Plasma Neurofilament Light and Glial Fibrillary Acidic Protein Levels over Thirty Days in a Porcine Model of Traumatic Brain Injury

Samuel S. Shin, Marco M. Hefti, Vanessa M. Mazandi, David A. Issadore, David F. Meaney, Andrea L. C. Schneider, Ramon Diaz-Arrastia, and Todd J. Kilbaugh

Abstract
To establish the clinical relevance of porcine model of traumatic brain injury (TBI) using the plasma biomarkers of injury with diffusion tensor imaging (DTI) over 30 days, we performed a randomized, blinded, preclinical trial using Yorkshire pigs weighing 7-10 kg. Twelve pigs were subjected to Sham injury (n = 5) by skin incision or TBI (n = 7) by controlled cortical impact. Blood samples were collected before the injury, then at approximately 5-day intervals until 30 days. Both groups also had DTI at 24 h and at 30 days after injury. Plasma samples were isolated and single molecule array (Simoa) was performed for glial fibrillary acidic protein (GFAP) and neurofilament light (NFL) levels. Afterwards, brain tissue samples were stained for β-APP. DTI showed fractional anisotropy (FA) decrease in the right corona radiata (ipsilateral to injury), contralateral corona radiata, and anterior corpus callosum at 1 day. At 30 days, ipsilateral corona radiata showed decreased FA. Pigs with TBI also had increase in GFAP and NFL at 1-5 days after injury. Significant difference between Sham and TBI animals continued up to 20 days. Linear regression showed significant negative correlation between ipsilateral corona radiata FA and both NFL and GFAP levels at 1 day. To further validate the degree of axonal injury found in DTI, β-APP immunohistochemistry was performed on a perilesional tissue as well as corona radiata bilaterally. Variable degree of staining was found in ipsilateral corona radiata. Porcine model of TBI replicates the acute increase in plasma biomarkers seen in clinical TBI. Further, long term white matter injury is confirmed in the areas such as the splenium and corona radiata. However, future study stratifying severe and mild TBI, as well as comparison with other subtypes of TBI such as diffuse axonal injury, may be warranted.

Keywords: diffusion tensor imaging; glial fibrillary acidic protein; neurofilament light; pig; porcine; traumatic brain injury

1Department of Neurology, 2Department of Anesthesiology and Critical Care Medicine, Children’s Hospital of Philadelphia, 3Department of Bioengineering, 4Department of Biostatistics, Epidemiology, and Informatics, 5Center for Brain Injury and Repair, University of Pennsylvania, Philadelphia, Pennsylvania, USA.
6Department of Pathology, University of Iowa, Iowa City, Iowa, USA.

*Address correspondence to: Samuel S. Shin, MD, Department of Neurology, Perelman School of Medicine, University of Pennsylvania, 3400 Spruce Street, 3 W Gates, Philadelphia, PA 19104, USA E-mail: samuel.shin@pennmedicine.upenn.edu
Introduction

Neurofilaments (NFs) are under the family called intermediate filaments found specifically in neurons as major components of axonal cytoskeleton that maintains its structural integrity and axon diameter. Specifically, neurofilament light (NFL) is a portion of this NF that comprises of 67-69 kDa subunit, currently under investigation as a biomarker for many neurological diseases, including Alzheimer’s disease, Huntington’s disease, multiple sclerosis, and stroke. In traumatic brain injury (TBI), NFs have been characterized in histological studies where they show increased packing at the site of axonal injury. As TBI results in axonal injury, these proteins, which are abundant in axons, may be released into cerebrospinal fluid and blood. Similarly, glial fibrillary acidic protein (GFAP) has gained much attention in TBI research. As a marker of astrocytic activation, neurological injury can lead to increase in GFAP levels near the site of injury.

