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

Keywords: traumatic brain injury, protein biomarkers, proteomics, neuroinflammation, blood-brain barrier, apolipoprotein E4, Glasgow Outcome Scale, human

Posted Date: October 29th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-96625/v1
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Version of Record: A version of this preprint was published on March 12th, 2021. See the published version at [https://doi.org/10.1186/s13054-021-03503-x](https://doi.org/10.1186/s13054-021-03503-x).
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 uid (CSF) to blood albumin quotient (QA) (11). An increased QA indicates albumin leakage due to loss of BBB integrity. Following TBI, QA 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 QA 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 chronic 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$_{\text{CSF}}$ and albumin$_{\text{serum}}$ samples; 0.88 (-2.27-9.15) hours for albumin$_{\text{CSF}}$ and the proteomic sample; and −2.83 (-3.82 - -2.08) hours for albumin$_{\text{serum}}$ 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](http://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, \( p_{\text{adjusted}} < 0.05 \)) and the Wilcoxon signed rank test (FDR, \( p_{\text{adjusted}} < 0.01 \)). 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, \( p_{\text{adjusted}} < 0.05 \)) 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, \( p_{\text{adjusted}} \leq 0.01 \)) 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, \( p_{\text{adjusted}} < 0.05 \)).

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 (\(P_{\text{adjusted}} \leq 0.05\)).

**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 QA) as independent variable in a proportional odds regression analysis, using the rms package (64) in R. We conducted univariable analysis, and if significant (FDR, \(P_{\text{adjusted}} \leq 0.05\) if multiple testing), multivariable analysis (FDR, \(P_{\text{adjusted}} < 0.05\) 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 (2.3–3.3)  | 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)        | count (%)   |
| APOE allele status            | 15 (17)    | No allele: 57 (63) | count (%)   |
|                               |            | Heterozygote: 16 (18) |
|                               |            | Homozygote: 2 (2) |
| GOS                           | 0 (0)      | GOS 1 (death): 12 (13) | 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) | 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; QA, 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).
QA was an independent significant predictor of GOS (p = 0.044, ΔNagelkerke’s pseudo-R² = 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 carri ership, as APOE4 variant was not associated with QA 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 QA, 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 QA were complement proteins, except VCAM1 (Table 2). In fact, the majority of proteins that correlated with QA 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 QA associated protein levels in either CSF or serum.

| 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 QA correlated proteins as deemed by correlation coefficient Kendall τ. Correlations were calculated between protein CSF/serum ratio and QA. Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; QA, albumin quotient. Full protein names are detailed in Table S1.

Cluster analysis of QA correlated proteins revealed that in CSF, but not in serum, protein levels paralleled QA (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 \( \Delta \text{Nagelkerke's pseudo-R}^2 \) 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 | $\Delta R^2$ | 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 $\Delta$ 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.

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, $p = 0.0143$, $\Delta R^2 = 7.4\%$) was the only protein retained. In serum, CFB ($p = 0.0031$, $\Delta R^2 = 9.2\%$) 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 pathophysiologicaly 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
| Term | Definition |
|------|------------|
| 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 |
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.

**Funding**

We would like to acknowledge funders who enabled this work. CL received funding for this study through the Karolinska Institute Funds Clinical Scientist Training Program and Research Internship Program (grant numbers K825715022, K825715052). EPT received salary from the Swedish Society for Medical Research Post-Doctoral Scholarship (grant number N/A) and the Swedish Brain Foundation (grant
number: FO2019-0006). BMB received grants from Stockholm County ALF (grant number 20120560). The funders did not participate in study design/conceptualization, data acquisition, data analysis/interpretation, manuscript compilation, or submission decision.

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.

Acknowledgements

Genotyping was performed by the SNP&SEQ Technology Platform in Uppsala (www.genotyping.se). The platform is part of Science for Life Laboratory at Uppsala University and supported as a national infrastructure by the Swedish Research Council.

The authors would like to thank Dr. Jacob Kjell for his advice and careful reading of our manuscript.

