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Time-Dependent Changes in Serum Level of Protein Biomarkers after Focal Traumatic Brain Injury

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Abstract

Serum biomarkers could indicate the pathological changes during the secondary injury process after traumatic brain injury (TBI). Furthermore, they could reflect specific pathological processes following different types of TBI. Here we analyzed time-dependent changes of select protein biomarkers in serum samples collected from a rodent model of penetrating type of injury (pen-TBI). The model is a controlled penetration of a 2 mm thick needle-shaped object, which is accelerated into the brain tissue with a bullet from an air gun. The results obtained in the current study were compared to previously reported results of levels of serum biomarker following a rotational acceleration injury that mimics mild TBI. A total of 24 animals were used, grouped in normal controls, sham-operated and injured animals. The rats were sacrificed at day 1, day 3 and day 14 post-injury and serum samples were analyzed for Tau, neurofilament heavy chain (NF-H), myelin basic protein (MBP), N-cadherin and S100B. We found that all markers but MBP showing a bi-phasic response to injury. Their serum levels significantly increased at day 1, dropped at 3 and increased again at day 14 post-injury. This was in contrast to rotational TBI model where the peak of biomarkers was found at day 3. Our study suggests that pen-TBI results in both acute axonal and neuronal damages as well as delayed changes likely part of the ongoing secondary injury process. These findings illustrate the dynamics of the injury process in pen-TBI and underline the importance of monitoring changes in serum biomarker levels for more accurate assessment of injury severity and outcome. In addition, comparison to rotational TBI model revealed distinctive temporal pattern of serum biomarker expression dependent on the type of injury.

Keywords: Traumatic brain injury; Biomarkers; Axonal injury; S100B; Tau; Neurofilament; N-cadherin; Penetrating head injury

Introduction

Traumatic brain injuries (TBI) range from mild to severe lesions that can be immediately lethal. TBI can be caused by different injury mechanisms [1] such as focal impact, acceleration-deceleration and blast waves. The primary physical impact may lead to focal tissue injury or more dispersed injuries, such as diffuse axonal injury (DAI) but in many clinical cases there is a complex mixture of both focal and diffuse lesions [2]. Furthermore, the primary impact triggers complex secondary injury processes such as neurotransmitter release, excitotoxicity, mitochondrial dysfunction and inflammation that may lead to further tissue impairment [3-5]. This second phase of injury can last and extended period of time that provides a time window for intervention and treatment. Current diagnostic and consequently treatment options are limited. Although imaging techniques have an obvious central role in the clinical assessment after TBI, additional tools such as serum biomarker can provide critical information. Serum biomarkers in TBI have been suggested both for initial triaging as well as a tool for monitoring outcome. In order to better understand time-dependent changes during the secondary injury process following pen-TBI we collected blood samples at an early (1 day) mid (3 days) and a late (14 days) post-injury time points and determined injury induced changes in the serum levels of biomarkers indicative of axonal, neuronal and glia damage and/or loss using reverse phase protein microarray (RPPM).

Neuronal and glial proteins, such as Tau, neurofilament-heavy chain (NF-H), myelin basic protein (MBP), S100B and N-cadherin detected in the serum may reflect the extent and type of structural damage after TBI. Proteins such as Tau and neurofilaments have

been suggested candidates as axon specific biomarkers [6-8]. Tau is a microtubule-associated protein that is abundant in neurons in central nervous system (CNS) and primarily located in axons [8]. Early after TBI neuronal damage leads to axonal cytoskeleton changes and rapid loss of microtubules and microtubule associated proteins [9, 10]. Neurofilaments maintain axonal structural integrity where a disruption of this could result in neurofilament proteins being released into the extracellular space. Elevated serum levels of NF-H following TBI has previously been reported and correlated with lesion severity [11].

N-cadherin is a trans-synaptic cell adhesion molecule and is abundant in axons and dendrites where it links the extracellular environment to the actin cytoskeleton [12,13]. As a part of secondary processes following TBI calpains are pathologically activated [14,15] and lead to cleavage of N-cadherin [16].

