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Radiological-Pathological Correlations Following Blast-Related Traumatic Brain Injury in the Whole Human Brain Using ex Vivo Diffusion Tensor Imaging

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14. ABSTRACT The objective of the project is to perform anatomically correlated high resolution DTI studies and quantitative immunohistochemical analyses of axonal injury in the human brain following traumatic brain injury. The specific aims are Aim 1) To optimize the resolution and signal quality of whole human brain ex vivo diffusion tensor imaging. Aim 2) To perform radiological-pathological correlations using whole human brains from civilian TBI fatalities and US military fatalities caused by blast-related injuries. Aim 3) To construct detailed atlases of the brain regions most frequently injured by blast-related TBI and non-blast-related TBI. During the third year of the project, we have made progress as follows: A) We have performed DTI on 59 blocks of brain tissue: 2-3 regions from each of 22 cases (5 blast-TBI, 8 non-blast TBI, 9 controls). B) We have performed blinded stereological analysis of axonal injury, microgliosis and astrogliosis in each of the 63 blocks of tissue. C) We found essentially no correlation between any of the DTI parameters and the histopathological markers. Possible explanations for this lack of correlation include heterogenous brain white matter fiber orientation, high variability in injury to death interval, and inconsistent brain tissue fixation. Ongoing work will address these possibilities.					
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INTRODUCTION:

Blast-related traumatic brain injury (TBI) continues to be a major cause of death and disability in the wars in Iraq and Afghanistan (Okie 2005; Warden and French 2005; Armonda et al. 2006; Warden 2006; Martin et al. 2008; Benzinger et al. 2009; Cernak and Noble-Haesslein 2009; Champion et al. 2009; Kochanek et al. 2009; Ling et al. 2009). However, very little is known about the pathological features of these injuries, and this has limited the analyses of the underlying mechanisms of injury (Mott 1916; Mott 1917; Cohen and Biskind 1946; Cramer et al. 1949; Guy et al. 2000; Taber et al. 2006; Benzinger et al. 2009; Kocsis and Tessler 2009).

In brain injuries caused by non-blast related trauma (e.g. falls, motor vehicle accidents, etc.), post-mortem pathological analyses have revealed that traumatic axonal injury in a set of characteristic locations is a consistent hallmark (Strich 1956; Strich 1961; Oppenheimer 1968; Clark 1974; Adams et al. 1982; Adams et al. 1989; Abou-Hamden et al. 1997; Jones et al. 1998). Of note, even in mild TBI with death due to other causes, axonal injury was prominent (Pilz 1983; Blumbergs et al. 1994; Blumbergs et al. 1995; Abou-Hamden et al. 1997). In animal models of blast-related TBI, axonal injury appears to be present (Stone and Young; Saljo et al. 2000; Bauman et al. 2009; Long et al. 2009) but the extent to which these animal models accurately reproduce the injuries suffered in human blast injury is unknown.

Axonal injury goes largely undetected on CT or conventional MRI (Jones et al. 1998), but a newer MRI modality called diffusion tensor imaging (DTI) has proven to be very sensitive to traumatic axonal injury in animal models (Kim et al. 2007; Loy et al. 2007; Mac Donald et al. 2007a; Mac Donald et al. 2007b; Zakaria et al. 2007). At Washington University (WU), recent advances in MRI technology now allow the possibility of performing DTI in human brain tissue at very high resolution. These innovative methods have the potential to allow detailed radiological-pathological correlations for traumatic axonal injury to be performed for the first time in the human brain. A central part of these advances is the use of high resolution imaging of blocks of human brain at 4.7 Tesla. This high resolution imaging has allowed visualization of both large and small white matter tracts, plus subcortical white matter regions close to the junctions between gray and white matter. In contrast, lower resolution approaches using commercial hardware mainly allow analyses of larger white matter tracts.

Specific Aims

Aim 1) To optimize the resolution and signal quality of whole human brain *ex vivo* diffusion tensor imaging. We will iteratively refine the design of the specialized MRI coils and MRI pulse sequences using whole normal human brains and MRI phantoms. Resolution at or below 0.75 mm will be the goal.

Aim 2) To perform radiological-pathological correlations using whole human brains from US military fatalities caused by blast-related injuries and civilian TBI fatalities. This will include a wide range of TBI severities, including mild to moderate TBI with death caused by extracranial injuries. A total of 30 brains will be assessed.

Aim 3) To construct detailed atlases of the brain regions most frequently injured by blast-related TBI and non-blast-related TBI. These atlases will be distributed to scientists and clinicians in both military and civilian sectors focusing on TBI.

BODY:

To recap, during year 2 of the project, we determined that *ex vivo* whole brain imaging would not be feasible with current technology, and turned our attention to high resolution *ex vivo* imaging of selected smaller blocks of brain tissue. During year 3 of the project, we performed DTI scans on 59 blocks of brain tissue: 2-3 regions from each of 22 cases.

The regions chosen were:

Middle Cerebellar Peduncle: A region suggested to be especially susceptible to blast-related TBI (Mac Donald et al NEJM 2011)

Orbitofrontal White Matter: A region suggested to be vulnerable in both blast-related and non-blast related severe TBI.

Parietal Subcortical White Matter: A region with similar anatomical structure to the orbitofrontal white matter but not expected to be especially vulnerable to TBI.

The cases are summarized in the following Table:

Group	Age Range	Mean Age	# of PSCs	# of OFs	# of MCPs
Blast TBI	22-45	30.8	4	5	5
Non-blast TBI	18-50	29	6	8	7
Control	18-70*	39.8	6	9	9

Causes of death by group were as follows:

Blast TBI:

Suicide drug overdose – blast years prior

Ruptured aneurysm – blast years prior

intraventricular hemorrhage – blast years prior

Civilian Blast DOA

Non-blast TBI:

MVA – DOA

MVA – DOS

Suicide – NFL – GSW to chest

Cardiac Arrest – NFL

Controls:

