An outlier analysis for acute blood biomarkers of moderate and severe traumatic brain injury

Otto Korhonen, BM[1,2,3], Malla Mononen, BM[1,2,3], Mehrbod Mohammadian, PhD[2, 3], Olli Tenovuo, MD, PhD[2, 3], Kaj Blennow, MD, PhD[9,10], Iftakher Hossain, MD, PhD[1, 2, 3, 4], Peter Hutchinson, PhD, FMedSci[4], Henna-Riikka Maanpää, MD[1, 2, 3], David K. Menon, PhD, FMedSci[7], Virginia F. Newcombe, MD, PhD[7], Jean-Charles Sanchez, PhD[14], Riikka S.K. Takala, MD, PhD[5], Jussi Tallus, MD[2, 3, 8], Mark van Gils, PhD[6], Henrik Zetterberg, MD, PhD[9, 10, 11, 12, 13,15], Jussi P. Posti, MD, PhD[1, 2, 3]

Names of Departments and Institutions: [1]Neurocenter, Department of Neurosurgery, Turku University Hospital, Finland; [2]Turku Brain Injury Center, Turku University Hospital, Finland; [3]Department of Clinical Neurosciences, University of Turku, Finland; [4]Department of Clinical Neurosciences, Neurosurgery Unit, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom; [5]Perioperative Services, Intensive Care Medicine and Pain Management, Turku University Hospital and University of Turku, Finland; [6]Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland; [7]Division of Anaesthesia, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom; [8]Department of Radiology, University of Turku and Turku University Hospital, Turku, Finland; [9]Institute of Neuroscience and Physiology, Department of Psychiatry and Neurochemistry, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden; [10]Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden; [11]Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London, UK; [12]UK Dementia Research Institute at UCL, University College London, London, UK; [13]Hong Kong Center for Neurodegenerative Diseases, Hong Kong, China; [14]Department of Specialities of Internal Medicine, Faculty of Medicine, University of Geneva, Geneva, Switzerland; [15]Wisconsin Alzheimer's Disease Research Center, University of Wisconsin School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, USA

Corresponding author: Jussi Posti, +358 2 313 0282 (tel), +358 2 313 3052 (fax), Neurocenter, Department of Neurosurgery, Turku University Hospital, P.O. Box 52, FI-20521 Turku, Finland

Running title: Outliers in moderate and severe TBI biomarker analysis

Key words: 1) blood biomarker, 2) diagnostics, 3) outcome, 4) traumatic brain injury

Abstract

Blood biomarkers have been studied to improve the clinical assessment and prognostication of patients with moderate–severe traumatic brain injury (mo/sTBI). To assess their clinical usability, one needs to know potential factors that might cause outlier values and affect clinical decision-making. In a prospective study we recruited patients with mo/sTBI (n = 85) and measured the blood levels of eight protein brain pathophysiology biomarkers, including glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), neurofilament light (NF-L), heart-type fatty acid-binding protein (H-FABP), interleukin-10 (IL-10), total tau (T-tau), amyloid β40 (Aβ40) and amyloid β42 (Aβ42), within 24h of admission. Similar analyses were conducted for controls (n = 40) with an acute orthopedic injury without any head trauma. The patients with TBI were divided into subgroups of normal vs. abnormal (n = 9/76) head computed tomography (CT) and favorable (Glasgow Outcome Scale Extended = GOSE 5-8) vs. unfavorable (GOSE < 5) (n = 38/42, 5 missing) outcome. Outliers were sought individually from all subgroups and the whole TBI patient population. Biomarker levels outside Q1 −1.5 IQR or Q3 +1.5 IQR were considered as outliers. The medical records of each outlier patient were reviewed in a team meeting to determine possible reasons for outlier values. A total of 29 patients (34%) combined from all subgroups and 12 patients (30%) among the controls showed outlier values for one or more of the eight biomarkers. 9 patients with TBI and 5 control patients had outlier values in more than one biomarker (up to 4). All outlier values were higher than Q3 +1.5 IQR. A logical explanation was found for almost all cases, except the amyloid proteins. Explanations for outlier values included extremely severe injury, especially for GFAP and S100B. In case of H-FABP and IL-10 the explanation was extracranial injuries (thoracic injuries for H-FABP and multi-trauma for IL-10), in some cases these also associated with abnormally high S100B. Timing of sampling and demographic factors such as age and pre-existing neurological conditions (esp. for T-tau), explained some of the abnormally high values especially for NF-L. Similar explanations also emerged in controls, where the outlier values were caused especially by pre-existing neurological diseases. To utilize blood-based biomarkers in clinical assessment of mo/sTBI, very severe or fatal TBIs, various extracranial injuries, timing of sampling and demographic factors such as age and pre-existing systemic or neurological conditions must be taken into consideration. Very high levels seem to be often associated with poor prognosis and mortality (GFAP and S100B).

Introduction

Traumatic brain injury (TBI) is considered one of the most complex and heterogeneous human diseases, which makes the clinical assessment a major challenge. TBIs are also recognized as a major global health issue with more than 50 million cases annually causing deterioration of quality of life and significant costs to society.[1] Moderate and severe traumatic brain injuries (mo/sTBI) represent only 10-20% of TBIs, but they have high mortality and disability rates, and also in many cases, they require neurosurgical intervention especially in case of sTBI.[2,3] It has been estimated that the acute care of sTBI costs more than 16 000€, but the direct medical costs represent only a minor portion of the total economic burden as indirect productivity losses account for the majority.[4] In the clinical management of TBI, one of the first steps is the assessment of severity, which is currently performed using imaging and clinical features such as level of consciousness (assessed with the Glasgow Coma Scale [GCS]) and duration of post-traumatic amnesia (PTA). However, these clinical features do not sufficiently reflect occurring complex pathophysiological processes, making the severity assessment and outcome prediction of TBI exceedingly challenging.

Biomarkers are being studied to improve prognostication, treatment monitoring, and severity assessment.[5] Astroglial biomarkers S100 calcium-binding protein B (S100B) and glial fibrillary acidic protein (GFAP) have been widely studied in the acute setting. The levels of both S100B and GFAP seem to correlate strongly with the severity of the initial injury, and GFAP also with functional outcome.[6–10] Also, blood levels of heart-type fatty acid-binding protein (H-FABP) and interleukin-10 (IL-10) have been able to distinguish patients with more severe TBIs from those with a mild TBI (mTBI), and they are shown to be promising candidates to predict the outcome.[7,8,11] Neurofilament light (NF-L) is abundantly expressed in axons thus reflecting axonal damage. Recent studies indicate that NF-L has shown promise in predicting outcome and severity in the subacute phase.[7,12] Along with NF-L, also less studied biomarkers β-amyloid isoforms 40 (Aβ40) and 42 (Aβ42) might have predictive value in the subacute phase for moderate and severe TBI (mo/sTBI).[13,14] Tau is a neurodegenerative biomarker that was initially used for neurodegenerative diseases and later found useful also in TBI diagnostics. It has been associated with severity based on clinical and radiological variables, and the plasma levels have also correlated with outcome.[8,12,13]

In mo/sTBI, the diagnosis does not require biomarkers, because the decision to perform a head computed tomography (CT) is usually clear and the findings are sufficient to make clinical decisions. The main need for biomarkers in mo/sTBIs lies in monitoring treatment and predicting outcome.[15] In patients with mo/sTBI it would be clinically important to identify those with an apparent unfavorable outcome and progressive brain injury to target treatment resources. Currently, the only clinical use of biomarkers is in assessing the need for head CT as this has attracted the most research interest in the field. However, several studies have reported outlier (exceptionally high or low) values in blood biomarker levels.[6,7,13,16–18] Many factors may affect these levels such as patient's age [16,19], integrity of the blood-brain barrier [20], glymphatic system functioning [21], extracellular proteolysis [22], and hepatic and renal functions [23,24]. Before biomarkers can be fully implemented into clinical practice, factors that may cause outlier biomarker levels that do not reflect actual injury have to be investigated to avoid misinterpretations.

It has been shown that several biomarker levels are consistently very high in patients with mo/sTBI [7], therefore we considered it clinically relevant to investigate the background of outlier levels in these patients. The aim of this study was to identify factors that might cause outlier plasma levels of S100B, GFAP, H-FABP, NF-L, IL-10, total tau (T-tau), Aβ40 and Aβ42 in a well-characterized cohort of patients with mo/sTBI.

Methods

Study population

In this post-hoc analysis of a prospective study, altogether 85 patients with moTBI [GCS 9-12, n=48] or sTBI [GCS 3-8, n=37] were recruited at Turku University Hospital between November 2011 and October 2013 as a part of the EU funded TBIcare (Evidence-based Diagnostic and Treatment Planning Solution for Traumatic Brain Injuries, EU FP7 Grant Agreement 270259) project. Injury Severity Score (ISS) was assessed for all patients to classify the presence and magnitude of extracranial injuries. Also, 40 control patients with acute orthopedic injuries in absence of head trauma were recruited.

Inclusion criteria for the study were age ≥ 18 years, clinical diagnosis of moTBI or sTBI (GCS <13), and indication for acute head CT according to the National Institute for Health and Care Excellence (NICE) criteria [25] assessed by the physician on call. Exclusion criteria were age < 18 years, blast-induced or penetrating injury, chronic subdural hematoma, inability to live independently due to pre-existing brain disease, TBI or suspected TBI not needing head CT, more than 2 weeks from the injury, not living in the district thereby preventing follow-up visits, not speaking the native language, or no consent received.[17] Inclusion criteria for control patients were age ≥ 18 years and orthopedic injury without any head trauma. Control patients with inability to live independently due to pre-existing brain diseases or not speaking the native language were excluded.

