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Plasma Protein Profiling by Proximity Extension Assay Technology Reveals Novel Biomarkers of Traumatic Brain Injury—A Pilot Study

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ARTICLE

Plasma Protein Profiling by Proximity Extension Assay Technology Reveals Novel Biomarkers of Traumatic Brain Injury—A Pilot Study

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Background: Traumatic brain injury (TBI) is a significant public health issue affecting nearly 69 million patients worldwide per year. Reliable diagnostic biomarkers are urgently needed to aid in disease diagnosis and prognosis and to guide patient aftercare. Blood biomarkers represent an attractive modality to quickly, cheaply, and objectively evaluate clinical status. We hypothesize that deep and quantitative plasma proteomic profiling with a novel technology, proximity extension assay, may lead to the discovery of diagnostic and/or prognostic biomarkers of TBI.

Methods: We used high-throughput proximity extension assays (PEA) to quantify the relative abundance of over 1000 unique proteins in plasma. PEA is a highly sensitive multiplex immunoassay capable of detecting very low-abundance proteins (down to fg/mL) in complex biological matrices. Our patient cohort consisted of severe TBI (sTBI) patients, matched healthy controls, and another non-TBI group that was included in the analysis to validate the specificity of the candidates during the selection process. The obtained protein quantification data was then filtered to identify candidate biomarkers through statistical analysis, literature searches, and comparison to our reference control groups.

Results: Overall, we identified 6 novel candidate TBI biomarkers. Candidates exhibit a significant increase in plasma protein abundance in sTBI when comparing between healthy controls and sTBI patients. Candidates generally had low expression in our reference groups compared with the sTBI group.

Conclusions: Our preliminary findings represent a starting point for future validation. These biomarkers, either alone or in combination, may have significant clinical utility in aiding in TBI diagnosis, prognosis, and/or management.

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IMPACT STATEMENT

TBI is a significant cause of morbidity and mortality. We tested the feasibility of the multiplexed proximity extension assay (PEA) for identifying non-invasive prognostic severe TBI biomarkers. Measuring 1104 proteins in plasma, we identified 6 candidate biomarkers that discriminated healthy adults from sTBI patients. Two candidates significantly correlated with clinical outcomes. The 6 candidates need to be validated with larger independent TBI groups, including mild/concussive cases, where they may prove helpful for triaging patients into surveillance or interventional groups. Furthermore, our study demonstrates the utility of the ultrasensitive PEA for simultaneous deep profiling 1000+ proteins in TBI plasma.

INTRODUCTION

Traumatic brain injury (TBI) is a significant cause of morbidity and mortality, affecting nearly 69 million people worldwide per year (1). Children and youth are particularly at risk for experiencing a TBI. TBI is caused by trauma to the head or transmitted forces from a blow to the body that causes neurologic damage and dysfunction. These mechanical injuries can vary in both the severity and the form of injury to the brain (2). Generally, presenting TBI may be classified as mild, moderate, or severe. Patients are primarily stratified using the Glasgow Coma Scale (GCS), a scoring system that evaluates verbal performance, motor function, and eye function. A lower GCS score indicates a more severe diagnosis and may therefore indicate the need for brain imaging or surgical intervention.

In severe TBI (sTBI; GCS 8-3), patients experience loss of consciousness (LOC), convulsions, pupil dilation, numbness in body extremities, and profound confusion (3). Symptoms of TBI can appear within seconds or hours from the initial trauma. In-hospital major adverse events (IMAEs), such as progressive hemorrhagic injury, acute traumatic coagulopathy, acute lung injury, and posttraumatic cerebral infarction, increase the risk of mortality in sTBI (4). Moreover, all severities of TBI can be associated with physical, cognitive, and/or behavioral symptoms that persist for months or even years, and can be a source of significant disability (5, 6). TBI survivors may experience lifelong deficits (7, 8) and be at an increased risk for the development of neurodegenerative diseases, including Alzheimer disease (AD), Parkinson disease, chronic traumatic encephalopathy (CTE), and motor neuron disease (9). Children who suffered a TBI have been shown to suffer from cognitive deficits even 10 years after injury and may experience negative psychosocial outcomes (10). Accurately and meaningfully predicting the risk of IMAEs, patient outcome, and long-term effects following sTBI remains an unmet clinical need (11).

One promising solution is the quantification of serum biomarkers, such as proteins, that can delineate the severity of trauma and/or prognostic outcome. These proteins enter circulation due to disruption of brain structures, such as the blood brain barrier and/or axons, leading to a neuroinflammatory response (12, 13). Although brain-specific or brain-enhanced proteins are particularly promising as blood-based biomarkers for TBI, since an increase in their protein expression in the serum/plasma is likely specific to injury to brain structures and/or axons, the presence of brain-related proteins in the plasma/serum may often be at very low concentrations and be hard to detect with conventional proteomics tools.

