Fluid Proteomics of CSF and Serum Reveal Important Neuroinflammatory Proteins in Blood-Brain Barrier Disruption and Outcome Prediction following Severe Traumatic Brain Injury: a Prospective, Observational Study

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Research

Abstract

Background: Severe traumatic brain injury (TBI) is associated with blood-brain barrier (BBB) disruption and a subsequent neuroinflammatory process. We aimed to perform a multiplex screening of brain enriched and inflammatory proteins in blood and cerebrospinal fluid (CSF) in order to study their role in BBB disruption, neuroinflammation and long-term functional outcome in TBI patients and healthy controls.

Methods: We conducted a prospective, observational study on 90 severe TBI patients and 15 control subjects. Clinical outcome data, Glasgow Outcome Score, was collected after 6-12 months. We utilized a suspension bead antibody array analyzed on a FlexMap 3D Luminex platform to characterize 177 unique proteins in matched CSF and serum samples. In addition, we assessed BBB disruption using the CSF-serum albumin quotient (Q_A), and performed Apolipoprotein E-genotyping as the latter has been linked to BBB function in the absence of trauma. We employed pathway-, cluster-, and proportional odds regression analyses. Key findings were validated in blood samples from an independent TBI cohort.

Results: TBI patients had an upregulation of structural and neuroinflammatory pathways in both CSF and serum. In total, 114 proteins correlated with Q_A, among which the top-correlated proteins were complement proteins. A cluster analysis revealed protein levels to be strongly associated with BBB integrity, but not carriage of the Apolipoprotein E4-variant. Among cluster-derived proteins, innate immune pathways were upregulated. Forty unique proteins emanated as novel independent predictors of clinical outcome, that individually explained ~10% additional model variance. Among proteins significantly different between TBI patients with intact or disrupted BBB, complement C9 in CSF (p = 0.014, DR^2 = 7.4%) and complement factor B in serum (p = 0.003, DR^2 = 9.2%) were independent outcome predictors also following step-down modelling.

Conclusions: This represents the largest concomitant CSF and serum proteomic profiling study so far reported in TBI, providing substantial support to the notion that neuroinflammatory markers, including complement activation, predicts BBB disruption and long-term outcome. Individual proteins identified here could potentially serve to refine current biomarker modelling or represent novel treatment targets in severe TBI.

Introduction

Traumatic brain injury (TBI) is a common cause of death and acquired disability worldwide (1). The initial trauma is followed by a series of secondary injury processes, which may lead to deterioration and irreversible brain damage (2). Increased knowledge of these might be of key relevance for long-term outcome and improved patient management. Among secondary injury pathologies, blood-brain barrier (BBB) disruption is of particular interest. The acute, mechanically-induced BBB injury has been shown to peak at 1-3 hours post-TBI (3,4) and contribute to the inflammatory activation of (CNS) inherent cells, such as astrocytes and microglia, but also in facilitating the infiltration of immune cells from the

systemic circulation (5,6). This generates an inflammatory cascade that can exacerbate BBB injury, thereby increasing the intensity of CNS neuroinflammation (7). Jointly, BBB injury and neuroinflammation propagate secondary injury pathologies, such as edema development, increased intracranial pressure, decreased cerebral perfusion, and consequent ischemia (4), presumably of importance for long-term outcome. It is unclear whether these acute TBI processes are influenced by the genetic set up, but in the absence of trauma the E4 variant of apolipoprotein E (APOE4) is associated with reduced BBB function and predicts risks of cognitive decline (8).

Even though there are radiological techniques that quantitatively assess BBB disruption (9), the current gold-standard metric within the field of clinical neuroscience is the cerebrospinal fluid (CSF) to blood albumin quotient (Q_A) (10). An increased Q_A indicates albumin leakage due to loss of BBB integrity. Following TBI, Q_A has shown to be associated with both structural (11), and neuroinflammatory (12–14) proteins, important as albumin does not confer information on underlying pathophysiology. Yet, as these studies included only a small selection of proteins, they potentially miss out on important biological information, pertaining to protein families and pathways that might confer joint or discrepant functions within the CNS. More comprehensive proteomic profiling efforts are warranted to deduce the pathophysiology causing BBB disruption (15,16).

Mass-spectrometry holds the largest capacity for simultaneous assessment of multiple proteins (17) and has been utilized in numerous TBI studies (18–26). Inherent limitations of mass-spectrometry entail its limited capacity to detect low-abundance proteins (e.g. cytokines) (17), thus obstructing detection of low- and high-abundant proteins within the same study. An alternative technique is affinity proteomics, combining microarray technology with affinity reagents (27) that is suitable for multiplexed protein screens in large numbers of samples (28,29) from both serum (28,29) and CSF (27). These broad advantages of affinity proteomics have not yet been utilized in the clinical TBI setting.

Collectively, although BBB disruption seems to be a key secondary injury event ensuing TBI, no systematic assessment of Q_A related protein alterations has yet been described. We therefore conducted a proteomic screen of neuroinflammatory, BBB-related, and CNS structural proteins in CSF and serum of neuro-critical care unit (NCCU) treated TBI patients and controls utilizing affinity-based proteomics, while also analyzing APOE4. The main objective was to determine to what degree changes in protein concentrations could be associated to BBB disruption, as well as their association with long-term outcome following severe TBI.

Methods

This was a prospective, observational study, part of two separate studies conducted at the Karolinska University Hospital, and Karolinska Institutet, Stockholm, Sweden. The first study included TBI patients between 2007 and 2015. Oral informed consent was granted by next-of-kin. The second study included healthy volunteers, used as control subjects here, between 2014 and 2015. All control subjects provided written, informed consent. All research activities were in accordance with Swedish law and the Declaration of Helsinki. Ethical approvals (#2005/1526-31/2; #2014/1201-31/1) were granted by the Swedish Ethical Review Authority.

Study participant inclusion and exclusion criteria

Inclusion criteria for TBI patients were: (i) severe TBI (as per Glasgow Coma Scale [GCS] 3-8 upon hospital admission or else a higher GCS score but with a significant risk for deterioration) in need of NCCU treatment and invasive intracranial monitoring, and (ii) age 18-75 years. Exclusion criteria comprised: (i) desolate prognosis precluding NCCU treatment, (ii) penetrating TBI, (iii) unconsciousness due to etiology other than TBI, (iv) underlying chronical condition precluding follow-up, or (v) other reason precluding follow-up. Inclusion criteria for control subjects were: (i) previously healthy, (ii) age 18-50 years, (iii) sufficient linguistic knowledge to participate in self-evaluation forms. Exclusion criteria were: (i) ongoing, or history of, psychiatric illness, (ii) family history of serious psychiatric comorbidity, (iii) somatic illness precluding physical activity, (iv) current pharmacological treatment interacting with the study intervention, (v) substance abuse (smoking or narcotic substances), or (vi) pregnancy. Sample size calculation was based on expected protein level difference between TBI patients and control subjects and was exerted as a two-sample t-test. We utilized Cohen's d (30,31) as effect size metric and set it to 0.8 (large effect) (30,31) in a power calculation utilizing the R package pwr (32). In order to obtain 80% power at the 0.05 significance level with n = 15 control patients, we needed to recruit n = 77 TBI patients. As this was not based on empirical data, we included patients continuously throughout the study period.

