Blood-brain barrier response in the context of sports-related TBI

A thesis submitted to the University of Dublin for the Degree of Doctor of Philosophy

By

Eoin O'Keeffe

2020

Under the supervision of Dr. Matthew Campbell

Department of Genetics

Trinity College, Dublin

Declaration

This thesis has not been previously submitted as an exercise for a degree at this or any other University. The work presented here is entirely my own. I agree to deposit this thesis in the university's open access institutional repository or allow the library to do so on my behalf, subject to Irish copyright legislation and Trinity College library conditions of use and acknowledgement.

_____

Eoin O'Keeffe

Summary

Traumatic brain injury (TBI) is one of the largest causes of mortality and disability globally. The severity of TBI is clinically categorised into mild, moderate and severe injuries. Moderate and severe TBI often present with clear diagnostic criteria, such as loss of consciousness, skull fractures and/ or structural damage to the brain detectable by neuroimaging modalities such as computed tomography (CT) or magnetic resonance imaging (MRI) scans. However, mild TBI (mTBI), which is thought to represent almost 70% of TBI cases, is often difficult to recognise. In contrast to moderate and severe TBI, mTBI is incurred by a non-penetrating blow to the head, which may or may not result in loss of consciousness, and by most definitions, does not display abnormal neuroimaging findings. The spontaneous resolution of symptoms has also led to the perception that mTBI is a benign condition with no lasting consequences. However, in recent years, several reports have detailed a dementia-like condition in athletes and military veterans exposed to repetitive mTBI during the course of their careers. The condition is currently called chronic traumatic encephalopathy (CTE) and bears a close resemblance to a dementia condition described in the 1930s found to be prevalent in boxers, termed "dementia pugilistica" or "Punch Drunk Syndrome". While moderate and severe TBI is known as one of the largest environmental risk factors in developing dementia, the link between mTBI and development of dementia-like conditions remains to be fully characterised. Accumulating evidence suggests that changes to the neurovasculature and the integrity of the blood-brain barrier (BBB) are early events in the development of several neurodegenerative conditions. The BBB is a highly specialised structure that maintains homeostasis of the central nervous system by limiting the passage of blood-based agents in to and from the neural space. As BBB dysfunction (BBBD) is a known consequence of TBI at all severities, changes to BBB integrity may result from repetitive mTBI and represent an underlying risk in dementia development. However, the magnitude and significance of changes in BBB integrity over time due to mTBI have yet to be fully explored.

In this study, changes in BBB integrity as a result of participation in contact sports was investigated. To that end, two of amateur rugby union teams were recruited to undergo an extensive baseline assessment prior to the start of the competitive season, as well as after the season's competition. A sub-group of individuals underwent assessment shortly after completion of a competitive rugby match, to investigate acute changes in the BBB

following exposure to mTBI. The assessment consisted of MRI screening for changes in BBB integrity via novel dynamic contrast-enhanced MRI (DCE-MRI) analysis techniques and changes in neuroanatomical structure via diffusion tensor imaging (DTI). Blood samples were also collected at the time of MRI scanning, allowing for matched screening of potential blood-based biomarkers of TBI, as well the collection of peripheral blood mononuclear cells (PBMCs) to gauge potential adaptive changes in immune response to neural antigens. Together, this assessment represented a holistic approach to mTBI research. A total of 18 players were retained throughout the study period, completing the entire rugby season without a reported mTBI. However, even in the absence of a diagnosed mTBI, significant changes in the BBB integrity were detected in our cohort after a season of rugby, with a sub-group of individuals showing significant increases in BBB permeability compared to baseline. Increases in fractional anisotropy (FA) of axonal fibre tracks within the body of the corpus callosum were also observed after a season of rugby. The serological screening of potential mTBI markers: S100β, brain-derived neurotrophic factor (BDNF) and monocyte chemoattractant protein 1 (MCP-1)/ chemokine (C-C motif) ligand 2 (CCL2), found a significant increase in BDNF after a season of rugby, accompanied by a significant decrease in S100β. However, post-match screening of plasma identified an increase in S100β even in the absence of injury. PBMC immune response, as measured by interleukin-1β (IL-1β) production, was found to be elevated in cells collected after a season of rugby compared to baseline cells when exposed to necrotic brain tissue.

In addition to the clinical aspect of this study, presented here are 3 case studies are of dementia patients, whose history of TBI was thought to play a role in the development of their conditions. Characterisation of tight junction (TJ) components and BBB integrity displayed loss of the TJ protein, claudin-5, within regions of dense phospho-tau (p-tau) deposits in all three cases. Accompanying the loss of claudin-5 and overlapping with p-tau deposition was extensive extravasation of blood-based components IgG and fibrinogen, suggesting BBBD within these regions.

The results obtained from in life assessment of the BBB suggest that the forces associated with contact sports, such as rugby, are sufficient to induced long term changes in BBB integrity. Serological results highlight the need for continued development of blood-based biomarkers for mTBI, particularly if they are to be used in the context of sport-related mTBI. Immune response also offers a possible avenue of future research to how

changes in PBMCs may feed into long-term sequelae of repetitive TBI. The accompanying findings of BBBD in individuals with a history mTBI also suggest that mTBI may pose some degree of risk to long term neural health.

Acknowledgements

Throughout my PhD work, I have had the privilege of working with many great people, whose help, support and guidance have been invaluable during my studies. Words cannot express the debt of gratitude I owe to each and every one of you. I would particularly like to thank my supervisor, Dr. Matthew Campbell, who gave me the opportunity to work on such a unique and interesting project. He has spearheaded research in the blood-brain barrier and TBI since the project's inception and has been a constant source of perseverance and positivity when faced with the difficulties of clinical research. I would also like to thank Dr. Campbell for allowing me to broaden my research skills into new areas of research. I would also like to thank the research team based in St. James' Hospital, Dr. Colin Dorthey, Dr. Eugene Wallace, the staff in the CAMI centre, Ms. Niamh Doyle and Dr. Eoin Kelly. I would like to thank Eoin Kelly in particular for his doggedness and dedication in the recruitment and management of participants throughout. Gratitude must also be extended to Dr. John Kealy, whose advice, teaching and support have been invaluable. I am deeply indebted to him for his support and friendship.

To my fellow lab members, with whom I have had the honour of working with during my time in the lab. Dr. Natalie Hudson, Dr. Chris Greene, Dr. Lucia Celkova, Mr. Conor Delany and Ms. Nicole Hanley. I am eternally grateful for your support and friendship. The discussions we have had and the advice you have given, as well as the patience you have shown me over the years; all of this I hope to have learnt from and allowed me to be counted among you as peers. Special thanks to Dr. Hudson, who does so much for the lab on a daily basis. To the members of the Humphries and Farrar labs, as well as the staff and students of the Smurfit Institute past and present, thank you for making my time here enjoyable as it has been. Words fail me in expressing the joy it has been to work with you all.

Finally, to my friends and family, both near and far, thank you. Erin, Lara, Cian, Ross, Jeff, Simon, Shane and Sarah – thank you for allowing me to share the laughter, picking me up and relieving stress. I'm lucky beyond measure to have met each of you. To Ema, who has put up with me for so long, offering advice and calm even in the most panicked times… mostly. Thank you for always trying. Finally, to my sister, Clodagh, for all the help she has provided and for setting the high standards I have always had to scrambled

to meet and my parents Pauline and Neil. Thank you for your unending love and support, for your advice and understanding, for listening, for providing me with every opportunity and for just being there. “Thank you” will never capture the feelings I hold for each of you.

Table of Contents

Declaration ii
Summary iii
Acknowledgements vi
Table of Contents viii
Table of Figures and Tables xi
Abbreviations xiv
Chapter 1: Introduction 1
Epidemiolgy and defining mild traumatic brain injury 2
1.1: mTBI and dementia 5
1.2: The neurovascular unit and TBI 9
1.2.1: Endothelial cells 9
1.2.2: Astrocytes 10
1.2.3: Pericytes 12
1.3: Tight Junctions of the BBB 15
1.3.1: Claudins 15
1.3.2: Occludin 16
1.3.3: JAMs and ZOs 17
1.4: Biomarkers of TBI 18
1.4.1: S100β 19
1.4.2: Tau protein 22
1.4.3: Ubiqutin carboxy-terminal hydrolase L1 23
1.4.4: Glial fibrillary acid protein 26
1.4.5: Neuron specific enolase 26
1.5: Inflammation and TBI 29
1.5.1: Neutrophils 29
1.5.2: Monocytes 31
1.5.3 Innate Immune training 32
1.5.4 Adaptive immunity 33
1.5.5: Cytokine response in TBI 35
1.5.6: Interleukin-1 35
1.5.7: Interleukin-6 37
1.6: Magnetic resonance imaging in TBI 39
1.6.1: T1 and T2-based analysis 39
1.6.2: Diffusion tensor Imaging (DTI) 42
1.6.3: Contrast enhanced MRI 45

Objective of this study 49
Chapter 2: Materials and Methods 51
2.1: Immunohistochemistry 49
2.2: Patient recruitment 50
2.3: PBMC isolation from human whole blood 55
2.4: Necrotic Brain Conditioned Media 55
2.5: PBMC stimulation with Necrotic Brain Conditioned Media 56
2.6: Enzyme-linked immunosorbent assay (ELISA) 56
2.7: Magnetic Resonance Imaging (MRI) 57
2.8: Statistical Analysis 60
Chapter 3: Case reports of dementia patients connected by a history of TBI 64
3.1: Abstract 65
3.2: Introduction 66
3.3: Results 68
3.3.1: Case Study 1: BBB dysfunction present in a boxer with CTE and schizophrenia 68
3.4.1.1: Patient History and pathology findings 68
3.3.1.2: BBB disruption and loss of TJ integrity 72
3.3.2: Case Study 2: BBB dysfunction present in rugby player with CTE and PSP 74
3.3.2.1: Patient history and pathology findings 76
3.3.2.2: BBBD and loss TJ integrity 79
3.3.3: Case Study 3: Neuropolypathology in patient with single severe TBI in history 84
3.3.3.1: Patient History and pathology findings 84
3.3.3.2: BBBD and loss of TJ integrity 92
3.3.4: Comparison of tau and BBBD pathology in reported cases studies with that of a control brain 95
3.4: Discussion 100
Chapter 4: Changes in serological markers of mTBI in contact sports 104
4.1: Abstract 105
4.2: Introduction 106
4.3: Results 108
4.3.1: Changes in potential biomarkers for TBI following a season of rugby 108
4.3.2: Changes immune response to necrotic brain tissue 110
4.3.3: Biomarker levels in plasma following a competitive rugby match 112
4.3.4: PBMC immune response is unaltered following competitive play 114
4.3.6: Changes in potential biomarkers for TBI following a competitive mixed martial arts bout 116
4.4: Discussion 118

Chapter 5: Changes to cerebral vasculature and microstructure associated with contact sports 121
5.1: Abstract 122
5.2: Introduction 123
5.3: Results 125
5.3.1: Changes in BBB integrity following a season of competitive rugby 125
5.3.2: Acute post-match assessment of BBB integrity 133
5.3.3: BBB changes in an injured player 136
5.3.4: Changes in neural tissue microstructure following a season of competitive rugby ... 139
5.3.5: Correlation of BBB permeability measurements with relative changes in serological markers of mTBI 142
5.3.6: BBB changes following competitive a mixed martial arts bout 144
5.3.7: *Changes in neural tissue microstructure following a season of MMA bout* 148
5.4: Discussion 150
Chapter 6: General discussion 154
General discussion 155
Reference List 162
Appendix I .....
Appendix II .....
Appendix III .....

Table of Figures and Tables

Figure No. ..... Page

Figure 1. 1: Schematic of the neurovascular unit and tight junction complexes of the CNS. 14

Figure 1. 2: Potential biomarkers for the use in detecting mild TBI and their neural cell source. 19

Table 2. 1: Demographics of participants engaged in rugby. 52

Table 2. 2: Demographics of participants engaged in mixed martial arts.....53

Figure 2. 1: : Flowchart of participant retention within the rugby cohort. 54

Figure 2. 2: A priori power analysis for the rugby phase indicates that 50 participants are required to power the study 61

Figure 2. 3: Post-hoc power analysis using the available number of participants for the rugby study found the study was underpowered using the initial parameters. 62

Figure 2. 4: A priori power analysis for the rugby phase indicates that 50 participants are required to power the study 63

Figure 3. 1: Macroscopic examination images of the brain of a former boxer diagnosed with CTE.....70

Figure 3. 2: Immunohistochemistry staining for p- tau deposition in multiple brain regions of a former boxer diagnosed with CTE.....71

Figure 3. 3: Dense phosphorylated-tau deposition is accompanied by absent or reduced CLDN5 immunoreactivity. 73

Figure 3. 4: hIgG extravasation is limited to larger vessels. 74

Figure 3. 5: CLDN5 staining with Fibrinogen dense regions suggests historic disruption and healing of the BBB.....75

Figure 3. 6: In life neuroimaging and post-mortem brain examination of amateur rugby player diagnosed with CTE.....78

Figure 3. 7: Immunohistochemical staining of tight junction protein claudin-5 (red) and p-tau (green) in the brain of a former amateur rugby player diagnosed with CTE. 80

Figure 3. 8: Immunohistochemical staining of tight junction protein ZO-1 (red) and p-tau (green) in the brain of a former amateur rugby player diagnosed with CTE. ZO-1 .81

Figure 3. 9: Immunohistochemical staining of endogenous IgG (red) and p-tau IgG (green) in the brain of a former amateur rugby player diagnosed with CTE.....82

Figure 3. 10: Immunohistochemical staining of p-tau (green) and endogenous fibrinogen (red) in the brain of a former amateur rugby player diagnosed with CTE. 83

Figure 3. 11: Initial neurological and neuroimaging assessment of a patient presenting with emotional instability and lapses in memory. 86
Figure 3. 12: Sections of brain for macroscopic examination from patient presenting with emotional instability and lapses in memory. 87
Figure 3. 13: Histology of cortical, sulcal depths and hippocampus patient presenting with emotional instability and lapses in memory. 89
Figure 3. 14: Immunohistochemistry for amyloid-β and p-tau in in patient presenting with emotional instability and lapses in memory. 90
Figure 3. 15: Immunostaining for α-synuclein-positive Lewy bodies. 91
Figure 3. 16: Immunohistochemical staining of tight junction protein claudin-5 (red) and p-tau (green) in the brain of an individual diagnosed with multiple neuropathologies ..93
Figure 3. 17: Immunohistochemical staining of tight junction protein claudin-5 (red) and endogenous IgG (green) in the brain of an individual diagnosed with multiple neuropathologies. 94
Figure 3.18: Normal claudin-5 immunoreactivity coincides with an absent of phosphorylated-tau immunoreactivity in control tissue. 97
Figure 3. 19: Absence of hIgG extravasation suggests normal BBB function in control tissue. 98
Figure 3. 20: Fibrinogen extravasation and immunoreactivity for p-tau is absent in control tissue. 99
Figure 4. 1: Changes in circulating levels of potential biomarkers for TBI following a season of rugby. 109
Figure 4. 2: Changes of inflammatory response following a season of rugby. 111
Figure 4. 3: Changes in circulating levels of potential biomarkers for TBI immediately following a competitive rugby match. 113
Figure 4. 4: Changes of inflammatory response following a competitive rugby match. 115
Figure 4.5: Changes in circulating levels of potential biomarkers for TBI following an MMA bout. 117
Figure 5. 1: Schematic and graphic representation of singal generation via the Toft's Extended Model and the Linear Dynamic Model. 115
Figure 5. 2: Changes in BBB integrity are detectable use Toft's Extended Model following a season of play in rugby. 117
Figure 5. 3: Baseline K^{trans}values do not correlate with post-season readouts. 118

Figure 5. 4: Changes in BBB integrity are not observed on a group's basis following a season of rugby using Linear Dynamic Modeling. 120
Figure 5.5: Analysis of trends in voxels displaying BBBD in rugby players following a season of play 121
Figure 5.6: Assessment of BBB permability in rugby players following a competitive rugby match using Toft's Extended Model. 123
Figure 5.7: Assessment of BBB permability in rugby players following a competitive rugby match using the Linear Dynamic Model. 124
Figure 5.8: BBB permability in an injured player over the course of a season, evalutated using Toft's Extended Model. 126
Figure 5.9: BBB permability in an injured player over the course of a season, evalutated using the Linear Dynamic Model 127
Table 5.1: Changes in measures of axonal integrity in rugby players following a season of play. 130
Figure 5.10: Correlation analysis of potential markers of mTBI in rugby players 132
Figure 5.11: Assessment of BBB permability in Mixed Martial Arts fighter following a competitive bout using Toft's Extended Model 134
Figure 5.12: Assessment of BBB permability in Mixed Martial Arts fighter following a competitive bout using the Linear Dynamic Model 136
Table 5.1: Changes in measures of axonal integrity in MMA fighters measured within 48 hours of a competitive bout 138

Abbreviations

Aβ	amyloid-beta
AD	Alzheimer`s disease
Ang-1	angiotensin-1
ANOVA	analysis of variance
APOE	apolipoprotein E
AQP4	aquaporin 4
ATP	adenosine triphosphate
AxD	axial diffusivity
BBB	blood brain barrier
BBBD	blood brain barrier dysfunction
BDNF	brain derived neurotrophic factor
Ca	calcium
CAMCOG	Cambridge cognition examination
CCI	closed cortical impact
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CI	Confidence Interval
CNS	central nervous system
CSF	cerebrospinal fluid
CT	computed tomography
CTE	Chronic Traumatic Encephalopathy
DAMP	damage associated molecular pattern
DAI	diffuse axonal injury
DCE-MRI	dynamic contrast enhanced magnetic resonance imaging
DLBD	Diffuse Lewy Body Disease
DMSO	dimethylsulfoxide
DTI	diffusion tensor imaging
DWI	diffusion weighted imaging
ECL	extracellular loop

ELISA	enzyme-linked immunosorbent assay
FA	fractional anisotropy
FBD	foetal bovine serum
FGF	fibroblast growth factor
fMRI	functional magnetic resonance imaging
FPI	fluid percussion injury
FTD	frontotemporal dementia
GCS	Glasgow Coma Scale
GDNF	glial-derived neurotrophic factor
GDNFRα-1	glial-derived neurotrophic factor receptor alpha 1
GFAP	glial fibrillary acidic protein
HD	Huntington's disease
Hh	Hedgehog
HR	Hazard Ratio
IFOF	inferior fronto-occipital fasciculus
IgG	immunoglobulin G
IL	interleukin
IQR	interquartile range
JAM	junction adhesion molecule
K	potassium
KO	knock-out
LDM	linear dynamic model
LOC	loss of consciousness
LPS	lipopolysaccharide
MAGUK	membrane associated guanylate kinase
MAPK	mitogen activated protein kinase
MARVEL	MAL and related proteins for vesicle trafficking and membrane link
MCP-1	monocyte chemoattractant protein-1
MD	membrane density
MD	mean diffusivity
Mg	magnesium

MHC	major histocompatibility complex
MMA	mixed martial arts
MMP	matrix metalloproteinase
MMSE	Mini mental state examination
MoCA	Montreal cognitive assessment
MRI	magnetic resonance imaging
MS	multiple sclerosis
MT	metallothionein
mTBI	mild traumatic brain injury
Na	sodium
NART2	National Adult Reading Test
NFL	National Football League
NFT	neurofibrillary tangle
NGF	nerve growth factor
NOX2	NADPH oxidase 2
NSE	neuron specific enolase
PBMC	peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCS	post-concussion syndrome
PD	Parkinson`s disease
PDGFβ	platelet-derived growth factor beta
PDGFRB	platelet-derived growth factor receptor beta
PET	positron emission tomography
PSP	Progressive Supranuclear Palsy
p-τ	phospho-tau
PTSD	post-traumatic stress disorder
Rag-1	recombination activating gene-1
RD	radial diffusivity
ROC	receiver operating characteristic
ROI	region of interest
ROS	reactive oxygen species

RPMI	Roswell Park Memorial Institute
SD	standard deviation
Shh	Sonic Hedgehog
SNP	single nucleotide polymorphism
SPECT	single-photon emission computed tomography
SSeCKS	Src suppressed C kinase substrate
TBI	traumatic brain injury
TEER	transendothelial electrical resistance
TJ	tight junction
TM	transmembrane
TNF	tumour necrosis factor
UCH	ubiquitin C-terminal hydrolase
VCI	vascular cognitive impairment
VCS	Veterinary Coma Scale
VEGF	vascular endothelial growth factor
VFA	variable flip angle
WHO	World Health Organisation
WM	white matter
WT	wild-type
ZO	zonula occludens

CHAPTER 1:
INTRODUCTION

Epidemiolgy and defining mild traumatic brain injury

Traumatic brain injury (TBI) is a major cause of mortality and disability around the world, with the latest available incidence figures suggesting that over 2 million TBIs occur each year in the US alone. Global incidence rates are difficult to accurately estimate, due to variation between regions in the criteria used to classify the severity of the injury. However, it is estimated that TBI accounts for roughly 200 injuries per 100, 000 people per year, with large variance in incident rates between countries (Centre of Disease Control, 2010, Bryan-Hancock & Harrison, 2010; Faul & Coronado, 2015). The commonality amongst these numbers is that it is estimated that most of the reported injuries (70-90%) are considered mild TBI (mTBI) (Cassidy *et al.,* 2004). However, these numbers may still be an underestimation of the real incidence of mTBI, as it is speculated that many injuries go unrecognised or unreported, potentially due to differences in the definition of the injury. Currently, there is confusion surrounding the use of terms "mTBI" and "concussion", as they are often used interchangeably and share diagnostic criteria. For example, the Zurich Consensus statement on Concussion in Sport proposed to classify mTBI and concussion as separate injuries, defining concussion as a "complex pathophysiological process affecting the brain" in the absence of any neuroimaging findings on standard structural studies (McCrory *et al.,*2012). In contrast, the American Academy of Neurology made no distinction between the two terms in their consensus guidelines, defining the condition as "a clinical syndrome of biomechanically induced alteration of brain function, typically affecting memory and orientation, which may involve loss of consciousness" (Sharp & Jenkins, 2015). The term "complicated" mTBI is also being used to describe cases that would otherwise be classified as a mTBI based on the Zurich Consensus definition. The original definition of "complicated mTBI" distinguished the injury from "uncomplicated" mTBI by the presence of a trauma-related intracranial abnormality, such as a haemorrhage, contusion or oedema, but otherwise share diagnostic criteria (Williams, Levin & Eisenberg, 1990). However, the presence of large structural damage, such as haemorrhage or contusions, more closely align "complicated mTBI" with definitions of moderate or severe TBI. Despite this, the term "complicated mTBI" is still used in clinical studies of TBI. In recent years, the term "mTBI" has increasingly been used in place of "concussion", due to changes in the perception that concussion refers to a benign condition. As symptoms normally resolve over the course of a few days to weeks, concussions were thought to have no long-term

effects on brain structure or neural health. However, variations in the time for patients to recover, in some instances extending beyond months to years, suggest that the injury does involve some degree of neurostructural change (Sharp & Jenkins, 2015).

Another term that has entered into the field of mTBI is the term "sub-concussive injury". This term has primarily been used within studies examining contact sports, for blows to the head that appear to occur with a substantial degree of force, yet do not result in detectable symptoms of mTBI. One of the early uses of the term was by Guskiewicz *et al.* (2009), in their study looking at the linear and rotational forces involved in concussive injuries in American football players. The study determined that mTBI occurs over a wide range of linear and rotational forces and acknowledged that repeated "subconcussive impacts", impacts that involved a degree of force but did not result in injury, could have been a confounding factor in determining a threshold for accelerating forces necessary for mTBI. While no official definition for "sub-concussive" forces has been put forward since its use in Guskiewicz *et al.*'s report, likely due to the degree of force required for a mTBI to occur remain undetermined, generally the term is used within the context of an impact-induced brain trauma that does not produce neurological symptoms and does not fall within the criteria of a mTBI (Concussion Legacy Foundation, 2019). However, a degree of confusion remains regarding the term. To date, several studies have detected changes in metabolism, biomarker release and structural changes to the brain in patients subjected to "sub-concussive" forces (Puvenna et al.,2014; Poole *et al.,*2015; Abbas *et al.,*2015; Mainwaring *et al.,*2018). A recent comparison of "sub-concussed" head trauma patients has also found that the biomarkers UCH-L1 and GFAP showed distinct patterns of release in "sub-concussed" mTBI patients (Papa *et al.,* 2019), while "sub-concussive forces" have also been attributed to the development of neurocognitive decline in boxers and suggested to play roles in cognitive changes in players of other contact sports (Jordan, 2000; Erlanger, 2013), although a definitive connection between "sub-concussive" forces and dementia has yet to be firmly established.

However, issues abound for the use of "sub-concussive" as a term, primarily arising from the ambiguity of the term. For example, in Guskiewicz *et al.*'s (2009) paper, they recorded mTBI occurring in individuals with forces as low as 60g and as high as 169g. If 60g is taken as a minimum threshold for mTBI to occur (which the author's do not suggest), it would be possible that an individual may be subjected to forces greater than this repeatably but not incur injury. However, under the current working understanding

of the term, they would still have sustained "sub-concussive" blows that may involve a greater degree of force that produced the injury. Indeed, data published in O'Keeffe *et al.* (2019) would suggest that individuals can sustain a considerable range of forces without being diagnosed with a mTBI. In addition, as suggested by Erlanger (2013), these "sub-concussive" blows may result in changes brain that are same or similar to those resulting for symptomatic mTBI and therefore may represent the same or similar injury. However, they are overlooked, or their significance underestimated due to the patient appearing normal, and only later, if/when symptoms manifest is the contribution of these blows considered.

While separating "sub-concussion" from mTBI patients may represent an attempt to better define the characteristics of mTBI, the advent of the term may have resulted in an inaccurate division within the mTBI spectrum. In the case of diagnostic criteria for mTBI, symptoms it is a combination of cognitive function and consciousness that allows for identifying the injury. However, the lack of a firmly established positive marker for mTBI raises the possibility that asymptomatic patients that have experienced "sub-concussive" force may be undergoing the same processes as those that are diagnosed with mTBI. The absence of loss of consciousness or post-traumatic amnesia does not rule out mTBI, so why should the absence of neurological symptoms but changes in potential biomarkers suggest that a mTBI has not occurred? As mentioned, Papa *et al.*'s 2019 study did find a distinction between "sub-concussed" individuals and those displaying signs of mTBI, however, much remains to be determined in regards the accuracy of biomarkers in detecting mTBI and distinguishing it from routine "jostles" of the brain.

In order not to devalue the potential contribution "sub-concussive blows" have to overall neural health, renaming the term may be required, similar to the move to mTBI from concussion. Erlanger (2013) suggested the term "repetitive brain trauma". This allows for the acknowledgment that mechanical forces applied to the brain may still have a detrimental effect even in the absence of neurological symptoms and may not represent a benign condition as previously thought. However, the term still bears some notable limitations, notably in the potential confusion in distinguishing it from symptomatic TBI. It also remains be seen if "sub-concussive blows" or "repetitive brain trauma" should be considered a distinct entity from that of mTBI, similar to the distinction between mTBI and moderate and severe injuries.

Currently, the term is an arbitrary one. One that serves the purpose of describing participants in studies who have been subjected to high mechanical forces but have not been medically recognised to have sustained an injury. A more accurate definition will likely require a more refined characterisation of the processes involved mTBI, in order to determine if the processes of "sub-concussive blows" just part of the mTBI spectrum or a distinct entity entirely.

Currently, standard neuroimaging modalities, such as computed tomography (CT) and magnetic resonance imaging (MRI) scans do not detect subtle changes in axonal tract or vascular integrity, and so may overlook mTBI-induced damage. Therefore, attempts to identify an objective marker of mTBI has been the focus of intense research in recent years. Such a marker could reduce the reliance on neurological assessments to diagnose injuries, facilitate a more precise classification of TBI and allow for greater accuracy in monitoring recovery or changes in cognitive health. Currently, recovery from mTBI is gauged based on resolution of symptom, which often resolve quickly even in the absence of intervention. However, underlying mechanisms may be at play that contribute to sequala of such an injury in the future. These mechanisms may continue even in the absence of overt symptoms of injury, and the treatment of symptoms may belittle the risk associated with mTBI. A objective biomarker would therefore afford greater insight into the process of TBI recovery. Increased attention has been focused on the potential long-term neurological sequelae of repetitive mTBI, such as those sustained in contact sports, however, the lack of an objective marker has made drawing definitive conclusions between mTBI and neurological outcome difficult. Identification of a marker of injury may provide the link between mTBI and neurocognitive decline and allow identification and monitoring of individuals at risk of dementia development because of their injuries. While dementia conditions present symptoms in life, it is widely thought that the neurodegenerative processes begin prior to clinical presentation. As TBI is considered one of the largest environmental risk factors for dementia, markers capable of detecting mTBI-induced changes may allow for detection of dementia-associated neural changes before onset, allowing for better patient management.

1.1: mTBI and dementia

A growing body of evidence links moderate and severe TBI to the development of dementia later in life, with some studies suggesting that a history of moderate or severe

TBI can increase the risk of dementia development by 2- to 4-fold (Ramalho & Castillo, 2015).

Development of Alzheimer's disease (AD) and TBI history appears to have the closest relationship with such injuries. One meta-analysis examining 15 case-control studies suggested that history of TBI resulted in a small increase in the likelihood of AD relative to controls (OR = 1.58, CI = 1.21-2.06) (Fleminger *et al,*2003). A history of TBI with loss of consciousness was also found to have the greatest association with AD development (OR = 2.26, 95% CI = 0.53-1.59), although this association was limited to the male cohort examined. A more recent meta-analysis found a similar degree of association between TBI history and AD development when considering 32 observational studies (RR = 1.51, 95% CI = 1.26-1.80), however, in contrast to Fleminger *et al.*'s analysis, no association was identified between the state of consciousness and development of a dementia-like condition (Li *et al.,* 2017). However, many of the studies included in these meta-analyses do not distinguish between injury severity, or often only include moderate or severe TBI. Therefore, the relationship between mTBI and the development of dementia has continued to be explored.

Assessment of recent studies has demonstrated a complicated relationship between mTBI and neurocognitive decline, with studies both suggesting and refuting mTBI's role in dementia development. In a study by Gardner *et al.* (2014) the risk of dementia development in patients over the age of 55 years with a history of TBI was assessed. TBI incidence rate was assessed using hospital inpatient and Emergency Department visitation data; and included over 160,000 participants, over 51,000 (~31%) of whom had suffered a TBI. Upon comparison of TBI patients to non-head trauma patients, it was found that the number of dementia diagnoses was significantly higher in the TBI group compared to the non-head trauma group (8.4% vs. 5.9%). Patients with a history of TBI also had a slightly earlier time of dementia onset post-trauma compared to non-head trauma patients (3.1 years post-trauma vs. 3.2 years). When patients were stratified by age, moderate and severe TBI appeared to confer the greatest risk of dementia development in patients that were within the 55-64 years category (HR = 1.72, 95% CI = 1.4-2.1). mTBI was found to have a significant association with dementia development if individuals had sustained the injury at the age 65 or above (HR = 1.25, 95% CI = 1.04-1.51 for ages 65-74).

In another large scale, long term study of over 800,000 ex-military personnel, of whom over 45,000 (~5.6%) had sustained at least one TBI, no associations between AD development and TBI history were identified over a median surveillance period of 33 years (Nordström *et al.,* 2014). However, after adjusting for age and co-varying factors, a significant association was found between the development of a general dementia phenotype in patients that had suffered 1 or 2 mTBIs (HR = 3.8, 95% CI = 2.8-5.2 and HR = 10.4, 95% CI = 6.3-17.2 respectively), as well as in individuals who had suffered a single severe TBI (HR = 11.4, 95% CI = 17.4-17.5). While these associations fell sharply when analysis was adjusted for additional covariates such as family history, mental illness and narcotic consumption, mTBI continued to represent a significant risk to dementia development (HR = 1.7 in the case of a single mTBI, HR= 1.7 in cases of at least 2 mTBIs and HR = 2.6 in those who had sustained 1 severe TBI). A similar association between mTBI and a general dementia phenotype was reported in a study of a mixed-aged population, which comprised of 720,933 patients, of which over 28,000 (~3.9%) were reported to have suffered a mTBI (Lee *et al.,* 2013). The study reported that mTBI patients showed an increased risk in developing dementia when compared to a control population that had no history of TBI, after correcting for covariates similar to Nordström *et al.*'s study (HR = 3.26, CI = 2.69-3.94). Those aged over 65 years also demonstrated an increased risk of dementia development if a mTBI occurred at this age (HR = 3.27, CI = 2.67-4.00). Individuals that required hospitalisation due to their injuries also displayed a greater risk of dementia development than non-injured controls (HR = 4.44, CI = 3.24-6.1), possibly reflecting a parallel in dementia risk and severity of TBI. However, the study failed to note the potential overlap between the over 65 years group and the hospitalisation groups. As the over 65 age bracket represents the largest demographic hospitalised due to TBI, their inclusion within the hospitalisation group may conflate the risk (CDC, 2019).

In contrast to these studies, several reports have suggested that mTBI confers no increased risk of development of a dementia condition. A study by Dams-O'Connor *et al.* (2013) examined 3,465 non-demented patients over the age of 65 with a follow-up period of 16 years, of which over 1,100 went on to develop either dementia or AD. From this subgroup, no association was found between TBI history and development of dementia, nor any interaction between TBI and the AD risk allele variant, Apolipoprotein E (APOE) ε4, and the development of AD. Similarly, a meta-analysis of 8 studies published between

2004 and 2011 found no association between mTBI and dementia or cognitive impairment (Godbolt *et al.,* 2012). The meta-analysis did suggest that "complicated" mTBI was associated with chronic cognitive impairment for a period of up to 12 months post-injury in paediatric populations, which lead to long term neurological consequences. However, follow-up beyond the 12-month period was carried out, and the consequences of paediatric TBI is yet to be fully explored. Also of note is the inclusion of "complicated" mTBI within the category of mTBI, which may represent a more moderate injury, confounding the risk.

Recently, a prolific examination of brains donated by deceased amateur and professional American football players suggested that repeated mTBI and sub-concussive blows incurred over the course of play may contribute to the development of Chronic Traumatic Encephalopathy (CTE) (Mez *et al.,* 2017). CTE has been closely associated with exposure to repetitive mTBIs since it was first detailed in retired boxers as "*Dementia Pugilistica*", and its subsequent rediscovery in a number of athletes and military veterans with a history of repetitive mTBI (Castellani & Perry, 2017; Omalu *et al.,* 2010; Goldstein *et al.,* 2013). However, the actual incidence rates of the disease remain to be determined. Mez *et al.* reported that of the 202 brains examined, 87% were given a post-mortem diagnosis of CTE based on recently outlined diagnosis guidelines for the condition (McKee *et al.,* 2016). In addition to the diagnosis of CTE, many of the cases that showed more advanced and severe disease progression also showed signs of co-morbid dementia conditions, such as AD, Lewy Body disease and motor neuron disease. A link between the extent of mTBI exposure and disease progression at the time of death was also inferred from the degree of disease severity and the length of the athletes' playing career. The highest proportion of severe CTE cases were found in professional players (101 out of 110 (86%) patients vs. 27 out of 48 (56%) college football players), suggesting an increased risk of disease development with increasing years of mTBI exposure. However, no definitive correlation between the number of mTBIs and disease state was drawn.

TBI's link with dementia is far from clear, as associations such as these are rife with difficulties regarding data collection. A common issue regarding TBI data collection is recall bias of severity and symptoms of mTBI upon clinical examination, an issue which all studies mentioned above acknowledge as a potential confounder and is currently unavoidable in a study involving a retrospective examination of head trauma. Studies such as Nordström *et al.* (2012) and Lee *et al.* (2013) make good attempts at accounting

for this by recruiting large numbers and by having a long follow up, however, some degree of bias will remain. The small number of TBI patients available for study may also hamper these studies, such as those included in Fleminger *et al.*'s (2003) meta-analysis. A selection bias may also be present among the population represented in post-mortem tissue banks of CTE, leading to a possible over-representation of the disease among athletes involved in contact sports. Many of the reported cases of CTE come from a limited group of individuals, with cases primarily representing former American football players. In recent years, additional cases have come to the fore, such as CTE diagnosed in professional wrestlers, military personnel and rugby union players, however, further research is warranted in individuals from a range of fields exposed to repetitive head trauma (Stewart *et al.,* 2015; Goldstein *et al.,* 2013; Omalu *et al.,* 2010; Omalu *et al.,* 2011).

