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Original research

Dynamics of synaptic damage in severe traumatic brain injury revealed by cerebrospinal fluid SNAP-25 and VILIP-1

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ABSTRACT

Background Biomarkers of neuronal, glial cells and inflammation in traumatic brain injury (TBI) are available but they do not specifically reflect the damage to synapses, which represent the bulk volume of the brain. Experimental models have demonstrated extensive involvement of synapses in acute TBI, but biomarkers of synaptic damage in human patients have not been explored.

Methods Single-molecule array assays were used to measure synaptosomal-associated protein-25 (SNAP-25) and visinin-like protein 1 (VILIP-1) (along with neurofilament light chain (NFL), ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillar acidic protein (GFAP), interleukin-6 (IL-6) and interleukin-8 (IL-8)) in ventricular cerebrospinal fluid (CSF) samples longitudinally acquired during the intensive care unit (ICU) stay of 42 patients with severe TBI or 22 uninjured controls.

Results CSF levels of SNAP-25 and VILIP-1 are strongly elevated early after severe TBI and decline in the first few days. SNAP-25 and VILIP-1 correlate with inflammatory markers at two distinct timepoints (around D1 and then again at D5) in follow-up. SNAP-25 and VILIP-1 on the day-of-injury have better sensitivity and specificity for unfavourable outcome at 6 months than NFL, UCH-L1 or GFAP. Later elevation of SNAP-25 was associated with poorer outcome.

Conclusion Synaptic damage markers are acutely elevated in severe TBI and predict long-term outcomes, as well as, or better than, markers of neuroaxonal injury. Synaptic damage correlates with initial injury and with a later phase of secondary inflammatory injury.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Synaptic damage has been demonstrated to be a consequence of primary and secondary injury mechanisms in experimental models of traumatic brain injury (TBI).
⇒ Markers of synaptic damage such as synaptosomal-associated protein 25 (SNAP-25), are actively investigated for diagnostic and prognostic applications in neurodegenerative diseases.

WHAT THIS STUDY ADDS

⇒ Longitudinal studies of synaptic damage markers in human severe TBI were so far missing and the dynamics of acute synaptic damage in vivo were unknown.
⇒ By using commercially available and newly established single-molecule array tests, we demonstrate the acute rise and decrease of SNAP-25 and VILIP-1 in ventricular cerebrospinal fluid (), along with their correlation with neuroaxonal damage markers, and their correlation at two points with early and subacute neuroinflammatory responses.
⇒ We show that day-of-injury SNAP-25 levels in CSF have high specificity and selectivity in predicting unfavourable outcomes.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Synaptic damage markers can be added to the biomarkers panel for monitoring patients with TBI.
⇒ Synaptic damage markers can be investigated in multicentric studies to validate their prognostic, stratification and treatment-response value.

INTRODUCTION

Traumatic brain injury (TBI) simultaneously affect neuronal cell bodies and their processes, glial cells, myelin and blood vessels through physical forces (primary injury) and through oxidative, excito-toxic, necrotic damage and neuroinflammation (secondary injury).[1]

Synapses constitute a substantial fraction of the brain parenchyma[2] and display unique sensitivity to mechanical damage, ischaemia, excitotoxicity and neuroinflammation.[3 4] Acute synaptic damage following TBI has been demonstrated in murine models[5] and may be relevant in acute and long-term evolution of TBI.[6] Thus, a set of biomarkers of synaptic damage would enable the exploration of their integrity in TBI.

Synaptosomal-associated protein 25 (SNAP-25) is a synaptic protein enriched in the presynaptic space,[7] while visinin-like protein 1 (VILIP-1) is enriched in both presynaptic and postsynaptic

structures.[8] SNAP-25 and VILIP-1 have been shown to be elevated in cerebrospinal fluid (CSF), indicating synaptic damage in Alzheimer's disease, Creutzfeldt-Jakob disease[9 10] and fronto-temporal dementia.[11] Thus, SNAP-25 and VILIP-1 may provide an entry point to monitor synaptic damage in TBI.

CSF and/or serum biomarkers reflect the integrity of different cell types in TBI: astrocytic glial fibrillar acidic protein (GFAP) is associated with acute injury and long-term prognosis[12]; the neuro-axonal protein neurofilament light chain (NFL) and neurocytoplasmic ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) correlate with the traumatic axonal injury and brain atrophy.[13 14] Inflammation biomarkers such as interleukin-6 (IL-6), interleukin-8 (IL-8) and matrix metallopeptidase 9 are strongly correlated with tissue damage, neuroimmunological response and prognosis.[15 16]

Here, we investigate the time course of SNAP-25 and VILIP-1 in the CSF of patients with severe TBI (from the Melbourne neurotrauma cohort, the subject of a previous publication)[3] and their correlation with markers of neuroaxonal damage (NFL, UCH-L1), inflammation (IL-6, IL-8) and clinical severity and outcome measures.

METHODS

Patients

Inclusion criteria were severe TBI with a postresuscitation Glasgow Coma Scale (GCS) ≤8 (unless initial GCS >9 was followed by deterioration requiring intubation) and, on CT imaging, the need for an extraventricular drain for Intra Cranial Pressure (ICP) monitoring and therapeutic drainage of CSF. CSF was collected over 24 hours and kept at 4°C; samples were obtained on admission (day 0; D0) and daily up to day 12 after injury. Within an hour from collection, samples were centrifuged at 2000×g for 15 min at 4°C and stored at −80°C until analysis. Exclusion criteria comprised pregnancy, known neurodegenerative diseases, HIV and other chronic infection/inflammatory diseases or history of TBI. As a control cohort, lumbar CSF samples from patients diagnosed with tension-type headache were used (Ulm reference). Clinico-demographic details for TBI and for the reference cohort are reported in table 1. The cohort biosamples have been subject of publication before.[3 17]

Single-molecule array assay for SNAP-25, UCH-L1, IL-6 and IL-8

SNAP-25, UCH-L1, IL-6 and IL-8 concentration was measured with the Quanterix (Quanterix, Lexington, USA) single-molecule array (SIMOA) HD-1 analyzer using commercially available assays (SNAP-25, IL-6 and IL-8 Advantage Kit, UCH-L1 Discovery Kit), according to manufacturer's instructions. For SNAP-25, samples were diluted with sample buffer (provided by the company) to a final concentration of 1:20 dilution (1:5 in the plate and 1:4 in the HD-1 analyzer). For UCH-L1, a final dilution of 1:60 dilution (1:15 in the plate and 1:4 in the HD-1 analyzer) was implemented. For IL-6, the final dilution was 1:100 (1:25 in the plate and 1:4 in the HD-1 analyzer) and for IL-8 the final dilution was 1:20 dilution (1:5 in the plate and 1:4 in the HD-1 analyzer). Groups were randomised on the different assay plates and CSF quality control (QC) samples were included in all runs. The intra-assay and inter-assay coefficients of variation (CV) for SNAP-25 were 15% and 22%, for UCH-L1 were 14% and 19%, for IL-6 were 15% and 17% and for IL-8 were 16% and 24%.

