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MEDICAL HYPOTHESIS

CNS disease-related protein variants as blood-based biomarkers in traumatic brain injury

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Abstract

Objective

To utilize a panel of 11 single chain variable fragments (scFvs) that selectively bind disease-related variants of TAR DNA-binding protein (TDP)-43, β-amyloid, tau, and α-synuclein to assess damage following traumatic brain injury (TBI), and determine if the presence of protein variants could account for the increased risk of various neurodegenerative diseases following TBI.

Methods

We utilized the panel of 11 scFvs in a sensitive ELISA format to analyze sera from 43 older veterans, 25 who had experienced at least 1 TBI incident during their lifetime (~29.4 years after TBI), and 18 controls who did not incur TBI, in a cross-sectional study.

Results

Each of the 11 scFvs individually could significantly distinguish between TBI and control samples, though they did not detect each TBI sample. Comparing the levels of all 11 variants, all 25 TBI cases displayed higher reactivity compared to the controls and receiver operating characteristic analysis revealed 100% sensitivity and specificity. Higher total protein variants levels correlated with TBI severity and with loss of consciousness. Oligomeric tau levels distinguished between single and multiple TBI incidents. While all TBI cases were readily selected with the panel, the binding pattern varied from patient to patient, suggesting subgroups that are at increased risk for different neurodegenerative diseases.

Conclusion

The panel of protein variants-specific scFvs can be used to identify blood-based biomarkers indicative of TBI even 20 years or more after the initial TBI. Being able to identify subgroups of biomarker profiles allows for the possibility of individually targeted treatments.

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Go to Neurology.org/N for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

Glossary

α-syn = α-synuclein; **Aβ** = β-amyloid; **AD** = Alzheimer disease; **ALS** = amyotrophic lateral sclerosis; **AUC** = area under the curve; **CTE** = chronic traumatic encephalopathy; **LOC** = loss of consciousness; **mTBI** = mild traumatic brain injury; **PD** = Parkinson disease; **ROC** = receiver operating characteristic; **scFv** = single chain variable fragment; **TBI** = traumatic brain injury; **TDP-43** = TAR DNA-binding protein 43.

Traumatic brain injury (TBI) affects over 1.7 million people each year. Even mild TBI (mTBI) may disrupt cognitive functioning[1,2] and lead to chronic traumatic encephalopathy (CTE) and an increased risk and earlier onset of a spectrum of neurodegenerative brain disorders, including Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia, and Lewy body dementia[3–6] (although not universally accepted). A common link between these diseases is the presence of misfolded and aggregated variants of β-amyloid (Aβ), tau, TAR DNA-binding protein (TDP)-43, and α-synuclein (α-syn). While these proteins can form fibrillar aggregates, they also form smaller soluble oligomeric aggregates, and increasing evidence implicates these oligomers in the onset and progression of these diseases.

TBI induces a variety of neuromorphologic and neurochemical changes that result in the generation of misfolded and aggregated protein variants,[7,8] which provides a link for the increased risk of neurodegenerative diseases. We previously generated antibody-based reagents that recognize different toxic variants of tau, Aβ, TDP-43, and α-syn,[9–19] and utilized these reagents in conjunction with a sensitive novel sandwich ELISA.[20] We show that different patterns of these variants are present in blood from TBI patients even many years post-injury, depending on the extent and type of injury. These variants in blood following TBI represent powerful diagnostic biomarkers to assess the type and extent of neuronal damage and may be useful to predict which patients are most susceptible to a particular neurodegenerative disease.

Methods

Study population

We acquired 25 TBI cases and 18 controls from the cross-sectional Brain Aging in Veterans (BRAVE) study (table 1), a study of independently living residents at the Veterans Home of California in Yountville. All participants were aged 50–95 years, and received an in-person evaluation including medical history, TBI history, and a blood draw. Excluded were individuals with a previous penetrating head injury and those with medical conditions or hearing or vision loss severe enough to impede participation. The only characteristic that was significantly different between the TBI and control groups was diabetes. To account for this variability, we completed analyses of our biomarker results with and without these cases. Similar results were acquired in both sets of analyses (data not shown). To help ensure that participants could provide consent, those with low cognition (Mini-Mental State Examination <20) were also excluded. The study was approved by the Institutional Review Board at the University of California, San Francisco, and all participants gave written informed consent.