1.2: The neurovascular unit and TBI

The association of TBI with the development of multiple neurodegenerative conditions suggests a common underlying mechanism by which the biomechanical forces associated with the injury result in a molecular pathology. Both TBI and several neurodegenerative conditions share an aspect of vascular pathology. TBIs of all severities have demonstrated a degree of blood brain barrier dysfunction (BBBD), both in animal models and human studies of TBI (Tagge *et al.,* 2018; Johnson *et al.,*2018). BBBD is also thought to precede symptom onset in conditions such as AD and Parkinson's disease, suggesting that changes to blood brain barrier (BBB) integrity may play an establishing role in dementia development (Desai *et al*., 2007; Nation *et al.,* 2019). Therefore, measuring changes in the integrity of the BBB and the neurovascular unit is currently one of the most common avenues of investigation in biomarker discovery for mTBI.

1.2.1: Endothelial cells

The endothelial cells of the neurovasculature form the first physical barrier of the BBB. The endothelial layer of the cerebral vasculature differs from that of lining capillaries of other organs of the body, as they lack the fenestrations that facilitate free diffusion of small molecules from the blood into the perivascular space (Erdö, Denes & de Lange, 2016). The free passage of molecules across the BBB via the paracellular space is impeded by the presence of TJ complexes that bind endothelia together, limiting passive diffusion to water molecules, gases and lipophilic molecules. Passage of most macromolecules therefore require other means by which to traverse the endothelial

barrier, such as osmotic diffusion, facilitated diffusion, carrier-mediated endocytosis and active transport via ATP-mediated hydrolysis or a carrier protein. The presence of transporter proteins that direct the flow of molecules to and from the neural space results in the polarisation of cerebral endothelial cells to apical (luminal) and basolateral (abluminal) membranes, further contributing to the selectivity of the BBB.

1.2.2: Astrocytes

The role of astrocytes in the neurovascular unit has evolved from what was perceived as a passive structural glial cell to an active unit in maintaining barrier integrity, contributing to synaptic formation, maturation and pruning, maintenance of ionic homeostasis, regulation of extracellular space and clearance of neurotransmitters (Dallérac & Rouach, 2016). In addition to these functions, astrocytic endfeet ensheath the endothelial cells of the brain vasculature, providing a link between neural tissue and cerebral capillaries, allowing for modulation of the BBB in response to injury or changes in homeostatic demands (Abbott, Rönnbäck & Hansson, 2006). Communication of neurons to astrocytes via neurotransmitters also influence cerebral blood flow, as astrocytic end processes relay neuronal signals to smooth muscle cells, surrounding arterioles, or pericytes, surrounding capillaries (Gordon, Howarth & MacVicar, 2011). Astrocytes may also play an important role in the maintenance of barrier integrity. Astrocytes have been reported to secrete that morphogen: Sonic Hedgehog (Shh), which plays an important role in the maturation and maintenance of BBB integrity. Shh is provides a large contribution to the normal development of animals, regulating the patterning and development of limbs, as well as the development of specialised organs such as the CNS and eye. Secretion of SHH induces Hedgehog (Hh) signalling via the Patched (PTCH) receptor, and upon activation of the Hh pathway within endothelial cells of the CNS, contributes to normal BBB phenotype via two means. The first is through reduced peripheral immune cell infiltration, by SHH-mediated downregulation of the intercellular adhesion molecule: ICAM-1. (Alvarez *et al.,*2011). Reduced expression of the adhesion molecule reduces the likelihood of immune-cell tethering to endothelial cells of the BBB and recruitment of cells to the neural space. The second means is by regulation of paracellular space via tight junction expression. *In vitro* experiments have demonstrated induction of BBB properties, such as tight junction (TJ) expression and increased trans-endothelial electrical resistance (TEER), following activation of the Hh pathway, while blocking the pathway has demonstrated increased barrier permeability and decreased TJ expression *in*

vivo (Alvarez *et al.,*2011; Alvarez, Katayama & Prat, 2013). Loss of Hh signalling was also accompanied by a reduced association of astrocytic endfeet with vascular endothelia, suggesting a reciprocal relationship between the two cell types in the maintenance of barrier integrity. Secretion of Src-suppressed C kinase substrate (SSeCKS) by astrocytes also seems to be important in orchestrating the maturation of BBB phenotype as development progresses, decreasing vascular endothelial growth factor (VEGF) and increased expression of TJ proteins and the anti-permeability factor; angiotensin-1 (Ang-1) (Lee *et al.,*2003). However, it has also been reported that secretion of Ang-1 in combination with VEGF can improve barrier integrity in developing vessels (Shen *et al.,*2011). VEGF, along with fibroblast growth factors (FGFs) and glial-derived neurotrophic factor (GDNF), are known to be either secreted from or signal to astrocytes. Astrocyte-derived VEGF plays a vital role in vascular remodelling and maintenance, while mice lacking FGF-2 or FGF-5 display increased permeability to serum albumin and decreased TJ expression (Reuss, Dono & Unsicker, 2003). However, the influence of FGF on BBB phenotype may be due to its role in astrocyte development and activity, rather than acting directly on endothelial cells, as FGF signalling has recently been shown to dampen astrocyte activity both in the resting state and following injury (Kang *et al.,* 2014). GDNF, which is expressed and secreted by astrocytes, as well as pericytes, can signal to GDNF receptor alpha 1 (GDNFRα-1) to stimulate TJ expression an *in vitro* model of the BBB (Iagrashi *et al.,*1999; Shimizu *et al.,*2011).

Within the context of TBI, astrocytic responses seem to be closely tied to that of vascular recovery. Glial scar tissue, made up of astrocytes, endothelial cells, fibroblasts and immune cells, is often seen in moderate and severe injuries, and has been shown to coincide with astrocytic-endothelia interactions following closed-cortical impact (CCI), while reactive astrocyte can upregulate matricellular proteins following injury (Jones & Bouvier, 2014; Villapol, Byrnes & Symes, 2014). This scar tissue may serve as a physical barrier to extravasating material or the diffusion of damage associated molecular patterns (DAMPs), limiting the spread of injury responses. Glial fibrillary acidic protein (GFAP) released from damaged astrocytes and into the peripheral circulation via a disrupted BBB is being investigated as a potential biomarker of mTBI. Reactive astrocytes have a dual role in modulating immune response following injury, capable of releasing pro-inflammatory cytokines, as well as matrix metalloproteinases (MMPs), which can contribute to the recruitment of additional immune cells and exacerbate BBB disruption,

as well as anti-inflammatory cytokines to taper immune responses (Carpentier *et al.,* 2004; Kim *et al.,*2005; Kim *et al.,*2010). In addition to directing immune response, the membranes of astrocytic endfeet are enriched with aquaporins, water-selective channel protein, encoded by the gene: aquaporin 4 (AQP4). AQP4 plays a crucial role in the regulation of water movement to and from the neural space under normal physiological and pathological conditions. Therefore, AQP4's role in the development and resolution of oedema, associated with severe TBI has been investigated. While AQP4 has been found to play a part in the development of both cytotoxic and vasogenic oedema, the role the water-channel plays in the pathophysiological response differs between the two processes. APQ4$^{-/-}$ mice have shown that AQP4 not only contributes to water accumulation in cytotoxic oedema; but also, actively contributes to the resolution of vasogenic oedema, a process that was previously thought to occur by bulk flow of water from the brain (Manley *et al*. 2000; Verkman *et al.,*2006). Increased expression of AQP4 has been observed in animal models of all severities of TBI, while human studies have identified a single nucleotide polymorphism (SNP) within the AQP4 gene associated with favourable outcomes following moderate and severe injury (Ren *et al.,*2013; Dardiotis *et al.,*2014), suggesting that the degree to which expression changes may reflect the severity of the injury.

1.2.3: Pericytes

Pericytes are mesodermal, perivascular cells that ensheath roughly 80% of the microvessels within the body and display the greatest density of coverage on vasculature of the central nervous system (CNS), covering an estimated 99% of the abluminal surface (Engelhardt & Sorokin, 2009; Winkler *et al.,*2012). Three morphological subgroups of pericytes have been identified and contribute to vascular function within the CNS; arterial pericytes, which are found to encompass arterioles and contribute to cerebral blood flow regulations; capillary pericytes, which run along capillary beds and aid BBB integrity; and venule pericytes, which have a radial morphology and modulate immune cell infiltration (Attwel *et al.,*2016). Within the context of the development and maintenance of the BBB, pericytes appear to play a larger role in the formation of TJs between endothelial cells than that of astrocytes. Endothelial cell/ pericyte co-culture models have shown that secretion of GDNF and Ang-1 increase TJ expression and TEER, even in the absence of coverage by astrocytic endfeet (Hori *et al.,*2004; Kim *et al.,*2009; Shimizu *et al.,* 2011). *In vivo* studies have also highlighted the importance of pericytes in BBB

formation during embryonic development, prior to the astrocyte-endothelia interactions. *Platelet-derived growth factor receptor beta* (*Pdgfrb)*$^{-/-}$ mice, which have greatly diminished pericyte coverage, display increased BBB permeability and decreased TJ protein expression shortly after birth (Daneman *et al.,*2011; Winkler *et al.,* 2012).

The role of pericytes in response to and recovery from TBI has yet to be explored in great depth. Pericyte response is thought to occur rapidly following injury, with the migration of cells from the vasculature of the injury site being observed within hours after CCI, dramatically reducing the ratio of pericytes-to-endothelial cells from an estimated 1:5 to 1:10-12 (Dore-Duffy *et al.,*2000). Platelet-derived growth factor beta (PDGFβ) has also been reported to be upregulated in a number of cells, including oligodendrocytes, astrocytes and endothelial cells, shortly after lateral fluid percussion injury. Increases in PDGFβ secretion may be a protective mechanism to maintain pericyte coverage and BBB regulation at the site of injury, as pericytes remaining at sites of injured vasculature showed signs of cell stress and degeneration, such as condensed chromatin and nuclear membrane folding. Evidence from ischemic injury suggests that pericytes may fulfil a similar role to that of astrocytes. Pericyte have been reported to influence cerebral blood flow and mediate immune cell infiltration during ischemic injury, while also supplying immunoreactive cytokines, such as IL-10, IL-12, IL-13, under physiological conditions and large amounts of reactive oxygen species and nitric oxide under immune challenge condition, in addition to clearing released cell debris (Cai *et al.,*2017).

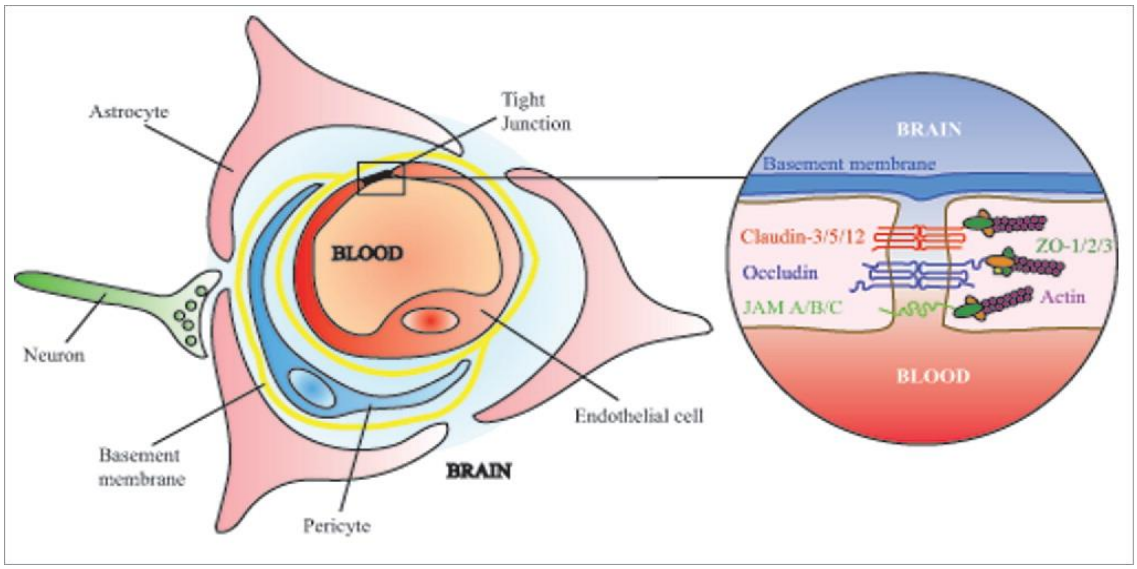


Figure 1. 2: Schematic of the neurovascular unit and tight junction complexes of the CNS.

The neurovascular unit is a highly specialised unit, developed to limit paracellular passage of molecules from the blood into the neural space. Composed of endothelial cells, astrocytes and pericytes, along with influences exherted by neurons and resident immune cells, the combination of which all impact the specific and dynamic properties of the blood-brain barrier. With endothelial cells of the central nervous system, specialised tight junction complexes, comprised of highly enriched claudin proteins, confers the unique diffusion properties of the endothelia. Combined with ensheathments of pericytes and astrocyte endfeet, paracellular passage is limited to >700 Da, ions and lipid soluble molecules. Taken from Greene & Campbell, 2016.

1.3: Tight Junctions of the BBB

1.3.1: Claudins

Within the claudin family of proteins, 24 members have been identified (Tsukita, Furuse & Itoh, 2001). Members of the family share common characteristics such as molecular masses between 20-27 kDa, four membrane-spanning regions, an intracellular loop, two extracellular loops (ECLs), a short, cytoplasmic N-terminal domain and a cytoplasmic C-terminal domain. Contained within the extracellular loops are domains which dictate the protein's dimerization with other claudin proteins; a common GLW(2aa)C(8-10aa) motif containing charged amino acids within the first ECL, which dictate charge selectivity and are involved in homodimerization, while heterodimerization is thought to be orchestrated via the shorter, second ECL, comprised of a helix-turn-helix structure (Krause *et at.,* 2008; Piontek *et al.,* 2008). The cytoplasmic C-terminal domain contains a PDZ domain, allowing claudins to interact with other proteins, such as Zonula Occludens (ZO-), and connect TJ complexes to the cytoskeleton, as well as influencing protein stability and localisation within the TJ structure (Van Itallie & Anderson, 2004; Van Itallie, Colegio & Anderson, 2004). Members of the claudin family show unique expression patterns *in vivo,* with some showing widespread expression, such as claudin-1 and claudin-5 being found in endothelial cells of the brain and testis, as well as other organs, or more tissue-specific expression, such as claudin-16, found within the loop of Henle in the kidneys (Tuskia, Furuse & Itoh, 2001; Krause *et al.,* 2007). The use of gene manipulation studies has identified the role of specific claudins in regulating paracellular permeability and ion conductance across cell barriers, classifying claudins broadly into ion-channel forming or barrier subtypes. These studies have also identified the importance of claudin-based TJs in several organs through the phenotypes they produce. For example, knockdown of *claudin-16* results in renal wasting of magnesium $(Mg)^{2+}$ and calcium $(Ca)^{2+}$, replicating the hereditary human conditions; Familial Hypomagnesemia and Hypercalciuria and Nephrocalcinosis, while mutations in *claudin-19,* phenocopies *claudin-16* mutants with the addition of severe visual impairment (Hou *et al.,*2007; Konrad *et al.,*2008; Naeem, Hussain & Akhtar, 2011). Similarly, *claudin-14* regulates sodium $(Na)^{+}$ and potassium $(K)^{+}$ ion passage and the permeability of TJs of the outer hair cells of the ear's cochlea, with *claudin-14* knock- out (KO) mice becoming deaf due to rapid degeneration of hair cells (Ben-Yosef *et al.,* 2003). These KO mice mimic the autosomal recessive deafness of *DFNB2* mutations found in humans, caused by mutations in the orthologous gene

(Wilcox *et al.,*2001). Mice lacking *claudin-11*, also expressed within the cochlea, also gradually develop hearing loss, but appears to fall more within the barrier category of claudins, as K^+ ion concentrations of outer hair cells were indistinguishable from those of littermate controls (Gow *et al.,*2004). In addition to deafness, male mice lacking *claudin-11* are sterile, with Sertoli cells being unable to correctly develop, losing polarity and detaching from the basement membrane and being eliminated via the epididymis (Mazaud-Guittot *et al.,*2009). Regarding the BBB, claudin-5 is the most enriched claudin isoform expressed in the brain's endothelial cells, with expression levels ~51- and 62-fold greater than claudin-22 (barrier forming protein) and claudin-10 (ion-channel forming protein) respectively, the next most enriched isoforms found in brain endothelial cells (Ohtsuki *et al.,* 2007). The importance of this enrichment is highlighted by the phenotype of *claudin-5* KO mice, which die shortly after birth, possibly due to an influx of small molecules that prove toxic to neural tissue (Nitta *et al.,* 2003). The KO model found that loss of claudin-5 provided increased permeability to small molecules of < 800Da, which has also been replicated in RNAi mediated knockdown of the TJ junction protein, facilitating passage of small molecules into the neural space (Campbell *et al.,*2008). Recently, mutations in *claudin-5* have been implicated in neurological conditions such as schizophrenia and epilepsy. Mutations in the 3'-UTR of the *claudin-5* RNA strand have been found to be associated with schizophrenia development. The mutation was found to be over-represented in individuals with 22q11 deletion syndrome, who have an increased risk of developing schizophrenia, while the region of chromosome deletion includes the cldn5 gene, reducing claudin-5 levels further (Greene *et al.,* 2018). Post mortem examination of schizophrenic brains also found reduced expression of claudin-5 compared to controls, while prolonged knockdown of the TJ protein produced psychosis and epileptic symptoms in mice.

1.3.2: Occludin

Occludin is a 65kDa member of the MARVEL (MAL and related proteins for vesicle trafficking and membrane link) protein family, and one of the first components identified within TJs of brain endothelial cells (Furuse *et al.,*1993; Raleigh *et al.,* 2010). The protein is made up of four transmembrane (TM) domains, two ECLs, a long cytoplasmic C-terminal domain and a shorter, cytoplasmic N-terminal domain. Like claudins, occludin's C-terminal dictates membrane localisation and allows ZO-1 and ZO-2 interactions, linking the TJ complex to the actin cytoskeleton (Furuse *et al.,* 1993). Truncation of this

domain results in discontinuous localisation of occludin at cell interfaces and increased paracellular permeability, as well as a loss of cellular polarity (Balda *et al.,* 1996). Animal models have also illustrated barrier properties different to that of claudins. For example, occludin KO mice obvious developmental phenotype prior to dissection and microscopic inspection, survive to adulthood and retain normal TJ morphology and barrier properties within the intestine (Saitou *et al.,*2000). However, KO mice develop poorly after birth and show several abnormalities in several organs, including calcification in the brain, chronic gastritis, testicular atrophy and male sterility. Further research has found that occludin is capable of regulating epithelial differentiation, apoptosis and claudin-2 dependant TJ function via mitogen-activated protein kinase (MAPK) and Akt signalling pathways, (Schulzke *et al.,*2005; Murata *et al.,*2005). Such findings suggest that a possible means by which depletion of occludin results in widespread deficits during postnatal development

1.3.3: JAMs and ZOs

Junction adhesion molecule (JAM) proteins are TM members of the immunoglobulin G (IgG) superfamily, expressed in both endothelial and epithelial cell. Within the neurovasculature, JAM-1, -2 and -4 are the most enriched (Daneman *et al.,* 2010; Mariano *et al.,*2011). JAMs share a common structure of a single TM domain and an extracellular domain carrying two Ig-like domains, which facilitate endothelial/ epithelial interactions with circulating immune cells (Ebnet *et al.,* 2004). The intracellular C-terminus carries a PDZ domain-binding motif, allowing JAM to interact with TJ-associated proteins such as Zonula Occludens (ZOs) and orchestrate changes in the actin cytoskeleton, recruit additional TJ proteins and TJ formation, as well as regulate cellular polarity (Liu *et al.,*2000; Ebnet *et al.,* 2003; Shi *et al.,* 2018). Within a cell, the PDZ domains of JAMs, as well as other tight junction proteins such as claudins and occludin, facilitate binding of TJ-associated proteins such as the ZO scaffold proteins. ZO proteins belong to the membrane-associated guanylate kinase homologue (MAGUK) homologue family, of which three have been identified: ZO-1, ZO-2 and ZO-3. ZO proteins were among the first to be identified within TJ complexes, and carry three PDZ binding domains, a Src homology 3 domain and one guanylate kinase domain (Stevenson *et al.,* 1986; Shi *et al.,* 2018). Through PDZ domain interactions, ZO proteins link peripheral TJ proteins to the cytoskeleton both via direct interaction with actin filaments and the C-terminus of ZOs, as well as indirectly though actin-binding proteins (Bauer *et al.,* 2010).

Mutation studies suggest that ZO-1 at least is required for early formation of TJs complexes, as ZO-1 KO mice are embryonic lethal by day E11.5, displaying retarded growth and disrupted yolk sac angiogenesis (Katsuno *et al.,* 2008).

1.4: Biomarkers of TBI

The reliance of neurological symptoms to recognise and monitor the progression of mTBI can be seen a substantial limiting factor in the study of mTBI and any associated pathophysiological processes. Therefore, an objective biomarker of mTBI has been highly sought in order to improve diagnostic accuracy of neurological assessment, as well as gain an means to monitor recovery independent of neurological symptoms, which often resolve within hours or days after injury. In recent years, an increasing number of publications detailing potential candidate proteins, molecules and structural changes as a means of identifying mTBI quickly, efficiently and accurately. The advances in neuroimaging has facilitated a greater understanding of the structural changes associated with TBI and subsequent development of long-term changes associated with recognised injuries. However, medical imaging-based diagnosis is not always available nor applicable to the spectrum of injuries associated with TBI. The high cost of running scanners, limited availability of scanner time and potential ethical considerations associated with the use of certain scanning sequences all preclude the use of neuroimaging for mTBI, in addition to the specialised training to run and analyse the data generated. Cerebro-spinal fluid (CSF) is a valuable resource in the screening and identification of biomarkers in other neurological conditions, and the CSF-to-serum ratio of albumin is currently the gold standard for gauging BBBD in many neurological conditions. However, CSF collection is an invasive process and not without risk. Therefore, blood-based biomarkers are highly sought, as samples can be collected quickly, cheaply and with a minimally invasive procedure, while processing of samples can be carried out rapidly with basic scientific training. However, despite identification of several candidate proteins and molecules associated with TBI and BBBD, and continued investigation into their utility as a marker of TBI, none have progressed to clinical use due to issues sensitivity and specificity in identifying mTBI in the absence of other injuries (Kawata *et al.,* 2016).

Obvious biomarker candidates for TBI would naturally be specific or enriched to neural tissue. Currently, the most actively investigated candidate proteins include GFAP,

neuron-specific enolase, ubiquitin C-terminal hydrolase (UCH-L1), tau protein and S100β, and of these, S100β has been the most extensively studied (Papa *et al.,* 2015).

1.4.1: S100β

S100β is a calcium-responsive protein suggested to be involved in neurite and axonal growth in the developing nervous system, while circulating S100β is considered a marker of astrocyte damage and disruption of the BBB. It is primarily found in astrogila and Schwann cells, but is also expressed in small quantities in peripheral tissue, such as adipose tissue, white blood cells and skeletal muscle tissue (Olsson *et al.,*2011; Donato *et al.,* 2013). However, due to its high expression in neural tissue it remains at the forefront of TBI research as a potential marker of neural damage in many neurological conditions, such as multiple sclerosis (MS), stroke, mood disorders and psychiatric disorders, and is often held as one of the strongest candidates as a marker of BBBD following TBI (Schroeter *et al*., 2003; Undén *et al.,,* 2013; Barateiro *et al.,,*2016; Zaigham, Lundberg & Olofsson, 2017; da Rosa *et al.,,*2016). For example, S100β has demonstrated strong negative predictive value in detecting computed tomography (CT) positive lesion in the acute scenario of severe TBI, in addition to also increasing significantly in patients reporting with complicated mTBI (de Kruijk *et al.,* 2001; Egea-Guerrero *et al.,*2011). Comparison of 15 mTBI patients with an intracranial lesion to 128 without found that S100β levels were significantly increased 6 hours after injury (Egea-Guerrero *et al.,* 2011). A trend was observed of increasing S100β serum concentration with increasing numbers of observed intracranial lesions was also present. While the study deemed that patients had sustained a mTBI, the presence of abnormal CT or magnetic resonance imaging (MRI) findings would suggest the injured be classified as a "complicated mTBI", and therefore reflecting a more moderate injury. As such, the study may not be indicative of S100β's utility in milder injuries. Within the clinical setting, S100β was found to be significantly higher in emergency department mTBI patients compared to healthy controls (de Kruijk *et al.,* 2001). A study of 104 patients reporting mTBI found that S100B serum levels were significantly higher 6 hours after receiving a direct blow to the head and experiencing a brief period of post-traumatic amnesia.

However, many of the studies suggesting the utility of S100β in identifying mTBI have focused on this acute scenario after injury and within a clinical setting. In the context of sports-related mTBI, S100β has found mixed success. Rapid drop off in circulating levels after the first 2 hours post-injury, as well as findings of exercise-related increases in

blood-based S100β levels has limited it use an accurate marker of mTBI (Thelin *et al.,* 2017).

A study of 35 concussed ice hockey players found that S100β was significantly increased in blood 1 hour after injury compared to baseline levels (Shahim *et al.,* 2014). Elevated concentrations of circulating S100β at the 1-hours post-injury time point was also associated with loss of consciousness and delayed return to play in participants. Longitudinal examination of the protein found that S100β levels returned to normal within 12 hours post-injury, and changes from baseline levels at later time points, up to 144 hours post-injury, were not associated with delayed recovery of symptoms or likelihood of return to play. S100β was found to be repeatedly elevated in an American football player 1 hour after a game, even in the absence of a concussion (Marchi *et al.,*2013). Serum levels of S100β indicated BBBD (based on previous thresholds established by the group) in several individuals repeatedly over the course of a season, and a trend of increasing serum levels post-match in each instance. However, as noted in the study, individuals of African-American descent had higher baseline levels of S100β than their Caucasian counterparts, some even coming close to the indicated BBBD threshold. This suggests that baseline S100β levels may differ between ethnic backgrounds, which may limit its utility. The study does not indicate whether these players were the individuals experiencing the greatest increases in S100β post-match. Changes in S100B concentrations correlated with the number and severity of blows to the head sustained over the course of the season. Similar correlations have been made when observing the number of headers done during a football game, the number of collisions in a rugby game and the number of jumps done during a basketball game (Stålnacke, Tegner & Sojka, 2003; Stålnacke, Tegner & Sojka, 2004; Bouvier *et al.,* 2017). Marchi and colleagues also found increases in auto-antibodies to S100β in ~50% of players over the course of the playing season. Increases in S100β auto-antibody levels correlated with the extent of changes in white matter (WM) membrane density, measured via diffusion tensor imaging (DTI)-MRI, and may reflect a possible auto-immune mode of damage.

A prospective study of 46 athletes engaging in a range of sports, including American football, soccer, basketball, and ice hockey, found that S100β serum concentrations when measured within a 3- hour window after play were an accurate indicator of mTBI (Kiechle *et al.,*2014). Levels of circulating S100β were significantly different to post-exertion,

non-contact control. Over the course of the two-year study, 17 players were available to give blood samples within a 3- hours post-exertion and after incurring a concussive injury. S100β serum concentrations were significantly higher than matched base line levels in players reporting a mTBI, as well as higher than that of post-exertion athletes. S100β concentrations at this time-point were capable of distinguishing between participants reporting a mTBI and non-contact players. However, comparison of post-exertion S100β concentrations in contact players to that of concentrations found in injured players was not made, allowing for the possibility that the increases observed may be a result of repeated sub-concussive blows associated with the sport rather than mTBI alone.

Several other issues are associated with the use of S100β as a potential biomarker of mTBI. While many of the studies mentioned so far have found that the protein is a good negative indicator of mTBI, S100β's positive predictive value in identifying mTBI remains limited. (Bouvier *et al.,* 2017; Shahim *et al.,* 2014; Kiechle *et al,* 2014).

Several studies have also highlighted whether S100β is a suitable biomarker for use in the context of non-complicated mTBI and sport. Several studies have found that circulating S100β did not have any correlation with imaging findings following mTBI or long-term outcome. Two follow-up studies, looking at heading the ball in football, one in which the ball was dropped repeatedly onto the head, and another simulating the force of heading the ball during play, found no changes in CSF or serum concentrations of S100β as a consequence of the repeated headers (Zetterberg *et al.,* 2007; Stålnacke & Sojka, 2008). S100β levels were not associated with abnormal CT or MRI findings in mTBI patients, nor with long term outcomes, such as delayed recovery when taken 3 hours after injury (Bazarian *et al.,* 2006; Metting *et al.,*2012). However, these observations cannot be directly compared to many of the other studies previously mentioned, as many involved sampling times several hours after the time of injury, which may limit the utility of S100β as a biomarker. Studies have found S100β screening appears to show little use when examined outside the acute-injury time frame of 1-2 hours post-injury. Neselius *et al.,* (2013) found there was no difference in S100β blood concentrations between boxers and age matched controls in the days following a fight, nor were S100β levels significantly different within boxers following a 14-day rest period. A prior study had shown that S100β was not significantly different between boxers and controls after 2 months of non-participation (Zetterberg *et al.,*2009). Exertion is also a factor when considering measurement of S100β serum level. As mentioned previously,

as several studies have seen increases in the potential biomarker during the post-exertion phase in participants of non-contact sports such as swimming and running (Dietrich *et al.,*2003; Hasselblatt *et al.,* 2004; Watson *et al.,* 2006).

1.4.2: Tau protein

The microtubule-associated protein, tau, is one of the hallmark features of the mTBI-associated disease CTE. Therefore, changes in levels of total tau or phosphorylated tau protein in the CSF or blood is thought to provide a possible early biomarker of mTBI sequela, such as axonal injury. However, animal studies and data collected from patients presenting to emergency departments with mTBI seemed to suggest tau having limited utility as a potential biomarker (Bulut *et al.,* 2006; Kavalci *et al.,* 2007; Liliang *et al*. 2010)

In the study of concussed hockey players previously mentioned, total tau was significantly increased above pre-season levels in concussed players (Shahim *et al.,* 2014). This increase was most evident immediately after injury (> 1 hour), with serum concentrations decreasing significantly after 12 hours. However, unlike S100B, total tau remained significantly higher than pre-season levels up to 144 hours post-injury, possibly due to an observed trend of a second spike in tau concentration between 12 and 36 hours. The presence of a secondary release of tau into the blood may be due to a possible biphasic opening of the BBB on a 24- hours cycle (Başkayaa *et al.*1997). However, so this pattern of phasic opening has only been demonstrated in animal models. Neselius *et al.,* (2012) demonstrated the potential of circulating tau as a post-acute-injury biomarker in a cohort of 30 post-bout Olympic boxers. Blood samples were taken at 1-6 days (average 3 days) after a fight and again after a 14-day rest period, which included no fights or sparing with head blows. Peripherally circulating levels of total tau was significantly higher in the blood post-bout boxers than those of age-matched controls, despite no knock-outs being recorded. This was case despite the boxers being asymptomatic of TBI symptoms, neuropysch evaluation returning a negative result for mTBI and no adverse findings reported on medical imaging scans. While in most boxers, tau concentrations dropped to those comparable of the control group upon the day 14 sampling time, a subgroup of six individuals was identified with continued total tau levels exceeding concentrations of 2.7ng/L. These individuals retained significantly higher total tau plasma levels up to 14-days post-injury, when compared to the 24 boxers below this threshold, however, no other stratifying parameters were identified. The authors mention

the possibility that the inefficacy of tau as a biomarker outside of acute scenarios (including a study in boxers previously carried out by the group) may have been due to plasma levels falling below conventional assay sensitivities (Zetterberg *et al.,*2009). In this context, sensitivity to very low sera concentrations may have hampered current investigations of biomarkers for mTBI. The subtle changes in neuroanatomy and microstructure may be reflected in similarly subtle changes in concentrations of fluid biomarkers. A follow-up study by the same research group found that circulating tau levels shortly after injury could estimate recovery time and segregate individuals who would go on the develop post-concussion syndrome (PCS) (Shahim *et al.,*2014). In a cohort of ice hockey players, plasma concentrations of total tau 1- hours post-injury were capable of estimating the number of days required for resolution of symptoms, with the highest plasma concentrations being found in a player unable to return to play. As well as this, plasma concentration at 144 hours post-injury was significantly higher in players with PCS that lasted more than 6 days compared to players whose PCS resolved within that time frame. Plasma levels of total tau were also significantly higher than pre-season levels in concussed players at 1-, 12-, 36- and 144-hours post-injury. Plasma concentrations of total tau also showed an increasing trend with the severity of mTBI when measured within 1 hour after injury. The greatest levels of tau correlated most strongly with injuries resulting in loss of consciousness. While a similar pattern was observed with S100β and neuron-specific enolase (NSE) plasma levels, these proteins were unable to predict recovery time. Plasma levels of tau at 1 hour and 144 hours post-injury were also able to distinguish between players with persistent PCS lasting longer than 6 days and displayed greater accuracy in matching with a diagnosis of concussion than S100β and NSE.

1.4.3: Ubiqutin carboxy-terminal hydrolase L1

Ubiquitin carboxy -terminal hydrolase L1 (UCH-L1) is a neuron enriched protein associated numerous processes, including cell survival, axonal transport and ubiquitin-proteasome mediated protein degradation (Bishop, Rocca & Henley, 2016). While negligible levels of the protein can be detected in peripheral tissue such as the gonads and, under wound healing conditions, fibroblasts, UCH-L1 has primarily been used as a neuronal specific marker, and since being identified in the CSF and serum of severe TBI patients, a candidate for a mTBI biomarker (Papa et al.,2010; Mondello et al.,2012). In addition to be a highly enriched neuronal protein, the UCH-L1 also demonstrates a

greater half-life than that of proteins such as S100β, with serum concentrations continuing to be elevated up to 7 days following injury in 96hrs post-injury (Mondello et al., 2012).

To-date, UCH-L1's utility has primarily been explored in the context of moderate and severe TBI, in which the biomarker has experienced some degree of success, both as positive marker of CT-detectable lesions, as well as a prognostic marker of recovery. A meta-analysis focusing on 13 clinical studies using UCH-L1 levels in blood and CSF found that the neuron enriched protein proved highly accurate in predicting CT scan findings across the spectrum of TBI severity (Shajouei et al.,2017). Use of UCH-L1 in combination with other putative TBI biomarkers has also demonstrated utility as a prognostic marker in moderate and severe TBI. Early studies found that serum levels of UCH-L1 could distinguish between moderate TBI patients that required neurosurgical intervention and those that did not (Papa et al., 2012). When combined with the astrocytic enriched protein; GFAP, elevated levels of the two proteins on day two post-injury served as accurate predictors of negative outcomes in severe TBI patients, as well as a distinction in circulating biomarker levels between GOS score 1-3 and GOS score 4-5 patients (Takala et al., 2016). The combination of UCH-L1 and GFAP has severed as the basis of clinically-relevant biomarker panels used in several clinical trials, as well as the first FDA-approved biomarker panel for TBI.

In the context of sport's related mTBI, UCH-L1 has also demonstrated a degree of promise. In a small, prospective study of collage American football players, Puvenna et al. (2014) found that UCH-L1 levels, along with those of S100β, were significantly elevated compared to pre-game levels following exposure to sub-concussive forces associated with play. However, it was noted that serum levels of UCH-L1 was not correlated with the number of hits sustained during a match, in contrast to S100β, possibly suggesting either peripheral release of UCH-L1 or a more delayed temporal release of UCH-L1 across the BBB following injury. Similar studies examining UCH-L1 in the context of contact sport have also shown promise, with UCH-L1 serums levels measured within 6hrs post American football match being higher than both matched pre-season values, as well as being significantly higher than serum levels of time-match non-contact athletes (Meier et al., 2017). UCH-L1 serum levels measured at 6hrs post-match within this cohort was also capable of distinguishing between players that had sustained a mTBI and healthy athletes over the course of the game, indicating UCH-L1 potential as a diagnostic tool for sport's related mTBI.