Table 1 Clinico-demographic characteristics of patients with TBI and control subjects

Variables	Trauma group Melbourne	Control group Ulm
Age, years, median (range)	25 (15–66)	66 (57–80)
Male/Female (%)	33/9 (78.6)	8/14 (36.4)
GCS, median (range)	5 (3–13)	–
Hypoxia, n (%)	18 (42.9)	–
ISS, median (range)	35 (30–43)	–
GOSE, median (range)	3 (1–8)	–

GCS, Glasgow Coma Scale; GOSE, Glasgow Outcome Scale Extended; ISS, Injury Severity Score; TBI, traumatic brain injury.

Optimisation of SIMOA assay and determination of VILIP-1 levels in CSF

CSF VILIP-1 concentrations were measured with the Quanterix SIMOA HD-1 analyzer in Ulm, using a VILIP-1 assay as previously reported.[18] Briefly, the capture antibody was coated to carboxylated paramagnetic beads, in a concentration of 0.2 mg/mL, according to manufacturer's instructions (Quanterix, Massachusetts, USA). A reduction and replacement of coupled active beads for helper beads was performed, using approximately 350 000 helper and 150 000 active beads per sample. The beads were washed twice, with 1% bovine serum albumin (BSA), 1× phosphate-buffered saline (PBS) and 0.05% Tween-20, on a magnetic separator and subsequently diluted in the same buffer. The biotinylated VILIP-1 detection antibody was diluted to a concentration of 0.5 µg/mL, in the same buffer. Streptavidin-β-galactosidase concentrate (SβG) was diluted to a final concentration of 25 pM in SβG-diluent (Quanterix, Lexington, USA). Resorufin-β-D galactopyranoside substrate was used as provided by the manufacturer's instructions (Quanterix, Lexington, USA). CSF samples were diluted 1:10 (in 1% BSA in PBS with 0.05% Tween-20) in a 96-well plate (Quanterix, Lexington, USA) and placed into the HD-1 analyzer. A two-step assay protocol was chosen for assay configuration. In the first step, the samples were incubated with 25 µL of bead and 20 µL biotinylated antibody solution for 30 min (40 cadences) in a reaction cuvette, followed by several wash steps. In the second step, the samples were incubated with 100 µL of SβG for 5 min and 15 s (7 cadences), followed by substrate addition and automated imaging. The analysis was performed on the HD-1 analyzer software V.1.5 (Quanterix, Lexington, USA). A four-parameter logistic curve with 1/y weighting was applied. Groups were randomised on the different assay plates and CSF QC samples were included in all runs. The intra-assay and inter-assay CV were 5% and 9%.

ELLA assay for NFL determination

CSF NFL concentrations were measured with the ProteinSimple Ella instrument (Bio-Techne, Minnesota, USA) as previously reported,[19] using the human NFL Kit according to manufacturer's instructions with a 1:10 dilution for controls and 1:50 for patients. Groups were randomised on the different assay plates and CSF QC samples were included in all runs.

ELISA for GFAP determination

CSF GFAP concentration was measured with the Human GFAP ELISA (BioVendor, Czech Republic) according to manufacturer's instructions with a 1:2 dilution for controls and 1:750 for patients. Groups were randomised on the different assay plates and QC samples were included in all runs. The intra-assay and inter-assay CV were 11% and 14%.

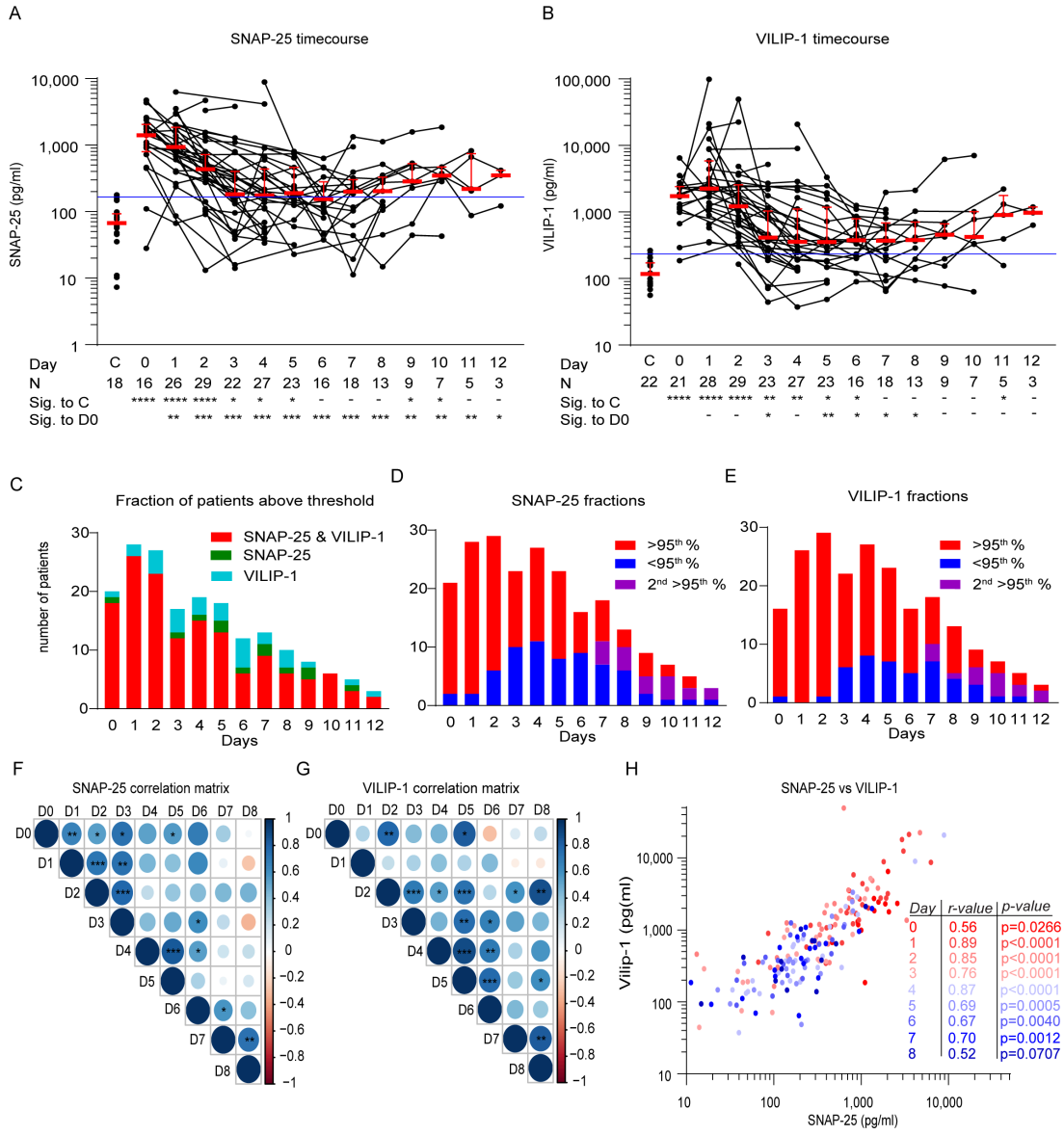

Figure 1 Synaptic proteins synaptosomal-associated protein-25 (SNAP-25) and visinin-like protein 1 (VILIP-1) are elevated in cerebrospinal fluid on severe traumatic brain injury (TBI). (A–B) Timecourse of SNAP-25 (A) or VILIP-1 (B) in the ventricular cerebrospinal fluid (CSF) of patients with severe TBI. (C) Fraction of patients with only SNAP-25 (green), only VILIP-1 (cyan) or both (red) above the normal range (95th percentile of the reference cohort). (D–E) Fraction of patients with SNAP-25 (D) or VILIP-1 (E) levels below (blue) or above (red) the upper limit of the normal range or displaying a second elevation after initial normalisation (purple). (F–G) Correlation of SNAP-25 levels (F) or VILIP-1 levels (G) across the timepoints matrix. Colour and size code for the Spearman's correlation coefficient (blue=positive) and significance shown with asterisks. (H) Pairwise correlations of SNAP-25 with VILIP-1 across multiple timepoints; each dot corresponds to a single patient/timepoint. Spearman's correlation coefficient and statistical significance are reported in the table. In A–B: median (red bar) and IQR are depicted. *P<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Statistical analysis

