# vCSF Danger-associated Molecular Patterns After Traumatic and Nontraumatic Acute Brain Injury: A Prospective Study

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**Background:** Danger-associated molecular patterns (DAMPs) may be implicated in the pathophysiological pathways associated with an unfavorable outcome after acute brain injury (ABI).

**Methods:** We collected samples of ventricular cerebrospinal fluid (vCSF) for 5 days in 50 consecutive patients at risk of intracranial hypertension after traumatic and nontraumatic ABI. Differences in vCSF protein expression over time were evaluated using linear models and selected for functional network analysis using the PANTHER and STRING databases. The primary exposure of interest was the type of brain injury (traumatic vs. nontraumatic), and the primary outcome was the vCSF expression of DAMPs. Secondary exposures of interest included the occurrence of intracranial pressure $\geq 20$ or $\geq 30$ mm Hg during the 5 days post-ABI, intensive care unit (ICU) mortality, and neurological outcome (assessed using the Glasgow Outcome Score) at 3 months post-ICU discharge. Secondary outcomes included associations of these exposures with the vCSF expression of DAMPs.

**Results:** A network of 6 DAMPs (*DAMP_trauma*; protein-protein interaction [PPI] $P = 0.04$) was differentially expressed in patients with ABI of traumatic origin compared with those with nontraumatic ABI. ABI patients with intracranial pressure $\geq 30$ mm Hg differentially expressed a set of 38 DAMPS (*DAMP_ICP30*; PPI $P < 0.001$). Proteins in *DAMP_ICP30* are involved in cellular proteolysis, complement pathway activation, and post-translational modifications. There were no relationships between DAMP expression and ICU mortality or unfavorable versus favorable outcomes.

**Conclusions:** Specific patterns of vCSF DAMP expression differentiated between traumatic and nontraumatic types of ABI and were associated with increased episodes of severe intracranial hypertension.

**Key Words:** acute brain injury, cerebrospinal fluid, protein-protein interactions, danger-associated molecular patterns (DAMPs), ICU outcomes, systems biology

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Protein-protein interactions (PPI) are involved in a wide range of biological processes, including cell-to-cell interactions and metabolic control.[1] After primary acute brain injury (ABI), necrotic and injured neurons release danger-associated molecular patterns (DAMPs), which bind to pattern recognition receptors on the cell membrane.[2] This binding leads to signal transduction, nuclear factor-κB gene induction pathway activation, and release of proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-6.[3,4] These events serve to coordinate local and controlled responses to tissue injury with remodeling, neovascularization, and activation of repair pathways.[5] However, with large amounts of tissue injury, the inflammatory response can become dysregulated and contribute to vascular dysregulation, cerebral edema, and intracranial hypertension.[4,6] Cytokines then activate the endothelium, causing chemotaxis of other inflammatory cells, such as neutrophils and macrophages, to the tissue injury site and the release of reactive oxygen species, vasoactive agents, and toxic granular components; these events lead to secondary brain injury.[7]

Current evidence suggests that single proteins can independently activate specific parts of inflammatory, proapoptotic, and redox-related pathways (among others) and that activation of these pathways may be related to

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unfavorable outcomes.[7–10] Previous attempts have been made to identify proteins responsible for traumatic[11,12] and ischemic[13] ABI, but mainly in retrospective studies. In a recent prospective study by our group, differences in the expression of specific ventricular cerebrospinal fluid (vCSF) proteins after traumatic and nontraumatic ABI were identified. These proteins were largely related to structural damage, complement activation, and cholesterol metabolism, and some were associated with a greater risk of severe intracranial hypertension.[9]

However, proteins do not act individually in vivo but as part of multiprotein complexes.[14–16] We therefore hypothesized that networks of vCSF proteins, notably DAMPs, might be associated with the type of injury, the severity of brain dysfunction, and unfavorable outcomes in patients with ABI. We used proteomic and computational analysis of vCSF proteins from ABI patients to evaluate this possibility.

## METHODS

This is a secondary analysis of a previously studied cohort.[9] The study was approved by the ethics committee of the Hospital Universitaire de Bruxelles, Belgium (#2014/170-2015/130; November 6, 2013). All patients or their next of kin provided written informed consent for participation in the study. Consecutive adult (above 18 y) patients with ABI who required an intraventricular catheter as part of their standard of care were considered for inclusion during the study period, April 1, 2014, to October 31, 2015. Patients with acute central nervous system infections or malignant lesions and pediatric patients were excluded.