Suicide drug overdose

Suicide blunt trauma - fall

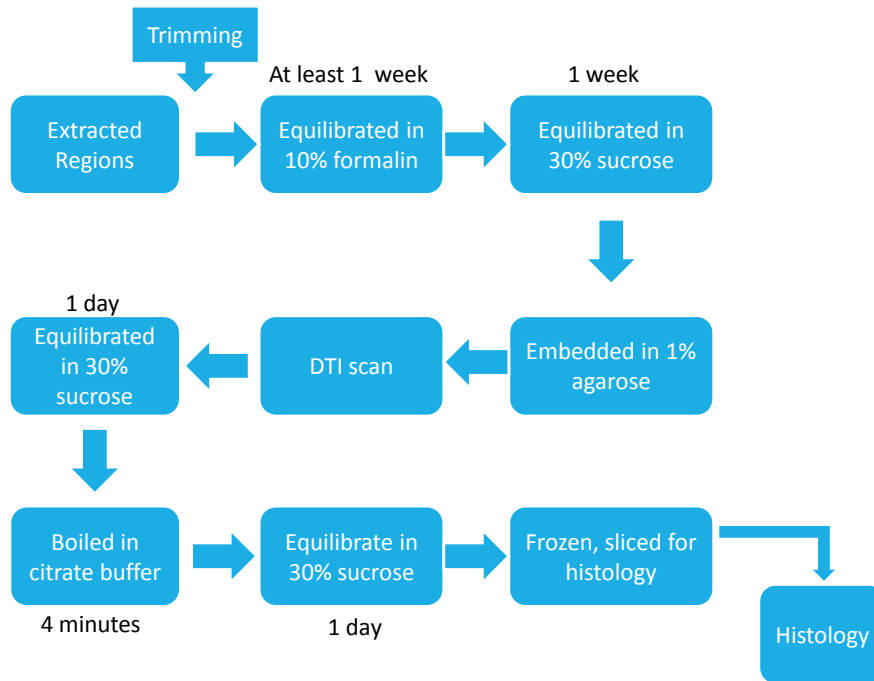
Cancer

Cardiac Arrest

Tissue fixation was highly variable because cases were obtained from 4 different sources:

Location	Fixation Protocol
St. Louis County Medical Examiners Office	10% formalin
Harris County Institute for Forensic Sciences	10-20-10% graded formalin series, with added salt to keep tissue buoyant
Boston University/Boston VA	Periodate-lysine-paraformaldehyde (4% PFA)
Uniformed Services University of the Health Sciences	10% formalin

Workflow is summarized on **Figure 1**



Examples of the process are shown in **Figures 2-3**:



Figure 2: Workflow exemplar. The upper left panel shows a slab of cerebellum. The upper right panel shows a series of isolated regions containing the middle cerebellar peduncle. The lower left panel shows a trimmed tissue block containing the middle cerebellar peduncle. The lower right panel shows a histological slide containing the middle cerebellar peduncle white matter.

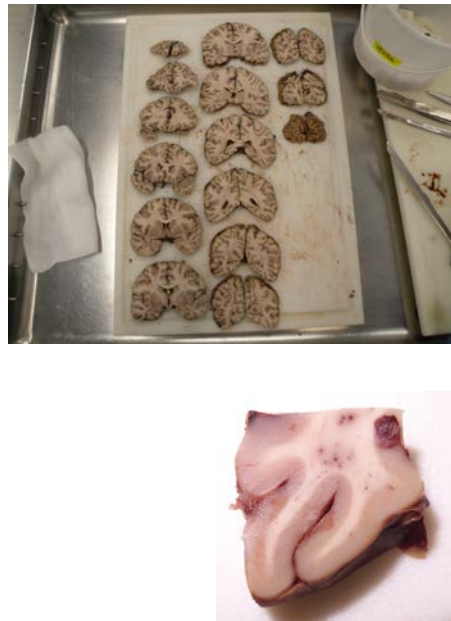


Figure 3 Workflow exemplar. The upper left panel shows a whole human brain from a non-blast TBI case. The upper right panel shows coronal slabs of the brain. The lower left panel shows a frontal slab. The lower central panel shows the removal of a block of tissue corresponding to the orbitofrontal region. The lower right panel shows a magnified view of the orbitofrontal tissue block, including a region of punctuate hemorrhage in the white matter.

DTI Methods:

All scans were performed on a 4.7T Varian small bore scanner.

12-direction spin-echo diffusion weighted images

b0, b2000

TE 40ms, TR 1500ms

In-plane resolution 500x500 microns

Total acquisition time ~3hours

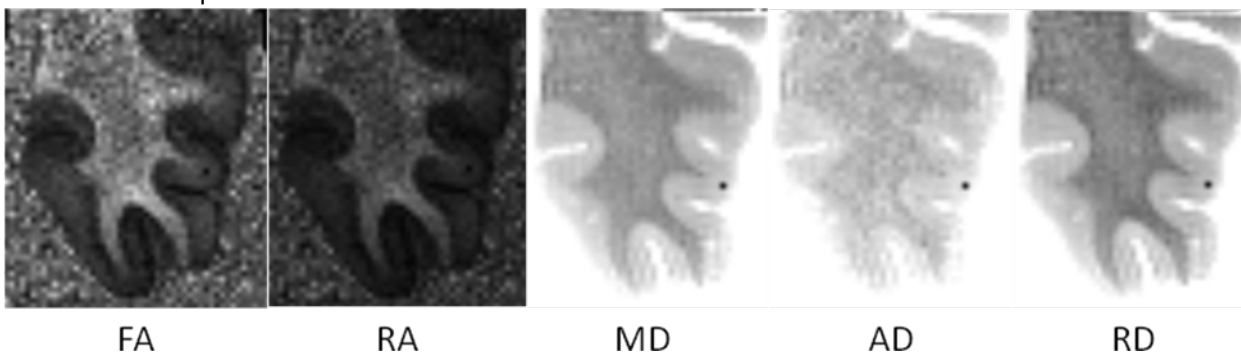


Figure 4: DTI exemplars:

The figure shows an exemplar of the diffusion tensor imaging from a block of orbitofrontal brain tissue.

FA and RA: fractional anisotropy and relative anisotropy, indicators of overall white matter integrity.

MD: mean diffusivity: the overall rate of diffusion of water.

AD: axial diffusivity: the rate of diffusion parallel to the main fiber direction.

RD: radial diffusivity the rate of diffusion perpendicular to the main fiber direction.