Data analysis was performed with the GraphPad Prism software V.8. Two individual comparisons were made: first, groups were compared with the reference group (control group) by performing a Kruskall-Wallis test with Dunn's multiple correction and second, a mixed linear model with false discovery rate (FDR) (0.05) correction was used to compare the various timepoints to D0. Correlation matrices were performed using the Spearman's rho correlation comparing each target with SNAP-25 and VILIP-1 from D0 to D8. Later timepoints (D9–D12) were disregarded for this analysis, due to the limited amount of data points. The upper normal reference value was set at the 95th percentile of the reference group; this value was depicted as a blue line in the graphs of each marker. Data points above this value were considered abnormal and data points below this value were considered normal. Analysis of SNAP-25 and VILIP-1 with the clinical data was performed using the median value of the variables (Glasgow Outcome Scale Extended (GOSE); GCS and Injury Severity Score (ISS)) as separation of the two groups, Mann-Whitney U tests (one per row), with FDR (0.05) correction was used to compare the clinical variables. Receiver operating characteristic (ROC) analysis, using the Wilson-Brown method, was performed to identify a threshold value for the prognosis of unfavourable outcome at D1 and D5. The Youden index was calculated to determine the most optimal cut-off value, providing optimal sensitivity and specificity for that value.

The principal component analysis (PCA) was performed using R-studio software (V.4.3.1). A 'leave-one-out cross-validation'

has been performed for the estimation of optimal number of components, followed by imputation with iterative PCA to estimate missing values based on the identified components.

The data were presented as scatter plot with median and IQR on a log(10) scale, with each patient depicted as a single data point in the graphs. Statistical significance was defined as $p<0.05$.

RESULTS

Synaptic proteins SNAP-25 and VILIP-1 are elevated in cerebrospinal fluid on severe TBI

We measured SNAP-25 and VILIP-1 in ventricular CSF samples longitudinally obtained from patients with severe TBI during the course of their stay in the intensive care unit (ICU). As a normal reference, lumbar CSF from patients with tension-type headache were considered. The clinico-demographic characteristics of the cohorts are reported in table 1.

On admission (D0), patients with TBI displayed a strong increase in SNAP-25 and VILIP-1 in CSF compared with reference values (SNAP-25 and VILIP-1: $p<0.0001$; figure 1A,B and table 2), although with substantial interindividual variability. Median SNAP-25 values peaked already at D0, whereas median VILIP-1 peaked 24 hours later (D1), although in the latter the median increase between D0 and D1 was due to a small proportion of patients with upward trends. Both proteins displayed an overall decreasing trend over time: median SNAP-25 levels became significantly lower than D0 from D1 onward (figure 1A), whereas median VILIP-1 remained statistically comparable to D0 across all timepoints (except for D3 and D5–D8; figure 1B). Most patients displayed elevation (above the 95th percentile of the reference cohort) of both proteins at the same timepoint, although a small number of patients displayed elevation only in one of the two (figure 1C).

Table 2 Median and IQR of all markers over the complete timecourse

	Median (IQR, pg/mL); n						
Markers	**Control**	**D0**	**D1**	**D2**	**D3**	**D4**	**D5**
SNAP-25	57 (50–89) 18	1404 (877–2029) 16	904 (555–1724) 26	434 (324–717) 29	183 (73–399) 22	176 (112–400) 27	190 (143–463) 23
VILIP-1	117 (86–168) 22	1735 (1080–2358) 21	2135 (1175–4105) 28	1190 (639–2490) 29	412 (221–966) 23	355 (196–932) 27	353 (182–950) 23
NFL	1014 (806–1268) 19	4571 (2423–8203) 19	6323 (3781–16 229) 28	9352 (4554–17 937) 29	9755 (3929–13 378) 22	8059 (4431–15 147) 28	9343 (6712–20 969) 25
UCH-L1	317 (0–355) 19	31 036 (10 235–62 269) 21	19 905 (10 801–47 658) 29	6646 (3019–13 710) 29	2360 (727–7523) 23	1085 (539–4609) 28	1250 (596–5294) 24
GFAP (ng/mL)*	2 (1–3) 24	5191 (3045–5765) 21	2434 (1100–4550) 26	983 (350–2797) 27	220 (65–558) 21	116 (62–569) 27	157 (84–591) 20
IL-6	2 (1–8) 24	3524 (584–6753) 20	2749 (416–4771) 27	726 (269–5434) 29	114 (26–214) 23	170 (58-610) 28	121 (22–583) 24
IL-8	29 (9–92) 20	6265 (2181–12 201) 19	2186 (664–5438) 29	203 (86–720) 28	1099 (476–1780) 22	99 (36–831) 29	269 (35–1299) 24
	D6	**D7**	**D8**	**D9**	**D10**	**D11**	**D12**
SNAP-25	156 (108–255) 16	200 (103–288) 18	204 (121–309) 13	285 (227–517) 9	348 (299–423) 7	220 (205–664) 5	352 (237–386) 3
VILIP-1	372 (228–761) 16	372 (193–627) 18	380 (196–704) 13	456 (197–632) 9	423 (331–892) 7	903 (394–1310) 5	975 (804–1083) 3
NFL	12 358 (9400–22 921) 18	17 507 (5463–34 151) 19	25 786 (6830–37 364) 14	22 552 (6749–26 904) 8	21 551 (13 795–41 010) 6	35 523 (33 419–56 858) 5	56 279 (39 874–66 545) 3
UCH-L1	1913 (1519–3152) 17	1383 (615–3700) 19	1127 (635–1967) 12	–	–	–	–
GFAP (ng/mL)*	93 (55–253) 16	92 (39–662) 16	83 (48–301) 12	–	–	–	–
IL-6	80 (11–632) 18	93 (17–471) 19	264 (21–2967) 13	–	–	–	–
IL-8	37 (18–342) 18	1151 (479–3988) 19	438 (27–642) 13	–	–	–	–

*all analyte concentrations are reported in pg/mL, only for GFAP the concentration is reported in ng/mL
GFAP, glial fibrillar acidic protein; IL, interleukin; NFL, neurofilament light chain; SNAP-25, synaptosomal-associated protein-25; UCH-L1, ubiquitin carboxy-terminal hydrolase L1; VILIP-1, visinin-like protein 1.

