Blood-Based Brain and Global Biomarker Changes after Combined Hypoxemia and Hemorrhagic Shock in a Rat Model of Penetrating Ballistic-Like Brain Injury

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Abstract
Penetrating traumatic brain injury (pTBI) often occurs with systemic insults such as hemorrhagic shock (HS) and hypoxic (HX). This study examines rat models of penetrating ballistic-like brain injury (PBBI) and HX+HS to assess whether the blood levels of brain and systemic response biomarkers phosphorylated neurofilament-heavy protein (pNF-H), neurofilament-light protein (NF-L), αII-spectrin, heat shock protein (HSP70), and high mobility group box 1 protein (HMGB1) can distinguish pTBI from systemic insults and guide in pTBI diagnosis, prognosis, and monitoring. Thirty rats were randomly assigned to sham, PBBI, HS+HX, and PBBI+HS+HX groups. PBBI and sham groups underwent craniotomy with and without probe insertion and balloon expansion, respectively. HX and HS was then simulated by blood withdrawal and fraction of inspired oxygen (FIO2) reduction. Biomarker serum concentrations were determined at one (D1) and two (D2) days post-injury with enzyme-linked immunosorbent assay (ELISA) methods. Axonal injury-linked biomarkers pNF-H and NF-L serum levels in PBBI groups were higher than those in sham and HX+HS groups at D1 and D2 post-injury. The same was true for PBBI+HS+HX compared with sham (D2 only for pNF-H) and HX+HS groups. However, pNF-H and NF-L levels in PBBI+HS+HX groups were not different than their PBBI counterparts. At D1, αII-spectrin levels in the HX+HS and PBBI+HS+HX groups were higher than the sham groups. αII-spectrin levels in the HX+HS group were higher than the PBBI group. This suggests HX+HS as the common insult driving αII-spectrin elevations. In conclusion, pNF-H and NF-L may serve as specific serum biomarkers of pTBI in the presence or absence of systemic insults. αII-spectrin may be a sensitive acute biomarker in detecting systemic insults occurring alone or with pTBI.

Keywords: biomarker; brain injury; global injury markers; organ injury

Introduction
The incidence and mortality of penetrating traumatic brain injury (pTBI) have more than doubled in the civilian population between 2010 and 2014. Roughly half of these patients suffered severe TBI, with a mortality rate of 43.8%.1 These patients often require extensive time in the intensive care unit (ICU) and require extensive rehabilitation services. Unfortunately, limited data exist for pTBI due to its relatively low prevalence.2 Further, polytrauma commonly occurs with pTBI after
improvised explosive device attacks. Patients who suffered from pTBI and polytrauma face physical, cognitive, and psychological impairments and have a higher mortality rate than those with pTBI alone.3–5

A high prevalence of concomitant hypoxemia (HX) and hemorrhagic shock (HS) exists in patients with pTBI and polytrauma. Experimental TBI combined with HX and HS results in worsened neurological outcomes, increased metabolic disturbances, and neuronal cell death.9 For example, our previous study demonstrated the cumulative effects of HS and HX on the physiological, metabolic, and neurobehavioral outcomes in a penetrating ballistic-like brain injury (PBBI) model.10–12 Therefore, these systemic insults are often incorporated into the existing pTBI models to increase translational research’s clinical relevance.

Brain imaging modalities, including computed tomography (CT) and magnetic resonance imaging (MRI), provide a useful but limited assessment of the injury severity of pTBI. For example, CT exposes patients to radiation and is insensitive for the diagnosis of diffuse axonal injury.13 Although MRI better detects axonal injury and subtle neuronal damage, it is not suitable for patients with hemodynamic instability, is more difficult to perform on patients receiving ventilation, and is generally reserved for the subacute stage.14 These limitations leave a clinical gap where important data need to be garnered for prognosis and treatment. Readily accessible blood-based biomarkers found within the serum, plasma, or whole blood may play a role in the diagnosis and prognosis of PBBI with or without systemic insults. Additionally, they may provide pathophysiological information of injury severity of pTBI throughout a patient’s disease course in the intensive care setting. Further, they may play a role in evaluating the efficacy of treatments of TBI in the setting of polytrauma. The ability to predict mortality and injury severity may facilitate the development of clinical guidelines for managing pTBI.15

Several biomarkers are reported to be altered after PBBI, including GFAP (glial fibrillary acidic protein; astrogliosis/astroglial injury),16–18 brain-derived neurotrophic factor (BDNF; neurogenesis/neuroprotection),19 ubiquitin C-terminal hydrolase-L1 (UCH-L1; neuronal cell body injury),20 cathepsin B (CatB; apoptosis/cell necrosis),21 and microRNA (miRNA; astrogliosis/inflammation/neurodegeneration).22,23

Considering that pTBI and polytrauma often occur concurrently in the setting of emergency medicine, we hypothesized that the use of brain-specific and global injury biomarkers can reliably identify HX+HS and frontal PBBI with and without systemic insults (HX+HS) to diagnose and differentiate combined injury from isolated pTBI. The brain-specific biomarkers of interest include the phosphorylated axonal form of the heavy neurofilament subunit (phosphorylated neurofilament-heavy protein [pNF-H]; axonal injury)15,24,25 and the light neurofilament subunit (neurofilament-light protein [NF-L]; axonal injury).26,27 The global injury biomarker panel includes the high mobility group box 1 protein (HMGB1; alarmin stress response/cell necrosis)28–31 and heat-shock protein (HSP70; organ stress/shock response).32 Although levels of zII-spectrin and its breakdown products (axonal injury/cell necrosis) have been reported to change in the setting of PBBI, they were also included in this global injury marker panel considering their abundant expression in non-central nervous system (CNS) organs.15,16,18,33,34 We also hypothesize that polytrauma will exacerbate brain injury and cause rising levels in brain-specific biomarkers.

