**Study Protocol**

**Biomarkers in the Brain Oxygen Optimization in Severe Traumatic Brain Injury Trial (Bio-BOOST).**

A multicenter, observational study of the effect of derangements in brain physiologic parameters on brain injury biomarker levels in patients with severe traumatic brain injury.

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# Table of Contents

**PRECIS**

<u>Study Title:</u>  Biomarkers in the Brain Oxygen Optimization in Severe Traumatic Brain Injury Trial (Bio-BOOST)

<u>Objectives</u>
Bio-BOOST is a multicenter, observational study of the effect of derangements in brain physiologic parameters on brain injury biomarker levels in patients with severe traumatic brain injury.

Primary Objective: To quantify the effect of total brain tissue hypoxia exposure on brain injury using biofluid-based biomarkers of brain injury.

Secondary Objectives:
- To determine the effect of total cerebral hypoperfusion exposure (defined as the depth and duration of cerebral perfusion pressure<60 mmHg within 48 hours of randomization, quantified using the AUC methodology) on peak levels of GFAP, UCH-L1, Total Tau and NFL.
- To determine whether treatment informed by PbtO2 monitoring results in a decrease in blood and CSF levels of GFAP, UCH-L1, Total Tau and NFL.
- To determine whether the initial CSF and blood levels of brain injury biomarkers (GFAP, UCH-L1, Total Tau and NFL) are associated with unfavorable functional outcome as measured by the Glasgow Outcome Scale Extended (GOSE) 6 months after severe TBI.
- To determine whether the rate of increase in brain injury biomarker levels during the first 24 hours after randomization are associated with 6-month functional outcome as measured by the GOSE.
- To determine the time-point at which GFAP, UCH-L1, Total Tau and NFL levels provide the best discriminative ability for functional outcome as measured by the GOSE.
- To create a biorepository of longitudinal serum, plasma, CSF, mRNA and DNA samples of severe TBI patients for validating novel brain injury biomarkers.

<u>Study Procedures</u>
This study is a prospective observational, multi-center study of subjects enrolled in the Brain Oxygen Optimization in Severe Traumatic Brain Injury—Phase 3 (BOOST-3) trial. BOOST-3 is a multicenter, randomized, blinded-endpoint, comparative effectiveness study of goal-directed critical care based upon monitoring of brain tissue oxygen and intracranial pressure versus monitoring of intracranial pressure alone in patients with severe traumatic brain injury.

We will obtain an initial set of biospecimens (serum, plasma, CSF, DNA and RNA) shortly after randomization into BOOST-3 and within 24 hours of injury. Subsequent biospecimens will be obtained every 8 hours for the first 24 hours post-enrollment. This will allow the characterization of acute changes in biomarker levels. On study days 2 through 5, biospecimens will be obtained twice a day to allow characterization of sub-acute changes in biomarker levels, without overburdening study teams or taking too much blood from individual subjects. On study days 7 and 14 and at 6-months post-enrollment, one set of biospecimen will be obtained, preferably in the morning. Biospecimens collected at each time point will consist of 6 ml of whole blood for serum extraction, 6 ml of whole blood for plasma extraction, 2.5 ml of whole blood for mRNA extraction (a total of 14.5 ml [one tablespoon] of blood) and 5 ml of cerebrospinal fluid (CSF).

Serum and plasma will be apportioned into aliquots and stored in a -80ºC freezer at enrolling sites within 2 hours of collection. During the separation of plasma samples from whole blood, the buffy coat suspension (a concentrated leukocyte suspension) will be extracted and stored

for DNA analysis to avoid wasting this valuable biospecimens. mRNA and CSF samples will also be collected, processed and stored. Periodically (once or twice a year), all biospecimens stored at enrolling sites will be shipped on dry ice to a biorepository at the University of Pittsburgh. Biospecimens will be collected, processed, stored and shipped according to the NINDS common data elements protocol for TBI biospecimen collection.[1]

Study Duration
Participants will be enrolled over a period of 2.5 years; however, each participant will remain in the study for 6 months.

Sample Size and Population
We plan to enroll a maximum of 300 male and female subjects among multiple clinical sites.

# 1 STUDY OBJECTIVES

To study the effect of derangements in brain physiologic parameters on brain injury biomarker levels in patients with severe traumatic brain injury.

## 1.1 Primary Objective

To quantify the effect of total brain tissue hypoxia exposure on brain injury using biofluid-based biomarkers of brain injury.

## 1.2 Secondary Objectives

- To determine the effect of total cerebral hypoperfusion exposure on peak levels of GFAP, UCH-L1, Total Tau and NFL.
- To determine whether treatment informed by PbtO2 monitoring results in a decrease in blood and CSF levels of GFAP, UCH-L1, Total Tau and NFL.
- To determine whether the initial CSF and blood levels of brain injury biomarkers (GFAP, UCH-L1, Total Tau and NFL) are associated with unfavorable functional outcome as measured by the Glasgow Outcome Scale Extended (GOSE) 6 months after severe TBI.
- To determine whether the rate of increase in brain injury biomarker levels during the first 24 hours after randomization are associated with 6-month functional outcome measured by the GOSE.
- To determine the time-point at which GFAP, UCH-L1, Total Tau and NFL levels provide the best discriminative ability for functional outcome measured by the GOSE.
- To create a biorepository of longitudinal serum, plasma, CSF, mRNA and DNA samples of severe TBI patients for validating novel brain injury biomarkers.

## 2 BACKGROUND

### 2.1 Rationale

**The societal burden of traumatic brain injury (TBI).** TBI is a major cause of death and disability in modern industrialized societies. The most recent estimates from the Centers for Disease Control and Prevention (CDC) indicate that in the United States 3.5 million individuals experience a TBI annually, of which 300,000 are hospitalized and discharged alive, and 52,000 died as a consequence of the TBI.[1;2] Among the 300,000 hospitalized survivors, over 40% experience long-term disability,[3] which limits their activities of daily living, such as grooming, eating, or walking. Because TBI often affects young people who survive for many years with serious functional limitations, the prevalence of TBI related disability is high, and it is estimated that 3.3 million people, or 1% of the US population, is living with long-term disabilities from TBI.[4] The annual cost to society resulting from TBI has been estimated to range from $83 billion[5] to $244 billion[6] (in 2014 dollars). The magnitude of this problem has led to numerous clinical trials aimed at improving survival or functional outcome after TBI, yet no effective therapies have been identified to date.