Clinical management, data, and sample acquisition

NCCU management of severe TBI at Karolinska University Hospital has been described elsewhere (33). In brief, Karolinska University Hospital employs an intracranial pressure (ICP-) driven approach, in accordance with the Brain Trauma Foundation Guidelines (34). ICP is monitored either through a closed external ventricular drain (EVD) (Medtronic, USA), or an intraparenchymal pressure monitor (Codman & Shurtleff Inc. Raynham, MA, USA or Rehau AG + CO, Rehay, Germany). While EVDs may be used to drain CSF in order to decrease ICP, the choice between monitoring device is multifactorial and not exclusively reliant on injury severity. At the NCCU, multi-modal monitoring data is automatically collected. Through the Karolinska University Hospital TBI Database, additional data is collected prospectively and comprise neurological variables, injury severity score variables, radiological variables, and outcome data, described in detail elsewhere (11). Functional outcome data (Glasgow Outcome Score, GOS) was collected at 6-12

months following hospital discharge, through structured questionnaires, or follow-up assessments in the outpatient clinic at the Neurosurgical Department. We collected CSF and serum, used for APOE genotyping, proteomic, and albumin analysis. The latter was assessed as Q_A, i.e. the CSF/serum albumin quotient (10), with the reference intervals (35): 15-29 years < 0.006; 30-49 years < 0.007; and ³ 50 years < 0.009. Sampling time points were not identical for $albumin_{CSF}$, $albumin_{serum}$ and the proteomic samples from CSF and serum. Time discrepancies were in median (interquartile range [IQR]): 4.3 (0-11.8) hours for $albumin_{CSF}$ and $albumin_{serum}$ samples; 0.88 (-2.27-9.15) hours for $albumin_{CSF}$ and the proteomic sample; and -2.83 (-3.82 - -2.08) hours for $albumin_{serum}$ and the proteomic sample.

Sample acquisition

Control subjects were recruited to a study on effects of a physical exercise intervention (36), of which only baseline samples were used. Participants were instructed to abstain from physical exercise seven days before sampling, performed by lumbar puncture and venipuncture, between 7.30 and 9 AM while fasting since midnight after a full night of bed rest. For TBI patients, blood was sampled through an arterial line and CSF through an EVD. TBI sample acquisition occurred in median at 60.8 hours (IQR 36.6-109.1) following trauma for CSF samples and 53.3 hours (30.5-91.1) for serum samples (**Figure S1A**). Samples were stored locally in 4°C in median 1 day (0-1) for both CSF and serum (**Figure S1B**), until delivery to a local biobank, where samples were vertically incubated for 30 min before centrifugation for 15 min at 2000g, aliquoting, and storage at -80°C until further analysis (37). No protein content alteration was seen per sample (**Figure S2A**) or analyte (**Figure S2B**, representative example) due to delayed biobank delivery.

Genotyping

Whole blood was collected together with serum in ethylenediaminetetraacetic acid (EDTA) tubes, and was frozen in the biobank until DNA extraction. Genotyping was performed with the SNP markers rs429358 (ApoE112) and rs7412 (ApoE158) using single base primer extension (SBE) with detection of the incorporated allele by ¨Fluorescent Polarization Template Dye Incorporation¨ (FP-TDI) (38). Signal intensities were read using a Tecan Genios Pro fluorescence absorbance reader. Raw data from the fluorescence polarization was converted to genotype data using the software AlleleCaller 4.0.0.1 and alleles ε2, ε3 or ε4 were identified.

Proteomic analysis

In total, 177 protein depicted through 220 antibodies were examined (**Table S1**, where the full protein name is provided). For 43 proteins, two antibodies targeted different regions of the same protein, i.e.

sibling antibodies (39). The protein panel was chosen based on CNS-enrichment (40), previous clinical/experimental/mass-spectrometry TBI studies, or previous neuroinflammation studies (20,24,26,41–45). Antibodies were selected from the Human Protein Atlas (HPA) (www.proteinatlas.org) (46).

Antibodies were immobilized onto color-coded magnetic beads (MagPlex, Luminex Corporation) as previously described (28). Briefly, the beads surface was activated by using 0.1 M sodium hydrogen phosphate (Sigma), 0.5mg of N-hydroxysulfosuccinimide (sulfo-NHS) (Nordic Biolabs) and 0.5mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (ProteoChem). Beads were then incubated with antibodies (16 μg/ml in 2-(*N*-morpholino)ethanesulfonic acid [MES] buffer, Sigma) for 2h at room temperature. Each antibody type was immobilized on a different bead identity (bead type with specific color-code). After incubation, the beads were washed with phosphate-buffered saline (PBS, Fisher Scientific) 0.05% Tween-20 (Fisher Scientific) (PBS-T) to eliminate the antibody excess, stored overnight in blocking buffer (Roche blocking reagent for ELISA, Roche), and combined into a suspension bead array.

Samples were processed as previously described, with minor adjustments (27,47). Serum and CSF samples were separately randomized into 96-well microtiter plates. CSF samples were diluted 0.6:1 in PBS (Fisher Scientific) with 0.5% bovine serum albumin (BSA, Sigma), 0.1% rabbit IgG (Nordic Biosite), and labeled with biotin (Fisher Scientific). The samples were then further diluted 1:8 in assay buffer (0.1% casein [Fisher Scientific], 0.5% polyvinyl alcohol [Sigma], 0.8% polyvinylpyrrolidone [Sigma] in PBS-T (0.05% Tween-20 [Fisher Scientific]), supplemented with 0.5 mg/ml rabbit IgG [Nordic Biosite]), heat treated (56∘C for 30min), and incubated with the bead array overnight at room temperature. Serum samples were diluted 1:10 in PBS (Fisher Scientific) prior to labeling with biotin (Fisher Scientific), and further diluted 1:50 in assay buffer (0.1% casein [Fisher Scientific], 0.5% polyvinyl alcohol [Sigma], 0.8% polyvinylpyrrolidone [Sigma] in PBS-T (0.05% Tween-20 [Fisher Scientific]), supplemented with 0.5 mg/ml rabbit IgG [Nordic Biosite]) after labeling, heat treated (56∘C for 30min), and incubated with the bead array for 2 hours at room temperature.

The captured proteins were cross-linked to the antibodies for 10 min at room temperature using 0.4% paraformaldehyde (Thermo Scientific). The antibody-protein immunocomplexes were detected by using a streptavidin-conjugated phycoerythrine (Fisher Scientific) and a FlexMap3D instrument (Luminex Corporation). The relative protein abundance was reported as median fluorescence intensity (MFI) for each bead identity and sample. Quality control assessments are described in **Supplementary Methods**. Briefly, bead counts were evaluated per sample and analyte (**Figure S3A-S3B**). Due to a small systematic increase in MFI_{CSF} samples (**Figure S4A**), background subtraction was conducted (**Figure S4B**). MFI

values varied across analytes (**Figure S4C**), of which one was excluded due to borderline non-detected signal (**Figure S4C**, inset). Antigen profiles were assessed per sample and analyte (**Figure S5-S6, Table S2**), resulting in the exclusion of a few sibling antibodies (**Supplementary Results**).

Statistical analysis

For inferential analysis, matched CSF-serum patient samples were compared. Validation analysis was exerted in the non-matched TBI cohort with serum-samples only. We used R (version 4.0.2) (48), through RStudio® (version 1.3.1056) and the tidyverse (49), RColorBrewer (50), cowplot (51), and gridExtra (52) packages. Continuous data were presented as median (IQR). Categorical data were presented as count (%). For multiple testing correction, we used the Bonferroni, Holm (53) or the false-discovery rate (FDR) (54) method. A p-value < 0.05 was considered significant, unless otherwise stated.

