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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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 Scale, 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.

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 ($p = 0.014$, $\Delta R^2 = 7.4\%$) and complement factor B in serum ($p = 0.003$, $\Delta R^2 = 9.2\%$) 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). Paradoxically, current TBI outcome prediction variables comprise solely admission data and these variables merely explain ~ 40% of model variance (3, 4). This indicates that secondary injury processes are of key relevance for long-term outcome and that an increased understanding of these might improve patient management.

Among secondary injury pathologies, blood-brain barrier (BBB) disruption is of particular interest. The TBI inflicts an immediate, acute injury to the BBB (5), contributing to inflammatory activation of (CNS) inherent cells, such as astrocytes and microglia, but also facilitates the infiltration of various immune cells from the systemic circulation (6, 7). Together, this generates an inflammatory cascade that will propagate several secondary injury mechanisms (8). This inflammatory cascade can also exacerbate BBB injury, thereby further increasing the intensity of CNS neuroinflammation (9). It is still unclear whether these processes are also influenced by the genetic set up in the acute phase of TBI. However, in the absence of trauma, recent data show that the E4 variant of apolipoprotein E (APOE4) is associated with reduced BBB function and predicts risks of cognitive decline (10).

Clinically, the gold-standard metric for BBB disruption is the cerebrospinal fluid (CSF) to blood albumin quotient (Q_A) (11). 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 (12), and neuroinflammatory (13–15) proteins, of importance as albumin per se does not confer information on the 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 protein pathways that might confer joint or discrepant functions within the CNS. In order to deduce the pathophysiology causing BBB disruption, more comprehensive proteomic profiling efforts are therefore warranted (16, 17).

Among available techniques, mass-spectrometry holds the largest capacity for simultaneous assessment of multiple proteins (18) and has been utilized in numerous TBI studies (19–27). However, mass-spectrometry has inherent limitations, such as its limited capacity to detect low-abundance proteins (e.g. cytokines) (18), thus obstructing concomitant detection of low- and high-abundant proteins within the same study. An alternative technique is affinity proteomics, combining microarray technology with affinity reagents (28). Affinity proteomics is suitable for multiplexed protein screens in large numbers of samples (29, 30) from both serum (29, 30) and CSF (28). However, 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, originally 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. For study inclusion, oral informed consent was granted by next-of-kin.

The second study was conducted between 2014 and 2015 on healthy volunteers, used as control subjects in the current study. All control subjects provided written, informed consent. All research activities undertaken were in accordance with Swedish law and the Declaration of Helsinki. Ethical approval (#2005/1526-31/2 and #2014/1201-31/1) was granted through the Swedish Ethical Review Authority.

Patient inclusion

Inclusion criteria for TBI patients were: (i) severe TBI (Glasgow Coma Scale [GCS] 3–8), in need of NCCU treatment and invasive intracranial monitoring, and (ii) age between 18 and 75 years. Exclusion criteria comprised: (i) desolate prognosis precluding the patient from NCCU treatment, (ii) penetrating TBI, (iii) unconsciousness due to etiology other than TBI, (iv) underlying chronical condition precluding adequate follow-up, or (v) patient that for other reasons would not be possible to follow-up. Inclusion criteria for control subjects were: (i) previously healthy, (ii) age 18–50 years, (iii) sufficient linguistic knowledge to be able to participate in self-evaluation forms. Exclusion criteria were: (i) ongoing, or history of, psychiatric illness, (ii) family history of serious psychiatric comorbidity, (iii) ongoing somatic illness precluding physical activity, (iv) current pharmacological treatment interacting with the planned study intervention, (v) substance abuse (smoking or narcotic substances), or (vi) pregnancy. TBI patient sample size calculation was based on the expected protein level alterations between TBI patients and healthy controls and was exerted as a two-sample t-test (two patient samples with unequal number of participants). Due to the sparsity of similar studies, we utilized Cohen's d (31, 32) as effect size metric and set it to Cohen's d = 0.8 (large effect) (31, 32) in a power calculation utilizing the R package pwr (33). 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 calculation was not based on any previous studies, we set out to include patients continuously throughout the entire study period.

Clinical management, data, and sample acquisition

NCCU management of severe TBI at Karolinska University Hospital has been described elsewhere (34). In brief, Karolinska University Hospital employs an intracranial pressure (ICP-) driven approach, in accordance with the Brain Trauma Foundation Guidelines (35). At the NCCU, patients are managed using multi-modal monitoring from which data is automatically collected. Through the Karolinska University Hospital TBI Database, additional clinical data is collected prospectively. For the current study, clinical data collection comprised neurological variables, injury severity score variables, radiological variables, and outcome data, described in detail elsewhere (12). 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. Uniquely for this study, we collected CSF and serum, that was used for APOE genotyping, proteomic, and albumin analysis. The latter was assessed as Q_A, i.e. the CSF/serum albumin quotient (11), with the reference intervals (36): 15–29 years < 0.006; 30–49 years < 0.007; and ≥ 50 years < 0.009. Sampling time points were similar, but 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 (37), of which only baseline samples were used here. Participants were instructed to abstain from any physical exercise seven days before sampling, which was performed by lumbar puncture and venipuncture, respectively, in the morning between 7.30 and 9 AM while fasting since midnight and after a full night of bed rest. For TBI patients, blood was sampled through an arterial line and CSF through an external ventricular drain (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**). Following acquisition, samples were stored locally in 4°C at the clinic 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 to allow coagulation followed by centrifugation for 15 min at 2000 g, aliquoting, and storage at -80°C until further analysis (38). No protein content alteration was seen per sample (**Figure S2A**) or per 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) (39). 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 targeting different regions of the same protein were used, onwards referred to as *sibling antibodies* (40). The protein panel was chosen based on CNS-enrichment (41) or else if relevant with regard to clinical/experimental TBI, previous TBI mass-spectrometry studies, or neuroinflammation (21, 25, 27, 42–46). Antibodies were selected from the Human Protein Atlas (HPA) (www.proteinatlas.org) (47).