This proposal focuses on the most severely injured victims of TBI, those with prolonged unresponsiveness and extensive intracranial pathology, such as contusions, hemorrhages, edema, and diffuse axonal injury. Since surveillance databases do not typically include data elements commonly used to assess severity,[7] epidemiologic studies specific to severe TBI are sparse. One detailed studied in Aquitaine, France, which included reviews of hospital records of all patients admitted to one of 5 trauma centers over one calendar year concluded that the incidence of severe TBI was 17.3/100,000 population,[8] and the incidence of traumatic coma (severe TBI which resulted in coma lasting longer than 24 hours) was 8.5/100,000.[9] Extrapolating the latter number to the US population, a reasonable estimate of the annual number of cases of traumatic coma in the US is 27,000. These patients experience high mortality and morbidity rates, and less than 20% make a good recovery.[9] They require sophisticated care in intensive care units (ICUs), and the burden to society in direct and indirect costs is very high. Average lifetime costs per TBI survivor in the US has been estimated to be $533,000 in 2014 dollars,[6] but since this estimate is not specific to those with prolonged traumatic coma, the per patient costs for patients who are the target of this proposed study is likely to be significantly higher. Thus, the potential payoff to society from improved care of these most severely injured TBI patients is potentially very high.

**TBI is a heterogeneous condition that lacks adequate tools to assist in management.** TBI is a heterogeneous disorder that is usually classified by physical examination (the Glasgow Coma Scale, GCS), mechanism of injury (blunt versus penetrating) and neuroimaging.[10] The primary diagnostic tool for evaluating TBI is the brain CT scan. While CT is often life-saving by identifying patients who need emergent neurosurgical interventions, it is insensitive to pathologies that account for a substantial fraction of disability after severe TBI, such as diffuse axonal injury (DAI), diffuse vascular injury, and cytotoxic edema.[11] Further, neuroimaging requires transfer from the neurological ICU to the scanning suite, which is cumbersome and potentially dangerous for critically ill patients, and is thus poorly suited to evaluate hour-by-hour changes in neurological status. Accordingly, novel strategies are needed to improve the management of critically ill patients with TBI.

**Management of severe TBI currently relies on invasive neuromonitoring.** Invasive neuromonitoring, using devices implanted into brain parenchyma to measure intracranial pressure (ICP) and brain tissue oxygen ($PbtO_2$), is widely used in neurological ICUs to identify risk factors for secondary neural injury while they are still preventable. Invasive neuromonitoring is the focus of the recently funded BOOST-3 (Brain Oxygen Optimization in

Severe TBI—Phase 3) Study (NIH/NINDS U01 NS099046).[12]  This study offers a unique opportunity to accelerate our understanding of the pathophysiology of severe TBI and promote the development of effective interventions.  BOOST-3 will be carried out through the SIREN (Strategies to Innovate Emergency Clinical Care Trials Network).  This study will enroll 1094 participants with severe TBI from 2019 – 2023, across approximately 45 clinical sites in the US and Canada  and represents a $32.5 M federal investment. The primary hypothesis of BOOST-3 is that a treatment based on $PbtO_2$ and ICP monitoring improves neurologic outcome measured by the Glasgow Outcome Scale-Extended (GOS-E) 6 months after injury compared to treatment based on ICP monitoring only.

Although invasive neuromonitoring is the clinical standard of care for severe TBI, it has several important limitations which need to be addressed by carefully assessing the relationship between intracranial hypertension, brain tissue hypoxia, and molecular biomarkers of neural injury.  First, although it is clear that high ICP and low $PbtO_2$ are associated with poor outcomes after TBI, the thresholds for ICP and $PbtO_2$ beyond which brain cellular death occurs remain controversial, and empirical evidence of cellular death, assessed by measuring brain injury biomarkers, is critically needed.  Second, $PbtO_2$ is measured within a small area (7.1 – 15 $mm^2$) of brain tissue (usually in area not visibly injured)[13;14] and may not accurately represent global risk for ischemia.  Brain injury biomarkers provide important data on this issue, as they reflect the global burden of brain injury. Third, invasive neuromonitoring is justifiably used only in patients who are in coma (GCS $\leq$ 8) in whom the neurologic exam cannot be reliably followed.  However, secondary neural injury also occurs in patients with milder injuries, and since the neurological evaluation, based on the GCS, has limited sensitivity and dynamic range, noninvasive tools to measure secondary neural injury in patients who do not require intracranial monitors are desperately needed.  Measuring molecular biomarkers serially is an attractive strategy for identifying secondary harmful events which may guide changes in therapy.[15] Finally, there are no available tools for monitoring individual patient responses to novel treatment strategies, hindering the development of novel therapies. In summary, there is an unmet clinical need for brain injury biomarkers to complement the information obtained from neuroimaging, invasive neuromonitoring, and other clinical assessments to optimally inform the management of patients with TBI.

Capitalizing on the infrastructure and the rich study population for BOOST-3, we propose conducting an ancillary biomarker study, Bio-BOOST. Bio-BOOST will profile longitudinal changes in well-validated molecular biomarkers measured in blood and cerebrospinal fluid (CSF) to identify molecular signatures that classify severe TBI with improved precision. BOOST-3 will study severe TBI only; therefore, Bio-BOOST will fill an important gap in the field, since ongoing biomarker collections efforts through the Transforming Research and Clinical Knowledge in TBI (TRACK-TBI) and TBI Endpoints Development (TED) efforts are primarily focused on mild TBI.

**Biomarkers provide a minimally invasive approach for diagnosing and monitoring TBI.** Blood and cerebrospinal fluid (CSF) levels of structural protein components of brain cells that are released in the aftermath of brain injury are a promising adjunct for detecting and monitoring secondary brain injury, including in the neurological ICU setting.[15-22] The proposed study will examine the diagnostic and prognostic value of four TBI biomarkers which have been widely studied in severe[15] as well as mild TBI[23]: Glial Fibrillary Acidic Protein (GFAP), Ubiquitin C-terminal hydrolase 1 (UCH-L1), total Tau, and Neurofilament light chain (NF-L).  While we believe these are currently the most promising biomarkers for TBI, which justifies their inclusion in this proposal, we are convinced that other promising biomarkers will be discovered over the next several years.  Thus, an important goal of Bio-BOOST, in addition to measuring the four

biomarkers listed here, is to create a Biofluids Bank of well-characterized samples that can be used in validation studies of future biomarkers of TBI.