A few variables (pre-hospital hypotension, Q_A, APOE allele status) had a substantial number of missing values (**Table 1, Figure S7**). When applicable, we conducted multiple imputation using n = 200 imputations in the mice package (55). Reported p-values were calculated as the unadjusted median p-value from all imputations.

Protein Characterization

Analytes were characterized using the HPA (46,56) version 19.1 (release date 2019/12/19, Ensembl version 92.38), using the protein tissue data, RNA tissue data (Consensus data set), and Brain Atlas (57) RNA data (**Supplementary Methods, Supplementary Results**).

Parallel assessments in CSF, serum, and relationship with BBB disruption

T-distributed stochastic neighbor embedding (t-SNE) (58,59) was employed to examine if proteins pertained to compartment (CSF or blood) and disease characteristics among study subjects (**Supplementary Methods**). We assessed protein levels in CSF and serum under control conditions and following TBI using the Wilcoxon rank sum test (FDR, $p_{adjusted} < 0.05$) and the Wilcoxon signed rank test (FDR, $p_{adjusted} < 0.01$).

Cluster analysis within CSF and serum was conducted for proteins that had a CSF/serum ratio significantly correlated (Kendall correlation, Holm method, $p_{adjusted} < 0.05$) with Q_A (**Supplementary**

Methods). Clusters were visualized using the ComplexHeatmap package (60). Proteins significant upon linear regression (FDR, $p_{adjusted} \leq 0.01$) compared with the reference cluster (containing the majority of control patients) were deemed significantly altered. For CSF (n = 3 clusters), proteins needed to be concurrently significant in all clusters compared with the reference cluster. Protein levels between TBI patients with disrupted/intact BBB were compared using the Wilcoxon Rank Sum Test (FDR, $p_{adjusted} <$ 0.05). Linear regression models were used to examine if APOE4 carriership was important for Q_A, or protein levels (FDR, p £ 0.05). Age, gender and injury scores were used as covariates in addition to APOE variant.

Pathway and outcome analysis

Pathway analysis through the pathfindR package (61) and pipeline (62), was conducted for proteins altered following TBI or that pertained to a BBB integrity related cluster. For protein input, p-value thresholds were set to 0.05. For enrichment analyses, the Biocarta gene set and the Bonferroni method ($p_{adjusted} \leq 0.05$) for multiple correction were used.

Proteins of interest for outcome analysis were: i) protein intersects between CSF cluster analysis and TBI-induced altered proteins in CSF, ii) protein intersects between CSF cluster analysis and TBI-induced altered proteins in serum, and iii) significantly elevated/decreased proteins following BBB disruption. Protein intersects were visualized using the VennDiagram package (63) in R. We used GOS as dependent variable and protein levels of an individual protein (or other variable of interest such as Q_A) as independent variable in a proportional odds regression analysis, using the rms package (64). Only TBI patients were included, as healthy control subjects by definition had no GOS data. We conducted univariable analysis, and if significant (FDR, $p_{adjusted}$ £ 0.05 or ≤ 0.01 if multiple testing, the latter for dichotomized GOS/short-term mortality), multivariable analysis (FDR, $p_{adjusted} < 0.05$ if multiple testing or $p_{imputed} \leq 0.05$ if imputed). We used age, GCS motor score, pupillary reactions, hypoxia, hypotension and Stockholm computerized tomography (CT) score as covariates in accordance with the International Mission for Prognosis and Clinical Trial (IMPACT) database studies (65). We used the Stockholm instead of the Marshall CT score, as the former has been shown to be superior (66,67). When applicable, we conducted step-down modelling to see how the proteins performed jointly in the regression models.

Results

Patient demographics

In total, 190 NCCU TBI patients and 15 control patients were included. Of these, n = 4 TBI patients were excluded due to low bead counts (**Figure S3A**). Of the remaining, data analysis was conducted on the 90 TBI patients and 15 healthy controls that had matched CSF and serum samples. The n = 96 TBI patients

that merely had serum samples were used for validation analyses and are referred to as the "validation cohort". Patient demography is depicted in **Table 1**. TBI patients comprised predominantly middle-aged men among whom n = 2 (2%) were homozygotes for APOE4. Even though 32 % of patients suffered a multi-trauma, the CNS trauma was the dominant pathology as deemed by a head-Abbreviated Injury Score (AIS) of 5 ("critical") among 48% of patients. In total, 51% of patients suffered an unfavorable outcome (GOS 1-3). TBI patients and the validation cohort differed in type of surgery performed and long-term prognosis. Notably, while all patients in the TBI cohort had EVDs, the validation cohort had fewer (n = 25, 31%), but higher degree of intraparenchymal ICP monitors.

Table 1
Study Participant Demography

Variable	TBI cohort		Control cohort		Unit/Metric
	Missing	Data	Missing	Data	
Age	0 (0)	57 (41−62)	0 (0)	25 (22−29)	years
Male	0 (0)	67 (74)	0 (0)	7 (47)	count (%)
GCS admission	0 (0)	7 (3−9)	15 (100)		scale 1−15
GCS motor admission	0 (0)	4 (1−5)	15 (100)		scale 1−6
Pupils	3 (3.3)	bilaterally responsive: 67 (74)	15 (100)		count (%)
		unilaterally unresponsive: 11 (12)			
		bilaterally unresponsive: 9 (10)			
Head AIS	7 (7.8)	1 (minor): 0 (0)	15 (100)		score 1−6
		2 (moderate): 0 (0)			
		3 (serious): 10 (11)			
		4 (severe): 30 (33)			
		5 (critical): 43 (48)			
		6 (maximum): 0 (0)			
ISS	7 (7.78)	25 (19−29)	15 (100)		scale
Multitrauma	0 (0)	29 (32)	15 (100)		count (%)
Hypotension	24 (27)	2 (2.2)	15 (100)		count (%)
Hypoxia	4 (4.4)	15 (17)	15 (100)		count (%)
Stockholm CT score	0 (0)	2.5 (2-3.3)	15 (100)		scale
Q_A	19 (21.1)	0.0041 (0.0018−0.011)	0 (0)	0.0040 (0.0035−0.0060)	quotient

Variable	TBI cohort		Control cohort	Unit/Metric
APOE4 carrier	15 (17)	18 (20)	15 (100)	count (%)
APOE allele status	15 (17)	No allele: 57 (63)	15 (100)	count (%)
		Heterozygote: 16 (18)		
		Homozygote: 2 (2)		
GOS	0 (0)	GOS 1 (death): 12 (13)	15 (100)	score 1−5
		GOS 2 (vegetative): 0 (0)		
		GOS 3 (severe disability): 34 (38)		
		GOS 4 (moderate disability): 28 (31)		
		GOS 5 (good recovery): 16 (18)		
Unfavorable GOS	0 (0)	GOS 1−3: 46 (51)	15 (100)	count (%)
Patient demographics are summarized for the whole TBI cohort. Data is depicted as median (interquartile range [IQR]) if continuous and otherwise as count (%). Abbreviations: AIS, Abbreviated Injury Scale; APOE, ApoE lipoprotein; CT, computerized tomography; GCS, Glasgow Coma Scale; ISS, injury severity score; GOS, Glasgow Outcome Scale; Q_A, albumin quotient.				