Glial proteins such as MBP and S100B have been broadly studied in serum after TBI. MBP is a myelin membrane proteolipid produced by oligodendroglial cells. It is abundant in white matter and has been

examined as a marker of axonal damage in neurointensive care. S-100B is a cytosolic calcium-binding protein that is predominantly found in glial cells and mainly in astrocytes. S100B has been considered as a promising biochemical marker of brain damage based on several studies showing a positive correlation between high S-100B and severity and outcome of TBI patients [17-21]. However, there are several studies showing no correlation between serum S100B levels and injury severity [22]. Furthermore, S100B has low specificity since it is present in many different cell types [23].

We have previously reported on the serum levels of Tau, NF-H, MBP and S100B in a rotational acceleration TBI (rot-TBI) model that mimics mild TBI [24,25]. Axonal injury detected by β-APP was a hallmark of this injury with no signs of contusion or hemorrhages. The behavioral outcome of this model showed transient memory impairment indicating a mild-TBI. The serum levels were measured at 1 day, 3 days and 14 days postinjury and there was a significant increase at all-time points except in NF-H at day 14. The biomarkers peaked at day 3 following injury. In the current study we have used a model of focal penetrating TBI by inducing controlled, reproducible and quantifiable injury [26] and monitored changes in the serum levels of the above protein biomarkers including N-cadherin. Our aim was to investigate if focal penetrating TBI induces release of serum biomarkers and if it varies in level of expression or temporal pattern compared to a mild diffuse TBI. We hypothesized that the level of expression and/or the temporal pattern of these biomarkers will be different dependent on the biomechanical elements inducing TBI as well as the resulting level of injury.

Materials and Methods

Animals

A total of 24 adult Sprague-Dawley male rats were used, there were 6 normal control rats and 18 sham and injured rats described in detail below. The animals were deeply anaesthetized by intra-abdominal injection of a 2.4 ml/kg of a mixture of 1 ml Dormicum® (5 mg/mlMidazolam, Roche), 1 ml Hypnorm® (Janssen) and 2 ml of distilled water. Thereafter, the animals were given 0.2 ml/kg intra-muscular injections of mentioned mixture every 0.5 hours until the trauma and following surgery was completed. The work was performed in accordance with the Swedish National Guidelines for animal experiments, which was approved by the Animal Care and Use Ethics Committee in Stockholm. The methods have been described in detail previously [25,27,28].

Penetration injury model (pen-TBI)

A midline incision was made through the skin and a 2.8 mm craniotomy was drilled with a centre about 3 mm posterior and 3 mm lateral to the bregma. The animals were placed in a frame in order to avoid acceleration injury. A lead bullet was accelerated by air pressure in a specially designed rifle and impacted a secondary projectile. The second projectile consisted of a metal cylinder with an attached carbon fiber pin (length 24 mm, diameter 2 mm) with a tip shaped like a pencil, tip angle of 30 degrees. The pin of the secondary projectile, guided by a narrow tube, penetrated into the brain of the rats with a speed of 90 m/s. The design of the narrow tube provided good control of the penetration depth into the brain, which was 5.4 ± 0.4 mm (mean ± SD) mm from the dura. This model produces a widespread axonal lesion that can be detected within 2 hours with immunohistochemistry for b-amyloid precursor protein (APP) [25]. A zone with a mixture of dying and surviving neurons and glial cells surrounds the focal lesion and there is

a manifest Blood-Brain Barrier (BBB) disturbance and an extracellular perivascular edema [25]. A total of 18 animals with different surviving times, were used for this TBI group. Injury groups: 1day (n=3), 3 days (n=3), 14 days (n=3) and sham groups; 1day (n=3), 3 days (n=3), 14 days (n=3).

Reverse phase protein microarray (RPPM)