Antibodies were immobilized onto color-coded magnetic beads (MagPlex, Luminex Corporation) as previously described (29). Briefly, the beads surface was activated by using 0.1 M sodium hydrogen phosphate, 0.5 mg of N-hydroxysulfosuccinimide (sulfo-NHS) (Nordic Biolabs) and 0.5 mg 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) for 2 h 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) 0.05% Tween-20 (PBS-T) to eliminate the antibody excess, stored overnight in blocking buffer (Roche blocking reagent for ELISA), and combined into a suspension bead array.

Samples were processed as previously described, with minor adjustments (28, 48). Serum and CSF samples were separately randomized into 96-well microtiter plates. CSF samples were diluted 0.6:1 in PBS with 0.5% bovine serum albumin (BSA), 0.1% rabbit IgG, and labeled with biotin. The samples were then further diluted 1:8 in assay buffer (0.1% casein, 0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone in PBS-T (0.05% Tween-20), supplemented with 0.5 mg/ml rabbit IgG), heat treated (56◦C for 30 min), and incubated with the bead array overnight at room temperature. Serum samples were diluted 1:10 in PBS prior to labeling with biotin, and further diluted 1:50 in assay buffer (0.1% casein, 0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone in PBS-T (0.05% Tween-20), supplemented with 0.5 mg/ml rabbit IgG) after labeling, heat treated (56◦C for 30 min), 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. The antibody-protein immunocomplexes were detected by using a streptavidin-conjugated phycoerythrine 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 of clinical and proteomic data are described in **Supplementary Methods**. In brief, bead counts were evaluated per sample (**Figure S3A**) and analyte (**Figure S3B**), resulting in the exclusion of n = 4 patients. Next, MFI was assessed (**Figure S4**). Due to a small systematic increase in MFI_{CSF} samples (**Figure S4A**, inset), background subtraction was conducted with improved results (**Figure S4B**, inset). MFI values varied across analytes (**Figure S4C**), of which one was excluded due to borderline non-detected signal (**Figure S4C, inset**). Antigen profiles were then assessed and validated 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, only CSF-serum matched patient samples were compared. For all analyses we used R (version 4.0.2) (49), through the interface RStudio® (version 1.3.1056). Generally, we used the tidyverse (50), RColorBrewer (51), cowplot (52), and gridExtra (53) packages. Additional packages are referenced below, where applicable. Continuous data were presented as median (IQR). Categorical data were presented as count (%). For multiple testing correction, we used the Bonferroni, Holm (54) or the false-discovery rate (FDR) (55) method, depending on the analytical scope. The FDR procedure is adapted

in R and employs a cumulative minimum function where the output can be used as an adjusted p-value. A p-value < 0.05 was considered significant, unless otherwise stipulated.

Missing Data

Missing data was depicted graphically (**Figure S7**, Table 1). As no variables of importance for downstream analysis had missing values exceeding 30% of the TBI cohort sample size, no imputation of missing values was attempted.

Protein Characterization

Analytes were characterized using the HPA (47, 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. Functional annotations were gathered manually. Methodological considerations are described in **Supplementary Methods** and missing data is described in **Supplementary Results**.

Parallel assessments in CSF and serum

We employed t-distributed stochastic neighbor embedding (t-SNE) (58, 59) to examine if our proteins pertained to compartment (CSF or blood) and disease characteristics among study subjects. Methodological considerations are described in **Supplementary Methods**. Next, 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, $p_{adjusted} < 0.01$). Protein levels that were significantly different in one compartment following TBI were used for pathway analysis.

Proteomic/genotypic alterations and relationship with BBB disruption

We calculated the Kendall correlation between the CSF/serum ratio of all our non-transformed protein levels and Q_A. Proteins significantly correlated with Q_A (Holm method, $p_{adjusted} < 0.05$) were used for cluster analysis within the CSF and serum compartment respectively. Methodological considerations are described in **Supplementary Methods**. Clusters were visualized using the R package ComplexHeatmap (60). We compared protein levels between clusters derived in CSF and serum using linear regression. For CSF (n = 3 clusters), we used the cluster containing the majority of all control patients (cluster #3) as reference category. Proteins that were significant (FDR, $p_{adjusted} \leq 0.01$) in both clusters were deemed as significantly altered. Similar operations were carried out in serum, albeit with n = 2 clusters for comparison. In CSF, the clustering pattern related to BBB integrity status among patients and was therefore subjected to pathway analysis. Finally, we compared protein levels between TBI patients with pathological/intact Q_A, using the Wilcoxon Rank Sum Test (FDR, $p_{adjusted} < 0.05$).