In the recent few years, many studies have demonstrated the utility of blood-based biomarkers in detection of TBI due to the increasing sensitivity of modern biomarker detection assays such as single molecule array (Simoa). For example, NFL was shown to have marker detection assays such as single molecule array of TBI due to the increasing sensitivity of modern biomarker detection assays such as single molecule array (Simoa).9,12–15 For example, NFL was shown to have injury severity dependent elevation. Also, there is a prolonged elevation of NFL and GFAP even years after injury. Higher levels of serum biomarker such as NFL may also predict the clinical course long-term, as it was correlated with severity of injury using Glasgow Outcome Score.14,18

However, a closely monitored time course of NFL/GFAP levels have not been described in the literature in a porcine model of TBI. Moreover, their correlation to imaging markers of white matter injury has not been described in detail. There is significant difference in the anatomy, genetic background, and the mechanics of injury between rodents and humans. Thus, a biomarker study using a porcine model of TBI that more closely resembles human injury will have significant clinical implications for designing effective therapeutic strategies in the future. Using a pediatric porcine model of TBI, we compared animals that were subjected to contused cortical impact (CCI) to sham animals by diffusion tensor imaging (DTI) at 1 day and 30 days after injury. Moreover, we compared the time course of plasma NFL and GFAP over a course of 30 days.

Methods

Study design and animal surgery

In this randomized, blinded pre-clinical trial, approximately 4-week-old female pigs weighing 7-10 kg were utilized. This study was performed in accordance with the guideline by the Institutional Animal Care and Use Committee of the University of Pennsylvania (protocol number: 806943). The care and handling of the animals in this study were in accord with the National Institutes of Health guidelines. There were seven pigs that were subjected to TBI by controlled cortical impact (CCI) device and five pigs subjected to Sham injury as previously described. Briefly, animals underwent intramuscular injection of ketamine (20 mg/kg) and xylazine (2 mg/kg). They were then intubated after induction with 4% inhaled isoflurane and maintained at 1% isoflurane during the operation. Pigs were then randomized into either TBI or Sham groups. They were subjected to central venous catheter (CVC) placement into cephalic veins, terminating in the superior vena cava. The CVC were then tunneled between the animals’ scapula and securely left inside a jacket to be used over a course of 30 days for blood draws.

Pigs in the TBI group (n = 7) then underwent right frontoparietal craniotomy and CCI was performed at 0.7 cm depth over the cortical rostral gyrus, reflecting mild-to-moderate focal contusion injury. However, pigs in the Sham injury group (n = 5) underwent only skin incision without craniotomy, with similar anesthetic dosages and exposure. After the operation, pigs were monitored for 24 h to ensure normal recovery prior to return to animal housing. Blood samples were collected prior to the operation, then at 1 h, 8 h, and 24 h after the operation. Subsequently, they were collected at 5 days, 10 days, 15 days, 20 days, and 30 days after the operation. Both Simoa and DTI analysis occurred in a blinded fashion, with the blood sample and imaging data processing by a blinded experimenter to the conditions of the experiments. The group designations were revealed only after the samples were processed to prevent subjective bias.

NFL and GFAP immunoassay

Blood samples were centrifuged at 4400 × g for 5 min to isolate plasma samples. Then, plasma aliquots were stored in −80°C until analysis. We utilized a custom-made Simoa 2-plex assay for GFAP and NFL developed by Quanterix Corporation for our plasma biomarker analysis. The assay was run in house by the Human Immunology Core service available at the University of Pennsylvania using Simoa HD-1 Analyzer (Quanterix). Simoa detection of porcine GFAP has been validated by Quanterix as previously indicated. To confirm that porcine GFAP can be detected, we used a commercially available purified porcine GFAP (Millipore, AG230) and confirmed that 1:3 serial dilutions from 9000 pg/mL to 1 pg/mL standards resulted in corresponding changes in average number of enzymes per bead.