TBI history ascertainment

TBI history was defined as at least one head injury requiring medical care (doctor's visit, emergency room visit, treatment from field medic, hospitalization). Using the Ohio State University TBI Identification Method (OSU-TBI-ID), a structured clinical interview, we determined the number of total TBIs for each participant, the cause of each TBI, the age at each TBI, and symptoms such as loss of consciousness (LOC) and memory loss. Complete medical records were not available for all participants, but we reviewed retirement home charts and validated TBI in 51% of the TBI participants and absence of TBI in the controls. Each TBI was categorized as mild (no LOC or LOC <30 minutes), moderate (LOC ≥30 minutes but <24 hours), or severe (LOC ≥24 hours).

Single chain variable fragments

We previously isolated single chain variable fragments (scFvs) that are reactive with neurodegenerative disease-associated protein variants of TDP-43, Aβ, tau, and α-syn. The scFvs AD-TDP-1, AD-TDP-2, AD-TDP-3, ALS-TDP-9, and ALS-TDP-11 recognize different variants of TDP-43 immunoprecipitated from human ALS brain tissue.[21,22] AD-TDP-1, AD-TDP-2, and AD-TDP-3 also show strong reactivity with human AD cases (references 22 and 23 and unpublished data), providing further evidence that TDP-43 may play an important role in AD. The A4 and C6T scFvs bind different oligomeric variants of Aβ.[15,17–19] The F9T and D11C scFvs bind different oligomeric tau variants,[24] and the 10H and D5 scFvs bind different oligomeric α-syn variants.[10,11,19] We have previously demonstrated the diagnostic value of this panel of scFvs for various neurodegenerative diseases including AD and PD[19,23] so they are promising tools to detect disease-related protein variants in patients incurring brain trauma. The scFvs were produced as previously described.[19–21]

Detection phages

For the detection antibody, we used an scFv that recognized a nonconformation-specific epitope of the target protein. The following 4 detection scFvs and their respective protein targets were used: TDPM1 (TDP-43), H1V2 (Aβ), TauM1 (tau), and D10 (α-syn).[11,13,22,23,25] The detection scFvs were produced as previously described attached to self-assembling phage particles and their coat proteins biotinylated to amplify

Table 1 Demographics and medical history of the veterans by traumatic brain injury (TBI) history

	Controls (n = 18)	TBI (n = 25)	*p* Value
Age, y	77.2 (8.2)	76.9 (11.9)	0.930
Male	72	88	0.198
Nonwhite race	5.6	8.0	0.763
Education, y	15.7 (1.6)	14.5 (2.3)	0.073
Military service, y	4.5 (4.4)	3.9 (3.9)	0.656
MMSE score	28.9 (1.5)	28.2 (2.0)	0.159
Medical history			
Hypertension	56	68	0.417
Stroke	16.7	16.7	1.000
Diabetes	5.6	32	0.036
TBI characteristics			
Age at first TBI, y	—	33.9 (28.0)	—
Age at most recent TBI, y	—	47.5 (26.5)	—
Participants with >1 TBI	—	60.0	—
At least 1 TBI with LOC	—	80	—
TBI severity (most severe per person)			
Mild	—	68	—
Moderate/severe	—	32	—

Abbreviations: LOC = loss of consciousness; MMSE = Mini-Mental State Examination.
Values are mean (SD) or %.

the signal (docplayer.net/30268507-Human-single-fold-scfv-libraries-i-j-tomlinson-i-j.html).[20]

Capture ELISA

For analysis of the sera samples, we used a capture ELISA essentially as described previously.[19,20]

Statistical analysis

The binding intensity between each scFv and the individual cases is expressed as relative SD, as previously described.[23] First, the raw absorbance of each sample was divided by the raw absorbance of the phosphate buffered saline control to generate a ratio (R). Next, we calculated the mean ratio (R_c) and SD (SD_c) of the control cases. The R_c was then subtracted from the R value for each case and the resulting value divided by the SD_c. Since the samples were tested in multiple batches, they were first normalized to the controls included in their trial run to account for plate to plate variations before collectively analyzing all the samples.