Protein characterization

The majority of proteins exhibited highest tissue enrichment in the CNS (**Figure 1A**), although several proteins exhibited high RNA expression in multiple different tissues (**Figure 1B**). Within the Brain Atlas, proteins exhibited top RNA expression in the cerebral cortex proteins (**Figure 1C**), but concurrent CNS tissue expression was common (**Figure 1D**).

TBI alters CSF and serum protein levels and upregulates neuroinflammatory pathways

Among control subjects, CNS-originating proteins (e.g. GAP43, $\log_2$ fold change [FC] 3.41, p < 0.001) were enriched in CSF, while for example complement proteins (e.g. C1QB, $\log_2$ FC -2.38, p < 0.001) were enriched in serum (**Figure S8**). Following TBI, t-SNE demonstrated that the patients' protein composition grouped along compartment (serum and CSF) and disease status (TBI and control) (**Figure 2A**). t-SNE 2 seemed related to BBB integrity in CSF (**Figure 2B**). This indicates that the CSF and serum proteomes are distinct in health and following TBI, and that injury characteristics may be reflected in protein composition. In fact, following TBI, n = 124 (unique) proteins were altered in either CSF or serum compared with controls (**Figure 2C-D, Table S3**). This allowed assessment of currently used TBI

biomarkers, comprising the astrocytic proteins S100B and glial fibrillary acidic protein (GFAP), as well as the neuronal proteins neuron-specific enolase (NSE, or ENO2), neurofilament-light (NFL), and ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) (37). We could confirm previous findings of upregulation of S100B, GFAP, NSE (ENO2), and NFL post-TBI **(Table S3)**.

Following TBI, far more proteins were altered in CSF (n = 109) than in serum (n = 35). In CSF, n = 81 (74%) of all altered proteins were CNS related, whereas n = 11 (10%) were immune system related. Proteins enriched in CSF following TBI were among else myelin basic protein (MBP) (DMFI = 3655, p < 0.001), and AQP4 (DMFI = 2208, p = 0.002). Similarly to CSF, the majority of altered proteins in serum were CNS related (n = 23, 66%), whereas n = 7 (20%) proteins were immune system related. The proteins in serum that exhibited the highest DMFI were the complement proteins CFB (DMFI = 2131, p < 0.001) and C9 (DMFI = 2000, p < 0.001). Top-altered pathways in CSF included the lectin-induced complement pathway, erythropoietin-mediated neuroprotection through Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells (NF-kB), synaptic proteins at the synaptic junction, and Role of Tob in T-cell activation **(Figure 2E)**. This was partially mimicked in serum with regard to the neuroinflammatory pathways, particularly the complement system **(Figure 2F)**, which also held true for our validation cohort **(Figure S9)**. Surprisingly, merely n = 19 proteins were concurrently altered in both CSF and serum following TBI. Among these, n = 12 proteins (63%) were CNS enriched and n = 4 (21 %) immune system related. Among immune system proteins, notably all but one (CXCL1) were complement system proteins (CFI, FCN1, MASP2).

BBB disruption yields a protein signature in CSF and is predictive of outcome

Under homeostasis, the amount of ventricular albumin comprises ~40% of lumbar albumin (68) and the Q_A reference interval is defined for lumbar albumin (35). In line with our previous work we did not attempt any rostro-caudal correction for the Q_A values (11,14), as ventricular albumin is expected to be higher than the lumbar ditto following a supratentorial trauma. As expected (69), a few control subjects exhibited pathological Q_A values **(Table 1).** In contrast, BBB disruption was present among n = 23 TBI patients (32 %), and median Q_A was 0.004 (0.002-0.011) **(Figure 3A)**. Q_A was an independent significant predictor of GOS (p = 0.044, DNagelkerke's pseudo-R^2 = 8.89%). This finding is novel and highlight BBB disruption as a prognostic marker for severe TBI. This finding could not be attributed to multi-trauma as multi-trauma patients had slightly lower Q_A values (p = 0.035), and Q_A was negatively correlated with multi-trauma ($r_{Spearman}$ = -0.25). APOE4 variant was not associated with Q_A adjusted for age and sex (p = 0.494), or if injury severity was added to the model (p = 0.634).

In total, 114 unique CSF/serum protein ratios correlated significantly with Q_A, conferring a median correlation coefficient t 0.33 (0.29-0.40) **(Table S4)**. The ten proteins with highest correlation coefficient t between CSF/serum ratio and Q_A were complement proteins, except VCAM1 **(Table 2)**. The majority of proteins that correlated with Q_A were either nervous system or immune system proteins **(Figure 3B, Table S4)**. Protein size had no obvious relationship with protein levels associated with Q_A **(Table 2)**. APOE4 was not a predictor of the Q_A associated protein levels in either CSF or serum.

Table 2
Complement Proteins Exhibited Highest Correlations with QA

Protein, Antibody	Specific function	τ	adjusted p-value
C1QB HPA052116	innate immunity/complement system	0.67	< 0.001
CFB HPA001817	innate immunity/complement system	0.66	< 0.001
C9 HPA029577	innate immunity/complement system	0.65	< 0.001
C9 HPA070709	innate immunity/complement system	0.65	< 0.001
C1QA HPA002350	innate immunity/complement system	0.64	< 0.001
MASP2 HPA029314	innate immunity/complement system	0.58	< 0.001
VCAM1 HPA069867	cell cell communication	0.54	< 0.001
FCN3 HPA071173	innate immunity/complement system	0.54	< 0.001
MASP2 HPA029313	innate immunity/complement system	0.52	< 0.001
C5 HPA075945	innate immunity/complement system	0.52	< 0.001
Top 10 Q_A correlated proteins as deemed by correlation coefficient Kendall τ. Correlations were calculated between protein CSF/serum ratio and Q_A. Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; Q_A, albumin quotient. Full protein names are detailed in **Table S1**.			

Cluster analysis of Q_A correlated proteins demonstrated that protein levels paralleled Q_A in CSF, but *not* in serum (**Figure 3C-D**). The protein levels exhibited an association with dichotomized GOS (in CSF), but not APOE4 (**Figure 3C-D**). Among proteins significantly different between CSF clusters, pathway analysis exhibited that structural and inflammatory pathways were upregulated (**Figure 3E**). Merely n = 7 of all Q_A associated proteins were altered dependent on intact or disrupted BBB . In CSF, the majority of these proteins were inflammatory (CFB, C9, IL6, FCN1), whereas the sole significant protein in serum was the structural protein OLIG1 (**Figure 3F-G**).

Proteins associated with BBB disruption are outcome predictors following TBI

There was an overlap between proteins that were significantly altered (in either CSF or serum) following TBI *and* that were altered in the CSF cluster analysis among Q_A associated proteins (**Figure 4A-B**). For these we performed outcome analysis (**Table S5**). In total, n = 40 proteins comprised independent outcome predictors (**Table S5**, the representative examples CASKIN1, and matrix metalloproteinase-(MMP-)9 are highlighted in **Figure 4C-D**). Importantly, numerous of these outcome proteins were also upregulated in our validation cohort following TBI (**Figure 4E**). The proteins from **Table S5** with highest DNagelkerke's pseudo-R^2 are summarized in **Table 3**.