**GFAP** is an intermediate filament protein that is responsible for maintaining the mechanical strength of the cytoskeleton of astrocytes in the central nervous system (CNS).[24] GFAP released into the CSF post-TBI may be absorbed into peripheral circulation through the sagittal sinus. GFAP also gains access to the circulatory system via the glymphatic system.[25] Although GFAP is predominantly expressed by CNS astrocytes, it can also be expressed in lesser quantities by non-myelinated Schwann cells of the peripheral nervous system[26] and in glial cells found in the gut.[27;28] As a consequence of these non-CNS sites of expression, extracranial traumatic injuries do cause elevations in blood GFAP levels.[29;30] Nonetheless, numerous studies have demonstrated that GFAP values obtained on the day of injury are capable of discriminating TBI subjects with intracranial hemorrhage from those without.[30-35] Furthermore, higher GFAP levels are associated with poor functional recovery.[35-38]

**UCH-L1** is an enzyme that is highly abundant in neuronal cells and accounts for 1-2% of total brain protein. It plays a key role in the removal of excessive, oxidized, or misfolded proteins.[39] Similar to GFAP, elevated blood concentrations of UCH-L1 occur following TBI in proportion to TBI severity. However, blood UCH-L1 levels peak shortly after injury and decline to near baseline levels within 24 hours of injury, whereas GFAP levels peak within 20 hours of injury and remain significantly elevated for approximately 72 hours.[40;41]

**NF-L** is an intermediate filament protein abundantly expressed in the long myelinated subcortical axons. It is the smallest and most abundant of the three major neurofilament subunits (NF-light chain, NF-medium chain, NF-heavy chain) and consequently the most likely to be found in circulation after brain injury.[42] NF-L has been found to be elevated in the cerebrospinal fluid (CSF) of boxers after a bout;[43] however until recently measurement of NF-L in circulation has been limited by the analytical sensitivity of existing immunoassays.[44] The single molecule array technology (Simoa HD-1 Analyzer from Quanterix) is capable of measuring NF-L with higher analytical sensitivity than existing immunoassays.[45] Serum NF-L measured by Simoa correlates with CSF NF-L with a correlation coefficient of 0.89.[46] Using this assay, a small study of American football players (n=20) reported elevated NF-L levels in starters compared to non-starters.[47] Additionally in another small study, severe TBI patients (n=72) had higher NF-L levels than healthy controls (n=35).[48] A third study demonstrated that serum and CSF NF-L levels are associated with functional recovery.[16] NF-L levels remain elevated in CSF up to 19 months after severe TBI.[49]

**Tau** proteins are microtubule associated proteins that are found predominantly in neurons and play an important role in the assembly of tubulin monomers into microtubules that are important in maintaining the cytoskeleton of axons.[50] Elevated blood levels of Total tau protein have been reported in concussed professional hockey players.[51] Furthermore, total tau levels were reported to be higher in TBI patients with intracranial hemorrhage on head CT, compared to those without intracranial hemorrhage on head CT.

### 2.2 Supporting Data

**Use of blood biomarkers in the management of severe TBI in the neuroICU.** There are limited data on the role of longitudinal measurements of brain injury biomarkers in monitoring secondary brain injury. In a cohort of 67 severe TBI patients who were subjected to hypothermia and had daily serum GFAP measurements during the first 5 days of injury, GFAP levels decreased gradually but increased when rewarming was started on day 4 (Figure 1)[36] This finding suggests that serial GFAP levels may be useful for monitoring secondary brain injury. Furthermore, in a cohort of predominantly severe TBI patients[18] (n=250, 70% severe Figure 2), subjects with progression in the size of traumatic intracranial lesions found on head CT were

likely to have a secondary increase in serum levels of S100B (a biomarker of astrocytic injury). In this proposal we have selected GFAP as the biomarker of astrocytic injury since S100B lacks brain specificity.[52]
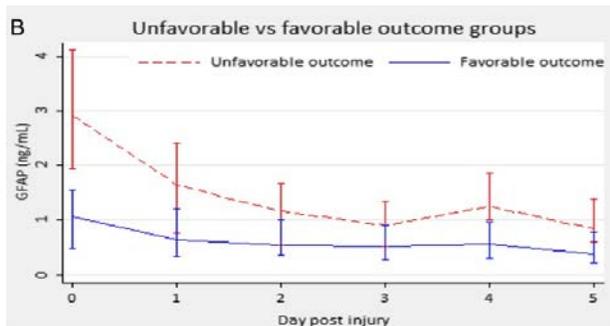


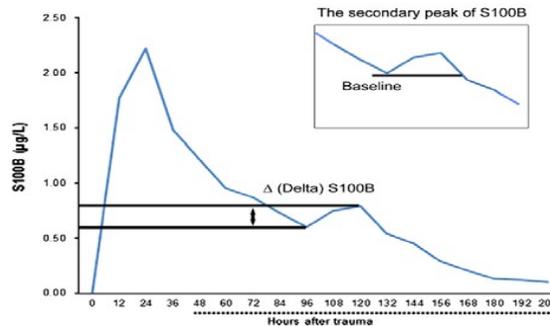| Figure 1: GFAP values decreased from days 1-3 and increased on day 4 when rewarming was started | Figure 2: A secondary peak in serum S100B levels occurred in TBI subjects with progression of intracranial lesions. |
|---|---|

**Ultrasensitive immunoassays can be multiplexed to detect GFAP, UCHL-1, NF-L, and Tau in blood from TBI patients.** GFAP, UCH-L1, total Tau and NF-L been widely studied in severe as well as mild TBI, and currently represent the most promising biomarkers for TBI. Since each biomarker is expressed in different cells or cell regions (GFAP in astrocytes, UCHL-1 in neuronal cell bodies, Tau in dendrites and axon terminals, NF-L in axons),[23;52] it is likely that each provides complementary information about primary and secondary neural injury.