All the graphs and statistical analyses were completed using the IBM SPSS (Chicago, IL) Statistics 24 program. Significance was based on one-way analysis of variance with least significant difference post hoc analyses at $p < 0.05$. Receiver operating characteristic (ROC) curves were generated using SPSS. Extreme outliers (few highly reactive TBI cases) were identified using boxplot analysis and excluded from some of the statistical assessments. Comparisons of demographics and medical history were completed using t tests.

Data availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

Results

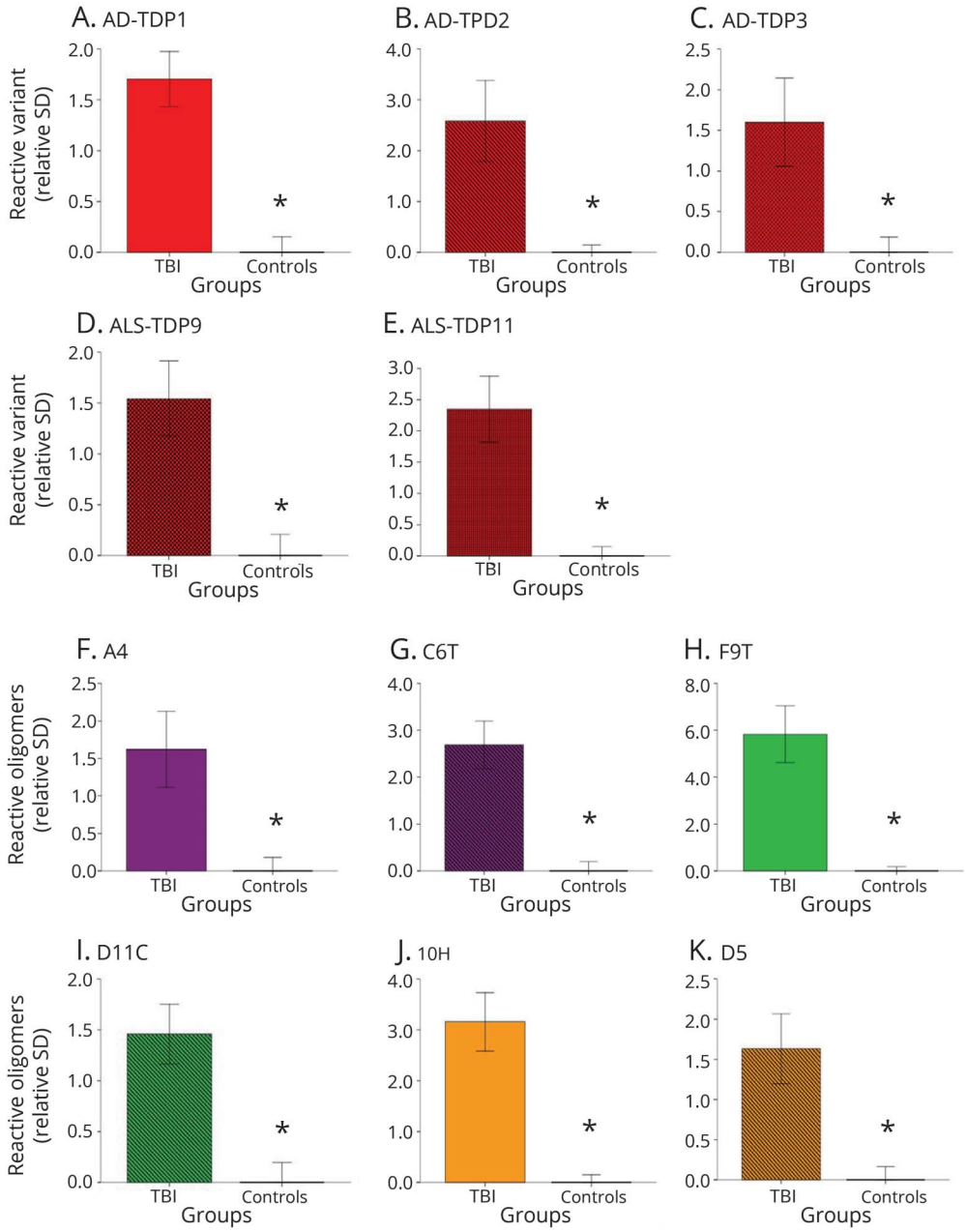
The 25 TBI and 18 control cases were analyzed with the panel of 11 scFvs using capture ELISA.[10,11,15,17–19,21–24] The 5 different TDP-43 reactive scFvs—AD-TDP-1, AD-TDP-2, AD-TDP-3, ALS-TDP-9, and ALS-TDP-11—showed significantly stronger reactivity with TBI cases relative to controls (figure 1, A–E). The oligomeric Aβ-reactive scFvs A4 and C6T also generated significantly stronger reactivity with the TBI cases relative to controls (figure 1, F and G), as did F9T and D11C, which are reactive with oligomeric tau variants (figure 1, H and I), and 10H and D5, which are reactive with oligomeric α-syn variants (figure 1, J and K). Even though the TBI cases represented patients with different time intervals from the first or last occurrence (the first TBI occurring on average ~43 years ago with the most recent first TBI incidence ~29.4 years ago) and patients with single or multiple TBIs, each of the 11 scFvs could distinguish between individuals who incurred TBI and those who did not.