While UCH-L1 holds all the bearings of a promising biomarker for mTBI, recent studies have raised some potential issues with its use in certain contexts, particularly those of contact sport and TBI with polytrauma. A recent animal study found that UCH-L1 was a poor maker for distinguishing TBI from peripheral injury in rats (Morris et al., 2019). The study found that serum levels of UCH-L1 were elevated 4hrs after weight drop-induced TBI, however, haemorrhagic shock and blunt tail injury also induced similar increases in the potential biomarker within the same time frame. While this is evidence from a single study, does suggest that UCH-L1 may be of limited use in determine TBI severity in cases of polytrauma or in contact sports where the whole body is subjected to mechanical forces. Another recent study also raised the question regarding the benefits of including UCH-L1 within TBI biomarker panels, as the study by Papa *et al*. (2019) found that GFAP was capable of distinguishing between mTBI patients, asymptomatic head trauma patients and body trauma patients. While blood levels of GFAP and UCH-L1 were elevated in mTBI patients and, to a lesser extent, asymptomatic patients, within 4 hours after injury compared to body trauma patients; only GFAP differed a significantly in mTBI patients from both asymptomatic and body trauma patients. When distinguishing between the three groups, GFAP outperformed UCH-L1 in detecting mTBI patients at every time point investigated, up to 180hrs post-injury, GFAP's accuracy alone was comparable to the accuracy provided by GFAP and UCH-L1 levels combined. Also interesting to note was distinct temporal release patterns of both biomarkers in cases of asymptomatic head trauma and mTBI, with initial increases in GFAP being followed by gradual decline after 12hrs only mild fluctuations in UCH-L1 levels in the case of asymptomatic patients, while mTBI patients showed initially rapid increases in UCH-L1 levels , followed by gradual decline, and delayed release of GFAP, reaching it peak almost 20hrs post-injury. These distinct release patterns may prove useful in distinguishing mTBI and lesser head trauma, however they do highlight the importance of sample timing when determining an biomarker's utility.

Despite these recent studies. UCH-L1 remains a promising biomarker for mTBI. While the studies by Morris (2017) and Papa et al. (2019) as questions of UCH-L1's use in the context of mTBI, further study is required to truly it out. However, an important contribution UCH-L1 has already made to the development of biomarkers for mTBI is its use in a panel, most of with GFAP. The complex nature of mTBI will likely require a multifaceted approach, and while biofluid-based proteins have been the primary focus in

biomarker development, UCH-L1 has been at the forefront of the development of a biomarker panel for mTBI.

1.4.4: Glial fibrillary acid protein

GFAP is an intermediate filament protein forming part of the astroglial cytoskeleton and is being investigated alongside S100β as a systemic marker in many neurological conditions. As a glial-specific protein, GFAP is one of the brain-specific proteins being investigated in order to circumvent the issues of extracranial sources of S100β contributing to measured serum levels, as well S100β's rapid fall off in circulating levels (Papa *et al.,* 2014; Papa *et al*, 2016a). However, so far GFAP has primarily been the focus of studies examining the post-acute scenario of mTBI and the long-term recovery window of TBI.

When compared to other candidates, GFAP appears to outperform S100β as a potential marker of TBI. In a study of patients admitted to emergency departments for mTBI, serum levels of GFAP were significantly higher in mTBI patients with CT positive lesions or MRI-detectable axonal injuries compared to controls, while levels of S100β displayed no discernible differences compared to that of controls (Metting *et al.,*2012). Patients that continued to show symptoms of injury also displayed a sustained increase in serum GFAP concentrations up to 6 months following the injury, with similar observations later being reported in a paediatric population of mTBI (Mannix *et al.,* 2014). The sustained increase in GFAP may offer a longer window after injury in which severity can be assessed, as well as a means of continuous monitoring of recovery outside of the acute injury phase. A similar study found GFAP was also a better predictor of the presence of CT-detectable lesions in polytrauma patients compared to S100β (Papa *et al.,* 2014; Papa *et al.,*2016b). GFAP serum levels above 0.067 ng/ml had a specificity of 55% for detecting intracranial lesions in patients with a skull fracture, compared to a 5% specificity associated with S100β. The study also found that S100β levels were significantly associated with the presence of extremity bone fractures, while GFAP concentrations remained independent, suggesting that S100β may not be as useful in polytrauma scenarios.

1.4.5: Neuron specific enolase

NSE is an isoform of the glycolytic enzyme enolase, and specific to neural-lineage cells. Due to its specific expression pattern, increases in concentrations within body fluids are taken as a maker of pathological conditions such as tumour-induced aberrant expression

or cell damage. Therefore, NSE is currently being investigated as a potential biomarker for a number of conditions, such as glioblastomas, ischaemic stroke and TBI (Isgro, Bottoni and Scatena, 2015). NSE has often been studied alongside S100B, with the protein showing similar patterns of increase following mTBI (Naeimi *et al.,* 2006; Honda *et al.,* 2010; Graham *et al.,* 2011). However, the use of NSE appears to be found in identifying potential mTBI outside of the acute scenario. A study examining a group of amateur boxers after a period of non-participation in the sport found that NSE was significantly higher in boxers compared to controls, possibly reflecting continued BBBD from mTBI despite a lack of exposure (Zetterberg *et al.,* 2009).

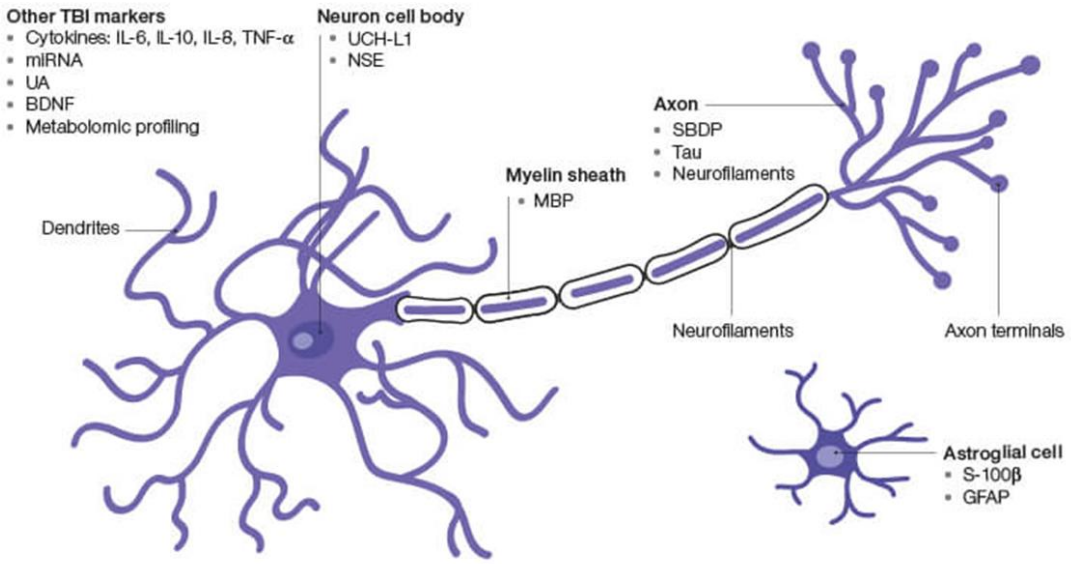


Figure 1. 4: Potential biomarkers for the use in detecting mild TBI and their neural cell source.

Neural-specific proteins have been the primarily focus in the search for markers of mTBI. The astrocytic proteins; S100β and GFAP, have been some of the most heavily studied potential markers. Circulating levels of both proteins have been found to increase following moderate and severe TBI within clinical contexts and have been demonstrated to fluctuating according to the number of head trauma s sustained in contact sports. Neurofilaments and the microtubule-binding protein, tau, found within axons and axonal terminals, have also been touted as potential markers due to the use of phosphor-tau being a defining feature of the mTBI-associated condition: Chronic Traumatic Encephalopathy. Their longer circulating half-life has also conferred an advantage over the somewhat short circulating half-life of S100β. Neuronal body proteins, such as UCH-L1 and NSE, have been used primary in conjunction with other potential markers such as GFAP. UCH-L1, in combination with GFAP, is one of the first FDA-approved biomarker s for mild and moderate TBI in a clinical setting. Other molecular markers, such as cytokine profiling, micro RNAs and metabolic profiling have also been investigated for their use in detecting and distinguishing mTBI. However, these markers have primarily been studied in a clinical context and their utility as prognostic markers of recovery.

Image taken form https://www.bioradiations.com/traumatic-brain-injury-a-controversial-and-deadly-epidemic/

1.5: Inflammation and TBI

Following the primary, mechanical injury and its associated damage, such as contusion, haemorrhage and shearing, a series of secondary processes, such as the release of DAMPs, metabolic dysfunction and cerebral blood flow changes, can occur within hours and days following TBI. Secondary processes result in inflammatory responses that are vital in terms of recovery from the injury but can also have profound effects on the prognosis of recovery. In recent years, the time course for immune response has been characterised to the extent that a rough time course of immune cell recruitment has been established after the release of injury-associated DAMPs and chemokines (McKee & Lukens, 2016; Gyoneva & Ransohoff, 2015). Recent studies have also found that the mismanagement of inflammation following TBI can lead to long-term cognitive consequences, feeding into a cycle of secondary injury processes. Therefore, inflammation is one possible link to the development of long-term sequalae of TBI, such as WM damage, delayed recovery of BBB integrity and possible development of neuropathologies such as AD and epilepsy (Herman, 2002; Johnson *et al.,* 2013; Jellinger *et al.,*2001).

1.5.1: Neutrophils

Neutrophils are the most abundant type of leukocyte within the blood and form the first innate response to infection and injury across most tissue types. Neutrophils are also present within the CNS in low numbers under physiological conditions, confined in small numbers primarily to the CSF, meninges and pia membrane (Wilson, Weniger & Hunter, 2010; Roth *et al.,* 2014). Following the primary insult, neutrophils are recruited within hours of injury and contribute to the early immune response and recruitment and activation of immune cells (Soares *et al.,*1995; Whalen *et al.,*1999; Perez-de-Puig *et al.,*2015). Trafficking of neutrophils to the injury site can also be aided by reductions in cerebral blood flow, such as in moderate and severe TBI, facilitating improved tethering to blood vessel endothelia (Kelly *et al.,*1997; Walcheck *et al.,* 1996; Kelly *et al*. 1996). Upon recruitment to the injury site, neutrophils help establish an early pro-inflammatory state through the release of tumour necrosis factor (TNF) cytokines and pro-inflammatory interleukins (ILs). Neutrophils have also been reported to play roles in recruiting additional immune cells through the release of CXC- and CC-chemokines, promoting neuron survival via neurotrophic factor release and potentially modulating BBB permeability through the release of MMPs, elastases and angiogenic factors (Bennett *et*

*al.,*1998; Mantovani *et al.,* 2011; Kolaczkowska & Kubes, 2013). In addition to mediating early inflammatory responses, the rapid activation and recruitment of neutrophils seem to play important roles in the development of oedema following TBI and influencing cell survival shortly following injury. Studies blocking or limiting neutrophil infiltration to the brain in animal models of TBI have found that reductions in infiltration numbers significantly reduce the water of content of injured brains. *Cxcr2* KO mice, which lack the primary receptor responsible for neutrophil migration, found that *Cxcr2*$^{-/-}$ mice had reduced cell death following a CCI (Semple *et al.,*2010). However, the abolition of *Cxcr2* resulted in no change in neurological outcome, nor did the loss of neutrophil infiltration alter changes in BBB integrity compared to wild-type (WT) mice. Similar findings reported using anti-Gr-1 depletion of neutrophils (Kenne *et al.,*2012). In addition to reductions in lesion size, blocking neutrophil infiltration also reduced levels of oedema and the number of activated microglia and macrophages at day seven post-injury. A more recent study has found that neutrophil recruitment is dependent on non-classical monocytes, and the means by which this sub-population mediates neutrophil responses dictates functional outcome (Makinde *et al.,*2017). Global monocyte depletion via clodronate pre-treatment reduced infiltrating neutrophil numbers within 24 hours of a CCI injury. Monocyte depletion was accompanied by reduced levels of oedema and improved functional outcomes in working memory and locomotive tasks. However, blocking early neutrophil infiltration may not provide a longer survival period for cells following TBI. Similar to previous studies, Semple and colleagues (2015) found that neutrophil elastase, while not impacting neutrophil infiltration following TBI, did reduce the levels of oedema or cell death 24 hours after TBI. However, cell death and volumetric changes within the cortex and hippocampus of elastase KO mice were comparable to that of WT mice on the ipsilateral hemisphere of the injury two months post-injury. Interestingly, bilateral volumetric changes and cell death were observed within regions of the hippocampus in both WT and elastase KO mice.

Shortly after neutrophil recruitment and activation at lesion sites, monocytes and microglia are considered to dominate the following phase of immune response in TBI, remaining in an activated state from days up to weeks and years (Johnson *et al*., 2013; Morganti *et al,* 2015; McKee & Lukens, 2016). Activation of infiltrating monocytes and microglia co-ordinate immune responses of recruited cells, and correct management of this response is considered of vital importance in dictating outcome following TBI.

Chronic activation of microglia and monocytes, which can perpetuate secondary responses, are thought to be one of the main contributors to long-term damage associated with TBI, with studies indicating that peripheral monocyte numbers and subtypes can predict clinical course and outcome in TBI and stroke (Urra *et al.,*2009; Li *et al.,* 2018).

1.5.2: Monocytes

Studies examining the role of monocytes in TBI have found that depletion of macrophages and microglia shortly after TBI can improve cognitive performance and limit inflammation as a result of an injury. For example, animals lacking C-C motif chemokine receptor 2 (CCR2), had reduced lesion size and reduction in associated secondary injury damage (Gyoneva *et al.,* 2015). CCR2 forms part of the monocyte-specific chemotaxis axis, along with monocyte chemoattractant protein-1 (MCP-1)/ C-C motif chemokine ligand 2 (CCL2). Abolishing CCR2-mediated signalling via gene insertion resulted in the absence of peripheral $CCR2^{+}$ monocytes infiltrating the injury site 3 days after lateral fluid percussion injury. This was accompanied by a significant reduction in injury-induced cavity volumes compared to heterozygotic mice, as well as reducing axonal swelling. Changes in the levels and localisation of p-tau staining following injury were also observed. Both *ccr2* heterozygous and homozygous deletion mice were observed to have diffuse p-tau staining within the cortex and hippocampus, however, homozygous *ccr2* deletion mice predominantly showed p-tau reactivity within the cell soma. This is in contrast to an earlier study in *mcp1/ccl2*$^{-/-}$ mice. Semple and colleagues (2010b) used a CCI model of TBI, which produces a different, more localised type of TBI to that of fluid percussion. However, they reported that lesion volume did not differ significantly from that of WT mice up to 7 days after injury, despite time points showing increased levels of cell death. However, a delayed reduction in lesion volume and neuronal cell loss compared to WT mice was observed 4 weeks after injury. This was in parallel to reduced numbers of infiltrating macrophages, reduced astrogliosis and inflammation over a longer period after injury. Similarly, specific blocking of macrophage infiltration through the use of the CCR2 antagonist; CCX872, resulted in improved post-acute cognitive function in spatial learning and memory (Morganti *et al.,*2015). Serial administration of the drug for 5 days after CCI injury resulted in improved hippocampal-dependant function up to 28 days post-injury. Improvement to cognitive function was accompanied by reductions in gene expression of IL-1β, IL-6 and MCP-1/CCL2; as well as reduced expression of NADPH oxidase 2 (NOX2), part of a

multi-unit complex associated with reactive oxidative species (ROS) production and prolonged proinflammatory signalling (Block *et al.,*2007).

A small study in humans examined microglia activation states 11 months after a moderate or severe TBI using positron emission tomography (PET) imaging (Ramlackhansingh *et al.,*2011). Activated microglia were detected within regions of the thalamus, putamen, occipital cortices, and posterior limb of the internal capsules at this point, with increased microglial activation associated with increased cognitive impairment. Despite a limited sample size, it was noted that in the majority of the participants examined, regions with increased microglia activation were not within the original lesion site, suggesting propagation of secondary responses outward from the site of injury, possibly as a result of diffuse injury along axonal tracts. Autopsy studies of patients who had sustained a single severe TBI found that activated microglia were enriched in regions of the corpus callosum and parasagittal cortex (Johnson *et al.,*2013). An increasing density of microglia occupying these regions was observed in sub-acute (2 weeks to 9 months) and long-term (1 year-47 years) injury groups compared to controls and the acute (10 hours-10 days) injury group. Distribution of activated microglia was also distinct between sub-acute and long-term injury groups. The sub-acute cohort had predominantly tightly packed clusters of activated microglia compared to the widespread distribution of the acute and control groups. In comparison, the long term-survival groups showed widespread distribution of these activated microglia throughout the investigated regions. Coupled with these observations were the findings that axonal pathology in sub-acute and long-term injury groups was considerably more widespread than that of controls. Decreased myelin staining in individuals with extensive activated microglia was also observed, and a trend of increased thinning of the corpus callosum with longer times of survival after injury was also noted.

1.5.3 Innate Immune training

Innate immunity is widely accepted as the first response to foreign pathogens as well as the processes involved in sterile inflammation. With the recent develops in the understanding of molecular processes involved in innate immunity, the capability of the innate arm to "retain" information and adapt to previous stimuli has come to the fore. Initially referred to as "innate immune memory", "trained immunity" was has been purposed as a concept to account for the additional immune-stimulation effects of the Bacillus Calmette-Guérin (BCG) vaccine outside of its intended role of tuberculosis

protection (Roth *et al.,* 2005). This allows the innate arm of the immune system to mount a greater response to familiar stimuli independent of the adaptive immunity mechanisms. Changes in metabolic and epigenetic profiles have been identified and associated with the acquisition of trained immunity, with a shift from oxidative phosphorylation to Akt/Mtor/HIF-mediated aerobic glycolytic metabolism and changes in TLR expression via histone medication (Kleinnijenhuisa l *et al.,*2012; Arts, Joonsten & Netea*.,* 2016). Trained immunity has grand implications for both development of new immune-based therapeutics, as well as understanding potential auto-immune conditions and responses.

To date, studies in trained immunity have primarily been concerned with positive non-specific effects of immune challenges such as those carried out using β-glucan stimulation, IL-1 cytokine pre-treatments or vaccination such as those observed with the BCG vaccine (Bekkring *et al.,* 2016; Moorlag, *et al.*, 2018; Černý & Stříž, 2019). However, trained immunity is thought to also play an important role in auto-inflammatory diseases and represent a potential avenue of development for chronic inflammatory conditions. Reprogramming of macrophages of within adipose tissue of obesity patients, recruitment and activation of monocytes associated with the progression of atherosclerosis and cholesterol-induced inflammation associated with cardiovascular diseases all represent the potential negative impacts of trained immunity to the host (Černý & Stříž, 2019).

This idea can also be expanded to potential long-term deleterious effects of mTBI. Il-1 has been previously mentioned as a potential prognostic marker of severe TBI recovery. A Growing number of studies suggest that IL-1 family cytokines play a key role in the acquisition of trained immunity (Moorlag *et al.,*2018). In addition, it has been suggested that a number of non-immune cells, such as endothelial cells and microglia, may allow for retention of the trained immune response beyond the limited life-cycle of immune cells (Hamada *et al.,*2018). In cases of repeated exposure of TBI; such as in cases of sports-related mTBI, circulating immune cells are in a favourable environment to develop a trained response to neural specific antigens, such as those investigated as potential biomarkers of mTBI.

1.5.4 Adaptive immunity

The adaptive arm of the immune system, comprised of T- and B-cell lymphocytes, is suggested to be a driving force in immune response in the long-term injury response to TBI. Activity of adaptive lymphocytes appears to be highest several days after initial

injury and is thought to contribute to recovery mechanisms for several weeks during recovery (McKee & Lukens, 2016). However, the role T-cells and B-cells play in the progression of post-injury inflammation remains mostly understudied (McKee & Lukens, 2016; Clausen *et al.,* 2007; Czigner *et al.,*2007). For example, *recombination activating gene-1 (Rag1)*$^{-/-}$ mice, which functionally lack both T-cells and B-cells, showed no significant differences to WT mice in regard to inflammatory cytokine profile or functional outcome 7 days after CCI-induced TBI (Weckbach *et al.,* 2012). These immune-deficient mice were observed to have significantly different levels of circulating complement factors, however, inflammatory cytokines such as TNF-α and IL-6 were comparable to levels seen in WT injured mice at time points ranging from 4 hours to 7 days post-injury. The lack of the adaptive arm also did not prevent early cell death or tissue damage within 24 hours of injury, nor was the permeability of the BBB significantly altered 4 hours after injury from that of control injured mice. In a separate study, the adoptive transfer of activated/ effector T-cells to *Rag1*$^{-/-}$ mice prior to TBI resulted in similar levels of tissue damage to that observed in WT mice, and greater than that in *Rag1*$^{-/-}$ mice supplemented with non-activated cells (Fee *et al.,* 2003). Following 24 hours after aseptic cerebral injury, *Rag1*$^{-/-}$ mice had significantly fewer apoptotic cells and smaller injury size compared to their WT counterparts. However, transfer of activated effector T-cells 24 hours prior to injury resulted in a larger lesion and increased cell death. The findings of the study suggest that the presence of activated T-cells may play a role in the early stages of TBI injury and contribute to secondary damage to tissue outside that done by the mechanical force. Pharmacologically blocking effector T-cell activity in WT mice was also capable of reducing lesion size and apoptosis to levels similar to that observed in *Rag1*$^{-/-}$ mice. A finding unique to the transfer of activated T-cells was an increase in neutrophil numbers within the injury site. Changes in neutrophil counts were not observed in *Rag1*$^{-/-}$ mice following injury, nor in WT mice with blocked T-cell activity. While this study and the *Rag1*$^{-/-}$ model provides the best indication that adaptive immunity plays a limited role in acute and sub-acute stages of TBI, several studies have reported differing effects and responses of T-cells in several models.

In contrast to these findings, a study found that CD74-deficient (CD74^{def}) mice were found to have smaller lesion volume 3 days after lateral fluid percussion TBI (Tobin *et al.,*2014). CD74 is required for the processing and sorting of major histocompatibility complex (MHC) class II molecules, and mice deficient in antigen presentation expressed

less pro-inflammatory cytokines within the 24 hours after injury. Decreases in cell death and lesion volume over 3 days following injury were also observed. An expansion of splenic B-cells and $CD4^+$ T-cell populations was also noted within 24 hours of fluid percussion injury (FPI). This expansion was accompanied by increases in Class II invariant peptide expression; an MHC II antigen presenting peptide, and MHC II molecules on B-cells and γδ T-cells respectively. However, cellular population expansion was abolished after TBI in $CD74^{def}$ mice. In a recent observational study, infiltrating T-cells appeared to linger within lesioned and perilesional areas of the cortex for up to 90 days after FPI TBI in rats (Ndode-Ekane, *et al.,*2018). While numbers of infiltrating T-cells peaked within 2 days of injury and subsequently dropped off thereafter, the number of cells remain 21-fold higher than that of controls at the same time point. In addition to this, animals that showed slower neurological and motor function recovery within the first 21 days after injury also showed great numbers of infiltrating T-cells at 30- and 90-days post-injury. Areas showing a continued presence of T-cell infiltration also displayed increased BBB permeability and a higher density of activated macrophages/ microglia.

While these studies suggest some role for the adaptive arm of the immune system in TBI response, immune studies involving *Rag1*$^{-/-}$ mice are wildly accepted as the definitive means by which to test adaptive immunity involvement in disease processes. Therefore, further study is required in order to fully elucidate the findings suggesting adaptive immunity involvement within TBI response.

1.5.5: Cytokine response in TBI

In addition to the immune cells involved in potentially pathological processes following TBI, several inflammatory mediators have also been investigated for the roles they play in acting out these processes. Understanding of the mechanistic pathways involved in TBI offer greater insight into the processes involved, denote potential biomarker targets useful in injury assessment and therapeutic targets.

1.5.6: Interleukin-1

IL-1β is a potent inflammatory mediator involved in a range of inflammatory disorders; and under strict regulation by two-step activation via the multiprotein inflammasome complex (Ozaki, Campbell & Doyle, 2015). Within the context of TBI, increases in levels of circulating IL-1β have been observed in both animal and human studies, and investigated as potential prognostic markers of TBI outcome (Helmy *et al.,* 2010; Fenn

*et al.,*2014; Qian, Li & Shi, 2016). However, these in the cases of human studies (Helmy *et al.,*2010), microdialysis was used to collect samples, which could have influenced cytokine levels, which ranged in the 5pg/ml level. Discussed here are some of the potential mechanisms by which these cytokines could prove detrimental in chronic inflammation. For example, increased levels of IL-1β have been reported to associate with increased numbers of immune cell recruitment following injury, as well being accompanied by increased BBB permeability (Andersson, Perry & Gordon, 1992; Ferrari *et al.,*2004; Shaftel *et al.,*2007). Macrophage and neutrophil numbers increased over the course of days within regions of the striatum transfected with human IL-1β, and this increased number of infiltrating leukocytes was accompanied by a large area displaying increased BBB permeability (Ferrari *et al.,*2004). A follow-up study also found an increased number of peripheral immune cells entering the brain parenchyma following overexpression of IL- 1β (Shaftel *et al.,*2007). Cell types included T-cells and dendritic cells and occurred within two weeks of gene activation. A sustained neutrophil presence was also observed for up to 1 year after activation. BBBD was also observed. However, the observation that BBB leakage was present in *Cxcr2*$^{-/-}$ mice, as well as WT mice, suggested that the compromised barrier was due to a neutrophil independent pathway. In subsequent studies, it has been found that IL-1β can induce changes in BBB integrity through induced expression of Matrix Metalloproteinase 9 (Alluri *et al.,*2014; Alluri *et al.,*2016, Michael *et al.,*2016). *In vitro* experiments have demonstrated that IL-1β induces hyperpermeability in endothelial cell monolayers that can be elevated via MMP-9 siRNA treatment (Alluri *et al.,*2016a). Increases in permeability were found to coincide with the disruption and possible redistribution of ZO-1 tight junction complexes, as expression levels were not altered following IL-1β treatment. A follow-up study using the CCI model of TBI further indicated that these changes in barrier permeability was ue mediated by MMP-9, as greater Evans blue dye extravasation was observed in vehicle-treated animals than those treated with MMP-9 inhibitors.

In addition to BBB integrity, IL-1β has been implicated in other secondary processes following TBI. The use of IL-1β antagonists have suggested both a potential therapeutic route for TBI treatment, as well as suggesting the larger role IL-1β plays in TBI. In two CCI studies, the use of the IL-1β neutralising agent: IgG2a/k, greatly reduced the number of infiltrating neutrophils and activated T-cells, as well as limiting the number of activated microglia (Clausen *et al.,* 2009; Clausen *et al.,*2011). Of note, however,

expression of chemokines *mcp1/ccl2* and *ccl3* were not altered 48 hours after injury, nor was the early pro-inflammatory cytokine; IL-6. Expression of the astrocyte activation marker: GFAP was also unaltered, suggesting some other possible means outside of IL-1β by which recruitment of circulating leukocytes to injury sites is limited. IL-1β-neutralised mice also showed significantly smaller lesion volumes and improved performance in memory tasks, suggesting a role in cell survival. Several other studies also suggest that IL-1β may contribute to cell death after brain injury. Use of IL-1α and IL-1β in ischemic brain injury models suggest that both forms of IL-1 contribute to neural damage after injury, while studies also display neuroprotective properties of IL-1 receptor antagonist, the endogenous inhibitor of both IL-1 proteins (Boutin *et al.,*2011; Lin *et al.,* 1995; Vezzani *et al.,*2000). A recent study, which inhibited inflammasome activity, the primary means of activating IL-1β, reported several improvements over vehicle-treated mice (Xu *et al.,*2018). IL-1β expression and protein levels were reduced following inflammasome inhibition, resulting in reduced lesion size 21 days after CCI compared to vehicle treated controls. Treated mice were also reported to have reductions in the degree of oedema, improvement in neurocognitive function and reduced BBB disruption. The study also reported reduced levels of apoptosis in treated mice, and that the neuroprotective effects of inflammasome inhibition required the presence of microglia. This suggests that IL-1β relays apoptotic signals to surrounding neural cells via the resident immune cells. However, chronic overexpression of IL-1β in the hippocampus of mice did not contribute to neurotoxicity up to a year after activation (Shaftel *et al.,*2007). This is in spite of diverse T-cell recruitment and BBB dysfunction in the region of transgene activation. The authors do suggest however, that sustained expression of IL-1β may make to tissue more susceptible to further injury. Overexpression of IL-1β also does not account for the requirement for a secondary signal to induce cleavage of the pro-form of IL-1β to its active form.

1.5.7: Interleukin-6

IL-6 is another heavily investigated cytokine and potential biomarker in recognising and informing prognosis of TBI. Despite the investigations surrounding its clinical use, the functional role of IL-6 in TBI is relatively unknown. Using IL-6 KO mice and a cold-induced TBI model, Penkowa and colleagues (1999) found that IL-6 increases rapidly following TBI and may play a protective role at lesion sites. Coinciding with peak IL-6 concentrations, WT mice displayed greater reactive astrogliosis and activated microglia/

macrophages numbers at 3 days post-injury, as well as increased glial scaring over a 3-week period compared to KO mice. In contrast, IL-6 KO mice showed increased levels of oxidative stress and decreased expression of anti-apoptotic factors; metallothionein I+II (MT-I+II). Perhaps unsurprisingly due to the reduced levels of MT-I+II, the extent of apoptotic cell death was also greater in IL-6 KO mice than WTs over the 3 weeks following injury, and the observation was made that surviving cells were those that retained some level of MT-I+II expression. A later study demonstrated that the lack of IL-6 can translate to behavioural deficits, suggesting that the cytokine may play a protective role following TBI. Ley *et al.,* (2011) found that IL-6 KO mice performed worse at locomotive and exploratory behaviours 24 hours after CCI-induced TBI. IL-6 KO mice as in displayed reduced neurological evaluation of sensory perceptions, righting reflex and stimuli-induced responses. These behavioural deficits were accompanied with a significant increase in IL-1β concentrations in the plasma of IL-6 KO mice following injury compared to WT mice, possibly suggesting compensatory injury response in the absence of IL-6. IL-6 may also promote vascular repair, which may, in turn, allow better regulation of immune cell infiltration. Comparing recovery following cold-induced TBI, Swartz *et al.,* (2001) demonstrated that IL-6 KO $^{-/-}$ mice had increased BBBD a week after injury, while GFAP-IL-6 transgenic mice showed accelerated recovery from injury. GFAP-IL-6 mice demonstrated greater infiltration of immune cells, restoration of normal tissue structure and improved re-vascularization. The protective role of IL-6 may stem from induction of neurotrophic factors. *In vitro* experiments have shown that IL-6 can improve the survival of retinal ganglion cells via A1 adenosine receptor signalling, as well as increasing in brain-derived neurotrophic factor (BDNF) expression (Perígolo-Vicente *et al*., 2013). An earlier study found that the concentration of nerve growth factor (NGF) in the CSF of severe TBI patients increased following IL-6 peak and correlated with injury severity (Kossmann *et al.,*1996). Treatment of mouse astrocytes with either TBI patients' CSF containing IL-6 or recombinant IL-6 resulted in increased production of NGF, which was abolished in the presence of IL-6 neutralising antibodies.

While it is suggested that IL-6 plays a positive role in recovery following TBI, there is evidence pointing to the cytokine having a detrimental effect on neural cells following injury. This seems to be the case following chronic IL-6 signalling. Treatment of developing cerebellar granule neuron cultures with physiologically and pathologically relevant concentrations of IL-6 resulting in reduced cluster size and protein content, as

well as reduced viability when treated with 5 ng/ml or above (Conroy *et al.,* 2004). Neutralisation of IL-6 produced improved motor coordination recovery, as well as potentially reducing the extent of BBBD in a weight drop model of TBI (Yang *et al.,*2013). Injured mice performed significantly worse on rota-rod tasks compared to sham mice 24 hours after injury. However, neutralisation of IL-6 resulted in injured mice retaining motor co-ordination comparable to that of sham injured mice. In addition to improved motor performance, serum levels of NSE, a protein currently investigated for its potential use as a biomarker of BBBD in TBI, was also decreased relative to vehicle-treated mice.

1.6: Magnetic resonance imaging in TBI

MRI and CT scans are powerful tools capable of assessing numerous aspects of brain pathology in living patients. Anatomical scans such as T1-3D sequences can be used to identify gross anatomical changes in brain volume. Diffusion tensor imaging (DTI) and diffusion-weighted imaging (DWI) focus on anatomical changes in the brain architecture, and functional MRI (fMRI) is capable of identifying regions involved in a range of cognitive tasks via changes in blood flow. In addition to these techniques, dynamic contrast-enhanced MRI (DCE-MRI) is useful in identifying areas of compromised blood vasculature, a possible hallmark of several neurological conditions (Bell & Zlokovic, 2009; Dorthey et al, 2016; Stolp & Dziegielewska, 2009). However, while MRI continues to be used to gain a better understanding of the minutia of several neurological conditions, its utility in identifying changes associated with TBI, especially in the context of mTBI, has yet to be fully established. This is most clearly illustrated in the WHO definition of mTBI, which does not include medical imaging findings among its diagnostic criteria, many other definitions include the caveat that lesions are not always found on CT or MRI scans of mTBI cases and some definitions suggesting that the presence of pathological findings upon medical imaging places the injury within the moderate to severe category (Cassidy et al., 2004, Kristman et al., 2014). Despite this, several studies, both pre-clinical and animal, have put forward possible means by which medical imaging can be used to identify structural damage to the brain and neurovascular unit as a result of TBI

1.6.1: T1 and T2-based analysis

A T1-3D sequence produces a series of images across axial, sagittal and coronal planes of the brain with high contrast between CSF, grey matter (GM) and WM. Analysis of these scans can be processed with the use of software packages such as FreeSurfer and

FSL-FIRST. Volumetric analysis can be used to identify potential early stages of atrophy associated with neurodegenerative diseases such as AD, PD and CTE (Gavett, Stern & McKee, 2011; Koga *et al.,* 2016; Weintraub *et al.,* 2011; Zeighami *et al.,* 2015; Migliaccio *et al.,* 2015). Cell loss and volumetric changes in specific brain regions, such as those associated with executive function, memory and impulse control, have been observed in human and animal studies of TBI. While these changes are most identifiable in moderate and severe injuries, differences in brain volume have been observed in mTBI patients in life with the use of T1 and T2-weighted sequences. Animal models of TBI, studies examining mild and moderate TBI have found reduced neuronal density, increased neuronal death and gliosis, up to a year post-injury (Balança *et al.,* 2016; Baratz *et al.,* 2015; Gao *et al.,* 2011; Hick *et al.,* 1993; Smith *et al.,*1997). Alongside this loss in viable neuronal cells is a reduction in neurological function, particularly in regions responsible for memory. In life measurement of neural cell density is capable via T1 and T2 MRI paradigms, and is being investigated as a potential marker of future onset of neurodegenerative conditions.

A study examining adolescents who had recovered from a moderate or severe TBI observed that changes in regions of the corpus callosum and ventricular area correlated with reduced performance in neurological tasks probing memory, as well as reduced general intelligence compared to age-matched controls (Verger *et* al, 2000; Verger *et* al., 2001). Atrophy in the head of the hippocampus was observed in an adult cohort of moderate TBI within a week of injury when compared to uninjured controls (Ariza *et al.,* 2006). This was in turn associated with reduced performance in memory tasks. In the context of mTBI, comparison of controls to recovered individuals found widespread compromises to both GM and WM integrity in those with a history of multiple mTBIs (Little *et al.,* 2014). Atrophy was observed for the right internal capsule and right ventrolateral prefrontal white matter, as well as parahippocampal and cerebellar grey matter. While changes in tissue density were present in individuals who had sustained a single mTBI, individuals with a history of multiple mTBIs showed the most widespread changes in tissue density, as well as greater loss of tissue integrity than those who had sustained a single TBI. Reduced neurological performance was also observed in both single and multiple mTBI individuals in domains associated with attention, executive function and memory. Accompanying these neurological findings were reductions in tissue density within regions of the parahippocampal gyri, anterior temporal lobe and

internal capsule, regions associated with memory performance. However, only 33% of mTBI individuals fell outside of 2 standard deviations of the control neurological scores when normalising for volume changes. This suggests that at the time of the study, changes in tissue density were not significantly impacting neurological performance; and may have represented a progressive state in mTBI-induced neurological decline.