Table 3
BBB correlated proteins improved outcome prediction independently following severe TBI

Protein, Antibody	Compartment	Highest Tissue Enrichment	Coefficient	ΔR^2	adjusted p-value	Q_A subgroup analysis
STMN4 HPA078407	CSF	cns	-0.00505	0.121	0.04548	no
C5 HPA075945	CSF	liver/gallbladder	-0.00095	0.106	0.04548	no
GPR26 HPA062736	CSF	cns	-0.00684	0.099	0.04548	no
CFB HPA001817	Serum	liver/gallbladder	0.00098	0.092	0.04548	yes
FCN1 HPA001295	Serum	blood	0.00303	0.082	0.04548	yes
C9 HPA070709	CSF	liver/gallbladder	-0.00123	0.074	0.04548	yes
IL6 HPA064428	Serum	adipose/soft tissue	0.00185	0.071	0.04548	yes

All proteins that comprised the intersect between CSF-altered proteins and CSF cluster-derived proteins or serum-altered proteins and CSF cluster derived proteins were used for outcome analysis. Outcome prediction was conducted by univariable followed by multivariable proportional odds regression analysis where GOS was used as dependent variable and the protein level as independent variable. The IMPACT variables were used as covariates. Here we show the n = 3 proteins that conferred the highest Δ Nagelkerke's pseudo- R^2 (decimal number) in CSF (row 1−3), in serum (row 4, 5, 7), and upon specific outcome analysis for proteins significantly different between patients with intact and disrupted BBB (row 4−6). Proteins that were significantly different between disrupted and intact BBB (CFB, FCN1, C9, IL-6) were subjected to a sub-group analysis ("Q_A subgroup analysis" column), for which adjusted p-values are described in **Table S6**. Abbreviations: BBB, blood-brain barrier injury; CNS, central nervous system; Coeff., Regression Coefficient; CSF, cerebrospinal fluid; GOS, Glasgow Outcome Score; IMPACT, International Mission for Prognosis and Analysis of Clinical Trials in TBI; TBI, Traumatic Brain Injury; Q_A, Albumin Quotient. All full protein names are listed in **Table S1**.

We also analyzed our proteins against the dichotomized GOS, for which no proteins were significant. As this might have been caused by a type II error due to the loss of power associated with ordinal variable dichotomization (70), we re-did this analysis on imputed data (**Table S6**). Both levels of structural proteins (e.g. MBP, $p_{imputed}$ = 0.002), and inflammatory proteins (e.g. C9, $p_{imputed}$ = 0.034) in CSF were predictive of outcome. We also conducted outcome analyses for the proteins significantly different between patients with intact and disrupted BBB (**Table S7**). Among proteins that had significantly altered levels if the TBI patient had a BBB injury we found independent outcome predictors (**Table S7, Table 3**). For these, we conducted a step-down analysis, comprising all proteins significant within the specific compartment upon multivariable analysis followed by sequential deletion until merely significant proteins were retained in the model. C9 (p = 0.0143, DR^2 = 7.4%) was the only protein retained in CSF and CFB (p = 0.0031, DR^2 = 9.2%) the only protein in serum.

Discussion

We conducted a prospective, proteomic study of 177 proteins analyzed in matched CSF and serum samples of 90 severe TBI patients and 15 control subjects. Being one of the largest proteomic studies yet conduced following severe TBI, it allows us to define protein pathway alterations in CSF and serum in parallel. Specifically, we analyzed neuroinflammatory protein alterations in relation to BBB disruption, two key secondary injuries following TBI. We show that BBB disruption is an important outcome predictor following TBI, and that a protein signature comprised of predominantly neuroinflammatory pathways in CSF coincide with BBB disruption, while also serving as novel proteins of clinical importance for prognosis.

A novel approach in TBI studies: targeting secondary injury mechanisms in large patient cohorts

We analyzed proteins of relevance for BBB disruption, a key TBI secondary injury for which there is currently no treatment (4,71). We utilized an antibody array (28), enabling multiplexing across a large range of protein concentrations, with low measurement variability (29). We included a larger patient cohort than previous proteomic studies in TBI (18–22,24–26,72–74), thus enabling outcome analyses and APOE genotyping. Two pediatric TBI studies on smaller patient cohorts (73,74) and one study on adult TBI patients (75) have employed similar approaches, albeit with methodological restrictions that precluded analysis of the relationship between BBB disruption and neuroinflammation, which we managed by concurrent serum and CSF sampling. We thus provide a novel framework for secondary injury studies following TBI.

TBI studies benefit from CSF, but warrant a new BBB disruption metric

We found important differences in protein composition between the two compartments CSF and serum, within which patients grouped depending on diagnosis and BBB integrity. This was more evident in CSF than in serum, indicating that CSF might confer pivotal pathophysiological information in TBI studies. Our approach enabled quantification of BBB disruption and we found that 32% of our TBI patients suffered a BBB injury, using Q_A. This is unexpectedly low in a severe TBI cohort. We hypothesize that albumin was possibly washed-out from CSF, as samples were in median obtained around two days following the trauma, a time-frame during which the acute vasogenic edema has been shown to be mixed with a concurrent cytotoxic edema and a delayed vasogenic edema has not yet occurred (3). This highlights that Q_A might be suboptimal to use as a BBB integrity metric following TBI. Yet, we could show that Q_A in itself was a strong outcome predictor. Taken together, CSF is key for proteomic studies following TBI and important injury features might be accidentally surpassed if exclusively considering blood. Further, even though Q_A is the current golden-standard method for BBB integrity, the TBI field would benefit from a new BBB integrity biomarker. Radiological tools, notably dynamic enhanced contrast magnetic resonance imaging, have been utilized in other neurological disorders to assess BBB disruption (9), but has as of yet been but sparsely utilized in the TBI setting (76,77). Meanwhile, we show that BBB disruption measured utilizing Q_A is a novel important outcome predictor following severe TBI.

Structural proteins altered following TBI and BBB disruption reflect pathophysiologically relevant biomarkers

We confirmed protein alterations of current TBI biomarkers as well as protein and pathway alterations of proteins less studied following TBI. The proteins MBP and AQP4 were both increased following TBI.

Unlike previous biomarkers, MBP has an oligodendrocytic origin and is a tentative TBI biomarker in the post-acute phase (78). AQP4 is an astrocytic protein, distributed along the astrocyte podocytes lining the BBB (79), thus presumably reflecting structural BBB pathophysiology in our material. Previous experimental work has shown that AQP4 is globally increased following TBI, but with a decreased perivascular expression pattern (80) in line with our findings. AQP4 has also been implicated in edema development and resolution (81). We also found upregulation of two structural protein pathways. First, we found the "synaptic proteins at the synaptic junction" pathway, entailing the spectrin proteins SPTAN1 and SPTBN1. The breakdown product of these proteins have been implicated in calpain- and caspase-mediated proteolysis and shown to be related to prognosis (82). We also found the pathway "hypoxia-inducible factor in the cardiovascular system", and in concordance (83) the proteins HIF1A, VEGFA, and LDHA to be upregulated, speculatively related to metabolic dysfunction. In summary, while corroborating earlier data on some of the previously known TBI biomarkers, we also provide data on novel structural proteins, which possibly reflects ongoing pathophysiology within the CNS and hence a valuable addition to the TBI biomarker literature.