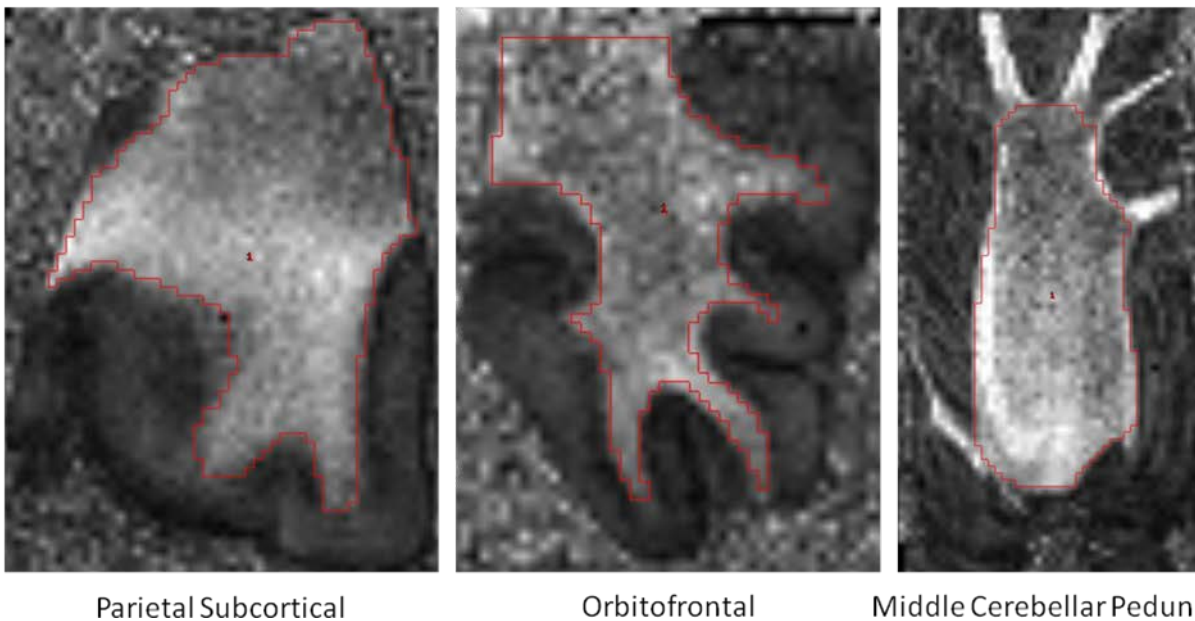


Figure 5: DTI Regions of Interest. The figure shows the white matter regions of interest analyzed on the diffusion tensor imaging (Fractional anisotropy maps) for the 3 anatomical regions assessed. Note that the signal in the white matter appears inhomogeneous in a manner that does not appear to reflect anatomical substructure. This indicates possible sub-optimal signal to noise

Stereological methods:

Stereoinvestigator software coupled to a motorized stage on a Nikon 80i upright microscope.

All tissue counted using a $250^2 \mu\text{m}$ counting frame and a grid size of 1500^2 , 2500^2 , or $3500^2 \mu\text{m}$ depending on tissue and marker.

Optical Fractionator ran at 10x objective, ROIs drawn at 1x

2 slices were used for each marker per region of interest

ROIs defined by grey/white matter boundaries

All white matter was taken as ROI except for middle cerebellar peduncle, where only non-branching white matter was selected

ROI Definition



Figure 6: Histological Regions of Interest. The figure shows the white matter region of interest in the middle cerebellar peduncle, including only non-branching white matter regions. The branching white matter regions cannot be resolved with DTI, and so were not counted using stereology.

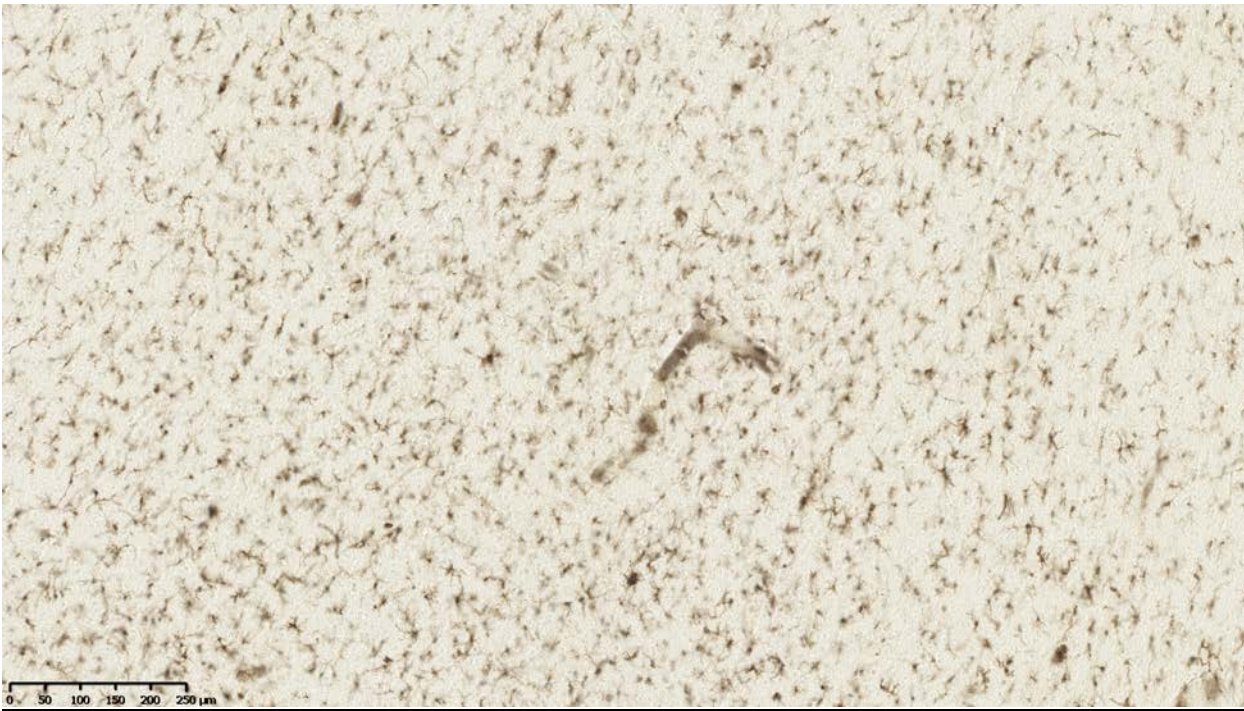


Figure 7: Microgliosis.The figure shows the morphological characteristics of Iba1-stained cells, used to indicate microglia.