Methods

Research subjects

Thirty male adult Sprague-Dawley rats (280–320 g, 3 months old; Charles River Labs, Raleigh, NC, USA) were used in these experiments. All protocols involving animal use were reviewed and approved by the Institutional Animal Care and Use Committee of Walter Reed Army Institute of Research. Research was conducted in compliance with the Animal Welfare Act, Guide for the Care and Use of Laboratory Animals (eighth edition, National Research Council), and other federal statutes and regulations. All surgical procedures were performed using aseptic technique. Animals were housed individually under a 12-h light-dark cycle in a temperature-controlled facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animals had access to food and water ad libitum post-injury.

PBBI, HX, and HS

Anesthesia was induced in an induction chamber with 3.5% isoflurane delivered in air/oxygen mixture (fraction of inspired oxygen [FIO2] = 0.26) and maintained using a nose cone at 1.5% throughout the surgeries. In all animals, the right femoral artery and vein were cannulated using number 2 French tubing (cat. no. BTPU-027; Instech Laboratories) for mean arterial blood pressure (MAP) monitoring and fluid resuscitation, respectively. Using the same tubing, the tail artery was cannulated for inducing HS by withdrawing blood and for blood gas
was induced by reducing FIO2 to 0.1 (10% oxygen balancing was sealed with sterile bone wax. Transient HX brain volume causing a temporary intracerebral cavity. an elliptical balloon calibrated to 10% of the total rat material [M-L] from bregma) to a distance of 12 mm (from dura). The elastic tubing on the probe was inflated by a cranial window (Mitre Corp., McLean, VA, USA) with a specially designed stainless-steel probe (Popper & Sons Inc., Hyde Park, NY, USA). The probe was mounted to a stereotaxic arm at an angle of 50 degrees from the vertical axis and 25 degrees counterclockwise from the anteposterior (A-P) axis. It was then manually inserted through the right frontal cortex of the anesthetized rat via a cranial window (+4.5 mm A-P, +2 mm mediolateral [M-L] from bregma) to a distance of 12 mm (from dura). The elastic tubing on the probe was inflated by a rapid (40 millisecond) water pressure pulse, forming an elliptical balloon calibrated to 10% of the total rat brain volume causing a temporary intracerebral cavity.

The probe was then gently retracted, and the cranial opening was sealed with sterile bone wax. Transient HX was induced by reducing FIO2 to 0.1 (10% oxygen balanced with 90% nitrogen), resulting in a PaO2 of less than 40 mm Hg (confirmed by blood gas analysis). Normoxia (FIO2 = 0.26) was restored after 30 min of HX. Transient HS was induced by withdrawing blood via the tail arterial catheter using a withdrawing pump (Harvard Apparatus, MA, USA) at a constant rate of 0.25 mL per 100 g/min to reduce the MAP by 5 mm Hg, from 45 mm Hg to 40 mm Hg (monitored via femoral artery catheter). HS was maintained for 30 min. The animals then received fluid resuscitation with lactated Ringer’s solution (B. Braun Medical, PA, USA) via the femoral vein catheter. The infusion volume was 3 times the blood volume withdrawn. In the HX+HS groups, HX started at 5 min after PBBI or sham PBBI, whereas HS started at 5 min after restoring normoxia from HX. The sham control animals received all surgical procedures (including catheterizations and craniotomy) with the exception of the PBBI probe insertion/expansion.

Serum preparation
Blood samples (2 mL) were collected at terminal end-points (D1 and D2 post-injury) by cardiac puncture using Z/1.3 clotting tubes (Sarstedt, Newton, NC, USA) and were allowed to clot at room temperature for 30 min before centrifugation at 1200 g for 10 min at 4°C. Serum was transferred to a storage tube and kept in −80°C. All samples were shipped via FedEx priority overnight (on dry ice) to the University of Florida for protein analyses.

Quantitation of protein biomarker
Dynamic evaluation of brain and global biomarkers was performed at D1 and D2 post-injury in the PBBI model. Serum concentrations of brain and global biomarkers pNF-H (cat no. RD191138300R; Biovendor), NF-L (item 103345; Quanterix), HSP70 (cat no. ab133060; abcam), and inflammation markers HMGB1 (ABIN416082; Antibodies-online.com) and zII-spectrin (cat no. ABIN1572517; Antibodies-online) were determined with sandwich enzyme-linked immunoabsorbent assays (ELISA) at the University of Florida. Sandwich ELISAs were conducted using standard 96-well, flat-bottom, Nunc Immuno Maxisorp plates (Fisher, Pittsburgh, PA, USA), according to the manufacturer’s protocol. Selected capture and detection antibody pairs were used.

