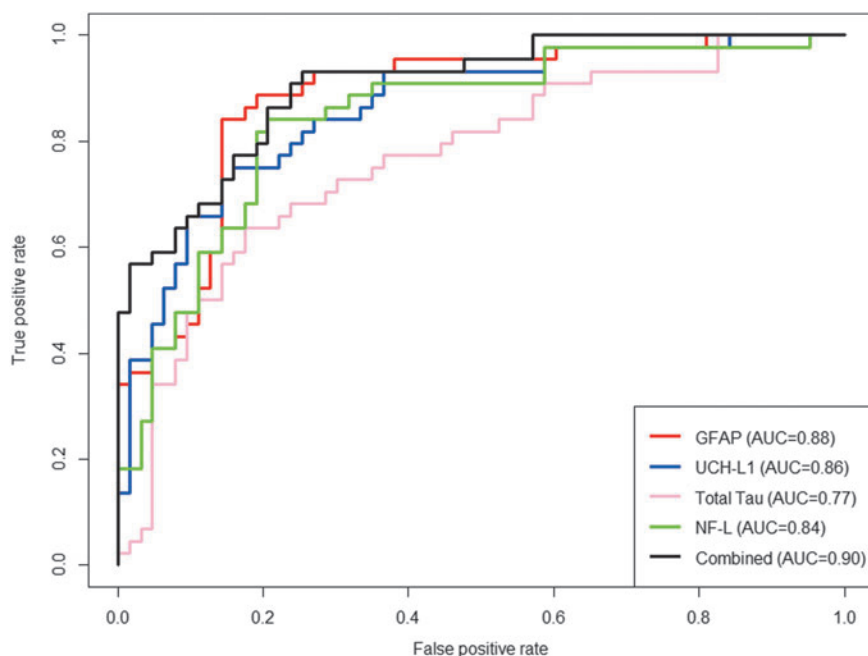


**FIG. 1.** Distribution of plasma glial fibrillary acidic protein (GFAP), ubiquitin c-terminal hydrolase L1 (UCH-L1), total tau, and neurofilament light chain (NF-L) levels according to head computed tomography (CT) findings. Plasma levels of GFAP, UCH-L1, total tau, and NF-L were higher in subjects with traumatic intracranial abnormality seen on head CT than subjects with normal head CT scans.

Progress in the field of TBI has been slowed by the lack of available biomarkers that can be used for clinical management and research. Studies over the past several years have identified several novel proteins that may have diagnostic and prognostic utility in TBI.<sup>17,18</sup> There are limited data, however, on simultaneous measurement of multiple TBI biomarkers within the same cohort of patients with TBI. In this first publication of results from a 4-plex

neurology assay developed by Quanterix corporation, we report a number of important findings.

The first is that there is very good correlation between NF-L values derived from a multiplex and singleplex Simoa assays. Multiplex assays save cost, time, and sample volume. They often have lower analytical sensitivity compared with singleplex assays, however. In this study, we have determined that for the purposes of measuring



**FIG. 2.** Discriminative ability of each of the biomarkers examined for distinguishing between those with normal and abnormal head computed tomography (CT) scans. Receiver operator curve illustrating the discriminative value of glial fibrillary acidic protein (GFAP), ubiquitin c-terminal hydrolase L1 (UCH-L1), total tau, and neurofilament light chain (NF-L) for distinguishing between subjects with traumatic intracranial lesions on head CT and those without such lesions. Color image is available online at [www.liebertpub.com/neu](http://www.liebertpub.com/neu)

NF-L, the analytic performance of the Simoa 4-plex neurology assay is similar to that of the singleplex assay. Multiplex immunoassays often have compromised analytical sensitivity because of interference between different antibodies, analytes, and assay diluents; variability in the manufacturing process; and incompatibility between different assay limits of quantification. Our finding of similar analytic performance between the singleplex and multiplex assays for measuring NFL represents an important technological advance and sets the stage for further evaluation of the differential expression of TBI biomarkers in different TBI subtypes.