The cumulative binding of all 11 scFvs with each sample is also shown (figure 2A). Each of the 25 TBI cases showed stronger reactivity compared to the 18 controls, with most being dramatically different from the controls, including those controls showing slight reactivity. Interestingly, not every scFv reacted with every TBI sample, but all TBI samples were selected when using the entire panel, suggesting that different cellular processes may be affected in different patients following TBI, and showing the importance of detecting multiple protein variants for better selection of affected individuals.

Stratification of TBI cases

Since TBI may lead to a spectrum of neurodegenerative diseases, and since the different TBI cases had different protein binding fingerprints (figure 2A), we grouped them into subcategories based on these patterns. Since TDP-43 is linked to ALS, Aβ to AD, and α-syn to PD, we focused on the cumulative binding levels for these 3 proteins to create subgroups (to be more stringent, only cumulative bindings >3 SD were considered positive).[19,21,22,26–28] Patients with high TDP-43 levels were hypothesized to be at an increased risk for developing ALS and were placed in subgroup A (figure 2B). Patients with high TDP-43 and Aβ levels were considered to have an increased susceptibility to AD and placed in subgroup

Figure 1 Protein variants biomarkers in traumatic brain injury (TBI) sera



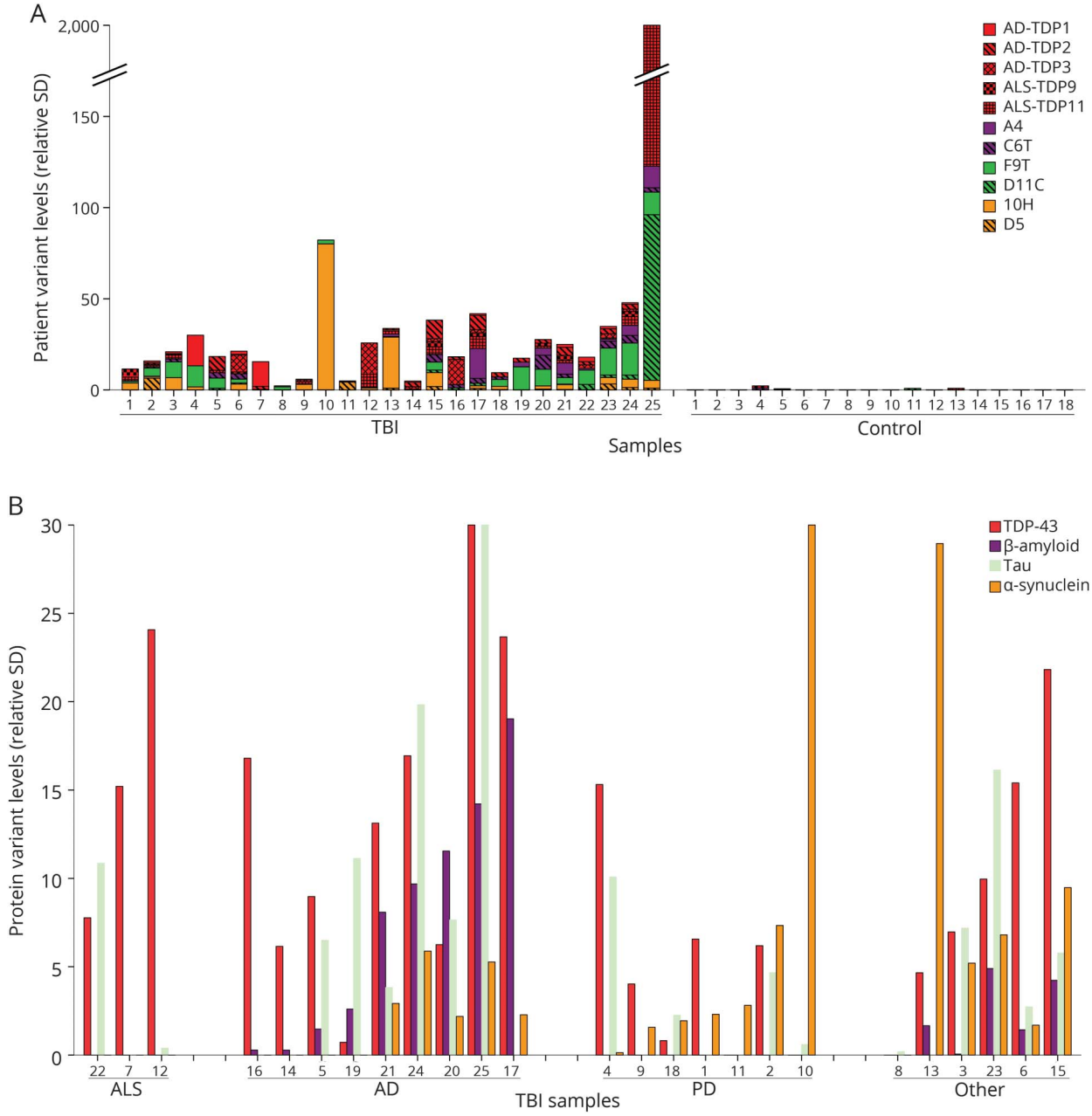
The protein variants levels in the 25 TBI and 18 control cases were assessed using a capture ELISA and panel of single chain variable fragments. There were significantly higher levels of (A) Alzheimer disease (AD)–TAR DNA-binding protein (TDP)–1, (B) AD-TDP-2, (C) AD-TDP-3, (D) amyotrophic lateral sclerosis (ALS)–TDP-9, (E) ALS-TDP-11, (F) A4, (G) C6T, (H) F9T, (I) D11C, (J) 10H, and (K) D5 reactive variants in the TBI samples compared to controls. The relative SDs were utilized and boxplot analysis identified outliers. Significance is based on one-way analysis of variance at $p < 0.05$. Errors bars ± 1 SE. * Significantly different from TBI.

B (figure 2B). Some of the samples in subgroup B also had high α-syn levels, which can occur in AD cases, but the levels of α-syn were always lower than that of Aβ.[19] Patients with high TDP-43 and α-syn levels were considered to have an increased susceptibility to PD and placed in subgroup C (figure 2B). All remaining patients were placed in subgroup D, which could potentially be at risk for AD, PD, ALS, or other neurodegenerative disease, including PD dementia, frontotemporal dementia, or CTE (figure 2B). The different subgroups provide a potential explanation for how TBI might result in the development of different neurodegenerative diseases.