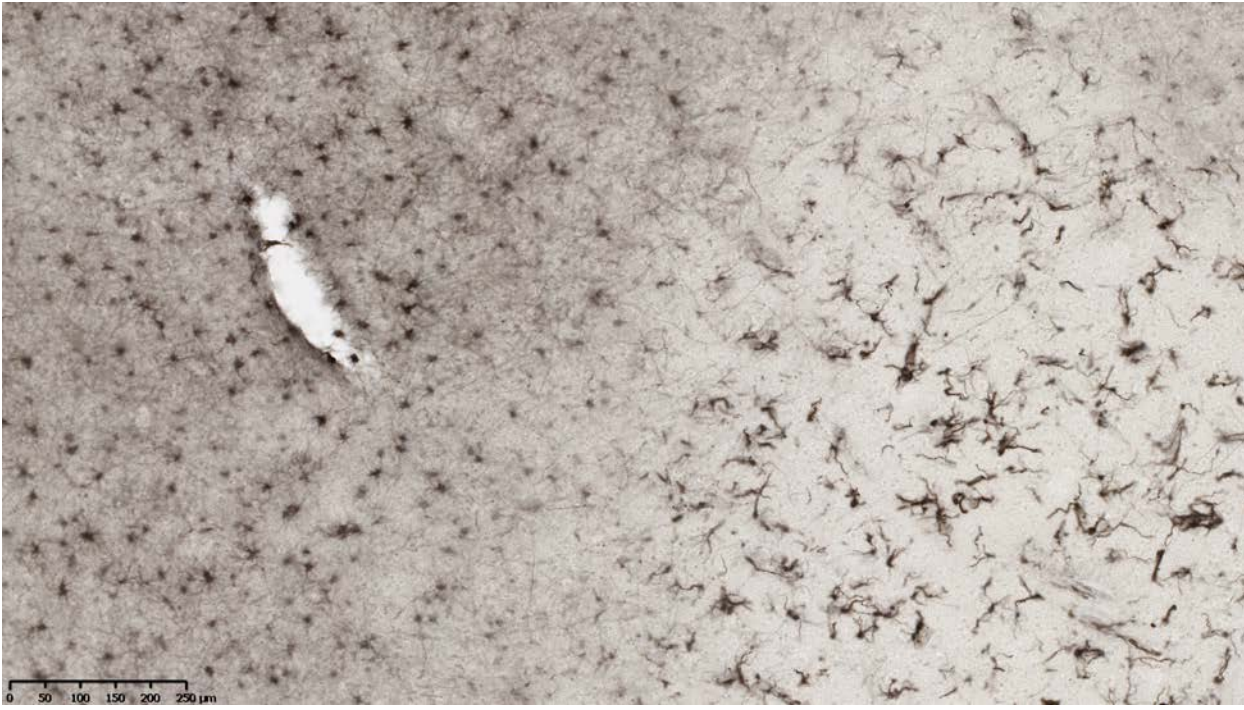


Figure 7: Astrogliosis The figure shows the morphological characteristics of GFAP-stained cells, used to indicate astrocytes. Note that there was a variety of observed morphologies, including small branched cells with fine processes (upper left portion) and larger cells with coarse dysmorphic processes (lower right portion)



Figure 9: Axonal Injury The figure shows the morphologies of neurofilament-heavy chain stained injured axons. The upper panel shows punctate staining indicating injured axons running in and out of the plane of the section. The lower panel shows elongated stained forms, indicating injured axons running within the plane of the section.

RESULTS

Ex vivo DTI

The main finding from the analysis of the ex-vivo diffusion tensor imaging was that the cases and controls did not differ substantially in any DTI parameter. In large part, this may have been due to the very wide variety of DTI parameter across cases. This was especially notable in the controls, which were expected *a priori* to have had high and relatively homogenous fractional anisotropy. (See discussion)

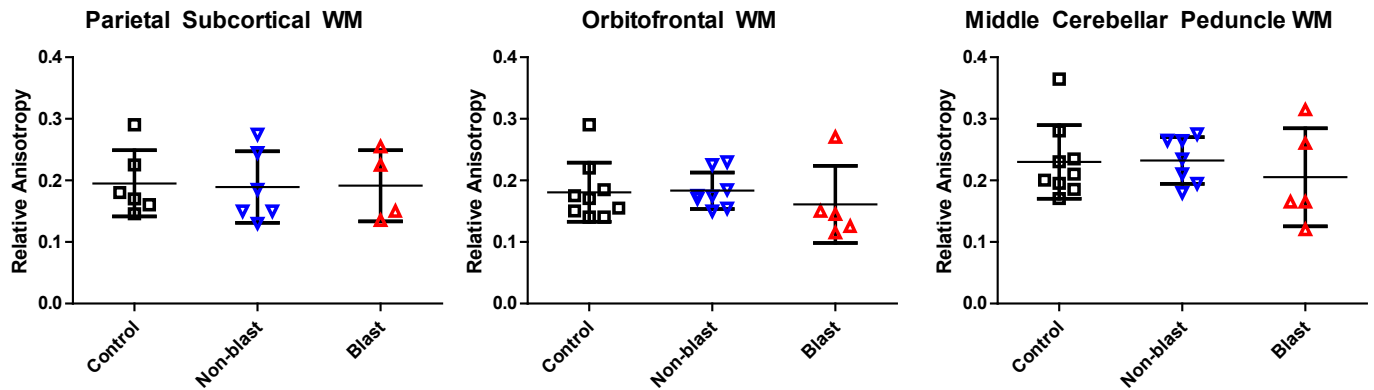


Figure 10: Relative Anisotropy across groups. The figure shows that there was no difference in relative anisotropy between any of the groups in any of the 3 regions of interest. The relative anisotropy was quite low in all cases, lower than expected for intact white matter based on *in vivo* and previous *ex vivo* studies.