The second important finding is that there is a strong correlation between blood levels of a biomarker of astrocytic injury (GFAP) and biomarkers of neuronal/axonal injury (UCH-L1 and NF-L). There is a weak correlation, however, between GFAP and another biomarker of axonal injury (total tau). The strong correlation between an astrocytic and neuronal/axonal biomarker of injury is not surprising because TBI results in concurrent injuries to the different brain cell types. Therefore, the weak correlation between GFAP and total tau may appear to be an aberrancy at first glance.

It is important to note, however, that although UCH-L1, tau, and NF-L are predominantly expressed in the central nervous system (CNS), they are also expressed by the peripheral nervous system (PNS). Tau is expressed predominantly by unmyelinated axons (Group C nerve fibers), which may be found in the somatosensory system; therefore, we postulate that weak correlation between GFAP and tau may be because of the additional release of total tau from extracranial sources in patients with TBI who have polytrauma. In addition, the kinetics of release and degradation of the different biomarkers may differ. For example, UCH-L1 is released early after injury and has a short half-life in blood, while GFAP release occurs slightly later and levels persist longer.<sup>19</sup> NF-L levels release occurs in a slower and more sustained fashion.<sup>20</sup> In this study, all samples were collected within 24 h of injury, and given the known differences in release and degradation kinetics, it is not surprising that the correlation between the different biomarkers is low.

The third important finding is that serum NF-L levels have excellent discriminative ability for distinguishing between persons with and those without intracranial hemorrhage on head CT. A number of studies have reported the association between GFAP, UCH-L1, total tau, and intracranial head CT abnormalities. Our report of the association between NF-L and intracranial head CT abnormalities is novel, however. NF-L is the smallest and most abundant of the three major neurofilament subunits (NF-light chain, NF-medium chain, NF-heavy chain) and consequently the most likely to be found in circulation after brain injury.<sup>21</sup>

Patients with traumatic intracranial hemorrhage are also likely to have concomitant axonal injury resulting in elevations in serum NF-L levels. Accordingly, elevations in serum NF-L may be correlated with TBI severity. Serum NF-L levels have been reported to be elevated in patients with both mild<sup>22</sup> and severe TBI,<sup>23</sup> and CSF NF-L levels may remain elevated for up to 19 months post-injury.<sup>24</sup> High serum NF-L levels are associated with worse functional recovery from TBI.<sup>25</sup> Future studies utilizing the TRACK-TBI dataset will examine the prognostic value of serum NF-L levels in risk-stratifying the clinical severity of and outcomes after TBI.

Our study has a number of strengths including the use of a novel multiplex biomarker assay, the use of rigorous statistical techniques for quantifying improvement in predictive ability, and the measurement of different biomarkers within the same cohort of patients. A major limitation of the study is the use of a modest sample size, which precluded evaluations of whether combined biomarker

values provide additional prognostic value over single biomarkers. We plan to overcome this limitation in a future study using data from the ongoing multi-center TRACK-TBI study (<http://tracktbi.ucsf.edu>).

We conclude that there is a very strong correlation between NF-L values derived from a singleplex and a multiplex Simoa assay. Further, GFAP, NF-L, and UCH-L1 values derived from the multiplex assay all have excellent discriminative ability for identifying patients with TBI who have abnormal head CT scans.

## Author Disclosure Statement

David H. Wilson and Kevin Hrusovsky are employees of Quantarix Corporation, which manufactures the multiplex assay studied. For the remaining authors, no competing financial interests exist.

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Address correspondence to:  
*Frederick K. Korley, MD, PhD*  
*Department of Emergency Medicine*  
*University of Michigan*  
*1500 East Medical Center Drive*  
*Ann Arbor, MI, 48109*  
  
*E-mail: korley@med.umich.edu*