Briefly, ELISA plates were passively coated overnight at 4°C with capture antibody, then washed and blocked. Serum samples were added and incubated at room temperature with shaking. After washing, peroxidase-conjugated detection antibody or HRP-conjugated streptavidin were added, which catalyzed the reaction with a colorimetric substrate (TMB; Pierce). The product was quantified by absorbance at 450 nm in a microplate spectrophotometer. Standard curves were generated using recombinant proteins corresponding to the biomarker measured in each assay. Four parameter-fit non-linear regression analyses were applied to determine biomarker quantities.

Statistical analysis
Statistical analysis was performed using GraphPad Prism (version 9.0.0). Data were presented as mean ± standard error of the mean (SEM). The differences in biomarker concentration among the groups were analyzed with one-way analysis of variance (ANOVA) followed by post hoc comparisons using Tukey’s test. The criterion for significance was set at \( p < 0.05 \).

Results
Tissue specificity for biomarkers associated with brain injury and polytrauma
We first conducted omics-based data mining to ascertain specific tissue expression of the five biomarkers of interest. The relative biomarker mRNA expression
levels were retrieved from the GeneCards human gene database.\textsuperscript{60} pNF-H (NEFH) and NF-L (NEFL) show high specificity to the CNS (brain, spinal cord). mRNA for HSP70 (HSPA4), and HMGB1 is ubiquitously expressed in all organs and peripheral blood cells.\textsuperscript{15,33,34} Although our data mining show that zII-spectrin (SPTAN1) levels are highest in the CNS, this biomarker is widely distributed among other organs.

We next verified the protein distribution of the same markers in various human organs and cell types. The results are based on mass spectrometry detection of the tryptic peptides derived from the five respective parent proteins shown on the Human Proteome Map portal.\textsuperscript{61} NF-L and NF-H are highly specific and enriched in the brain and spinal cord and are minimally concentrated in other organs or cells. In contrast, HMGB1 and HSP70 show strong protein signals throughout all organs and cells examined. Lastly, zII-spectrin (SPTAN1) protein is enriched in the CNS but is also expressed in other tissues.

Temporal survey of serum biomarkers
The serum levels of the brain-specific axonal injury marker pNF-H were analyzed at D1 and D2 in all groups (Fig. 1A,B). pNF-H serum levels in animals subjected to PBBI were significantly higher than those in the sham control ($p<0.01$) and HX+HS ($p<0.001$) groups at D1. Similarly, pNF-H levels in animals subjected to PBBI were significantly higher than those in the sham control ($p<0.0001$) and HX+HS ($p<0.0001$) groups at D2. There was also a significant increase in pNF-H levels in the P+HX+HS groups compared with the HX+HS groups at D1 and D2. There was a non-significant increase at D1 in pNF-H levels in the PBBI group compared with the sham group.

\begin{figure}[h]
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\caption{Serum pNF-H levels at D1 (A) and D2 (B) post-injury. The levels of pNF-H showed a significant increase in PBBI at D1, and in both PBBI and P+HX+HS groups at D2 when compared with sham (**$p<0.01$, ****$p<0.0001$). The levels of pNF-H significantly increased in PBBI and P+HX+HS groups at D1 and D2 when compared with HS+HX (##$p<0.01$, ###$p<0.001$, ####$p<0.0001$). Biomarker concentration differences were analyzed with ANOVA followed by post hoc comparisons using Tukey’s test. Data are presented as mean ± SEM. ANOVA, analysis of variance; D1, one day; D2, two days; HS, hemorrhagic shock; HX, hypoxemia; P, penetrating ballistic-like brain injury; PBBI, penetrating ballistic-like brain injury; pNF-H, phosphorylated neurofilament-heavy protein; SEM, standard error of the mean.}
\end{figure}
that reached significance at D2. No significant differences were detected between the HX+HS and sham groups. At D1 and D2 post-injury, pNF-H levels trended lower in the P+HX+HS group compared with those in the PBBI group, but the difference was not significant.

The serum levels of NF-L were significantly higher in the PBBI (p<0.01) and P+HX+HS (p<0.05) groups at D1 and D2 post-injury. NF-L serum levels were also significantly higher in both PBBI (p<0.01 at D1 and D2) and P+HX+HS (p<0.05 at D1; p<0.001 at D2) groups when compared with HX+HS. There were no statistically significant differences between PBBI and P+HX+HS groups at either day post-injury. (Fig. 2A,B)

aII-spectrin is a major cortical cytoskeleton protein located in the brain and in nucleated cells in most peripheral organs.15,33,37 aII-spectrin serum levels in animals subjected to HX+HS were significantly higher than those in the sham control (p<0.001) and PBBI (p<0.05) groups at D1 post-injury (Fig. 3A). Similarly, aII-spectrin levels were significantly higher at D1 in the PBBI+HX+HS groups compared with the sham groups (p<0.05) (Fig. 3A). No significant differences were detected between the PBBI alone and sham groups at D1 post-injury. By D2 post-injury, aII-spectrin levels in all groups returned to sham control levels. (Fig. 3B).