TBI and BBB disruption yields an innate immune response with marked increase of complement proteins

In both CSF and serum, TBI upregulated innate immune system pathways, which were also upregulated in CSF following BBB disruption. BBB disruption is intertwined with neuroinflammation (7), commencing when blood-borne factors leak across the disrupted BBB and tissue injury-mediated release of alarmins trigger CNS innate immune mechanisms (71,84,85). This yields microglial- and inflammasome-mediated production of the cytokines IL1-β, IL-6, TNF-α, and IL-18 (84). Both IL1-β and TNF-α can further increase BBB permeability (71). Moreover, microglia-mediated production of IL-1α, TNFα, and C1q was recently shown to activate astrocytes (86), known to respond by IL-6 and MMP-9 production. Both IL-1α, IL-1β, IL-6, and MMP-9 were increased following TBI in our material. MMP-9 stimulates BBB disruption through degradation of tight junction and extracellular matrix proteins, while also triggering further neuroinflammation (87). IL-6 has been suggested to be intertwined with TGF-β (12), one of the upregulated pathways that we observed. Previously, TGF-β has been shown to be increased following TBI, correlate with, and even cause BBB disruption (12,88). Finally, across all our comparisons complement pathways, a key element within the neuroinflammatory response (89), were implicated. Panels of elevated complement proteins have been found in blood (90), CSF (13,18,20,91), and brain parenchyma (24,42,92) following TBI. As we assessed all complement pathways, we can corroborate many of these findings. Complement activation following TBI has been shown to occur both through systemic complement leakage across the disrupted BBB and through local CNS complement activation (93). In line with this, and earlier data (13,14), complement CSF/serum ratios were highly correlated with Q_A. Among TBI patients with intact or disrupted BBB, a handful of primarily complement proteins were altered in CSF, congruent with descriptions that complement activation might aggravate BBB disruption (93). We did not find any relationship between APOE genotype and our proteins. Although important, this finding should be cautiously interpreted as few TBI patients were homozygotes for APOE4.

In summary, BBB disruption and neuroinflammation following TBI mutually stimulate and aggravate one another, which in our material can be quantitatively assessed in a more comprehensive fashion than before.

Altered proteins comprise novel predictors of long-term functional outcome

One application of our findings is to use structural proteins as markers of damaged parenchyma/BBB, and neuroinflammatory proteins as novel treatment targets. In total, we found 40 predominantly CNS enriched or neuroinflammatory proteins that comprised novel, independent outcome predictors following severe TBI. Individually, these proteins explained ~10% additional model variance, demonstrating that a large amount of unexplained variation in TBI outcome emanates from secondary injuries. The protein with highest additional variance was STMN4 in CSF, belonging to a protein family with microtubule-destabilizing capacity (94) but also of importance for neuronal regeneration (95). We hypothesize that STMN4 in this context serves as a metric for CNS cell death. Other proteins with high amount of additionally explained variance were neuroinflammatory proteins, notably from the complement system. Among proteins significantly different between patients with and without disrupted BBB, CFB and C9 were unique outcome predictors. Experimental TBI studies have linked variations in complement activation to worsened functional outcome (96). Knock-out and complement inhibition models have improved outcome (92,97–99), whereas inhibition of complement inhibition has worsened it (100). Recently, membrane-attack complex inhibition was shown to attenuate acute TBI deficits, whereas complement protein C3 inhibition was needed to improve long-term outcome. Overall, the alternative pathway was implicated as key following TBI (101). We cannot draw as extensive conclusions, but we note that several different complement pathway proteins comprised outcome predictors, indicating that a common therapeutic target is of interest for future studies. We thus link for the first-time proteomic data with BBB disruption, neuroinflammation, and clinical outcome within one TBI study.

Limitations

Several limitations must be acknowledged. The supervised protein selection, although hypothesis-driven, is biased by definition. Still, as the TBI literature on unbiased approaches is vast there is a need for secondary injury mechanism focused studies on larger patient cohorts, such as ours. Further, our study is limited to cross-sectional data, which is problematic as our sampling was not entirely synchronized between or even within patients. This might cause us to miss important longitudinal protein alterations, known to be time-sensitive from preclinical research (84). In contrast, our current findings become even more robust, as they manifest in spite of less stringent sampling. Other limitations concern discrepancies between the TBI and control subjects. Controls were younger than the TBI patients, thus possibly exaggerating the observed protein differences. Yet, they were healthy, which we considered superior compared with utilizing other patient groups with EVD/shunt treatment. Further, CSF was obtained

through an EVD among TBI patients and through lumbar puncture among control subjects. An EVD decreases the external validity of the study, as patients for ethical reasons cannot be randomized to EVD treatment and an EVD would not be ethical to insert in healthy controls. This warrants for caution in CSF proteome comparisons, as CSF protein content varies along the rostro-caudal axis (68,78). Moreover, CSF protein levels could fail to portray intracellular alterations (19). For this, one would need brain tissue biopsies, difficult to obtain in larger-scale quantities. Moreover, a small biopsy cannot confer global information on protein alterations within the CNS (19). The similar limitation holds true for microdialysis (102). Hence, CSF constitutes the state-of-the-art matrix within TBI studies of global CNS markers (18). For us, CSF was therefore the superior biofluid to use, but future, external validation on a smaller protein-panel ought to be conducted using microdialysis as has been done in other studies (23,103).

Conclusion

We have examined the interplay between BBB disruption and neuroinflammation that commonly ensue a severe TBI. We have found that neuroinflammatory processes are intimately linked with BBB disruption and that both BBB disruption and numerous neuroinflammatory proteins serve as novel outcome predictors, adding ~10% additional variance to TBI outcome prediction models, suggesting that future efforts should strive to develop therapeutic targets towards these secondary injuries.

Abbreviations

AIS	Abbreviated Injury Scale
APOE	Apolipoprotein E
APOE4	Apolipoprotein E epsilon 4-allele
BBB	Blood-Brain Barrier
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computerized Tomography
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EVD	External Ventricular Drain
FDR	False-Discovery Rate
FP-TDI	Fluorescent Polarization Template Dye Incorporation
GFAP	Glial Fibrillary Acidic Protein
GCS	Glasgow Coma Scale
GOS	Glasgow Outcome Score
HPA	Human Protein Atlas
ICP	Intracranial Pressure
IMPACT	International Mission for Prognosis and Clinical Trial
ISS	Injury Severity Score
IQR	Interquartile Range
MBP	Myelin basic protein
MES	2-(N-morpholino)ethanesulfonic acid
MFI	Median Fluorescence Intensity
MMP	Matrix metalloproteinase
NCCU	Neuro-Critical Care Unit
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NFL	Neurofilament-Light

NSE	Neuron-Specific Enolase
NX	Normalized Expression
PBS	Phosphate-Buffered Saline
PBS-T	Phosphate-Buffered Saline with Tween 20
Q_A	Albumin Quotient
SNP	Single-Nucleotide Polymorphism
sulfo-NHS	N-hydroxysulfosuccinimide
TBI	Traumatic Brain Injury
t-SNE	t-Distributed Stochastic Neighbor Embedding
UCHL1	Ubiquitin Carboxy-Terminal Hydrolase-L1

Across the manuscript all proteins are referenced utilizing their abbreviated gene name; a comprehensive overview of their full protein name is depicted in Table S1.

Declarations

Ethics approval and consent to participate

All research activities were undertaken in accordance with Swedish law and the Declaration of Helsinki. For inclusion of TBI patients, oral informed consent was granted by next-of-kin. All control subjects provided written, informed consent. Ethical approval (#2005/1526-31/2 and #2014/1201-31/1) was granted through the Swedish Ethical Review Authority.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request in a format that adheres to current Swedish and European Union legislation regarding study participant anonymity.