Reductions in the area of several regions were observed in mTBI patients up to two months post-injury compared to controls, including regions such as caudate, the right thalamus and right putamen (Zagorchev *et al.,* 2016). These regions continued to demonstrate significantly reduced volume over 1-year post-injury, in addition to regions of the amygdala that showed significant reductions in volume over the time period. Hippocampal volume also trended towards significant reductions 1-year post-injury. Changes within regions not highlighted upon initial assessment may reflect decay in associated regions and Wallerian degeneration within the brain, or regional-specific responses and preservation mechanisms due to injury.

In a study of military veterans who had sustained either a mild or moderate blast-induced TBI, cortical thickness was found to be significantly reduced in both groups compared to that of controls (Michael *et al*, 2015). Cortical thinning was significantly correlated with measures of depression and post-traumatic stress disorder (PTSD) but not with neuropsychological measures such as executive function or memory. In a small study examining young adults who had sustained at least 2 sports-related mTBIs and who were not currently recovering from a head injury, bilateral thinning of sub-cortical regions within the frontotemporal cortex was found when compared to non-injured controls (List *et al.,* 2015). The extent of cortical thinning within bilateral insula, the middle temporal cortex and right superior temporal cortex were correlated with the number of mTBIs reported. Despite observed volumetric changes in the mTBI group, mean fractional anisotropy (FA) and mean diffusivity (MD) values, measures of axonal tract health discussed below, were not significantly different to that of controls. Cognitive assessment also indicated no large differences between the groups. However, overall, the mTBI group tended to preform worse in almost every neuropsychological assessment.

These findings show that anatomical and macroscopic changes of the brain can be observed within life of TBI patients and are associated with neurological performance changes. However, as has been mentioned and will be discussed below, these changes

may reflect a more transient state than end-stage atrophy associated with neurological disease.

1.6.2: Diffusion tensor Imaging (DTI)

Diffuse Axonal Injury (DAI) is thought to be a hallmark pathology of TBI and is particularly evident in moderate and severe TBI (Gentleman *et al.,*1995; Meythaler *et al.,* 2001; Smith & Meaney, 2000). However, it is thought to be a result of injuries of all severities, as autopsies of mTBI patients reported similar axonal pathology to those of more severe injuries (Blumbergs *et al.,*1994; Oppenheimer, 1968,). The acceleration-deceleration forces applied to the head and brain during TBI causes contortion of the brain as it is compressed, rotated and stretched, resulting in shear stress being applied to axonal tracks (Bigler & Maxwell, 2011). Through the use of finite force models, several regions have been identified as being particularly at risk of shear damage due to their location and/or tissue make up, for example: areas comprised of grey-white matter junctions, the corpus callosum, the fornices and rostral brainstem, (Inglese *et al.,* 2005; Filley & Kelly, 2018). Shear stress resulting from these contortions can propagate along white matter fibres, resulting in compromised axonal tract integrity throughout the brain and within regions distal to the focal injury. DTI utilizes the diffusion of water molecules within the confines of neural tissue and long fibre tracts to estimate axonal tract integrity; via four output measurements; axial diffusivity (AxD), radial diffusivity (RD), mean diffusivity (MD) and fractional anisotropy (FA). Each of these values are used to estimate a different facet of axonal fibre integrity; such as AxD gauging fibre length and maturation, RD changing in response to axonal myelination and density, MD being an inverse measure of axonal membrane density and FA giving an overall measure of microstructure integrity (Alexander *et al*. 2007). The use of these measurements allows for the detection of subtle changes in brain structure, potential indicators of DAI in life. Therefore, DTI facilitates long-term follow-up of changes in the brain's microstructure after injury. To this end, DTI has been heavily investigated for its potential in detecting subtle changes in fibre tract integrity associated with mTBI in the absence of other diagnostic findings. However, while several studies have demonstrated changes in axonal tract integrity following mTBI, there remains a lack of consensus as to the direction in which these changes occur, and their pathological significance remains to be fully explored.

For example, increases in FA and reductions in RD within the regions of the corona radiata, the cerebellar peduncles and corpus callosum were reported in children assessed within 30 days post-injury (Van Beek *et al.,* 2015a). While the data indicated that FA was higher and RD lower in the injured cohort shortly after injury, no statistical differences between the groups within this time frame were reported. In an inter-hemisphere comparison, a small study found upon, individuals who had suffered a mTBI had a larger difference in FA values across 7 regions of interest shortly after injury (Arfanakis *et al.,* 2002). Changes in FA and MD were also observed in a prospective study comparing non-concussed ice hockey players to non-contact controls following a season of play (McAllister *et al.,* 2014). MD was significantly higher in the corpus callosum of returning players exposed to head trauma compared to their non-contact counterparts, in addition to significant changes in FA and MD within the amygdala of the contact group. Changes in axonal parameters within these regions, as well as changes within the cerebellum and hippocampus, were found to be associated with exposure to non-concussive head impacts incurred over the course of the study period. A detrimental effect on verbal learning and memory was observed in a sub-group of hockey players, which was associated with the largest change in MD within the corpus callosum. These findings build on an earlier, smaller study that found an increase in the number of WM voxels indicating a change in FA and MD at the end of a season of ice hockey, was dependant on the number of head traumas sustained during the season (Bazarian *et al*., 2012). Players exposed to head impacts showed the greatest number of voxels displaying a change in WM parameters, accumulating with the highest changes being observed in a single concussed player. Over the course of the study, 10 regions in which tract parameters had significantly changed were identified; including the corpus callosum, the inferior fronto-occiptial fasciculus, bilateral regions of the internal capsule and adjoining regions of the hippocampus. In most instances, members of the contact group displayed a larger proportion of voxels increasing in FA and decreasing in MD, while values for members of the control group remained largely similar to their baseline proportions.

Reductions in FA and increases in RD were identified in regions of the corpus callosum of retired American football players with an early exposure to head trauma (under the age of 12 years old at first exposure) (Stamm *et al,* 2015). These changes were matched with increased cognitive impairment and behavioural changes. Exposure to mTBI also appears to limit the development of axonal tracts in children, with FA and RD significantly

changing over time for control children but not for those exposed to mTBI. Controls showed significant increases in FA and decreases in RD within structures of the corpus callosum over a 6-month period, compared to children with recent exposure to mTBI, who displayed limited changes over that time (Van Beek *et al.,*2015b). This lack of change in mTBI patients was associated with decreased working memory scores compared to controls. Other studies have highlighted the impact of sub-concussive blows on tract integrity. Using a region of interest (ROI) approach, Bahrami *et al*. (2016) found that there was a significant correlation between reductions in FA within the fronto-occipital fasciculus and exposure to sub-concussive head injuries. Comparisons between pre- and post-season FA values found that overall, FA reductions within the inferior fronto-occipital fasciculus (IFOF) were due to decreases in FA within the central part of the fibre tracts, as well as at fibre terminals. This correlation was only observed unilaterally, possibly representing exposure of a single side to injury. No significant changes were observed within structures of the corpus callosum. A similar correlation had previously been observed for increases in abnormal WM voxels (measured via FA and MD changes) over the whole brain, decreases in verbal memory and cumulative head forces (Davenport *et al.,*2014). Long term analysis of mTBI patients also indicate that changes in axonal damage can persist and develop. A study of blast-induced mTBI patients found developing axonal damage in patients up to 12 months post-injury compared to controls (Donald *et al.,*2011). Relative anisotropy remained significantly lower in mTBI patients compared to controls, while AxD was found to be significantly reduced compared to initial assessment (up to 90 days post-injury), suggestive of delayed axonal damage. Another study found a significant interaction between age and blast exposure, and changes in tract integrity (Trotter *et al.,* 2015). A greater negative relationship between RD and FA and age was observed in blast-exposed individuals compared to unexposed controls, with changes being greater in older individuals. Lifespan trajectories of FA were plotted based on age and blast-exposure and found that blast-exposed individuals also showed accelerated loss of axonal tract integrity compared to controls. This relationship appeared to be unique to blast exposure, as when comparing TBI exposure-age interactions and diffusion parameters, no significant interactions were observed. The lack of an identified interaction suggested that blast-exposed mTBI may be a distinct injury to that of blunt force mTBI.

1.6.3: Contrast enhanced MRI

Dynamic contrast-enhanced MRI (DCE-MRI) allows for quantitative assessment of BBB permeability through measurement of an administered contrast agent, such as gadolinium- or ferumoxytol-chelates (Padhani *et al.,* 2004; Hope *et al*., 2015). The signal of the contrast agent is quantified, as well as other parameters such as blood flow and tissue perfusion; applied to one or more tracer-kinetic models for post-processing. The Tofts Extended Model is one of the most commonly applied models used in DCE-MRI assessments, the primary output of which is the volume transfer constant (K^{trans}). Secondary outputs of the model, such as interstitial volume (v_e), the rate constant (k_{ep}) and plasma volume (v_p), are also used to gauge aspects of vascular health (Sourbron & Buckley, 2011). K^{trans} is considered the primary endpoint by which changes to barrier integrity in anti-angiogenic or anti-neovascular therapies is assessed and is used to estimate barrier properties in conditions such as MS, AD, glioblastomas and stroke (Starr *et al.,* 2009). However, a limited number of studies have used K^{trans} measurements in the context of human TBI studies, due to considerations of high signal/ noise ratio, low spatial resolution and ethical considerations surrounding the administration of gadolinium chelates (Li *et al.,* 2014; Pinter *et al.,* 2016). Therefore, evidence collected from the use of DCE-MRI in conditions previously mentioned is an indicator of the potential DCE-MRI parameters have in assessing BBB dysfunction in TBI. For example, one study found that MS and vascular cognitive impairment (VCI) patients showed markedly higher volume transfer coefficient when compared to a threshold set by controls (Taheri *et al.,*2016). The control cohort was used to threshold for noise present in DCE scans, while the Patlak model was used to determine the equivalent volume transfer (K*i*). MS and VCI patients showed markedly higher K*i* within WM regions, as well trending to higher mean K*i* values compared to controls. In a sub-cohort of MS and VCI, CSF/Serum albumin ratios were available and correlated with mean K*i* values of white matter regions

In another study, patients with post-traumatic epilepsy were suggested to have increased barrier permeability compared to age-matched, non-epileptic TBI patients reporting some degree of PCS (Tomkins *et al.,* 2008). In both groups, most patients had their TBI classified as mild. Within both groups, post-traumatic lesions were identified; and using a semi-quantitative method of counting pixels with significant signal intensity changes post-contrast, barrier integrity was assessed. Of the initial cohort of 32, 14 (43.75%) were

identified as having increased BBB permeability, with 13 of 14 patients having a proximal old haemorrhagic contusion to regions of increased signals. Post-traumatic epilepsy patients were found to be more likely to present with a lesion, as well as have associated impaired barrier integrity than non-epileptic patients, in addition to having a significantly larger cortical volume displaying BBBD. In a sub-group of patients with higher barrier permeability, standardised Low-Resolution Brain Electromagnetic Tomography indicated that regions of abnormal delta activity were matched to the same cortical regions indicating disrupted BBB integrity.

Much of the data applicable to the use of DCE-MRI in detecting minor changes in barrier permeability come from animal models, which, while approximating a mTBI, often involve procedures or injuries, such as craniotomy or skull fracture, that may invalidate that term.

A study in rabbits found that K^{trans} values calculated by the extended Tofts model were significantly different in injured animals compared to controls, including those that received a mild injury (Wei *et al.,*2011). Utilizing a weight-drop model of TBI to produce mild, moderate and severe injuries, the study found that average K^{trans} values were greater at injury severities compared to sham injuries in animals 24 hours post-injury, with the greatest increases being observed in severe TBI. Along with this, increasing K^{trans} values correlated with the increasing size of lesion volume across all injury severities. Correlation of K^{trans} with lesion volume as a reference of injury suggests that within the weight-drop model, changes in BBB integrity may be detected within the acute stage of injury by neuroimaging and accurately reflect the extent of the injury. A similar study, also in rabbits and using a weight drop model, although this time performing a craniotomy 24 hours prior to TBI, found that increases in K^{trans} were accompanied by developing neurological deficits and WM changes (Li *et al.,* 2011). K^{trans} measurements recorded at 3- and 7-days post-injury were found to predict scores of the Veterinary Coma Scale (VCS), the animal equivalent to the Glasgow Coma Scale (GCS), at 30 days post-injury, as well as 3-days post-injury K^{trans} values correlating with neurological performance. This relationship is valid for both focal and perifocal lesion values, although most clear when considering focal lesion values. This increase in transport coefficient was accompanied by an increase in apparent diffusion coefficient, a general measure of water movement within tissue. However, while a similar model was employed, Li *et al.*'s (2011) model appears to more closely resemble a severe TBI, as noticeable swelling outside the skull

was observed in MRI scans, incidences of fatal injury were reported, and a craniotomy was performed prior to injury While these confounders may be somewhat mitigated by the delay in the application of TBI, they still represent observations more consistent with moderate and severe injuries.

In an attempt to mitigate some of the issues with applying the Tofts models to TBI research, namely the low spatial resolution and unfavourable signal-to-noise ratio, an extended DCE scan sequence showed improved localisation of BBBD, compared to non-specific oedema findings from T2 scans (Li *et al.,*2014). Rats were scanned 1 hour after receiving a CCI using a modified DCE scan protocol. The study extended the DCE sequence to include a pre-scan module, to allow mapping of flip angle and magnetization distributions, as well as compared and combined reversible and irreversible models of contrast agent leakage. Li *et al*.'s (2014) study found that application of a reversible model to non-injured regions increased the level of noise in K^{trans}. However, if an irreversible model is applied in assessing leakage within the focal region of the injury, barrier permeability is significantly underestimated compared to use of a reversible model. Conversely, application of reversible modelling to assess normal, non-injured tissue significantly increased the level of noise present throughout the rest of the scanned region. A combination of both modelling methods allowed for retention of accurate assessment of focal and perifocal barrier leakage, as well as reductions in the levels of noise present in non-injured tissue. This combined model suggested leakage to a similar extent to that found upon histopathological examination using Evans Blue extravasation. The model also indicated a non-linear relationship with the extent of oedema formation, as measured using T2 sequences.

Utilizing a similar model, a more recent study in rats also reported that increases in K^{trans} values following injury were accompanied with increased oedema and BBB break down (Lu *et al.,*2018). K^{trans} was higher in animals that received an injury compared to sham injured animals within 3 hours of the injury, and higher values were sustained for up to 72 hours. This matches corresponding findings of increased brain water contents over the 72 hours, as well a gradual decline in the TJ proteins; ZO-1 and Occludin, and increases in MMP-9, all important mediators in maintaining barrier integrity. However, while the speed of impact and impact depth in this model was low (1.5m/s and 2.5mm respectively), the study reflects more a moderate to severe model, due to the removal of the skull and direct impact of the impactor tip on to brain tissue.

As mentioned, the use of DCE-MRI and BBB assessment in mTBI cases is limited. However, some studies have investigated the utility of the technique in humans.

For example, one study found that repetitive blows associated with normal play of American Football can alter BBB permeability, even in the absence of a mTBI (Wiessberg *et al*. 2014). American football players and track-and-field athletes, who served as control athletes, were scanned after two months of competitive participation, at which point barrier integrity was assessed across the whole brain using a novel linear-fit model mentioned early in this section. The threshold value of high BBB permeability was set to the 95th percentile value of all slopes observed within the track-and-field athletes and BBBD was measured by the percentage of suprathreshold voxels was accessed for each participant. Application of Gaussian modelling clustered participants into two distinct groups; one with a low percentage of suprathreshold voxels and referred to as the "intact" BBB group, and a group with a high number of suprathreshold voxels, termed a "pathological" blood-brain barrier group. Within the intact group, 55% (11/20) were control athletes, compared to 14.2% (1/7) in the "pathological" group. The authors reported no differences in the number of head injuries sustained between the players and control groups, nor between intact and "pathological" groups, suggesting that repetitive sub-concussive force to the brain may be sufficient to induce changes in BBB integrity.

A recent case-control study found that individuals with Post-Concussion Syndrome (PCS) had higher K^{trans} and v_e (extravascular extracellular volume) values than that of controls (Yoo *et al.,*2018). Individuals with PCS, who were scanned at least 2 months following their injury, showing no signs of trauma related-haemorrhagic lesions, compared to controls that displayed no abnormal findings on MR scan, including WM lesions. Using a ROI approach, the study found that K^{trans} and v_e were significantly higher in both normal appearing WM and regions displaying DAI. Regions of DAI investigated included bilateral frontal GM matter interfaces and the dorsolateral midbrain. While regions of the corpus callosum and temporal GM matter interfaces were also highlighted as regions of DAI, they did not show significantly different K^{trans} values. However, median values did tend to be higher in these regions compared to controls. Also observed was a decrease in the difference between K^{trans} values at regions of DAI between PCS individuals and controls after 3 months post-injury, which the authors suggest may reflect re-establishment of integrity within damaged WM regions. In a sub-cohort of individuals with paired MR scans and neuropsychological testing, correlation analysis identified a

relationship between v_e values, T2 hyperintense WM lesions and performance in Verbal Learning Test (delayed recall). In addition, digit span tests Receiver operating characteristic (ROC) analysis showed that median v_e at regions of WM lesions was able to distinguish between controls and PCS patients with a sensitivity of 100% and specificity of 71%.

While these injuries are more severe than a mTBI and occurred during a dynamic time in brain development, they illustrate the long-lasting effects TBI have on brain structure and neurological function.

Objective of this study

Maintenance of the BBBD plays a critical role in neuronal homeostasis by limiting the passage of macromolecules, ions and immune cells into the neural space. Aberrant disruption of the barrier is being increasingly linked to the early processes involved in the development of a number of neurological and psychiatric conditions (Greene *et al.,* 2017, Nation *et al.,* 2018). However, TJ proteins of the BBB have also been demonstrated to display considerable plasticity within the regions of neural injury, capable of repair even in presence of large scale, long-term damage such as that observed in vasogenic oedema (Campbell *et al.,* 2012). As moderate and severe TBI is suggested to be one of the largest environmental risk factors in the development of a neurodegenerative disease, BBBD and loss of homeostasis may act as linking factor between physical injury and molecular pathology. However, in recent years attention has focused on the risk posed by repetitive mTBI, such as those incurred by military personnel or athletes engaged in contact sports. While epidemiology studies have yet to fully characterise a link between mTBI and dementia risk, the neurological condition, CTE (previously dubbed Dementia Pugilistica) has been closely associated with repetitive mTBI since its first description in retired boxers (Castellani & Perry, 2017). In recent years, case studies have put forth evidence of BBBD individuals engaged in contact sports and subsequently diagnosed with CTE (Doherty *et al.,*2016, Tagge *et al.,*2018). BBBD has been well characterised in moderate and severe, however, mTBI on the BBB have remained understudied, particularly in humans.

The primary aim of this study was to characterise the changes in BBB in the context of sports-related mTBI. To that end, the objectives of this study consisted of:

• Characterisation of TJs and BBB integrity in TBI-related dementia cases.

• Investigate the utility of blood-based biomarkers, S100β, BDNF and MCP-1/ CCL2 in identifying BBBD in contact-sports athletes over a period of 8 months in rugby athletes and within 48hrs of an organised bout in MMA athletes.

• Investigate the evolving immune response of peripheral immune cells of contact sports athletes to necrotic brain tissue.

• Establish the use of novel DCE-MRI analysis techniques and investigate their utility in detecting changes to BBB integrity in the context of sport-related mTBI over a period of 8 months in rugby athletes and within 48hrs of an organised bout in MMA athletes.

.

CHAPTER 2: MATERIALS AND METHODS

2.1: Immunohistochemistry

Previously prepared 10 μm paraffin-embedded sections, as well as cryosections of post-mortem human brain tissue from each individual reported in Chapter 3 were obtained with ethical approval from the Dublin Brain Bank at Beaumont Hospital in Dublin. Ireland. Neuropathological diagnoses for each subject are detailed in the reports of Chapter 3 were prepared by Dublin Brain Bank personal.

Paraffin-embedded sections underwent xylene-based deparaffination and sequential rehydration by submerging sections five times in solutions of 100%, 90%, 70% and 60% ethanol solutions before submersion in dH_2O. Following rehydration, sections underwent additional antigen retrieval. Antigen retrieval was performed by boiling the deparaffinated sections in sodium citrate buffer (l0 mM sodium citrate. 0.05% Tween-20. pH 6.0) for two 5-minute rounds.

Following deparaffination and antigen retrieval, sections underwent the protocol for immunohistochemistry. Sections were blocked in 5% normal goat serum (NGS, Sigma), 0.5% Triton X-100 in PBS for 20 minutes before overnight incubation with primary antibodies in dilutions of 1:100 to 1:250. Following overnight incubation in primary antibody solutions, sections underwent five 5-minute washes in PBS, before sections were incubated with fluorescently-conjugated secondary antibodies at a dilution of 1:500 for 3 hours at room temperature. After incubation in secondary antibody solutions, sections underwent five 5-minute washes in PBS. Following washing, sections were counterstained with Hoechst 33258 for 30 seconds at a dilution of 1:5,000 of a stock 1 mg/mL solution to visualise nuclei. After nuclei counterstaining, sections were mounted using Polymount Aqua Medium and a coverslip. Sections were stored at 4°C until imaged. Imaging of staining sections was carried out using a Carl Zeiss LSM 710 confocal microscope.

Primary antibodies used were rabbit anti-claudin-5 (1:250 dilution, Invitrogen), mouse anti-p-tau (1:250 dilution, Innogenetics), and rabbit anti-ZO-1 (1:250 dilution, Invitrogen). Labelling of human IgG and fibrinogen used Cy3-conjugated goat anti-mouse IgG (1:100, Abcam) and FITC-conjugated rabbit anti-human fibrinogen (1:100, DAKO) respectively. Secondary antibodies used were goat anti-rabbit Cy3 conjugated antibodies (1:500, Abcam) and goat anti-mouse Alexa Fluro-488 conjugated antibodies (1:500).

2.2: Patient recruitment

All ethical approvals were in place prior to initiation of studies on human subjects. Over the course of the study, a total of three participant groups were recruited to the study: a school-aged rugby cohort (aged 17-18), a university-age rugby cohort (aged 21-24) and an MMA cohort (aged 24-29). All participants were healthy at the time of recruitment. Participants were recruited through direct contact with coaches of the school and university teams, and through information evenings carried out by the research team in MMA gyms in the Dublin area. Participants were considered suitable for the study if: 1) they actively engaged in the contact sport under study (either rugby or MMA); 2) they had not been diagnosed with any mental health disorders; 3) they had no history of kidney problems; 4) they were good health at the time of enrolment. Demographic charts detailing mTBI history and timing of post-season MRI assessment for rugby participants (**Table 2.1**) and MMA participants (**Table 2.2**) were made from information collected during baseline, post-match and post-season assessment by medical personnel conducting physical assessment.

Rugby participants were assessed within a month of the beginning of the competitive season. The competive season lasted roughly 8 months, beginning in September 2015 and ending in late April 2016. Following the completion of the competitive season, returning participants were assessed with two months of the match. In the cases of rugby participants who underwent post-match assessment, this was carried out within 2 hours of the match. Post-match blood samples, as well as baseline and post-season samples, were processed immediately onsite following collection.

MMA participants underwent baseline assessment during a period in which they were one week detached from a competive bout and heavy-contact sparring sessions. This period was chosen to accommodate participants' training schedules. Post-bout assessments were carried within 48hrs following a competitive bout.

Assessments for all participants at all time points were carried out in a clinical research facility in St. James' Hospital, Dublin, by professional medical personal. Assessment at each time point consisted of participants undergoing a brief physical examination by medical personal to assess any external injuries, as well as noting any neurological complaints consistent with symptoms of mTBI present at the time of assessment. Blood samples collected via venepuncture were collected during the assessment by either

medical personal or trained research staff. Blood samples consisted of two 8ml EDTA-coated tubes for PBMC and plasma isolation, one 5ml “red capped” tube for serum collection and one 5ml “red capped” tube which was sent to St James’ haematology department for routine blood screening and creatine assessment. MRI scans carried out at the immediately after blood collection consisted of T1, T2 and FLAIR scans, as well as DCE-MRI and DTI sequences.

Initially, 22 participants for the school-aged cohort and 10 participants for the university-aged cohort, totalling 33 participants signed up for the study. However only 11 of the school-age cohort and 7 from the university-aged cohort were retained for post-season evaluation, resulting in a total of 18 participant available for comparison with baseline values (**Figure 2.1**). In addition, the university-based team participants (Initially n = 10 recruited but only n = 7 were scanned post-match) underwent an assessment within 2 hours of playing a full contact competitive rugby match.

To date, 18 participants have been recruited as part of the MMA cohort. However, at the time of submission only 5 participants had undergone baseline and post-bout assessment, and therefore only these 5 participants were eligible to be included in this work.

		Concussion History				MRI Inclusion				
School-aged cohort										
Subject	Age	# of Prior mTBIs	Most Recent mTBI	Time for symptom resolution	Hospitalisation	Baseline		Post-Season	Time until Post-season assessment	Medication taken at assessment
1	17	1	>6 months	≤ 1 hour	No	✓		✓	2 weeks	-
2	18	0	N.A.	-	N.A.	✓		✓	3 weeks	Ibuprofin
3	17	1	>3 months	≤ 24 hours	No	✓		✓	3 weeks	-
4	17	0	N.A.	-	N.A.	✓		✓	3 weeks	-
5	17	0	N.A.	-	N.A.	✓		✓	2 months	Ventolin
6	17	0	N.A.	-	N.A.	✓		✓	2 months	-
7	16	1	>2 years	1 week	No	✓		✓	2 weeks	-
8	18	0	N.A.	-	N.A.	✓		✓	3 weeks	-
9	17	0	N.A.	-	N.A.	✓		✓	3 weeks	-
10	19	0	N.A.	-	N.A.	✓		✓	1 week	-
11	18	0	N.A.	-	N.A.	✓		✓	1 week	-
University-aged cohort										
Subject	Age	# of Prior mTBIs	Most Recent mTBI	Time for symptom resolution	Hospitalisation	Baseline	Post-match	Post-season	Time until Post-season assessment	Medication taken at assessment
12	21	1	>6 months	5 days	No	✓	✓	✓	2 months	-
13	22	0	N.A	-	N.A.	✓	✓	✓	2 months	-
14	21	6	>1 yr.	7 weeks	No	✓	✓	✓	2 months	Ventolin
15	23	1	>2 yrs.	≤ 24 hours	No	✓	✓	✓	2 months	-
16	21	2	>1 yr.	1 week	No	✓	✓	✓	2 months	-
17	22	0	N.A.	-	N.A.	✓	✓	✓	2 months	-
18	23	0	N.A.	-	N.A.	✓	✓			-
19	24	3	>2 yrs.	6 days	No	✓	✓			-

TABLE 2. 1: DEMOGRAPHICS OF PARTICIPANTS ENGAGED IN RUGBY.

Clinical history of mTBI for both school-aged and university-aged participants engaged in rugby. History of mTBI, time for resolution of symptoms, hospitalisation status and currently taken medication was documented by medical personnel during baseline assessment and followed up at post-season assessment. Demographics were included for participants retained for post-season and post-match assessments.

		Concussion History				MRI Inclusion			
MMA cohort									
Subject	Age	# of Prior mTBIs	Most Recent mTBI	Time for symptom resolution	Hospitalisation	Baseline	Post-fight	Time until Post-season assessment	Medication taken at assessment
1	25	4	> 3 months	5 days	No	✓	✓	24 hours	-
2	26	0	N.A.	-	N.A.	✓	✓	24 hours	-
3	28	1	> 1 yr.	≤ 24 hours	No	✓	✓	48 hourss	-
4	24	1	>2 yrs.	≤ 24 hours	No	✓	✓	24 hours	-
5	29	3	>1 yr	≤ 1 hour	No	✓	✓	24 hours	-

TABLE 2. 2: DEMOGRAPHICS OF PARTICIPANTS ENGAGED IN MIXED MARTIAL ARTS.

Clinical history of mTBI for participants engaged in MMA. History of mTBI, time for resolution of symptoms, hospitalisation status and currently taken medication was documented by medical personnel during baseline assessment and followed up at post-season assessment. Demographics were included for participants available for inclusion in the study at time of submission.

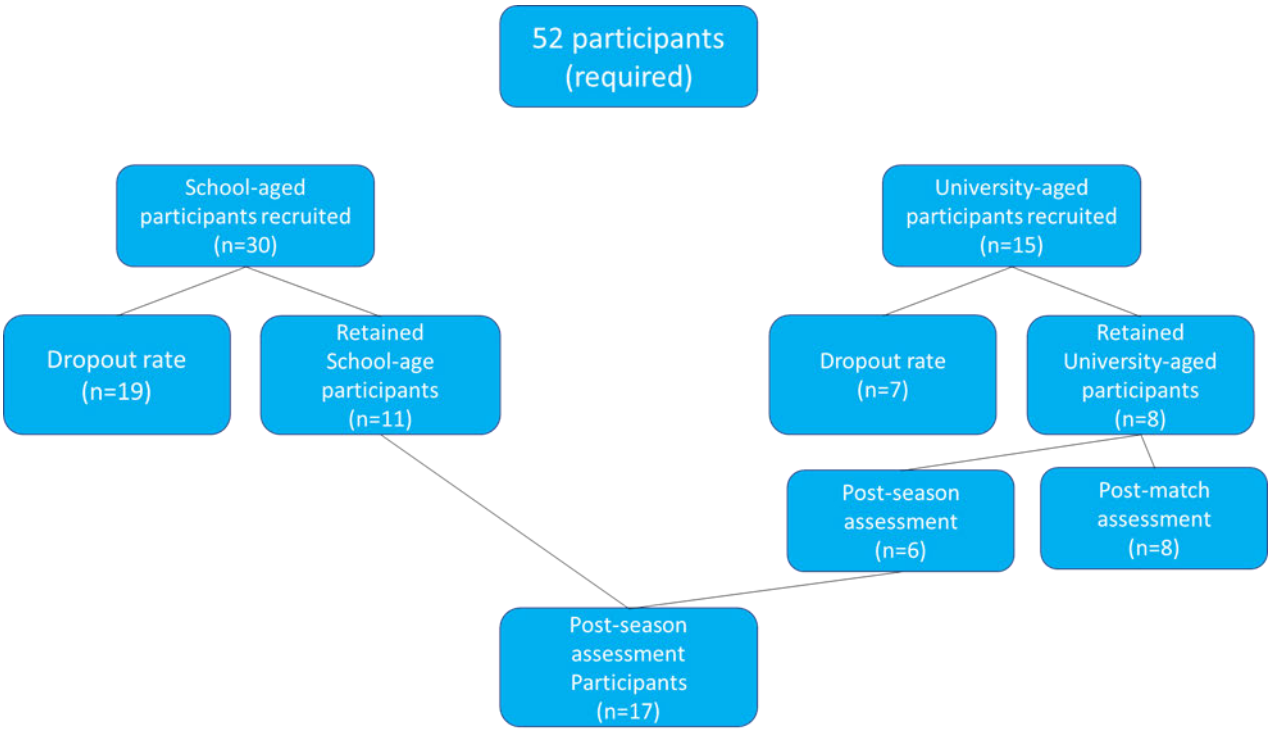


Figure 2. 1: Flowchart of participant retention within the rugby cohort.

A flowchart detailing participant retention with the school-aged and university-aged rugby cohorts. Prior to the commencement of the study, power analysis indicated a cohort size of 30 participants in order to detect a moderate effect size. Initial recruitment of within a local school rugby club garnered the required numbers for initial assessment. However, many participants dropped out of the study (n=19), either through not attending baseline assessment dates or choosing not to complete the post-season assessment. The university-age cohort was recruited to bolster cohort numbers and engaging with a local university team yielded an additional 15 participants. Within this cohort, dropout rates remained high (n=7), primarily due to participants choosing not to attend post-season assessment. Of the retained cohort, two participants did not attend post-season assessment, but did undergo post-match assessment. University-aged participants that completed post-season assessment were pooled with school-aged participants for a pre-post-season cohort of 17 participants. The pre-post-match cohort total 8 participants

2.3: PBMC isolation from human whole blood

Plasma, Peripheral Blood Mononuclear Cells and erythrocyte cell fractions were isolated via the density gradient separation protocol as follows.

Whole blood (~16mls) collected in EDTA-coated tubes was diluted in a 1:1 ratio with PBS. Diluted whole blood was gently layered atop 10ml of Lymphoprep (Stemcell Technologies, Vancouver, Canada). The buffy coat layer of whole blood containing the PBMCs was separated from whole blood by spinning the layered blood at 400G for 45mins. Acceleration and deceleration of the centrifuge was set to zero. Following this step, the whole blood separated to plasma, buffy coat/PBS and red blood cell layers. The plasma layer was gently removed in order to not to disturb the buffy coat layer. The collected plasma was stored at -80^{o}C until required. The buffy coat layer was gently removed in order to not collect any of the red blood fraction and transferred to a fresh falcon tube. The buffy-coat layer was washed three times by resuspending the layer in 40ml sterile PBS, and the spun down at 1000rpm for 15 minutes. Acceleration and deceleration of the centrifuge was set to maximum. Following washing the, the cell pellet made up of PBMCs were resuspended in RMPI culture media supplemented with 10% DMSO and frozen for later use at -80^{o}C overnight, before being transferred to liquid nitrogen for long-term storage.

2.4: Necrotic Brain Conditioned Media

In order to generate cell media supplemented with antigens of necrotic brain tissue, brains were dissected from healthy adult C57/BLJ mice. Following dissection, the olfactory blub and brain stem were removed, and the brain gently divided into units of one hemisphere each (0.2-.0.3g). One hemisphere per 3 ml of RMPI media was used to generate the conditioned media. RPMI media containing the dissected brain tissue was flash frozen in liquid nitrogen, before being allowed to thaw completely in a 37^{o}C water bath. This freeze-thaw process was repeated 3 times. Brain tissue that remain intact was homogenized. Particulate matter in the media was pelleted by centrifuging the brain-containing media at 1500 g for 30 min. The supernatant was gently removed from the tissue pellet and sterile filtered with 0.2-micron filter in to a fresh storage tube. Conditioned media was stored at -20^{o}C until required.

2.5: PBMC stimulation with Necrotic Brain Conditioned Media

After being brought up in 10% FBS/ RMPI media, PBMCs were counted and seeded in a 96-well plate at a density of 1x106 cells/ ml.

Cells incubated overnight at 37ºC/ 5% CO_2. Cells were then spun down at 1000rpm for 10min to ensure cells were at the base of the well before the media was carefully removed. Cells were supplied with either fresh 10% FBS/ RMPI, which served as the negative control, or 10% FBS/ RMPI supplemented with LPS (100ng/ml). The cells were primed with LPS supplemented media for 3 hours. After the 3-hour incubation, cells were spun down at 1000rpm for 10min and the media was removed. Fresh media containing before being spun down and treated with either ATP supplemented or conditioned media. Cells were incubated for 30mins at 37oC before being spun down and the supernatant collected.

Collected supernatants were analysed for release of IL-1β and IL-6 via ELISA (R&D Systems, Minneapolis, USA) per the manufacturer's recommended protocol (Section 2.6).

2.6: Enzyme-linked immunosorbent assay (ELISA)

Supernatants collected from PBMCs exposed to necrotic brain conditioned media and plasma samples collected via venepuncture were screened for various analytes via ELISA kit, the specifics of which are described below. The absorbance of each well was read at 450nm on a microplate photometer (Thermo Fisher Scientific Multiskan FC). Concentrations of the analytes were determined from a standard curve after correcting for background absorbance and dilution factors.