Serum levels of global stress response biomarker HSP70 were quantitated at D1 and D2 post-injury (Fig. 4). There was a non-significantly increasing trend of HSP70 levels in the injury groups compared with the sham control group at D1 post-injury (Fig. 4A). At D2 post-injury, the serum levels of HSP70 in all injury groups were comparable to that in the sham control group (Fig. 4B).

Lastly, serum levels of the inflammation biomarker, HMGB1, were quantitated at D1 and D2 post-injury (Fig. 5). No significant differences between groups were detected in the levels of HMGB1 at both time-points.

**FIG. 2.** Serum NF-L levels at D1 (A) and D2 (B) post-injury. The levels of NF-L showed a significant increase in both PBBI and P+HX+HS groups at D1 and D2 when compared with sham (*p<0.05, **p<0.01). The levels of pNF-H significantly increased in PBBI and P+HX+HS groups at D1 and D2 when compared with HX+HS (#p<0.05, ###p<0.01, ####p<0.001). Biomarker concentration differences were analyzed with ANOVA followed by post hoc comparisons using Tukey’s test. Data are presented as mean ± SEM. ANOVA, analysis of variance; D1, one day; D2, two days; HX, hemorrhagic shock; HX, hypoxemia; NF-L, neurofilament-light protein; P, penetrating ballistic-like brain injury; PBBI, penetrating ballistic-like brain injury; pNF-H, phosphorylated neurofilament-heavy protein; SEM, standard error of the mean.
Discussion

Our study investigates brain and global injury biomarkers following combined HX and HS (HX+HS) insults in a pTBI model to help differentiate combined injury from isolated TBI. Brain imaging, such as CT and MRI, provides consistent methods to assess the presence and severity of pTBI with or without the influence of polytrauma. However, these imaging techniques remain limited in critical conditions and have unique drawbacks and limitations especially within

![Image of Figure 3](http://online.liebertpub.com/doi/10.1089/neur.2021.0006)

**FIG. 3.** Serum global injury marker all-spectrin levels at D1 (A) and D2 (B) post-injury. The levels of all-spectrin showed a significant increase in HX+HS and P+HX+HS groups (compared with sham *p* < 0.05, ***p* < 0.001; #*p* < 0.05 compared between PBBI and HX+HS groups). Biomarker concentration differences were analyzed with ANOVA followed by post hoc comparisons using Tukey’s test. Data are presented as mean ± SEM. ANOVA, analysis of variance; D1, one day; D2, two days; HS, hemorrhagic shock; HX, hypoxemia; P, penetrating ballistic-like brain injury; PBBI, penetrating ballistic-like brain injury; SEM, standard error of the mean.

![Image of Figure 4](http://online.liebertpub.com/doi/10.1089/neur.2021.0006)

**FIG. 4.** Serum global injury marker HSP70 levels at D1 (A) and D2 (B) post-injury. There seems to be a trend of HSP70 toward an increase in injured groups than in sham control groups at D1. Otherwise, there are no statistically significant differences between any group at D1 and D2. D1, one day; D2, two days.
the acute to subacute period post-injury. Blood-based protein biomarkers may be used to augment the diagnosis, prognosis, and monitoring of pTBI. They may also help monitor evolution secondary injury. Treatments for pTBI are limited but may benefit from a better understanding of acute brain and global protein profiles as well as from identifying sensitive biomarkers to differentiate combined injuries from isolated TBI.

Brain-enriched biomarkers
Neurofilament (NF) proteins are crucial structural components of the axonal cytoskeleton. They are composed of three different subunits differentiated by molecular weight (neurofilament-heavy protein [NF-H], neurofilament-medium protein [NF-M], and neurofilament-light protein [NF-L]). NF-H is heavily phosphorylated (pNF-H). Several studies suggest that NF proteins may serve as sensitive markers of TBI. pNF-H was reported to be a good predictor of injury severity in patients with mild TBI and acute spinal cord injury. In another study using a PBBI model, serum levels of pNF-H at both D1 and D2 post-injury were correlated with the degree of histological injury in a rat model of controlled cortical impact (CCI). The NF-L isoform is important to consider as it is believed to accumulate more rapidly and have more sustained elevations than pNF-H. Further, NF-L levels have predicted TBI and patient outcomes. It is important to consider that both these NF proteins are present in the peripheral nervous system and in muscle. However, they are present in relatively low levels compared with the CNS. Further, there is no evidence that HX+HS or pTBI cause peripheral nerve injury. Our findings were consistent with our hypothesis that pNF-H and NF-L would serve as brain-specific markers for PBBI as pNF-H and NF-L levels in rats subjected to PBBI were elevated compared with the HX+HS and sham control groups.

Whereas we also anticipated an increase in pNF-H in the P+HX+HS group compared with sham, a statistically significant difference was only observed in D2, although it approached significance at D1. This is consistent with a previous study that demonstrated significantly higher levels of pNF-H in a PBBI model at D2 compared with D1 and suggests that pNF-H may serve as a more useful marker at D2.

Because post-traumatic HX and HS have been reported to exacerbate brain damage, it is surprising that these additional insults following TBI did not increase the levels of pNF-H and NF-L. The data may indicate the physiological compensatory response to handling mild to moderate HX and hypotension. Our previous studies demonstrated that the sensitivity capacity may account for the lack of additive effects of hypoxicemic and hypotensive insults in animals subjected to 10% PBBI. This issue may be resolved in future studies by reducing the injury severity in the PBBI model by reducing the volume of balloon expansion to a smaller percentage such as 5% of total brain volume.