Additionally, there are differences in the kinetics of release and clearance of each of these biomarkers, further supporting the need to obtain multiple biomarker measurements to fully assess TBI. These considerations have led to the development of multiplex assays, which allow simultaneous assay of GFAP, UCH-L1, total Tau, and NF-L from small volumes of biological fluids. For the Bio-BOOST study, we propose to use the Neurology 4PLEX A developed by Quanterix (Lexington, MA) as it is more cost-effective than running the four assays independently and minimizes the consumption of valuable biofluids.

We have experience using the Neurology 4-PLEX A in samples from humans with TBI. We measured serum levels of the four biomarkers in stored blood samples collected from subjects enrolled in the Transforming Research and Clinical Knowledge in Traumatic Brain Injury Pilot (TRACK-TBI Pilot) study (n=107).[53] The TRACK-TBI Pilot was a multicenter prospective observational study conducted at three Level I trauma centers in the U.S.[53] GFAP, UCH-L1, Total Tau and NF-L were measured in blood samples collected from consenting subjects within 24 hours of injury, using the Neurology 4-PLEX A (Quanterix).

The correlation matrix of all four biomarkers is presented in Figure 3. Among the four biomarkers studied, the strongest correlation was between NF-L and UCH-L1 and the weakest correlation was between GFAP and total Tau. In order to assess how each biomarker yielded useful information about TBI, we compared biomarker levels from TRACK-TBI patients with normal head CTs with those from patients whose head CT showed trauma-related abnormalities. Serum levels of all four biomarkers were higher in subjects with abnormal head CT than those with normal head CT (Figure 4). The discriminative ability of GFAP ($AUC_{GFAP}$) for distinguishing between subjects with and without abnormal head CT was 0.88 (95% CI: 0.81 – 0.95). $AUC_{GFAP}$ was not statistically significantly different from the $AUC_{UCH-L1}$ (0.86 [95% CI: 0.79

– 0.93], p=0.62) or the AUC$_{NF-L}$ (0.84 [95% CI: 0.77 – 0.92], p=0.44); however it was higher than the AUC$_{Tau}$ (0.77 [95% CI: 0.67 – 0.86], p=0.04). For each biomarker studied, there was a statistically significant linear association between biomarker value and probability of having an abnormal head CT. Higher biomarker values were associated with a higher probability of having an abnormal head CT.

**Pre-clinical studies indicate that GFAP and NF-L may be useful as pharmacodynamic biomarkers of neuroprotective therapies.** To date, the majority of the literature on TBI biomarkers has focused on the diagnostic and prognostic value of these biomarkers. Development of novel therapies for TBI will also require validated pharmacodynamic biomarkers. However, little is known on how TBI therapeutics modify blood levels of brain injury biomarkers. Our collaborators recently reported that in swine subjected to TBI and hemorrhagic shock only and to TBI, hemorrhagic shock and polytrauma, high dose valproic acid (VPA) treatment is safe, decreases brain lesion size and results in faster neurologic recovery when compared to resuscitation with normal saline alone. We subsequently conducted a post-hoc
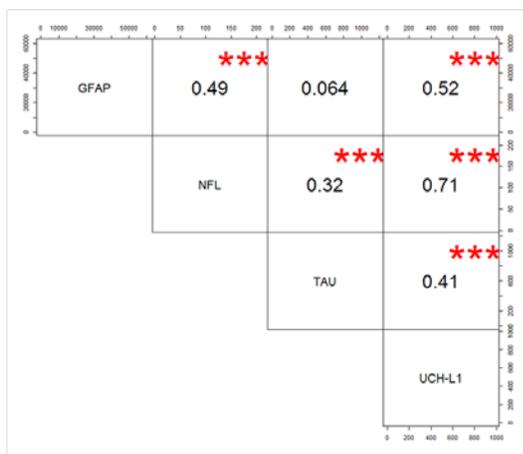


Figure 3: Correlation matrix of GFAP, UCH-L1, NF-L, Tau Correlations with *** have a significance value of <0.001



Figure 5: Longitudinal changes in GFAP and NFL in pigs randomized to valproic acid versus placebo

analysis of blood samples stored from these pigs to: 1) examine longitudinal changes in serum GFAP and NF-L levels post-TBI; 2) elucidate the effect of polytrauma on blood levels of GFAP and NF-L; 3) examine the treatment effect of VPA on serum levels of GFAP and NF-L. We studied a total of 15 Yorkshire Swine who were subjected to: controlled cortical impact (CCI) TBI + hemorrhagic shock + polytrauma and treated with normal saline (n=5); CCI TBI + hemorrhagic shock + polytrauma and treated with VPA (n=5); and CCI TBI + hemorrhagic shock only and treated with VPA (n=5). Blood samples were collected at baseline (prior to injury) and at 2, 4, 8, 24, 72 and 240 hours after injury. Neurocognitive testing was performed daily and consisted of complex integration of various long term memory processing and recall, integration of spatial memory, prioritization, and processing of color vision. Time to normalization of behavior was determined based on performance on neurocognitive testing. T2-weighted MRIs were completed under anesthesia on day 3 to determine lesion size. Serum GFAP and NF-L were measured in duplicates by blinded technicians from Quanterix using a digital immunoassay based on a single molecule array technology (Simoa).
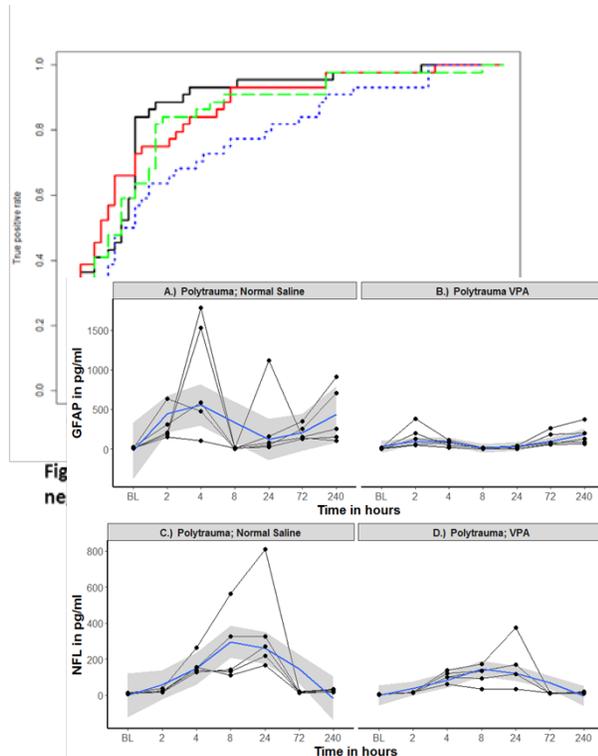
**Table 1: The Effects of Valproic Acid Treatment on Biomarker Levels: median biomarker values and their corresponding interquartile ranges in TBI + polytrauma swine models**