Protein biomarkers have classically been identified and quantified with enzyme-linked immunosorbent assay (ELISA) or mass spectrometry (MS). ELISA is often the clinical gold standard for measuring single protein targets in biofluids. However, multiplexing using general ELISA methods often suffer from antibody cross-reactivity and interference, thus limiting how many target proteins can be analyzed in a single assay (14). Performing separate ELISAs for many target proteins is labor-intensive, time-consuming, and most importantly, may not be feasible because of the large sample volume requirement per assay (up to 50–100 μL) (14). MS remains the core tool for large-scale proteomics (15–17). A single liquid chromatography (LC)-MS/MS experiment can detect up to several thousand proteins. However, the method may bias towards the detection of high abundance proteins and deliver less robust detection of low abundance proteins in biofluids, when performed without sample fractionation and enrichment steps to reduce the sample complexity (17). New MS-based workflows that focus on iterative data-dependent analysis have improved the sensitivity and coverage of protein detection in biofluids (18–21).

Another way to detect low abundance proteins is through targeted MS techniques, such as selected reaction monitoring (22). Although such targeted techniques have reached sensitivity as low as 50 to 100 pg/mL in biofluids, there is still limited multiplexing capability (about 20–30 analytes) in these methods (23, 24). Other limitations of MS-based proteomics analysis include the requirement of specialized equipment and skilled technical personnel, as well as restricted throughput, which can increase the time and cost (25). Advanced multiplex immunoassays, such as the newly developed proximity extension assay (PEA), may provide the multiplexing performance of MS (measuring >1000 protein simultaneously), while maintaining the high specificity and robustness of ELISA (sensitivity of low fg/mL to sub pg/mL). This is a novel technology that is based on antibody recognition of protein targets, and is able to simultaneously detect hundreds of targets with high sensitivity (down to low fg/mL) and low sample volume (26). This method is well suited to the detection of potentially low abundance biomarkers in the serum of patients with suspected mild TBI.

Here, we aim to identify novel candidate diagnostic and prognostic biomarkers of TBI using high-throughput, multiplex immunoassays (PEA). To this end, we probed over 1000 unique proteins in the plasma of patients with sTBI and healthy controls. We then apply both quantitative and qualitative filtering to identify preliminary biomarker candidates for future validation. We also investigate the correlation between these proteins and various TBI outcome measures. This is a proof-of-concept study to assess the feasibility of employing the innovative PEA technology to identify novel candidate biomarkers of TBI that may be present at low concentrations in the plasma. Our pilot study includes small patient numbers since the cost of this new technology is relatively high. However, given the highly promising data, further validation studies are warranted to assess candidate biomarker performance in independent, large cohorts of TBI patients with varying degrees of severity.

METHODS

Study Population and Sample Collection

In the sTBI cohort, plasma samples were obtained using strict standard operating procedures from the Translational Research Centre, London, Ontario, Canada (https://translationalresearchcentre.com/). Study protocols were approved by The Western University Health Science Research Ethics Board (REB # 16693) and by The Mount Sinai Hospital Research Ethics Board (REB #18-0069-E). Informed consent was obtained

either from the subject or a substitute decision maker.

Blood samples were drawn from adults with sTBI (n = 10), as defined by GCS ≤ 8 with pathological computed tomography findings, within 24 h of hospital admission. Patients were further separated into those who survived their brain injuries (sTBI (S)) and nonsurviving patients (sTBI (NS)). Samples from age-/sex-matched healthy controls (n = 10) were also obtained from the Translational Research Centre. Blood was collected into 3%, 0.109 M sodium citrate tubes, centrifuged for 15 min (1500 *g* at 4 °C), and the plasma was frozen at −70 °C. Detailed collection and handling protocols have been described previously (27, 28). Plasma samples were transferred on dry ice to Mount Sinai Hospital, Toronto, Ontario, Canada and stored at -80 °C until analysis.

Our reference cohort (no TBI) consisted of a cancer group and a dementia group. Although these groups are from unrelated studies, they were used as biological and technical reference since PEA analysis was performed in their respective plasmas for the same panels of analytes, in the same run, and by the same analytical provider (Olink Proteomics, Uppsala, Sweden) as the TBI study. These groups were not matched for any parameter with either the sTBI or control groups. In the dementia group, plasma from 30 patients were included. Clinical diagnoses of AD (n = 12) and amnestic mild cognitive impairment (MCI; n = 18) were made based on the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria (29) and the International Working Group on Mild Cognitive Impairment criteria (30), respectively. In the second reference group, serum samples from 32 ovarian cancer patients with high-grade serous carcinoma were retrospectively obtained from the University Health Network BioBank with approval by the Research Ethics Board of University Health Network, Toronto, Canada (REB #10-0591). Samples were collected and stored at -80 °C prior to analysis. Despite nonmatching of these latter control groups, they potentially provide specificity information if new biomarkers of TBI are identified.