We undertook uni-/multivariable linear regression models in order to examine whether APOE4 carriership was important for Q_A, or protein levels (FDR, $p \leq 0.05$). For analyses using Q_A or Q_A associated protein

levels as dependent variables, we used age, gender and injury scores (Stockholm computerized tomography [CT] scores, head Abbreviated Injury Scale [AIS], and Injury Severity Score [ISS]) as covariates in addition to APOE variant when applicable.

Pathway analyses

Pathway analysis was conducted using the pathfindR package (61) in R. We employed a similar pipeline as has been recommended by the package developer (62). The gene set used for enrichment analysis was Biocarta. Thresholds for (protein input) p-values were set to 0.05. For enrichment analyses, multiple correction was conducted using the Bonferroni method ($p_{adjusted} \leq 0.05$).

Outcome analyses

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) in R. We conducted univariable analysis, and if significant (FDR, $p_{adjusted} \leq 0.05$ if multiple testing), multivariable analysis (FDR, $p_{adjusted} < 0.05$ if multiple testing). For multivariable analysis, we used age, GCS motor score, pupillary reactions, hypoxia, hypotension and Stockholm CT score as covariates as these have previously been recommended by the International Mission for Prognosis and Clinical Trial (IMPACT) database studies (4). Notably, we used the Stockholm instead of the Marshall CT score, as the Stockholm CT score has been shown to be superior (3, 65). When applicable, we combined the list of significant proteins (generated from multivariable analysis) and conducted a step-down model, in order to see how the proteins performed jointly.

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 186 TBI patients, n = 96 were excluded as they did not have CSF samples, yielding 90 TBI patients and 15 controls with matched CSF and serum samples, eligible for further analysis.

TBI patient demography is depicted in Table 1. Patients comprised predominantly middle-aged men among which ~ 20% of patients were either hetero- or homozygotes for APOE4. Even though 32% of patients suffered a multi-trauma, the CNS trauma was the dominant pathology as deemed by the Stockholm CT score and a head-AIS of 5 (“critical”) among 48% of patients. Accordingly, 51% of patients suffered an unfavorable outcome (GOS 1–3).

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 vast majority of the selected proteins exhibited highest tissue enrichment in the CNS (Fig. 1A), although several proteins exhibited high RNA expression in multiple different tissues (Fig. 1B). Within the Brain Atlas, proteins exhibited top RNA expression in the cerebral cortex proteins (Fig. 1C), but concurrent CNS tissue expression was common (Fig. 1D). Accordingly, the majority of proteins were involved in nervous system functions (Fig. 1E).

TBI alters CSF and serum protein levels and upregulates neuroinflammatory pathways

Using t-SNE, data grouped along compartment (serum and CSF) and disease status (TBI and control) (Fig. 2A). In general, t-SNE 1 corresponded to compartment (CSF/serum), and t-SNE 2 to individual patient characteristics. Interestingly, BBB integrity seemed to be related to t-SNE 2, particularly in CSF (Fig. 2B). This indicates that the CSF and serum proteomes are distinctly different following TBI, and that certain injury characteristics may be reflected in protein alterations.

Next, we examined which specific protein levels that changed following TBI. Among control subjects, CNS-originating proteins (e.g. GAP43, $\log_2$ fold change [FC] 3.41, $p < 0.001$) were enriched in CSF compared with serum, while for example complement proteins (e.g. C1QB, $\log_2$ FC -2.38, $p < 0.001$) were enriched in serum (**Figure S8)**. Following TBI, n = 124 (unique) proteins were altered in either CSF or serum compared with controls (Fig. 2C-D, **Table S3**). This allowed us to assess 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, also referred to as ENO2), neurofilament-light (NFL), and ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) (38). We could confirm previous findings of upregulation of S100B, GFAP, NSE (ENO2), and NFL post-TBI **(Table S3)**.

Next, we characterized all proteins altered following a severe TBI. As expected, far more proteins were altered in CSF (n = 109) than in serum (n = 35) following TBI. In CSF, n = 81 (74%) of all altered proteins were CNS enriched, whereas n = 11 (10%) were immune system function related. Proteins notably enriched in CSF following TBI were among else MBP (ΔMFI = 3655, $p < 0.001$), and AQP4 (ΔMFI = 2208, $p = 0.002$). In contrast, there were n = 7 (20%) altered proteins with immune system function in serum following TBI. Similarly to CSF, the majority of altered proteins were predominant CNS enriched (n = 23, 66%). The proteins in serum that exhibited the highest ΔMFI were the complement proteins CFB (ΔMFI = 2131, $p < 0.001$) and C9 (ΔMFI = 2000, $p < 0.001$). Pathway analysis of these revealed that 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-κB), synaptic proteins at the synaptic junction, and Role of Tob in T-cell activation (Fig. 2E). This was partially mimicked in serum with regard to the neuroinflammatory pathways, entailing both signal transduction through IL1R, cytokines and inflammatory response, as well as the complement system (Fig. 2F).

Surprisingly, merely n = 19 proteins were altered concurrently in both CSF and serum following TBI. Among these, n = 12 proteins (63%) were CNS enriched and n = 4 (21%) had an immune system related function. Among immune system proteins, notably all but one (CXCL1) were complement system proteins (CFI, FCN1, MASP2).