The study protocol was approved by the ethical review board of the Hospital District of South-West Finland. Written informed consent was obtained from all patients or their legal guardians.

Biomarker analysis

Blood levels for Aβ40, Aβ42, GFAP, H-FABP, IL-10, NF-L, S100B, and T-tau were analyzed in this study. Blood samples were obtained within 24h of admission, however, they were not necessarily obtained within 24h of injury. Time elapse from injury to sampling was included as a dichotomized (within 24h or over 24h) variable. Blood was collected in EDTA-tubes, kept in cold ice and plasma separated within 1h and stored at −80 °C until the biochemical analyses. Hemolyzed samples were rare but when present discarded from further analyses.

H-FABP and IL-10 plasma levels were analyzed using the K151HTD and K151QUD kits, respectively (Meso Scale Diagnostics, Rockville, MD). For H-FABP and IL-10, the lower limits of detection (LLoD) were 0.103 ng/mL and 0.04 pg/mL with the calibration ranges of 0.137-100 ng/mL and 0.0774-317.0 pg/mL, respectively. The lower limit of quantification (LLoQ) for IL-10 was 0.298 pg/mL. The H-FABP test has not yet been fully validated by Meso Scale, therefore there is no established lower limit of quantification (LLoQ).

Plasma levels of S100B were measured using EZHS100B-33K kit (Millipore, Billerica, MA) with a LLoD of 2.7 pg/mL and the calibration range of 2.7-2000.0 pg/mL. One patient was below the detection range of S100B, and we decided to attribute a concentration of 1 pg/mL to this patient. This did not affect the statistics obtained. Plasma Aβ40 and Aβ42 levels were measured with a duplex Simoa immunoassay (Quanterix). For Aβ40, the LLoD was 0.045 pg/mL and the LLoQ was 0.142 pg/mL, with a calibration range between 0 pg/mL and 90.0 pg/mL. Corresponding concentrations for Aβ42 were LLoD of 0.142 pg/mL, LLoQ of 0.69 pg/mL, and with a calibration range of 0 -11.0 pg/mL.

The GFAP, NF-L, and T-tau plasma concentrations were measured using the Human Neurology 4-Plex A assay (N4PA) on an HD-1 Single molecule array (Simoa) instrument according to instructions from the manufacturer (Quanterix, Billerica, MA). For GFAP, the LLoD was 0.221 pg/mL, while the LLoQ was 0.467 pg/mL and the calibration range was 0.987 pg/mL to 725.0 pg/mL. Respective values for NF-L were 0.104 pg/mL (LLoD), 0.241 pg/mL (LLoQ), and a calibration range between 0.533 pg/mL and 453.0 pg/mL. For T-tau the respective figures were 0.024 pg/mL (LLoD), 0.053 pg/mL (LLoQ) and with a calibration range between 0.136 pg/mL to 112.0 pg/mL.

All patients, excluding the one patient with S100B level below detection range, were over the lower detection range. The measurements were performed according to instructions from manufacturers by board-certified laboratory technicians who were blinded to clinical data.

TBI severity and CT scan grading

The severity grading of TBI was initially based on the lowest GCS score before the intubation, evaluated at the scene of the accident or in transport by paramedics, or at the Emergency Department (ED) by the treating physician.[6,17] Patients with GCS of 9-12 were considered having moTBI and those with GCS of 3-8 sTBI. The duration of PTA[26] was assessed at the follow-up visit using the Rivermead scores [27]. Analysis of CT scans was conducted according to the descriptive system proposed by Marshall et al. [28]. Class I or no visual pathology was considered CT- and the classes II-VI were considered CT+, as they included diffuse injuries (class II-IV) and/or mass lesions (V-VI). CT scans were double read by a neuroradiologist and a neurosurgeon.

Outcome

The outcome was assessed at 6-12 months after the injury at a follow-up visit using the Glasgow Outcome Scale-Extended (GOSE).[29] Outcomes were classified as favorable (GOSE 5-8) or unfavorable (GOSE < 5) outcome. All patients were evaluated by the same experienced neurologist at the Turku Brain Injury Center.

Cohort characteristics

Normality of the numeric variables, including age, GCS, ISS and biomarkers, was assessed by visually examining histograms and with the Kolmogorov-Smirnov test. Age was normally distributed and is presented as mean ± standard deviation. Differences between groups are analyzed with independent samples t-test. Biomarker levels, GCS and ISS were not normally distributed and are presented as medians and inter-quartile ranges (IQRs). Differences between groups are analyzed with Mann-Whitney U test. Chi-square test is used for assessing the differences of categorical variables, including sex, pupil reactivity, isolated-TBI, hypoxia, hypotension, hypoglycemia, anemia, outcome (dichotomized), TBI-related deaths and Marshall grading, between groups. There was missing data on pupil reactivity, hypoxia, hypotension, hypoglycemia, anemia and outcome. Patients with missing data were excluded from the comparison analyses, which were conducted with IBM SPSS Statistics version 28 (IBM Corp, New York).

Outlier analysis

For the analysis all patients with mo/sTBI were divided into subgroups of normal (CT -, n=10) vs. abnormal (CT+, n=75) head CT, and favorable (GOSE 5-8, n=42) vs. unfavorable (GOSE < 5, n=38) outcome. Patients with missing outcome data were excluded from the outcome subgroups (n=5). Statistical outliers were sought individually for each biomarker from these four subgroups and solely from the whole population (n=85) based on biomarker levels. In this study we used the common definition of statistical outliers, Tukey's fences [30], to identify the outliers. Therefore, biomarker levels outside Q1 – 1.5 IQR or Q3 + 1.5 IQR were considered outliers. To identify possible clinical reasons for outlier values, the medical records of each patient were systematically reviewed in a team meeting and the clinical reasons were determined. The clinical reasons were classified as obvious or probable, as well as the following categories: TBI severity, timing of sampling, extracranial injuries, demographic, or unknown. Categories were created based on the obvious clinical reasons. The classification of each patient into these categories was independently cross-checked by a senior neurosurgeon (JPP) and a senior neurologist (OT), and potential conflicts were solved by discussion. For the tables, we have listed all clinical reasons for each biomarker, so that an outlier value could be explained by several concurrent clinical reasons. Pre-existing neurological conditions category included patients with clear evidence of white matter disease on magnetic resonance imaging (MRI) images, alcoholism, and neurodegenerative disease that did not affect the ability to live at home. Also, pre-existing systemic conditions category included hepatic cirrhosis, lymphoma, and cachexia. A corresponding analysis was also performed for control patients. Outlier analysis was performed with Rstudio software version 1.4 (RStudio, PBC, Boston, USA).

Results

A total of 85 mo/sTBI patients and 40 control patients were enrolled. The characteristics of whole TBI patient population and control patient population are presented in Table 1. Also, the characteristics of the CT and outcome subgroups are presented in Table 2. Blood samples of each patient were obtained within 24 h of admission. Exact time of the injury was available for 26 patients and the average time elapse from injury to sampling (mean ± SD) was 16.5 ± 11.1 h. In patients whom the exact time of injury was unavailable, 22 patients were sampled within 24 h and 37 patients after 24 h of injury. To assess the contribution of delays in sampling to the outlier values, we conducted the analysis excluding patients sampled >24h of injury and found that outliers were nearly identical, obviously excluding the outliers with delays. We also conducted an analysis for isolated-TBI only (n = 49) and found a different set of outliers (n = 20). One NF-L outlier overlapped with those of the whole TBI population. Results of the analysis are presented shortly in supplementary materials (Supplementary Table 1) and further elaborated in the discussion.

Of the 85 patients with mo/sTBI 29 (34%) showed outlier values when outliers from all subgroups were combined. From the whole TBI group we found 17 patients (20%) with outlier values in one or more and up to four biomarkers. Of the 29 outlier patients 9 (31%) had outlier values in several biomarkers. The outliers in different subgroups were strongly overlapping. In the CT subgroups, 21 patients (25%), and in outcome subgroups, 23 patients (27%) showed outlier values. All outlier values were on the higher spectrum, meaning there were no low outliers. Cut-off values are presented in supplementary materials (Supplementary Table 2). Across all subgroups, IL-10 showed most often outlier values, but also for GFAP, NF-L, and S100B a considerable number of outlier values were found. Fewest outliers were seen for Aβ40 and Aβ42.

Clinical explanations for TBI outliers

Clinical explanations for TBI outliers are presented in Table 3 and the boxplots for the biomarker values in the outcome and CT subgroups are presented in Figures 1 and 2, respectively. Also, the clinical explanations for the outcome and CT subgroups, as well as the whole TBI patient population, are presented in supplementary materials (Supplementary Table 3, 4 and 5, respectively).

Across all subgroups, extremely sTBIs (= large and widespread intracranial lesions with a respective clinical state) explained most of the outlier plasma GFAP levels. Outlier S100B levels were mainly explained either with extracranial injuries or extremely sTBIs. In the case of IL-10 extracranial injuries, especially multi-trauma, explained most outlier values. Extracranial injuries, especially thoracic injuries and multi-trauma, accounted for all of the outlier H-FABP levels.

Timing of sampling, especially delay in sampling, and demographic factors, including old age and pre-existing neurological conditions, explained almost all the outlier NF-L levels. Outliers for T-tau were explained mainly with demographic factors, including age and pre-existing neurological conditions. Pre-existing systemic conditions were associated with a few outlier values for IL-10, including one case with hepatic cirrhosis. However, we did not observe any association between outlier values and renal function. No plausible explanation could be found for the outlier Aβ40 and Aβ42 levels, except for one patient whose outlier values were considered to be caused by multi-trauma.