### Data Collection

Diagnosis, pupil reactivity, the motor component of the Glasgow Coma Scale score, systolic arterial pressure ($>$ or $<$ 90 mm Hg), and arterial oxygen saturation ($SpO_2$ $>$ or $<$ 92%) were recorded on admission. Intracranial pressure (ICP) was recorded immediately after placement of an intraventricular catheter and hourly thereafter; episodes of ICP $\geq$ 20 or $\geq$30 mm Hg over the following 5 days were noted. Intensive care unit (ICU) mortality was also recorded. The Glasgow Outcome Score was assessed in all patients at 3 months after ICU discharge by telephone interview. The results are presented according to the STROBE statement.[17]

### Cerebrospinal Fluid Sample Preparation

Cerebrospinal fluid samples (3 to 4 mL) were collected from the ventriculostomy catheter within the first 24 hours after insertion and then every 24 hours for 5 days. Samples were centrifuged, frozen, and stored at −80 °C until the time of analysis. vCSF (250 µL) was then concentrated to 25 to 30 µL on Amicon 3 kDa (Merck, Darmstadt, Germany). The concentrated samples were diluted to 100 µL with 25 mM $NH_4HCO_3$, then reduced with 10 mM DL-dithiothreitol for 30 minutes at 56°C and alkylated with 55 mM iodoacetamide for 20 minutes at room temperature. Trypsin (2 µg) (Promega) was added, and the sample was incubated overnight at 37 °C. Formic acid was added for a final concentration of 0.1%, and peptides were purified using StageTips C18 (Thermo Fisher Scientific) according to the manufacturer's instructions. After evaporation, peptides were resuspended in 15 µL of 5% ACN/0.1% HCOOH.

### Data-independent Acquisition Mass Spectrometry

Proteins were identified from vCSF samples by label-free data-independent acquisition mass spectrometry (TripleTOF 5600 SWATH, Sciex) coupled to an Eksigent NanoLC Ultra 2D HPLC System (Eksigent). Peptides (5 µL) were injected with a loading buffer (5% ACN/0.1% HCOOH) and concentrated on a trap column (Waters Symmetry C18 NanoAcquity 2G v/v, 20 mm×180 µm, 5 µm). After 10 minutes, separation was performed by applying a 100-minute hydrophobic gradient on a separation column (Waters Acquity UPLC HSS T3, 250 mm×75 µm, 1.8 µm) using a 2-step ACN gradient (5% to 25% ACN/0.1% HCOOH for 90 min then 25% to 60% ACN/0.1% HCOOH for 40 min). Subsequently, peptides were ionized by electrospray ionization and injected into the mass spectrometer. MS1 spectra were collected in the 400 to 1500 m/z range for 250 ms; the 20 most intense precursors with a charge state of 2 to 4 were selected for fragmentation. MS2 spectra were then collected in the 100 to 2000 m/z range for 100 ms; precursor ions were excluded from reselection for 12 seconds.

### Sequential Window Acquisition of all Theoretical Mass Spectrometry

Targeted data extraction of the MS/MS spectra generated by the data-independent acquisition method was used as previously described.[12,13] Sequential Window Acquisition of all THeoretical (SWATH) acquisitions was performed using 34 windows of fixed effective isolation width to cover a mass range of 400 to 1250 m/z. SWATH MS2 spectra were collected from 400 to 1500 m/z. The collision energy for each window was determined according to the calculation for a 2+ charged ion centered upon the window with a spread of 15. An accumulation time of 96 ms was used for all fragment-ion scans in high-sensitivity mode and for the survey scans in high-resolution mode acquired at the beginning of each cycle, resulting in a duty cycle of ~3.3 seconds. Confidence settings for spectral peak identification included mass error $\leq$5 ppm, retention time $\leq$5% error, isotope ratio $\leq$10% difference and library hit $\geq$70 purity score.

### Outcome Measures

The primary exposure of interest was the type of brain injury (traumatic vs. nontraumatic), and the primary outcome was vCSF expression of DAMPs. Secondary exposures of interest included the occurrence of ICP $\geq$ 20 or $\geq$30 mm Hg during the 5 days post-ABI, ICU mortality, and neurological outcome at 3 months post-ICU discharge. Secondary outcomes included associations of these exposures with vCSF expression of DAMPs.