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

Median Q_A was 0.004 (0.002–0.011) (Fig. 3A) and BBB disruption was present among 23 TBI patients (32%). The Q_A reference interval is defined for lumbar albumin (36), and due to the CNS rostro-caudal gradient, the amount of ventricular albumin comprises ~ 40% that of lumbar albumin under homeostasis (66). As one would expect the rostro-caudal gradient to be inverted following a supratentorial trauma with ventricular albumin consequently higher than the lumbar ditto, we did not attempt any rostro-caudal correction for the Q_A reference interval, in line with our previous work (12, 15). A few control subjects exhibited pathological Q_A values (Table 1), in accordance with previous work where ~ 15% of healthy subjects exhibit a pathological Q_A in the absence of neurological disorder (67).

Q_A was an independent significant predictor of GOS (p = 0.044, ΔNagelkerke'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 APOE4 carriership, as 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 proteins had a CSF/serum ratio significantly correlated with Q_A, conferring a median correlation coefficient τ 0.33 (0.29–0.40) (**Table S4**). The ten proteins with highest correlation coefficient τ between CSF/serum ratio and Q_A were complement proteins, except VCAM1 (Table 2). In fact, the majority of proteins that correlated with Q_A were either nervous system or immune system proteins, where the latter entailed (aside from the complement system proteins) for example the cytokines IL-1α, IL-1β, and IL-6 (Fig. 3B-C, **Table S4**). 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 revealed that in CSF, but *not* in serum, protein levels paralleled Q_A (Fig. 3D-E). Of note, the protein levels did not exhibit any association with APOE4 (Fig. 3D-E). Among proteins significantly different between the CSF clusters, pathway analysis exhibited that structural (synaptic proteins at the synaptic junction) and inflammatory pathways (complement pathway, lectin-induced complement pathway, IL5-signaling, Role of Tob in T-cell activation, signal transduction through IL1R, TGF-β signaling pathway) were upregulated (Fig. 3F). Finally, we examined whether any proteins

were significantly different between TBI patients dependent on intact or disrupted BBB. In total, merely n = 7 of all our Q_A associated proteins were significantly altered dependent on Q_A status in both CSF and serum (Fig. 3G-H). In CSF, the majority were inflammatory (CFB, C9, IL6, FCN1), whereas in serum the only significant protein was the structural protein OLIG1.

Proteins associated with BBB disruption comprise outcome predictors following severe 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 (Fig. 4A-B). For these, and also for the proteins that were significantly different between patients with intact and disrupted BBB, we performed outcome analyses (**Table S5-S6**). Among protein intersects between cluster/bicompartmental analyses, n = 40 proteins comprised independent outcome predictors (the representative examples CASKIN1, MMP9, and complement C5 are highlighted in **Fig. 4C-E**). The proteins with highest ΔNagelkerke's pseudo-R^2 from both analyses are summarized in Table 3. Of these, the majority were CNS enriched (n = 23, 58%) as compared to (neuro)inflammatory (n = 9, 23%) proteins.

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 ΔNagelkerke'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**.

Among proteins that had significantly altered levels if the TBI patient had a BBB injury we also found independent outcome predictors (**Table S6**, Table 3**)**. In order to see which of these proteins that were particularly important, we made a step-down analysis, which comprised all proteins that were significant within the specific compartment upon multivariable analysis followed by sequential deletion until merely significant proteins were retained in the model. In CSF, C9 (Fig. 4F, p = 0.0143, ΔR^2 = 7.4%) was the only protein retained. In serum, CFB (p = 0.0031, ΔR^2 = 9.2%) was the only protein retained.

Discussion

We present an observational, prospective, proteomic study of 177 proteins analyzed in matched CSF and serum samples of 90 severe TBI patients and 15 control subjects. This is one of the largest proteomic studies conducted following severe TBI. Uniquely, it allows us to define protein pathway alterations in CSF and serum in parallel following severe TBI. 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. In summary, we show that BBB disruption is associated with an increased neuroinflammatory response, of prognostic importance for long-term outcome.

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 (5, 68). We utilized an affinity based multiplex suspension bead antibody array (29), that allows multiplexing across a large dynamic range of protein concentrations, while maintaining a low measurement variability (30). We included a larger patient cohort than previous proteomic studies in TBI (19, 20, 71, 21–23, 25–27, 69, 70), which enabled outcome analyses. Additionally, genetic polymorphism has been shown to affect proteomic status and BBB integrity in the absence of trauma (10, 72), why we incorporated APOE genotyping. Two pediatric TBI studies on smaller patient cohorts (69, 70) have employed a similar approach, albeit with fewer proteins and no concurrent CSF/serum sampling. In addition, one study on adult TBI patients (73) combined microarray analysis and enzyme-linked immunosorbent assay (ELISA) on serum samples. All three studies thus preclude 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.