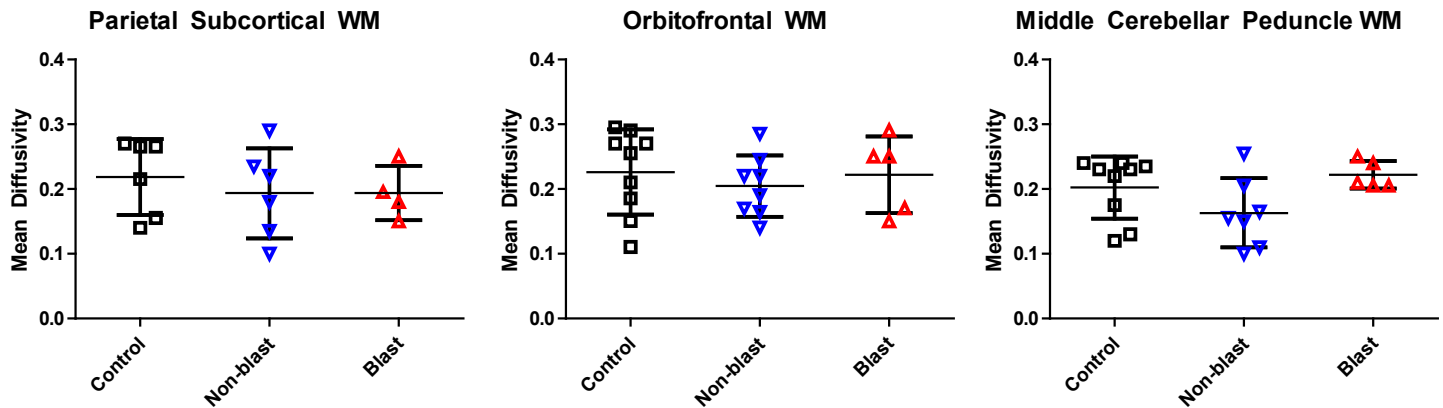


Figure 11: Mean Diffusivity across groups The figure shows that there was no difference in mean diffusivity between any of the groups in any of the 3 regions of interest.

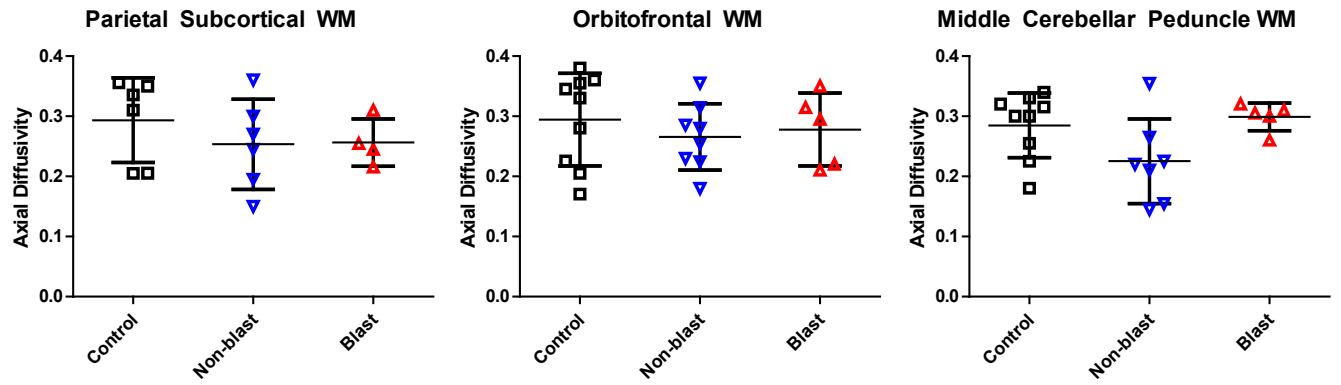


Figure 12: Axial Diffusivity across groups: The figure shows that there was no difference in axial diffusivity between any of the groups in any of the 3 regions of interest.

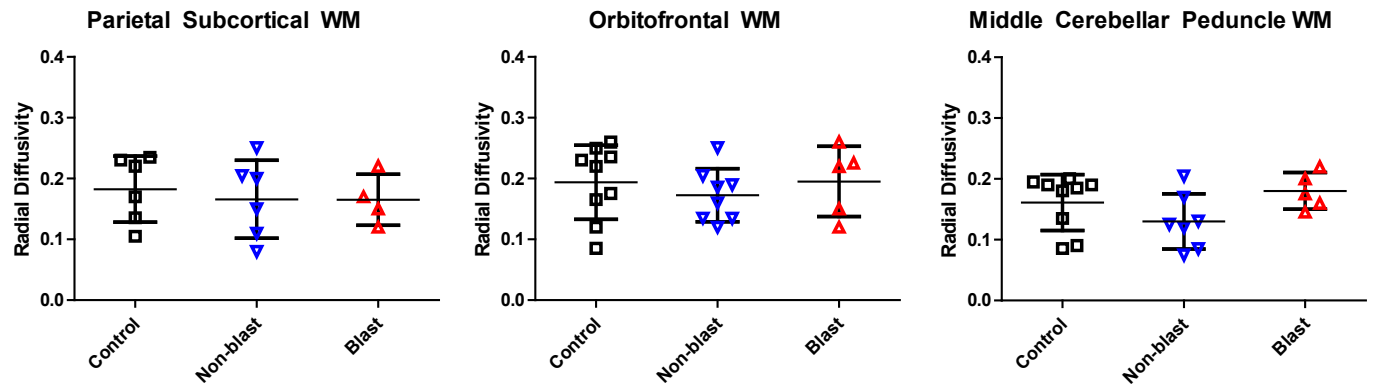


Figure 12: Radial Diffusivity across groups: The figure shows that there was no difference in radial diffusivity between any of the groups in any of the 3 regions of interest.

Quantitative Histology

Overall the main finding from the histological analysis was that the cases and controls did not substantially differ in the density of the pathological markers assessed. There was a wide variety in all of the pathological counts.

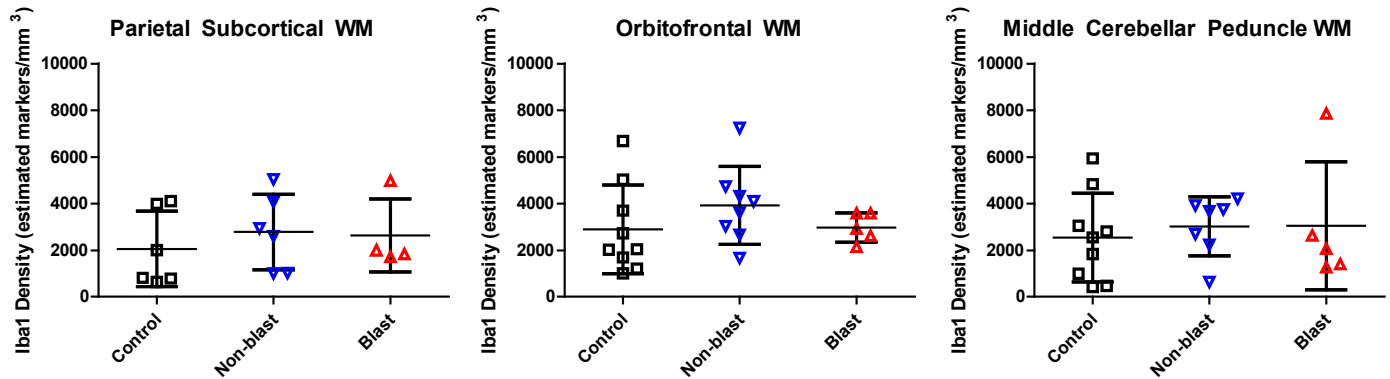


Figure 13: Microgliosis across groups: The figure shows that the density of Iba1 stained activated microglia was not different between controls, non-blast TBI cases, and blast-TBI cases in any of the 3 regions: parietal subcortical white matter, orbitofrontal white matter, and middle cerebellar peduncle white matter.