Multiplex Immunoassay

PEAs were conducted using all 12 currently available panels on the Olink Proteomics platform: cardiometabolic, cell regulation, cardiovascular III, cardiovascular II, development, immune response, inflammation, metabolism, neurology, neuroexploratory, oncology II, and organ damage. A total of 1104 analytes, available online at https://www.olink.com/products/complete-protein-biomarkers-list/, were queried for relative differences in abundance between patient groups (sTBI vs matched healthy controls). As cost is a limiting factor in the use of this technology for screening a large sample size, it is notable to mention that the list price is around 1 USD per analyte per sample. Therefore, screening for the entire library of 1140 analytes costs over $1000 USD per sample, thus highly limiting the number of replicates that can be feasibly run. Detailed methodology and quality control data for the PEA technology has been published earlier, and is also available online at www.olink.com/downloads (26, 31). Briefly, 1 μL of plasma sample is incubated with a pool of oligonucleotide-labeled antibody pairs that bind to specific target proteins. In this configuration, proximity-dependent DNA polymerization occurs and amplicons are subsequently quantified by real-time PCR (BioMark™ HD System, Fluidigm Corporation). Each sample was run in singleton, as recommended by the manufacturer, since various internal controls are included by the manufacturer in each sample and plate to ensure minimal intra- and inter-assay variability (described in detail at www.olink.com/downloads) (26, 31). Identical aliquots of 1 male and 1 female control plasmas from healthy volunteers were also

included multiple times on all assay plates without vendor knowledge, to check for blinded, unbiased assay reproducibility. The raw data was provided as $\log_2$ transformed normalized protein expression (NPX) values. NPX values represent relative protein concentrations in comparison to vendor calibrators, where comparisons may only be made for the same protein across samples. Values below the limit of detection (LOD; in the sub pg/mL concentration for most analytes as defined by the manufacturer) were reported as equal to the LOD.

Candidate Selection

We conducted a quantitative and qualitative selection process to narrow down the complete list of 1104 analytes to a subset of candidates suitable for further consideration. Proceeding statistical analyses were conducted using R (version 3.3.3). Our criteria for the selection of our TBI candidate diagnostic markers (defined by us) were for proteins with the following characteristics:

1. Median expression value in sTBI group is greater than 2 X LOD to ensure reliable quantification.
2. Stringent statistical significance for difference in expression level between healthy controls and sTBI group (Mann Whitney U test; $P < 0.01$).
3. Step-wise increase in median expression value from i) healthy controls to sTBI (S) and ii) from sTBI patients who survived to those who did not.
4. Low expression, indicated by lower median expression value, in both independent reference groups (dementia and cancer) compared with the sTBI group.
5. Literature search to identify candidates with brain-expression, with previous associations to brain disorders, or previous suggestions of being candidate TBI biomarkers.

Validation of PEA Data

We selected 2 biomarker candidates, interleukin 1 receptor-like 1 (ST2) and tumor necrosis factor receptor 2 (TNFR-2) for orthogonal validation in order to assess the technical reliability of our PEA data. All sTBI and matched control samples were analyzed in duplicate using the commercially available ELISA kits, DRT200 TNFR2 Quantikine kit and the DST200 ST2 Quantikine kit (R&D Systems), according to manufacturer's instructions. Samples were thawed at room temperature and measured in one run. Controls were diluted according to manufacturer specifications for normal serum/plasma samples, while sTBI patient plasmas were diluted 100-fold for ST2 analysis and 20-fold for TNFR2 analysis. Absorbance values were read at 450 nm with a microplate reader (EnVision, PerkinElmer).

Statistical Analysis

In addition to the statistical analyses utilized in the candidate selection process, Kruskal-Wallis test with Dunn's multiple correction was utilized to compare reference groups to the sTBI controls and patients. Spearman's correlations (r_s) were also conducted for all analytes to assess the relationship between NPX levels and lowest documented GCS score (within 24 h of hospital admission), as well as hospital length of stay (LOS). In addition, Spearman's correlations were utilized to determine potential concordance between the PEA data with ELISA validation data. All above statistics were performed using GraphPad Prism (version 6.0e).

Receiver operating characteristic (ROC) curves were conducted and area-under-the-curve (AUC) was calculated for each biomarker to assess their ability to predict sTBI and survival outcome. Logistic regression analyses were also conducted with nonsurvival as the outcome and the top 3 markers by AUC were entered as predictors; the predicted values from the logistic regression models were then saved for use in ROC curve analyses to determine the combination of markers with the greatest combined AUC. All analyses were conducted using SPSS version 25 (IBM Corp.).

RESULTS

Patient Population

Demographics and pathological characteristics of recruited subjects are summarized in Table 1 and Supplemental Table 1.

sTBI Biomarker Candidates

We used novel multiplexed proximity extension assays to assess protein abundance changes in the plasma of sTBI patients compared with healthy controls. We queried the expression of 1104 proteins in the plasma of sTBI patients, healthy controls, and our two reference groups. From these data, we applied quantitative and qualitative filtering to identify a list of six candidate TBI biomarkers that met our stringent pre-set criteria (Table 2). All candidate markers are significantly increased in sTBI (S) patients in comparison with controls (Fig. 1). Furthermore, we observed a significant increase in plasma ST2, interleukin-6 (IL6), troponin I (TNNI3), chitinase-3-like protein 1(CHI3L1), and TNFR-2 in sTBI (NS) patients vs. controls (Fig. 1, A–E). There was no statistically significant difference in marker abundance between sTBI (S) and sTBI (NS) patients; however, as per our filtering criteria, we observed an increasing trend in candidate protein expression from surviving patients to non-surviving patients in this small pilot study. In all candidates except TNFR-2 (Fig. 1, D), protein abundance was markedly lower in the reference cancer and dementia groups. We observed increased abundance of TNFR-2 in the MCI dementia group, ($P < 0.05$). This finding was not investigated further since it was outside the scope for this project.