IL-1β in PBMC supernatant was measured using Human IL-1β/ IL-1F2 DuoSet ELISA Kit (R&D Systems). The range of detection was between 3.9 and 250 pg/ml.

IL-6 in PBMC supernatant was measured using Human IL-6 DuoSet ELISA Kit (R&D Systems). The range of detection was between 9.4 and 600 pg/ml.

Concentrations of biomarkers contained in plasma samples of both rugby and MMA participants were analysed using the following ELISA kits. Absorbance of each well was read at 450nm on a microplate photometer (Thermo Fisher Scientific Multiskan FC).

Concentrations of the analytes were determined from a standard curve after correcting for background absorbance and dilution factors

S100β in human plasma samples was measured using Human S100β DuoSet ELISA Kit (R&D Systems). The range of detection was between 46.9 and 3000 pg/ml.

BDNF in human plasma samples was measured using Human/ Mouse BDNF DuoSet ELISA Kit (R&D Systems). The range of detection was between 23.4 and 1500 pg/ml.

MCP-1 in human plasma samples was measured using Human MCP-1 (CCL2) Standard ABTS ELISA Kit (R&D Systems). The range of detection was between 15.62 and 1000 pg/ml.

2.7: Magnetic Resonance Imaging (MRI)

MRI was performed using a 3T Philips Achieva scanner in the Center for Advanced Medical Imaging, St. James' Hospital, Dublin. The scanning protocol included a T1-weighted anatomical scan (3D gradient echo, TE/TR =3/6.7 ms, acquisition matrix 268x266, voxel size: 0.83x0.83x.9 mm), T2-weighted imaging (TE/TR =80/3000 ms, voxel size: 0.45x0.45x.4 mm), FLAIR (TE/TR =125/11000 ms, voxel size:0.45x0.45x4 mm) and DTI sequence (TE/ TR=55/ 12877 ms, Flip Angle=90 0, acquisition matrix= 124×120, FOV= 250×250 mm2, b0=1000 s/mm2).

In the school-aged cohort, the calculation of pre-contrast longitudinal relaxation time (T10), the variable flip angle (VFA) method was used (3D T1w-FFE, TE/TR = 2.78/5.67 ms, acquisition matrix: 240x184, voxel size: 0.68x0.68x5 mm, flip angles: 2,10,16 and 24°). DCE sequence was the acquired (Axial, 3D T1w-FFE, TE/TR = 2.78/5.6 ms, acquisition matrix: 240x184, voxel size: 0.68x0.68x5 mm, flip angle: 6°, Δt = 6.5 Sec, temporal repetitions: 70, total scan length: 7.6 minutes). An intravenous bolus injection of the contrast agent gadobentate dimeglumine (Gd-BOPTA, Bracco Diagnostics Inc., Milan, Italy) was administered using an automatic injector after the first three DCE repetitions.

For the second, university-aged cohort, T1-weighted, T2-weighted and FLAIR imaging parameters were kept the same. For the calculation of pre-contrast longitudinal relaxation time (T10), the VFA method was used (3D T1w-FFE, TE/TR = 2.78/5.67 ms, acquisition matrix: 208x,204 voxel size: 0.86x0.86x6 mm, flip angles:10,15, 20, 25 and 30°). DCE sequence was the acquired (Axial, 3D T1w-FFE, TE/TR = 2.78/5.6 ms, acquisition

matrix: 208x,204 voxel size: 0.86x0.86x6 mm, flip angle: 20°, Δt = 22.2 Sec, temporal repetitions: 61, total scan length: 22.6 minutes). Intravenous bolus injection of the contrast agent gadobentate dimeglumine was administered using an automatic injector after the first five DCE repetitions.

DCE-scan protocols for the MMA participants was the same as the university-aged cohort.

Post-acquisition image analysis of DCE-MRI images was done using a custom Matlab script that estimated contrast agent extraversion using the LDM, as reported in Chassidim et al. (2013) and Wissberg et al. (2014), as well as Tofts-extend Model. Training in the use of the script was carried out in person with the authors of the script; Dr. Ronel Veksler of Ben Gurion University, Israel.

Post-acquisition image analysis of DCE-MRI images was done using a custom Matlab script that estimated contrast agent extraversion using the LDM, as reported in Chassidim *et al.* (2013) and Wissberg *et al.* (2014), as well as Tofts-extend Model. The LDM estimated the Normalised Perfusion Index (pixel intensity/ time) by calculating the linear slope of contrast agent intensity for each voxel and normalising to the Venous Input Function. The Tofts-extended model calculates (Formula 1) Volume Transfer Constant (Ktrans), which, in the case of the brain, is equivalent to the Permeability Surface Product (PS)

$$i(t) = vp\delta(t) + Ktranse - tKtrans/ve$$

Derivation of the Ktran is done using Formula 2:

$$Ktrans = FpPSFp + PS$$

For a further explanation as to the derivation of the formulae and each of its components, one is directed to Sourbron and Buckley (2011). Processing of the images involved use of the script through Matlab and consisted of the following steps:

1. Converting DICOM images of the MRI scan to. nii format and preforming image alignment.
2. Setting repetition time of T1 and DCE scans.
3. Selecting the flip angles to be included in the analysis of DCE signal.

4. Setting the time between image acquisition during the acquisition time. During this study, image acquisition was acquired every 20 seconds during the DCE scan.

5. Running the script to allow for selection of Regions of Interest (ROI) to serve as the reference point for Arterial Input Function (AIF) and Venous Input Function (VIF). For the entirety of this study, the AIF was taken from the carotid artery and the VIF was set to the signal from the sagittal sinus at the posterior of the brain.

6. Set the time during the DCE scan after which to analyse the signal for the LDM. This only affects the LDM results. For the entirety of this study, this was set to 360 seconds.

7. Run script. Enter applicable participant details when prompted.

Diffusion weighting was consisting of 32 non-collinear directions, and a non-diffusion tensor image whose b0=0 s/mm2. Nineteen axial sections of scanning in 5mm thickness without gap, covering the whole brain were obtained.

Post-scan DTI analysis was carried out using the ExploreDTI suite. Training in the use of the software and analysis was carried out in person with Dr. Kathy Ruddy of Trinity College Dublin, Ireland. Processing of the images consisted of the following steps:

1. Converting the DICOM files to. nii format using the third-party software "dcm2niigui.mat".

2. With the ExploreDTI suite, compute the B-matrix from BVEC and BVAL files.

3. Align .nii files for further analysis by ExploreDTI analytics. Use the "Flip/permute dimensions in 3D/4D *.nii file(s)" plugin function to ensure correct alignment.

4. Create a diffusion tensor file for the scan from the flipped DTI scan file and corresponding b-matrix file.

5. An optional correction step can be carried out at this point. Correct for motion artefacts by registering the DTI scan to reference scans (i.e. T1 or T2 structural scans). Use the SM/ EC/ EPI correction function to perform an EPI (non-rigid correction). For T1 reference scans, the options of "Avg(DWIs)" or "FA" are

available. For T2 scans, the correction option of "non-DWI" is available. Once set, use the "Correct for subject motion & EC/ EPI distortions" function and select the folder contain both the flipped DTI scans and the reference scans. For the entirety of this study, this step was carried out using T1 scans as reference and using the "FA" correction option.

6. Whole brain tractography was performed on the corrected DTI files using the "wholebrain tractography (DTI)" function.

7. Analysis of tractography parameters was performed using a pre-determined brain atlas. This was done via the "network analysis tool (get connectivity matrices)" function and selecting the "from atlas template/ labels" option and selecting the desired reference atlas. For the entity of this study, the ICBM brain atlas was used as the reference atlas.

2.8: Statistical Analysis

Statistical analyses were performed using two-tailed Wilcoxon t test, with statistical significance being considered at $P \leq 0.05$. For correlation analysis, association strength 46 was set at 0-0.39 for no association, 0.4-.0.59 for a weak association, 0.6-0.79 for a moderate association and 0.8-1 for a strong association. Prior to the commencement of the study and recruitment of participants, power analysis was performed using the G*Power software suite. A priori analysis set with a power value of 0.85 and assuming a moderate effect size ($\rho=0.4$) and α error probability of 0.05. This gave a required sample size of 50 participant (**Figure 2.2**). However, following the large attrition in participation numbers, post-hoc power analysis was recalculated following final assessment of the university participants in order to determine the power of the study with the number of individuals available (n=18) (**Figure 2.3**). This gave a greatly diminished power value ($1-\beta$ error probability = 0.41).

As part of the study focusing on the MMA participants, power analysis was calculated using G*Power using an expected sample size of 30 participants and a large effect size ($\rho=0.5$) (**Figure 2.4**).

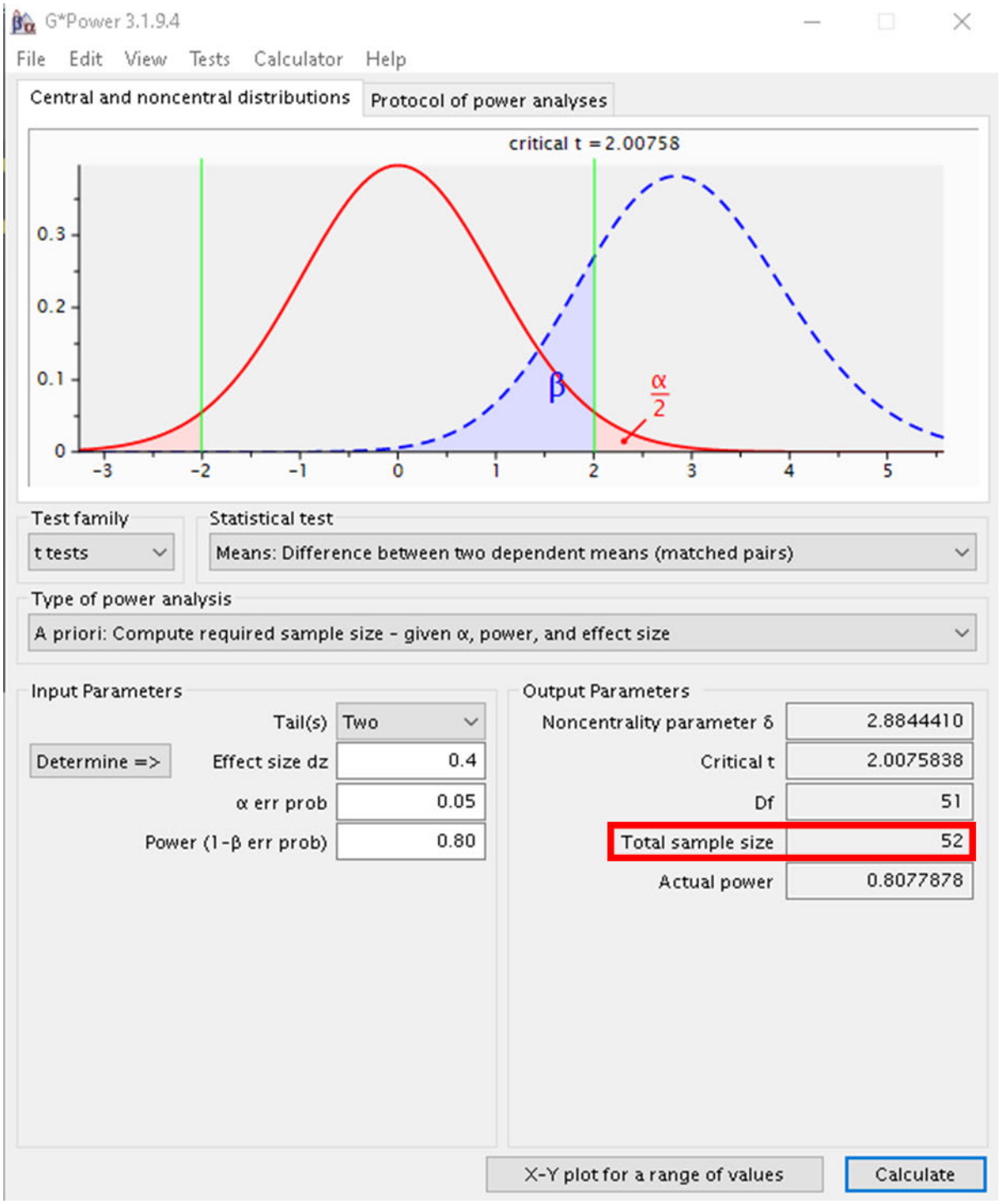


FIGURE 2. 2: A PRIORI POWER ANALYSIS FOR THE RUGBY PHASE INDICATES THAT 50 PARTICIPANTS ARE REQUIRED TO POWER THE STUDY

Prior to the start of the study, it was calculated that 52 participants would be required to suitably power the study (red box). Based number of required participants was based on a moderate effect size (ρ=0.4), with an α-error probability of 0.05, and an acceptable degree of power (1-β error probability 0.8).

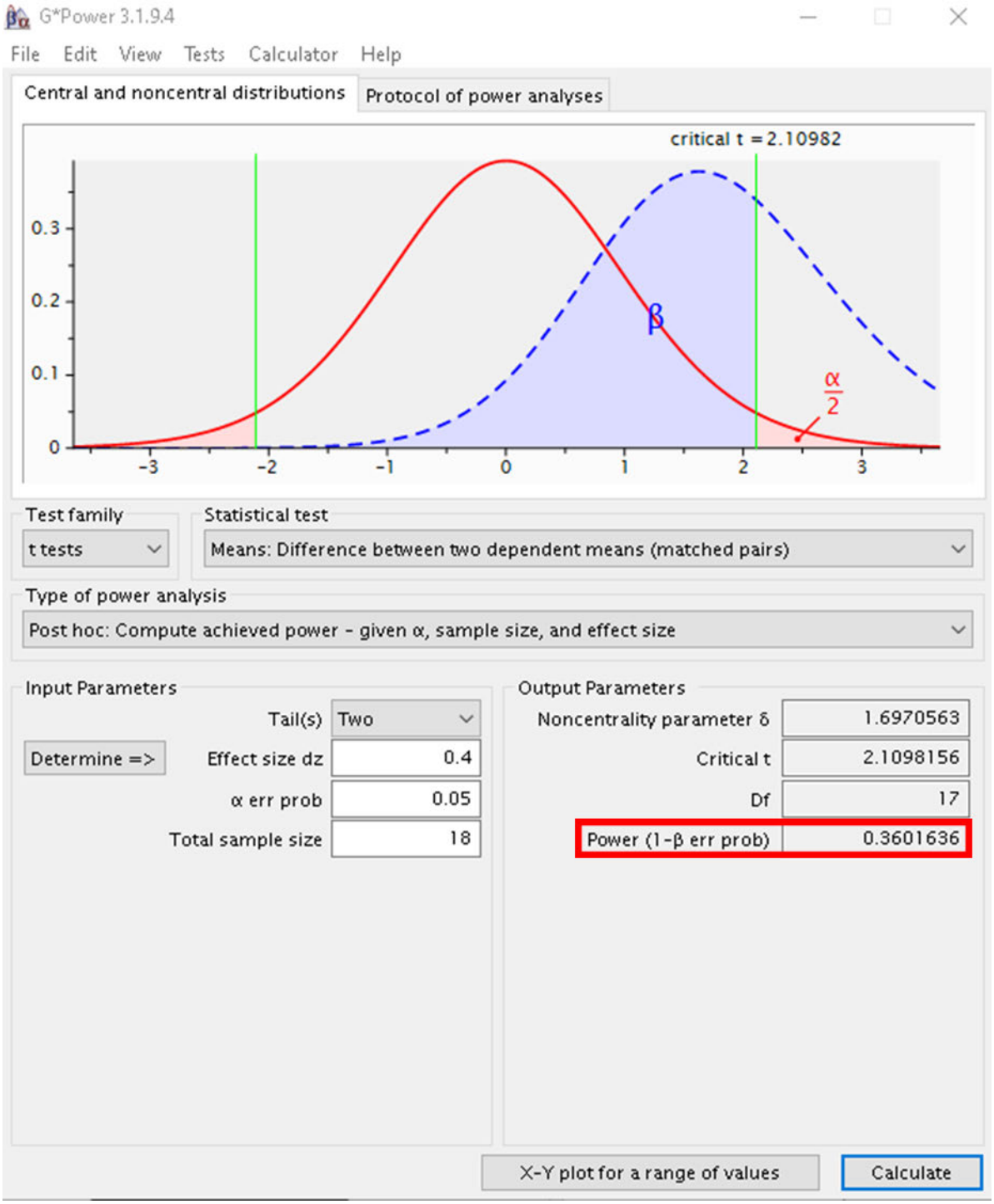


FIGURE 2. 3: POST-HOC POWER ANALYSIS USING THE AVAILABLE NUMBER OF PARTICIPANTS FOR THE RUGBY STUDY FOUND THE STUDY WAS UNDERPOWERED USING THE INITIAL PARAMETERS.

Post-hoc power analysis using the number of available participants (n=18) as part of both schoolboy- and university-aged cohorts for the rugby phase of the study found that given the prior set parameters, the study was under powered (1-β error probability = 0.41). Effect size (ρ=0.4) and α-error probability (set to 0.05) were retained from a priori analysis.

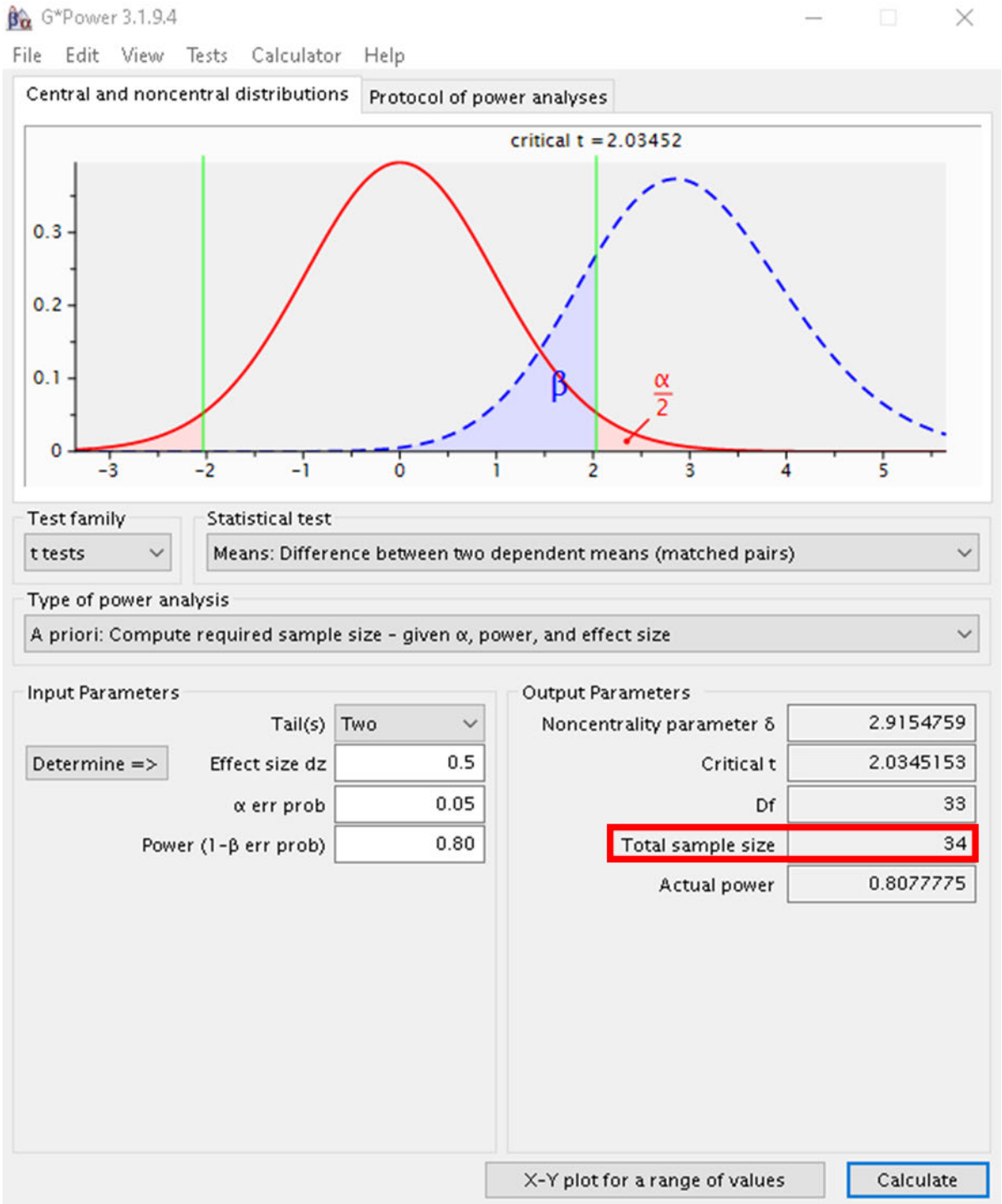


FIGURE 2. 4: A PRIORI POWER ANALYSIS FOR THE RUGBY PHASE INDICATES THAT 50 PARTICIPANTS ARE REQUIRED TO POWER THE STUDY

Prior to commencement of the MMA study, power analysis was carried out to determine the number of participants required. Taking in to context results seen from the rugby phase of the study, the effect size was changed to reflect a large effect size (ρ=0.4). The α-error probability was kept to 0.05, and an acceptable degree of power (1-β error probability 0.80). The required number of participants for this sort of study was determined to be n-34 (red box).

CHAPTER 3: CASE REPORTS OF DEMENTIA PATIENTS CONNECTED BY A HISTORY OF TBI

3.1: Abstract

A history of head trauma has long been associated with the development of dementia later in life, with diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), serving as the strongest environmental risk factor for dementia development, and linking traumatic brain injury (TBI) and neurodegenerative disease. In recent years, attention has shifted to the risk posed by repetitive, mild TBI (mTBI) due to the re-emergence of the neurodegenerative condition Chronic Traumatic Encephalopathy (CTE). The publication of several high-profile case reports of former athletes in contact sports being diagnosed with CTE has ignited an increased interest in the risks posed by mTBI. Additional reports of CTE in military veterans exposed to blast-induced mTBI, as well as football players who frequently "header" the ball, suggest that physical forces of mTBI contribute to the mechanisms by which the dementia condition develops (Goldstein *et al.,*2012; Ling *et al.,* 2017). Presented in this section is a series of case reports examining the state of the BBB in individuals with a history of head trauma diagnosed with a neurodegenerative disease. Two of these cases (Case 1 and Case 2) were diagnosed with CTE and were noted to have had a long career in contact sports in which they incurred multiple mTBI over their active years. The third (Case 3) was of a unique case in which the individual was diagnosed with multiple neurodegenerative conditions, the only finding of note in their medical history was a single severe TBI incurred in their youth. In each of these cases, the level of the tight junction (TJ) protein claudin-5, as well as phosphorylated tau (p-tau) pathology was examined. In addition, the extent of BBB dysfunction (BBBD) was explored by examining the degree of endogenous immunoglobulin G (IgG) and fibrinogen extravasation in post-mortem sections of each case. In both Case1 and Case 2, discontinuous or absent claudin-5 immunoreactivity was observed in regions of intense p-tau deposition, accompanied by evidence of hIgG and fibrinogen extravasation, suggesting BBBD at these sites. Reduced levels of claudin-5 reactivity was also observed in Case 3, along with hIgG extravasation and p-tau deposition patterns unique from CTE. These case reports are among the first evidence of BBB dysfunction in CTE and suggest that disruptions to the BBB may arise as a result of mTBI acquired in contact sports.

3.2: Introduction

TBI is a major cause of death worldwide, representing roughly 9% of all worldwide deaths, a leading cause of mortality in children and young adults globally and represents a large injury burden in the elderly, with age-standardised injury rates increasing by 8.4% in 16 years (WHO, 2014; CDC, 2004). Cerebral oedema, BBB dysfunction (BBBD) and subsequent pathophysiological reactions, such as inflammation and neurometabolic changes, resulting from moderate and severe TBI are well characterised processes evolving from the injury (O'Leary & Nichol, 2018). However, the physiological responses arising from mTBI are less well characterised, although animal models suggest that BBB dysfunction is at least a common process (Nag *et al.,* 2007). While mTBI is an injury that by some definitions does not produce gross neuroanatomical changes, the implications of sustaining multiple injuries or subclinical injuries for the long-term brain health of individuals has become increasingly pertinent. Athletes participating in contact sports are suggested to be at a particularly high risk of mTBI sequelae. This is due in part to recent high-profile case studies concerning early onset neurodegenerative diseases in athletes engaged in contact sports and the return of "Punch Drunk" syndrome to the public domain in the form of CTE (Parker, 1934; Omalu *et al.,* 2010a; Omalu *et al.,* 2010b; Alosco *et al.,* 2018; Tagge *et al.*, 2018).

Increased risk of dementia is suggested to be a consequence of exposure to TBI, with the risk of developing AD has been found to increase up to 82% in men with a history of TBI, while the risk of developing PD or non-AD dementia estimated to increase by 30% and 40% respectively (Mortimer *et al.,* 1991; Fleminger *et al.,* 2003; Rosso *et al.,* 2003; Gardber *et al.,* 2014). However, meta-analyses of TBI studies propose a less than clear relationship between TBI and its long-term risks for dementia development, with recent reports indicating conflicting findings regarding mTBI's contribution to dementia risk (Godbolt *et al.,* 2014; Perry *et al.,* 2016). Some large-scale meta-analysis found a non-specific association between TBI and development (Huang *et al.,* 2018). The lack of a clear link between TBI and dementia, particularly mTBI, mirrors the confusion surrounding an accurate definition of the condition. Numerous health bodies have put forward definitions for the injury, but there is a lack of consistency between these definitions. This is then reflected in a possible underdiagnosis of the condition, or dissuade individuals from reporting injuries to health practitioners, which may, in turn,

lead to difficulties in recruiting individuals with mTBI or stratifying mTBI from other severities.

Within many of these neurodegenerative diseases, aberrant aggregation of proteins to form plaques or neurofibrillary tangles (NFTs). These protein aggregates are often identifiable hallmarks of the disease and used as confirmation markers of a given condition upon autopsy. NFTs of p-tau protein and amyloid-β (Aβ) plaques within the neocortex and hippocampus are classic markers of AD, while diffuse Aβ plaques are found in the frontal and temporal lobes of frontotemporal dementia (FTD). Perivascular p-tau and NFTs within the sulcal depths have become the defining hallmark of the seemingly TBI-unique condition CTE (Baugh *et al.,* 2014).

Currently, CTE is only diagnosable post-mortem, as a number of the clinical presentations of the condition overlap with other neurodegenerative diseases, most notably AD and FTD (Prince *et al.*, 2013; Tartaglia *et al.,* 2014). However, at least within the limited number of CTE cases reported to date, pathological examination appears to indicate a unique pattern of findings, enough to warrant proposal of potential diagnostic and disease progression criteria (Baugh *et al.,* 2014; McKee *et al.*, 2016). These include dilated ventricles, swollen cortical and subcortical axons and focal, perivascular NFTs within the sulcal depths in the early stages. Disease progression is marked by widespread p-tau, increased cerebral and medial temporal atrophy, enlarged ventricles and severe cortical axonal loss. However, perivascular p-tau aggregates are currently the only required diagnostic criteria, with the other pathological markers being deemed as supportive findings (McKee *et al.,* 2016).

Changes in BBB integrity and TJ complexes in the context of chronic neurodegenerative disease, while implicated, remains ill-defined in regard to its role in disease development and progression. Truncated capillaries, increases in microvascular density and loss of pericyte and basement membrane coverage have been reported in brain tissue of AD, PD and HD cases (Drounin-Ouellet et al., 2015; Pienaar et al., 2015; Halliday et al., 2016), while post-mortem evidence of leaky blood vessels has been identified in AD, PD, HD and ALS (Sweeney, Sagare & Zlokovic, 2018). Endogenous blood components, such as IgG, fibrinogen and albumin, have been found upon post-mortem examination of disease brains, localising with Aβ deposits in AD, suggesting a link between BBBD and the disease (Sengillo *et al.,*2013). Changes to the make-up of the neurovascular unit may represent a cyclical process, in which the loss of pericyte coverage deprives endothelial

cells of soluble factors required for vascular maintenance, resulting in changes in cell thickness, length or density. Aberrant angiogenesis may arise due to this reduction in the endothelial cell population. The increases in pro-angiogenic factors, potentially generated by hypoxia-responsive pathways, identified in AD and PD patients are suspected to produce the increase in permeable vasculature seen in these diseases when coupled with reduced pericyte coverage (Bradaric et al., 2012; Grammas., 2011). Additionally, disruptions in TJ complexes and protein expression have also been reported in cases of AD, PD and Huntington's disease (HD) cases, resulting in increased paracellular permeability of the BBB. TJ protein expression has also been shown to be downregulated by the presence of Aβ monomers, suggesting that neurotoxic aggregates present in these proteinopathies contribute to BBBD in these diseases, and demonstrating the intimate link between the two (Keaney *et al.,* 2015).

In each of the following cases reported within this chapter, in life medical and neurological assessment was carried out by medical personal at either St. James' Hospital Dublin, or St. Vincent's Hospital, Dublin. Preparation of post-mortem tissue, dissection and documentation of gross anatomy of brain sections, sectioning of tissue samples and light microscopy immunohistochemical analysis was carried out by personnel at the Dublin Brain Bank, Beaumount Hospital, Dublin. Immunofluorescent histochemistry was performed by the candidate: Eoin O'Keeffe

3.3: Results

3.3.1: Case Study 1: BBB dysfunction present in a boxer with CTE and schizophrenia

The results presented in this section were published in Farrell, Michael, Susan Aherne, Sean O'Riordan, Eoin O'Keeffe, Chris Greene, and Matthew Campbell. 2019. "Blood-Brain Barrier Dysfunction in a Boxer with Chronic Traumatic Encephalopathy and Schizophrenia." Clinical Neuropathology 38 (03): 51–58. doi:10.5414/NP301130.

See Appendix I

3.4.1.1: Patient History and pathology findings

In 2009, a 64-year-old man presented to Neurology services in St. Vincent Hospital, Dublin, with multiple symptoms, including short-term memory loss, slurred speech, gait

disturbances and bradykinesia. Prior to presenting to neurological services, the patient was reported to be healthy and engaging in regular exercise. Over the course of 8 years following the initial clinical presentation, the patient's bradykinesia worsened, the frequency of falling increased, gait became abnormal, dysarthria worsened, cognitive impairment increased with worsening short-term memory and increasing emotional instability. Neurological assessment suggested that the patient had a progressive neurodegenerative disorder with elements of cognitive impairment and Parkinsonism. Single-photon emission computed tomography (SPECT) imaging found scintigraphic features suggesting PD according to Catafau Classification (Catafau *et al.,* 2004). Medical imaging assessment found prominent involution changes on CT scan and mild periventricular and left frontal subcortical deep white matter (WM) ischemia.

The patient's medical history indicated excessive alcohol consumption for more than 30 years, type 2 diabetes mellitus and hypercholesterolemia. Initial indication of mental issues was reported in 1989 with psychiatric symptoms, including impulsive behaviour, paranoia and bouts of anger. The patient was diagnosed with paranoid schizophrenia in 1991.

Personal history also reported that the patient had a 19-year history of competitive professional boxing between the ages of 16 and 35. In addition to this, the patient was also a military physical trainer in the Middle East. Cause of death was identified during autopsy as severe acute traumatic subarachnoid haemorrhage as a result of a fall suffered at home. Examination of the brain reported no signs of atrophy in the temporal lobes, hippocampus, caudate, putamen or globus pallidus (**Figure 3.1 a-c**), although a prior brain injury was noted by the presence of a chronic subdural hematoma membrane (**Figure 3.1 d**).

Upon immunohistochemical examination by the attending pathologist, p-τ tangles in the pyramidal layer and dentate fascia of the hippocampus were noted, in addition to p-tau deposits in the substantia nigra (**Figure 3.2**). Perivascular astrocytic p-tau was also identified in the sulcal depths of the neocortex (**Figure 3.2**). Immunohistochemistry staining Aβ was negative. These findings, in the context of the patient's history mTBI exposure, led to a neurological diagnosis of CTE.

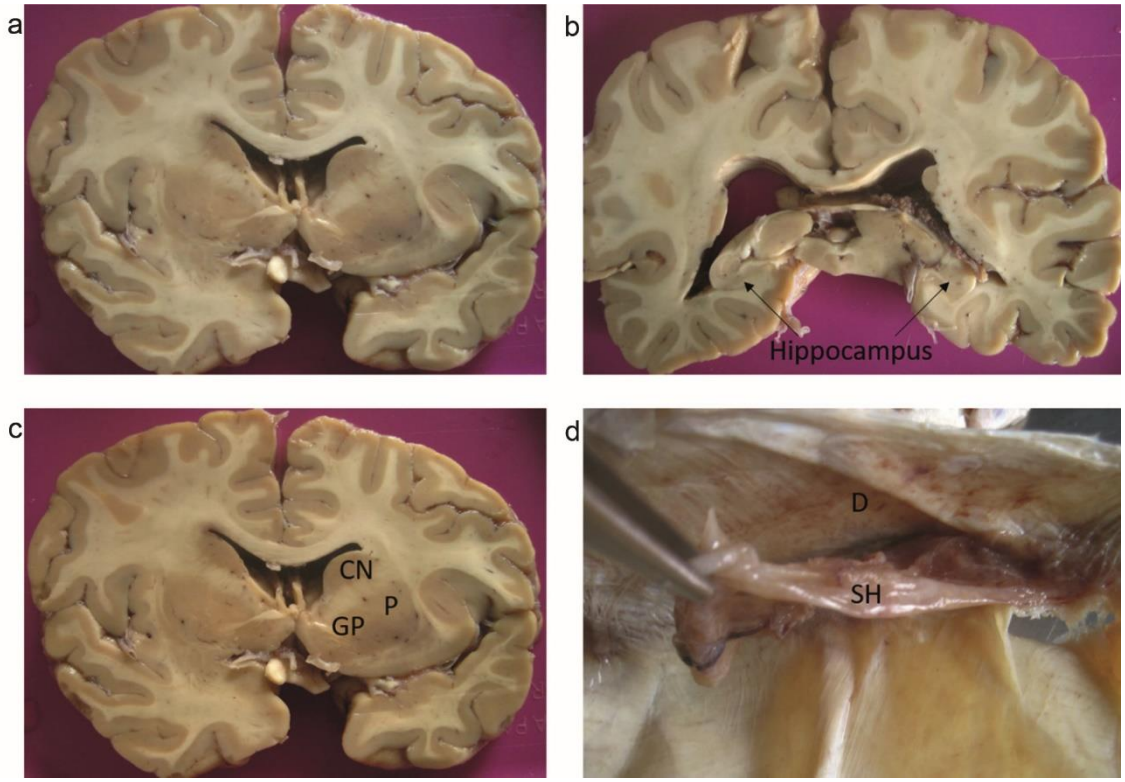


FIGURE 3. 1: MACROSCOPIC EXAMINATION IMAGES OF THE BRAIN OF A FORMER BOXER DIAGNOSED WITH CTE.

Macroscopic examination the brain a pathologist indicated no signs of atrophy in the **a**) temporal lobes, **b**) hippocampus or **c**) caudate, putamen and global pallidus. **d**) Pathological examination did note evidence of prior trauma with a chronic subdural hematoma membrane. CN = caudate nucleus; GP = globus pallidus; P =putamen; D = dura; SH = subdural hematoma. Image used with permission from Campbell lab publications (Farrell *et al.,*2018).