The survival time of traumatized and sham animals was 1 day, 3 days and 14 days after injury. The animals were deeply sedated by 2.4 ml/kg intra-abdominal injections of a mixture of 1ml Dormicum® (5 mg/ml Midazolan, Roche), 1 ml Hypnorm® (Janssen) and 2 ml of distil water and sacrificed through vast drainage of peripheral blood. The blood was drained and centrifuged and serum was removed, aliquoted and frozen on dry ice. The degree of hemolysis was performed by visualization. The detailed method of proteomics is described previously [29]. Briefly, tissue samples were pulverized in liquid nitrogen, transferred into T-per lysis buffer (#78510 Thermo Fisher, Waltham, MA) with EDTA-free Halt protease inhibitor cocktail (#78441 Thermo Fisher), sonicated, then the supernatants of the centrifuged samples were aliquoted, and stored at -80°C until use. Then samples were transferred into a JANUS Varispan Integrator and Expanded Platform Workstation (PerkinElmer, Waltham, MA) for serial dilution and transferred into Genetix 384-well plates (X7022, Fisher Scientific, Pittsburg, PA). Plates were centrifuged at 4°C at 1,500 g for 5 minutes and transferred into a Q-array Mini microarray printer (Genetix, Boston, MA). Nitrocellulose-coated glass slides (Whatman FAST, Fischer) were stained with primary antibodies for neurofilament heavy chain (NF-H, 1:20, Sigma N4142), the microtubule associated protein total-Tau (Tau, 1:20, Santa Cruz sc-1995P), N-cadherin (N-Cadh 1:20 Santa Cruz sc-31031) myelin basic protein (MBP, 1:20, Santa Cruz sc-13914) and S100B (s100b 1:20 Abcam ab-41548). The slides were incubated with primary antibodies in a humidity chamber at 4 °C overnight, while gently rotated. The slides were finally incubated with secondary antibodies gently rotated for 1 hour at room temperature. The fluorescent signals were measured by scanning the slides with a 633 wavelength laser using a 647 nm filter in a Scan Array Express microarray scanner (Perkin Elmer, Waltham, MA). Data were imported into a Microsoft Excel-based bioinformatics program developed in house for analysis. The tool imports intensity data from the scanner output and calculates the total net intensity after local background subtraction for each spot. The background subtracted intensity data from the dilution series of each sample are then plotted against dilution on a $\log_{10}$ graph. Linear regression of the $\log_{10}$ data is done after removal of flagged data. Flagged data include spot intensities in the saturation range or noise range, signal to noise ratio less than 2, or high variability between duplicate spots (>10-15%). The total amount of the antigen is determined by the Y-axis intercept *i.e.*, extrapolating the regression to zero (the undiluted sample). The Y-intercept values are given in log10 hence the power of each fold change is vast.

Statistical analysis

Statistical analysis was carried out using SPSS 19.0 and alpha was set to $p \leq 0.05$ for all analyses. For each of the biomarkers, a one-way analysis of variance (ANOVA) was performed to compare values of sham and normal controls. After that no significant difference were obtained between sham and controls the log10 values obtained for each biomarker in injured animals were normalized (z-transformation) in comparison to values obtained from normal controls. In order to compare the two different TBI models previous data from rotational TBI model [24] was compared to results obtained in current study. For each of the biomarkers, a two-way ANOVA on log10 values was performed with Time (day1, day3 and day14) and group (rot-TBI,