All markers exhibited significant predictive ability to discriminate healthy adults from sTBI patients; AUC estimates ranged from 0.840 to 1.000 (Supplemental Table 2). IL6 and ST2 demonstrated the highest AUC (1.000; 95% CI 1.000–1.000; $P < 0.001$).

Table 1. Demographic and clinical information of severe TBI (sTBI) patients and healthy controls.

Variable	sTBI (n = 10)	Healthy controls (n = 10)
Age, years*	35.1 (14.4)	35.5 (14.3)
Sex, male/female	7/3	7/3
Lowest documented GCS*†	5.1 (1.45)	
Outcome, dead/alive	4/6	
ICU LOS, days*	9.1 (6.7)	
Hospital LOS, days*	15.4 (18.0)	
Etiology, n (%)		
Motor vehicle collision	6 (60)	
Falls	3 (30)	
Abuse/assaults (nonaccidental)	1 (10)	
Admission CT abnormalities, n (%)		
Subarachnoid hemorrhage	5 (50)	
Subdural hemorrhage	4 (40)	
Epidural hemorrhage	2 (20)	
Intraventricular hemorrhage	3 (30)	
Intraparenchymal hemorrhage	5 (50)	
Diffuse axonal injury	6 (60)	
Midline shift	2 (20)	
Tonsillar herniation	1 (10)	
Cerebral contusion	8 (80)	
Cerebral edema	2 (20)	
Basal skull fractures	6 (60)	
Hydrocephalus	1 (10)	
Pneumocephalus	2 (20)	

Abbreviations: ICU, intensive care unit; GCS, Glasgow Coma Score; LOS, length of stay.
* median (SD).
† within 24 h of hospital admission.

Correlation to Other Clinical Measures

We assessed whether candidate biomarker levels were correlated with other severity and outcome measures. Plasma S100 calcium binding protein A12 (EN-RAGE) was significantly negatively correlated to the lowest documented GCS score within 24 h of hospital admission (r_s = -0.6441, $P < 0.0398$; Fig. 2, A). Additionally, we observed a

Table 2. Candidate plasma biomarkers of sTBI

Gene name	Uniprot ID	Olink panel
ST2	Q01638	Olink CARDIOVASCULAR III
EN-RAGE	P80511	Olink INFLAMMATION
IL6	P05231	Olink CARDIOVASCULAR II
TNNI3	P19429	Olink ORGAN DAMAGE
TNFR-2	P20333	Olink CARDIOVASCULAR III
CHI3L1	P36222	Olink CARDIOVASCULAR III

significant and positive association between plasma CHI3L1 and LOS in surviving sTBI patients ($r_s = 0.9856$, $P < 0.0056$; Fig. 2, B).

Orthogonal Validation

Finally, we aimed to determine the reliability of the commercial PEA assay, used as a discovery tool for this study. We conducted an independent validation of two candidate biomarkers, ST2 and TNFR-2, for which commercially available ELISAs were available. In both assays, the plasma biomarker concentrations were significantly elevated in the sTBI group in comparison with healthy controls (Fig. 3). In all groups, and for both proteins, the NPX quantification from PEA significantly correlated with ELISA data (Fig. 4). We observed a highly positive correlation between the two assay types in both healthy and sTBI plasmas.

DISCUSSION

We used the highly sensitive PEA technology to quantify the relative difference of 1104 proteins in the plasma of adult sTBI patients compared with age- and sex-matched healthy controls. To further increase our stringency during candidate selection and increase the specificity of our markers for TBI, we also compared the expression of all biomarkers in 2 independent reference groups of ovarian cancer and dementia patients that were subjected to the same PEA analysis from a previously performed project. Using a quantitative and qualitative selection process, including statistical analysis, literature search, and comparison with the reference groups, 6 biomarker candidates were subsequently selected (Table 2). These proteins include both known and novel TBI biomarkers and represent a promising starting point for a larger and more targeted validation.

We identified 2 well-known TBI biomarkers, IL6 and TNNI3, which serve as analytical validation that the multiplex proteomic assay was able to identify increased expression of TNNI3 and IL6 in sTBI plasma compared to controls, among the >1100 analytes. The role of IL6 in the inflammatory response of the brain to TBI is well-studied, with novel therapeutics that target IL-6-involved signaling pathways being a popular topic (32, 33). Numerous studies have established the prognostic value of TNNI3 elevation in TBI, where it is associated with all-cause in-hospital mortality in sTBI (34–36). All candidate biomarkers exhibit an increasing trend when comparing healthy controls, sTBI (S), and sTBI (NS), as well as demonstrate generally low abundance in the reference cancer and dementia reference groups. Furthermore, some of the candidate markers (ST2, EN-RAGE, TNFR-2, CHI3L1) are supported by literature to have protein expression in the brain and/or have been linked to brain injuries in some manner but have not been well-studied as biomarkers of TBI (37). One study demonstrated the potential prognostic ability of ST2 in TBI, where it showed higher correlation with in-hospital major adverse events compared to IL6 (37). TNFR-2 has not been investigated in TBI. Our overall goal was to select for candidate biomarkers with specificity to TBI events, which are informative in diagnosing, and even more importantly, in prognosing TBI.