## Statistical Analysis

Proteomic data preprocessing included sample outlier detection, missing value imputation (k-nearest neighbor approach), and sample-wise normalization. Statistical analysis of differentially expressed proteins included parametric and nonparametric models for protein differences between traumatic and nontraumatic ABI, those with and without intracranial hypertension, ICU survivors versus nonsurvivors, and good versus poor 3-month neurological outcome, and 2-sided *t* test and Wilcoxon test for significance during the 5 days after ICU admission as appropriate. Differences in proteins with a *P*-value of <0.05 were considered significant. A specific test for the correction of confounders was performed, with a global alpha of 0.01. The correction was made by dividing into 529, which corresponded to the number of comparisons, giving an adjusted alpha of 0.0000189. No adjustment was made for confounding factors across groups (age, sex, and primary disease) in the multivariable statistical models. Uniprot IDs of proteins with a statistical log2 fold difference between the groups were entered in the Protein ANalysis THrough Evolutionary Relationships (PANTHER) database for protein identification and then in the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database for PPI functional, pathway and enrichment analysis using the STRING database interaction confidence score (ICS). A DAMP was defined as a protein that had more interactions than expected for a random protein of similar size, which after binding to a pattern recognition receptor, activates pathophysiological pathways involved in downstream signaling and immune response.[18] The ICS ranges from 0 to 1, with 1 being the highest possible confidence. PPIs with an ICS > 0.4 were selected for further analysis. A PPI *P*-value of <0.05 was considered significant. Proteins with a node degree > 1 (ie, proteins with > 1 connection in the network structure) were selected, if available, to evaluate how much of the structure was captured by the node degree distribution.

# RESULTS

Demographic and baseline data of the included patients are shown in Table 1. The ABI was the result of spontaneous subarachnoid hemorrhage (n = 23, 46%), trauma (n = 15, 30%), intracranial hemorrhage (n = 6, 12%), ischemic stroke (n = 3, 6%), or other causes (n = 3, 6%). Disease severity was illustrated by the low median (interquartile range Glasgow Coma Scale score on admission of 6 [interquartile range 3 to 14], the 3-month Glasgow Outcome Score of 2 [1 to 4], and a 40% ICU mortality [n = 20]).

**TABLE 1.** Patient Characteristics

| Characteristic | Value (n = 50) |
|---|---|
| Male, n (%) | 23 (46) |
| Age (y) | 52 [36-57] |
| Admission diagnosis, n (%) | |
| Spontaneous subarachnoid hemorrhage | 23 (46) |
| Fisher grade 3 | 1 (4) |
| Fisher grade 4 | 22 (96) |
| Traumatic brain injury* | 15 (30) |
| Intraparenchymal hematoma | 7 (47) |
| Subdural hematoma | 5 (33) |
| Diffuse brain edema | 2 (13) |
| Traumatic subarachnoid hemorrhage | 6 (40) |
| Epidural hematoma | 2 (13) |
| Intracranial hemorrhage | 6 (14) |
| Hypertensive | 2 (33) |
| AV malformation | 4 (67) |
| Ischemic stroke | 3 (6) |
| Hemispheric | 3 (100) |
| Other† | 3 (6) |
| Admission motor component of the Glasgow Coma Scale score | 4 [1-6] |
| Pupil reactivity on admission, n (%) | |
| Bilateral | 36 (72) |
| Unilateral | 14 (28) |
| Admission systolic arterial pressure <90 mm Hg | 6 (3) |
| Admission $SpO_2$ <92% | 16 (8) |
| Patients with episode of ICP ≥ 20 mm Hg during study period | 45 (90) |
| Patients with episode of ICP ≥ 30 mm Hg during the study period | 21 (42) |
| ICU mortality | 20 (40) |
| 3-month Glasgow Outcome Score | 2 [1-4] |

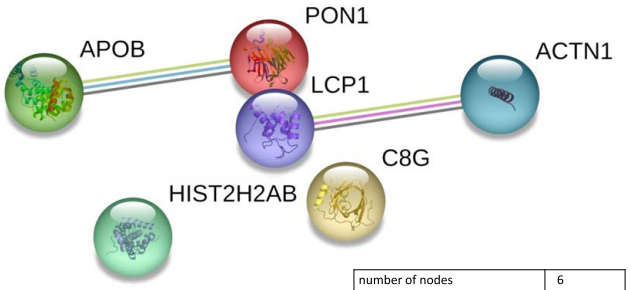
Data presented as number (%) or median [interquartile range].
*Some TBI patients had multiple contributory factors.
†Others included acute hydrocephalus (MAV) (n = 2), acute demyelinating encephalopathy (n = 1).
ICP indicates intracranial pressure; $SpO_2$, arterial oxygen saturation; TBI, traumatic brain injury.