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization and study design: FP, BMB, PN, MS, EPT, CL.

Study supervision: FP, BMB, PN, MS, EPT.

Data acquisition: FAN, EP, DJ, BMB, FP, EPT.

Data quality control: CL, EP, DJ.

Data analysis: CL.

Data interpretation: All authors.

Manuscript draft: CL, EP, FAN, EPT.

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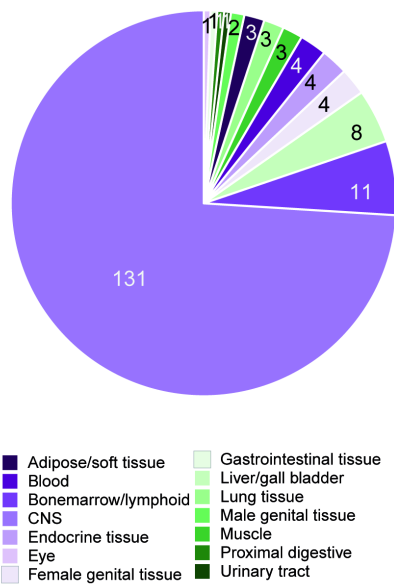
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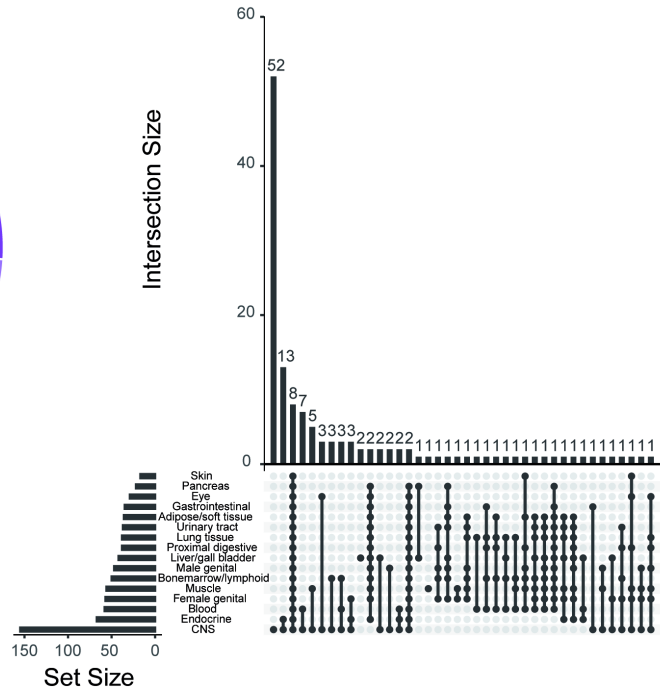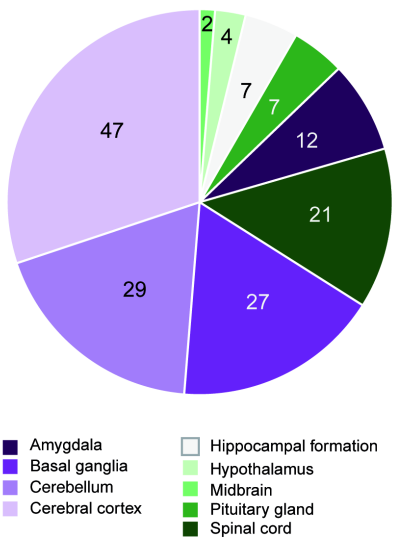# Figures

Figure 1

A Highest tissue enrichment

B Tissue enrichment distribution

C Highest CNS enrichment

D CNS enrichment distribution

Figure 1

A severe TBI induces protein alterations in CSF and serum. Individual patient proteomic profiles were different in CSF compared with serum, utilizing tSNE. Following a severe TBI, additional proteomic alterations occur within both of these compartments (A). Individual patient attributes, such as BBB disruption, seemed associated with some of TBI patient heterogeneity, predominantly in CSF (B). At the individual protein level, this was mimicked by altered protein levels in both CSF and serum (C-D).

Graphical significance threshold was set to log2 FC |0.5| and adjusted p-value < 0.05, and values not fulfilling these criteria were diminished in size and shaded in light-gray. In CSF, both CNS structural and neuroinflammatory protein levels were increased following a severe TBI (C). This was reflected in pathway upregulations of structural, metabolic, and inflammatory pathways (E). In contrast, fewer protein were altered in serum (D), and upregulated pathways were predominantly neuroinflammatory (F). Abbreviations: CSF, cerebrospinal fluid; TBI, traumatic brain injury; tSNE, t-distributed stochastic neighbor embedding. All full protein names are given in Table S1.

Figure 2

Figure 2

Assessed proteins were predominantly CNS structural proteins. The vast majority of proteins exhibited highest tissue enrichment in the CNS, with the second most frequent category being immune-system organs (A). Notably, numerous proteins were concomitantly expressed in multiple tissues (B). Within the Brain Atlas, the majority were cerebral cortex enriched (C), but few proteins were exclusively expressed within one CNS-niche (D). Protein characterization data was obtained from the Human Protein Atlas. Abbreviations: CNS, central nervous system.
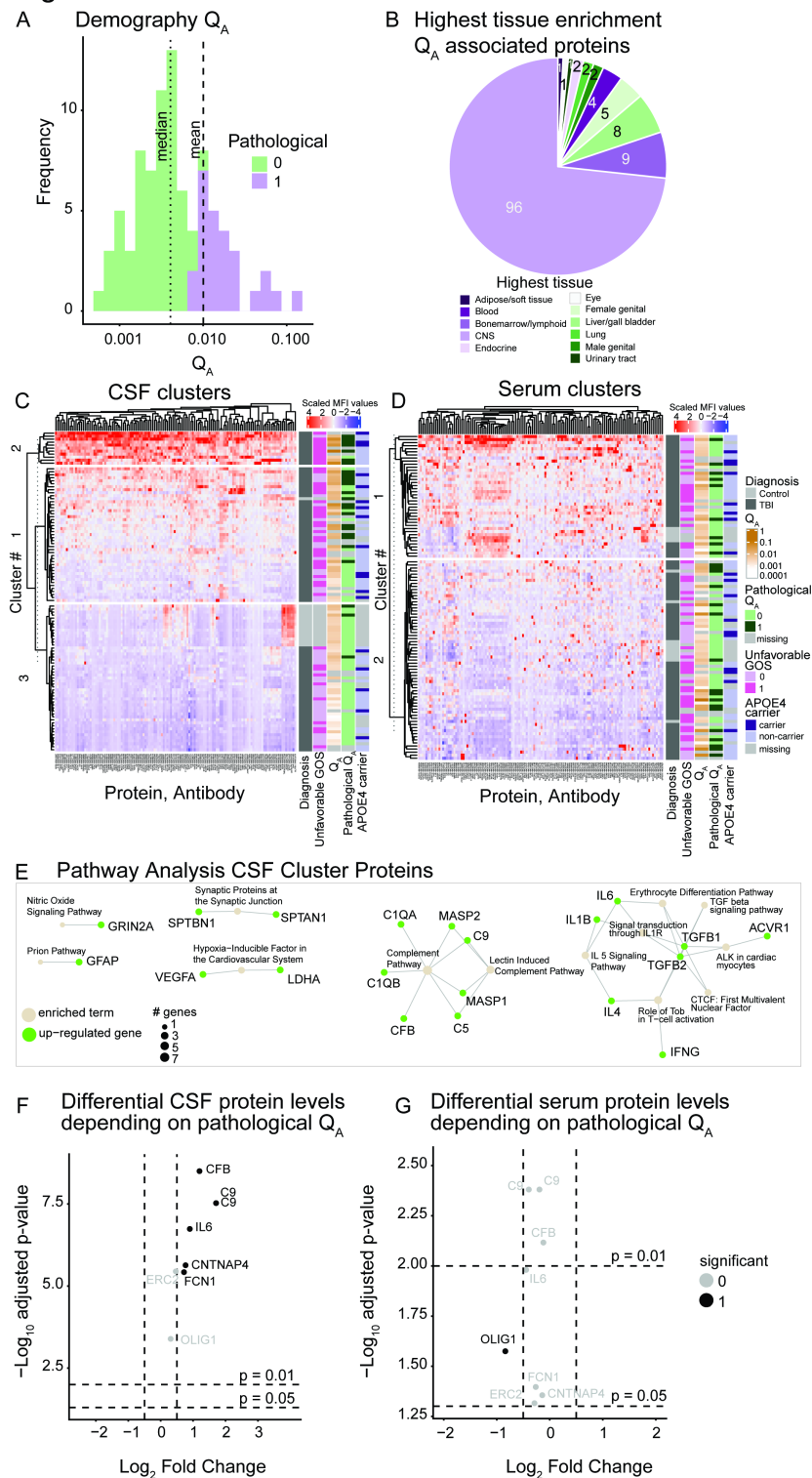
Figure 3