Proteomic studies following TBI benefit from access to CSF, but TBI studies warrant a new BBB disruption metric

We found that the predominant protein composition difference between samples was between CSF and serum. Within each compartment patients grouped depending on diagnosis and BBB integrity, again more evident in CSF than in serum. Hence, CSF is pivotal in TBI studies. Our approach also enabled quantification of BBB disruption. We observed 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 could have been falsely too low in CSF, as samples were in median obtained more than two days following the trauma, and albumin thus possibly washed-out. 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, not associated with APOE variant. 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. In the absence of such, 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 could confirm protein alterations of currently used TBI biomarkers. As each one of these are hampered by inherent advantages and limitations (34, 74) there is an interest for additional TBI biomarkers. We found protein level and pathway alterations following TBI. The proteins MBP and AQP4 were both increased following TBI. Unlike previous biomarkers, MBP has an oligodendrocytic origin and has been suggested to be a tentative TBI biomarker in the post-acute phase (75). AQP4 is an astrocytic protein, uniquely dense at the astrocyte podocytes lining the BBB (76), thus presumably reflecting BBB pathophysiology. 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 (77). We also found the pathway “hypoxia-inducible factor in the cardiovascular system”, and in concordance (78) 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

Aside from structural protein alterations, TBI upregulated markers of inflammatory pathways particularly pertaining to the innate immune system in both CSF and serum. Similar pathways were upregulated in CSF following BBB disruption. BBB disruption is intimately related to neuroinflammation (9), that commences when tissue injury-mediated release of e.g. alarmins trigger the innate immune mechanisms of the CNS (79, 80). This yields microglial- and inflammasome-mediated production of the cytokines IL1-β, IL-6, TNF-α, and IL-18 (79). Recently, microglia-mediated production of IL-1α, TNFα, and C1q was shown to activate astrocytes (81), known to respond by IL-6 and MMP-9 production, where the latter might be detrimental for BBB function (82). Both IL-1α, IL-1β, IL-6, and MMP-9 levels were increased following TBI in our material. IL-6, interestingly, has been suggested to be intimately intertwined with TGF-β (13), 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 (13, 83). Moreover, complement pathways were implicated across all our comparisons, and CSF/serum ratios of complement proteins were highly correlated with Q_A, congruent with earlier data (14, 15). Importantly, among TBI patients with intact and disrupted BBB, a handful of proteins, of which the majority were complement proteins, were altered in CSF. Complement is a key element within the neuroinflammatory response (84) following TBI and panels

of elevated complement proteins have been found in blood (85), CSF (14, 19, 21, 86), and brain parenchyma (25, 43, 87) of TBI patients. As we assessed all complement pathways, we can corroborate many of these findings systematically. Importantly, we could not find any relationship between genetic APOE genotype and any of our proteins, somewhat counter-intuitively given recent work (10) demonstrating how APOE4 causes BBB dysfunction. This highlights the discrepancy between traumatic and non-traumatic BBB injury, or less likely that APOE4 may affect functional outcome after more than 12 months.

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. The proteins of choice then need to be of prognostic importance. 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 variance, demonstrating that a large amount of unexplained variance in TBI outcome prediction models emanates from secondary injuries. The protein with highest additional variance was STMN4 in CSF, belonging to a protein family with microtubule-destabilizing capacity (88) but also of importance for neuronal regeneration (89). We hypothesize that STMN4 in this context serves as a metric for CNS cell death. Other proteins with high amount of additional explained variance were neuroinflammatory proteins, notably from the complement system. In fact, 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 between genetically distinct animal strains to worsened functional outcome (90). Further, knock-out and complement inhibition models have improved outcome (87, 91–93), whereas inhibition of complement inhibition has worsened it (94). Recently, membrane-attack complex inhibition alone was shown to merely 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 (95). We cannot draw as extensive conclusions, but we note that several of the 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 explored above, the TBI literature on unbiased approaches is vast and there is a need for secondary injury mechanism focused studies on larger patient cohorts. Further, our study is limited to cross-sectional data, why future studies should focus on longitudinal proteomics, possibly on the smaller panel of outcome-related proteins that we present. This would enable refined outcome modeling.

Other limitations concern discrepancies between the TBI patients and the healthy controls. Importantly, controls were younger than the TBI patients, thus possibly exaggerating some of the observed protein differences. Yet, they were healthy, which we considered superior compared with utilizing for example normal pressure hydrocephalus patients, as has been done elsewhere (20). Further, CSF was obtained through an EVD among TBI patients and through lumbar puncture among control subjects. An EVD itself 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 (66, 75), thus possibly exaggerating the proteomic differences between TBI patients and control subjects. Moreover, CSF protein levels could fail to portray intracellular alterations (20). In order to hamper this, one would need brain tissue biopsies, which however is difficult to obtain in larger-scale quantities. Moreover, a small tissue biopsy cannot confer global information on protein alterations within the CNS (20), which CSF does. The similar limitation holds true for microdialysis extracting brain extracellular fluid (96). With this in mind, CSF constitutes the state-of-the-art matrix within TBI studies of global CNS markers (19). For our study, CSF was therefore the superior biofluid to use, but future, external validation on a smaller protein-panel probably ought to be conducted using microdialysis as has been done in other studies (24, 97).

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
MES	2-(*N*-morpholino)ethanesulfonic acid
MFI	Median Fluorescence Intensity
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.

Manuscript revision and approval of manuscript: all authors.