rot-TBI-sham, pen-TBI and pen-TBI-sham) as variables of interest. Following this we performed multiple comparisons with Bonferroni correction to investigate the significant difference at each time point between each group. The non-normalized results obtained from each group and biomarkers are presented in Table 1. The normalized values of each marker are graphically illustrated in the Figures 1-3. It should be noted that the Y-intercept values in the table and figures are given in $\log_{10}$ hence the power of each fold change is vast. The $\log_{10}$ presentation has shown to be the most appropriate way to present data obtained from RPPM [29].
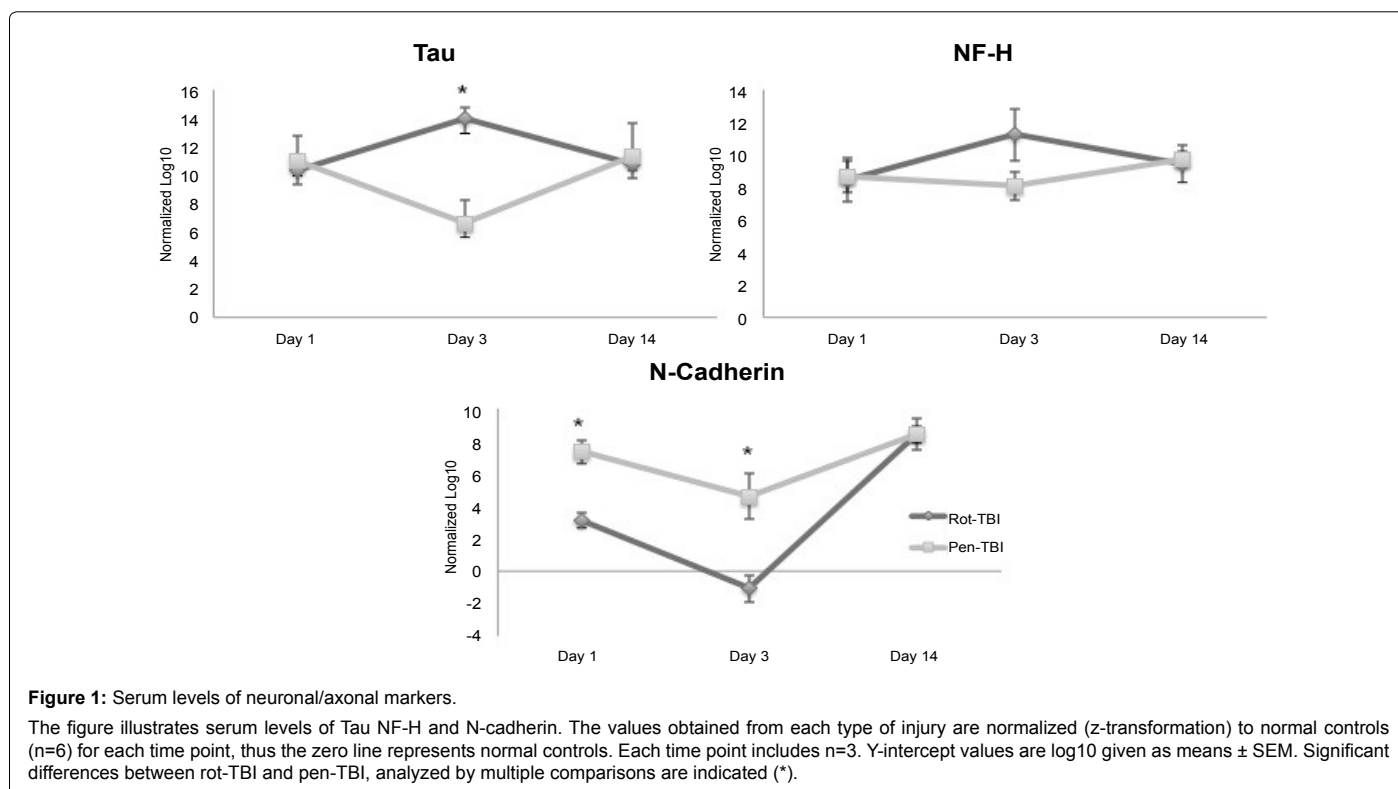
Results

Comparing sham to injured animals, we found significantly increased serum levels of both Tau and NF-H both showing time-dependent changes (Figure 1). Following pen-TBI, serum values of Tau showed a bi-phasic pattern; values peaked at day 1, dropped significantly at day 3 and returned to maximum level at day 14. Serum NF-H values showed a similar trend and mirrored the changes seen in serum Tau levels. Serum levels of N-cadherin, S100B showed similar bi-phasic patterns (Figure 2). The only marker that showed different temporal profile was MBP. It showed a gradual increase between and its serum level was the highest at day 14 (Figure 2). For comparison we have included data from our previous study on rotational TBI. That type of injury results in traumatic axonal injuries in the white matter [24] resembling diffuse axonal injuries (DAI). As seen in Table 1 and Figure 1-3 this injury also results in increase of Tau, NF-H, N-cadherin,