Clinical explanations for control patients

Of the 40 control patients, 12 (30%) showed outlier values in at least one and up to four biomarkers. Also, here all outlier values were abnormally high, and 5 patients (42%) showed outlier values in more than one biomarker. Most outlier values were observed for T-tau and NF-L, and the smallest number of patients with outlier values were found for S100B. Two control patients had overlapping outlier levels with TBI outliers. One had higher IL-10 levels than any of the TBI outliers (obviously because of severe multi-trauma and internal bleedings), and this same patient had also very high H-FABP levels overlapping with those of TBI outliers. Another control patient had high NF-L levels overlapping with the TBI CT subgroups, obviously due to a cerebrovascular disease. All controls have been sampled within 24 h of admission, however the exact sampling times are not available for all the controls.

The clinical explanations for controls are presented in Table 4 and boxplots for biomarker values in Figure 3. Outlier levels of GFAP were explained by demographic factors, especially pre-existing neurological conditions, and old age. In case of S100B and IL-10, the explanation was often multi-trauma, but for some there were no obvious reasons. As for the H-FABP, thoracic injuries explained most of the outlier values. Outlier values for NF-L were explained by demographic factors, including pre-existing neurological conditions and age. In most cases there was no obvious reason for outlier levels of T-tau, but some were explained with demographic factors or multi-trauma.

Discussion

The aim of this study was to examine the incidence of outlier biomarker levels in patients with mo/sTBI and if there was a reasonable explanation for these outlier values for S100B, GFAP, H-FAPB, NF-L, IL-10, Aβ40 and Aβ42 in a well-characterized cohort. We found that a substantial part (25-34%) of patients showed outlier values in at least one and up to four biomarkers and that all the outlier values were abnormally high. Also, an obvious or probable clinical explanation was found for almost all outlier values, except the Aβ40 and Aβ42 outlier levels.

We also identified and described the most obvious clinical explanations for the outlier values, which included extremely sTBIs, especially for GFAP and S100B. In case of IL-10 and H-FABP, a common explanation was extracranial injuries. Outlier NF-L levels were mainly explained by demographic and timing of sampling. Also, high T-tau was explained by demographic factors. We did not find obvious reasons for high levels of Aβ40 and Aβ42. Similar explanations occurred among control patients for NF-L, IL-10, H-FABP, and S100B, but for GFAP the outlier values were associated with demographic factors, and for T-tau we did not find any probable explanation.

Patients with extremely sTBIs showed often outlier values and especially high outliers plasma levels of GFAP were seen, which is reasonable considering that several studies suggest that GFAP is strongly associated with the severity of the initial injury and intracranial findings.[6,31–33] Consequently, in this study we also observed an association between high outlier levels of GFAP and poor outcome, as all outlier patients in the CT+ -subgroup or in the whole population died because of their injury. Interestingly, in those with favorable outcome many of the GFAP outliers had a disproportion between low admission GCS and high GOSE, which was explained with multiple small cortical contusions causing the high GFAP release. There are many studies indicating that GFAP has potential in outcome prediction.[8,9,17] In some cases, outlier levels of S100B were also seen, and we observed a respective connection with high outlier levels of S100B and poor outcome. S100B has been associated with the severity of the injury in the acute phase [7,8,10], and some studies suggest that S100B has predictive value for unfavorable outcome and death [34–37]. We find this observation significant as this could be useful in combination with other tools to determine whether we should withdraw or continue care in mo/sTBI patients. This could have a positive impact on resource management and improve the level of ethical treatment.

Timing of sampling, including delayed or very rapid sampling and sampling after surgery, were also identified as one of the potential contributors for unexpected levels. Especially majority of the outlier values for NF-L were associated with the timing of sampling, usually delay in sampling. NF-L is released slowly from the axons, peaking at 1-2 weeks after the injury, which is in line with our observations.[38] In some cases, outlier levels of S100B and GFAP were also associated with either delays in sampling (>24h), or in case of S100B also with rapid sampling (<12h). Previous studies have suggested, that peak values for S100B and GFAP are at approximately 2-6 h and 24-48 h, respectively [38,39], which would also explain our findings. In case of IL-10, delay in sampling and sampling after surgery explained some of the outlier values. However, there is no clear consensus on the kinetic profile of IL-10 - some studies suggest the plasma levels peaking at 3 h [40] and others at 5-6 days after injury [41]. We also found that in some cases outlier levels of IL-10 were associated with samples being drawn after surgery. Several studies have shown that IL-10 is not a brain-specific biomarker, therefore it might be released also from extracranial sources.[40,42] It is however unclear if surgical measures affect the levels significantly.

Our study suggests that extracranial injuries are the most common confounder for TBI biomarkers. In this respect, IL-10, S100B, and H-FABP were particularly associated. As mentioned previously, elevated IL-10 levels are common also in trauma patients without head trauma [31,40,42], which supports our suggestions of multi-trauma causing the outlier values. Several studies have reported that S100B also has extracerebral sources, such as bone fractures, and that high levels have been seen in critically ill patients without head trauma, which is also consistent with our observations of high outlier levels of S100B in patients with multi-trauma. [43–45] Outlier patients with high H-FABP had thoracic injuries and/or multi-trauma. H-FABP is not TBI-specific; it has been also identified as a biomarker for myocardial infarction [46,47] and elevated levels have also been present in patients with multi-trauma, especially affecting the thoracic area [31,48]. Spinal cord injury (SCI) was associated with outlier values for GFAP and NF-L, and also the current literature suggests elevated levels in patients with SCI.[49,50] To evaluate the significance of extracranial injuries to outlier values, we also analyzed patients with only isolated TBI, and found expectedly a different set of outliers compared to the whole TBI cohort. The more severe cases with more trauma energy tend to also have extracranial injuries. Hence, if non-isolated TBI patients are excluded, the more severe cases are excluded at the same time. This also explains why some CT-negative patients may have outlier values. Almost all of the CT-negative outlier patients had severe multi-trauma possibly causing the abundant release, except for one NF-L outlier patient with earlier TBI probably causing outlier levels.

Demographic factors, including age as well as pre-existing neurological and systemic conditions, were also found as obvious sources for confounding values. Previous studies suggest that age affects the levels of GFAP, NF-L, Tau, and S100B [16,19,51,52], and accordingly we found that age was associated with some outlier values for these biomarkers. At least for some TBI-related biomarkers the normal range should be age dependent. For example, the NF-L levels in the blood increase with age and should therefore have a specific age-adjusted reference values.[19] Chronic neurological conditions, including earlier TBIs, epilepsy, and cerebral atrophy, have been associated with elevated NF-L and T-tau levels. After a TBI, NF-L may be elevated for up to one year [38,53], why it can be assumed that earlier TBIs might cause permanently abnormally high NF-L blood levels in some patients. Tau has been used as a biomarker for Alzheimer's disease (AD) [54], and elevated plasma levels have been associated with neurodegeneration of grey matter [12,18]. Hence, we suspected that an observed T-tau elevation was caused by AD-type neurodegeneration. Some IL-10 outliers were possibly associated with pre-existing systemic conditions, which included hepatic cirrhosis, lymphoma, and cachexia. One study has reported that IL-10 could be produced by lymphoma cells leading to elevated serum levels [55], but it is not certain if these systemic conditions were the cause for outlier levels seen in these subjects. Some studies suggest that kidney function also affects biomarker clearance [23,24], which might lead to accumulation. However, we did not observe any connection between outlier biomarker levels and kidney function. Instead, we observed a connection between outlier IL-10 levels and hepatic function in one case.

Similar potential confounders for biomarkers were observed among our control patient outliers. In general control patients were not exposed to significant acceleration/deceleration forces. Most of them had a simple orthopedic trauma, such as bone fracture, which explained for example outlier levels of S100B. GFAP was the only biomarker having a different explanation in controls vs. patients with TBI, as most of the GFAP control outliers had a cerebrovascular disease. An earlier study suggests an association with cerebrovascular diseases and blood levels of GFAP.[56] For control outliers as well as for TBI outliers we did not find any obvious reason for outlier levels of Aβ40 and Aβ42, except for one patient where a severe multi-trauma was a possible explanation. Both amyloid outliers with TBI were CT negative and had favorable outcome.

Our study has some strengths and limitations. The study population is small, therefore a larger patient cohort would be needed to strengthen our observations. Due to the small number of observed associations, statistical analyses to establish the connections could not be done, why our findings remain merely on an observational level and should thus be interpreted with caution. Also, one considerable limitation in our study was that the severity assessment was performed with lowest recorded GCS. Classification of TBI is complex as it is more of a dynamic state, meaning that severity may change over time. Nevertheless, GCS is commonly used in current biomarker literature. A strength of this study is a prospectively collected well-characterized cohort where numerous biomarkers have been measured from these very same patients. Understanding the background of outlier values is very important for the use of biomarkers in the clinical setting, because unexpectedly high biomarker levels caused, for instance, by advanced age or extracranial injuries may lead to an incorrect assessment of the patient's injury burden and outcome. In the worst case this could lead to limiting treatments or to treatment discontinuation. These facts also speak in favor of the simultaneous use of multiple TBI biomarkers.