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Figures

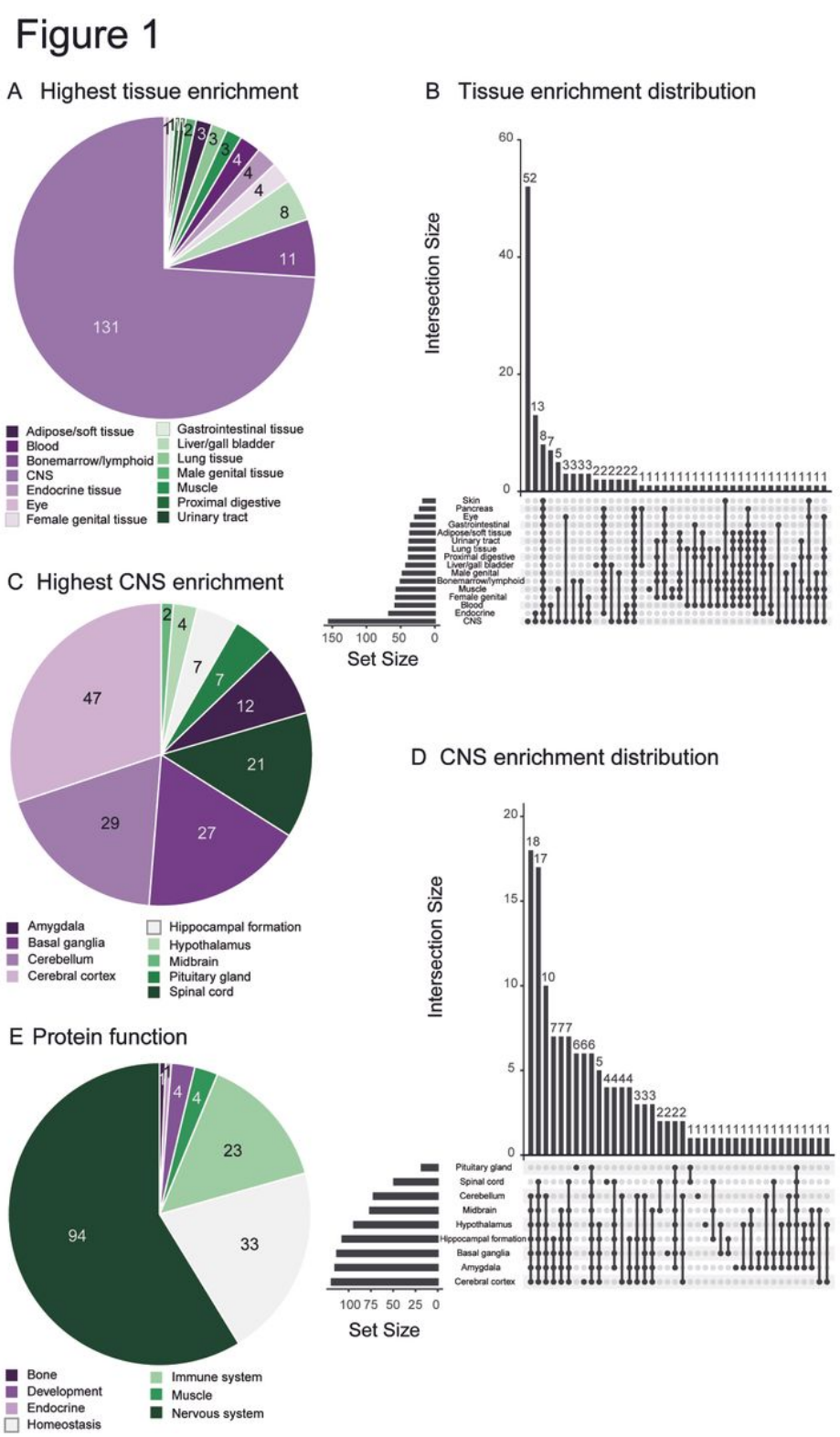

Figure 1

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). In accordance, the majority of proteins had a dedicated neurological function,

followed by a homeostatic function or immune system related function (E). Protein characterization data was obtained from the Human Protein Atlas. Abbreviations: CNS, central nervous system.