DTI

The first magnetic resonance imaging (MRI) with DTI sequence was performed at 24 h from the time of injury, and repeated imaging was performed at 30 days after injury. Imaging was performed using a 3T Tim Trio...
whole-body magnetic resonance scanner (Siemens, Germany) with 12-channel phased array head coil. Diffusion tensor imaging was performed using 64 noncollinear/noncoplanar directions with single-shot spin-echo, echoplanar imaging. The sequence parameters used were: repetition time (TR) = 4200 msec, echo time (TE) = 103 msec, flip angle = 180 degrees, bandwidth = 1186 Hz/pixel, field of view (FOV) = 192 mm, slices thickness = 2 mm, number of slices = 24, voxel size = 2 × 2 × 2 mm, b-values = 0, 1000, 2000, sec/mm². Data were analyzed using track-based spatial statistics method using FSL software. Eddy current and motion induced distortions were corrected. After a composite skeleton of the entire group was made, region of interest (ROI) was drawn for anterior corpus callosum, splenium, ipsilateral (right), and contralateral (left) corona radiata by a blinded analyzer. For each ROI, FA and mean diffusivity (MD) values were calculated and compared between Sham and TBI groups. The voxel-wise map of DTI results was corrected for multiple comparisons. Although seven TBI animals were scanned, data from only six were used as one of the DTI scans was lost in data handling process. Data from all five Sham animals were used for this study. Example DTI scans are shown in Supplementary Figure S1.

**Histology**

After euthanasia, 5-mm coronal sections were fixed in 10% formalin for immunohistochemistry. The tissue sections underwent dehydration and were embedded in paraffin. Using a microtome, 10-μm sections were cut. Sections were then deparaffinized and rehydrated in ethanol at different concentrations. Antigen retrieval was performed in Tris–ethylenediaminetetraacetic acid buffer, followed by 3% hydrogen peroxide block, and incubated in avidin biotin blocking kit. To label β-amyloid precursor protein (β-APP), recombinant anti-amyloid precursor protein (Y188) was used at 1:10,000 (Abcam, AB32136) in 3% bovine serum albumin/1× phosphate-buffered saline/0.1% Triton-X for 1 h. Then, anti-rabbit horseradish peroxidase secondary antibody was used at 1:500 (Invitrogen, A16104) for 1 h followed by BD 3,3′-diaminobenzidine substrate kit (BD 550880). Sections were then counterstained, dehydrated, clarified with xylenes, and cover-slipped. A pathologist (MH) who was blinded to the group assignment analyzed APP-stained slides, labeling them with the following scale: ‘−’ for no staining, ‘+’ for less than 3 β-APP positive spheroids, ‘++’ for 3-10 β-APP positive spheroids, and ‘+++’ for greater than 10 β-APP positive spheroids in a single 100× field. Example staining is shown in Supplementary Figure S2. Perilesional white matter, as well as ipsilateral and contralateral corona radiata were inspected.

**Statistical analysis**

Single molecule array data was compared between Sham and TBI group at each time-point along the 30-day course using repeated measured Mann-Whitney U test. The DTI parameters (FA and MD) were also analyzed using Mann-Whitney U test for each white matter region and each time-point to compare Sham and TBI groups. Correlation between NFL and DTI or GFAP and DTI parameters were then inspected, using Spearman Rank correlation coefficient. For all tests in this study, p < 0.05 was considered a significant cut-off value.

**Results**

**DTI findings**

When composite FA values were compared between Sham (n = 5) and TBI (n = 6) animals, major area that showed significant (p < 0.05) FA decrease in TBI animals was ipsilateral corona radiata (CR) for both 1 day and 30 days (Fig. 1). To a lesser extent, small areas of FA decrease was also noted in the contralateral CR. At 30 days, ipsilateral CR still showed areas of FA decrease, and splenium also showed some areas of decrease. Each region’s FA is reported in (Fig. 2). At 1 day, there was a widespread FA reduction in TBI animals. Specifically, this involved anterior corpus callosum, contralateral CR, and ipsilateral CR (Fig. 1). Ipsilateral CR of TBI showed a significant FA reduction compared with sham pigs at both 1 day and at 30 days. Splenium did not show a significant FA decrease but showed a decreasing trend at both 1 day and 30 days. Further clarification of this data is shown in Supplementary Figure S3, showing the mean value of each group.