**FIG. 5.** Serum HMGB1 levels at D1 (A) and D2 (B) post-injury. There are no statistically significant differences between any groups. D1, one day; D2, two days.
Further, technical issues, such as from HS, may have contributed to variation in the data, secondary to altered diffusion rates or distributions of biomarkers. It is possible that the HS was not severe enough to aggravate the TBI. It is also possible that the pNF-H and NF-L induced by combined PBBI and HS+HX might increase in a more delayed fashion in our model as seen in other injury types such as delayed cerebral ischemia and diffuse anoxic brain injury. If so, detection of pNF-H at more delayed time-points in future studies might provide novel mechanistic insight. A study in humans demonstrated similar results with no differences in pNF-H levels in patients with TBI with and without polytrauma.25

Global injury biomarkers
zII-spectrin is a cortical cytoskeletal protein with essential roles in neuronal development, synaptic plasticity, and cytoskeletal remodeling.50 It is abundant in neurons but is also found in most other organs.15,33,34,37 During cell apoptosis, zII-spectrin is processed into breakdown products of 150i and 120 kDa fragments (SBDP150 and SBDP120) by caspase 3. In cell necrosis, and to a lesser extent during cell apoptosis, zII-spectrin is truncated to 150 and 145 kDa fragments (SBDP150 and SBDP145) by calpain.50 Various studies have demonstrated that zII-spectrin may potentially serve as a biomarker in TBI.51–57 In the present study, the zII-spectrin ELISA assay detected intact zII-spectrin and its various SBDPs. Our data mining analysis shows that both mRNA levels and protein levels of zII-spectrin (SPTAN1) are abundant in the CNS tissues. Yet, compared with NEFH and NEFL, zII-spectrin levels are also high in various other organs such as the heart, retina, ovary/testis, kidney, and adrenal gland.

Our study suggested that zII-spectrin is a sensitive biomarker for sensitive insults but not PBBI. This was the case as HX+HS served as the common factor for elevated zII-spectrin levels. Further, PBBI levels were not significantly elevated compared with sham in our study despite its high CNS concentration. This is similar to the results from another rat PBBI study reported by Zoltekewicz and colleagues20 in which plasma SBDP150 was comparable to sham control levels at D1 post-injury. Thus, this serum biomarker’s sensitivity to HX+HS and lack of sensitivity and specificity to PBBI may be due to the expression of zII-spectrin in other organs besides the brain and may indicate subtle peripheral organ damage.15,33,34 It is important to note that PBBI+HX+HS levels were unexpectantly insignificantly elevated compared with the PBBI group. This may again represent an issue of sensitivity capacity and should be further investigated in future studies by reducing the volume of balloon expansion.

HMGB1 proteins are involved in DNA binding and contribute to chromatin architecture.58 They are released from the cell nucleus during cellular necrosis,31 serve as an alarmin signaling mediator during inflammatory responses,30,59 and may serve as biomarkers in TBI.29 HSP70, a protein activated in physiological stress, may also serve as a biomarker in TBI.32 No significant changes in these two non-brain organ-specific protein biomarkers33,34 were detected. The brain is highly susceptible to HS and HX insults. TBI itself can cause hypoperfusion, decreased oxygen delivery, metabolic dysfunction, and perturbed autoregulation of cerebral blood flow, all which account for the increased vulnerability of the injured brain to hypotensive and/or hypoxic insults. It is plausible that the durations of HX and HS were not long enough to cause multiple organ dysfunction. In the field, patients can be hypotensive or hypoxemic for significant periods of time prior to the arrival of emergency medical personnel. Further, the HX and HS may have not been severe enough. The primary focus of this study is the effect of combined PBBI and systemic insults on the brain. The impact on other organs requires further analyses in addition to the serum biomarkers studied herein.

Limitations and future directions
A limitation of this study is the exclusive use of serum; biomarker profiles in cerebrospinal fluid and brain tissue (e.g., microdialysate) could provide a more complete picture. These biofluids are often readily accessible in patients with severe TBI in the ICU setting who often undergo placement of intraventricular catheters.15 Further, only acute post-injury time-points (D1 and D2) were examined. More extended time periods could be obtained with clinical samples. Although pNF-H may have a delayed effect following PBBI, further studies should examine subacute to chronic time-points. Considering earlier time-points at 6- or 12-h post-injury for zII-spectrin levels may reveal valuable information considering the return to baseline by D2. The small sample size also serves as a limitation, especially considering the presence of outliers in the data. Finally, reducing PBBI severity may provide a more sensitive platform for evaluating the cumulative effects of combined HX and HS insults. This may be more
clinically relevant as well as it would target a representation of patients who are more likely to survive to discharge. Biomarkers profile patterns compared between different severities of PBBI should be considered in further studies. Future research may include behavioral studies to examine biomarkers’ role in predicting neurological deficits especially in the context of memory formation and impulsivity.