Five of the 6 candidate biomarkers identified were also significantly correlated to TBI clinical measures, thus strengthening their potential for clinical prognostic utility. Increasing abundance of plasma EN-RAGE was associated with higher TBI

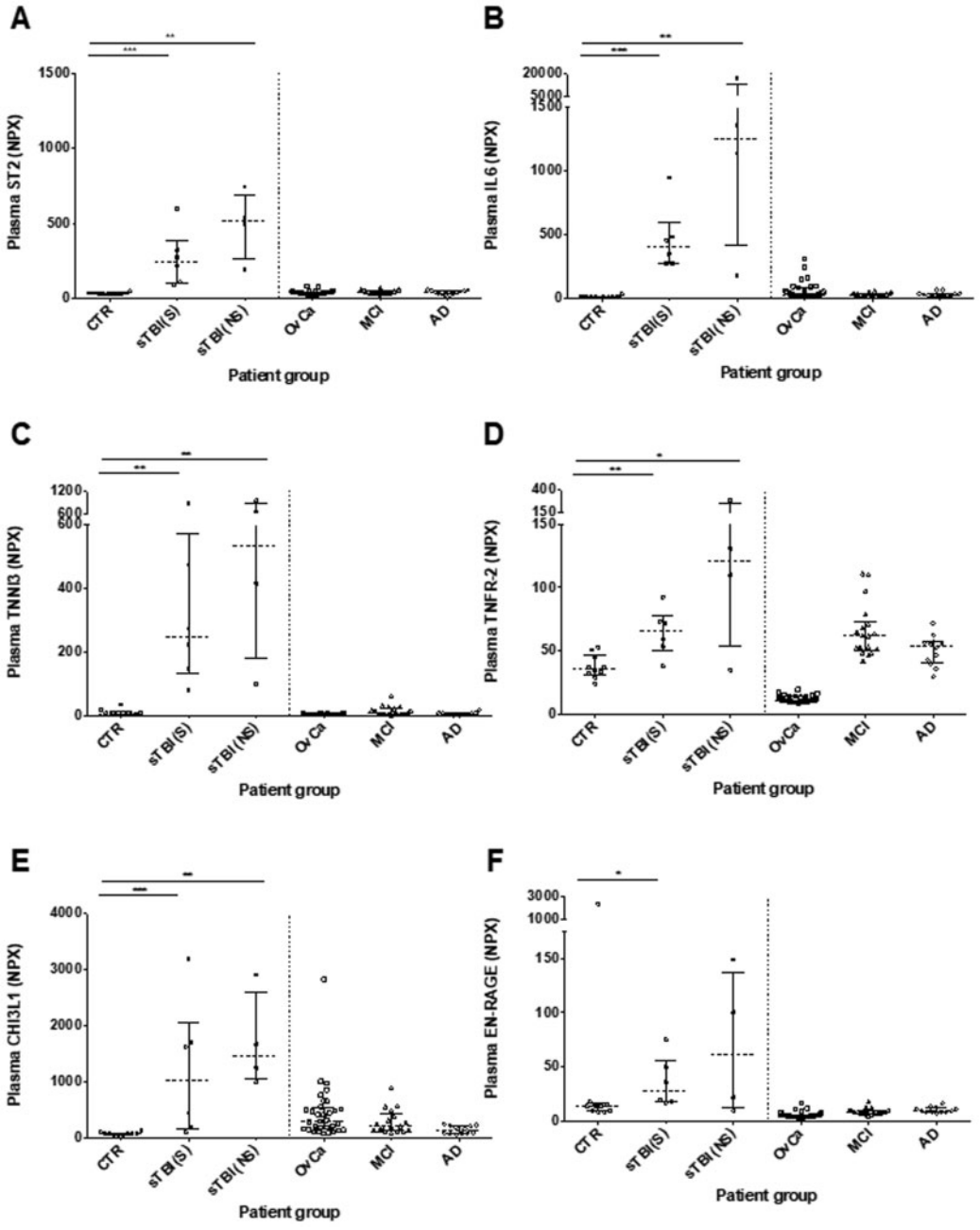

Fig. 1. Normalized protein expression (NPX) of plasma biomarkers in surviving [sTBI (S); n = 6] and non-surviving sTBI patients [sTBI (NS); n = 4] compared with matched healthy controls (CTR, n = 10). Biomarkers shown are (A) ST2, (B) IL6, (C) TNNI3, (D) TNFR-2, (E) CHI3L1, and (F) EN-RAGE. Reference groups shown are ovarian cancer patients (OvCa, n = 32) and dementia patients (mild cognitive impairment, MCI, n = 18; Alzheimer disease, AD, n = 12). Dotted lines represent group median, bars show interquartile range. Mann Whitney U test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant. For more discussion see text.