References

1. Hyder AA, Wunderlich CA, Puvanachandra P, Gururaj G, Kobusingye OC. The impact of traumatic brain injuries: A global perspective. NeuroRehabilitation. 2007;22(5):341–53.
2. Werner C, Engelhard K. Pathophysiology of traumatic brain injury. Br J Anaesth. 2007;99(1):4–9.
3. Thelin EP, Nelson DW, Vehvilainen J, Nystrom H, Kivisaari R, Siironen J, et al. Evaluation of novel computerized tomography scoring systems in human traumatic brain injury: An observational, multicenter study. PLoS Med. 2017/08/05. 2017;14(8):e1002368.
4. Murray GD, Butcher I, McHugh GS, Lu J, Mushkudiani NA, Maas AI, et al. Multivariable prognostic analysis in traumatic brain injury: results from the IMPACT study. J Neurotrauma. 2007/03/23. 2007;24(2):329–37.
5. Chodobski A, Zink BJ, Szmydynger-Chodobska J. Blood-Brain Barrier Pathophysiology in Traumatic Brain Injury. Transl Stroke Res. 2011;2(4):492–516.
6. Schwarzmaier SM, Zimmermann R, McGarry NB, Trabold R, Kim SW, Plesnila N. In vivo temporal and spatial profile of leukocyte adhesion and migration after experimental traumatic brain injury in mice. J Neuroinflammation. 2013;10(1):808.

7. Turtzo LC, Lescher J, Janes L, Dean DD, Budde MD, Frank JA. Macrophagic and microglial responses after focal traumatic brain injury in the female rat. 2014;

8. Hinson HE, Rowell S, Schreiber M. Clinical evidence of inflammation driving secondary brain injury: A systematic review. J Trauma Acute Care Surg. 2015;78(1):184–91.

9. Jassam YN, Izzy S, Whalen M, McGavern DB, El Khoury J. Neuroimmunology of Traumatic Brain Injury: Time for a Paradigm Shift. Neuron. 2017/09/15. 2017;95(6):1246–65.

10. Montagne A, Nation DA, Sagare AP, Barisano G, Sweeney MD, Chakhoyan A, et al. APOE4 leads to blood–brain barrier dysfunction predicting cognitive decline. Nature. 2020;581(7806):71–6.

11. Tibbling G, Link H, Ohman S. Principles of albumin and IgG analyses in neurological disorders. I. Establishment of reference values. Scand J Clin Lab Invest. 1977/09/01. 1977;37(5):385–90.

12. Lindblad C, Nelson DW, Zeiler FA, Ercole A, Ghatan PH, von Horn H, et al. Influence of Blood–Brain Barrier Integrity on Brain Protein Biomarker Clearance in Severe Traumatic Brain Injury: A Longitudinal Prospective Study. J Neurotrauma. 2020;11:1–11.

13. Morganti-Kossmann MC, HANS VHJ, LENZLINGER PM, DUBS R, LUDWIG E, TRENTZ O, et al. TGF-β Is Elevated in the CSF of Patients with Severe Traumatic Brain Injuries and Parallels Blood-Brain Barrier Function. J Neurotrauma. 1999 Jul;16(7):617–28.

14. Stahel PF, Trentz O, Kossmann T, Morganti-Kossmann MC, Perez D, Redaelli C, et al. Intrathecal levels of complement-derived soluble membrane attack complex (sc5b-9) correlate with blood-brain barrier dysfunction in patients with traumatic brain injury. J Neurotrauma. 2001;18(8):773–81.

15. Bellander BM, Olafsson IH, Ghatan PH, Bro Skejo HP, Hansson LO, Wanecek M, et al. Secondary insults following traumatic brain injury enhance complement activation in the human brain and release of the tissue damage marker S100B. Acta Neurochir. 2010/08/06. 2011;153(1):90–100.

16. Wang KKW, Ottens AK, Liu MC, Lewis SB, Meegan C, Oli MW, et al. Proteomic identification of biomarkers of traumatic brain injury. Expert Rev Proteomics. 2005;2(4):603–14.

17. Martinez BI, Stabenfeldt SE. Current trends in biomarker discovery and analysis tools for traumatic brain injury. J Biol Eng. 2019;13(1):1–12.

18. Kingsmore SF. Multiplexed protein measurement: Technologies and applications of protein and antibody arrays. Nat Rev Drug Discov. 2006;5(4):310–20.

19. Sjödin MOD, Bergquist J, Wetterhall M. Mining ventricular cerebrospinal fluid from patients with traumatic brain injury using hexapeptide ligand libraries to search for trauma biomarkers. J Chromatogr B Analyt Technol Biomed Life Sci. 2010 Jul 15;878(22):2003–12.