Conclusions

Our study showed that in patients with a mo/sTBI, outlier levels of several biomarkers are found often, and that in most cases there seems to be a plausible clinical explanation for such a finding. These include very severe or fatal TBIs, various extracranial injuries, timing of sampling and demographic factors including age and pre-existing systemic or neurological conditions. Similar potential explanations could be seen also in orthopedic injury controls. Our study suggests that further research efforts are needed to establish age-adjusted reference values for biomarkers of TBI and highlights the need for more rigorous studies on the kinetics of different biomarkers to determine a clinically acceptable time window for each biomarker. Furthermore, in patients with non-isolated TBI, additional consideration must be given to the extent of extracranial injury and their impact on biomarker levels. Knowledge on potential confounders when using TBI-related biomarkers for clinical decision-making is mandatory to avoid misleading diagnostics and false conclusions. The observations found in this study need to be confirmed in other or larger cohorts.

Transparency, Rigor, and Reproducibility Summary

This study was not formally registered because at the time the study was conducted, observational studies were not routinely registered outside research institutions. The analysis plan was not formally pre-registered, but JPP and OT with primary responsibility for the study and analysis certify that the analysis plan was pre-specified. A sample size of 85 patients and 40 controls was planned based on the outlier definition and availability of 203 patients and 40 controls from 620 potential participants were screened, samples were obtained and successfully analyzed in 160 patients and 40 controls.

Human participants were blinded to results of the fluid biomarker measurements. Handling of biofluid samples was performed by team members who were aware of relevant characteristics of the participants. Fluid biomarker measurements were performed by investigators blinded to relevant characteristics of the participants. Fluid biomarker quality control decisions and analyses were performed by investigators blinded to relevant characteristics of the participants. Fluid biomarkers were labeled using codes that were not linked to participant identifying information. Samples were acquired within 24 h of admission at the Turku University Hospital, Turku, Finland. The samples were centrifuged for 10 minutes at 10 000 rpm at 4 C, and the plasma was immediately frozen at -70 C for further analysis. Freeze-thaw cycles were performed one time prior to analysis. All samples were analyzed at the same time in a single batch. All equipment and analytical reagents used to perform measurements on the fluid biomarkers are widely available from commercial sources. Additional characteristics of the primary fluid biomarker analyses are presented in the Methods section. The key inclusion criteria (e.g., primary diagnosis or prognostic factor) are established standards in the field. The statistical tests used were based on the assumptions of variable distributions and outliers were defined as described in the Methods section. Data is available for qualified investigators upon request from the corresponding author.

Conflict of interest:

KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, BioArctic, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Ono Pharma, Pharmatrophix, Prothena, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. VFJN holds a grant with Roche Pharmaceuticals. HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

Acknowledgements

KB is supported by the Swedish Research Council (#2017-00915), the Swedish Alzheimer Foundation (#AF-930351, #AF-939721 and #AF-968270), Hjärnfonden, Sweden (#FO2017-0243 and #ALZ2022-0006), the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986 and #ALFGBG-965240), the European Union Joint Program for Neurodegenerative Disorders (JPND2019-466-236), and the Alzheimer's Association 2021 Zenith Award (ZEN-21-848495). IH is supported by the Finnish Medical Foundation, the Päivikki and Sakari Sohlberg Foundation, the Paulo Foundation, and the Finnish Cultural Foundation. VFJN is supported by a National Institute of Health and Care Research (NIHR) Advanced Fellowship. HZ is a Wallenberg Scholar supported by grants from the Swedish Research Council (#2022-01018), the European Union's Horizon Europe research and innovation programme under grant agreement No 101053962, Swedish State Support for Clinical Research (#ALFGBG-71320), the Alzheimer Drug Discovery Foundation (ADDF), USA (#201809-2016862), the AD Strategic Fund and the Alzheimer's Association (#ADSF-21-831376-C, #ADSF-21-831381-C, and #ADSF-21-831377-C), the Bluefield Project, the Olav Thon Foundation, the Erling-Persson Family Foundation, Stiftelsen för Gamla Tjänarinnor, Hjärnfonden, Sweden (#FO2022-0270), the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860197 (MIRIADE), the European Union Joint Programme – Neurodegenerative

Disease Research (JPND2021-00694), and the UK Dementia Research Institute at UCL (UKDRI-1003). JPP is supported by the Academy of Finland (grant no. 17379) and by the Maire Taponen Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors thank our research nurses Patricia Bertenyi and Satu Honkala for their valuable contribution to this study.

Author contribution statement

- Korhonen: Conceptualization; Writing – Original draft; Formal analysis; Interpretation of data
- Mononen: Conceptualization; Writing – review & editing; Interpretation of data
- Mohammadian: Formal analysis; Writing – review & editing
- Tenovuo: Conceptualization; Data curation; Resources; Writing – review & editing
- Blennow: Resources; Writing – review & editing; Interpretation of data
- Hossain: Writing – review & editing; Interpretation of data
- Hutchinson: Resources; Writing – review & editing; Interpretation of data
- Maanpää: Writing – review & editing; Interpretation of data
- Menon: Resources; Writing – review & editing; Interpretation of data
- Newcombe: Resources; Writing – review & editing; Interpretation of data
- Sanchez: Resources; Writing – review & editing; Interpretation of data
- Takala: Resources; Data curation; Writing – review & editing; Interpretation of data
- Tallus: Resources; Data curation; Writing – review & editing; Interpretation of data
- Zetterberg: Resources; Writing – review & editing; Interpretation of data
- Posti: Conceptualization; Data curation; Resources; Writing – review & editing; Supervision

References

1. Maas AIR, Menon DK, Adelson PD, et al. Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. Lancet Neurol 2017;16(12):987–1048; doi: 10.1016/S1474-4422(17)30371-X.

2. Capizzi A, Woo J, Verduzco-Gutierrez M. Traumatic Brain Injury: An Overview of Epidemiology, Pathophysiology, and Medical Management. Med Clin North Am 2020;104(2):213–238; doi: 10.1016/j.mcna.2019.11.001.

3. Vella MA, Crandall ML, Patel MB. Acute Management of Traumatic Brain Injury. Surg Clin North Am 2017;97(5):1015–1030; doi: 10.1016/j.suc.2017.06.003.

4. Tuominen R, Joelsson P, Tenovuo O. Treatment costs and productivity losses caused by traumatic brain injuries. Brain Inj 2012;26(13–14):1697–1701; doi: 10.3109/02699052.2012.722256.

5. Posti JP, Tenovuo O. Blood-based biomarkers and traumatic brain injury-A clinical perspective. Acta Neurol Scand 2022; doi: 10.1111/ane.13620.

6. Posti JP, Takala RSK, Runtti H, et al. The Levels of Glial Fibrillary Acidic Protein and Ubiquitin C-Terminal Hydrolase-L1 during the First Week after a Traumatic Brain Injury: Correlations with Clinical and Imaging Findings. Neurosurgery 2016;79(3):456–463; doi: 10.1227/NEU.0000000000001226.

7. Koivikko P, Posti JP, Mohammadian M, et al. Potential of heart fatty-acid binding protein, neurofilament light, interleukin-10 and S100 calcium-binding protein B in the acute diagnostics and severity assessment of traumatic brain injury. Emerg Med J EMJ 2022;39(3):206–212; doi: 10.1136/emermed-2020-209471.

8. Thelin E, Al Nimer F, Frostell A, et al. A Serum Protein Biomarker Panel Improves Outcome Prediction in Human Traumatic Brain Injury. J Neurotrauma 2019;36(20):285–2862; doi: 10.1089/neu.2019.6375.

9. Anderson TN, Hwang J, Munar M, et al. Blood-based biomarkers for prediction of intracranial hemorrhage and outcome in patients with moderate or severe traumatic brain injury. J Trauma Acute Care Surg 2020;89(1):80–86; doi: 10.1097/TA.0000000000002706.

10. Watt SE, Shores EA, Baguley IJ, et al. Protein S-100 and neuropsychological functioning following severe traumatic brain injury. Brain Inj 2006;20(10):1007–1017; doi: 10.1080/02699050600909698.

11. Lagerstedt L, Azurmendi L, Tenovuo O, et al. Interleukin 10 and Heart Fatty Acid-Binding Protein as Early Outcome Predictors in Patients With Traumatic Brain Injury. Front Neurol 2020;11.

12. Graham NSN, Zimmerman KA, Moro F, et al. Axonal marker neurofilament light predicts long-term outcomes and progressive neurodegeneration after traumatic brain injury. Sci Transl Med 2021;13(613):eabg9922; doi: 10.1126/scitranslmed.abg9922.

13. Bogoslovsky T, Wilson D, Chen Y, et al. Increases of Plasma Levels of Glial Fibrillary Acidic Protein, Tau, and Amyloid β up to 90 Days after Traumatic Brain Injury. J Neurotrauma 2017;34(1):66–73; doi: 10.1089/neu.2015.4333.

14. Graham NSN, Jolly A, Zimmerman K, et al. Diffuse axonal injury predicts neurodegeneration after moderate-severe traumatic brain injury. Brain J Neurol 2020;143(12):3685–3698; doi: 10.1093/brain/awaa316.

15. Wang KK, Yang Z, Zhu T, et al. An update on diagnostic and prognostic biomarkers for traumatic brain injury. Expert Rev Mol Diagn 2018;18(2):165–180; doi: 10.1080/14737159.2018.1428089.

16. Gardner RC, Rubenstein R, Wang KKW, et al. Age-Related Differences in Diagnostic Accuracy of Plasma Glial Fibrillary Acidic Protein and Tau for Identifying Acute Intracranial Trauma on Computed Tomography: A TRACK-TBI Study. J Neurotrauma 2018;35(20):2341–2350; doi: https://doi.org/10.1089/neu.2018.5694.