**Plasma biomarker study**

To assess the time course of NFL and GFAP over a time course of 30 days, Simoa assay was used for high sensitivity detection of these biomarkers (Fig. 3). The two biomarkers show a subacute increase to peak values at 1 day and 5 days for GFAP and NFL, respectively. Following this initial peak, there is a gradual decrease for both biomarkers over the 30 days. Although GFAP showed a fast normalization after 5 days, NFL in TBI animals continued to have increased trend even at 20 days (p = 0.06). We then assessed the correlation between acute biomarker levels and imaging findings at 30 days (Fig. 4). To assess for each biomarker’s prediction of the degree of injury, acute NFL and GFAP values at 1 day was compared with white matter injury at 30 days assessed by FA values. Fractional anisotropy of ipsilateral CR at 30 days were plotted, given that this was an area of FA loss. Both NFL (r = 0.87, p = 0.002) and GFAP (r = 0.73, p = 0.02) had significant negative correlation with FA values in the ipsilateral CR.
Histology
To further validate the biomarker and imaging findings, tissue sections from CR and perilesional cortical tissues were taken for β-APP staining to indicate the level of injury (Fig. 5). Both perilesional tissue and ipsilateral CR showed frequent β-APP positive axons. However, whereas all TBI animals had positive β-APP in perilesional tissue, only half of the injured animals had positive β-APP axons in ipsilateral CR indicating large variability of injury in remote white matter tracks.

Discussion
To this date, there is a relative lack of reported data on blood-based biomarkers for TBI in an animal model. There is one report showing the elevation of NFL in the pig model of TBI, looking at the therapeutic effect of valproic acid, but the detailed time course of plasma biomarkers and its validation with imaging and histological findings have not been performed. Biomarker levels are important in monitoring the disease severity as well as the mechanistic insight it provides. Since this information can guide our future efforts to develop therapeutic strategies, we aimed to characterize the time course and imaging correlate of serum NFL levels in the pig model of TBI.

This study demonstrated the 30-day time course of plasma biomarkers of neurological injury NFL and GFAP in a pediatric porcine model of TBI. Similar to the human data, NFL has a delayed increase in pigs over the time course whereas GFAP showed an early peak. However, while human data shows peak levels around 10 days, in the current study pig plasma NFL peaked around 5 days. This time course seems to be midway between the human and mouse time course of NFL levels after TBI, given the fact that mice have peak NFL levels at 3 days. Although the exact peak timing may depend also on injury modality and severity, this finding supports the notion that pig model of TBI more closely approximates human TBI than the rodent model. This is expected given the much better anatomical similarity between humans and pigs compared with rodents. Pig brain has gyrencephalic complexity much closer to humans and a large difference exists between the gray-white matter ratio as well as neuronal count between humans and rodents.

FIG. 1. Areas of significant fractional anisotropy (FA) decrease (red-yellow regions) in traumatic brain injury animals compared with Sham animals, co-registered with T1 pig brain map. Top three panels are maps of FA values at serial axial cuts at Day 1, and bottom three panels are maps of FA values at serial axial cuts at Day 30. Color image is available online.
Despite the significant difference in biomarkers between the Sham and TBI groups, a notable finding was a high variability of biomarker levels among the injured animals. In Figure 3 and Figure 4, a wide range for NFL as well as GFAP is noted. For example, despite having a uniform level of CCI injury amongst the TBI animals, NFL levels 5 days ranged widely between 30 and 600 pg/mL. However, this wide range in the current animal data is consistent with the general range of NFL seen in a much larger human TBI cohort. For example, there was a wide range of 10-300 pg/mL among subjects with severe TBI in this prior clinical study. Even in our animal model of homogeneous subject pool and injury severity, a wide range resulted with a 10- to 100-fold difference between the lowest and the highest biomarker value. Since a contributing factor to these biomarker levels may have been the variability of injury by CCI device, we validated the injury severity using DTI and showed that FA values show reliable decrease at the major white matter regions. Specifically, the most affected regions were CR at the ipsilateral side, although splenium of the corpus callosum also had a delayed FA decrease at 30 days. In the composite FA map (Fig. 1), small regions of contralateral CR also had significant FA decrease. However, these differences were not significant enough to lead to overall decrease in FA for the entire ROI for contralateral CR.