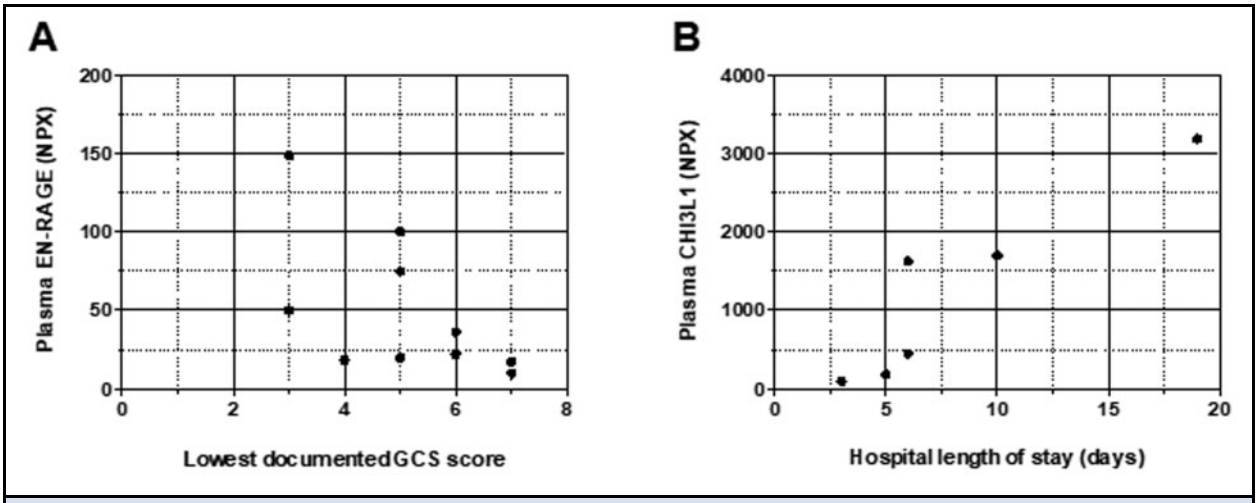

Fig. 2. Correlation between clinical measures and plasma biomarker abundance in sTBI patients. Spearman's correlation (r_s) is shown for (A) Plasma EN-RAGE vs Glasgow Coma Scale (GCS) score (n = 10); r_s = -0.6441, P < 0.0398, and (B) Plasma CHI3L1 vs hospital length of stay (days) in surviving patients only (n = 6); r_s = 0.9856, P < 0.0056.

severity, as indicated by lower GCS scores during the first 24 h of hospitalization. EN-RAGE, also known as S100A12, is a 10.4 kDa protein involved in pro-inflammatory signaling (38, 39). Although this marker has been previously associated with a range of noncranial inflammatory diseases, a recent study has shown that it may be an indicator for post-sTBI prognosis (4, 40). Similar to our findings, this study also found a significant increase in serum EN-RAGE in sTBI patients compared with healthy controls and a correlation with intake GCS. Given this, it appears that EN-RAGE may be an informative biomarker in the context of TBI. Notably, Petrone et al identified EN-RAGE as a biomarker for mild TBI or concussion (41). We found that increased CHI3L1 was significantly correlated with longer hospital stays in surviving sTBI patients, which may indicate its potential as a prognostic marker of TBI. CHI3L1 is a 40 kDa protein that has been hypothesized to be involved in CNS inflammation (42). Previous studies have shown that CHI3L1 is elevated in the cerebral spinal fluid of patients with sTBI. Mirroring our findings, the authors observed that nonsurviving patients have higher levels of CHI3L1 than those who survived (43). Furthermore, this protein may be a therapeutic target in TBI treatment (44). Interestingly, we observed that combining plasma measurements of ST2 and either IL6 or TNFR-2 was associated with survival outcome in sTBI patients (AUC 0.792). Further studies are required to validate this finding in a larger cohort of sTBI patients.

PEA data correlated well with commercial ELISA, in both pathological plasma and healthy controls. Although we only conducted orthogonal validation for 2 biomarker candidates (for the lack of other commercial ELISAs), which showed high concordance between the 2 technologies, our group had also assessed the intra-assay reproducibility of the PEA platform, in an independent and blind fashion to the vendor, with excellent results (45). Overall, the data gathered from this technology appear to provide robust quantification of low abundance plasma proteins (at concentrations as low as low fg/mL). Of all quantified proteins, 97.3%