17. Takala RSK, Posti JP, Runtti H, et al. Glial Fibrillary Acidic Protein and Ubiquitin C-Terminal Hydrolase-L1 as Outcome Predictors in Traumatic Brain Injury. World Neurosurg 2016;87:8–20; doi: 10.1016/J.WNEU.2015.10.066.

18. Shahim P, Politis A, van der Merwe A, et al. Neurofilament light as a biomarker in traumatic brain injury. Neurology 2020;95(6):e610–e622; doi: 10.1212/WNL.0000000000009983.

19. Iverson GL, Reddi PJ, Posti JP, et al. Serum Neurofilament Light Is Elevated Differentially in Older Adults with Uncomplicated Mild Traumatic Brain Injuries. J Neurotrauma 2019;36(16):2400–2406; doi: https://doi-org.ezproxy.utu.fi/10.1089/neu.2018.6341.

20. Villaseñor R, Lampe J, Schwaninger M, et al. Intracellular transport and regulation of transcytosis across the blood–brain barrier. Cell Mol Life Sci CMLS 2018;76(6):1081–1092; doi: 10.1007/s00018-018-2982-x.

21. Plog BA, Dashnaw ML, Hitomi E, et al. Biomarkers of traumatic injury are transported from brain to blood via the glymphatic system. J Neurosci 2015;35(2):518–526; doi: 10.1523/JNEUROSCI.3742-14.2015.

22. George N, Geller HM. Extracellular matrix and traumatic brain injury. J Neurosci Res 2018;96(4):573–588; doi: 10.1002/jnr.24151.

23. Akamine S, Marutani N, Kanayama D, et al. Renal function is associated with blood neurofilament light chain level in older adults. Sci Rep 2020;10(1):20350–20350; doi: 10.1038/s41598-020-76990-7.

24. McDonald SJ, Shultz SR, Agoston DV. The Known Unknowns: An Overview of the State of Blood-Based Protein Biomarkers of Mild Traumatic Brain Injury. J Neurotrauma 2021;38(19):2652–2666; doi: 10.1089/neu.2021.0011.

25. National Clinical Guideline Centre (UK). Head Injury: Triage, Assessment, Investigation and Early Management of Head Injury in Children, Young People and Adults. National Institute for Health and Clinical Excellence: Guidance. National Institute for Health and Care Excellence (UK): London; 2014.

26. Holm L, Cassidy JD, Carroll LJ, et al. Summary of the WHO Collaborating Centre for Neurotrauma Task Force on Mild Traumatic Brain Injury. J Rehabil Med 2005;37(3):137–141; doi: 10.1080/16501970510027321.

27. King NS, Crawford S, Wenden FJ, et al. Measurement of post-traumatic amnesia: how reliable is it? J Neurol Neurosurg Psychiatry 1997;62(1):38–42; doi: 10.1136/jnnp.62.1.38.

28. Marshall LF, Marshall SB, Klauber MR, et al. A new classification of head injury based on computerized tomography. J Neurosurg 1991;75(Supplement):S14–S20; doi: 10.3171/sup.1991.75.1s.0s14.

29. Wilson JTL, Pettigrew LEL, Teasdale GM. Structured interviews for the glasgow outcome scale and the extended glasgow outcome scale: Guidelines for their use. J Neurotrauma 1998;15(8):573–580; doi: 10.1089/neu.1998.15.573.

30. Tukey JW. Exploratory Data Analysis. Reading, Mass. : Addison-Wesley Pub. Co.; 1977.

31. Posti JP, Takala RS, Lagerstedt L, et al. Correlation of Blood Biomarkers and Biomarker Panels with Traumatic Findings on Computed Tomography after Traumatic Brain Injury. J Neurotrauma 2019;36(14):2178–2189; doi: 10.1089/neu.2018.6254.

32. Honda M, Tsuruta R, Kaneko T, et al. Serum Glial Fibrillary Acidic Protein Is a Highly Specific Biomarker for Traumatic Brain Injury in Humans Compared With S-100B and Neuron-Specific Enolase. J Trauma Acute Care Surg 2010;69(1):104–109; doi: 10.1097/TA.0b013e3181bbd485.

33. Luoto TM, Raj R, Posti JP, et al. A Systematic Review of the Usefulness of Glial Fibrillary Acidic Protein for Predicting Acute Intracranial Lesions following Head Trauma. Front Neurol 2017;8.

34. Thelin EP, Nelson DW, Bellander B-M. Secondary Peaks of S100B in Serum Relate to Subsequent Radiological Pathology in Traumatic Brain Injury. Neurocrit Care 2014;20(2):217–229; doi: 10.1007/s12028-013-9916-0.

35. Murillo-Cabezas F, Muñoz-Sánchez MA, Rincón-Ferrari MD, et al. The prognostic value of the temporal course of S100beta protein in post-acute severe brain injury: A prospective and observational study. Brain Inj 2010;24(4):609–619; doi: 10.3109/02699051003652823.

36. Thelin EP, Johannesson L, Nelson D, et al. S100B is an important outcome predictor in traumatic brain injury. J Neurotrauma 2013;30(7):519–528; doi: 10.1089/neu.2012.2553.

37. Di Battista AP, Buonora JE, Rhind SG, et al. Blood Biomarkers in Moderate-To-Severe Traumatic Brain Injury: Potential Utility of a Multi-Marker Approach in Characterizing Outcome. Front Neurol 2015;6.

38. Thelin EP, Zeiler FA, Ercole A, et al. Serial Sampling of Serum Protein Biomarkers for Monitoring Human Traumatic Brain Injury Dynamics: A Systematic Review. Front Neurol 2017;8.

39. Welch RD, Ellis M, Lewis LM, et al. Modeling the Kinetics of Serum Glial Fibrillary Acidic Protein, Ubiquitin Carboxyl-Terminal Hydrolase-L1, and S100B Concentrations in Patients with Traumatic Brain Injury. J Neurotrauma 2017;34(11):1957–1971; doi: 10.1089/neu.2016.4772.

40. Hensler T, Sauerland S, Riess P, et al. The effect of additional brain injury on systemic interleukin (IL)-10 and IL-13 levels in trauma patients. Inflamm Res 2000;49(10):524–528; doi: 10.1007/s000110050626.

41. Helmy A, Antoniades CA, Guilfoyle MR, et al. Principal Component Analysis of the Cytokine and Chemokine Response to Human Traumatic Brain Injury. PLOS ONE 2012;7(6):e39677; doi: 10.1371/journal.pone.0039677.

42. Shiozaki T, Hayakata T, Tasaki O, et al. CEREBROSPINAL FLUID CONCENTRATIONS OF ANTI-INFLAMMATORY MEDIATORS IN EARLY-PHASE SEVERE TRAUMATIC BRAIN INJURY. Shock 2005;23(5):406–410; doi: 10.1097/01.shk.0000161385.62758.24.

43. Undén J, Bellner J, Eneroth M, et al. Raised Serum S100B Levels after Acute Bone Fractures without Cerebral Injury. J Trauma Acute Care Surg 2005;58(1):59–61; doi: 10.1097/01.TA.0000130613.35877.75.

44. Routsi C, Stamataki E, Nanas S, et al. Increased levels of serum S100B protein in critically ill patients without brain injury. Shock Augusta Ga 2006;26(1):20–24; doi: 10.1097/01.shk.0000209546.06801.d7.

45. Hasselblatt M, Mooren FC, Ahsen N von, et al. Serum S100β increases in marathon runners reflect extracranial release rather than glial damage. Neurology 2004;62(9):1634–1636; doi: 10.1212/01.WNL.0000123092.97047.B1.

46. Glatz JFC, van der Vusse GJ, Simoons ML, et al. Fatty acid-binding protein and the early detection of acute myocardial infarction. Clin Chim Acta 1998;272(1):87–92; doi: 10.1016/S0009-8981(97)00255-6.

47. Erenler AK, Yardan T, Duran L, et al. Usefulness of heart-type fatty acid binding protein in the emergency department. JPMA J Pak Med Assoc 2013;63(9):1176–1181.

48. Walder B, Robin X, Rebetez MML, et al. The Prognostic Significance of the Serum Biomarker Heart-Fatty Acidic Binding Protein in Comparison with S100b in Severe Traumatic Brain Injury. J Neurotrauma 2013;30(19):1631–1637; doi: 10.1089/neu.2012.2791.

49. Leister I, Altendorfer B, Maier D, et al. Serum Levels of Glial Fibrillary Acidic Protein and Neurofilament Light Protein Are Related to the Neurological Impairment and Spinal Edema after Traumatic Spinal Cord Injury. J Neurotrauma 2021;38(24):3431–3439; doi: 10.1089/neu.2021.0264.

50. Kuhle J, Gaiottino J, Leppert D, et al. Serum neurofilament light chain is a biomarker of human spinal cord injury severity and outcome. J Neurol Neurosurg Psychiatry 2015;86(3):273–279; doi: 10.1136/jnnp-2013-307454.

51. Chiu M-J, Fan L-Y, Chen T-F, et al. Plasma Tau Levels in Cognitively Normal Middle-Aged and Older Adults. Front Aging Neurosci 2017;9.