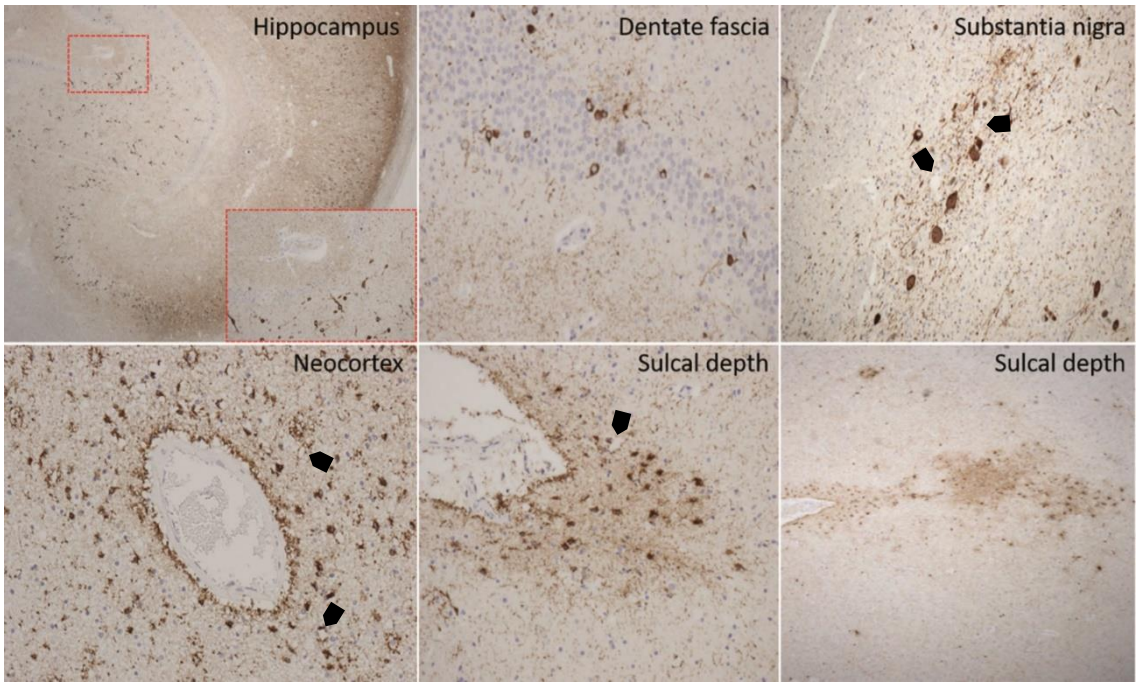


FIGURE 3. 2: IMMUNOHISTOCHEMISTRY STAINING FOR P- TAU DEPOSITION IN MULTIPLE BRAIN REGIONS OF A FORMER BOXER DIAGNOSED WITH CTE.

Photomicrographs of p-tau immunostaining in multiple regions of the brain. Perivascular p-tau deposition was noted in the sulcal depths, as well as around vessels of the neocortex and substanita nigra (**arrowheads**). Immunostaining was carried out by personnel within the Dublin Brain Bank. Image used with permission from Campbell lab publications (Farrell *et al.,*2018).

3.3.1.2: BBB disruption and loss of TJ integrity

Fluorescent immunohistochemical examination of the TJ protein, claudin-5, in the context of p-tau found that the TJ appeared disrupted in the presence of the microtubule-binding protein (**Figure 3.3**). In areas where there was dense deposition of p-tau, immunoreactivity for CLDN5 was greatly reduced or even completely absent. However, in the absence of p-tau immunoreactivity, CLDN5 reactivity appeared normal, forming long continuous strands. The diminished presence of CLDN5, the most abundant TJ protein present in the TJ complexes of the BBB, in areas of dense p-tau deposition suggest that BBBD is present in these areas.

To investigate the extent of BBBD, immunohistochemistry staining was performed for the endogenous blood proteins hIgG (**Figure 3.4**) and fibrinogen (**Figure3.5**).

Immunoreactivity for hIgG was appeared greatest in perivascular areas surrounding large vessels. Surrounding smaller vessels, there was no evidence of hIgG extravasation. This was supported by CLDN5 staining, which mostly showed continuous, strand-like staining, suggesting normal TJ complexes. However, some small vessels did show signs a impairments to TJ complexes, with globular CLDN5 immunoreactivity. These were accompanied with evidence of extravasated IgG.

There was a high level of immunoreactivity to endogenous fibrinogen, particularly surrounding large vessels, suggesting disruption of the BBB. However, CLDN5 immunoreactivity was also detected within these regions, suggesting an intact BBB. Smaller vessels also showed continuous, strand-like patterns indicative of a normal BBB. Therefore*, fibrinogen detected surrounding these vessels likely represents a historic disruption of the BBB, one that has been restored to homeostatic levels.

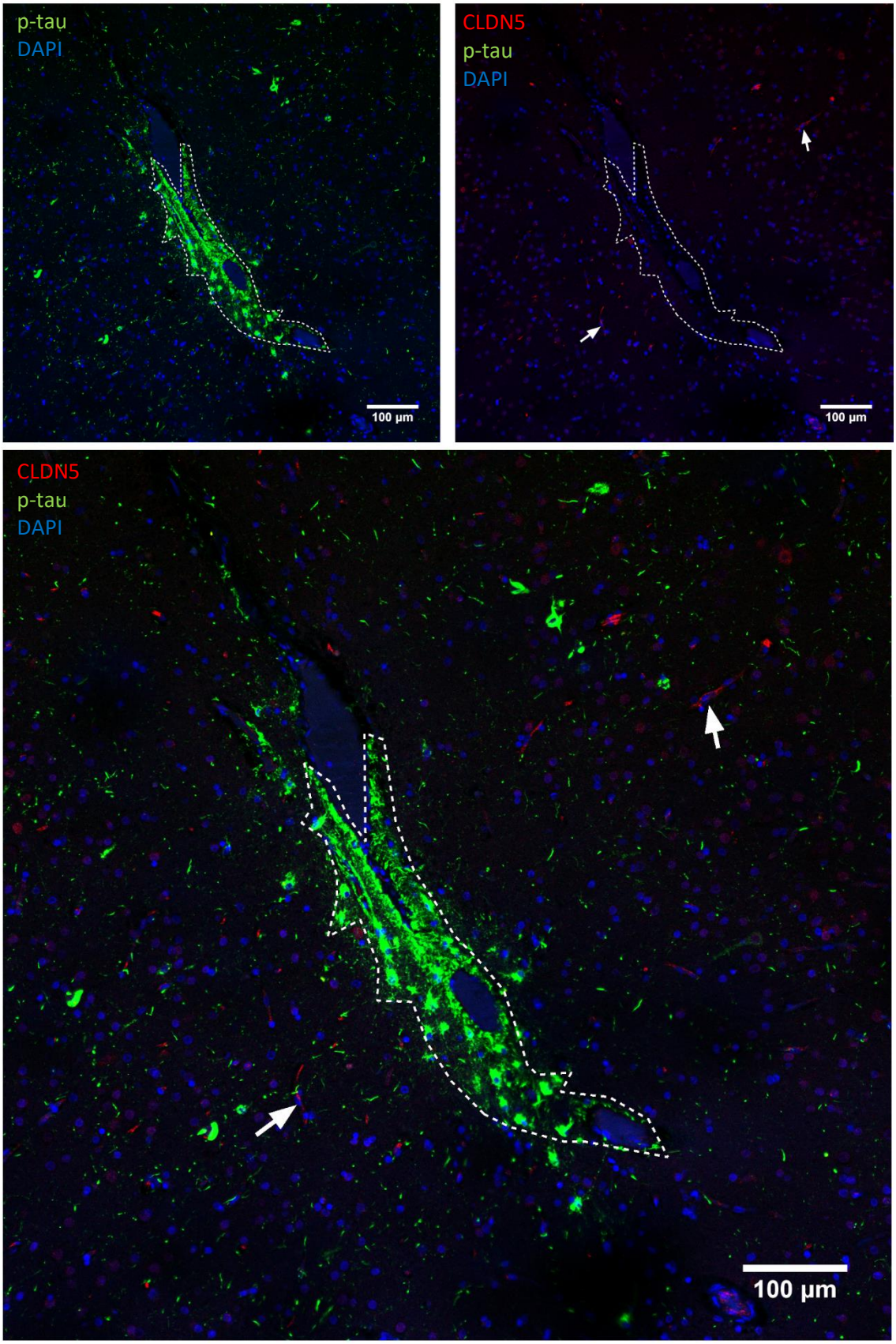


FIGURE 3. 3: DENSE PHOSPHORYLATED-TAU DEPOSITION IS ACCOMPANIED BY ABSENT OR REDUCED CLDN5 IMMUNOREACTIVITY.

Areas of dense p-tau deposition (dotted white line) localised around large vessels (**some form of arrow**). Areas of dense p-tau staining coincided with reduced or absent immunoreactivity to the TJ protein, CLDN5. In the absence dense p-tau deposition, CLDN5 staining appears normal as long, continuous strands (flat head arrows).

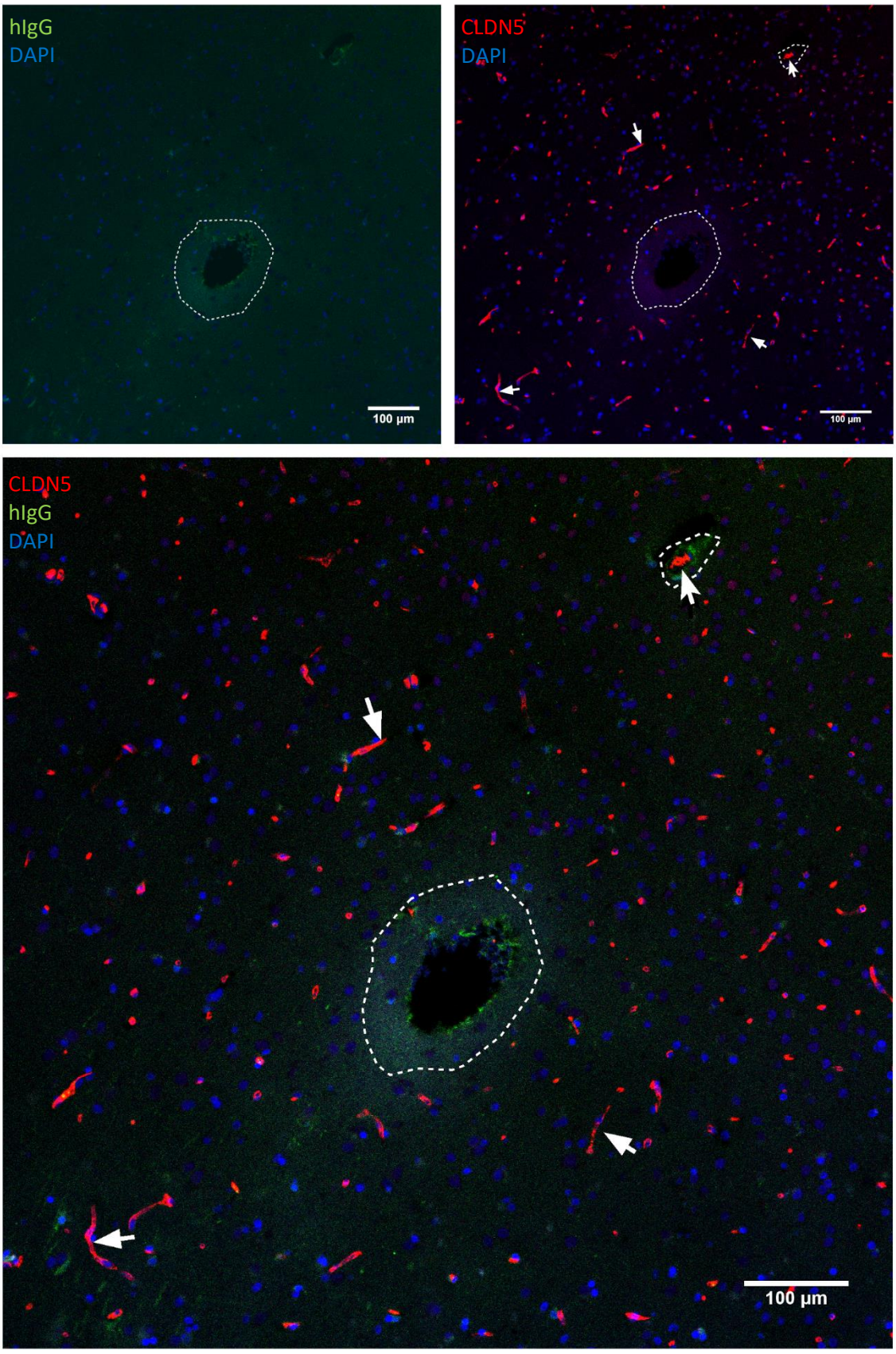


FIGURE 3. 4: HIGG EXTRAVASATION IS LIMITED TO LARGER VESSELS.

hIgG extravasation was largely limited to perivascular regions of large vessels (white dotted line). hIgG staining was not observed in the area surrounding smaller vessels, which coincided with continuous, strand-like staining of CLDN5 (flat arrow head), suggesting an intact BBB. However, some small vessels do show some impairment to integrity, with globular CLDN5 immunoreactivity surrounded by hIgG extraversion (notched arrow).

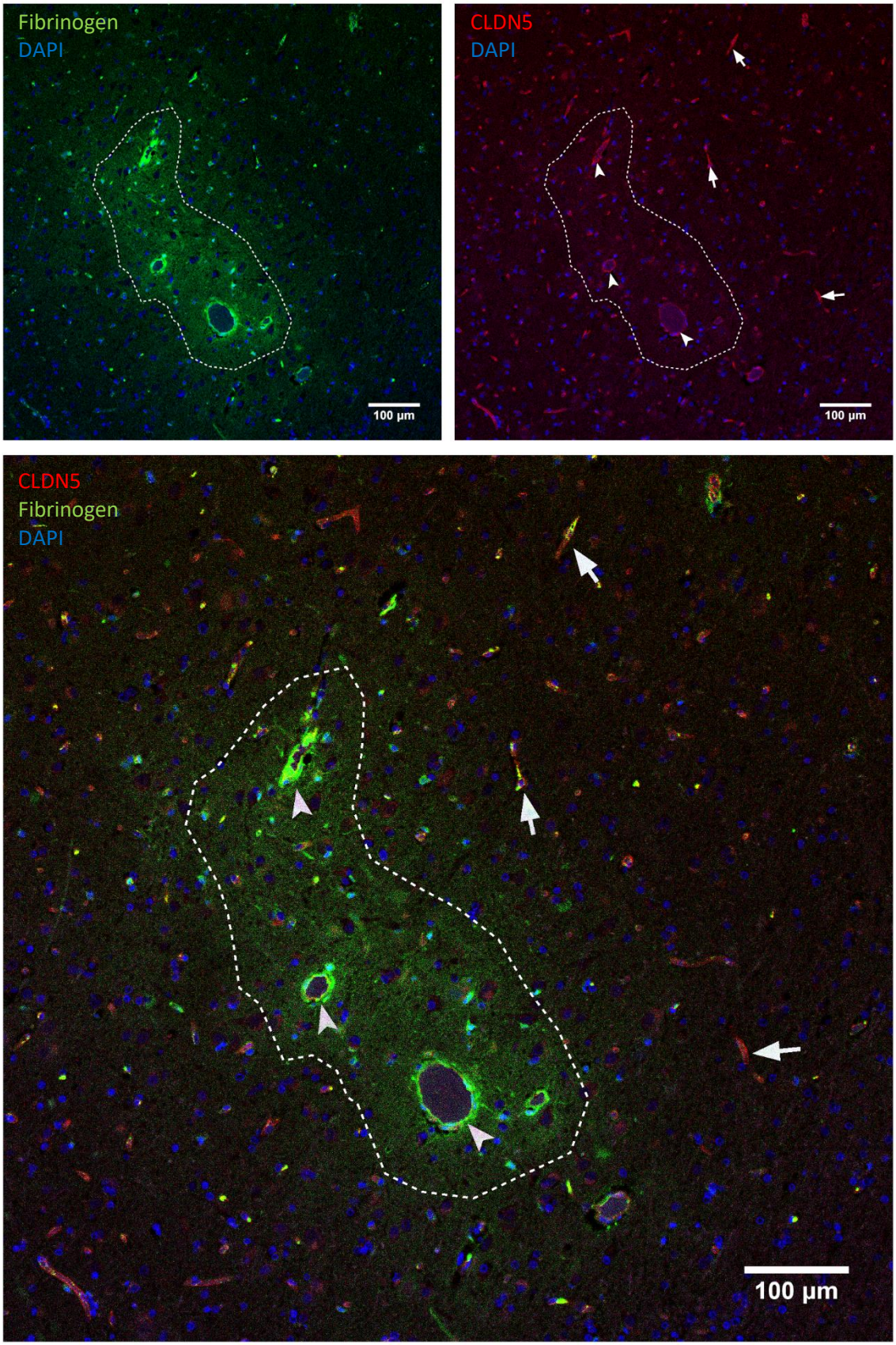


FIGURE 3. 5: CLDN5 STAINING WITH FIBRINOGEN DENSE REGIONS SUGGESTS HISTORIC DISRUPTION AND HEALING OF THE BBB.

A high level of immunoreactivity to endogenous blood-based protein fibrinogen was detected surrounding larger vessels (dotted white line). Despite this, claudin-5 immunoreactivity was detected within this region, suggesting that the BBB remained intact (notched arrows). Smaller vessels continuous, strand-like patterns of CLDN5 staining, in the absence of fibrinogen leakage (flat arrows). Therefore, fibrinogen staining detected suggests a previous breach in the BBB that has subsequently healed.

3.3.2: Case Study 2: BBB dysfunction present in rugby player with CTE and PSP

The results presented in this section were published in Doherty, Colin P, Eoin O'Keefe, Eugene Wallace, Teresa Loftus, James Keaney, John Kealy, Marian M Humphries, et al. 2016. "Blood–Brain Barrier Dysfunction as a Hallmark Pathology in Chronic Traumatic Encephalopathy." Journal of Neuropathology & Experimental Neurology 75 (7): 656–62. doi:10.1093/jnen/nlw036.
See Appendix II

3.3.2.1: Patient history and pathology findings

In 2011, a 56-year-old man presented to Neurology services in St. James' Hospital, Dublin, following an acute bout of confusion. A medical history enquiry found a progressive worsening of attention and memory issues and difficulty in task organisation going back 5 years prior to clinical presentation. Neurological findings at initial presentation included axial rigidity, hypomimia, positive glabellar tap, asymmetrical upper limb rigidity, bradykinesia and ideomotor apraxia. Cognitive performance as measured by the Montreal Cognitive Assessment (MoCA) yielded a score of 17/30, with difficulty in verbal fluency and recall tasks. Following an initial diagnosis of dysexecutive mild cognitive impairment, the patient experience rapid progression of cognitive symptoms and the development of Parkinsonism within a year after the initial assessment. Due to this progression of symptoms, a diagnosis of Progressive Supranuclear Palsy (PSP) was made. Symptom progression continued up to time of death one year from initial presentation. Cause of death was respiratory failure secondary to aspiration pneumonia.

Family history indicated that the patient's mother developed a psychotic depressive order in her 70's and went on to develop mild cognitive difficulties within a decade. Late-onset AD was diagnosed in the patient's maternal uncle. Alcohol consumption was noted as 20-30 units a week. Past medical history indicated asthma, hypertension, hypertrophic cardiomyopathy, gout and left myringotomy. Medication taken were ramipril, aspirin and atorvastatin.

Personal history noted that the patient had engaged in a long career of amateur rugby union, which was reported to have lasted from early teens to the age of 50. A retrospective

investigation using testimonies from first-degree relatives indicated the patient had sustained numerous head injuries consistent with mTBI over the course of his amateur rugby career. A single work-related head injury was also noted, with no loss of consciousness or formal medical attention reported.

Initial MRI scans indicated normal age-related WM changes with no other significant findings. However, retrospective review following death and prior to autopsy identified anteriorly cavum, posteriorly fenestrated septum pellucidum and bilateral hyperintensities on T1 images within the medial temporal lobe, indicative of gliosis (**Figure 3.6a**). Positron Emission Tomography (PET) scans indicated hypometabolism in the frontal and posterior cingulate (**Figure 3.6b**), while a dopamine transport scan showed signs of reduced uptake of fluorodeoxyglucose bilaterally within the caudate and putamen nuclei.

Gross anatomical examination on autopsy found no notable findings, with no evidence of focal lesions or generalised anatomical changes resulting from injury (**Figure 3.6c**), observed in the basal ganglia.

Upon immunohistochemical examination by the attending pathologist, p-tau tangles were reported in the neocortex, hippocampus and striatum, with notable perivascular, subpial and sulcal depth p-tau deposits. Astrocytic plaques staining positive p-tau were found primarily in perivascular and subpial regions (**Figure 3.6d**). Microscopic examination also revealed substantial neuronal loss in the CA4, CA3 and CA2 regions of the hippocampi and superficial line spongiosis of the cortex (**Figure 3.6e**). Based on these finding a diagnosis of CTE was put forward.

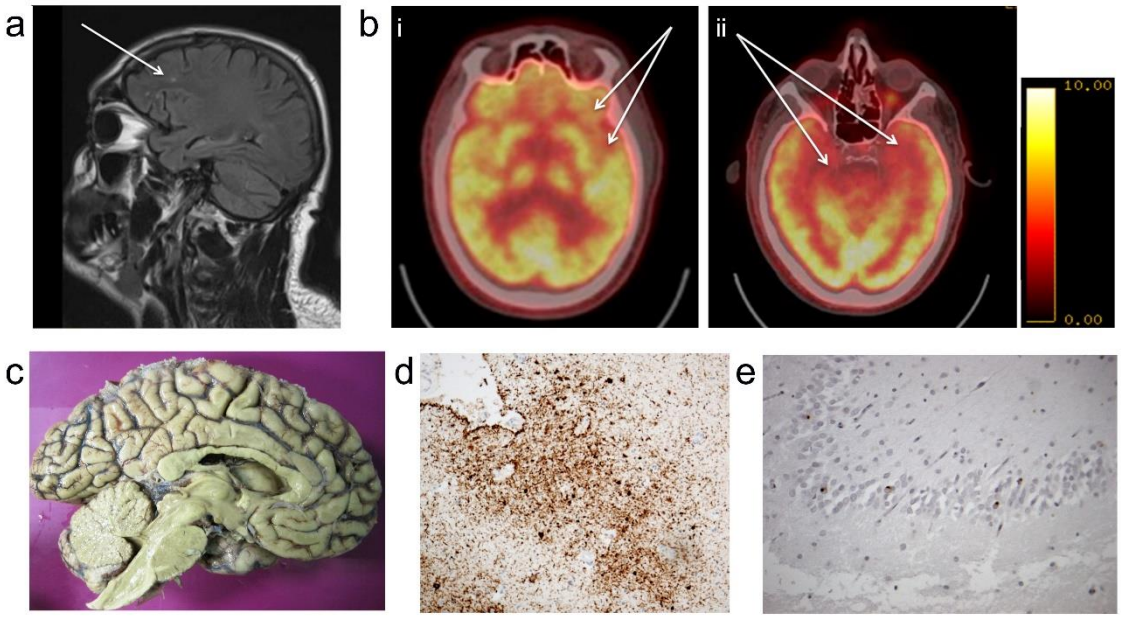


FIGURE 3. 6: IN LIFE NEUROIMAGING AND POST-MORTEM BRAIN EXAMINATION OF AMATEUR RUGBY PLAYER DIAGNOSED WITH CTE.

a) Sagittal FLAIR images of the patient carried out upon initial workup of diagnosis. Retrospective assessment of FLAIR images suggested white matter abnormalities (**arrow**) within areas of sampling. **b**) PET imaging using fluorodeoxyglucose as a tracer. Areas of hypometabolism were identified in the **i**) inferior left frontal and anterior temporal lobes and **ii**) bilateral medial temporal lobes. **c**) Macroscopic examination of the left cerebral hemisphere indicated minimal anterior frontal atrophy. d) Immunostaining for p-tau within the depths of sulci indicated widespread immunoreactivity surround blood vessels. e) Immunohistochemical staining for TDP-43 indicated cytoplasmic accumulation in neurons of the dentate fascia.

3.3.2.2: BBBD and loss TJ integrity

Immunohistochemical examination found that p-tau staining was widespread throughout the sections, with the densest deposits begin identified surrounding blood vessels (Figure 3.7). Within regions of dense perivascular p-tau deposition, immunoreactivity to CLDN5 was greatly diminished or absent. The regions of perivascular p-tau were also devoid of any evidence of normal, strand-like CLDN5 staining, suggesting a major disruption to the BBB within these areas. However, in the absence of p-tau immunoreactivity, CLDN5 appeared normal, forming strands along blood vessels, suggesting that BBB integrity was retained within these areas. Similar staining patterns were found for the TJ protein: ZO-1, in the context of p-tau (**Figure 3.8**). Areas with the densest p-tau immunoreactivity showed a marked reduction or even complete absence of ZO-1 staining. However, in the absence of p-tau deposition, ZO-1 staining remained intact. This suggests that the presence of p-tau can reduce the level of TJ proteins, leading to BBBD.

To determine the degree of BBBD within these regions of dense perivascular p-tau, sections was stained for endogenous blood protein hIgG (**Figure 3.9**) and fibrinogen (**Figure 3.10**).

hIgG immunoreactivity was present surrounding vessels, corroborating observations made using TJ immunohistochemistry that an impairment to BBB integrity was present. Within these areas of extravasating hIgG, dense deposits of p-tau were also present, indicating that BBBD was present in vessels surrounded by p-tau. Extravasating fibrinogen was also found within sections stained for p-tau and fibrinogen. Examination of immunoreactivity for endogenous fibrinogen showed similar finding to that of hIgG/ p-tau staining, with extravasating fibrinogen overlapping with areas of p-tau deposition. In vessels that displayed lumen-contain fibrinogen immunoreactivity, p-tau deposition was noted in areas distant from the vessel and less densely deposited.

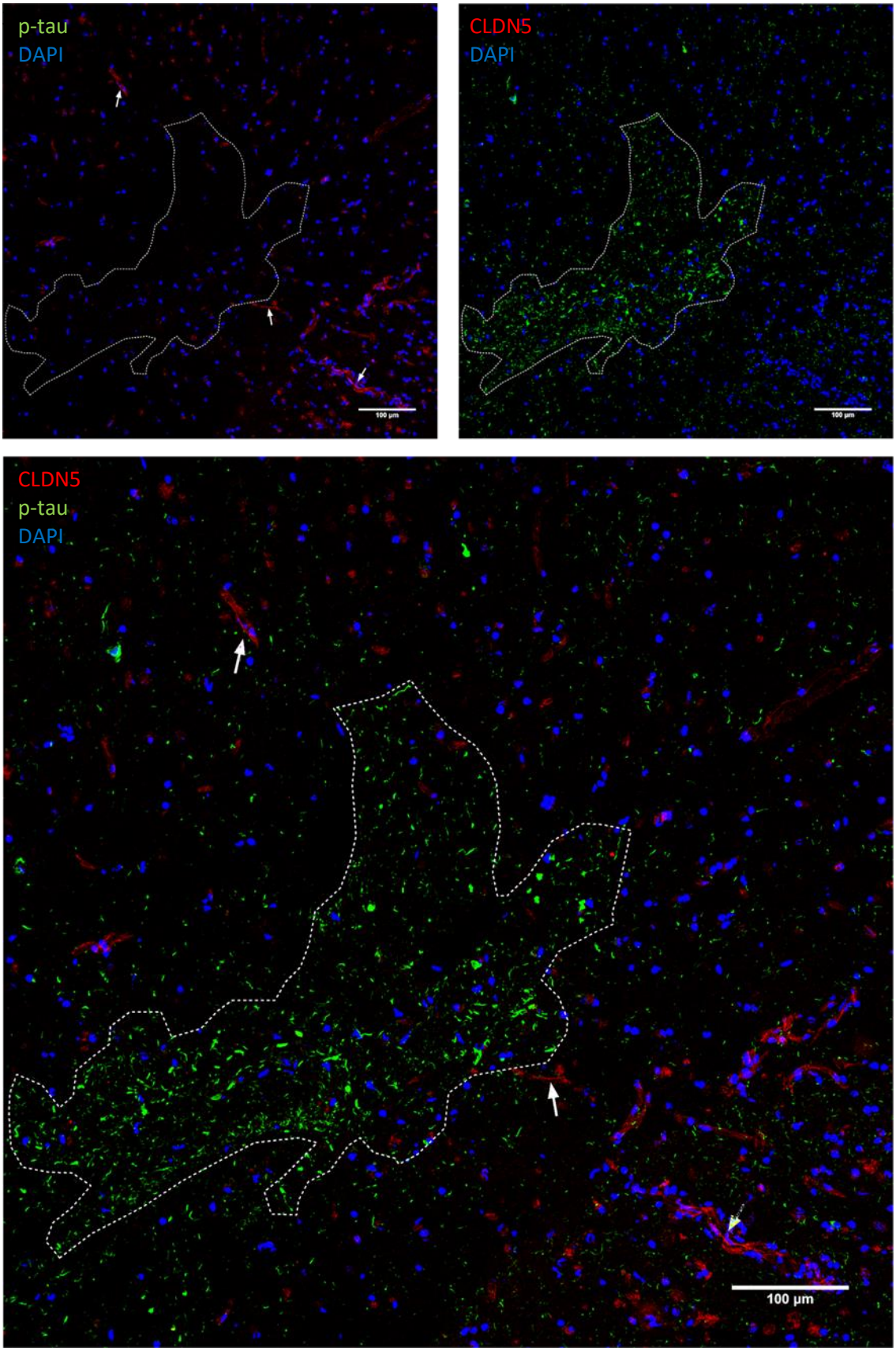


FIGURE 3. 7: IMMUNOHISTOCHEMICAL STAINING OF TIGHT JUNCTION PROTEIN CLAUDIN-5 (RED) AND P-TAU (GREEN) IN THE BRAIN OF A FORMER AMATEUR RUGBY PLAYER DIAGNOSED WITH CTE.

Areas of dense p-tau immunoreactivity coincided with an absence of CLDN-5 immunoreactivity (dotted white line), suggesting loss of BBB integrity within these regions. In the absence of p-tau deposition, CLDN-5 staining appeared in normal, strand-like pattern (flat arrows), indicative of an intact barrier.

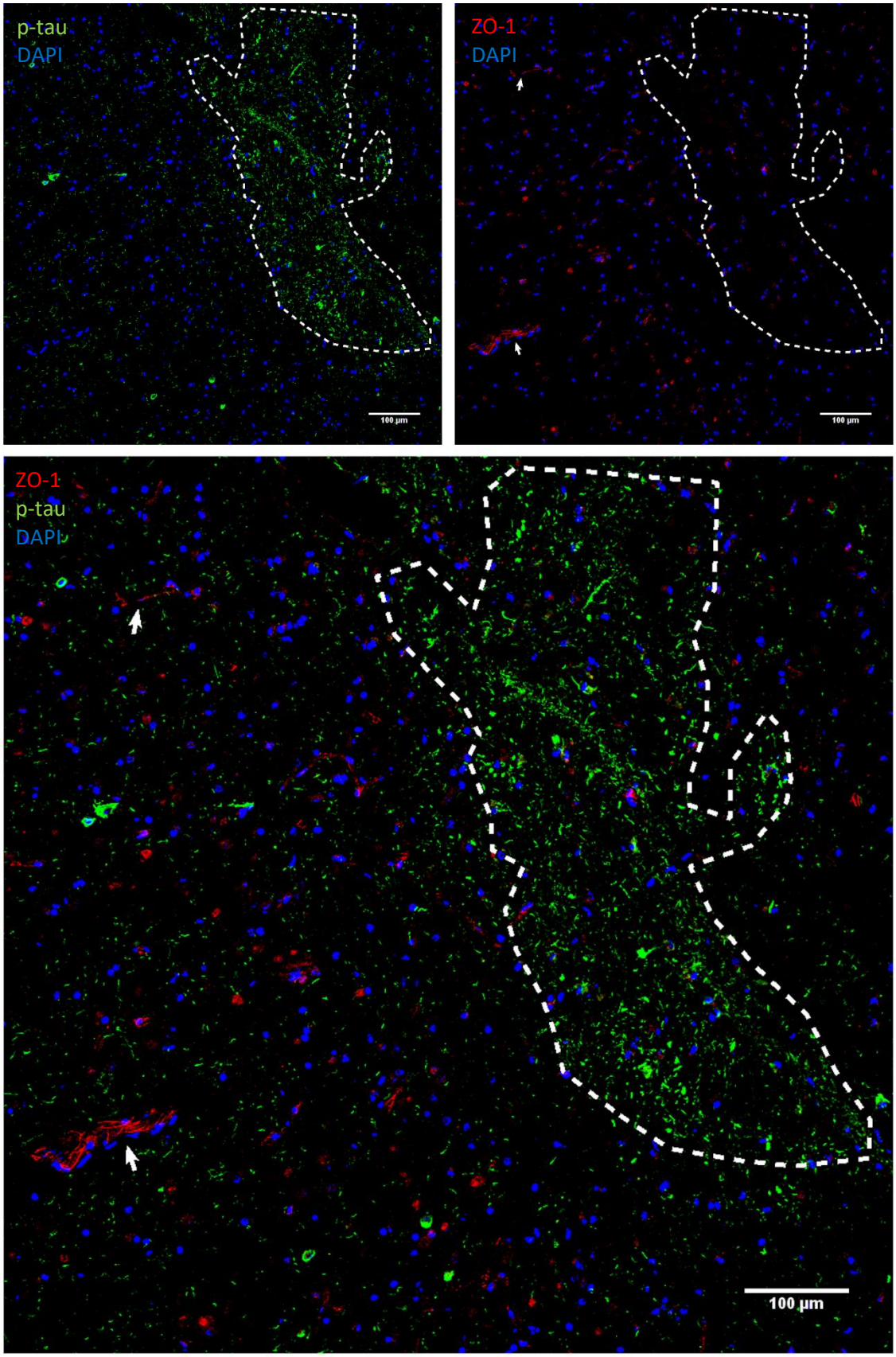


FIGURE 3. 8:IMMUNOHISTOCHEMICAL STAINING OF TIGHT JUNCTION PROTEIN ZO-1 (RED) AND P-TAU (GREEN) IN THE BRAIN OF A FORMER AMATEUR RUGBY PLAYER DIAGNOSED WITH CTE.
Areas of dense p-tau immunoreactivity coincided with a loss of the tight junction protein: ZO-1 immunoreactivity (dotted white line), similar to the patterning found with CLDN-5 immunoreactivity (Figure 3.7). ZO-1 immunoreactivity was diminished and showed discontinuous patterning within these regions. Similar to CLDN-5 immunoreactivity, in the absence of p-tau reactivity, ZO-1 immunoreactivity appeared normal and strand-like patterning (flat arrow).