Marker	Time	Normal Control	Pen-TBI	Sham	Rot-TBI	Sham
S100B	Day 1	5.98 ± 0.11	6.73 ± 0.15	5.96 ± 0.19	6.75 ± 0.16	5.95 ± 0.04
	Day 3		6.48 ± 0.12	6.02 ± 0.03	7.00 ± 0.09	6.02 ± 0.07
	Day 14		6.74 ± 0.14	5.93 ± 0.14	6.61 ± 0.16	6.20 ± 0.12
N-cadherin	Day 1	4.50 ± 0.07	5.67 ± 0.11	4.47 ± 0.07	5.00 ± 0.07	4.22 ± 0.18
	Day 3		5.23 ± 0.22	4.45 ± 0.06	4.34 ± 0.06	4.32 ± 0.06
	Day 14		5.84 ± 0.17	4.57 ± 0.16	5.82 ± 0.08	4.44 ± 0.03
MBP	Day 1	5.02 ± 0.01	5.36 ± 0.10	5.10 ± 0.03	5.33 ± 0.05	5.09 ± 0.01
	Day 3		5.46 ± 0.07	5.15 ± 0.04	5.73 ± 0.11	5.16 ± 0.02
	Day 14		5.57 ± 0.10	5.16 ± 0.03	5.63 ± 0.07	5.17 ± 0.07
NF-H	Day 1	5.20 ± 0.03	5.80 ± 0.07	5.24 ± 0.10	5.79 ± 0.09	5.24 ± 0.05
	Day 3		5.76 ± 0.06	5.23 ± 0.05	5.98 ± 0.11	5.25 ± 0.04
	Day 14		5.88 ± 0.03	5.26 ± 0.06	5.86 ± 0.08	5.20 ± 0.05
Tau	Day 1	4.43 ± 0.05	5.74 ± 0.21	4.80 ± 0.01	5.66 ± 0.09	5.01 ± 0.08
	Day 3		5.08 ± 0.10	4.74 ± 0.09	6.10 ± 0.10	4.84 ± 0.09
	Day 14		5.79 ± 0.27	4.97 ± 0.06	5.72 ± 0.08	4.63 ± 0.05

Table 1: The results for each biomarker are given as the Y-intercept values obtained from the RPPM analysis. The Y-intercept values are given in $\log_{10}$ hence the power of changes are vast. In normal control group *n*=6 and in each injured and sham group *n*=3.

Figure 1: Serum levels of neuronal/axonal markers.

The figure illustrates serum levels of Tau NF-H and N-cadherin. The values obtained from each type of injury are normalized (z-transformation) to normal controls (n=6) for each time point, thus the zero line represents normal controls. Each time point includes n=3. Y-intercept values are log10 given as means ± SEM. Significant differences between rot-TBI and pen-TBI, analyzed by multiple comparisons are indicated (*).
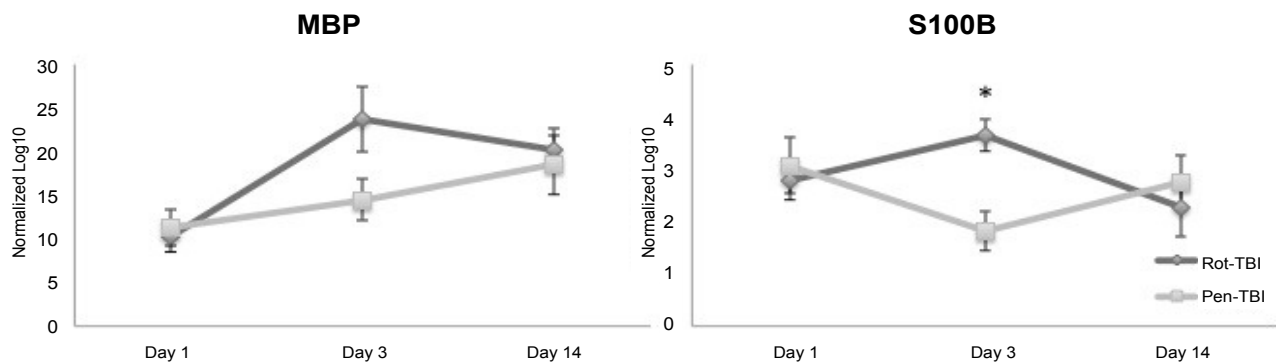
Figure 2: Serum levels of glial markers MBP and S100B.