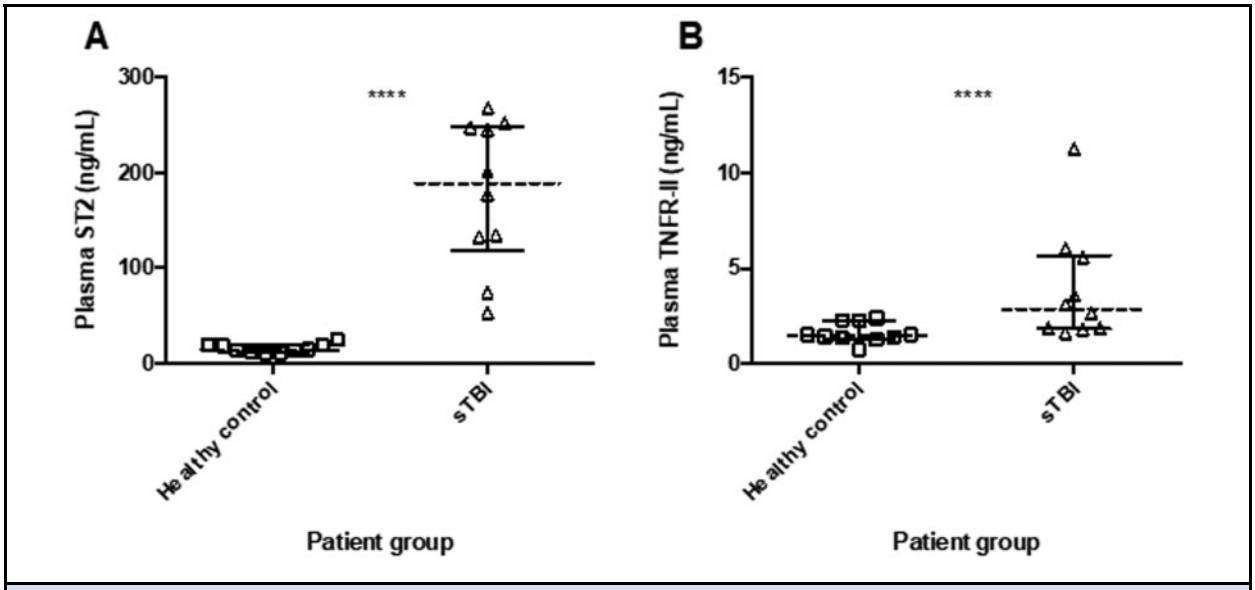

Fig. 3. Plasma concentration of ST2 and TNFR-2 in healthy controls (n = 10) and sTBI patients (n = 10), as measured by commercially available ELISAs. Mann Whitney U test; ****$P < 0.0001$. For more details see text.

have intra-assay coefficients of variation below 15%, and 89.3% of all proteins have CVs below 10% (our unpublished data). From this pilot study, we conclude that PEA is a strong proteomics platform to conduct biomarker discovery, particularly in the detection of low abundance changes in patient blood with minimal requirement for sample volume.

The current pilot study has important limitations. First, all samples were run in singlicate for cost containment. Although immunoassays should typically be performed with replicates, the Olink PEA, as mentioned, has low intra-assay coefficients of variability, as we demonstrated in blind experiments. Therefore, biological, not analytical variation, should account for much of the changes we see in these experiments. Second, the limited sample size in this pilot study warrants caution in the interpretation of the data. The findings presented here represent a starting point for the validation phase of biomarker discovery, where candidate biomarkers can be tested in a larger independent cohort with more targeted assays of lower cost. The limitations of our 2 unmatched control cohorts were mentioned already, along with the justification for including them. These cohorts provide some reassurance that our identified biomarkers are specific to TBI.

CONCLUSIONS

In this pilot study, we uncover potential novel diagnostic and prognostic TBI biomarkers that may prove useful, in combination with other modalities such as imaging, for triaging patients into surveillance or interventional clinical practices. It is remarkable that some of these biomarkers exhibit near perfect sensitivity/specificity profiles. Our findings need to be validated with larger and independent patient groups, particularly focusing on TBI of different severities including mild/