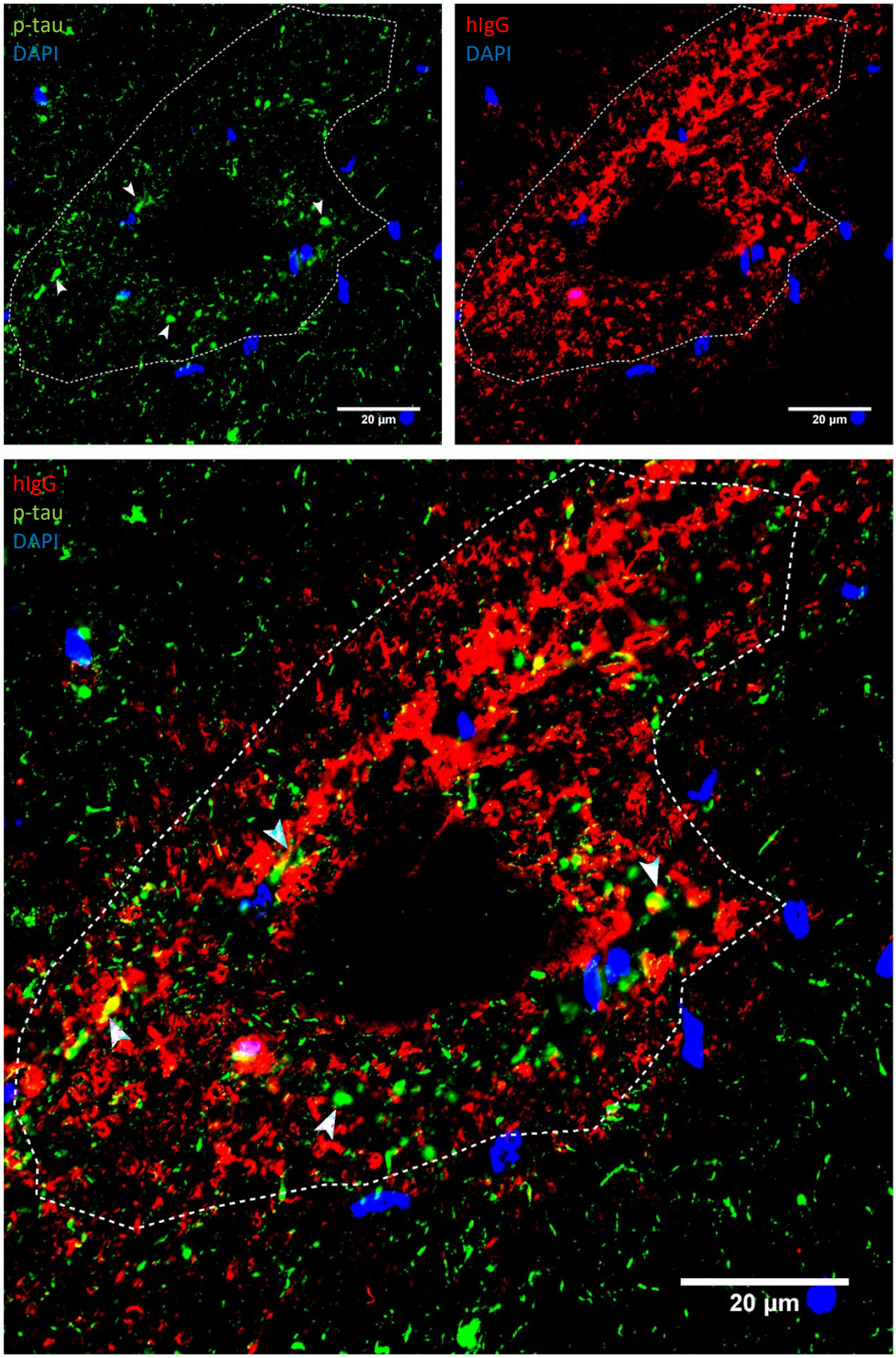


FIGURE 3. 9: IMMUNOHISTOCHEMICAL STAINING OF ENDOGENOUS IGG (RED) AND P-TAU IGG (GREEN) IN THE BRAIN OF A FORMER AMATEUR RUGBY PLAYER DIAGNOSED WITH CTE.
hIgG immunoreactivity was detected surrounding vessels (dotted white legends). Within these regions of hIgG immunoreactivity, dense p-tau immunoreactivity was detected (notched arrows), suggesting BBB disruption is present in regions with of p-tau deposition.

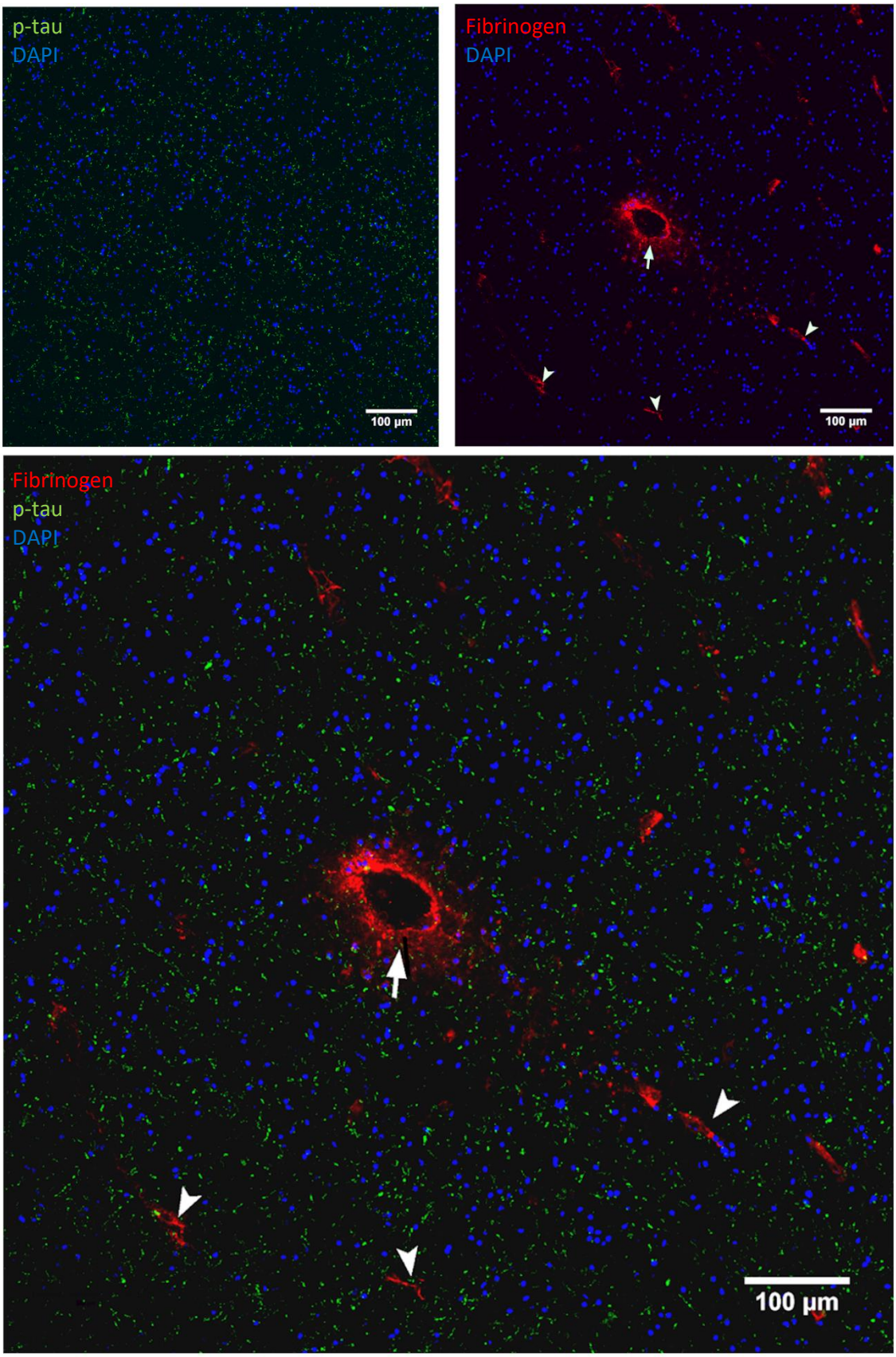


FIGURE 3. 10: IMMUNOHISTOCHEMICAL STAINING OF P-TAU (GREEN) AND ENDOGENOUS FIBRINOGEN (RED) IN THE BRAIN OF A FORMER AMATEUR RUGBY PLAYER DIAGNOSED WITH CTE.
Immunoreactivity for the endogenous blood-based protein, fibrinogen, was detected in regions with the densest p-tau immunoreactivity (flat arrow). In areas where p-tau immunoreactivity was sparse and distal to blood vessels, fibrinogen was retained within the luminal space (notched arrow). This suggests BBB disruption is evident in areas with p-tau deposition.

3.3.3: Case Study 3: Neuropolypathology in patient with single severe TBI in history

The findings presented in this section were published in Doherty, Colin P., Eoin O'Keeffe, James Keaney, Brian Lawlor, Robert F. Coen, Michael Farrell, and Matthew Campbell. 2019. "Neuropolypathology as a Result of Severe Traumatic Brain Injury?" Clinical Neuropathology 38 (01): 14–22. doi:10.5414/NP301131.

See Appendix III

3.3.3.1: Patient History and pathology findings

In 2009, a 66-year-old man presented to neurological services, reporting repetitive, unexplained bouts of anger and frustration, as well as memory and concentration lapses in regard to language and name usage. The patient also reported episodes of depression, brought about due to a recent bereavement; and was subsequently treated for the same. Neurological exam noted no abnormal findings in cranial nerves, limbs gait and balance. Upon cognitive assessment using the MoCA, the patient scored 25 out of 30, losing marks on the delayed recall (2/5), orientation (5/6) and minor flaws in the Clock-Draw test (**Figure 3.11g**). A thorough follow-up neuropsychological assessment was carried out at the end of 2010, including the National Adult Reading Test (NART2) (which marked the patient as high average intelligence), Cambridge Cognition Examination (CAMCOG) (resulting in a score of 94/107) and Mini Mental State Examination (MMSE) (resulting in a score of 27/30). No language difficulties were reported on the Boston Naming Test, although fluency was reduced. The patient's memory was reported as occasionally patchy, but not indicative of the rapid memory loss associated with AD. Mild executive function deficits were noted on verbal fluency tests regarding interference/ intrusion and reduced mental flexibility.

Personal medical history was unremarkable save for a single severe TBI incurred at the age of 12 as a result of a kick from a horse to the head. This injury resulted in loss of consciousness, hospitalization and coma for several days. However, prior to presentation, no adverse behavioural or neurological symptoms were noted.

MRI scans showed mild general atrophy in the absence of focal atrophy (**Figure 3.11a-c**), while PET imaging showed widespread cortical hypometabolism (**Figure 3.11d-f**), which was suggestive of reduced neural function.

A follow-up assessment in 2011 noted significant progression in psychiatric and cognitive symptoms, a repeat of the MoCA2 test resulted in a reduced score of 16/30 (**Figure 3.11h**). The patient was admitted to psychiatric care within a year of this assessment, and an in-life diagnosis of atypical AD was made. The patient's health gradually declined until his death in 2016 due to hypostatic pneumonia.

Post-mortem examination of the brain found frontal atrophy, ventriculomegaly and enlargement of the Sylvian fissure. Pallor of the substantia nigra and post-traumatic cicatrix was also reported (**Figure 3.12a-e**).

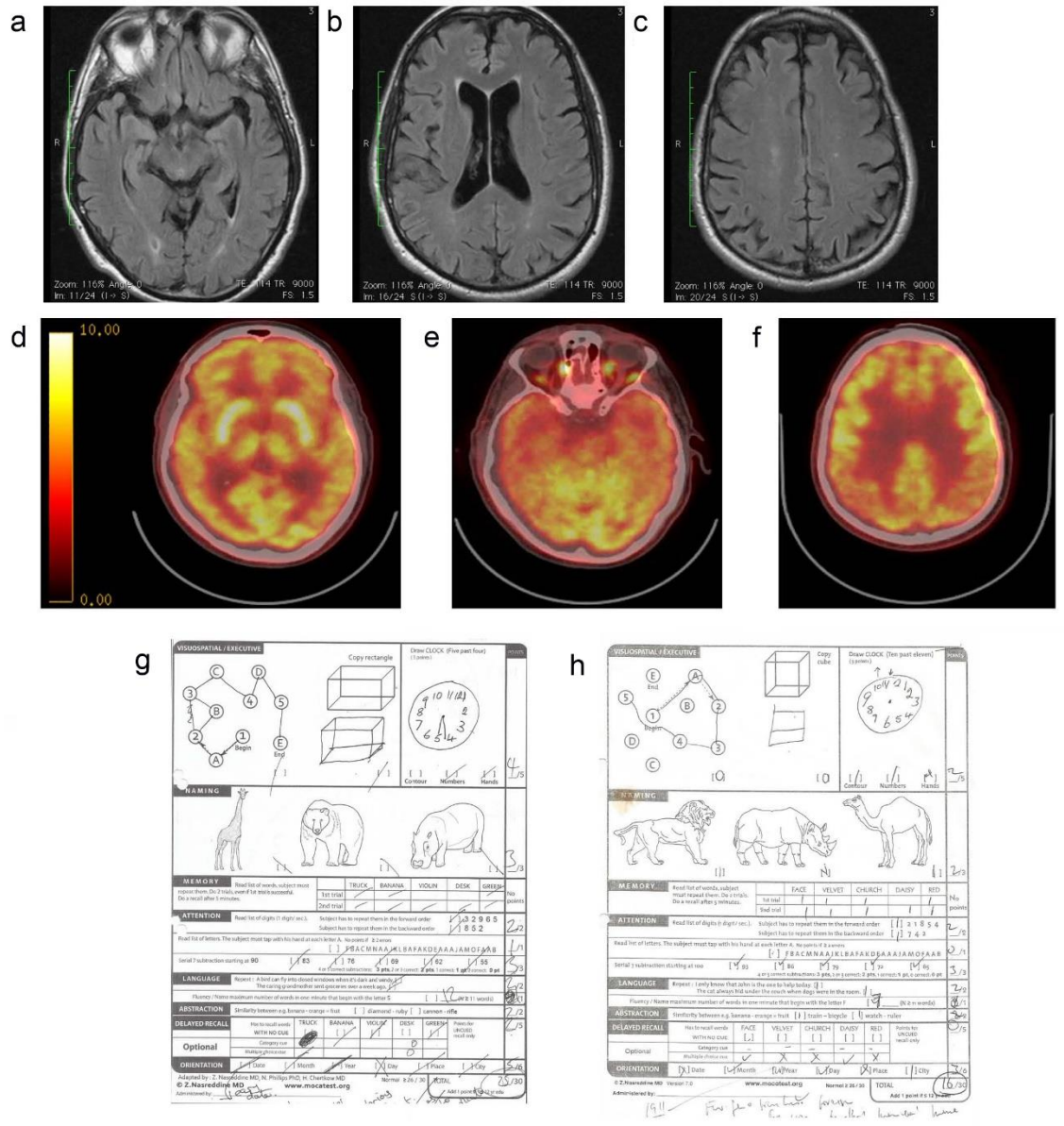


FIGURE 3. 11: INITIAL NEUROLOGICAL AND NEUROIMAGING ASSESSMENT OF A PATIENT PRESENTING WITH EMOTIONAL INSTABILITY AND LAPSES IN MEMORY.

MRI and PET imaging carried out between 2009 and 2011. a-c) FLAIR MRI images of a) the temporal lobes, b) the lateral ventricles and c) centrum semi-ovale. Generalised atrophy was noted, in the absence of any other abnormal finding. d-f) PET scan images using a radiolabelled fluorodeoxyglucose tracer of the c) caudate, f) temporal lobes and d) the caentrum semiovale. Generalized hypometabolism of the cortex was reported. g & h) The MOCA exam completed by the patient in g) 2009 and h) 2011. Hand placement for the clock was incorrect on both occasions. All assessments were carried out by medical personnel in St. James's Hospital, Dublin. Used with permission from Campbell lab publications (Doherty *et al.,* 2018).

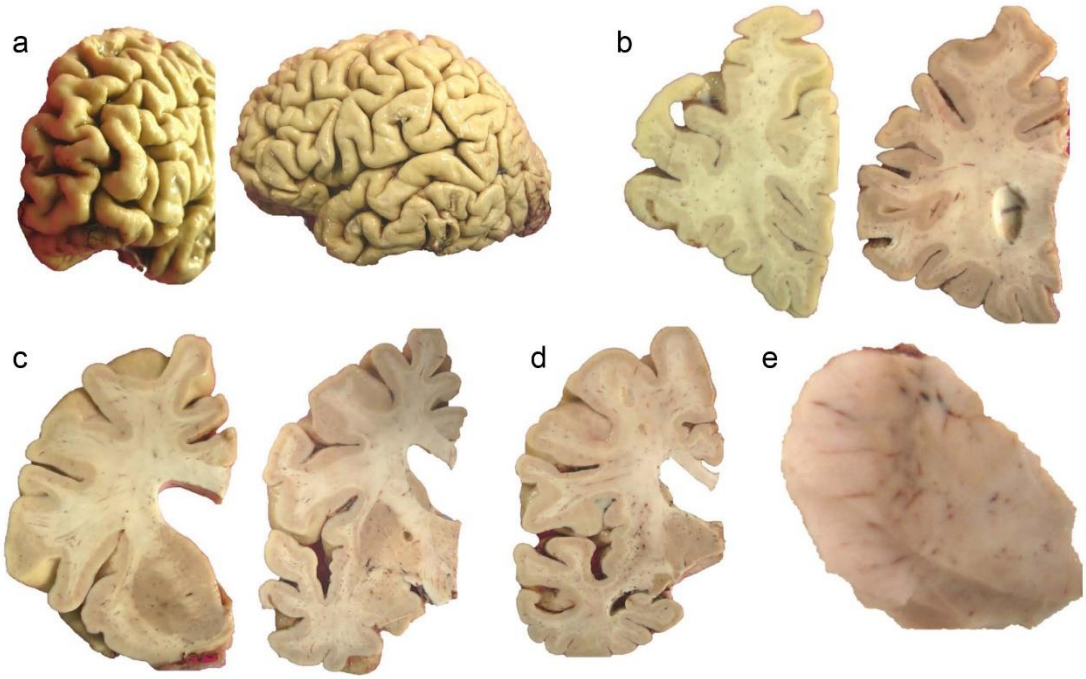


FIGURE 3. 12: SECTIONS OF BRAIN FOR MACROSCOPIC EXAMINATION FROM PATIENT PRESENTING WITH EMOTIONAL INSTABILITY AND LAPSES IN MEMORY.

a) Frontal whole brain sections indicated to have generalized atrophy upon pathologist examination. **b**) Coronal sections of frontal atrophy section. **c**) Ventriculomegaly (**arrow head**) and enlarged Sylvian fissure (*****)**. d**) Normal appearing hippocampus (**arrow head**). **e**) Pallor of the substantia nigra. Sections were prepared by personnel from the Dublin Brain Bank, Beaumont Hospital, Dublin. Used with permission from Campbell lab publications (Doherty *et al.,* 2018).

Histological examination of the brain found hyaline changes to large vessels within cortical regions and the parenchyma of the sulcal depths, accompanied by superficial spongiosis of cortical tissue (**Figure 3.13a-b**). examination of hippocampus histology showed an absence of atrophy (**Figure 3.13c**), however, neurofibrillary tangles (NFTs) and pyramidal inclusions were identified within the hippocampus and the neocortex (**Figure 3. 13c.i** & **c.ii**).

Immunostaining for p-tau and amyloid-β found diffuse staining of p-tau within the sulcal depths as well as intense amyloid-β staining surrounding larger vessels, indicative of severe cerebral amyloid angiopathy (**Figure 3. 14a.i** and **a.ii**). Diffuse p-tau staining, as well as NFTs and dystrophic neurites was identified in sub-cortical white matter, in addition to perivascular p-τ deposits (**Figure 3.14b-e**). Immunostaining for α-synuclein-positive Lewy bodies displayed positive staining within the superior temporal gyrus, the amygdala, the lateral frontal lobe and the substanita nigra, as well as regions of the neocortex (Figure 3.15 a-d). Based on these findings, the attending pathologist gave an ABC score of dementia progression as A3, B3, C2, based on pathology scoring criteria (Kovacs & Gelpi, 2012).

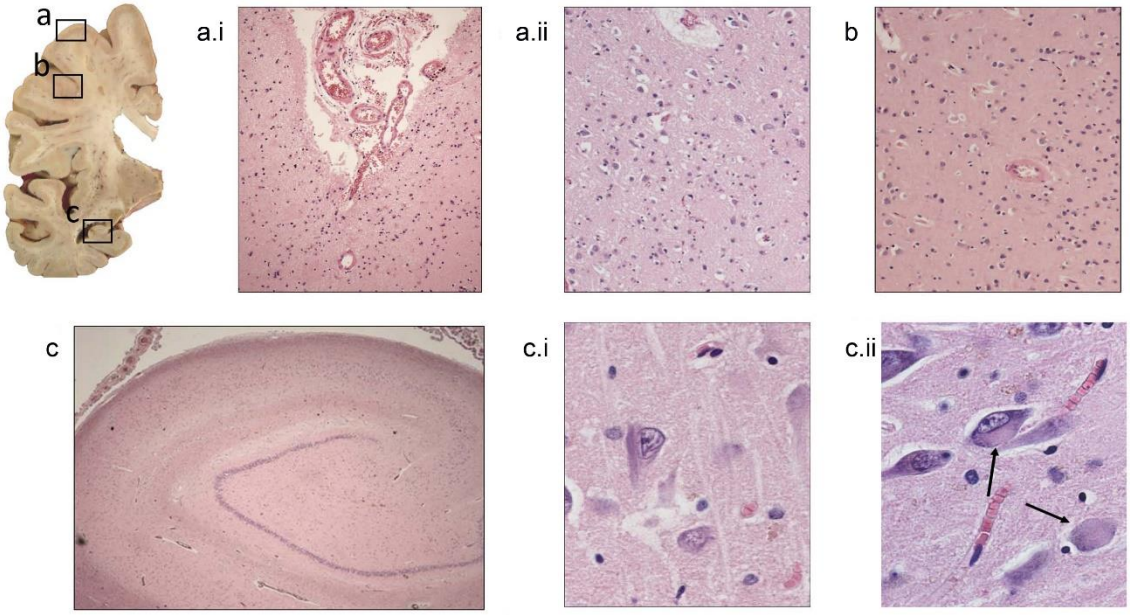


FIGURE 3. 13: HISTOLOGY OF CORTICAL, SULCAL DEPTHS AND HIPPOCAMPUS PATIENT PRESENTING WITH EMOTIONAL INSTABILITY AND LAPSES IN MEMORY.

a.i) Hyaline changes in large vessels within cortical regions. **a.ii**) Superficial spongiosis of cortical tissue. **b**) Hyaline changes in vessels of the parenchyma of the sulcal depths. **c**) Histology of hippocampus indicating an absence of atrophy. **c.i**) Neurofibrillary tangles present within the hippocampus. **c.ii)** Pyramidal inclusions (arrows) within the hippocampus. Sections and histology were prepared by personnel from the Dublin Brain Bank, Beaumont Hospital, Dublin. Used with permission from Campbell lab publications (Doherty *et al.,* 2018).

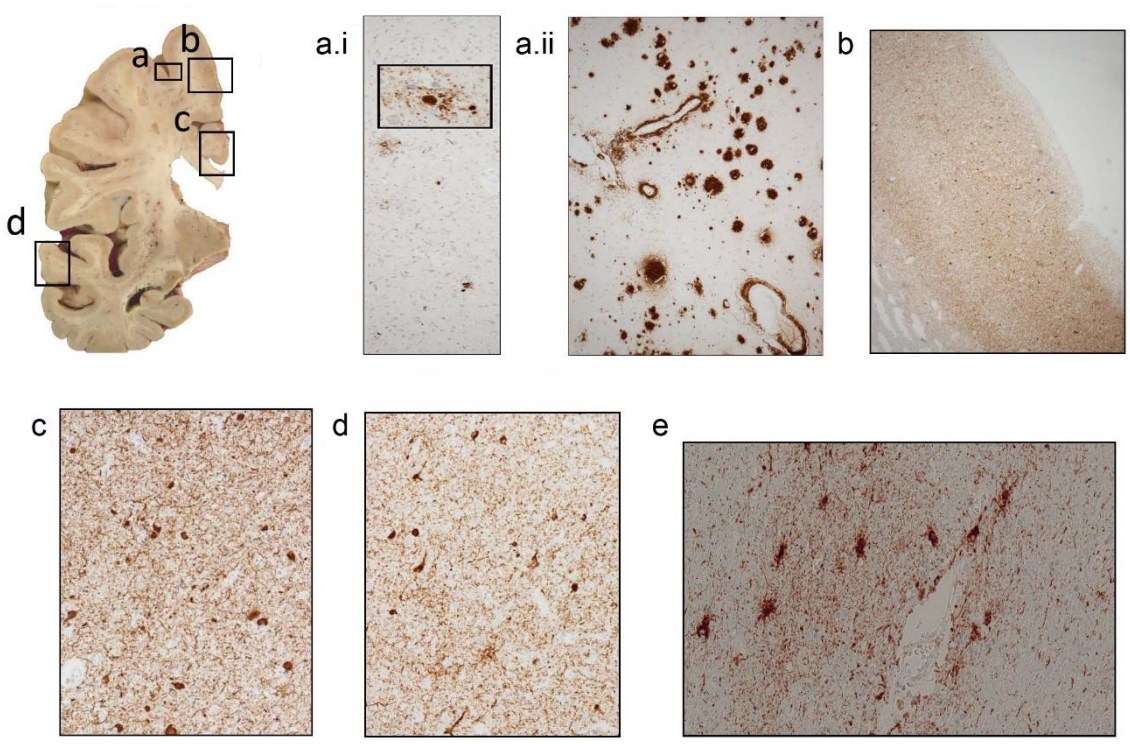


FIGURE 3. 14: IMMUNOHISTOCHEMISTRY FOR AMYLOID-B AND P-TAU IN IN PATIENT PRESENTING WITH EMOTIONAL INSTABILITY AND LAPSES IN MEMORY.

a.i) Diffuse staining of p-**tau** plaques within sulcal depths. **a.ii)** Amyloid-β staining surrounding large vessels, noted for severe cerebral amyloid angiopathy (CAA). **b**) Immunostaining for p-tau, neurofibrillary tangles and dystrophic neurites. **c-d**) Immunostaining for p-tau, neurofibrillary tangles and dystrophic neurites. **e**) Perivascular p-tau immunostaining present within sub-cortical white matter. Sections and histology were prepared by personnel from the Dublin Brain Bank, Beaumont Hospital, Dublin. Used with permission from Campbell lab publications (Doherty *et al.,* 2018).

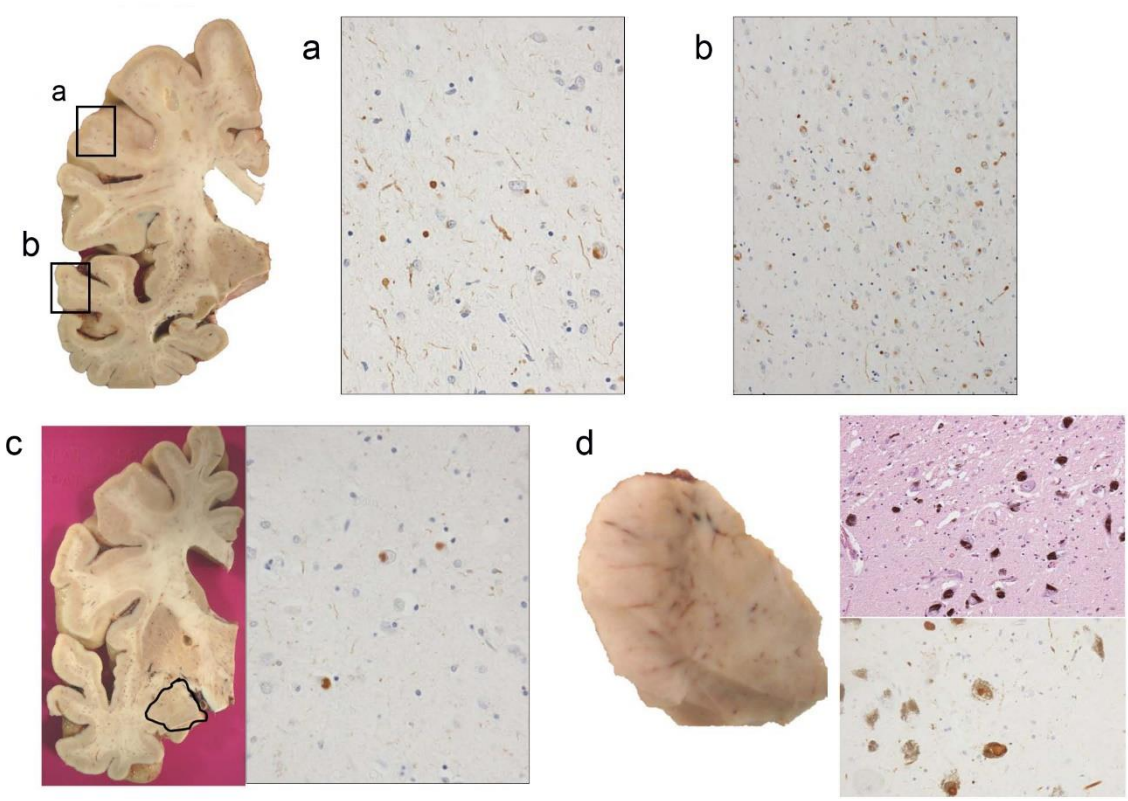


FIGURE 3. 15: IMMUNOSTAINING FOR A-SYNUCLEIN-POSITIVE LEWY BODIES.

Immunostaining for α-synuclein indicated the presence of Lewy bodies within the a) superior temporal gyrus (STG), b) lateral frontal lobe, c) the amygdala and d) the substantia nigra. Pallor of the substantia nigra was also reported. Sections and histology were prepared by personnel from the Dublin Brain Bank, Beaumont Hospital, Dublin. Used with permission from Campbell lab publications (Doherty *et al.,* 2018).

3.3.3.2: BBBD and loss of TJ integrity

Immunostaining for p-tau found that immunoreactivity formed dense clusters of p-tau, rather that more widespread tau immunoreactivity previously outlined in this chapter (**Figure 3.16**). However, similar to the previously detailed case studies, CLDN5 immunoreactivity was absent when within to these dense clusters of p-tau, in the surrounding proximity. In the absence of p-tau clusters, or where p-tau immunoreactivity was sparse, CLDN5 appeared normal long blood vessels. It was noted however that larger vessels had large gulfs of space between vessel wall and neighbouring cells. In these vessels, staining patterns of CLDN5 appeared more globular and discontinuous, suggesting some change to BBB integrity within these regions. However, these gulfs in neighbouring cells did not coincide with an enrichment of p-tau deposition.

Upon investigating the extent of BBBD within this individual, it was found that hIgG immunoreactivity was mostly contained within the lumen of blood vessels (**Figure 3.17**). This was accompanied with CLDN5 patterning, suggesting that BBB was mostly intact across the sections examined. However, that staining pattern for CLDN5 appeared globular and discontinues within these regions, suggesting some degree of disruption to CLDN5 localisation within the endothelial cells. There was limited diffuse hIgG immunoreactivity in areas noted of being devoid of CLDN5 immunoreactivity, suggesting that the BBB has been disrupted within limited areas. However, taken together, the globular staining of CLDN5 and limited extravasation of hIgG may indicate historic disruptions to the BBB rather than ongoing disruption present at time of death.

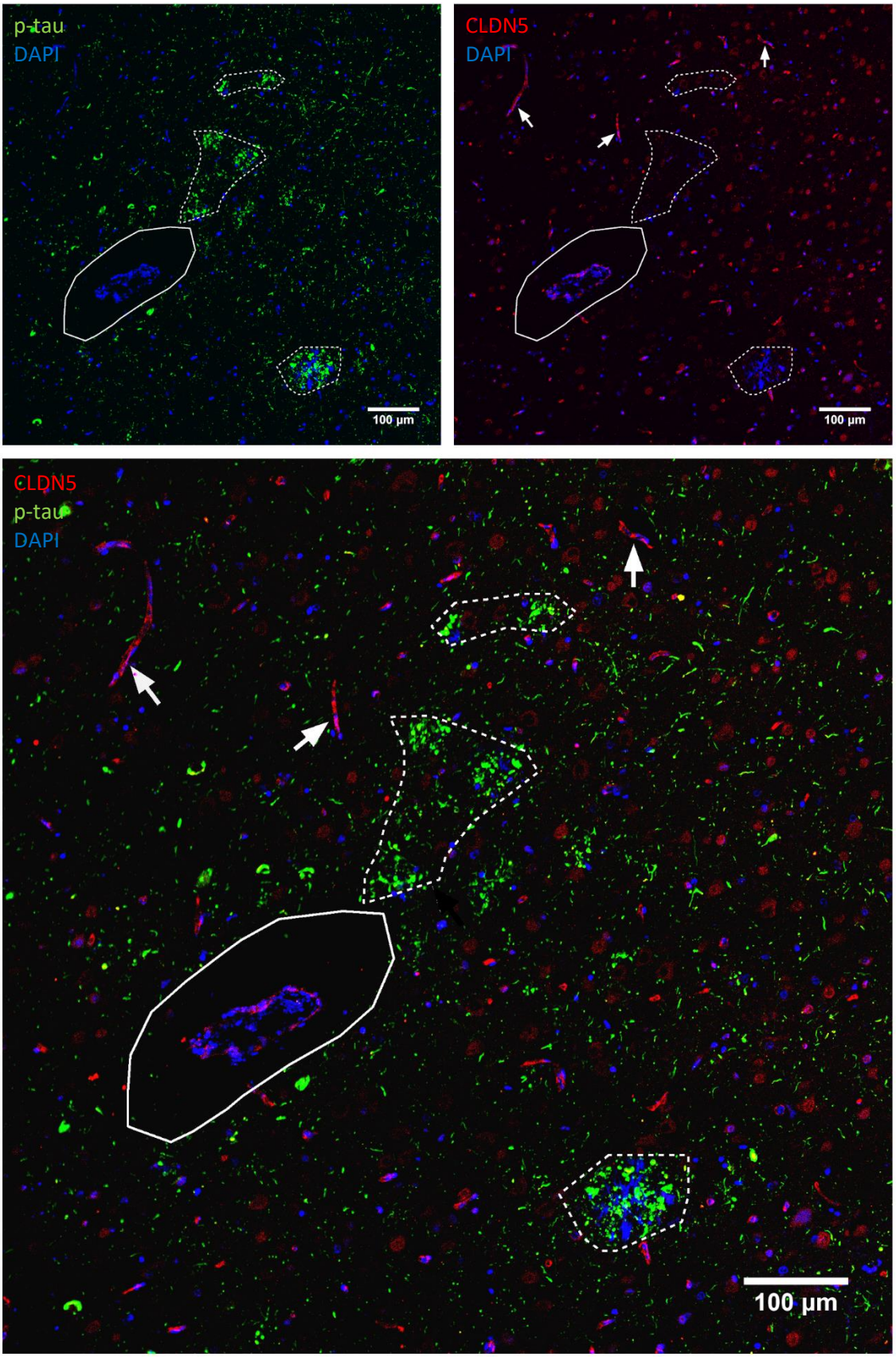


FIGURE 3. 16: IMMUNOHISTOCHEMICAL STAINING OF TIGHT JUNCTION PROTEIN CLAUDIN-5 (RED) AND P-TAU (GREEN) IN THE BRAIN OF AN INDIVIDUAL DIAGNOSED WITH MULTIPLE NEUROPATHOLOGIES

Immunoreactivity for p-tau was noted for appearing in dense clusters. Within these regions, immunoactivity for the tight junction protein: CLDN-5 was absent or reduced (white dotted line). In the absence p-tau deposition, CLDN-5 appeared normal and strand-like (flat arrow). It was also noted that large gaps were present between larger vessels and surrounding cells (solid white line). CLDN-5 immunoreactivity discontinuous and globular, suggesting a possible vascular pathology. Immunoreactivity for p-tau was not observed in these regions.