## Protein Expression and Type of Brain Injury

In total, 517 proteins (present in 75% of the patients) were quantified by the SWATH mass spectrometry method. Six proteins were differentially expressed in patients with traumatic ABI compared with those with nontraumatic ABI during the study period; mapped IDs were found for these 6 proteins using the PANTHER database (Supplemental Digital Content 1, http://links.lww.com/JNA/A584: Table showing Panther list of differential protein expression). Using the STRING database for PPI, these 6 proteins formed a DAMP network ($DAMP_{\_trauma}$; PPI, $P = 0.04$) (Fig. 1). Supplemental Digital Content 2 (http://links.lww.com/JNA/A585) shows the $DAMP_{\_trauma}$ network functional enrichment analysis from traumatic and nontraumatic ABI patients. The PPIs in this network included the interaction between serum paraoxonase/arylesterase 1 with apolipoprotein B-100 (ICS, 0.92) and the PPI between alpha-actinin-1 and lymphocyte cytosolic protein 1 (ICS = 0.53) (Supplemental Digital Content 3, http://links.lww.com/JNA/A586: Table showing PPI between traumatic vs. nontraumatic ABI). Functional analysis of the $DAMP_{\_trauma}$ network revealed significant enrichment for the high-density lipoprotein particle, actin filament bundle, and extracellular exosome.

## Protein Expression and Secondary Outcomes

There were no significant differences in vCSF expression of protein DAMPs between patients with ICP ≥ 20 mm Hg (n = 45) and those with ICP <20 mm Hg (n = 5) at any timepoint during the 5-day study period. However, 40 individual proteins were differentially




| | |
|---|---|
| number of nodes | 6 |
| number of edges | 2 |
| average node degree | 0.667 |
| avg. local clustering coefficient | 0.667 |
| expected number of edges | 0 |
| PPI enrichment p value | 0.0398 |

**FIGURE 1.** Systems biology construct showing ventricular cerebrospinal fluid protein-protein interaction (*DAMP_trauma*) in patients with traumatic acute brain injury and interaction confidence score (ICS) > 0.4. Node: protein of interest; edges: connections between nodes; degree (of a node): number of edges incident to that node; clustering coefficient: local density of connections. Protein IDs correspond to Uniprot IDs—LCP1, plastin-2; Hist2H2AB, histone H2A type 2-B; ACTN1, alpha-actinin-1; APOB, apolipoprotein B-100; PON1, serum paraoxonase/arylesterase 1; C8G, complement component C8 gamma chain. PPI indicates protein-protein interaction.

expressed in the 21 patients with ICP ≥ 30 mm Hg (DAMP_*ICP30*) compared with those with ICP <30 mm Hg (n = 40) during the study period (Supplemental Digital Content 4, http://links.lww.com/JNA/A587: Table showing the DAMP_*ICP30* network functional enrichment analysis).

Using the PANTHER database, mapped IDs were found for 31 of these 40 proteins (Supplemental Digital Content 5, http://links.lww.com/JNA/A588: Table showing protein IDs with differences in expression in patients with ICP ≥ 30 mm Hg and ICP <30 mm Hg). Using the STRING database for PPI, a 38-protein DAMP network with brain tissue specificity and located mostly in the extracellular space was observed in patients with ICP ≥ 30 mm Hg (PPI, $P < 0.001$) (Fig. 2A). A table showing the node degree of significant proteins in *DAMP_ICP30* network is available in Supplemental Digital Content 6, http://links.lww.com/JNA/A589). Pathway analysis of the DAMP_ *ICP30* network suggested significant interactions between ankyrin-1 and spectrin beta chain, nonerythrocytic 1 (ICS = 0.86), and of the post-translational modifier polyubiquitin-C ubiquitin with proteasome activator complex subunit 1 (ICS = 0.91). Details of the PPIs in patients with ICP ≥ 30 mm Hg and with ICP <30 mm Hg are available in Supplemental Digital Content 7, http://links.lww.com/JNA/A590. Interestingly, molecular mechanisms of post-translational modifiers, including glycation and sulfation, were also enriched. Another PPI included the constituent of the membrane attack complex complement component C8 alpha chain with the inhibitor of the membrane attack complex action CD59 glycoprotein (ICS = 0.75). Such PPIs persisted even after selecting those with a node degree ≥ 1.5 (Fig. 2B). Further functional enrichment analysis of the DAMP_*ICP30* network suggested biological processes associated with the regulation of inflammation and immune system activation.