ROC curves

An ROC analysis of the positive cumulative binding intensities for the TDP-43, Aβ, tau, and α-syn-reactive scFvs was performed to assess the diagnostic value of the pool of scFvs (table 2). Cumulatively, all 5 TDP-43-reactive scFvs gave an

Figure 2 Cumulative protein variants in individual traumatic brain injury (TBI) and control cases and reclassification of TBI cases



(A) The cumulative binding intensity of all protein variants for each sample was calculated with binding intensities included only when the relative SD was >1.5. All 25 TBI cases displayed cumulative levels higher than the 18 controls. Reactivity was observed in some controls, although the values were lower than in the TBI cases. (B) The TBI cases were grouped based on cumulative TAR DNA-binding protein (TDP)–43, β-amyloid, tau, or α-synuclein levels. Amyotrophic lateral sclerosis (ALS) group: high TDP-43 levels suggest an increased risk for ALS. Alzheimer disease (AD) group: high TDP-43 and β-amyloid levels suggest an increased risk for AD. Parkinson disease (PD) group: high TDP-43 and α-synuclein levels suggest an increased risk of PD. Other group: high levels of multiple proteins suggest an increased risk for overlapping diseases including AD, PD, ALS, or other neurodegenerative disease including PD dementia, frontotemporal dementia, or chronic traumatic encephalopathy. Some samples in subgroup B also had high α-synuclein levels, but they were lower than the β-amyloid levels. Only samples with cumulative binding levels >3 SD were utilized.

area under the curve (AUC) of 0.987, with a sensitivity and specificity of 100% and 88.9%, respectively. Cumulative Aβ variants gave an AUC of 0.898, with 84% sensitivity and 88.9% specificity; cumulative oligomeric tau levels gave an AUC of 0.926, with 88% sensitivity and 88.9% specificity; and cumulative α-syn variant levels gave an AUC of 0.890, with 80% sensitivity and 94.4% specificity. Together, all 11 scFvs gave an AUC of 1.000, with 100% sensitivity and specificity.

Protein variants levels and TBI history

We compared the protein variants levels of the TBI patients with TBI severity (mild or moderate/severe) and whether or