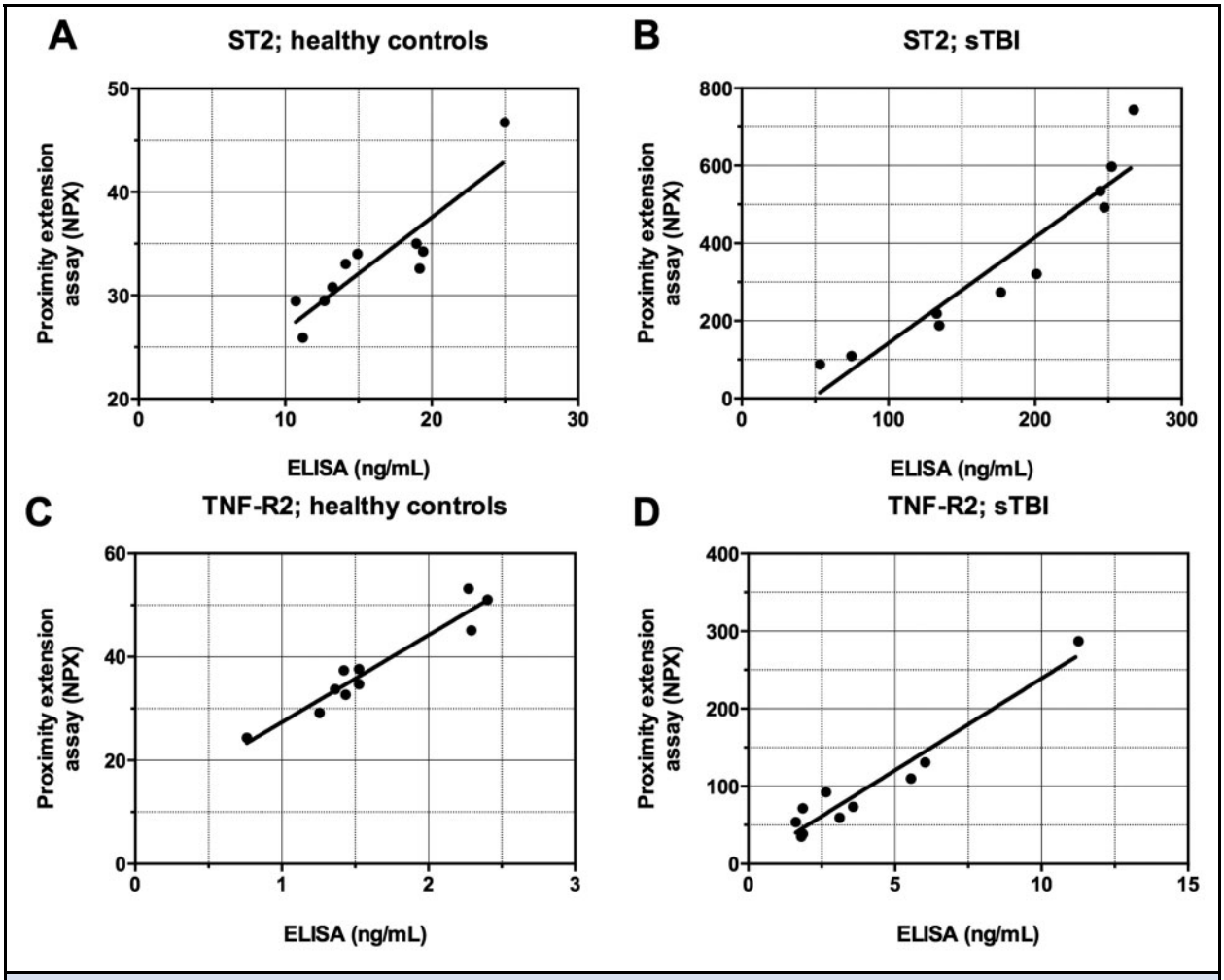

Fig. 4. Comparison between commercially available proximity extension assays (Olink Proteomics) and ELISAs. Plasma ST2 was measured in (A) healthy controls (n = 10); Spearman's correlation (r_s) = 0.8909, $P = 0.0011$, and (B) sTBI patients (n = 10); $r_s = 0.9758$, $P < 0.0001$. Plasma TNFR-2 was measured in (A) healthy controls (n = 10); $r_s = 0.9030$, $P = 0.0008$, and (B) sTBI patients (n = 10); $r_s = 0.8667$, $P = 0.0022$. Black line shows line of best fit.

concussive cases to evaluate the abilities of our candidate biomarkers to predict various clinical outcomes.

Moving forward, future studies to assess the efficacy and value of the candidate markers in an independent cohort of plasma from mild and moderate TBI patients and healthy controls could also reveal clinical utility for risk stratification and prognosis. It will also be highly informative to include a set of noncranial injury controls, such as patients with orthopedic injuries, in order to rule out nonspecific injury markers from our candidate list. A future goal will be to see whether our findings can be replicated in a larger and longitudinal cohort, and importantly, to determine whether these markers, individually or as a multiparametric panel, are informative of outcome, recovery, or duration of hospitalization in TBI of

various severity. Correlation with other measures of injury severity and clinical characteristics such as duration of coma, duration of posttraumatic amnesia and imaging patterns will need to be addressed in subsequent studies. Overall, the findings and methodology presented here lay the groundwork for identifying meaningful novel biomarkers that may have significant translational impact for the diagnosis and clinical management of TBI. Furthermore, our study exemplified the utility of an innovative multiplex proteomics tool for the simultaneous deep profiling of over 1000 proteins, which is an unprecedented scale, to detect potential markers of TBI, including brain-specific and brain-enhanced proteins, in the circulation of patients. Beyond the scope of TBI, the methodology of our study is important for biomarker discovery efforts in various diseases, such as neurodegenerative diseases, where brain-related proteins in the circulation may hold important information regarding disease diagnosis and prognosis.

SUPPLEMENTAL MATERIAL

Supplemental material is available at *The Journal of Applied Laboratory Medicine* online.

Nonstandard Abbreviations: AD, Alzheimer disease; AUC, area under the curve; CHI3L1, chitinase-3-like protein 1; CTE, chronic traumatic encephalopathy; CTR, matched healthy controls; ELISA, enzyme-linked immunosorbent assay; EN-RAGE, S100 calcium binding protein A12; GCS, Glasgow Coma Scale; ICU, intensive care unit; IL-6, interleukin-6; LOC, loss of consciousness; LOD, limit of detection; LOS, hospital length of stay; MCI, mild cognitive impairment; MS, mass spectrometry; NPX, normalized protein expression; OvCa, ovarian cancer patients; PEA, proximity extension assay; ROC, receiver operating characteristic; rs, Spearman's correlation; TBI, traumatic brain injury; ST2, interleukin 1 receptor-like 1; sTBI, severe traumatic brain injury; sTBI (NS), nonsurviving severe traumatic brain injury patients; sTBI (S), surviving severe brain injury patients; TNFR-2, tumor necrosis factor receptor 2; TNNI3, troponin I.

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