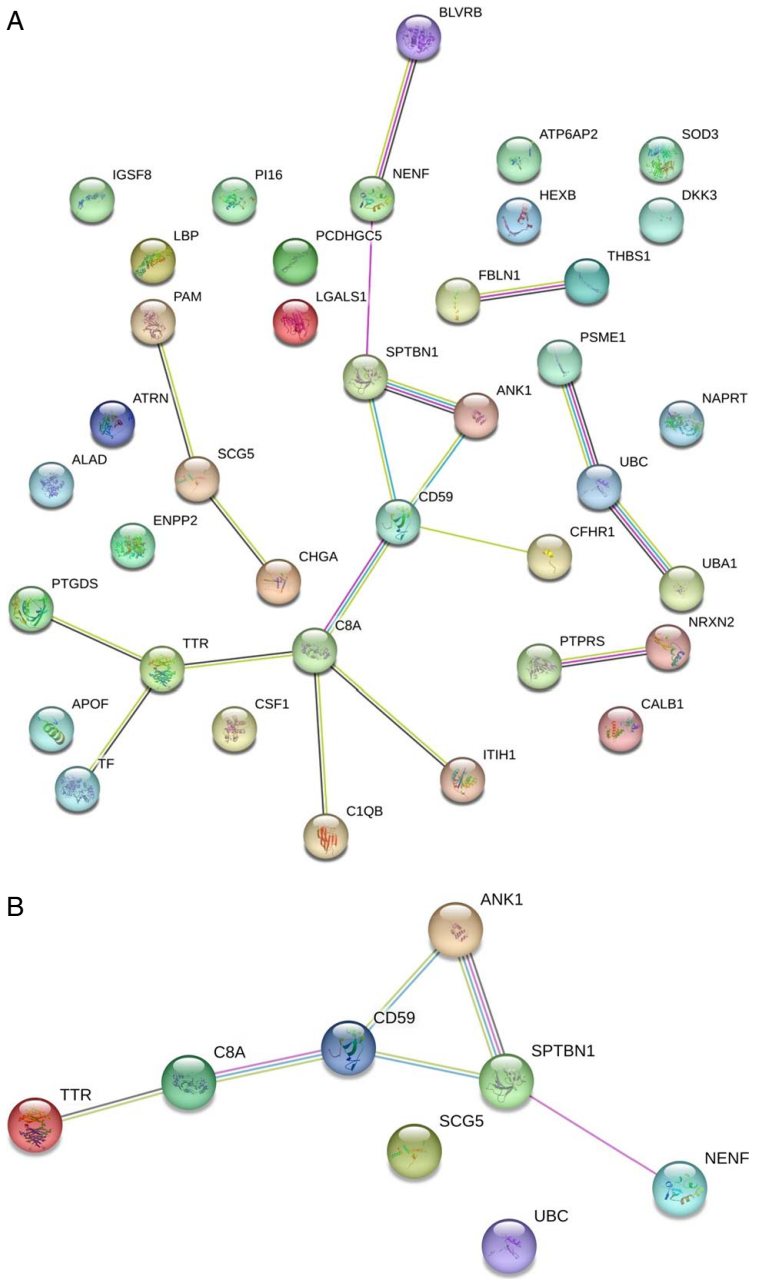
No significant differences in DAMPs were observed between survivors and nonsurvivors or between those with unfavorable and favorable neurological outcomes.

## DISCUSSION

Several proteins that are acutely expressed in vCSF after ABI interact differently according to the type of injury (ie, traumatic or nontraumatic) and to the severity of intracranial hypertension. The presence of the *DAMP_trauma* network distinguished ABI of traumatic origin from that of nontraumatic origin, suggesting a possible distinctive proteomic response in these ABI types. Three nodes in the *DAMP_trauma* network were related to the neuronal cytoskeleton, and 2 were related to the oxidative stress response. Functional enrichment of the *DAMP_trauma* network also documented the expression of complement-related immune system activation. This enrichment was also found in the *DAMP_ICP30* network and may indicate a major influence of complement-mediated activation processes in cell proteolysis and clinical outcomes, as previously described.[9,19,20]