| Time | NF-L | | | GFAP | | |
|---|---|---|---|---|---|---|
| | Saline | VPA | p-value | Saline | VPA | p-value |
| Baseline | 10.6 (9.0-12.9) | 4.6 (3.8-5.2) | <0.01 | 10.1 (6.6-16.7) | 5.4 (3.5-10.2) | 0.17 |
| 2 hours | 28.4 (18.3-31.2) | 14.7 (11.6-17.6) | 0.03 | 252.0 (126.2-810.1) | 129.4 (75.7-286.3) | 0.17 |
| 4 hours | 15.2 (14.3-18.8) | 11.0 (9.2-13.8) | <0.01 | 152.1 (137.0-303.3) | 78.4 (58.4-224.7) | 0.25 |
| 8 hours | 22.6 (17.2-31.1) | 15.8 (14.1-16.1) | <0.01 | 207.9 (165.9-470.1) | 125.0 (52.6-288.8) | 0.17 |
| 1 day | 153.0 (133.2-208.7) | 101.2 (80.9-129.3) | 0.02 | 586.7 (292.2-1657.4) | 79.7 (27.8-102.8) | 0.02 |
| 3 days | 270.2 (190.9-568.3) | 169.7 (75.1-272.6) | 0.17 | 83.1 (34.6-639.9) | 6.5 (2.7-31.7) | 0.03 |
| 10 days | 143.4 (121.5-444.5) | 114.8 (48.9-162.5) | 0.22 | 8.7 (4.0-12.1) | 2.9 (.4-15.6) | 0.33 |

We found that TBI + polytrauma swine treated with VPA had GFAP values pre-injury, 2, 4 and 8 hours post-injury that were similar to those treated with normal saline, however, median GFAP values at 1 and 3 days were significantly lower in the VPA group than in the normal saline group (see Figure 5A and 5B and Table 1). Each set of curves in Figure 5 represents a different animal. GFAP values in both groups returned to pre-injury levels at 10 days post-injury. Similarly, compared to swine treated with normal saline, swine treated with VPA had lower NF-L values at all time points, with the exception of at 3 and 10 days when the difference in NF-L between the two groups was not significantly different (Figure 5C and 1D and Table 1). Compared to GFAP values obtained at other time points, GFAP values obtained at 24 hours post-injury and the peak GFAP values (which are the same in most but not all cases) have the strongest correlation with time to normalization of behavior and lesion size detected by MRI (Table 2).

| | Correlation with Time to Normalization of Behavior (r) | Correlation with Lesion Size (r) |
|---|---|---|
| **GFAP** | | |
| 2 hour | 0.37 | 0.27 |
| 4 hour | 0.21 | 0.21 |
| 8 hour | 0.25 | 0.36 |
| 24 hour | 0.34 | 0.59 |
| 72 hour | 0.32 | 0.45 |
| 10 day | -0.22 | 0.08 |
| Peak | 0.38 | 0.59 |
| $AUC_{0-10day}$ | 0.09 | 0.25 |
| **NF-L** | | |
| 2 hour | 0.81 | 0.59 |
| 4 hour | 0.78 | 0.68 |
| 8 hour | 0.89 | 0.84 |
| 24 hour | 0.92 | 0.98 |
| 72 hour | 0.79 | 0.53 |
| 10 day | 0.79 | 0.48 |
| Peak | 0.79 | 0.53 |
| $AUC_{0-10day}$ | 0.86 | 0.67 |

Table 2: Correlation Analysis Between Biomarkers at Different Time-Points and Outcome Measures in Swine with TBI + Polytrauma

Relative to peak GFAP values, the area under the biomarker concentration versus time curve during the first 10 days ($AUC_{0-10days}$) was weakly correlated with lesion size and behavioral outcome. Similarly, NF-L values obtained at 24 hours post-injury and peak NF-L had the strongest correlation with time to normalization of behavior and lesion size. Irrespective of time point NF-L outperformed GFAP with regards to correlation with both lesion size and normalization of behavior. These findings support the potential of these proteins as pharmacodynamic biomarkers of neuroprotective therapies.

## 3 STUDY DESIGN

Bio-BOOST is a prospective observational study that will collect biospecimen during a 6-month period of observation, from subjects enrolled in the BOOST-3 trial.

## 4 SELECTION AND ENROLLMENT OF SUBJECTS

## 4.1 Inclusion Criteria

| Criteria | Rationale | Metric |
|---|---|---|
| Enrolled in BOOST-3 | This is an ancillary study to the BOOST-3 trial | Randomization to a BOOST-3 study arm. |
| BOOST-3 participant is enrolled at a Bio-BOOST site | Subjects can only be enrolled at BOOST-3 sites that have been approved to enroll in Bio-BOOST | Bio-BOOST site activation letter. |
| Able to obtain initial blood sample within 24 hours of injury | Time between injury and blood draw influences blood levels of some biomarkers | Study coordinators assessment |
| Provide proxy informed consent | Informed consent is required prior to enrollment in Bio-BOOST | Signed informed consent document |

## 4.2 Exclusion Criteria

| Criteria | Rationale | Metric |
|---|---|---|
| Profoundly anemic | Subjects who are profoundly anemic require blood transfusion. | Hemoglobin < 8 mg/dl |
| Age less than 18 years | These subjects will constitute a small subset of the parent BOOST-3 trial and therefore we may not be sufficiently powered to make scientifically sound inferences. | Age less than 18 years |

### 4.3 Study Enrollment Procedures

4.3.1  Identification and screening process

Subjects will be recruited from BOOST-3 participants enrolled at a Bio-BOOST site. Each site will have a system for identification and early notification of potential participants who qualify for this ancillary study. The early notification system will result in timely arrival of the study coordinator or other trained study personnel, who will evaluate participant eligibility. Once notified, study personnel will review the potential participant's information and screen the patient according to the inclusion and exclusion criteria.