Figure 2

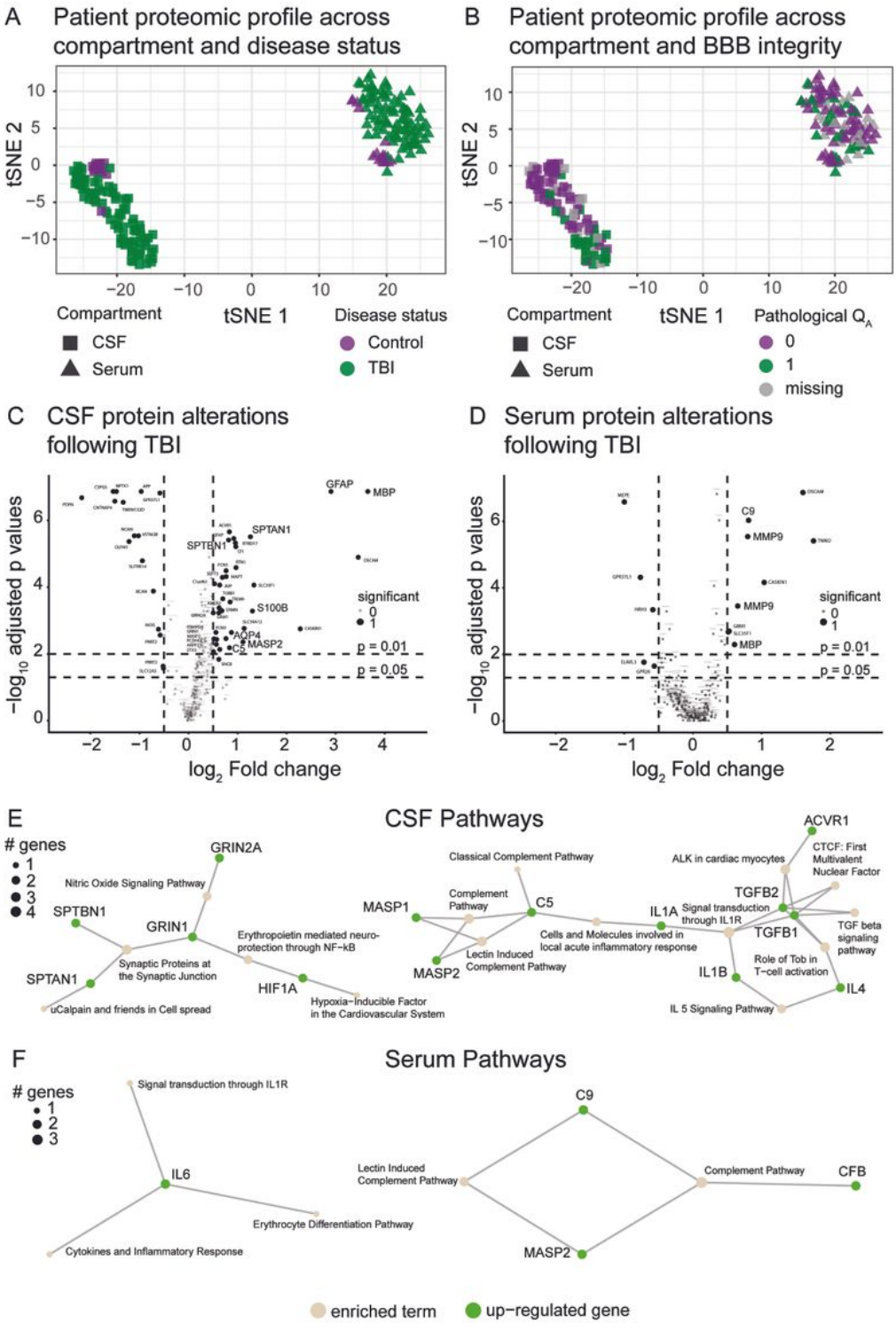

Figure 2

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 3

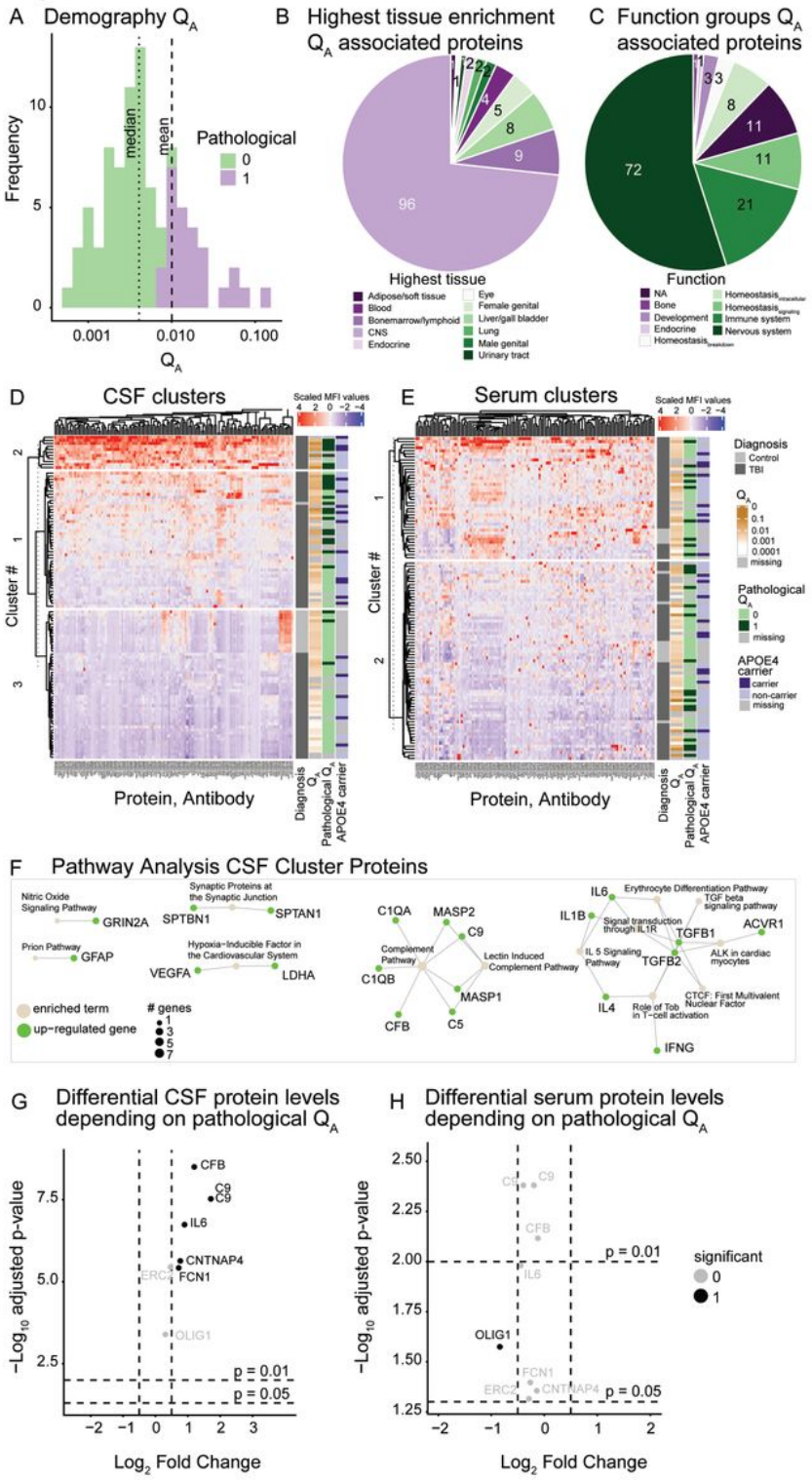

Figure 3

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), exhibiting functions corresponding to tissue enrichment (C). Using hierarchical clustering on CSF and serum protein measurements respectively, protein level clearly

clustered depending on BBB integrity status in CSF (D), but less so in serum (E). APOE carrier status was not associated with protein levels in either group (D, E). In CSF, this corresponded to pathway upregulation of predominantly innate immune mechanisms (F). Examining proteomic profiles between patients with disrupted and intact BBB, a handful of proteins were significant in CSF (G) and merely one in serum (H). In panel D and E, the categories QA, Pathological QA and APOE4 carrier