Table 2 Receiver operating characteristic analysis of protein variants levels

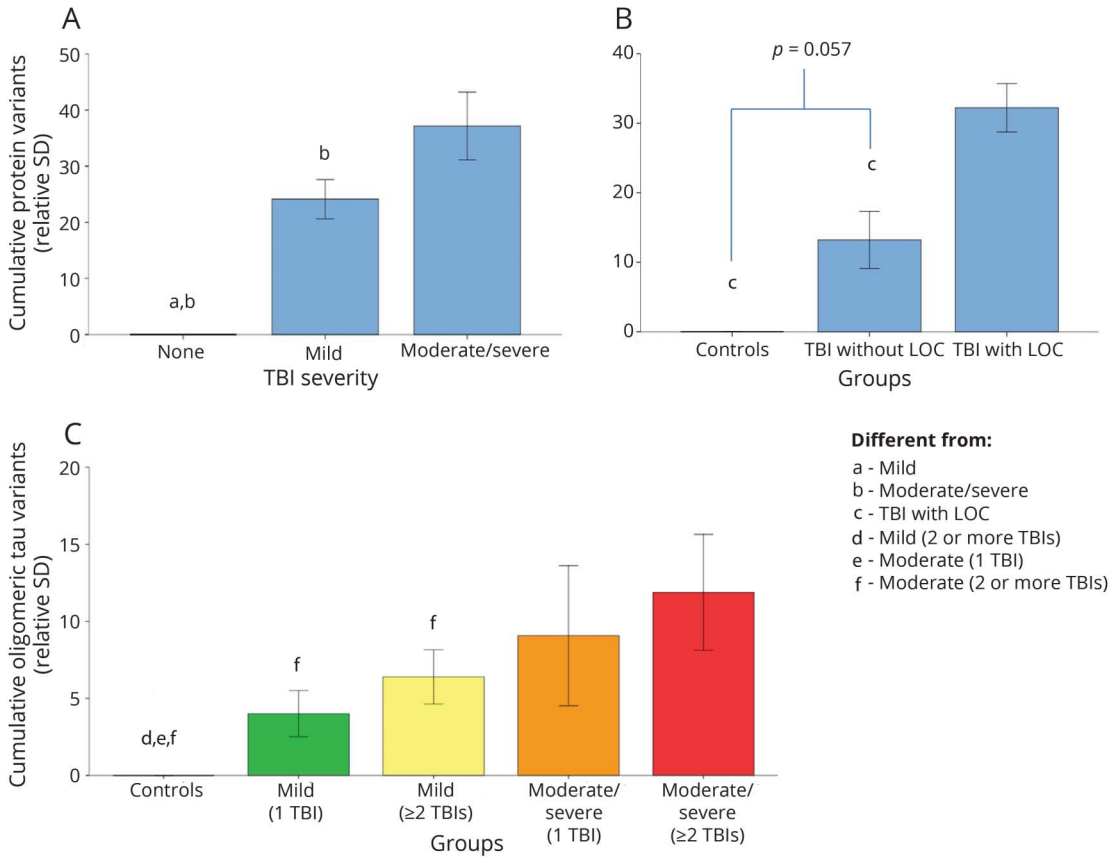
Protein variants	AUC	Sensitivity, %	Specificity, %
Cumulative TDP-43 variants	0.987	100.0	88.9
Cumulative oligomeric β-amyloid	0.898	84.0	88.9
Cumulative oligomeric tau	0.926	88.0	88.9
Cumulative oligomeric α-synuclein	0.890	80.0	94.4
Cumulative protein variants	1.000	100.0	100.0

Abbreviations: AUC = area under the curve; TDP = TAR DNA-binding protein.

not they lost consciousness. The average cumulative protein variants level of all 11 scFvs was significantly higher in both the mild and moderate/severe groups compared to the controls and significantly higher in the moderate/severe TBI group compared to the mTBI group (figure 3A). Cumulative protein variants levels also correlated with LOC, where TBI cases with LOC produced significantly higher levels compared to TBI cases without LOC and controls (figure 3B). There was an almost significant difference (p = 0.057) between the TBI cases without LOC and the controls.

Levels of tau aggregates have been previously correlated with repetitive mTBI,[29] so we compared oligomeric tau levels as a function of number and severity of TBIs (figure 3C). Oligomeric tau levels increased consistently starting with individuals who experienced a single mTBI, increasing in individuals with multiple mTBIs, then individuals with single moderate/severe TBI, with the highest levels in individuals with multiple moderate/severe TBIs. There were significant differences between the controls and those with multiple mTBIs, those with one moderate/severe TBI, and those with multiple moderate/severe TBIs, and also between individuals who experienced multiple moderate/severe TBIs and both mTBI groups.

Figure 3 Relationship between protein variants content and traumatic brain injury (TBI) history



The cumulative protein variants levels were compared based on severity of TBI and loss of consciousness (LOC) using one-way analysis of variance with least significant difference post hoc analyses at $p < 0.05$. (A) Significantly higher protein variants level was observed in the TBI cases with moderate/severe TBI compared to mild TBI (mTBI) and the control cases and between mTBI cases and controls. Error bars ± 1 SE. (B) TBI cases experiencing LOC had significantly higher protein variants content compared to those who did not experience LOC and controls. There was also an almost significant difference between the TBI cases without LOC and the controls (p = 0.057). Error bars ± 1 SE. (C) Oligomeric tau variants increased with number and severity of TBI. There were statistically significant differences between the controls and those who experienced multiple mTBIs, those with a single moderate/severe TBI, and those with multiple moderate/severe TBIs. In addition, there were significant differences between those with multiple moderate/severe TBIs and both mild groups whether encountering one or multiple TBIs. The relative SD values were utilized and boxplot analysis identified outliers. Error bars ± 1 SE.