Conclusion
This study demonstrates that PBBI increases the serum levels of pNF-H and/or NF-L in rat models. Further, HX and HS, alone or in combination with PBBI, are associated with acute increases in serum zII-spectrin levels, suggesting its utility as a global insult marker that is not brain specific. Therefore, when used together, pNF-H/NF-L and zII-spectrin can serve as promising biomarkers to distinguish PBBI from other insults, to identify systemic insults with or without brain trauma, and to augment clinical monitoring and prognosis of the two conditions.

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Authors’ Contributions
All authors have substantially contributed to the final version of this work and have approved the final version.

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References
1. Skarpupa, D.J., Khan, M., Hsu, A., Madbak, F.G., Ebler, D.J., Yorkgitsi, B., Rahmathulla, G., Alcindor, D., and Joseph, B. (2016). Trends in civilian penetrating brain injury: a review of 26,871 patients. Am. J. Surg. 18, 255–260.
2. Carney, N., Totten, A.M., O’Reilly, C., Ullman, J.S., Hawryluk, G.W., Bell, M.J., Bratton, S.L., Chesnut, R., Harris, D.A., Kissoon, N., Rubiano, A.M., Shutter, L., Tasker, R.C., Vavilala, M.S., Wilberger, J., Wright, D.W., and Ghajar, J. (2017). Guidelines for the Management of Severe Traumatic Brain Injury, Fourth Edition. Neurosurgery 80, 6–15.
3. Manley, G., Knudson, M.M., Morabito, D., Damron, S., Erickson, V., and Pitts, L. (2001). Hypotension, hypoxia, and head injury: frequency, duration, and consequences. Arch. Surg. (Chicago, IL: 1960) 136, 1118–1123.
4. Mchugh, G.S., Engel, D.C., Butler, I., Steyerberg, E.W., Lu, J., Mushkudiani, N., Hernandez, A.V., Marmarou, A., Maas, A.I., and Murray, G.D. (2007). Prognostic value of secondary insults in traumatic brain injury: results from the IMPACT study. J. Neurotrauma 24, 287–293.
5. Peek-Asa, C., McArthur, D., Hovda, D., and Kraus, J. (2001). Early predictors of mortality in penetrating compared with closed brain injury. Brain Inj. 15, 801–810.
6. Bramlett, H.M., Dietrich, W.D., and Green, E.J. (1999). Secondary hypoxia following moderate fluid percussion brain injury in rats exacerbates sensorimotor and cognitive deficits. J. Neurotrauma 16, 1035–1047.
7. Shear, D.A., Lu, X.-C.M., Pedersen, R., Wei, G., Chen, Z., Davis, A., Yao, C., Dave, J., and Tortella, F.C. (2011). Severity profile of penetrating ballistic-like brain injury on neurofunctional outcome, blood–brain barrier permeability, and brain edema formation. J. Neurotrauma 28, 2185–2195.
8. Blanie, A., Vigue, B., Benhamou, D., Duranteau, J., and Geeraerts, T. (2012). The frontal lobe and thalamus have different sensitivities to hypoxia-hypotension after traumatic brain injury: a microdialysis study in rats. J. Neurotrauma 29, 2782–2790.
9. Bramlett, H.M., Green, E.J., and Dietrich, W.D. (1999). Exacerbation of cortical and hippocampal CA1 damage due to posttraumatic hypoxia following moderate fluid-percussion brain injury in rats. J. Neurosurgery 91, 653–659.
10. Leung, L.Y., Deng-Bryant, Y., Shear, D., and Tortella, F. (2015). Combined hypoxic and hypotensive insults altered physiological responses and neurofunction in a severity-dependent manner following penetrating ballistic-like brain injury in rats. J. Trauma Acute Care Surg. 79, S130–S138.
11. Leung, L.Y., Deng-Bryant, Y., Cardiff, K., Winter, M., Tortella, F., and Shear, D. (2016). Neurochemical changes following combined hypoxemia and hemorrhagic shock in a rat model of penetrating ballistic-like brain injury: a microdialysis study. J. Trauma Acute Care Surg. 81, 860–867.
12. Leung, L.Y., Wei, G., Shear, D.A., and Tortella, F.C. (2013). The acute effects of hemorrhagic shock on cerebral blood flow, brain tissue oxygen tension, and spreading depolarization following penetrating ballistic-like brain injury. J. Neurotrauma 30, 1288–1296.
13. Amyot, F., Arciniegas, D.B., Braziatis, M.P., Curley, K.C., Diaz-Arrastia, R., Gandjbakhche, A., Herscovitch, P., Hinds, S.R., 2nd, Manley, G.T., Pacifico, A., Razumovsky, A., Riley, J., Salzer, W., Shi, H., Smimiotopoulos, J.G., and Stocker, D. (2015). A review of the effectiveness of neuroimaging modalities for the detection of traumatic brain injury. J. Neurotrauma 32, 1693–1721.
14. Lee, B., and Newberg, A. (2005). Neuroimaging in traumatic brain imaging. NeuroRx 2, 372–383.
15. Wang, K.K., Yang, Z., Zhu, T., Shi, Y., Rubenstein, R., Tyndall, J.A., and Manley, G.T. (2018). An update on diagnostic and prognostic biomarkers for traumatic brain injury. Expert Rev. Mol. Diagn. 18, 165–180.
16. DeDominicis, K., Hwang, H., Cartagena, C.M., Shear, D.A., and Boutte, A.M. (2018). Cerebrospinal fluid biomarkers are associated with glial fibrillary acidic protein and alphaii-spectrin breakdown products in brain tissues following penetrating ballistic-like brain injury in rats. Front. Neuro. 9, 490.
17. Madhathil, S.K., Deng-Bryant, Y., Johnson, D., Tortella, F.C., Dave, J.R., Shear, D.A., and Schmid, K.E. (2015). Serum glial fibrillary acidic protein predicts tissue glial fibrillary acidic protein breakdown products and therapeutic efficacy after penetrating ballistic-like brain injury. J. Neurotrauma 33, 147–156.
18. Madhathil, S.K., Deng-Bryant, Y., Wilfred, B.S., Leung, L.Y., Gildorf, J.S., and Shear, D.A. (2017). Alterations in brain-derived neurotrophic factor and insulin-like growth factor-1 protein levels after penetrating ballistic-like brain injury in rats. J. Trauma Acute Care Surg. 83, S16–S24.
20. Zoltewicz, J.S., Mondello, S., Yang, B., Newsom, K.J., Kobeisy, F., Yao, C.,
Lu, X.C., Dave, J.R., Shear, D.A., Schmid, K., Rivera, V., Cram, T., Seaney, J.,
Zhang, Z., Wang, K.K., Hayes, R.L., and Tortella, F.C. (2013). Biomarkers track
damage after graded injury severity in a rat model of penetrating brain
injury. J. Neurotrauma 30, 1161–1169.