The values obtained from each type of injury are normalized (z-transformation) to normal controls (n=6) for each time point, thus the zero line represents normal controls. Each time point includes n=3. Y-intercept values are log$_{10}$ given as means ± SEM. Significant differences between rot-TBI and pen-TBI, analyzed by multiple comparisons are indicated (*).
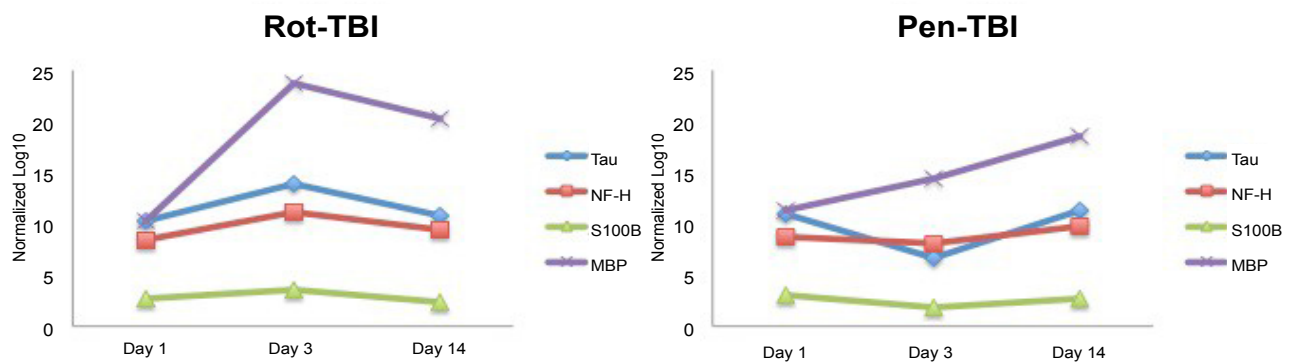

Figure 3: Panel of biomarkers.

Summary of serum markers that showed to discriminate between the two different TBI models and that can be used in a palette of biomarkers. Y-intercept values are log$_{10}$ given as means normalized to normal controls, thus the zero line represents normal controls.

S100B and MBP. However, the bi-phasic pattern that was seen in the present study was not observed after rotational TBI where all markers seemed to have a peak at 3 days. Thus, the timetable seems different when these two types of injury are compared.

Discussion

The results from the present study contrast our previous observations in the rotational type TBI [24]. The two different models result in different types of tissue damage [24-26]. The rotational acceleration TBI induces axonal injury detected by β-APP staining as early as 2h post-injury. The axonal injuries are more widespread and found in larger quantity in corpus callosum and the border of white and grey matter. No contusion or hemorrhages was detected when lower acceleration impact was used. In animals with high acceleration trauma β-APP positive axons were detected in the brain stem. There were also signs of axonal swelling and bulbs in the brain stem of these animals detected by FD silver staining. No signs of BBB changes could be detected. The behavioral outcome of the low acceleration impact produced modest and transient memory impairment indicating a mild TBI. In contrast, the penetrating injury induces impairment in motor function, reference memory and anxiety following focal tissue damage with laceration including bleeding and BBB disruption. Our gene array analysis of brain tissue obtained from these two different models also

revealed distinctive gene expression profiles [30]. We found greater inflammatory response and apoptosis after pen-TBI compared to rot-TBI.

Using RPPM, we detected significant changes in the serum levels of select biomarkers both after rot-TBI [24] as well as after pen-TBI (this paper) suggesting neuronal and glial cell damage, altered cell adhesion and axonal pathologies.

Axonal injury

Previous studies have suggested Tau and neurofilaments as biomarkers for axonal pathologies after TBI [6-8]. The axonal injury after TBI consist of an immediate axotomy caused by the direct mechanical insult and a secondary axotomy resulting from a progressive cascade of pathological events [10,31]. After axonal injury Tau is cleaved by calpains and this proteolytic activity has shown to have an early peak of minutes to hours following a second peak at 4 days [14,15]. In this study we detected a bi-phasic pattern of serum Tau levels, suggesting that the initial penetrating impact results in a rapid axonal damage and loss that can be detected in serum by day 1. By contrast, Tau levels peaked at 3 day following rot- TBI suggesting that the rotational injury probably initiates a cascade of events over days leading to the serum peak value of Tau at day 3. Previous experiments showed that using Controlled

Cortical Impact (CCI) model of TBI, Tau levels in serum peaked as early as 6h post-injury with no significant increase at later time points [32].