Discussion

Some of the key biochemical changes observed in the brain following TBI are similar to changes seen in patients with neurodegenerative diseases. For example, brain injury and the resulting high sheer forces and mechanical deformation[7,30–33] can induce biochemical changes resulting in increased formation of β-amyloid,[34–36] accumulation of tau,[7,37] and generation of stress granules containing cytoplasmic aggregates of TDP-43, all of which have been correlated with neurodegenerative diseases. Therefore, brain injury and the neuronal stress and cellular changes resulting from the injury can lead to generation of a variety of neurodegenerative disease-associated protein variants that are potential biomarkers to assess neuronal damage and potential risk of different neurodegenerative diseases. We previously generated a panel of 11 scFvs that selectively recognize variants of Aβ, tau, TDP-43, and α-syn associated with different neurodegenerative diseases and utilized this panel to analyze sera samples from a cohort of veterans, 25 of whom had incurred TBI and 18 without TBI. Each scFv by itself could statistically distinguish between the TBI and control groups (figure 1, A–K), although not every scFv showed significant binding with each sample (figure 2A). However, with all 11 scFvs, we could distinguish between the TBI and control samples with 100% sensitivity and specificity (table 2). These results demonstrate the value of utilizing multiple biomarkers to identify individual cases, as we reported previously.[19,22] The total protein variants levels present also correlated with severity of TBI (figure 3A) and between TBI cases with or without LOC (figure 3B), indicating that detection may be used to signify not only brain trauma but also the level of damage. In addition, the number and severity of TBIs correlated with the level of cumulative oligomeric tau variants (figure 3C).[29]

The pattern of reactivity between each sample and the panel of scFvs varied (figure 2A), suggesting that prospective neurologic outcomes may be different. Since TDP-43 is linked to ALS, Aβ to AD, and α-syn to PD, we divided patients into different risk pools based on cumulative binding levels of these 3 proteins and generated 4 subgroups (figure 2B). This heterogeneity in protein variants produced following injury provides evidence as to how TBI might lead to an increased incidence of several different neurodegenerative diseases. Since our sample population is small and cross-sectional, larger, more controlled longitudinal studies are warranted. In the future, we intend on testing larger longitudinal sample sets to determine if these preliminary findings of biomarker variations and biomarker profiles specific to each neurodegenerative disease persist over time. In addition, we intend to investigate the clinical and neuropsychological phenotypes of these groups to see if they are consistent with the generated biomarker profiles. There was an abnormally high rate of diabetes in our TBI population, and removal of these affected cases did not significantly influence the outcomes of our experiments; however, future TBI sample sets with a more normal distribution of diabetic patients will be required to further validate the absence of confounding effects on our data.

Even though the number of years since the first or last TBI varied greatly, we could readily distinguish all the TBI samples from control samples. Because of the long time period since the last TBI in many of the samples, the continued presence of these protein variants suggests that they persist many years postinjury. Therefore, a patient who experiences TBI may be able to receive an assessment of damage over an extended time frame. However, since detection of specific disease-related protein variants can indicate specific individualized therapeutic approaches, early detection could be advantageous for ameliorating potential long-term consequences.[15,17,38]

Author contributions

Dr. Williams: drafting/revising the manuscript, ELISA analysis of the blood samples, statistical analysis and interpretation of data. Dr. Peltz: drafting/revising the manuscript, provided blood samples and demographics of the study cohort, analysis and interpretation of data. Dr. Yaffe: drafting/revising the manuscript, obtain funding, study concept and design, provided blood samples and demographics of the study cohort, analysis and interpretation of data. P. Schulz: drafting/revising the manuscript. Dr. Sierks: drafting/revising the manuscript, obtain funding, study concept and design, analysis and interpretation of data.

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Disclosure

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