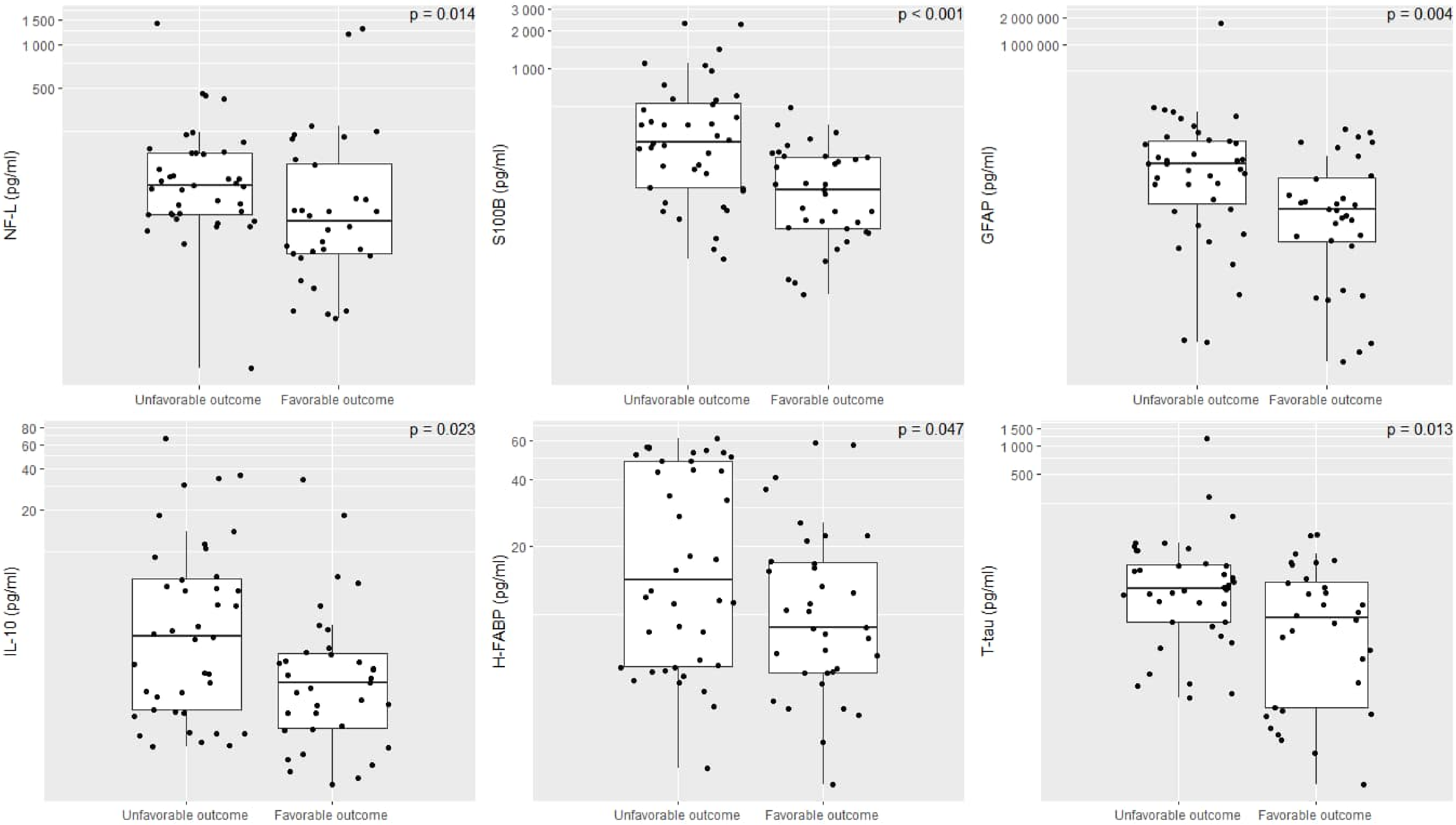
52. Calcagnile O, Holmén A, Chew M, et al. S100B levels are affected by older age but not by alcohol intoxication following mild traumatic brain injury. Scand J Trauma Resusc Emerg Med 2013;21(1):52–52; doi: 10.1186/1757-7241-21-52.

53. Shahim P, Gren M, Liman V, et al. Serum neurofilament light protein predicts clinical outcome in traumatic brain injury. Sci Rep 2016;6(1):36791; doi: 10.1038/srep36791.

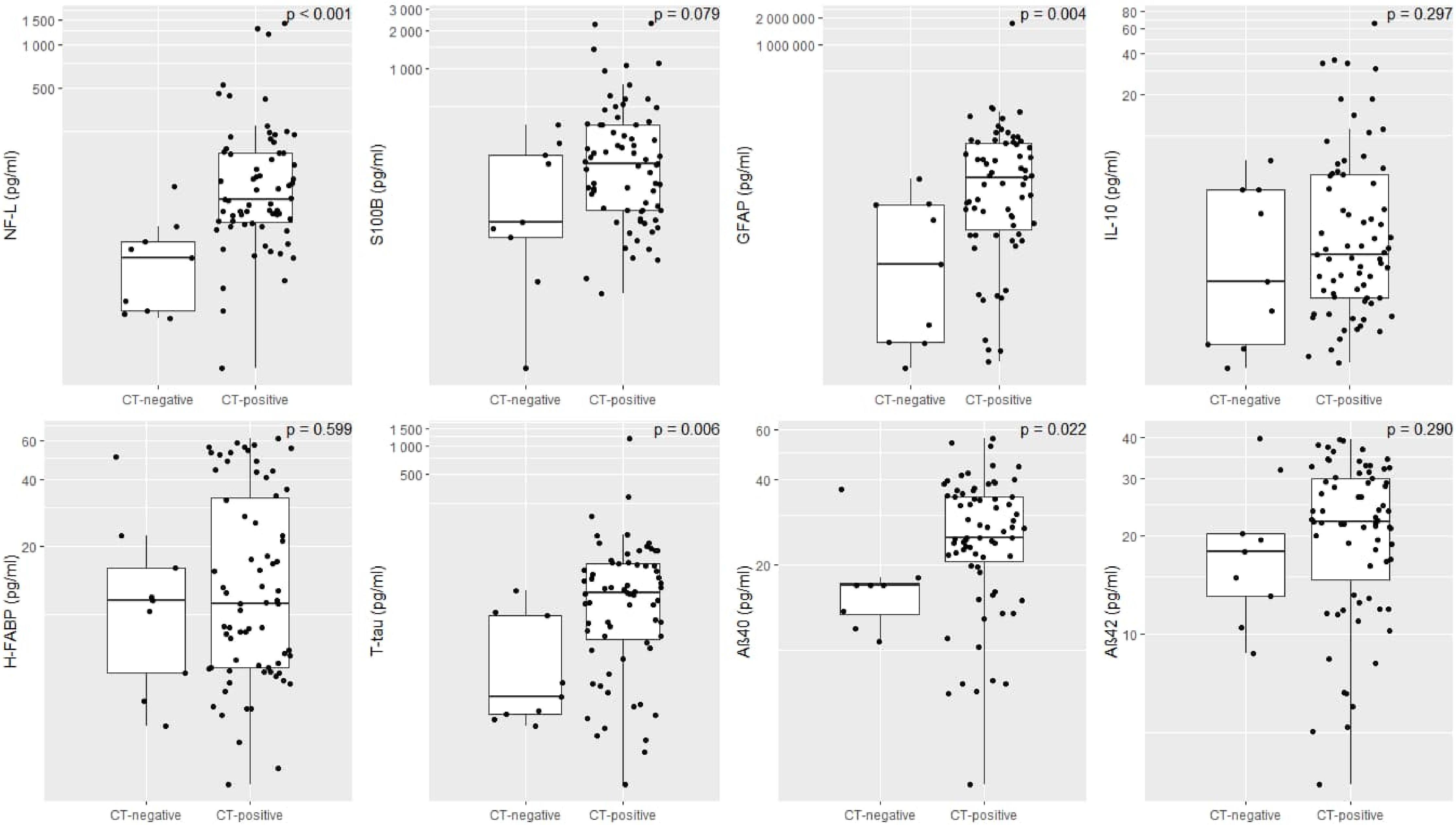
54. Blennow K, Zetterberg H. Biomarkers for Alzheimer’s disease: current status and prospects for the future. J Intern Med 2018;284(6):643–663; doi: 10.1111/joim.12816.

55. Voorzanger N, Touitou R, Garcia E, et al. Interleukin (IL)-10 and IL-6 are produced in vivo by non-Hodgkin’s lymphoma cells and act as cooperative growth factors. Cancer Res 1996;56(23):5499–5505.