A unique finding were the different patterns of injury severity between CR and splenium over the 30 days. Corona radiata had a large area of FA decrease at 1 day, although these areas reduced in size by 30 days. However, splenium, which showed minimal injury at 1 day, developed a larger area of decrease in FA at 30 days. Reduction in the area of low FA may be due to repair and recovery of white matter, but contribution of tissue edema in decreasing in FA should also be
considered.26 As it has been demonstrated that increase in interstitial fluid content reduces FA values,27-29 tissue edema in the setting of acute injury can exaggerate the degree of axonal injury as detected by DTI acutely. It is possible that as edema reduced over the period of 30 days, the ipsilateral CR had smaller area of FA deficit. As shown in Supplementary Figure S4, minor edema at the peri-contusion site acutely after injury had been previously noted. Although there is general elevation of radial diffusivity as well as axial diffusivity at 1 day among TBI pigs, these changes normalized by 30 days (Supplementary Fig. S5). In contrast, splenium had larger area of FA decrease over time, which may have developed as delayed disintegration of axons consistent with the time course of Wallerian degeneration.

When the biomarker levels at 1 day were plotted against FA values of the CR, there was a significant negative correlation (Fig. 4). Higher levels of NFL and GFAP reflected lower FA values, as higher injury severity was related to reduced axonal integrity. However,
there was still a wide distribution of NFL levels, with several injured animal’s NFL levels at the same range as those of the Sham animals. Given this large variability, we performed β-APP staining in these regions. Just as the biomarker levels indicated, there was still a wide distribution in the severity of injury in the CR shown by β-APP staining (Fig. 5C). Although the biomarkers and DTI findings show significantly different values between Sham and TBI animals, future experiments may require a larger number of animals to reduce the variability further. This initial study validating the biomarkers of TBI supports its future use as an early indicator of injury severity given its validation with imaging. Moreover, it further supports the use of porcine model of TBI given many of the findings that parallel human injury.

**Limitations**

There are several limitations in the current study that can be explored in the future. Given the limitation of resources, only one type of injury (CCI) has been investigated. However, it remains in question how other types of TBI can affect the levels of these plasma biomarkers across time. Time course of biomarkers for rotational injury, repetitive closed head injury, and blast injury which all have their own unique relevance to various types of clinical TBI will be important to clarify. Additionally, we only investigated one severity of injury. Understanding the profile of biomarkers for different severities of injury as well as looking at longer time course will also be helpful in future endeavors.

**Conclusions**

The findings in this study indicate that much like human studies, there is a delayed increase in NFL to peak levels whereas GFAP reaches peak levels early on. The elevation of NFL remains significantly prolonged until 20 days. Correlating with these findings, there was significant degree of white matter injury in areas such as ipsilateral corona radiata and splenium. These findings

---

**FIG. 5.** Histological validation of axonal injury. β-APP-stained perilesional cortex (A) and ipsilateral corona radiata in an injured animal (B), both with a grade of “+”. Black bar represents 50 μm. Black arrow head represents β-APP positive axonal processes. The degree of staining amongst the subjects is displayed in (C). The scale is described in the Methods section. N/A = not available, as specified region was not available for analysis. CR, corona radiata. Color image is available online.
validate the use of plasma biomarkers in a porcine model of TBI, which can be an important platform for future preclinical studies.

Acknowledgments
We acknowledge the technical support provided by Rina Degani and tissue dissection and handling by Jonathan Starr. Additionally, we would like to thank Thomas Hallowell on his help handling animals and surgical procedures. We would also like to thank Human Immunology Core at the University of Pennsylvania for their help in development and processing of single molecule array for NFL and GFAP.

Authors’ Contributions

Funding Information
This study was supported by Resuscitation Science Center institutional discretionary funds at the Children’s Hospital of Philadelphia.

Author Disclosure Statement
No competing financial interests exist.

Supplementary Material
Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5

References