21. Boute`e, A.M., Hook, V., Thangavelu, B., Sarkis, G.A., Abbatibelli, B.N., Hook,
G., Jacobsen, J.S., Robertson, C.S., Gil christ, J., Yang, Z., Wang, K.K., and
Shear, D.A. (2020). Penetrating traumatic brain injury triggers dys-
regulation of cathepsin B protein levels independent of cysteine pro-
tease activity in brain and cerebral spinal fluid. J. Neurotrauma 37,
1574–1586.

22. Johnson, D., Cartagena, C.M., Tortella, F.C., Dave, J.R., Schmid, K.E.,
and Boute`, A.M. (2017). Acute and subacute microRNA dysregulation is
associated with cytokine responses in the rodent model of pene-
trating ballistic-like brain injury. J. Trauma Acute Care Surg. 83,
S145–S149.

23. Thangavelu, B., Wilfred, B.S., Johnson, D., Gilchrist, J.S., Shear, D.A.,
and Boute`, A.M. (2020). Penetrating ballistic-like brain injury leads to
microRNA dysregulation, BACE1 upregulation, and amyloid pre-
cursor protein loss in lesioned rat brain tissues. Front. Neurosci. 14,
915–915.

24. Zajek, J. and Fedora, M. (2012). The usefulness of S100B, NSE, GFAP,
NF-H, secretagogin and Hsp70 as a predictive biomarker of outcome
in children with traumatic brain injury. Acta Neurochir. (Wien) 154,
93–103.

25. Shibahashi, K., Doi, T., Tanaka, S., Hida, H., Chikuda, H., Sawada, Y., Takasu,
Y., Chiba, K., Nozaki, T., Hamabe, Y., and Ogata, T. (2016). The serum
phosphorylated neurofilament heavy subunit as a predictive marker for
outcome in adult patients after traumatic brain injury. J. Neurotrauma 33,
1826–1833.

26. Gao, W., Zhang, Z., Lv, X., Wu, Q., Yan, J., Mao, G., and Xing, W. (2020).
Neurofilament light chain level in traumatic brain injury: a system review
and meta-analysis. Medicine (Baltimore) 99, e22363–e22363.

27. Siedler, D.G., Chuah, M.I., Kirkcaldie, M.T., Vickers, J.C., and King, A.E.
(2019). Blood biomarkers for traumatic brain injury: a quantitative
assessment of diagnostic and prognostic accuracy. Front. Neurol. 10,
44. Martı´nez-Morillo, E., Childs, C., Garcı ´a, B.P., A´ lvarez Mene´ndez, F.V.,
and Boutte´ , A.M. (2020). Penetrating ballistic-like brain injury leads to
microRNA dysregulation, BACE1 upregulation, and amyloid pre-
cursor protein loss in lesioned rat brain tissues. Front. Neurosci. 14,
915–915.

28. Zhang, Z., Zhu, T., Mondello, S., Akel, M., Wong, A.T., Kothari, I.M., Lin, F.,
Shear, D.A., Gilchrist, J.S., Leung, L.Y., Bramlett, H.M., Dixon, C.E., Dietrich,
W.D., Hayes, R.L., Povlishock, J.T., Tortella, F.C., Koc heanek, P.M., and
Wang, K.K. (2018). Serum-based phospho-neurofilament-heavy
protein as theranostic biomarker in three models of traumatic brain
injury: an Operation Brain Trauma Therapy study. J. Neurotrauma 36,
348–359.

29. Li, Y., Zhang, L., Kakukari, S., Cohen, A., and Cavanaugh, J.M. (2015). Cor-
relation of mechanical impact responses and biomarker levels: a new
model for biomarker evaluation in TBI. J. Neurol. Sci. 359, 280–286.