The trajectories of SNAP-25 and VILIP-1 in individual patients revealed distinct subgroups. Approximately half of patients had SNAP-25 levels returning below the upper normal reference threshold by D6 (56%; figure 1D). By contrast, 44% of patients displayed persistently elevated SNAP-25 levels. Notably, 22% of patients displayed a second elevation of SNAP-25. For VILIP-1, most patients stayed above the reference threshold for the duration of the follow-up (at D7 only 38% were below the reference, figure 1E). Also, in this case 16% showed a second peak of VILIP1 during their ICU stay.

SNAP-25 levels over several consecutive days were highly correlated, in particular at the earlier timepoints (notably between D0–D3 and D4–D6; figure 1F): values at D0 predicted the values at D1, D2, D3 and D5. VILIP-1 was not correlated across the early days (D0–D1) but displayed a strong correlation across consecutive days between D2 and D5 (figure 1G). The levels of SNAP-25 and VILIP-1 in each patient and at each timepoint were highly correlated (figure 1H).

Synaptic damage marker SNAP-25 predicts later neuronal and axonal injury

We explored the correlation between SNAP-25 and VILIP-1 and three biomarkers of neuronal damage: NFL,[13] UCH-L1 and GFAP.[14]

CSF NFL was elevated at D1 (compared with the reference) and increased over time (median-D0=4571 pg/mL vs median-D12=56 279 pg/mL, p<0.001; figure 2A and table 2). Conversely, UCH-L1 was significantly elevated at D0 but declined towards the reference threshold thereafter (median-D0=31 036 pg/mL vs median-D12=1126 pg/mL, p<0.0001; figure 2B and table 2). On the other hand, GFAP was elevated at D0 and remained so through the monitoring period, although with a steady declining trend (figure 2G and table 2).

Although the overall trend of SNAP-25 and VILIP-1 were more closely related to UCH-L1 and GFAP (early peak and downward slope) than to NFL, synaptic and neuroaxonal/glial biomarkers displayed a significant pairwise correlation at each timepoint (figure 2C–F, H–I). Levels of SNAP-25 at D0 and D2 predicted the values of NFL and UCH-L1 at D5 and D7 (figure 2C,D) and of GFAP at D3 and D6 (figure 2H,I), whereas NFL and UCH-L1 at D0–D2 values did not predict future SNAP-25 levels. VILIP-1 and NFL levels broadly cross-correlated at the majority of timepoints considered (figure 2E) but correlated with UCH-L1 and GFAP values at the same timepoint (figure 2F and I).

Synaptic damage revealed by VILIP-1 and SNAP-25 correlates with acute and with subacute neuroinflammatory response

Neuroimmunological responses contribute to synaptic damage.[4 20 21] We thus investigated the correlation between VILIP-1 and SNAP-25 levels and the extent of neuroinflammation as assessed by IL-8 and IL-6 levels.

IL-6 was already elevated at D0 compared with the reference thresholds and remained high across all timepoints (figure 3A and table 2). On the other hand, IL-8 was significantly increased at D0 and D1 in patients with TBI but oscillated later on: a second elevation was detected at D3 and a third at D7 (figure 3B and table 2). SNAP-25 and IL-6 displayed a peculiar correlation pattern: the two analytes were significantly correlated at D0 and D1, and then again at D4 (figure 3C). Similarly, SNAP-25 correlated with IL-8 in the early stages (D0 and D1) and, although to a lesser extent, with D4 and D5 (figure 3D). Likewise, VILIP-1 levels displayed two peaks of correlation with IL-6: at D1–D2 and again from D4 to D7 (figure 3E). Interestingly, VILIP-1 correlated with IL-8 at D1–D2 and again at D4–D5 (figure 3F). At least two correlation peaks were also detected between UCH-L1 and IL-6 (D0–D1, D4 and, although with fewer available samples D7; figure 3G) and IL-8 (figure 3H) and between NFL and IL-6 or IL-8 (D1 and D4–D8; figure 3I,J). GFAP levels correlated with IL-6 and IL-8 at D1–D2 and again D5–D6 (figure 3K,L).

Acute synaptic damage markers predict long-term recovery after TBI

Finally, we investigated whether the synaptic damage biomarkers correlate with clinical measures of TBI and overall injury severity (GCS and ISS at admission) or with long-term outcomes (GOSE). We divided the patients according to the median value of GCS (3–5 vs 6–10), or of ISS (<35 vs >35) or GOSE (1–3 vs 4–8) and compared the two groups across the timepoints of the follow-up. No significant difference in SNAP-25 or VILIP-1 was found between patients with GCS 3–5 or 6–10, neither on admission nor at later timepoints; likewise, SNAP-25 and VILIP-1 were comparable in patients in the two ISS groups (online supplemental figure 1A–D).

When patients were divided according to the median GOSE score at 6 months (1–3 vs 4–8), we found that very strong trends (q=0.06) towards lower levels of SNAP-25 in patients with more favourable prognosis (GOSE 4–8) at D2–D6 (figure 4A). Furthermore, significantly lower levels of VILIP-1 were observed for patients with favourable prognosis at D5 and D6 (q=0.024; figure 4B). Interestingly, patients displaying secondary elevation in SNAP-25 and VILIP-1 had an unfavourable prognosis.

We further investigated if the combination of synaptic, neuroaxonal, glial and inflammation markers could better separate patients with favourable versus unfavourable prognosis. PCA (as previously applied[22]) revealed that the two main components accounted for approximately 74% of variance at D1 and 78% at D5 (figure 4C and 4E). At D1, the vectors corresponding to UCH-L1, NFL, GFAP and IL-6 clustered together in the first component, whereas VILIP-1, SNAP-25 and IL-8 diverged, being closer to the second component (figure 4D); conversely, at D5 most markers clustered more toward the first component, with the notable exception of IL-6 (figure 4F).

We investigated if a threshold could be set either at D1 (earliest timepoint after patient stabilisation) or at D5 (stable patient in ICU), which could predict unfavourable prognosis (GOSE 1–3). We used the Youden index to determine the optimal cut-off value at D1 (1034 pg/mL for SNAP-25 and 2415 pg/mL for VILIP-1), which displayed for SNAP-25 a 78% sensitivity and 80% specificity, and for VILIP-1 a 75% sensitivity and 67% specificity. In comparison, UCH-L1, NFL and GFAP at D1 displayed a comparable or lower specificity and selectivity (table 3). Combinations of multiple analytes (SNAP-25+VILIP-1, SNAP-25+VILIP-1+UCH-L1, SNAP-25+VILIP-1+GFAP) did not result in a substantial change in the sensitivity and specificity values compared with SNAP-25 alone. Overall, SNAP-25 displayed the larger value of area under the curve for ROC curve and the highest likelihood parameter.