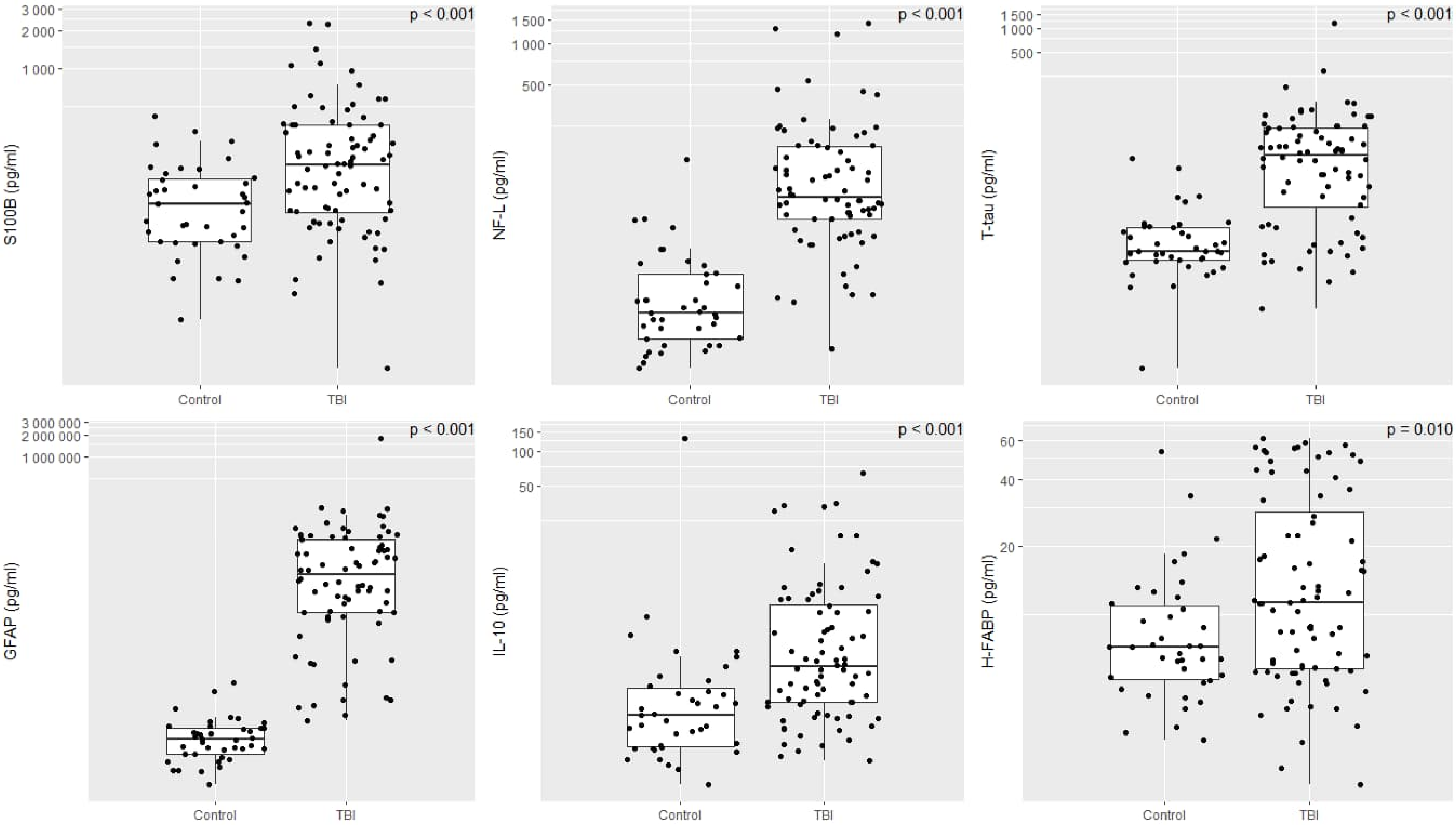
56. Stanca DM, Mărginean IC, Soriţău O, et al. GFAP and antibodies against NMDA receptor subunit NR2 as biomarkers for acute cerebrovascular diseases. J Cell Mol Med 2015;19(9):2253–2261; doi: 10.1111/jcmm.12614.



NF-L (pg/ml)
1 500
1 000
500
p = 0.014
Unfavorable outcome
Favorable outcome
S100B (pg/ml)
3 000
2 000
1 000
p < 0.001
Unfavorable outcome
Favorable outcome
GFAP (pg/ml)
2 000 000
1 000 000
p = 0.004
Unfavorable outcome
Favorable outcome
IL-10 (pg/ml)
80
60
40
20
p = 0.023
Unfavorable outcome
Favorable outcome
H-FABP (pg/ml)
60
40
20
p = 0.047
Unfavorable outcome
Favorable outcome
T-tau (pg/ml)
1 500
1 000
500
p = 0.013
Unfavorable outcome
Favorable outcome



NF-L (pg/ml)
p < 0.001
1 500
1 000
500
CT-negative
CT-positive
S100B (pg/ml)
p = 0.079
3 000
2 000
1 000
GFAP (pg/ml)
p = 0.004
2 000 000
1 000 000
IL-10 (pg/ml)
p = 0.297
80
60
40
20
H-FABP (pg/ml)
p = 0.599
60
40
20
T-tau (pg/ml)
p = 0.006
1 500
1 000
500
Aß40 (pg/ml)
p = 0.022
60
40
20
Aß42 (pg/ml)
p = 0.290
40
30
20
10



S100B (pg/ml)
3 000
2 000
1 000
p < 0.001
Control
TBI
NF-L (pg/ml)
1 500
1 000
500
p < 0.001
Control
TBI
T-tau (pg/ml)
1 500
1 000
500
p < 0.001
Control
TBI
GFAP (pg/ml)
3 000 000
2 000 000
1 000 000
p < 0.001
Control
TBI
IL-10 (pg/ml)
150
100
50
p < 0.001
Control
TBI
H-FABP (pg/ml)
60
40
20
p = 0.010
Control
TBI

Figure 1. Levels of neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10) and heart-type fatty acid-binding protein (H-FABP) in patients with favorable (GOSE 5-8) and unfavorable (GOSE <5) outcome. Boxplots describe medians and interquartile ranges measured in picograms per milliliter. The y-axis coordinate system is log10.

Figure 2. Levels of neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10), heart-type fatty acid-binding protein (H-FABP), amyloid β40 (Aβ40) and amyloid β40 (Aβ42) in patients with traumatic intracranial findings (CT-positive) and without findings (CT-negative) in head CT. Boxplots describe medians and interquartile ranges measured in picograms per milliliter. The y-axis coordinate system is log10.

Figure 3. Levels of neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10) and heart-type fatty acid-binding protein (H-FABP) in control patients and in patients with moderate/severe TBI. The y-axis coordinate system is log10.

Supplementary materials

	GFAP	IL-10	NF-L	S100B	H-FABP	T-tau	Amyloid β42	Amyloid β40
Number of outliers whole TBI population	4 (14%)	9 (30%)	7 (23%)	7 (23%)	0	3 (10%)	0	0
Number of outliers isolated TBI only	2 (10%)	4 (20%)	4 (20%)	3 (15%)	5 (25%)	2 (10%)	0	0

Table 1. Outliers for the whole TBI patient population and isolated TBI patient population. Biomarkers examined: neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10), heart-type fatty acid-binding protein (H-FABP), amyloid β40 (Aβ40) and amyloid β40 (Aβ42).

Cut-off values (pg/ml)	**NF-L**	**GFAP**	**IL-10**	**H-FABP**	**T-tau**	**S100B**	**Amyloid β40**	**Amyloid β42**
Upper limit	362,379	166944,9	11,17101	66,46743	131,0275	793,0883	61,6	55,5275
Lower limit	-134,251	-89305,06	-5,661655	-30,74025	-70,57715	-365,4058	-9,77	-12,3325
Unfavorable								
Upper limit	346,742	184183,8	15,03148	112,2019	124,2219	1201,899	61,66	50,62
Lower limit	-101,018	-87624,65	-7,903769	-58,00963	-52,59473	-539,0032	-9,94	-4,42
Favorable								
Upper limit	331,051	70030,7	-1,481582	34,54277	92,87082	422,293	63,495	53,565
Lower limit	-142,217	-33058,58	3,823522	-12,06785	-52,88261	-167,881	-7,825	-13,115
CT-positive								
Upper limit	362,878	182058,7	12,02766	75,707	133,4196	789,442	57,92	55,55
Lower limit	-124,413	-97624,87	-6,167386	-36,69333	-66,01042	-354,153	-2,245	-10,76
CT-negative								
Upper limit	86,780	37474,33	-5,28378	31,975	39,30932	443,0325	24,625	31,455
Lower limit	-29,158	-21815,58	9,627622	-10,42019	-21,12417	-193,1955	5,785	1,975
Controls								
Upper limit	41,106	308,6972	1,852841	19,98776	5,990566	277,296		
Lower limit	-13,695	-69,02101	-0,7033847	-4,036528	-1,720469	-100,1175		

Table 2. Cut-off values for each biomarker in each subgroup. Biomarkers examined: neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10), heart-type fatty acid-binding protein (H-FABP), amyloid β40 (Aβ40) and amyloid β40 (Aβ42).

	GFAP	IL-10	NF-L	S100B	H-FABP	T-tau	Amyloid β42	Amyloid β40
Number of outliers	9 (24%)	10 (26%	6 (15%)	4 (11%)	4 (11%)	5 (13%)		
(1) TBI severity	**8**	**2**		**3**				
(2) Timing of sampling	**2**	**2**	**4**	**2**		**1**		
(2a) Rapid sampling				1				
(2b) Delay in sampling	2	1	3	1				
(2c) Sampling after surgery		1	1			1		
(3) Extracranial injury	**1**	**7**	**1**	**2**	**4**			
(3a) Thoracic injury					1			
(3b) Multi-trauma		7		2	3			
(3c) Spinal cord injury (SCI)	1		1					
(4) Demographic	**1**	**3**	**2**	**1**		**3**		
(4a) Age	1		1	1		2		
(4b) Pre-existing neurological conditions			1			1		
(4c) Pre-existing systemic conditions		3						
(5) Unknown						**1**		

Table 3. Clinical explanations for outcome subgroups. Biomarkers examined: neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10), heart-type fatty acid-binding protein (H-FABP), amyloid β40 (Aβ40) and amyloid β40 (Aβ42).