4.3.2.  Recruitment and informed consent

This protocol and the informed consent document and any subsequent modifications will be reviewed and approved by the Central IRB and the Department of Defense's Human Research Protection Office (HRPO). A signed consent form will be obtained for every subject. Since subjects in this trial cannot consent for themselves, a LAR, or person with power of attorney, must sign the consent form. The consent form will describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation. Every attempt will be made to contact the subject's family as soon as possible after the subject's admission, and in accordance with the individual hospital's protocol. To the extent possible, consent discussions should be carried out in a private setting without distraction.  No coercion will be applied.  The LAR and other family members will be provided a verbal description of this ancillary study and all the items described in the consent form will be reviewed and explained.  The LAR will be given an opportunity to read the informed consent document, ask and have answered any questions they may have about the study.

4.3.3.  Consent/assent and Other Informational Documents Provided to participants

A copy of the consent form will be given to the LAR, and this fact will be documented in the subject's record.

4.3.3.  Consent Procedures and Documentation

Consent is obtained by either the clinical site PI or by individuals to whom the clinical site PI has delegated authority to obtain informed consent. The delegation of authority is documented and maintained in WebDCU™. As with most clinical trial responsibilities delegated by the clinical site PI, it is his/her responsibility to ensure that the delegation is made only to those individuals who are qualified to undertake the delegated tasks, and that there is adherence to all applicable regulatory requirements and Good Clinical Practices (GCP) Guidelines. Additionally, it is the investigator's responsibility to ensure that the subject's legally authorized representative (LAR) has been given an adequate explanation of the purpose, methods, risks, potential benefits and subject responsibilities of the study.

## 5  BIOSPECIMEN

### 5.1  Biospecimen Collection

The initial set of biospecimens (serum, plasma, CSF, DNA and RNA) will be obtained as soon as feasible after randomization to a BOOST-3 study arm, but no later than 24 hours from injury. Given the logistical challenges of timely identification of an LAR, we will obtain the initial set of biospecimen as soon as it is logistically feasible; however, we will only utilize biospecimens for

research purposes after informed consent has been obtained. For subjects in whom the initial set of biospecimens is obtained before informed consent, the study team will have up to 24 hours after blood draw to obtain informed consent. If informed consent cannot be obtained, biospecimens will be discarded and will not be utilized in Bio-BOOST. Once informed consent is obtained, subsequent biospecimens will be obtained every 8 hours (+/- 1 hour) for the first 24 hours post-enrollment. This will allow the characterization of acute changes in biomarker levels. Our preliminary data from pig experiments (Figure 5 and Table 2) suggests that peak GFAP and NF-L values have the strongest correlation with neurological outcome post-TBI, compared to GFAP and NF-L values obtained at other time points. On study days 2 through 5, biospecimens will be obtained every 12 hours (+/- 2 hours) to allow characterization of sub-acute changes in biomarker levels, while minimizing the amount of blood collected from individual subjects. On study days 7 (+/-1 day), 14 (+/- 1 day) and at 6 months (+/- 30 days) post-enrollment, a set of biospecimen will be obtained. These samples will also allow the characterization of subacute and long-term changes in biomarker levels. On study Days 7 and 14 samples will be obtained only if the subject remains hospitalized during those days. In addition, during days 2 – 5, a one-time additional set of biospecimens will be obtained 2 hours after the start of a sustained hypoxic event in subjects randomized to PbtO2 guided treatment. A sustained hypoxic event is defined as a period of brain tissue hypoxia ($PbtO_2$ < 20 mmHg) that lasts 30 minutes or greater. In subjects randomized to ICP guided treatment only, a one-time additional set of biospecimens will be obtained 2 hours after the start of a sustained cerebral hypoperfusion event. A sustained cerebral hypoperfusion event is defined as a period of CPP<55mmHg that lasts 30 minutes or greater.

It has been suggested that brain injury biomarkers are transported to the blood via the glymphatic system, and sleep deprivation manipulates glymphatic activity.[19] Accordingly, it is possible that biomarker values are affected by circadian rhythm. For this reason, on days 2 through 5, biospecimens will be collected at 8 am (+/- 2 hours) and 8 pm (+/- 2 hours). On days 7, and 14 after injury, biospecimens will be obtained at 8 am local time if feasible. The 6-month sample will be drawn in conjunction with their follow-up visit. If the 6-month visit for the parent BOOST-3 study is conducted remotely, then no 6-month blood sample will be collected. Table 3 presents a tabulation of the sequence of biospecimen collection.

Biospecimens collected at each time point will consist of 6 ml of whole blood for serum extraction, 6 ml of whole blood for plasma extraction, 2.5 ml of whole blood for RNA extraction, collected in a PaxGENE tube (a total of 14.5 ml [one tablespoon] of blood) and 5 ml of cerebrospinal fluid (CSF). Since subjects are unlikely to have an EVD after the first week post-injury, CSF samples will be collected only for as long as the EVD is in place. During the separation of plasma samples from whole blood, the buffy coat suspension (a concentrated leukocyte suspension) will be extracted and stored for DNA analysis. This will be done each time plasma is extracted from whole blood, in order to increase the DNA yield.

Research coordinators participating in Bio-BOOST will participate in a training session performed by the biorepository staff at the University of Pittsburgh. This session will occur via a webinar (or site visit depending on the availability of funds) and will teach appropriate blood draw technique and storage techniques.

**Table 3: Sequence of biospecimen collection**

|  | **Day 1** | **Days 2 - 5** | **Day 7** | **Day 14** | **6 month** |
|---|---|---|---|---|---|
| **Frequency** | ASAP after randomization and 16 and 24 | Every 12 hours and once post-sustained ischemic event | Once | Once | Once |

| | | | | | |
|---|---|---|---|---|---|
| | hours post-injury | | | | |
| **Timing** | See above | Q8am and Q8pm (+/- 2 hours) and once post-sustained ischemic event | Q8 am | Q8am | During follow-up visit |
| **Specimen Type** | Serum, Plasma, CSF, DNA, RNA | Serum, Plasma, CSF, DNA, RNA | Serum, Plasma, CSF, DNA, RNA | Serum, Plasma, DNA, RNA | Serum, Plasma, DNA, RNA |

*Timing of Sample Collection.*

Day 1 is defined as the remainder of the calendar day after randomization. The first set of Day 1 samples should be collected at the time of enrollment into Bio-BOOST (and no later than 24 hours post-injury). The second sample should be collected 16 hours after injury, and the 3rd sample collected 24 hours after injury.