At D5, the calculated cut-off for SNAP-25 (294 pg/mL) delivered a 67% sensitivity and 91% specificity. For VILIP-1, the cut-off (556 pg/mL) resulted in 67% sensitivity and 90% specificity. At D5, UCH-L1, NFL or GFAP had a similar or lower specificity than SNAP-25 (91%, 83% or 78%, respectively) but better sensitivity (78%, 89% and 75%, respectively). A combination of

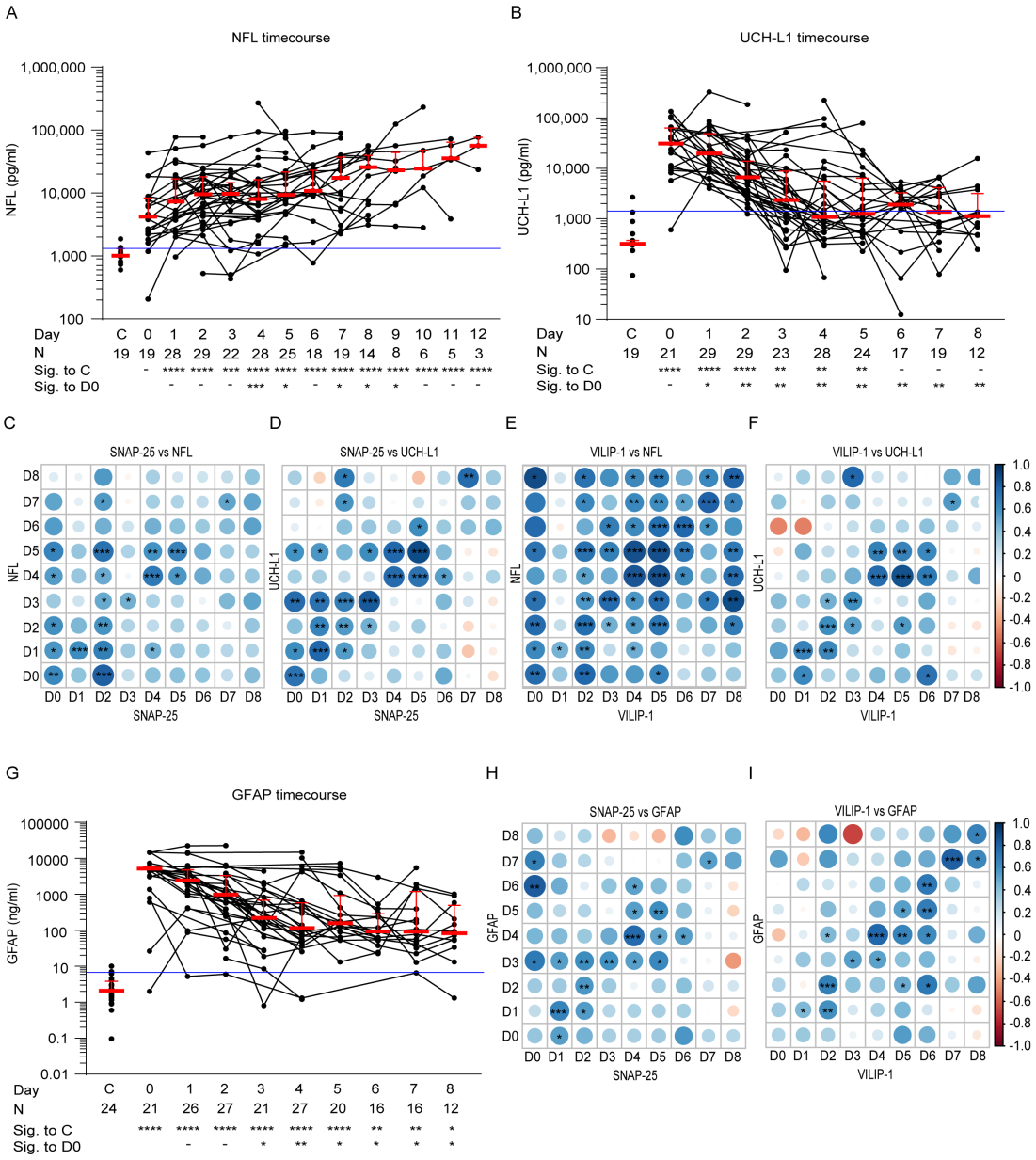

Figure 2 Synaptic damage marker synaptosomal-associated protein-25 (SNAP-25) predicts later neuronal and axonal injury. (A–B) Timecourse of neurofilament Light chain (NFL) (A) or ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) (B) in the ventricular cerebrospinal fluid (CSF) of patients with severe traumatic brain injury (TBI). (C–D) Cross-correlation (Spearman's) of SNAP-25 and NFL (C) or UCH-L1 (D) across timepoints. (E–F) Cross-correlation (Spearman's) of visinin-like protein 1 (VILIP-1) and NFL (E) or UCH-L1 (F) levels across timepoints. (G) Timecourse of glial fibrillar acidic protein (GFAP) in the ventricular CSF of patients with severe TBI. (H–I) Cross-correlation (Spearman's) of GFAP versus SNAP-25 (H) or VILIP-1 (I) across timepoints. In A–B and G: median (red bar) and IQR are depicted. Colour/Size code for Spearman's correlation coefficient (blue=positive) and statistical significance is indicated by asterisks. *P<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

several analytes increased sensitivity to 78%–89% but did not increase further the specificity.

Taken together, these data show that CSF SNAP-25 is the best prognostic marker at D1 compared with other damage markers and remains highly specific, although less sensitive, at D5.

DISCUSSION

We show that the synaptic biomarkers SNAP-25 and VILIP-1 are upregulated in the CSF of patients with TBI at D0 and decline between D1 and D8. Synaptic damage markers correlate with neuroinflammation markers at two distinct timepoints, D0–D1 and D5. Synaptic damage markers correlate with neuroaxonal markers up to D5 and predict later elevations of NFL. High levels of SNAP-25 and VILIP-1 at D1 and (to a lesser extent) at D5 predict unfavourable outcomes, as well as or better than neuroaxonal markers.

Although synaptic involvement in TBI has been ascertained in human samples[4 23] and in murine models,[5] their monitoring in humans has been limited to date. Our data demonstrate a multiphasic effect of TBI on synaptic markers. Overall, our findings are in agreement with the consistent elevation of neurogranin, beta-synuclein and other synaptic proteins in serum or CSF of TBI previously reported.[17 24 25] The SIMOA platform enabled the quantitative determination of SNAP-25 and VILIP-1 in individual samples and therefore demonstrated the previously unexplored individual kinetics.