20. Abu Hamdeh S, Shevchenko G, Mi J, Musunuri S, Bergquist J, Marklund N. Proteomic differences between focal and diffuse traumatic brain injury in human brain tissue. Sci Rep. 2018 Dec 1;8(1):1–15.
21. Hanrieder J, Wetterhall M, Enblad P, Hillered L, Bergquist J. Temporally resolved differential proteomic analysis of human ventricular CSF for monitoring traumatic brain injury biomarker candidates. J Neurosci Methods. 2009;177(2):469–78.

22. Connor DE, Chaitanya GV, Chittiboina P, McCarthy P, Scott LK, Schrott L, et al. Variations in the cerebrospinal fluid proteome following traumatic brain injury and subarachnoid hemorrhage. Pathophysiology. 2017;24(3):169–83.

23. Conti A, Sanchez-Ruiz Y, Bachi A, Beretta L, Grandi E, Beltramo M, et al. Proteome Study of Human Cerebrospinal Fluid following Traumatic Brain Injury Indicates Fibrin(ogen) Degradation Products as Trauma-Associated Markers. J Neurotrauma. 2004;21(7):854–63.

24. Orešič M, Posti JP, Kamstrup-Nielsen MH, Takala RSK, Lingsma HF, Mattila I, et al. Human Serum Metabolites Associate With Severity and Patient Outcomes in Traumatic Brain Injury. EBioMedicine. 2016 Oct 1;12:118–26.

25. Xu B, Tian R, Wang X, Zhan S, Wang R, Guo Y, et al. Protein profile changes in the frontotemporal lobes in human severe traumatic brain injury. Brain Res. 2016 Jul 1;1642:344–52.

26. Halford J, Shen S, Itamura K, Levine J, Chong AC, Czerwieniec G, et al. New astroglial injury-defined biomarkers for neurotrauma assessment. J Cereb Blood Flow Metab. 2017;37(10):3278–99.

27. Harish G, Mahadevan A, Pruthi N, Sreenivasasamurthy SK, Puttamallesh VN, Keshava Prasad TS, et al. Characterization of traumatic brain injury in human brains reveals distinct cellular and molecular changes in contusion and pericontusion. J Neurochem. 2015 Jul 1;134(1):156–72.

28. Pin E, Sjöberg R, Andersson E, Hellström C, Olofsson J, Jernborn Falk A, et al. Array-Based Profiling of Proteins and Autoantibody Repertoires in CSF. In: Santamaría E, Fernández-Irigoyen J, editors. Cerebrospinal Fluid (CSF) Proteomics: Methods and Protocols. New York: Springer Science + Business Media LLC; 2019. p. 303–18.

29. Schwenk JM, Gry M, Rimini R, Uhlén M, Nilsson P. Antibody Suspension Bead Arrays within Serum Proteomics. J Proteome Res. 2008;7(8):3168–79.

30. Schwenk JM, Nilsson P. Antibody Suspension Bead Arrays. In: Wu CJ, editor. Protein Microarray for Disease Analysis Methods and Protocols. New York: Springer Science + Business Media LLC; 2011. p. 29–36.

31. Cohen J. Statistical Power Analysis for the Behavioral Sciences. 2nd ed. Lawrence Erlbaum Associates; 1988.

32. Lakens D. Calculating and reporting effect sizes to facilitate cumulative science: A practical primer for t-tests and ANOVAs. Front Psychol. 2013;4(NOV):1–12.

33. Champely S. pwr: Basic Functions for Power Analysis. version 1. R package; 2018.

34. Thelin EP, Johannesson L, Nelson D, Bellander BM. S100B is an important outcome predictor in traumatic brain injury. J Neurotrauma. 2013/01/10. 2013;30(7):519–28.

35. Carney N, Totten AM, O’Reilly C, Ullman JS, Hawryluk GWJ, Bell MJ, et al. Guidelines for the Management of Severe Traumatic Brain Injury, Fourth Edition. Neurosurgery. 2017;80(1):6–15.
36. Karolinska University Hospital Laboratory. Albuminkvot, Csv/S- [Internet]. [Internet]. Stockholm: Klinisk kemi och KUL 24Sju; Available from: https://www.karolinska.se/KUL/Alla-anvisningar/Anvisning/9993

37. Isung J. Neuroinflammatory biomarkers in suicidal behavior [dissertation on the Internet]. Stockholm: Karolinska Institutet [cited 2020 Sep 28]; 2016.