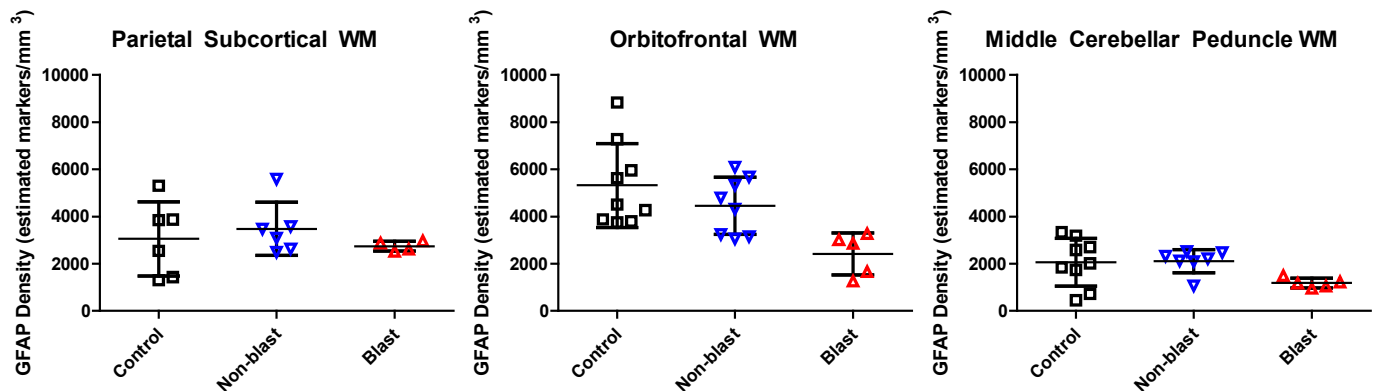


Figure 14: Astrogliosis across groups: The figure shows that the density of GFAP stained reactive astrocytes was not different between controls, non-blast TBI cases, and blast-TBI cases in any of the 3 regions. There was a trend towards *reduced* astrocytosis in the blast-TBI cases.

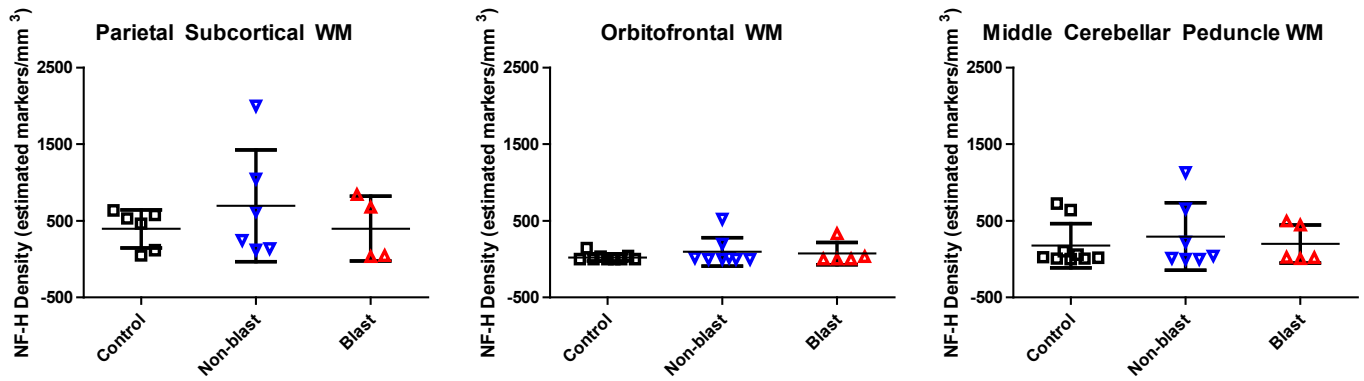


Figure 15: Axonal Injury across groups: The figure shows that the density of neurofilament-heavy chain stained injured axons was not different between controls, non-blast TBI cases, and blast-TBI cases in any of the 3 regions. In a substantial number of cases, there were no injured axons detected in the orbitofrontal white matter or middle cerebellar peduncle. This is in contrast to our initial hypothesis that these regions would be especially vulnerable to axonal injury.

Correlations between DTI and quantitative histology:

Overall, the main finding was that there were few significant correlations between the DTI parameters and the quantitative histological markers of microgliosis, astrogliosis or axonal injury. The strongest correlations indicated that 1) higher astrocyte counts correlated with lower anisotropy (indicating less intact white matter microstructure). 2) Higher NF-H counts indicative of injured axons correlated with lower mean, axial and radial diffusivity. The finding regarding the astrogliosis is in the expected direction, but the correlations were substantially weaker than would be expected for a clinically useful radiological-pathological correlation. The finding regarding the neurofilament heavy chain is difficult to reconcile with previous studies or theoretical models of diffusivity changes following traumatic axonal injury. It may be due to covariates such as overall tissue integrity or fixation quality.