30. Martinez-Morillo, E., Childs, C., Garcia, B.P., Alvarez Menendez, F.V.,
Romасhин, A.D., Cervelli, G., Lippi, G., and Diamandis, E.P. (2015).
Neurofilament medium polypeptide (NFM) protein concentration is increased
in CSF and serum samples from patients with brain injury. Clin. Chem.
Lab. Med. 53, 1575–1584.

31. Neselius, S., Zetterberg, H., Blennow, K., Marcusson, J., and Brifsy, H.
(2013). Increased CSF levels of phosphorylated neurofilament protein
following bout in amateur boxers. PLoS One 8, e81249.

32. Olivera, M., Jones, M.T., Kirk, K.M., Gable, D.A., Rajab, J.T., Johnson, T.A.,
Andréasson, U., Norgren, N., Blennow, K., and Zetterberg, H. (2016). Serum
neurofilament light in american football athletes over the course of a
season. J. Neurotrauma 33, 1784–1789.

33. Al Nimer, F., Thelin, E., Nystrom, H., Dring, A.M., Svennssing, A., Piehl, F.,
Nelson, D.W., and Bellander, B.-M. (2015). Comparative assessment of the
prognostic value of biomarkers in traumatic brain injury reveals an
independent role for serum levels of neurofilament light. PLoS one 10,
e0132177–e0132177.

34. Gatson, J.W., Barillas, J., Hynan, L.S., Diaz-Arrastia, R., Wolf, S.E., and Minei,
P.J. (2014). Detection of neurofilament-H in serum as a diagnostic tool
to predict injury severity in patients who have suffered mild traumatic brain
injury. J. Neurosurg. 121, 1322–1328.

35. Singh, A., Kumar, V., Ali, S., Mahdi, A.A., and Srivastava, R.N. (2017).
Phosphorylated neurofilament heavy: a potential blood biomarker to
evaluate the severity of acute spinal cord injuries in adults. Int. J. Crit. Illn.
Inj. Sci. 7, 212–217.

36. Yan, X.K., Jeromin, A. and Jeromin, A. (2012). Spectrin Breakdown Pro-
ducts (SBDPs) as Potential Biomarkers for Neurodegenerative Diseases.
Curr. Transl. Geriatr. Exp. Gerontol. Rep. 1, 85–93.

37. Pike, B.R., Flint, J., Dutta, S., Johnson, E., Wang, K.K., and Hayes, R.L. (2001).
Accumulation of non-erythroid alpha II-spectrin and calpain-cleaved
alpha II-spectrin breakdown products in cerebrospinal fluid after tra-
matic brain injury in rats. J. Neurochem. 78, 1297–1306.

38. Mondello, S., Robicsek, S.A., Gabrielli, A., Brophy, G.M., Papa, L., Tepas, J.,
Robertson, C., Buki, A., Scharf, D., Jiexiang, M., Akinyi, L., Muller, U., Wang,
56. Berger, R.P., Hayes, R.L., Richichi, R., Beers, S.R., and Wang, K.K. (2012). Accumulation of calpain and caspase-3 proteolytic fragments of brain-derived alphal-spectrin in cerebral spinal fluid after middle cerebral artery occlusion in rats. J. Cereb. Blood Flow Metab. 24, 98–106.

57. Brophy, G.M., Pineda, J.A., Papa, L., Lewis, S.B., Valadka, A.B., Hannay, H.J., Heaton, S.C., Demery, J.A., Liu, M.C., Tepas, J.J., Akle, V., Brophy, G.M., Tepas, J.J., Wang, K.K., Robertson, C.S., and Hayes, R.L. (2007). Clinical significance of alphal-spectrin breakdown products in cerebrospinal fluid after severe traumatic brain injury. J. Neurotrauma 24, 354–366.

58. Brophy, G.M., Pineda, J.A., Papa, L., Lewis, S.B., Valadka, A.B., Hannay, H.J., Heaton, S.C., Demery, J.A., Liu, M.C., Tepas, J.J., 3rd, Gabrielli, A., Robicsek, S., Wang, K.K., Robertson, C.S., and Hayes, R.L. (2009). alphal-Spectrin breakdown product cerebrospinal fluid exposure metrics suggest differences in cellular injury mechanisms after severe traumatic brain injury. J. Neurotrauma 26, 471–479.

59. Siman, R., Giovannone, N., Hanten, G., Wilde, E.A., McCauley, S.R., Hunter, J.V., Li, X., Levin, H.S., and Smith, D.H. (2013). Evidence that the blood biomarker SNTF predicts brain imaging changes and persistent cognitive dysfunction in mild TBI patients. Front. Neurol. 4, 190.

60. Baxevanis, A.D., and Landsman, D. (1995). The HMG-1 box protein family: classification and functional relationships. Nucleic Acids Res. 23, 1604–1613.

61. Okuma, Y., Liu, K., Wake, H., Zhang, J., Maruo, T., Date, I., Yoshino, T., Ohtsuka, A., Otani, N., Tomura, S., Shima, K., Yamamoto, Y., Yamamoto, H., Takahashi, H.K., Mori, S., and Nishihori, M. (2012). Anti-high mobility group box-1 antibody therapy for traumatic brain injury. Ann. Neurol. 72, 373–384.