38. Thelin EP, Al Nimer F, Frostell A, Zetterberg H, Blennow K, Nystrom H, et al. A serum protein biomarker panel improves outcome prediction in human traumatic brain injury. J Neurotrauma. 2019/05/11. 2019;

39. Chen X, Levine L, Kwok PY. Fluorescence polarization in homogeneous nucleic acid analysis. Genome Res. 1999 May;9(5):492–8.

40. Neiman Kungliga Tekniska Högskolan M. Bead based protein profiling in blood.

41. Sjostedt E, Fagerberg L, Hallstrom BM, Haggmark A, Mitsios N, Nilsson P, et al. Defining the Human Brain Proteome Using Transcriptomics and Antibody-Based Profiling with a Focus on the Cerebral Cortex. PLoS One. 2015/06/16. 2015;10(6):e0130028.

42. Woodcock T, Morganti-Kossmann MC. The role of markers of inflammation in traumatic brain injury. Front Neurol. 2013;4 MAR(March):1–18.

43. Bellander B-M, Singhrao SK, Ohlsson M, Mattsson P, Svensson M. Complement Activation in the Human Brain after Traumatic Head Injury. J Neurotrauma. 2001 Dec;18(12):1295–311.

44. Helmy A, Carpenter KLH, Menon DK, Pickard JD, Hutchinson PJA. The cytokine response to human traumatic brain injury: Temporal profiles and evidence for cerebral parenchymal production. J Cereb Blood Flow Metab. 2011;31(2):658–70.

45. Thelin EP, Just D, Frostell A, Häggmark-Månberg A, Risling M, Svensson M, et al. Protein profiling in serum after traumatic brain injury in rats reveals potential injury markers. Behav Brain Res. 2018;340:71–80.

46. Salim A, Hadjizacharia P, Brown C, Inaba K, Teixeira PGR, Chan L, et al. Significance of troponin elevation after severe traumatic brain injury. J Trauma - Inj Infect Crit Care. 2008;64(1):46–52.

47. Collaborators. HPA. The Human Protein Atlas [Internet]. 2005.

48. Drobin K, Nilsson P, Schwenk JM. Highly Multiplexed Antibody Suspension Bead Arrays for Plasma Protein Profiling. In: Bäckvall H, Lehtiö J, editors. Methods in Molecular Biology. New York: Springer Science + Business Media LLC; 2013. p. 137–45.

49. Team RC. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2018.

50. Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, et al. Welcome to the Tidyverse. J Open Source Softw. 2019 Nov 21;4(43):1686.

51. Neuwirth E. RColorBrewer: ColorBrewer Palettes. 2014.

52. Wilke CO. cowplot: Streamlined Plot Theme and Plot Annotations for "ggplot2." Comprehensive R Archive Network (CRAN); 2019.
53. Auguie B. gridExtra: Miscellaneous Functions for “Grid” Graphics. Comprehensive R Archive Network (CRAN); 2017.

54. Holm S. A Simple Sequentially Rejective Multiple Test Procedure A Simple Sequentially Rejective Multiple Test Procedure. Scand J Stat. 1979;6(6):65–70.

55. Benjamini Y, Hochberg Y. CONTROLLING THE FALSE DISCOVERY RATE - A PRACTICAL AND POWERFUL APPROACH TO MULTIPLE TESTING. J R Stat Soc Ser B-Methodological. 1995;57(1):289–300.

56. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. Science (80- ). 2015/01/24. 2015;347(6220):1260419.

57. The human brain - The Human Protein Atlas [Internet]. [cited 2020 May 19]. Available from: https://www.proteinatlas.org/humanproteome/brain

58. Van Der Maaten L, Hinton G. Visualizing Data using t-SNE. J Mach Learn Res. 2008;9:2579–605.

59. Krijthe JH. Rtsne: T-Distributed Stochastic Neighbor Embedding using a Barnes-Hut Implementation. GitHub; 2015.

60. Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data.

61. Ulgen E, Ozisik O, Sezerman OU. PathfindR: An R package for comprehensive identification of enriched pathways in omics data through active subnetworks. Front Genet. 2019;10(SEP):1–33.