We require that at least one sample should be obtained within 24 hours of injury.

For Days 2 - 5, the timing of blood collection should switch to a twice daily schedule, generally 8 AM and 8 PM. If sample #3 (24 hours after injury) is collected between 4 PM and 4 AM, sample #4 should be collected at 8 AM. If sample #3 is collected between 4 AM and 4 PM, sample #4 should be collected at 8 PM. Thus, the time between sample #3 and #4 will be variable and could be as short as 4 hours and as long as 16 hours. Time of sample collection will be documented in Case Report Forms.

While this introduces some variability into the scientific analysis, the excessive burden on research staff of maintaining a blood sample collection clock tied to the highly variable time of injury carries excessive risk of protocol deviations, which will impact the scientific integrity of the analysis to an even greater degree.

## 5.2 Biospecimen Processing and Storage

Whole blood and CSF samples will be centrifuged, aliquoted and stored in a -70 or 80 degree Celsius freezer within 2 hours of phlebotomy. They will then be shipped periodically (once or twice a year) from Bio-BOOST sites to a Biorepository housed at the University of Pittsburgh for long-term storage. This biorepository stores biospecimens collected in other ongoing TBI studies such as the Transforming Research and Clinical Knowledge in TBI (TRACK-TBI) study. Specimen collection and storage kits will be shipped from the biorepository to study sites.

See Bio-BOOST manual of procedures for detailed instructions on sample collection, processing and storage.

## 5.3 Biomarker Assays

Serum/plasma and CSF samples will be analysed for simultaneous measurement of GFAP, UCH-L1, tau, and NF-L. These procedures will be carried out in Dr. Diaz-Arrastia's laboratory at the University of Pennsylvania, by a research scientist blinded to the clinical and physiologic data. There will be no difference in the distribution of samples by BOOST-3 treatment group. Other novel brain injury biomarkers will be assayed when they become available.

Blood samples collected in the study may be used for both TBI research and the study of other medical conditions.

## 6   BOOST-3 DATA
### 6.1 Demographic and Clinical Data

Bio-BOOST will utilize data collected in the BOOST-3 trial. This data includes: demographic data and clinical data such as injury characteristics, vital signs, head CT findings, laboratory data and data on physiologic parameters such as intracranial pressure (ICP), partial pressure of brain tissue oxygen (PbtO2), mean arterial pressure (MAP), and cerebral perfusion pressure (CPP), among others.

### 6.2  Outcome Data

Bio-BOOST will also utilize outcome assessment data collected from BOOST-3 participants at 6 months after injury (180 Days ± 30 days). Trained study personnel who are blinded to the treatment arm will administer the outcome assessments, which will include the measures listed below. The battery includes measures of functional status (GOSE), cognition, and emotional health.  The 6-month follow-up interview will be done in person whenever possible.  It may be done by telephone or video conference with participants where an in-person interview is not possible.

| Table 4.  Outcome Assessments | | |
|---|---|---|
| Functional Status | Formal Measures of Cognition | Emotional Health Measures |
| ● Glasgow Outcome Scale-Extended (GOSE)<br>● Structured Interview | ● Rey Auditory Verbal Learning Test<br>● Trail Making Test Part A+B<br>● WAIS IV Processing Speed Index | ● Rivermead Post-Concussion Symptom Questionnaire<br>● Brief Symptom Inventory 18<br>● Satisfaction with Life Scale |
| For explanations, citations, and expected testing durations of each component of the battery please refer to the BOOST-3 study protocol. | | |

# 7   STATISTICAL ANALYSIS PLAN

## 7.1 Analytic Plan for Primary Objective

Our primary objective is to quantify the effect of total brain tissue hypoxia exposure on brain injury using fluid-based biomarkers of brain injury.

**Hypothesis 1:**  We hypothesize that brain tissue hypoxia is associated with higher peak levels of biomarkers of astrocytic (GFAP) and axonal (UCH-L1, total Tau and NF-L) injury.  This hypothesis will be tested via linear regression model, with peak biomarker level as the response variable and hypoxia exposure as the predictor of interest.  Hypoxia exposure will be defined as the depth and duration of $PbtO_2$ < 20 mmHg during the first 48 hours of injury, quantified using AUC methodology.  Model assumptions of normality and constant variance will be assessed.  If the validity of the model assumptions is questioned, even after appropriate transformation of the response variable, nonparametric methods may be considered.  As an exploratory analysis related to this objective, alternative definitions of hypoxia (including $PbtO_2$ < 15 mmHg and $PbtO_2$ < 10 mmHg) will also be considered.

**Additional analyses will be detailed in the Statistical Analysis Plan.**

## 7.3 Sample Size Justification

The primary analysis will evaluate the effect of total brain tissue hypoxia exposure on brain injury biomarker levels.  Because the primary analysis considers each of the blood-based biomarkers independently, a conservative Bonferroni correction was assumed in order to maintain the overall family-wise error rate at 0.05.  We assume the standard deviation of the hypoxia exposure is 291 hour x mmHg,[12] as observed in BOOST-2.  The power of the planned linear regression model thus depends on the assumed slope under the alternative hypothesis and the standard deviation of the model residuals.  The relevant variables do not currently exist in the same data set, so there is little information on which to base these assumptions.  Instead, the power of the regression model is shown in Figure 6 for varying scenarios.
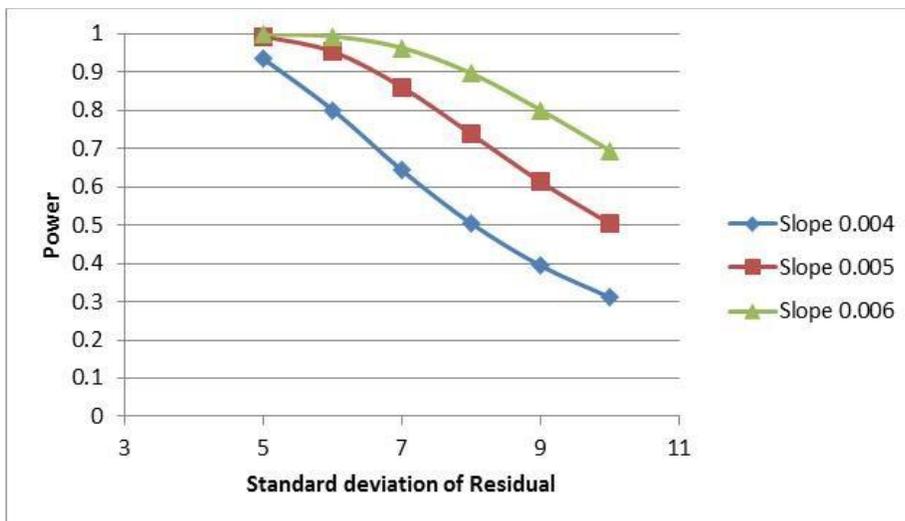