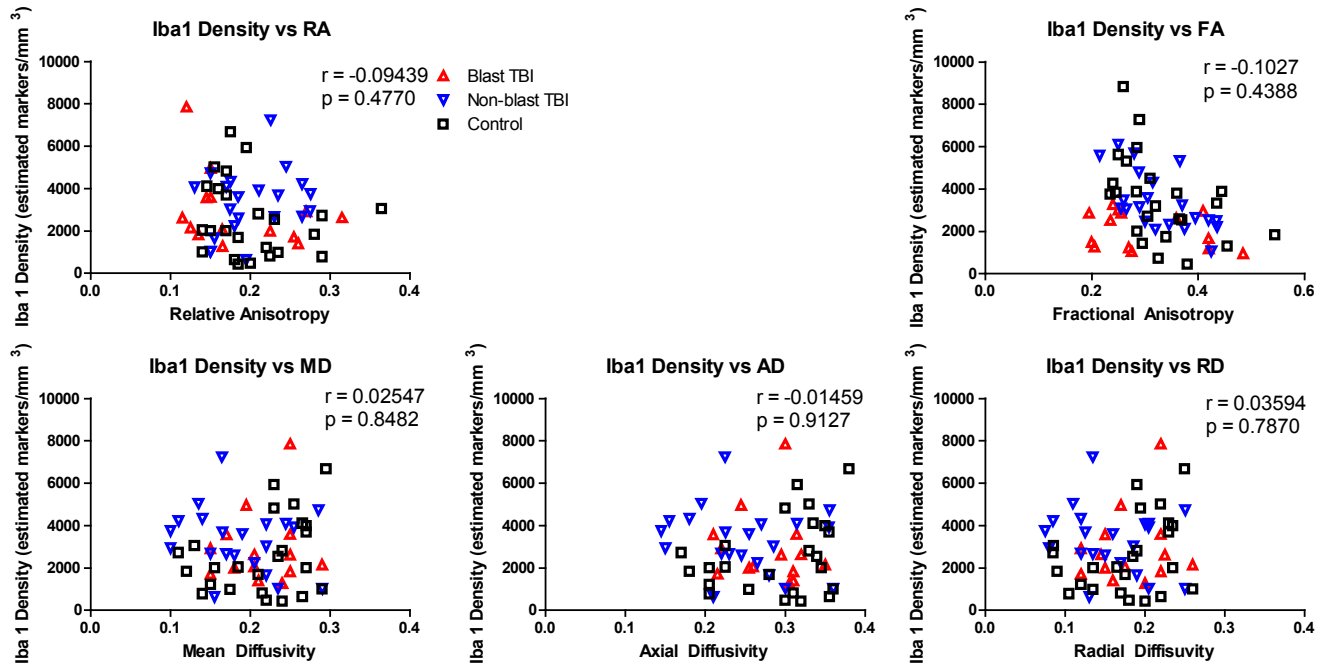


Figure 16: Correlations between DTI parameters and microgliosis: The figure shows the lack of correlations between the 5 diffusion tensor imaging parameters and activated microglia counts as assessed by Iba1 immunohistochemistry.

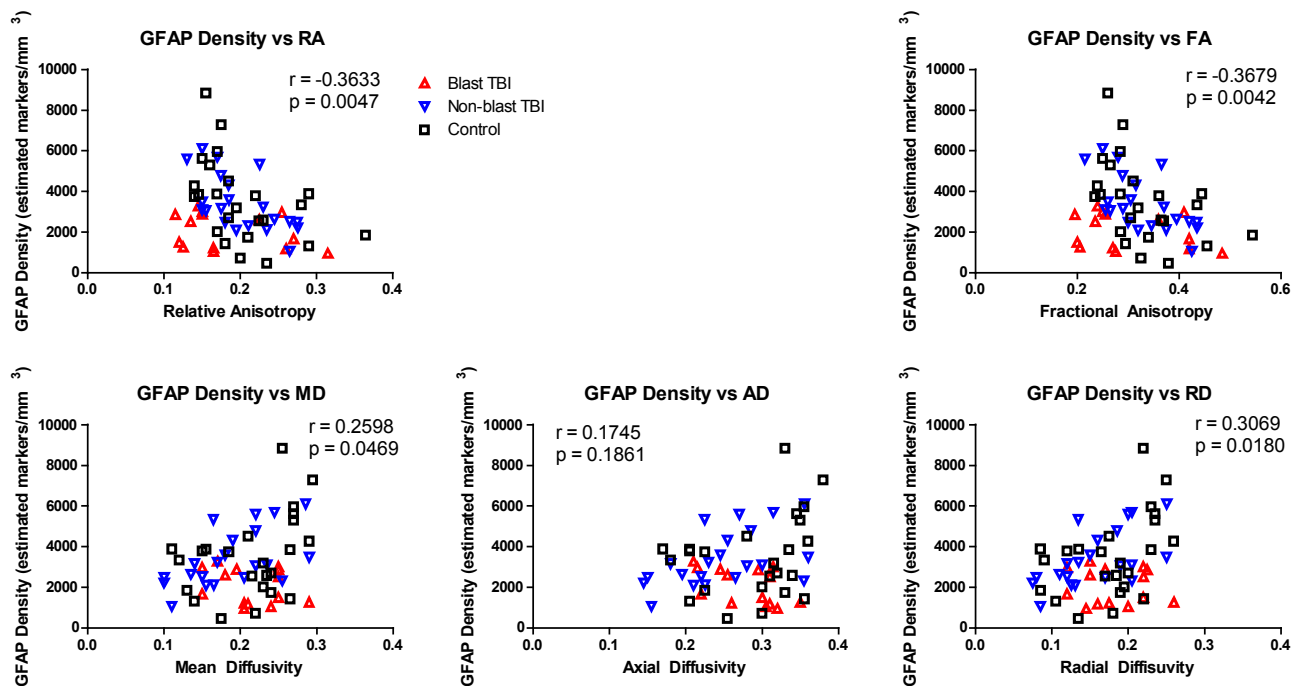


Figure 17: Correlations between DTI parameters and astrogliosis: The figure shows only modest correlations between the diffusion tensor imaging parameters and reactive astrocyte counts as assessed by GFAP immunohistochemistry.