62. Step-by-Step Execution of the pathfindR Enrichment Workflow [Internet]. [cited 2020 Jul 30]. Available from: https://cran.r-project.org/web/packages/pathfindR/vignettes/manual_execution.html

63. Chen H. VennDiagram: Generate High-Resolution Venn and Euler Plots. CRAN; 2018.

64. Harrell Jr FE. rms: Regression Modeling Strategies. 2019.

65. Nelson DW, Nystrom H, MacCallum RM, Thornquist B, Lilja A, Bellander BM, et al. Extended analysis of early computed tomography scans of traumatic brain injured patients and relations to outcome. J Neurotrauma. 2009/08/25. 2010;27(1):51–64.

66. Weisner B, Bernhardt W. Protein fractions of lumbar, cisternal, and ventricular cerebrospinal fluid. J Neurol Sci. 1978 Jul;37(3):205–14.

67. Brettschneider J, Claus A, Kassubek J, Tumani H. Isolated blood-cerebrospinal fluid barrier dysfunction: Prevalence and associated diseases. J Neurol. 2005;252(9):1067–73.

68. Shlosberg D, Benifla M, Kaufer D, Friedman A. Blood-brain barrier breakdown as a therapeutic target in traumatic brain injury. Nat Rev Neurol. 2010/06/17. 2010;6(7):393–403.

69. Buttram SDW, Wisniewski SR, Jackson EK, Adelson PD, Feldman K, Bayir H, et al. Multiplex Assessment of Cytokine and Chemokine Levels in Cerebrospinal Fluid following Severe Pediatric Traumatic Brain Injury: Effects of Moderate Hypothermia. J Neurotrauma. 2007 Nov;24(11):1707–18.

70. Berger RP, Ta’asan S, Rand A, Lokshin A, Kochanek P. Multiplex assessment of serum biomarker concentrations in well-appearing children with inflicted traumatic brain injury. Pediatr Res.
71. Stefini R, Catenacci E, Piva S, Sozzani S, Valerio A, Bergomi R, et al. Chemokine detection in the cerebral tissue of patients with posttraumatic brain contusions. J Neurosurg. 2008;108(5):958–62.
72. Gorgoraptis N, Li LM, Whittington A, Zimmerman KA, Maclean LM, McLeod C, et al. In vivo detection of cerebral tau pathology in long-term survivors of traumatic brain injury. Sci Transl Med. 2019;11(508).
73. He XY, Dan QQ, Wang F, Li YK, Fu SJ, Zhao N, et al. Protein network analysis of the serum and their functional implication in patients subjected to traumatic brain injury. Front Neurosci. 2019;13(JAN):1–15.
74. Thelin EP, Jeppsson E, Frostell A, Svensson M, Mondello S, Bellander BM, et al. Utility of neuron-specific enolase in traumatic brain injury; relations to S100B levels, outcome, and extracranial injury severity. Crit Care. 2016/09/09. 2016;20:285.
75. Zetterberg H, Blennow K. Fluid biomarkers for mild traumatic brain injury and related conditions. Nat Publ Gr. 2016;
76. Abbott NJ, Rönnbäck L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci. 2006;7(1):41–53.
77. Mondello S, Robicsek SA, Gabrielli A, Brophy GM, Papa L, Tepas J, et al. αII-Spectrin Breakdown Products (SBDPs): Diagnosis and Outcome in Severe Traumatic Brain Injury Patients. J Neurotrauma. 2010 Jul;27(7):1203–13.
78. Ramakrishnan S, Anand V, Roy S. Vascular endothelial growth factor signaling in hypoxia and inflammation. J Neuroimmune Pharmacol. 2014;9(2):142–60.
79. Gadani SP, Walsh JT, Lukens JR, Kipnis J. Dealing with Danger in the CNS: The Response of the Immune System to Injury. Neuron. 2015;87(1):47–62.
80. Hammad A, Westacott L, Zaben M. The role of the complement system in traumatic brain injury: A review. J Neuroinflammation. 2018;15(1):1–15.
81. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. Neurotoxic reactive astrocytes are induced by activated microglia. Nature. 2017/01/19. 2017;541(7638):481–7.
82. Abdul-Muneer PM, Pfister BJ, Haorah J, Chandra N. Role of Matrix Metalloproteinases in the Pathogenesis of Traumatic Brain Injury. Mol Neurobiol. 2016;53(9):6106–23.
83. Shen W, Li S, Chung SH, Zhu L, Stayt J, Su T, et al. Tyrosine phosphorylation of VE-cadherin and claudin-5 is associated with TGF-β1-induced permeability of centrally derived vascular endothelium. Eur J Cell Biol. 2011;90(4):323–32.
84. Dinet V, Petry KG, Badaut J. Brain–Immune Interactions and Neuroinflammation After Traumatic Brain Injury. Front Neurosci. 2019;13(November).
85. Bao W, He F, Yu L, Gao J, Meng F, Ding Y, et al. Complement cascade on severe traumatic brain injury patients at the chronic unconscious stage: implication for pathogenesis. Expert Rev Mol Diagn. 2018 Aug 3;18(8):761–6.
86. Kossmann T, Stahel PF, Morganti-Kossmann MC, Jones JL, Barnum SR. Elevated levels of the complement components C3 and factor B in ventricular cerebrospinal fluid of patients with traumatic brain injury. J Neuroimmunol. 1997;73(1–2):63–9.