had missing values, depicted in gray. Abbreviations: APOE, Apolipoprotein E; CSF, cerebrospinal fluid; CNS, central nervous system; MFI, median fluorescence intensity; QA, albumin quotient; TBI, traumatic brain injury. All full protein names are given in Table S1.

Figure 4

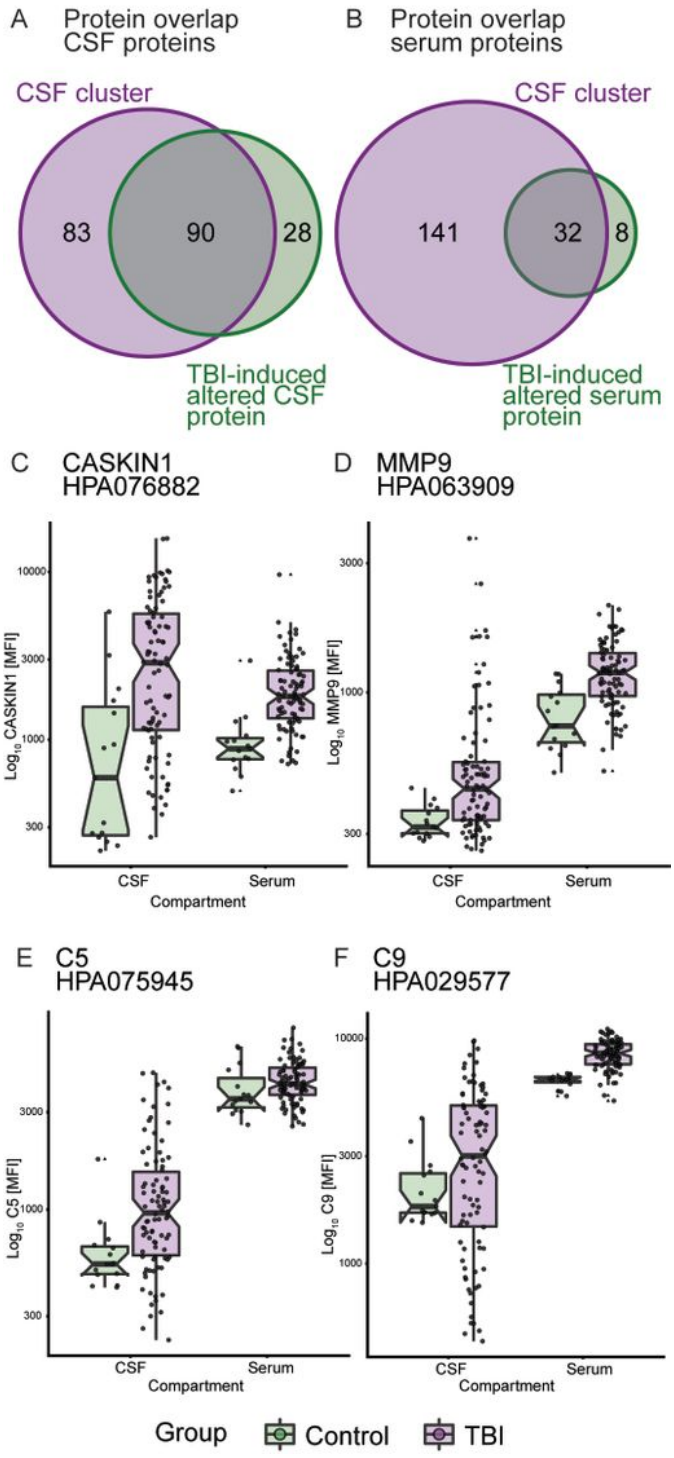

Figure 4

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-E). A subgroup outcome analysis was also conducted on proteins significantly different between patients with intact and disrupted BBB, where complement proteins (F) emanated independently significant. All analyses were multivariable, meaning that our outcome predictors are independently significant even when adjusting for previously known prognostic covariates following a severe TBI. 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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