	GFAP	IL-10	NF-L	S100B	H-FABP	T-tau	Amyloid β42	Amyloid β40
Number of outliers	3 (9%)	8 (25%)	8 (25%)	6 (19%)	1 (3%)	3 (9%)	2 (7%)	1 (3%)
(1) TBI severity	**3**	**2**		**4**				
(2) Timing of sampling		**3**	**5**	**2**		**1**		
(2a) Rapid sampling				2				
(2b) Delay in sampling		1	4					
(2c) Sampling after surgery		2	1			1		
(3) Extracranial injury	**1**	**6**	**1**	**4**	**2**		**1**	**1**
(3a) Thoracic injury					2			
(3b) Multi-trauma		6		4			1	1
(3c) Spinal cord injury (SCI)	1		1					
(4) Demographic	**1**	**2**	**3**	**1**		**2**		
(4a) Age	1		1	1		1		
(4b) Pre-existing neurological conditions			2			1		
(4c) Pre-existing systemic conditions		2						
(5) Unknown							**1**	

Table 4. Clinical explanations for imaging (CT) subgroups. Biomarkers examined: neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10), heart-type fatty acid-binding protein (H-FABP), amyloid β40 (Aβ40) and amyloid β40 (Aβ42).

	GFAP	IL-10	NF-L	S100B	H-FABP	T-tau	Amyloid β42	Amyloid β40
Number of outliers	4 (14%)	9 (30%)	7 (23%)	7 (23%)		3 (10%)		
(1) TBI severity	4	3	1	5				
(2) Timing of sampling	1	4	5	3		1		
(2a) Rapid sampling				2				
(2b) Delay in sampling	1	2	4	1				
(2c) Sampling after surgery		2	1			1		
(3) Extracranial injury		5	1	4				
(3a) Thoracic injury								
(3b) Multi-trauma		5		4				
(3c) Spinal cord injury (SCI)			1					
(4) Demographic	1	2	2	1		2		
(4a) Age	1		1	1		1		
(4b) Pre-existing neurological conditions			1			1		
(4c) Pre-existing systemic conditions		2						
(5) Unknown								

Table 5. Clinical explanations for biomarker outliers from the whole TBI patient population. Biomarkers examined: neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10), heart-type fatty acid-binding protein (H-FABP), amyloid β40 (Aβ40) and amyloid β40 (Aβ42).

Variable		TBI (n = 85)	Control (n = 40)	p-value
Age (years, mean ± SD)		54.94 ± 19.37	52.15 ± 18.83	0.45
Sex (male/female)		67 (79%) / 18 (21%)	18 (45%) / 22 (55%)	**<0.001**
GCS [median (range)]		10 (3-15)	-	-
Pupil reactivity	Unreactive / sluggish / reactive	17 (22%) / 3 (4%) / 57 (74%)[1]	-	-
ISS [median (range)]		17 (1-75)	-	-
Isolated TBI		49 (58%)	-	-
Hypoxia		7 (9%)[2]	-	-
Hypotension		4 (5%)[3]	-	-
Hypoglycemia		0 (0%)[4]	-	-
Anemia		5 (6%)[5]	-	-
Outcome				-
	Favorable (GOSE 5-8)	38 (48%)[6]	-	
	Unfavorable (GOSE 1-4)	42 (52%)[6]	-	
TBI-related deaths		17 (20%)	-	-
Marshall				-
	I	9 (11%)	-	
	II	21 (25%)	-	
	III	9 (11%)	-	
	IV	0 (0%)	-	
	V	32 (37%)	-	
	VI	14 (16%)	-	

Table 1. Demographics of the whole study cohort and controls.

Statistically significant p values are in bold. SD, standard deviation; GCS, Glasgow Coma Scale; ISS, Injury Severity Score; TBI, traumatic brain injury; Isolated TBI, TBI without concomitant extracranial injuries; Hypoxia, event of hypoxia after injury; Hypotension, hypotension after injury; Anemia, anemia after injury; GOSE, Glasgow outcome scale extended; [1], data missing on eight patients; [2], data missing on seven patients; [3], data missing on four patients; [4], data missing on one patient; [5], data missing on one patient; [6], outcome data missing on five patients

Variable		CT-positive (n = 76)	CT-negative (n = 9)	p-value	Favorable outcome (n = 38)[9]	Unfavorable outcome (n = 42)[9]	p-value
Age (years, mean ± SD)		55.63 ± 19.75	49.11 ± 15.62	0.343	48.63 ± 17.80	61.52 ± 18.43	**0.002**
Sex (male/female)		60 (79%) / 16 (21%)	7 (78%) / 2 (22%)	0.935	31 (82%) / 7 (18%)	32 (76%) / 10 (24%)	0.556
GCS [median (range)]		10 (3-15)	11 (3-15)	0.503	11 (3-15)	9 (3-15)	0.257
Pupil reactivity	Unreactive / sluggish / reactive	16 (24%) / 2 (3%) / 50 (73%)[1]	1 (11%) / 1 (11%) / 7 (78%)	0.38	5 (15%) / 1 (3%) / 27 (82%)[10]	11 (28%) / 2 (5%) / 27 (67%)[11]	0.381
ISS [median (range)]		17.5 (4-75)	6 (1-48)	**0.002**	12 (4-48)	24 (1-75)	**0.022**
Isolated TBI		46 (61%)	3 (33%)	0.118	21 (55%)	24 (57%)	0.866
Hypoxia		7 (10%)[2]	0 (0%)[3]	0.349	3 (8%)[12]	3 (8%)[13]	0.973
Hypotension		4 (6%)[4]	0 (0%)	0.468	2 (5%)[14]	2 (5%)[15]	0.936
Hypoglycemia		0 (0%)[5]	0 (0%)	-	0 (0%)[16]	0 (0%)	-
Anemia		4 (5%)[6]	1 (11%)	0.489	3 (8%)[17]	2 (5%)	0.542
Outcome				0.184			-
	Favorable (GOSE 5-8)	33 (45%)[7]	5 (71%)[8]		-	-	
	Unfavorable (GOSE 1-4)	40 (55%)[7]	2 (29%)[8]		-	-	
TBI-related deaths		17 (22.4%)	0 (0%)	0.113	-	17 (41%)	-
Marshall				-			0.067
	I	-	-		5 (13%)	3 (5%)	
	II	21 (28%)	-		13 (34%)	8 (17%)	
	III	9 (12%)	-		5 (13%)	5 (9%)	
	IV	0 (0%)	-		0 (0%)	1 (0%)	
	V	32 (42%)	-		12 (32%)	19 (43%)	
	VI	14 (18%)	-		3 (8%)	12 (26%)	

Table 2. Demographics of the study cohort divided into CT-positive vs. CT-negative and favorable (GOSE 5-8) vs. unfavorable (GOSE 1-4) outcome

Statistically significant p values are in bold. SD, standard deviation; GCS, Glasgow Coma Scale; ISS, Injury Severity Score; TBI, traumatic brain injury; Isolated TBI, TBI without concomitant extracranial injuries; Hypoxia, event of hypoxia after injury; Hypotension, hypotension after injury; Anemia, anemia after injury; GOSE, Glasgow outcome scale extended; [1], data missing on eight patients; [2], data missing on six patients; [3], data missing on one patient; [4], data missing on four patients; [5], data missing on one patient; [6], data missing on one patient; [7], data missing on three patients; [8], data missing on two patients; [9], outcome data missing on five patients; [10], data missing on five patients; [11], data missing on two patients; [12], data missing on one patient; [13], data missing on four patients; [14], data missing on one patient; [15], data missing on two patients; [16], data missing on one patient; [17], data missing on one patient

	GFAP	IL-10	NF-L	S100B	H-FABP	T-tau	Amyloid β42	Amyloid β40
Number of outliers	10 (19%)	12 (23%)	8 (16%)	8 (16%)	5 (10%)	5 (10%)	2 (4%)	1 (2%)
(1) TBI severity	**9**	**2**	**1**	**6**				
(2) Timing of sampling	**3**	**4**	**5**	**4**		**1**		
(2a) Rapid sampling				2				
(2b) Delay in sampling	3	2	4	2				
(2c) Sampling after surgery		2	1			1		
(3) Extracranial injury	**1**	**7**	**1**	**4**	**5**		**1**	**1**
(3a) Thoracic injury					2			
(3b) Multi-trauma		7		4	3		1	1
(3c) Spinal cord injury (SCI)	1		1					
(4) Demographic	**1**	**3**	**3**	**1**		**3**		
(4a) Age	1		1	1		2		
(4b) Pre-existing neurological conditions			2			1		
(4c) Pre-existing systemic conditions		3						
(5) Unknown						**1**	**1**	

Table 3. Clinical explanations for TBI outliers in the whole patient population, also including subgroups. Biomarkers examined: neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10), heart-type fatty acid-binding protein (H-FABP), amyloid β40 (Aβ40) and amyloid β40 (Aβ42).

	GFAP	IL-10	NF-L	S100B	H-FABP	T-tau
Number of outliers	3 (15%)	3 (15%)	4 (20%)	2 (10%)	3 (15%)	5 (25%)
(1) Unknown		**1**	**1**	**1**	**1**	**3**
(2) Extracranial injury		**1**		**1**	**2**	**1**
(2a) Thoracic injury					2	
(2b) Multi-trauma		1		1		1
(2c) Spinal cord injury (SCI)						
(3) Demographic	**4**		**3**			**2**
(3a) Age	1		1			1
(3b) Pre-existing neurological conditions	3		2			1

Table 4. Clinical explanations for control patient outliers. Biomarkers examined: neurofilament light (NF-L), glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), Total tau (T-tau), interleukin-10 (IL-10) and heart-type fatty acid-binding protein (H-FABP).