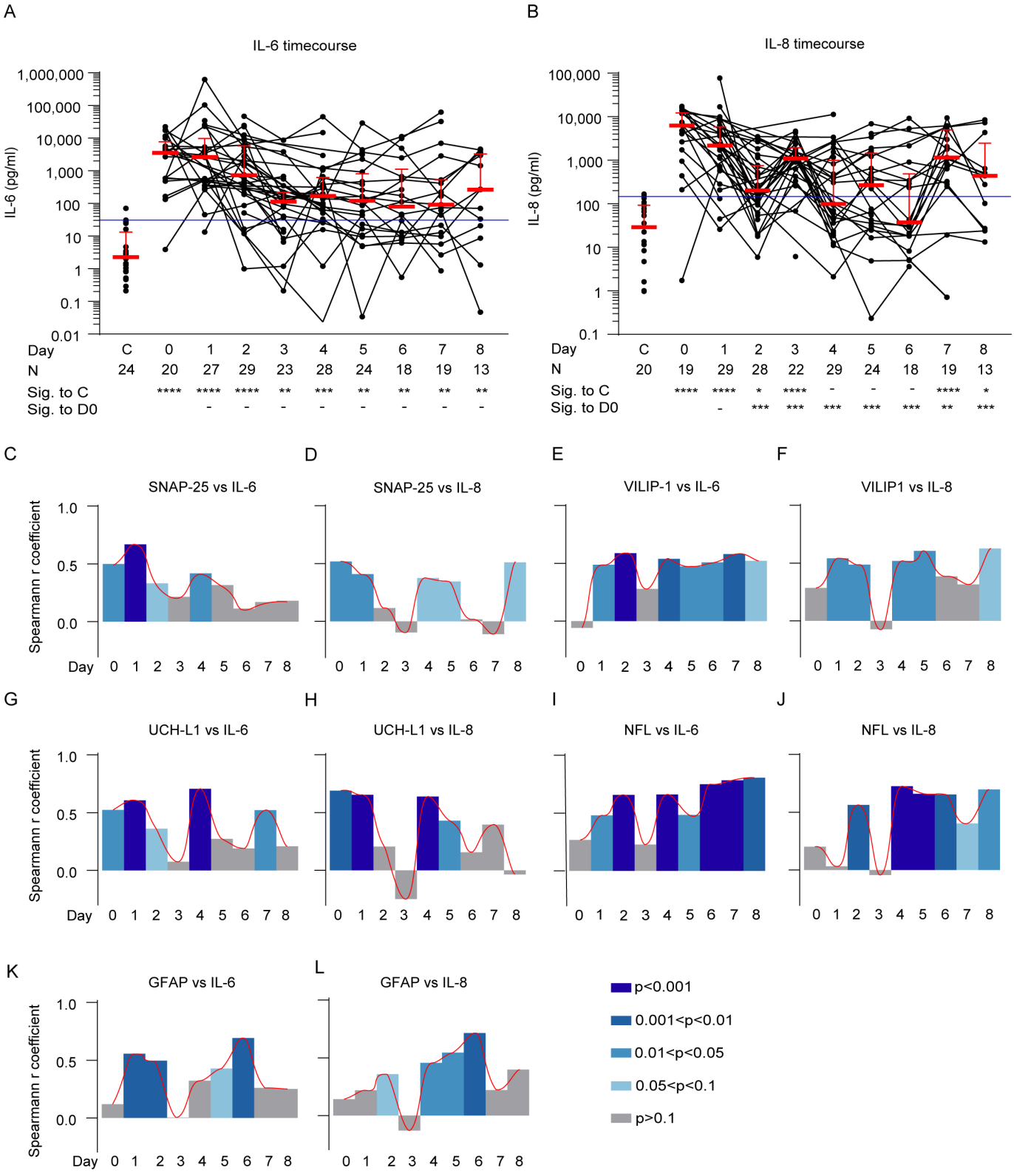

Figure 3 Early and late correlation of visinin-like protein 1 (VILIP-1) and synaptosomal-associated protein-25 (SNAP-25) and cytokines interleukin (IL)-6 and IL-8. (A–B) Timecourse of IL-6 (A) and IL-8 (B) in the ventricular cerebrospinal fluid (CSF) of patients with severe TBI. (C) SNAP-25 levels correlate with IL-6 levels at D0–D1 and again at D5. (D) SNAP-25 levels correlate (Spearman's) with IL-8 levels at D0–D1 and show a strong trend at D4–D5. (E) VILIP-1 levels correlate (Spearman's) with IL-6 at D1–D2 and again at D4–D7. (F) VILIP-1 correlates with IL-8 at D1–D2 and again at D4–D5. (G–H) Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) correlates with IL-6 (G) and IL-8 (H) at D0–D1 and again at D4–D5. (I–J) Neurofilament light chain (NFL) correlates with IL-6 (I) or IL-8 (L) at D1–D2 and again at D4–D8. (K–L) Glial fibrillar acidic protein (GFAP) correlates with IL-6 (K) or IL-8 (L) at D1–D2 and again at D5–D6. In A–B: median (red bar) and IQR are depicted. In C–L: Spearman's correlation coefficient was depicted by histogram height and statistical significance was coded as dark blue ($p<0.05$) or light blue ($p<0.1$). *P<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