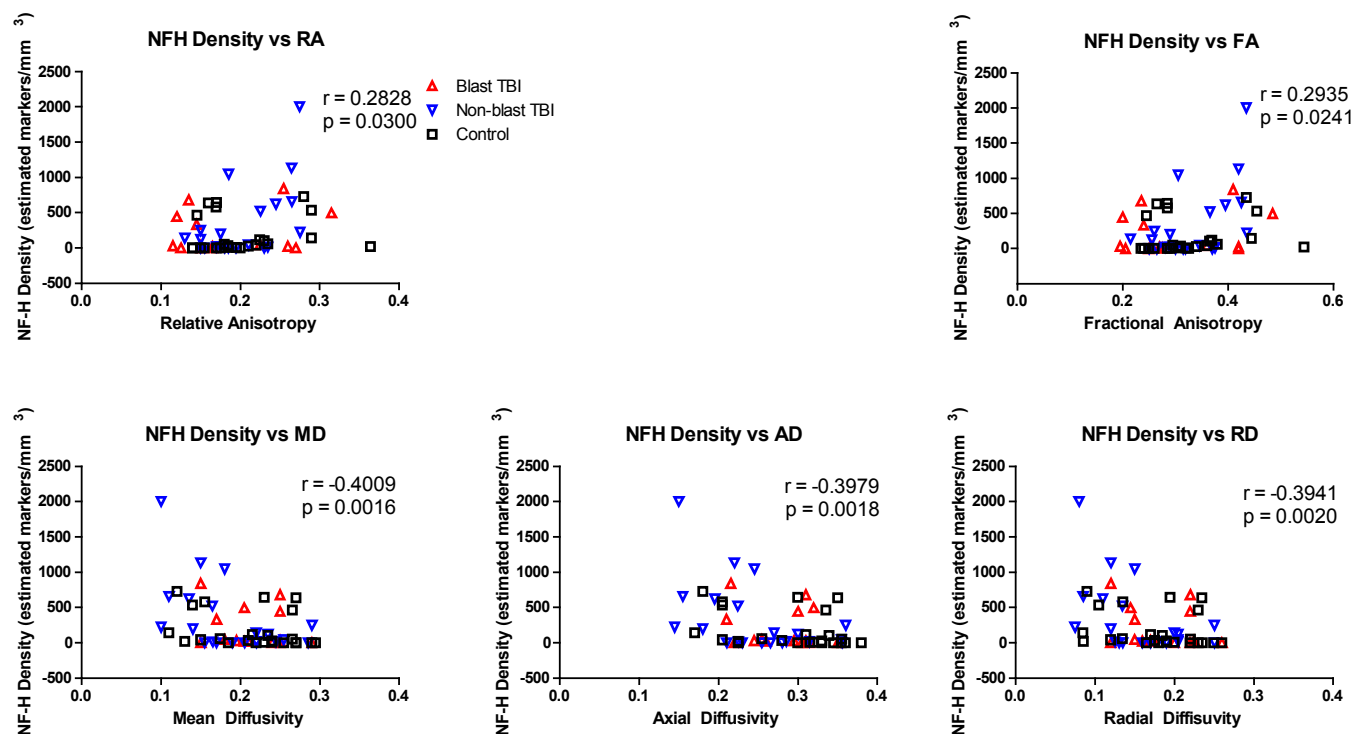


Figure 18: Correlations between DTI parameters and axonal injury: The figure shows only modest correlations between the diffusion tensor imaging parameters and axonal injury as assessed by neurofilament-heavy chain immunohistochemistry.

As a methodological control, we assessed whether the measured tissue volumes from the DTI and histological analyses were correlated.

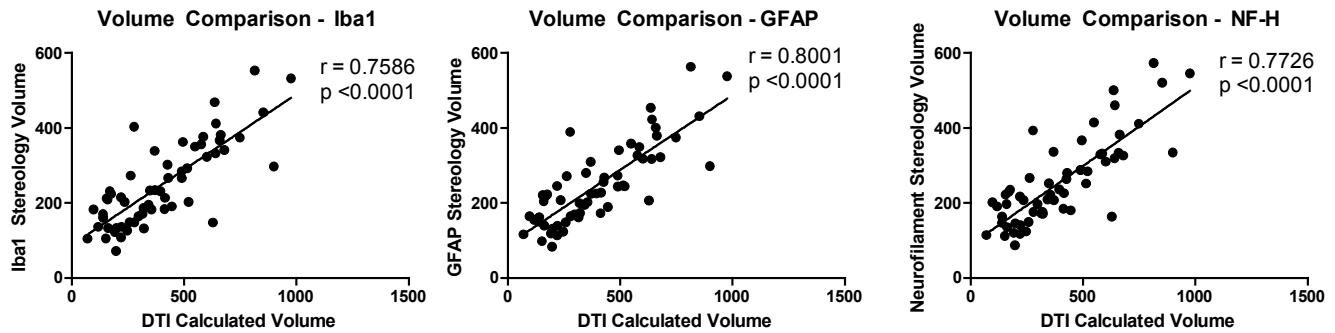


Figure 19: Methodological control: Correlations between DTI parameters histological region of interest volumes: TThe figure shows that the tissue volumes assessed during the DTI analysis were highly correlated with the volumes assessed during the stereological analysis. This suggested that bias in the way the regions of interest were assessed did not contribute substantially to the overall lack of correlation between DTI parameters and histological marker counts.

KEY RESEARCH ACCOMPLISHMENTS:

- We have identified many of the methodological issues required to perform high quality radiological-pathological correlations using diffusion tensor imaging in human brain tissue.
- We detected a statistically significant correlation between anisotropy and the extent of astrogliosis, but this correlation was too weak to be clinical useful.

REPORTABLE OUTCOMES:

Results from this project were used as preliminary data to support the PI's successful application for additional funding from the NIH. (See below).

CONCLUSION: The primary conclusion from this line of investigation is that while the concept of ex-vivo radiological pathological correlation analysis remains valid, the high degree of variability in the tissue samples analyzed complicates the analysis substantially. Specifically, variability in time from injury to death, age, fixation methods, and causes of death may have obscured the relationships between pathological markers and DTI signal characteristics. In addition, the highly variable fiber direction in the white matter regions chosen may make the relative simple diffusion tensor model inappropriate. We detected a statistically significant correlation between anisotropy and the extent of astrogliosis, but this correlation was too weak to be clinical useful.

We plan to address these concerns in future studies. Specifically, we plan to address the following methodological issues:

- 1) Selection of control cases: we will select only young, otherwise healthy patients who died from non-head trauma and had a short post-mortem interval.
- 2) Fixation: we will obtain all cases and controls from the same source using the same fixation methods
- 3) Scanning method: we will attempt to use more advanced diffusion methods such as diffusion kurtosis imaging and high angular resolution imaging to help address the problem of variable fiber direction
- 4) Signal to noise: it is possible that our signal to noise was too low to get reliable DTI information from some of the cases. We will build new custom coils with improved field homogeneity, soak tissues in gadolinium, and increase scan times to improve signal to noise.

While the current project is essentially complete, we have obtained funding from the NIH through a U01 mechanism (PI Dr. Ann McKee) to continue to advance our development of radiological-pathological correlation methods. With additional development, we plan to assess the effects of both repetitive concussive injury and blast-related TBI over the next 5 years.

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