Elevated serum NF levels have been observed after various forms of TBI and the temporal patterns of the increases varied depending on the type of injury. It was 7 days after subarachnoid hemorrhage [33], 3 days after spinal cord injury [7], 2-3 days following CCI [11] and 6h following blast TBI [34]. In this study highest serum NF levels were detected at day 1 and 14. This is in contrast with findings from our previous study where serum NF levels peaked at day 3 suggesting a different axonal pathology triggered by rotational forces. The second peaks of Tau and NF-H in pen-TBI at day 14 can indicate additional pathological process such as inflammation that can trigger additional – delayed- axonal damage. It should be pointed out that the intracerebral milieu after closed head, such as rotational and after open, such as penetrating TBI are very different. Intracranial bleeding after pen-TBI can majorly alter the half-life of protein biomarkers and may very well affect the elimination of protein biomarkers. The half-life of Tau and neurofilaments in the serum are not known but the half-life of (cleaved) Tau in the CSF is up to 5 days [35]. Accordingly, the observed peaks at 2 weeks could be a result of altered elimination of released proteins as well as continues release from damaged axons.

Elevated serum MBP levels are indicator of white matter tract and also axonal injury [34]. In this study, we observed a time-dependent increase of serum MBP levels reaching maximum at the termination of the experiment (day 14). The underlying cause of this temporal profile, which contrasts all the other markers measured in this study, can be multifold. These can include slow axonal degeneration similar to Wallerian degeneration, which may take months to years (in human), slow clearance of myelin debris [36,37], ongoing pathological changes such as inflammation and the combination of the above.

Neuronal and glial injury

Increased serum levels of N-cadherin can be an indicator of neuronal and /or synaptic loss [38,39]. Similar to most of the other biomarkers, serum N-cadherin levels also showed a biphasic pattern with peaks by day 1 and 14. The underlying cause(s) for this temporal pattern can involve several of the same changes as discussed above. We would like to point out that this is one of the first report showing injury induced changes in serum N-cadherin levels following TBI thus N-cadherin may be a valuable biomarker to consider in future TBI studies.

The bimodal increase of serum S100B level with peaks at day 1 and day 14 also supports the notion of additional secondary injury processes resulting in delayed astroglia damage and or loss. Increased levels of S100B in TBI patients have been correlated to secondary events [19,40]. A second, delayed peak in serum S100B levels has been found indicative of poor outcome after severe TBI [41]. The short half-life of S100B, 30-90 min, also indicates that its elevated serum levels should reflect ongoing damage of astroglia with the subsequent release of the protein into the serum [42,43]. A meta-analysis on mild-TBI including 12 studies showed that the pooled negative predictive value was more than 99% (95% CI 98%-100%) [44]. However, the interpretation of serum S100B in moderate to severe TBI is still under discussion. It is much dependent on collecting time following trauma and increased serum S100B levels have been found in other conditions and S100B can be of extracranial origin [45, 46].

Elevated serum levels of S100B have been shown to correlate with BBB dysfunction albeit but not at late post-injury time point [40,47].

In our rot-TBI model we observed highly elevated S100b serum levels even though no BBB defects could be detected [25]. A biphasic BBB breakdown after TBI with an early rapid phase, within hours, followed by a second phase 3-7 days after injury have been previously demonstrated [48,49]. How BBB dysfunction and elevated serum protein biomarker levels are correlated is currently not well understood.

The primary traumatic brain injury is the result of mechanical forces that put a strain on the brain parenchyma at the time of impact. Although these forces may be of various forms e.g. penetrating injuries or rotational acceleration they result in secondary pathological processes that can deteriorate the initial injury and the clinical status of TBI patients. It is highly valuable to be able to detect secondary injury processes by blood sampling and be able to take preventive measures in order to improve patient outcome. This study shows that injury severity and different types of TBI generate different level and temporal expression of serum biomarkers. This indicates that a panel of serum biomarkers may be used to aid clinician in diagnosis of type and level of injury. Furthermore, the temporal pattern may indicate ongoing secondary processes that could be prevented.

The current study has several limitations, most importantly is the low number of animals in each group thus further studies, with more animals and additional time points and additional biomarkers are needed to firmly establish the temporal pattern of changes of protein biomarkers following pen-TBI.

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