**Figure 6.**  Power estimates based on different scenarios for slope

Figure 7 provides a visual representation, using simulated data, of the scenario where the slope is 0.004 with a residual standard deviation of 6.
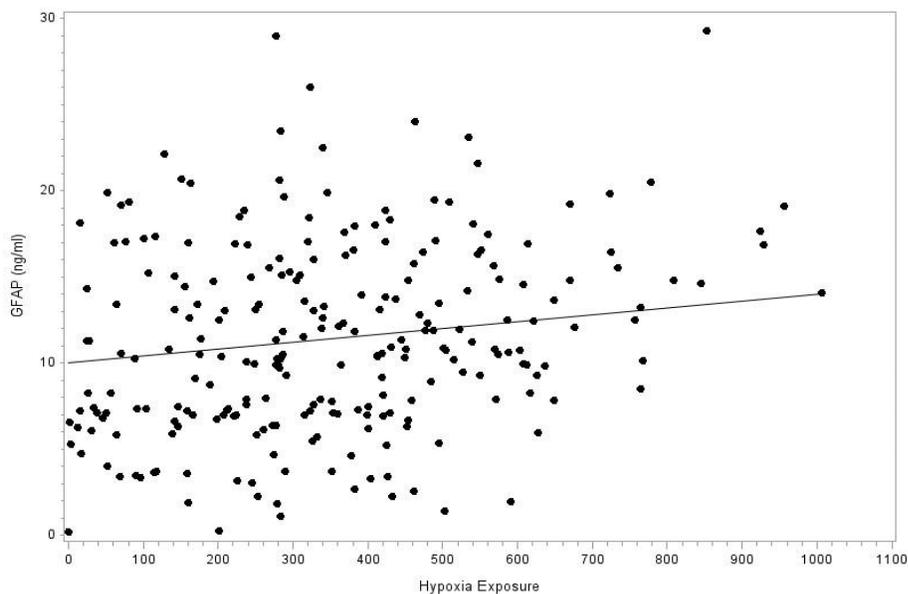
**Figure 7.** Simulation of relationship between GFAP and hypoxia exposure, based on slope of 0.004 and standard deviation of residual 6.

## 8  DATA MANAGEMENT AND QUALITY ASSURANCE

Data management will be handled by the BOOST-3 DCC, which is housed in the Data Coordination Unit (DCU) of the Department of Public Health Sciences at the Medical University of South Carolina. All study activities will be conducted in coordination with the study co-PIs, the hubs/spokes, and the CCC and DCC, and will use an electronic data acquisition method where all clinical data on enrolled subjects will be entered by site personnel. The latest version of each CRF will be available as a PDF file in the study database for use as worksheets and source documents by study personnel.

The study data will be managed by the DCC using the WebDCU™ system. This user-friendly web-based clinical trial management system, developed by the DCC, will be used for regulatory document management, data entry, data validation, project progress monitoring, subject tracking, user customizable report generation and secure data transfer.  Upon entry of CRFs into the study database, quality control procedures will be applied at each stage of data handling in order to ensure compliance with GCP guidelines, integrity of the study data and document processing system reliability.

## 9 HUMAN SUBJECT PROTECTION

### 9.1 Informed Consent Process

Written informed consent will be obtained from legally authorized representatives. See section 4.3.2.

### 9.2 Withdrawal from Participation

Participation in Bio-BOOST is voluntary. Participants or LARs may refuse to participate in the study at any time. Withdrawal from the study will not result in any penalty. If a participant withdraws from the study, they may request that any unused sample be destroyed. After the study is completed, it will not be possible to remove samples because they may no longer be identified with the participant.

### 9.3 Confidentiality

To protect against risks related to loss of confidentiality, clinical information will be kept coded in a secure database (WebDCU). Records collected will be confidential, unless required to be disclosed to oversight bodies, funders, regulators, or by state or federal law. Subjects will not be personally identified in any publications resulting from this project.  Numerous safeguards are maintained at all levels of the trial, including standard data management procedures at the DCC.

## 10 RESOURCE SHARING

Bio-BOOST will create a highly phenotyped biorepository of longitudinal serum, plasma, CSF, RNA and DNA samples from severe traumatic brain injury (TBI) patients. These samples will serve as a national resource for validating the diagnostic and prognostic accuracy of novel brain injury biomarkers. Investigators across the country will be invited to submit meritorious scientific proposals for the use of these samples. The use of these samples will be restricted to rigorous clinical validation of biomarkers that have already demonstrated diagnostic/prognostic value in preliminary investigations.

The primary results of this study will be disseminated by publication in the peer reviewed medical literature.  In accordance with the Department of Defense's Public Access Policy, the investigators will submit an electronic version of their final, peer-reviewed manuscripts (directly or through the publisher) to the National Library of Medicine's PubMed Central, no later than 12 months after the official date of publication.

The study will be registered with http://www.clinicaltrials.gov, and results of Bio-BOOST will be reported there within a year of trial completion.  Submission of results to http://www.clinical trials.gov will be performed consistent with the requirements for applicable clinical trials per FDAAA 801 requirements.

All manuscripts, abstracts and press releases using the study data must acknowledge BOOST-3/SIREN investigators and the Department of Defense as the study sponsor with the relevant grant numbers.

## 12 PUBLICATION OF RESEARCH FINDINGS

Publication of the results of this trial will be governed by the policies and procedures developed by the Executive Committee consistent with the SIREN publication policy. Any presentation, abstract, or manuscript will be made available for review by the Executive Committee prior to submission for publication.

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