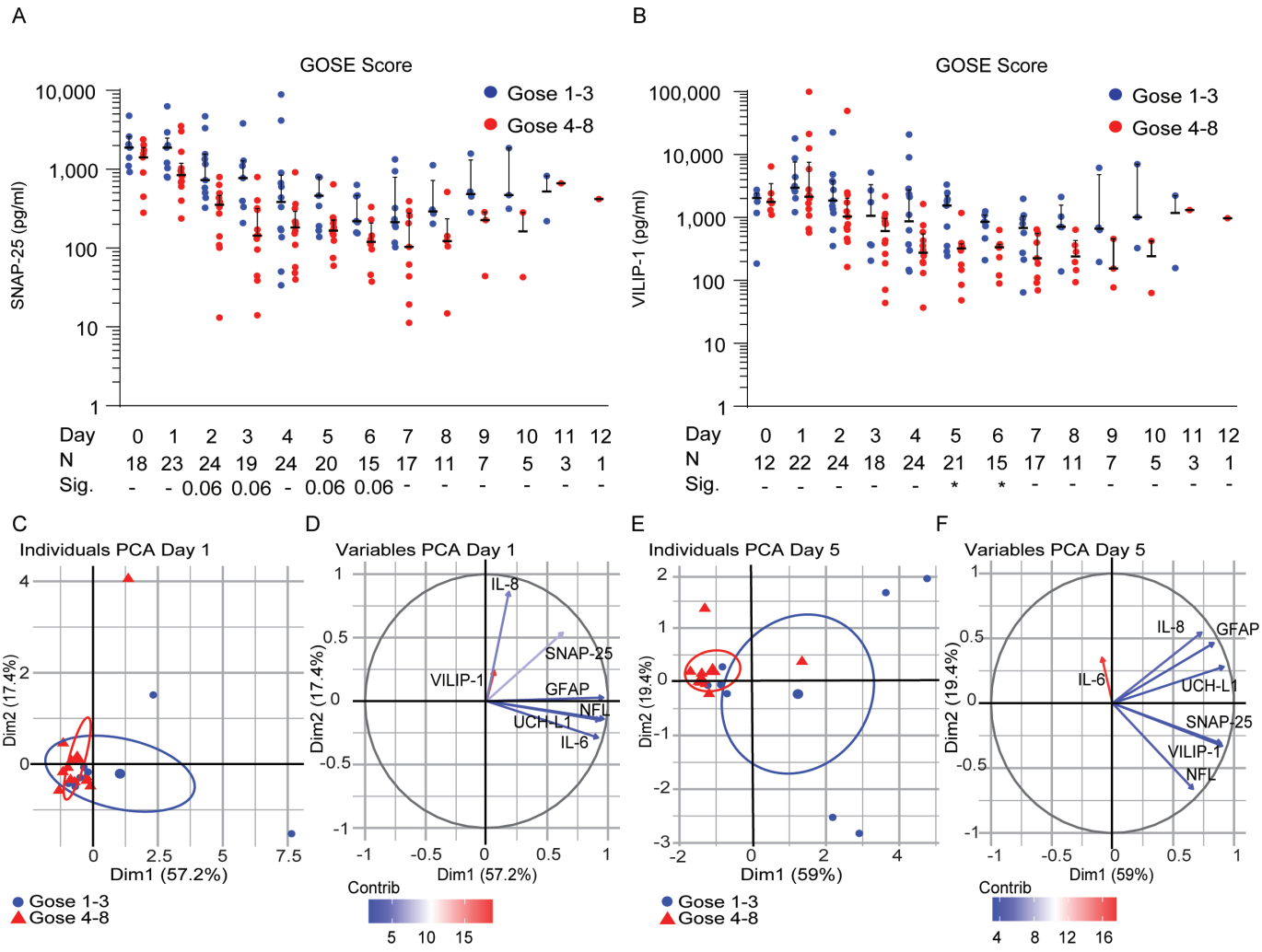

Figure 4 Early synaptosomal-Associated protein 25 (SNAP-25) long-term prognosis on severe traumatic brain injury (TBI). (A) Strong trend towards higher levels of SNAP-25 at D2–D3 and D5–D6 in patients with unfavourable versus favourable outcome (Glasgow Outcome Scale Extended (GOSE) at 6 months 1–3 vs 4–8). (B) Comparison of visinin-like protein 1 (VILIP-1) values between favourable (4–8) or unfavourable (1–3) GOSE. (C–F) Principal component analysis (PCA) (C,E) and PCA vector (D–F) analysis for the favourable versus non-favourable outcome groups. The first two dimensions explain approximately 74% (C) to 78% (E) of variance. SNAP-25 and VILIP-1 vectors diverge from the other neuroaxonal and glial damage markers at D1 (D) but not at D5 (F). Each data point represents one patients/timepoint; median and IQR are depicted (black bars). Group size and significance are reported in the graphs. *P<0.05.

SNAP-25 and VILIP-1 largely correlate at each timepoint; however, the faster rise of SNAP-25 and the correlation of early SNAP-25 with late VILIP-1 levels (but not the opposite, suggesting either a slower build-up of VILIP-1 or a slower effect of initial synaptic damage on VILIP-1 levels) suggest that SNAP-25 and VILIP-1 provide a differential assessment of synaptic damage in TBI.

SNAP-25 and VILIP-1 display a downward trend more closely resembling UCH-L1 and GFAP (displaying an early rise and a rapid decline, as also previously reported[14 26]) rather than NFL (which increases steadily over time).[13] This likely reflects the acute release of cytoplasmic and synaptic proteins and their faster turnover compared with the slower build-up of neurofilament proteins in the CSF. SNAP-25 enabled a better sensitivity and specificity than UCH-L1 (and VILIP-1) at D1 in terms of long-term prognosis, suggesting that synaptic damage may be a better day-of-injury read-out of overall tissue involvement than a neurocytoplasmic damage. Furthermore, in the PCA analysis, UCHL-1, GFAP and NFL vectors tend to converge towards the first component, whereas SNAP-25 and VILIP-1 are more divergent, suggesting that the synaptic markers explore a damage domain complementary (and only partially overlapping) to that of established neuroaxonal and glial damage markers.

SNAP-25 and VILIP-1 display a remarkable double-peak correlation with the neuroinflammation revealed by IL-6 and IL-8 (the smaller number of samples available for D8 and beyond makes conclusions about later timepoints unreliable). This finding is compatible with the conceptual framework of an early synaptic damage resulting from primary injury and a later synaptic damage resulting from inflammatory mechanisms. In fact, synaptic damage caused by reactive microglia, soluble mediators and infiltrating immune cells has been demonstrated in TBI models: ischaemia results in increased microglia-synaptic contact and then synaptic elimination[27] and reducing microglial reactivity also preserves synaptic contacts.[20 28] Moreover, complement-mediated synapse elimination[29–31] may contribute to the relationship between neuroinflammation and synaptic damage. Interestingly, the timing of the second peak corresponds

Table 3 Sensitivity and specificity of SNAP-25 and VILIP-1 and neuroaxonal injury markers for unfavourable prognosis at D1 and D5

Day 1	Cut-off	AUC (95% CI) (%)	P value	Sensitivity (95% CI) (%)	Specificity (95% CI) (%)	Likelihood	Youden index (%)
SNAP-25	>1034	78 (59 to 97)	0.025	78 (45 to 96)	80 (55 to 93)	3.89	58
VILIP-1	>2415	70 (50 to 90	0.107	75 (41 to 96)	67 (44 to 84)	2.25	42
UCH-L1	>11 139	53 (29 to 77)	0.815	100 (72 to 100)	29 (12 to 55)	1.40	29
NFL	>10 280	73 (52 to 94)	0.068	67 (35 to 88)	71 (45 to 88)	2.33	38
GFAP	>2434	72 (49 to 95)	0.096	63 (31 to 86)	62 (36 to 82)	1.63	24
SNAP-25+VILIP-1	–	74 (54 to 95)	0.061	88 (53 to 99)	60 (36 to 80)	2.19	48
SNAP-25+VILIP-1+UCH-L1	–	70 (47 to 93)	0.133	75 (41 to 96)	64 (39 to 84)	2.10	39
SNAP-25+VILIP-1+NFL	–	79 (59 to 98)	0.029	88 (53 to 99)	71 (45 to 88)	3.06	59
SNAP-25+VILIP-1+GFAP	–	74 (50 to 97)	0.088	57 (25 to 84)	85 (58 to 97)	3.71	42
Day 5	**Cut-off**	**AUC (95% CI) (%)**	**P value**	**Sensitivity (95% CI) (%)**	**Specificity (95% CI) (%)**	**Likelihood**	**Youden index (%)**
SNAP-25	>294	79 (58 to 100)	0.030	67 (35 to 88)	91 (62 to 100)	7.33	58
VILIP-1	>556	82 (63 to 100)	0.017	67 (35 to 88)	91 (62 to 100)	7.33	58
UCH-L1	>2130	84 (66 to 100)	0.011	78 (45 to 96)	91 (62 to 100)	8.56	69
NFL	>9460	87 (71 to 100)	0.005	89 (57 to 99)	83 (55 to 97)	5.33	72
GFAP	>157.2	72 (46 to 99)	0.124	75 (41 to 96)	78 (45 to 96)	3.38	53
SNAP-25+VILIP-1	–	87 (71 to 100)	0.006	78 (45 to 96)	82 (52 to 97)	4.28	60
SNAP-25+VILIP-1+UCH-L1	–	86 (69 to 100)	0.007	78 (45 to 96)	91 (62 to 100)	8.56	69
SNAP-25+VILIP-1+NFL	–	92 (80 to 100)	0.002	89 (57 to 99)	82 (52 to 97)	4.89	71
SNAP-25+VILIP-1+GFAP	–	82 (61 to 100)	0.027	75 (41 to 96)	89 (57 to 99)	6.75	64

AUC, area under the curve; GFAP, glial fibrillar acidic protein; NFL, neurofilament light chain; SNAP-25, synaptosomal-associated protein-25; UCH-L1, ubiquitin carboxy-terminal hydrolase L1; VILIP-1, visinin-like protein 1.

to the infiltration of macrophages[32 33] and the induction of reactive microglia.[34] Thus, SNAP-25 levels may provide a window into neuroinflammation-mediated synaptic damage in human patients.