87. Longhi L, Orsini F, De Blasio D, Fumagalli S, Ortolano F, Locatelli M, et al. Mannose-binding lectin is expressed after clinical and experimental traumatic brain injury and its deletion is protective. Crit Care Med. 2014;42(8):1910–8.

88. HPA Collaborators. STMN4 protein expression summary - The Human Protein Atlas [Internet]. [cited 2020 Aug 3]. Available from: https://www.proteinatlas.org/ENSG00000015592-STMN4

89. Nakazawa T, Morii H, Tamai M, Mori N. Selective upregulation of RB3/stathmin4 by ciliary neurotrophic factor following optic nerve axotomy. Brain Res. 2005;1061(2):97–106.

90. Al Nimer F, Lindblom R, Strom M, Guerreiro-Cacais AO, Parsa R, Aeinehband S, et al. Strain influences on inflammatory pathway activation, cell infiltration and complement cascade after traumatic brain injury in the rat. Brain Behav Immun. 2012/10/10. 2013;27(1):109–22.

91. Leinhase I, Rozanski M, Harhausen D, Thurman JM, Schmidt OI, Hossini AM, et al. Inhibition of the alternative complement activation pathway in traumatic brain injury by a monoclonal anti-factor B antibody: A randomized placebo-controlled study in mice. J Neuroinflammation. 2007;4:1–12.

92. Rich MC, Keene CN, Neher MD, Johnson K, Yu ZX, Ganivet A, et al. Site-targeted complement inhibition by a complement receptor 2-conjugated inhibitor (mTT30) ameliorates post-injury neuropathology in mouse brains. Neurosci Lett. 2016;617:188–94.

93. Fluiter K, Opperhuizen AL, Morgan BP, Baas F, Ramaglia V. Inhibition of the Membrane Attack Complex of the Complement System Reduces Secondary Neuroaxonal Loss and Promotes Neurologic Recovery after Traumatic Brain Injury in Mice. J Immunol. 2014;192(5):2339–48.

94. Stahel PF, Flierl MA, Morgan BP, Persigehl I, Stoll C, Conrad C, et al. Absence of the complement regulatory molecule CD59a leads to exacerbated neuropathology after traumatic brain injury in mice. J Neuroinflammation. 2009;6:1–11.

95. Alawieh A, Langley EF, Weber S, Adkins D, Tomlinson S. Identifying the role of complement in triggering neuroinflammation after traumatic brain injury. J Neurosci. 2018;38(10):2519–32.

96. Stocchetti N, Carbonara M, Citerio G, Ercole A, Skrifvars MB, Smielewski P, et al. Severe traumatic brain injury: targeted management in the intensive care unit. Lancet Neurol. 2017;16(6):452–64.

97. Dyhrfort P, Shen Q, Clausen F, Thulin M, Enblad P, Kamali-Moghaddam M, et al. Monitoring of Protein Biomarkers of Inflammation in Human Traumatic Brain Injury Using Microdialysis and Proximity Extension Assay Technology in Neurointensive Care. J Neurotrauma. 2019 Oct 15;36(20):2872–85.

Figures
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

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

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.
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.

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