The *DAMP_ICP30* network also had specific characteristics, which persisted even after including only nodes with a higher degree of connections.[21] In the 38-node-DAMP_ *ICP30* network, significant enrichment of the ankirin-1 and spectrin beta chain PPI may suggest early cellular structural instability due to anchoring proteolysis with intracellular structure displacement, induced voltage-gated sodium-channel malfunctioning and cell integrity disruption.[22] This PPI persisted in the analysis of the 8-node DAMP_*ICP30* pathway (with node degree ≥ 1.5), suggesting a hierarchical role of these pathways in the initial phases after ABI. Post-translational modifications of regulatory proteins may be present as suggested by the high polyubiquitin-C (UBC)-ubiquitin-like modifier-activating enzyme 1 (UBA1) and polyubiquitin-C (UBC)-proteasome activator complex subunit 1 PPI scores. Functional enrichment analysis of the 38-node *DAMP_ICP30* network suggested that molecular mechanisms involved in post-translational modifications after ABI may include tyrosine sulfation and protein glycation.[23,24] However, such interactions were lost when analyzing the *DAMP_ICP30* network nodes with a higher degree of connectivity.

The clinical implications of these results are 2-fold. First, DAMP networks may be important therapeutic targets in conditions where inflammation pathologically increases endothelial permeability, endothelial cell damage, and microcirculatory disturbances.[25,26] Second, DAMP networks could be used as biomarkers to identify patients at higher risk of more severe disease or complications.[9,27,28] Moreover, nodes with higher ICSs are often informative with respect to reaction interplay and reversibility of the ability of DAMPs to induce cell injury.[29] The notion of multiple adverse protein pathways is important as it could potentially help to develop




| | |
|---|---|
| number of nodes | 38 |
| number of edges | 18 |
| average node degree | 0.947 |
| avg. local clustering coefficient | 0.408 |
| expected number of edges | 8 |
| PPI enrichment p value | 0.000867 |

| | |
|---|---|
| number of nodes | 8 |
| number of edges | 6 |
| average node degree | 1.5 |
| avg. local clustering coefficient | 0.458 |
| expected number of edges | 1 |
| PPI enrichment p value | 0.000301 |

**FIGURE 2.** Systems biology construct showing ventricular cerebrospinal fluid protein-protein interaction (*DAMP_ICP30*) in patients with intracranial pressure ≥ 30 mm Hg and interaction confidence score > 0.4. A, With all nodes. B, With an average node degree of 1.5. Protein IDs correspond to Uniprot IDs (for expansions of abbreviations, see Supplemental Digital Content 5, http://links.lww.com/JNA/A588). Node: protein of interest; edges: connections between nodes; degree (of a node): number of edges incident to that node; clustering coefficient: local density of connections. PPI indicates protein-protein interaction.

future targeted therapeutic strategies, enabling a multifaceted, individualized treatment approach for ABI.[30,31] Moreover, genetics studies have begun to identify individual differences in polymorphisms that could affect recovery and cognitive and social processing outcomes after traumatic brain injury.[32]

The study has some limitations. First, the identification of DAMPs can be problematic because the consistency and accuracy of DAMP identification and alleged function may depend on the method used for protein identification.[33] Second, the identification of DAMPs depends on the presence of an external ventriculostomy for vCSF analysis; however, ABI patients requiring an intraventricular catheter do not represent the full spectrum of those with ABI, creating a potential patient selection bias. Moreover, the translation of these results to clinical practice is currently limited because

these proteins are not measured on a regular basis. Third, the heterogeneity of the groups may be viewed as a cause of bias; no adjustment for confounding factors was made across groups in the multivariable analyses because of the small sample sizes in the subgroups. Fourth, contamination of vCSF, for example, with blood,[34] after ABI may affect the CSF protein content and thus, potentially, have impacted our results, especially if not correlated with serum samples. Fifth, dichotomizing the patients into ICP $\geq$ 20 mm Hg and $\geq$ 30 mm Hg groups could be viewed as a crude assessment of the burden of intracranial hypertension; the area under the ICP curve may be a more robust method of assessing episodes of high ICP due to high cerebral blood volume, brain edema, or hydrocephalus.[6] Moreover, because of our methodology, we were unable to determine causality for the secondary outcomes. Finally, we cannot state that local factors, such as therapy and/or surgery, had no influence on the formation of the vCSF DAMPs, which may therefore not be related solely to the underlying ABI.

## CONCLUSIONS

PPIs among DAMPs were different between patients with traumatic and those with nontraumatic ABI and between patients with severe intracranial hypertension compared with those with lower ICP values. Evaluating a panel of biomarkers depicting different but complementary biological pathways could provide a more comprehensive view than a single biomarker analysis on the ability of the neuron to respond to secondary insults and on the prediction of severe intracranial hypertension.

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