The SNAP-25 and VILIP-1 trajectories identify a small group of patients who experiences a second elevation of synaptic damage. The sample size is insufficient to draw conclusions on these patients, and the clinical data recorded at the time of sampling do not include cues to the possible causes. One may speculate that infections[35] or status epilepticus[36] may be responsible for the secondary damage. Interestingly, these patients are not identifiable by the UCH-L1 or NFL patterns, suggesting that the synaptic damage may be a prominent pathophysiological event leading to the unfavourable prognosis.

The present work has some intrinsic limitations. First, the size of the cohort; it must be noted that longitudinal CSF samples datasets are uncommon and can be obtained only in tertiary neurosurgical ICUs and that the placement of Intra CerebroVentricular (ICV) catheters is constrained by the clinical indication. Second, related to the first limitation, only lumbar CSF was used as a reference cohort due to the unavailability of ventricular CSF samples from uninjured controls. Third, IL-6 and IL-8 levels served as proxy measures of the neuroimmunological response to the TBI; although these cytokines are well-characterised mediators of neuroinflammation in TBI,[37 38] they do not fully recapitulate the complexity of the neuroimmunological response to TBI.[39] Finally, we have not included a glial damage marker such as GFAP; since pristine samples (subject to less than one freeze-thawing cycle) were not available, the reliability of these assays may have been questionable.

Thus, this study is an exploratory investigation of synaptic damage markers in TBI. SNAP-25 levels appear to identify patients with unfavourable prognosis at the earliest timepoints and identify subgroups of patients with unfavourable course at later stages. These findings require extension in a larger, multicentre cohort to enable the characterisation of patient subsets and the use of stricter outcome measures. Nonetheless, these findings are promising and may prove to be useful for prognosis in the future.

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Contributors Conception and design of the study: FR, CM-K, MO, TMB, ACL. Sample collection and analysis: FoH, ZL, DR, SH, PO, SS, BS, HT. Data management and coordination: FR, CMK, MO, TB, PO. Statistical methods and analysis: FoH, ZL, NG, FR, BM. Interpretation of results: FR, CM-K, MO, TMB, ACL, HT, FoH. Manuscript writing (first draft): FR, CM-K, FoH, MO, PO. Critical revision of the manuscript: FoH, ZL, DR, SH, PO, SS, BS, ACL, TMB, DR, MO, CM-K, FR. All authors made substantial contributions to conception and design, and/or acquisition of data and/or analysis and interpretation of data. All authors gave final approval of the version to be submitted and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. FR is the guarantor.

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Competing interests None declared.

Patient consent for publication Consent obtained from next of kin.

Ethics approval The recruitment of patients and the use of CSF samples were authorised by the Alfred Hospital Human Ethics Committee with approval no. 194-05 and the analysis at Ulm University was authorised by the Ulm University Ethical committee with licence no. 264/22. The patients were prospectively recruited at the Alfred Hospital. Because TBI is an unpredictable condition, consent could not be obtained prior to the study. Because of the severity of the neurological condition with associated lack of consciousness, consent to the sampling was obtained by the next of kin. Forms are available on request.

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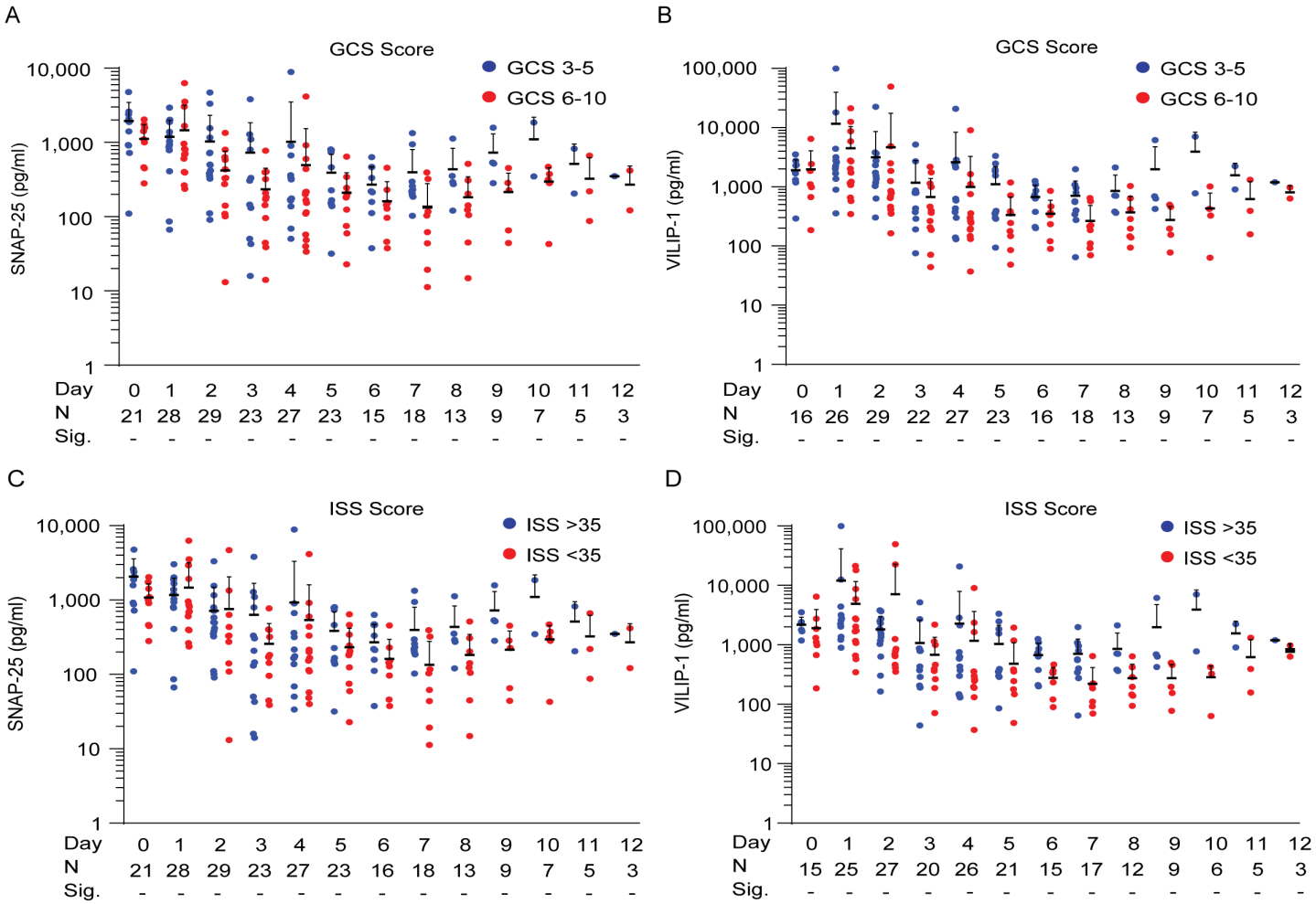
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GCS 6-10
SNAP-25 (pg/ml)
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1,000
100
10
1
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GCS Score
GCS 3-5
GCS 6-10
VILIP-1 (pg/ml)
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