Figure 3

BBB disruption co-occurs with upregulation of innate immune pathways, notably the complement cascade. A severe TBI elicited an acute BBB disruption among a subset of patients, quantified using QA (A). Among the n = 114 proteins significantly correlated with QA, the majority were nervous system or immune system enriched (B). Using hierarchical clustering on CSF and serum protein measurements respectively, protein levels clearly clustered depending on BBB integrity status in CSF (C), but less so in serum (D). APOE carrier status was not associated with protein levels in either group (C, D). In CSF, this corresponded to pathway upregulation of predominantly innate immune mechanisms (E). Examining proteomic profiles between patients with disrupted and intact BBB, a handful of proteins were significant in CSF (F) and merely one in serum (G). Abbreviations: APOE, Apolipoprotein E; CSF, cerebrospinal fluid; CNS, central nervous system; GOS, Glasgow Outcome Score; MFI, median fluorescence intensity; QA, albumin quotient; TBI, traumatic brain injury. All full protein names are given in Table S1.

Figure 4

A Protein overlap CSF proteins

CSF cluster

83 | 90 | 28

TBI-induced altered CSF protein

B Protein overlap serum proteins

CSF cluster

141 | 32 | 8

TBI-induced altered serum protein

C CASKIN1 HPA076882

Log$_{10}$ CASKIN1 [MFI]

CSF | Serum
Compartment

D MMP9 HPA063909

Log$_{10}$ MMP9 [MFI]

CSF | Serum
Compartment

Group   ⊟ Control   ⊟ TBI

E Serum protein alterations following TBI (Validation Cohort)

−log$_{10}$ adjusted p values

log$_2$ Fold change

DSCAM
MMP9
MEPE
C9
TNNI2
CASKIN1
MMP9
GPR37L1
PRRT2
p = 0.01
ELAVL3
MBP
p = 0.05
GPR26

Matched Cohort
Outcome Protein
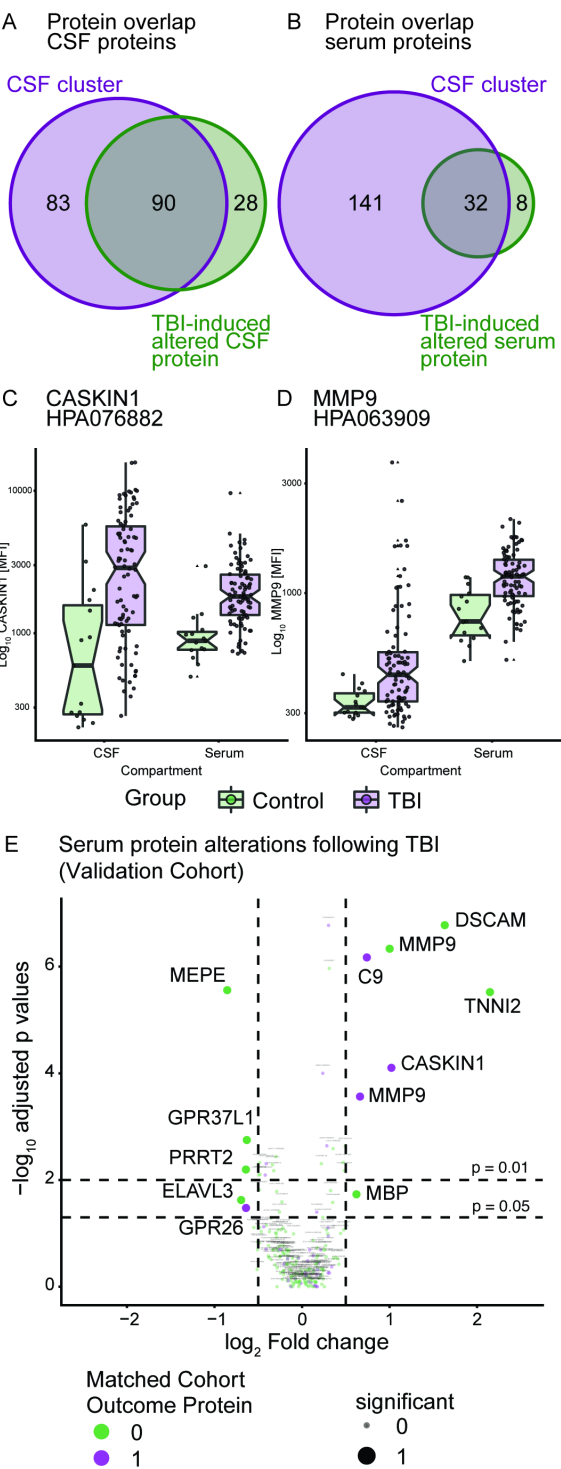● 0
● 1

significant
· 0
● 1

Figure 4

Proteins associated with BBB disruption and TBI-induced protein level alterations were outcome predictors following TBI. Using the hierarchical clustering depicted in Figure 3D, QA associated proteins significantly different between clusters were derived. Of these, n = 90 proteins were found to overlap with proteins altered in CSF following TBI as portrayed in Figure 2C (A). Similar assessments between CSF clusters and TBI-induced protein alterations in serum yielded an overlap of n = 32 proteins (B). Among

these, n = 40 proteins comprised novel outcome predictors following severe TBI, of which an excerpt of proteins with different features are shown (C-D). These analyses were multivariable, meaning that outcome predictors are independently significant even when adjusting for previously known prognostic covariates following a severe TBI. Validation of results were conducted in an independent TBI cohort without CSF samples. Following TBI, many of the matched cohort outcome proteins were upregulated in this validation cohort as well (E). Abbreviations: CSF, cerebrospinal fluid; MFI, median fluorescence intensity; TBI, traumatic brain injury. All full protein names are given in Table S1.

Supplementary Files

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