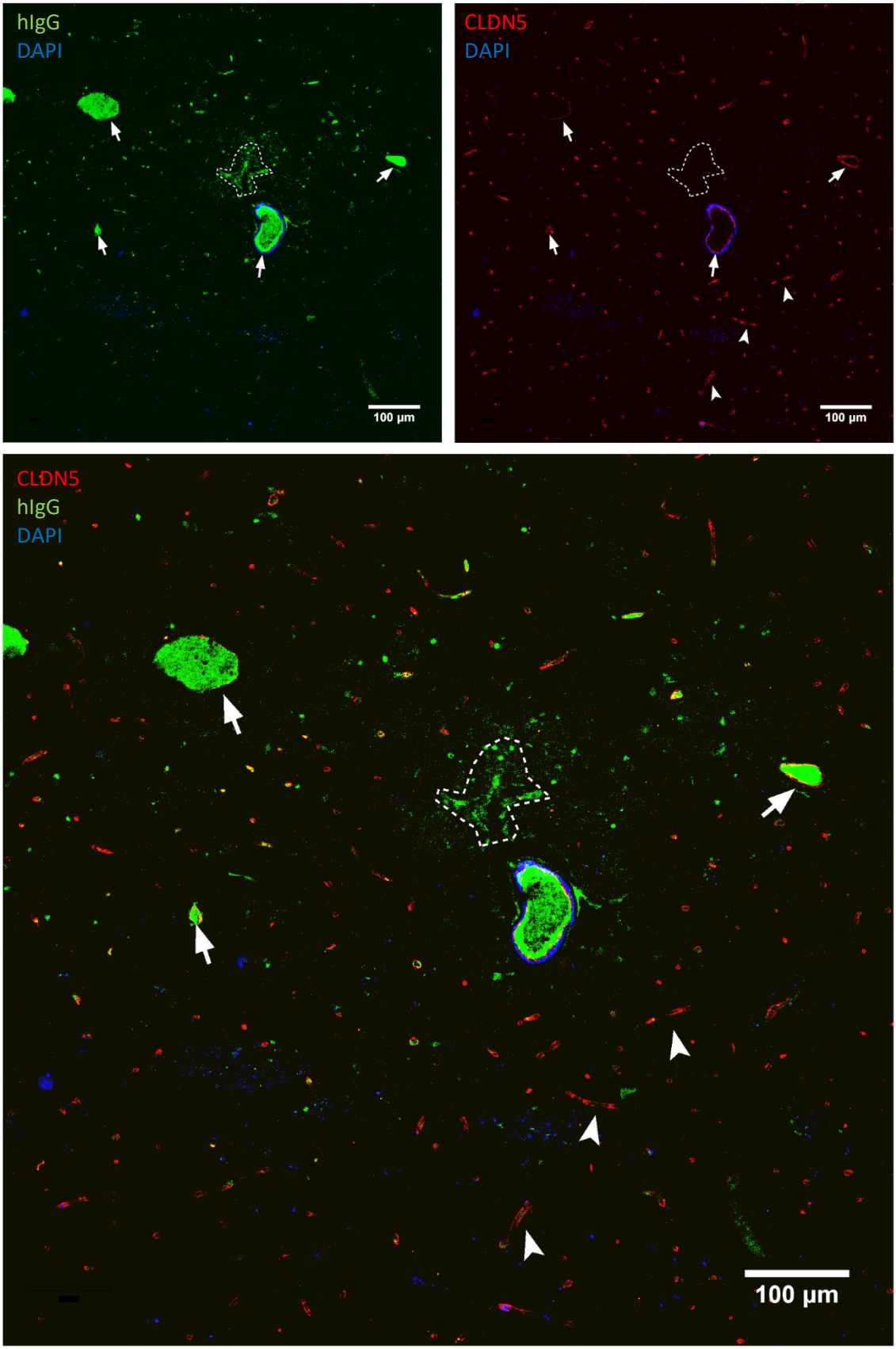


FIGURE 3. 17: IMMUNOHISTOCHEMICAL STAINING OF TIGHT JUNCTION PROTEIN CLAUDIN-5 (RED) AND ENDOGENOUS IGG (GREEN) IN THE BRAIN OF AN INDIVIDUAL DIAGNOSED WITH MULTIPLE NEUROPATHOLOGIES. Immunoreactivity for endogenous hIgG appeared mostly within the luminal space. Immunoreactivity for the tight junction protein: CLDN-5, was largely intact in these areas, however, signal appeared diminished and globular (flat arrows). CLDN-5 immunoreactivity appeared normal and strand-like in areas devoid of extravasating hIgG (notched arrows). Diffuse immunoreactivity for hIgG was noted in areas lacking CLDN-5 immunoreactivity, suggesting limited BBB disruption (white dotted line).

3.3.4: Comparison of tau and BBBD pathology in reported cases studies with that of a control brain

The Dublin brain bank prepared and provided a limited number of frozen sections of brain tissue from an individual that had no reported diagnosis of neurological conditions or pathologies prior to their death. Information provided from brain bank along with the tissue indicated that the individual was man in his mid-50's, with no notable history of head trauma. However, the possibility of the individual having sustained an unreported mTBI was not documented at the time of donation. This tissue served as a limited control in investigating the staining pattern of the TJ protein CLDN5, the presence or absence of p-tau deposition and the presence or absence of BBBD through the extravasation of endogenous, blood-based proteins.

Immunohistochemical staining for the TJ protein: CLDN5, showed long, continuous strand-like patterns of the protein (Figure 3.18 A). In addition to the continuous strands of CLDN5, immunoreactivity of p-tau was almost completely absent from control tissue sections (Figure 3.18 B). This contrasts with findings made in all three case studies. In case study 1 and case study 2, both involving individuals diagnosed with CTE, CLDN5 immunoreactivity is greatly diminished in areas of dense p-tau deposition (Figure 3.18D & G respectively, dotted white line). The is also notable widespread immunoreactivity for p-tau in both case study 1 and 2 (Figure 3.18 E & H respectively). Reduced CLDN5 immunoreactivity within p-tau deposits was also observed in case study 3 (Figure 3.18 J, dotted white line), a case of neuropolypathology brought about by a historic severe TBI. Widespread p-tau immunoreactivity is also present, along with the dense clusters of p-tau unique to case study 3 (Figure 3.18 K). In all three a case studies, areas that show a relatively reduced density of p-tau deposition show normal, strand-like CLDN5 (Figure 3.18 F, I & L), similar to control sections (Figure 3.18 C). Such a finding suggests that the presence of p-tau deposits in close proximity to blood vessels can influence the presence of CLDN5 within the neurovasculature.

A limited comparison of the integrity the BBB was also made between the control tissue and that of the case studies. Immunostaining for endogenous hIgG proteins found that hIgG was confined with CLDN-5 postive blood vessels (Figure 3.19 A-C). No areas were noted for diffuse staining of hIgG indicative of extravasated protein into the perivascular space. In case study 1 however, area lacking CLDN5 displayed diffuse immunoreactivity

of hIgG, while areas that showed control-like patterning of CLDN5 had hIgG immunoreactivity confined within these blood vessels (Figure 3.19 D-F). Case study 3 displayed hIgG immunoreactivity confined mostly within CLDN5-postive vessels (Figure 3.19 J-L). However, post0ve hIgG immunoreactivity was noted near a CLDN5-postive vessel (Figure 3.19 L, dotted white line), which may be indicative of a historic disruption to the BBB.

Similarly, immunoreactivity for the blood-based protein fibrinogen displayed a structural pattern, indicative of confinement with blood vessels (Figure 3.20 A-C). The absence of p-tau was also evident in these sections. In case study 2, fibrinogen extravasation, indicated by diffuse immunoreactivity surround larger vessels, was evident and coincided with p-tau deposits near these vessels (Figure 3.20 D-F).

These comparisons suggest the BBBD is not present in control brain tissue that lacks a history of TBI, and that the history of head trauma likely contributed to the findings presented for each case study.

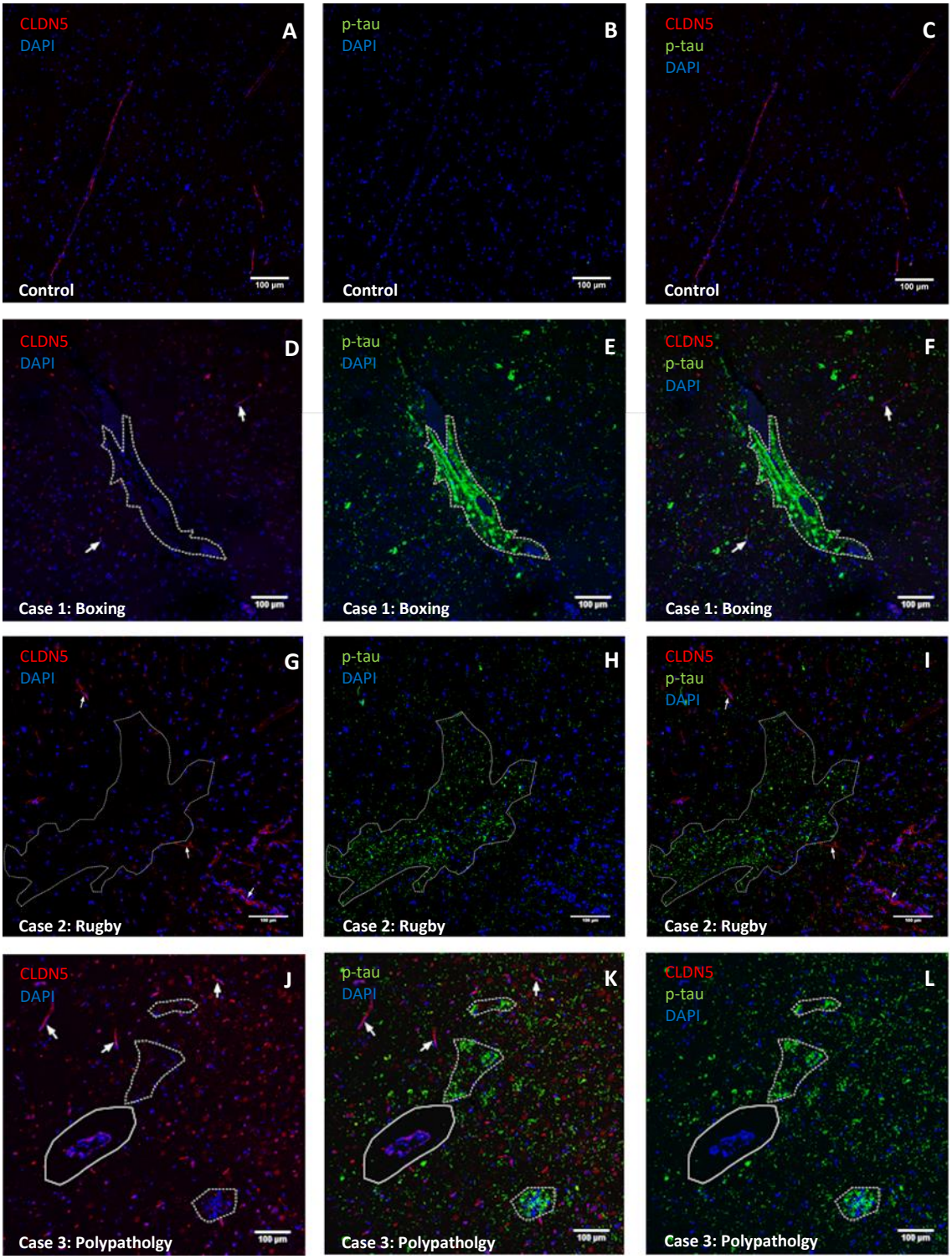


FIGURE 3. 18: NORMAL CLAUDIN-5 IMMUNOREACTIVITY COINCIDES WITH AN ABSENT OF PHOSPHORYLATED-TAU IMMUNOREACTIVITY IN CONTROL TISSUE.
Immunohistochemical staining for the TJ protein: CLDN5, and tau in brain tissue of control (**A-C**), Case Study 1 (boxer, **D-F**), Case Study 2 (rugby player, **G-I**) and Case Study 3 (polypathology, **J-L**). Immunoreactivity for CLDN5 appeared in long, continuous, strand-like patterns in control tissue (**A**), while p-tau immunoreactivity was almost entirely absent (**B**). The staining pattern for CLDN5 suggests that normal localisation can occure in the absence of p-tau depostion (**C**). The absence of p-tau and the strand-like patterning of CLDN5 contrasts with the immunohistochemistry findings made within each case study. Within case study 1 and 2, p-tau immunoreactivity is widespread (**E** and **H** respectively, dotted white line), while CLDN5 patterning was absent or diminished within regions of dense p-tau deposition and similar to the control section in areas distant to p-tau deposition (**D** and **G** respectively, flat-headed arrows). Phosphorylated-tau deposition was also widespread in case study 3, as well as forming dense clusters (**K,** dotter white line). CLDN5 staining also appeared similar to that of control sections when distal to p-tau depositions (**J,** flat-headed arrows). The large gaps between blood vessels (solid white line) was also absent in control tissue.

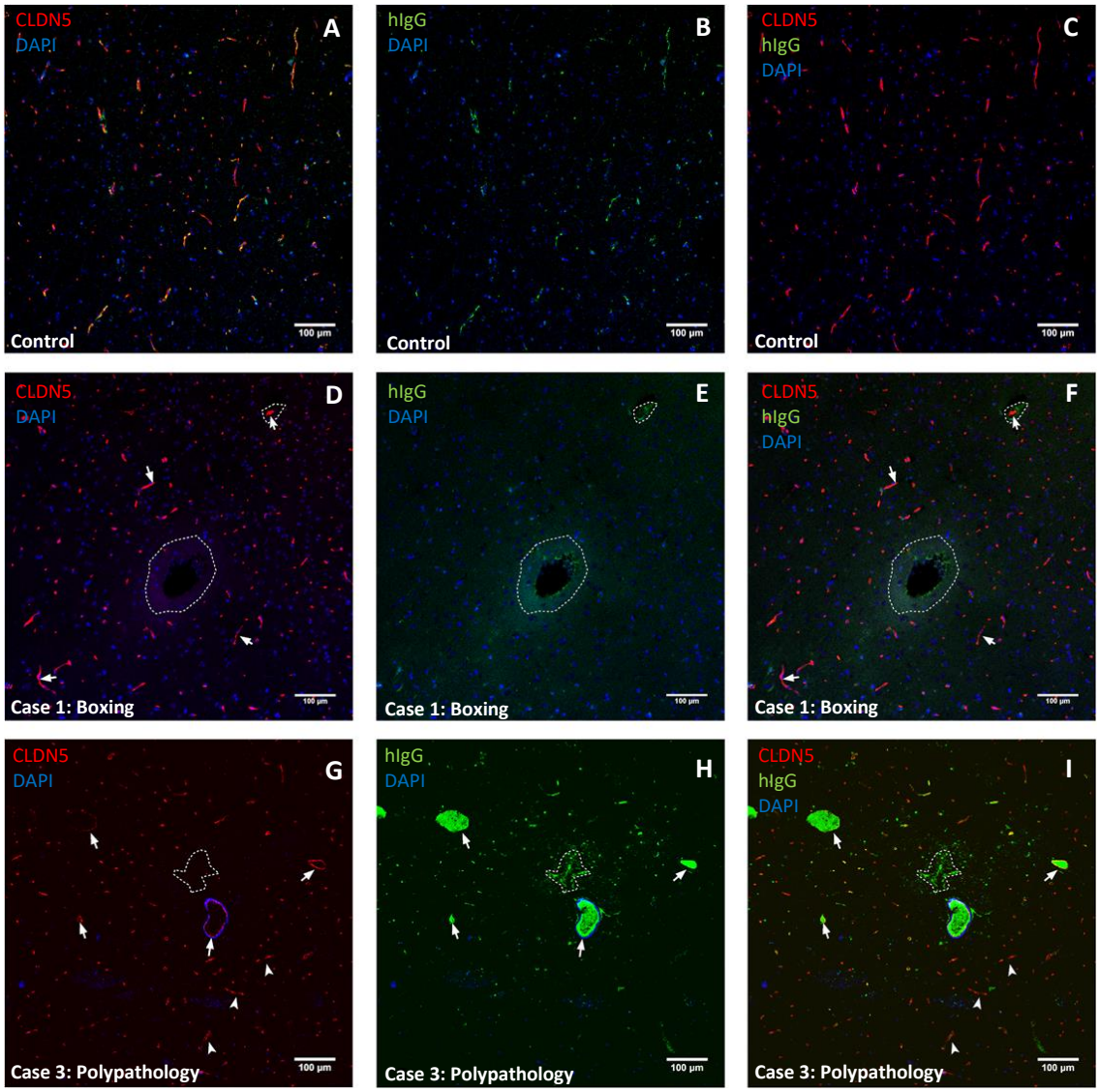


FIGURE 3. 19: ABSENCE OF HIGG EXTRAVASATION SUGGESTS NORMAL BBB FUNCTION IN CONTROL TISSUE. Immunohistochemical staining for the TJ protein: CLDN5, and tau in brain tissue of control (**A-C**), Case Study 1 (boxer, **D-F**), Case Study 3 (neuropolypathology, **G-I**). CLDN5 immunoreactivity appeared continuous and strand-like, along the blood vessels (**A**). Immunoreactivity for hIgG appeared to be confined within blood-vessels in control tissue (**B**). CLDN5 immunoreactivity surrounded areas of hIgG immunoreactivity, indicating that hIgG confined within blood vessels in control tissue. There was no evidence of hIgG extravasation in control tissue suggest an absence of BBBD (**C**). In contrast, case study 2 demonstrates hIgG extravasation near vessels lacking CLDN5 immunoreactivity (dotted white line) (**D-E**). Areas without signs of hIgG extravasation show CLDN5 staining similar to that found in control tissue (flat-headed arrows) (**D** & **F**). In case study 3, hIgG was mostly confined to blood vessles surrounded by CLDN5 immunoreactivity (flat-headed arrows) (**G** & **I**). Potential evidence of a historic disruption to the BBB is identified in case study 3 by immunoreactivity for hIgG near to a blood vessel that stained positive for CLDN5 (dotted white line) (**H**).

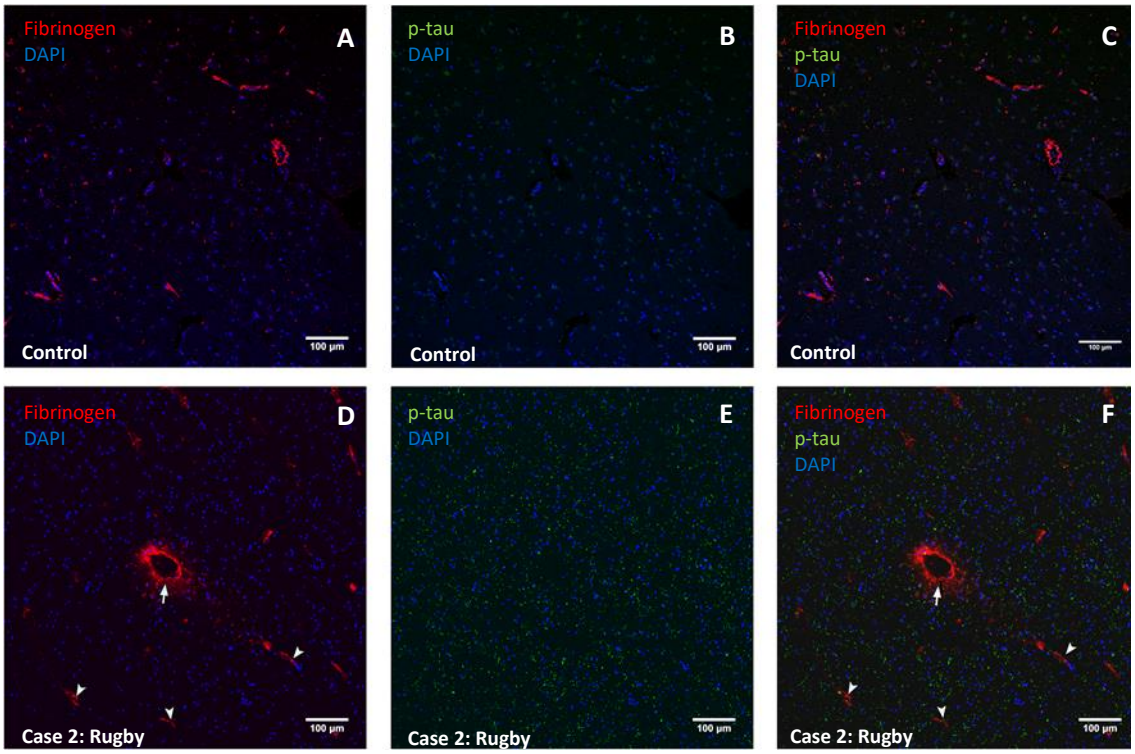


FIGURE 3. 20: FIBRINOGEN EXTRAVASATION AND IMMUNOREACTIVITY FOR P-TAU IS ABSENT IN CONTROL TISSUE.

Immunohistochemical staining for the TJ protein: CLDN5, and tau in brain tissue of control (**A-C**), Case Study 2 (rugby player, **D-F**). Immunostaining for endogenous fibrinogen within control tissue found that the protein was confined within blood-vessels (**A** & **C**). In addition to this, p-tau immunoreactivity was almost completely absent (**B**). The absence of fibrinogen extravasation and p-tau immunoreactivity suggests a link between BBBD and deposition of the microtubule-binding protein. In contrast, case study 2 showed diffuse immunoreactivity for fibrinogen suggesting extravasation for the protein (**D**). This coincided with areas of the densest p-tau deposits (**E**, flat-headed arrow). Immunoreactivity suggested confinement of fibrinogen within blood vessels in areas of sparse absence of p-tau staining (**D** & **F**, notched arrows).

3.4: Discussion

BBBD has been demonstrated in a number of animal studies as an early event in TBI, and recent case reports have shown that all levels of TBI can result in disruption of the BBB (Hay *et al.,* 2015; Tagge *et al.,* 2018). Recent findings have indicated that focal BBBD is accompanied with potential early markers of CTE development, strengthening the possibility that BBB dysfunction is an early inciting event to disease development (Tagge *et al.,* 2018). Presented in this chapter are three case studies linked by BBBD, distinct tauopathies and a history of TBI.

Cases 1 and 2 both had long careers in competitive contact sports and accompanying history of mTBI exposure. The clinical history of Case 1 and 2 is in keeping with our current understanding of individuals at risk of developing CTE; previously healthy individuals with no prior history of dementia-like illness, no gross morphological changes and mild cognitive impairment upon presentation. Autopsy and subsequence molecular examination found that perivascular p-tau was widespread and readily apparent in both cases, accompanied by loss of TJ protein expression in areas proximal to p-tau deposits. However, TJ expression appeared normal in the absence of phosphorylated tau aggerates, even within the space of microns. The proximity of BBB dysfunction and pathological aggregates suggest an intimate link between disease progression and compromised BBB integrity.

Case 3 presents an interesting possibility with regards to the consequences of severe TBIs. As noted, the individual had an unremarkable medical history, save for a single severe TBI incurred over 4 decades prior to presentation of emotional instability, memory and concentration deficits. However, upon autopsy examination was diagnosed with multiple neuropathologies, including Diffuse Lewy Body Disease (DLBD), FTD and AD, accompanied by BBB dysfunction in areas dense with p-tau. While the diagnoses and presentation of this case is distinct from that of Case 1 and 2, it nonetheless highlights in a link between compromises to the BBB and the development of dementia later in life.

Cases 1 and 2 both display clear BBB dysfunction in regions of dense, perivascular p-tau deposition, and current CTE diagnostic guidelines stipulate perivascular p-tau in the depths of the sulci as a requirement for the diagnosis of CTE to be made (McKee *et al.,* 2016). However, confounding factors in both cases must be considered when dealing with what remains a controversial condition. Both cases were reported to have a high intake

of alcohol on a regular basis, which may have contributed to decreased levels of TJ proteins (Yu *et al.,* 2017). Case 1 was also notable for a previous diagnosis of schizophrenia, a condition for which there is increasing evidence to support a cerebrovasculature role underlying the condition. The potential biomarker for BBBD; S100β, has been shown to be increased in serum of schizophrenic patients; mutations resulting in reduced expression of TJ proteins have been associated with schizophrenia development and knockdown of the TJ protein; claudin-5 has produced schizophrenic-like phenotypes in mice (Schroeter *et al.,* 2003; Enwright *et al.,* 2017; Greene *et al.,* 2017). However, CTE has been diagnosed in individuals with schizophrenia previously (Shively *et al.,* 2017). The case series reported by Shively *et al.* detailed findings of perivascular p-tau deposition consistent with CTE in institutionalised schizophrenic patients, up to 40 years after undergoing a leucotomy. In these cases, it is proposed the single, localised axonal injury lead to the subsequent development of CTE. However, it must be acknowledged that the injuries described by Shively *et al.* differ substantially from those of our case. In addition to the diagnosis of schizophrenia, the individual of Case 1 died via subarachnoid haemorrhage brought about by a fall. Severe TBI has a known influence on BBB integrity, demonstrated both in animal models and human cases (Başkaya *et al.,*1997; O'Leary & Nichol, 2018). All these factors and their potential influence should be considered when examining the BBBD and cognitive degeneration observed in these cases. Despite this, the long history of repetitive mTBI in both cases should not be overlooked; and could pose as a potential inciting factor to neurocognitive decline, as well as the development of a psychiatric condition in regard to Case 1.

Case 3 represents the potential long-term consequences of a single, severe TBI. It may also suggest a potential difference between a single, severe injury and repetitive, mTBI. As noted in Case 3; widespread amyloid plaques were observed, which were absent in Cases 1 and 2. A study of moderate and severe TBI survivors also reported widespread amyloid pathology in roughly a third of TBI cases, but absent or normal amounts for their age bracket in age-matched controls (Johnson, Stewart & Smith, 2011). However, polypathologies have also been diagnosed in footballers who repeatably header the ball, which could suggest that both mTBI and severe TBI follow similar disease trajectories (Ling *et al.,*2017). Therefore, environmental factors, secondary to the injury, may play a role in the direction of disease development. However, of note is the much shorter survival time after TBI in the individuals of Johnson, Stewart & Smith's study than that

of the case study presented here (Mean 8.2 years compared to 51 years.). The long survival time after injury is also a confounding factor. While it may be possible that BBBD lingered since the injury, it may also be the case that the BBBD due to the severe TBI influenced BBB changes over the course of ageing, ultimately leading the development of multiple dementia conditions. A recent study has demonstrated increased permeability of the BBB to endogenous blood-based proteins in non-pathological human brains, in the absence of changes in microvascular density or pericyte coverage (Goodall *et al.,* 2018). In addition, a recent case reported attributed the development of CTE to a single severe TBI incurred by an individual 42-years prior to death (Tribett *et al.,*2019). Similar to Case 3, the manifestation of the disease state occurred rapidly after many years of injury and in the absence of other notable medical findings, suggesting that the effects TBI may linger for many years and develop to a critical mass until a neurodegenerative condition can manifest.

Control tissue was in very limited supply during the course of the study, with only single non-pathological brain available for sectioning purposes during preparation of this work. While comparisons made with the tissue available is limited, they do make the findings within the pathological case studies all the more striking. In contrast to all three case studies, p-tau appears to be entirely absent in the sections provided. Accompanying this observation is the patterning of CLDN5 within the sections. Immunohistochemistry for the TJ protein appears continuous long the membranes of blood vessels, in contrast to case study 1, 2 and 3, in which CLDN5 can appear discontinuous, diminished or absent entirely in areas in proximity to p-tau. Comparison with control tissue also highlights the "normal" staining pattern of CLDN5 in pathological tissue in areas of sparse p-tau deposition. Complimenting the continuous patterning of CLDN5 is the noted lack of observable extravasation of endogenous blood-base proteins; fibrinogen and hIgG. The confinement of these proteins within vasculature structures marked by CLDN5 indicates that BBBD does not appear to be present within sections of tissue in this individual, in contrast to case study 1 & 2, which displayed signs of BBBD at time of death. However, among the limitations of the inclusion of this control tissue is the lack of an accurate head trauma history for the donating individual. While it was not indicated that the donor had a history of head trauma, at the time of donation, these details were not heavily investigated. Future studies could benefit from great documentation of prior medical

history, especially in cases of control specimens. However, it must be acknowledged that such enquiries may be difficult to make at the time of donation.

The differences between Cases 1 and 2 and that of Case 3 also poses the possibility that repetitive mTBI and a single, more severe TBI may trigger distinct pathological pathways. The observation of widespread amyloid plaques in Case 3, but their absence in Cases 1 and 2 may lend itself to this possibility. This is in part strengthened by a study of moderate and severe TBI survivors who died at least 1 year after injury, although this is confounded by findings of polypathologies in repetitive mTBI case and the CTE in cases of single severe TBI (Johnson, Stewart & Smith, 2011; Shively *et al.* 2017; Ling *et al.,* 2017; Tribett *et al.,*2019). In roughly a third of TBI cases, widespread amyloid plaque pathology is present, while it is largely absent in age-matched controls. In cases where amyloid plaques were present in controls, they were limited to individuals over 60 years old and considered as normal for their age bracket. However, this study also found that cases with amyloid pathology also displayed widespread NFT deposition in keeping with that observed in CTE cases reported to date. The survival time following TBI on average was also much less than that of Case 3, averaging 8.2 years compared to the 51 years of our case study.

The findings presented in this chapter suggest that loss of the TJ protein CLDN5 and the ensuing development of BBBD, is linked with the accumulation of p-tau deposits. In the cases of case study 1 & 2, both reports are linked by a diagnosis of CTE as a result of repetitive “mild” head trauma. In the case of case study 3, the rapid neuro-decline and subsequent development of multiple neuropathologies was attributed by a severe TBI incurred several years prior to diseases manifestation. Taken in that light, the finding presented here suggest that BBBD is closely associated with head trauma of all severities and appears to be linked with tau pathology. This is highlighted by the absence of BBBD and p-tau pathology in a non-injured control specimen.

Chapter 4: Changes in serological markers of mTBI in contact sports

4.1: Abstract

Current diagnosis and segregation of traumatic brain injury (TBI) rely largely on the Glasgow Coma Scale and neuropsychiatric assessment by trained medical personnel, supplemented with the use of medical imaging techniques to determine the extent of damage to the brain as a result from the injury. However, this assessment retains a degree of subjectivity, and when repeated assessments are conducted, such as in the contact sports, learned behaviour can compromise the accuracy of such assessments, while medical imaging often reports no findings in mild TBI (mTBI) cases. Therefore, the search for a fluid biomarker by which to diagnose mTBI has been highly sought. In this section, the utility of potential biomarkers S100β, brain-derived neurotrophic factor (BDNF) and monocyte chemoattractant protein-1 (MCP-1)/C-C motif chemokine ligand 2 (CCL2), as well inflammatory cytokines interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) are explored, as well as the potential in gauging changes in individual immune response to necrotic neural tissue. Over the course of two years, a total of 18 amateur rugby players (aged 17-22) were recruited and followed for mTBI over the course of a competitive season. Plasma samples were collected for serological analysis of blood-based biomarkers before the commencement of the season, as well as shortly after the season's end. Significant increases were observed in circulating BDNF levels post-season compared to baseline, while a significant decrease was found for circulating S100β levels. No mTBIs were reported over the study period. Peripheral immune cells also showed increased IL-1β secretion in response to necrotic brain tissue. Plasma samples were collected for a subset of players (n=7) within two hours of completing a competitive match. Serological analysis found significant increases in circulating S100β and MCP-1/ CCL2 levels following competitive play. In order to directly tie serological readout and immune response to head trauma, a cohort of mixed martial arts (MMA) fighters were recruited (n=15). Presented here are the findings from a limited number of participants (n=5) when comparing post-fight biomarker levels with that of matched baseline. For the MMA study within the limited cohort available so far, no significant changes have been identified between baseline and post-fight samples in any biomarker to date.

4.2: Introduction

Part of the difficulty in the diagnosis of mTBI is the lack of a reliable, quantifiable means to measure the injury. Moderate and severe injuries present with clear physical lesions when examined using CT or MRI techniques, and several batteries of tests can be utilized in the acute phases of milder injuries, with more extensive neuropsychiatric and functional balance assessment can be carried out given time and a suitable environment (Feddermann-Demont *et al.,* 2017; Raftery *et al.,* 2017). However, while these tools are useful in making a diagnosis of mTBI, neuropsychiatric results often require specialised training to interpret, while neuroimaging assessment requires dedicated facilities to perform. In addition to these limitations, screening by neuropsychiatric testing or neuroimaging are often limited to a single assessment; and offer no prognostic information on injury outcome. Therefore, blood-based biomarkers have been heavily investigated for their potential as a rapid point-of-care test to further facilitate mTBI diagnosis as well as their potential prognostic value. Currently, there are several potential blood-based biomarkers under investigation for their use in the context of mTBI, although many have failed to be robust enough to progress to clinical or commercial use.

S100β is heavily researched as a candidate biomarker due to its utility as a negative prediction of CT positive lesions following head trauma (Townend & Ingebrigtsen, 2006). Studies have found that serum levels of S100β increase following engagement in contact sports, and some suggest that circulating protein levels correlate with the number of blows incurred during play (Graham *et al.,* 2011; Shahim *et al.,* 2014; Rogatzki *et al.,* 2016; Bouvier *et al.,* 2017). These findings are often reported to be observed in the acute phases of injury (1-3 hours) following match play, and circulating S100β levels have been noted to return to baseline levels within roughly 6 hours, placing a limit on its utility outside of the context of acute injury assessment. Several other factors also limit the validity of S100β as a suitable biomarker for concussion; peripheral sources of S100β, such as those found in bone or adipose tissue, and their contribution to circulating protein levels must be considered, especially in cases of poly-trauma or sports with a large degree of full body contact, such as rugby or American football. In addition to this, exertion has also been shown to increased S100β levels in the blood, even in non-contact sports such as running, rowing or resistance training (Stocchero *et al.,* 2014; O'Connell *et al.,* 2018; Rogatzki *et al.,* 2018).

BDNF is involved in synaptic plasticity and neuronal survival; and highly expressed in the central nervous system. However, the neurotrophin has also been found to be expressed in peripheral tissue, such as the lung, testis, and heart, as well as being secreted from vascular endothelial cells (Saruta *et al.,* 2012). BDNF has been investigated as a marker of the secondary injuries associated with TBI, however, an association between serum levels and TBI remains to be determined (Simon *et al.,* 2017; Korley *et al.,* 2016). Due to the neurotrophin's role in synaptic plasticity and memory function, increases in circulating levels are thought to be an indicator of neuroprotective processes and restoration of synaptic connections in animal models of TBI (Mahmood *et al.,* 2009; Chen *et al.,* 2005.; Allison & Ivanco, 2018). In humans, reductions in serum BDNF levels following severe TBI were associated with poor recovery in memory function; and thought to represent neuronal degradation (Failla *et al.,* 2016; Korley *et al.,* 2016). In the context of sports-related concussion, BDNF has been largely understudied. A recent meta-analysis of concussion-related biomarkers identified only two studies of 13 that measured BDNF serum levels, and only one identified a significant increase from controls (Zetterberg *et al.,* 2009 Papa *et al.,* 2015).

The inflammatory response following TBI can contribute to a number of secondary injury processes resulting from the injury; and auto-immune responses against antigens released from damaged neural tissue have been reported following repetitive mTBI in American football players (Marchi *et al.,* 2013; Lozano *et al.,* 2015). Disruption of the BBB, as well as neural-target antigen response, could, therefore, be an inciting event to chronic neural inflammation and gradual neural degradation, such as that identified in autopsies of TBI patients (Johnson *et al.,* 2013). As such, serum cytokines and chemokine levels have been included in the investigation for potential biomarker of TBI, although primarily as prognostic markers of functional recovery in moderate and severe injuries. Elevations in blood concentrations of IL-1β and IL-6 have been found in individuals shortly after injury, as well as associations between high serum levels within the first 24 hours following injury and poor 6-month neurological outcome and mortality (Tasçı *et al.*, 2003; Chiaretti *et al.,* 2005; Biattista *et al.,* 2016). Chemokines are also thought to fill a central role in post-TBI immune responses, and MCP-1/ CCL2 has been a primary target of investigation for its use as a biomarker (Jaerve & Müller, 2012). Animal studies have found that MCP-1/CCL2 levels increase shortly following TBI and contributes to long-term neuronal loss (Semple *et al.,* 2009; Dalgard *et al.,* 2012; Liu *et al.,* 2013; Wang *et*

al., 2017). Early elevations in MCP-1/ CCL2 have also been observed in the serum of moderate and severe TBI patients, however, its potential role as a useful predictor of outcome has yet to be firmly established (Rhodes, Sharkey & Andrews, 2009; Buonora *et al.*, 2015; Biattista *et al.,* 2016). However, little work has been done to investigate the use of inflammatory changes in mTBI cases. In the context of sport-related concussion, the use of inflammatory biomarkers is also hindered due to the potential of low-grade polytrauma associated with contact sports.

4.3: Results

4.3.1: Changes in potential biomarkers for TBI following a season of rugby

Three biomarkers were chosen for investigation in the full cohort of rugby players prior to completion of participant recruitment. These included the astrocytic-enriched protein; S100β, the neurotrophic protein; BDNF, and the chemokine; MCP-1/ CCL-2. Levels of these biomarkers were measured via ELISA as detailed in Chapter 2. Significant changes in S100β and BDNF were observed from baseline to post-season. S100β levels were dramatically lower than the levels observed in participants at baseline, with many samples falling below the detectable levels of the assay (mean 985.3 pg/ml (SEM ± 244.8) vs. 415.3 pg/ml (SEM ± 222.7, $P \leq 0.05$) (**Figure 4.1 A-C**). Conversely, BDNF levels showed an increasefrom baseline following a season of play (mean 1213 pg/ml (SEM ± 257.5) vs. 2081 pg/ml (SEM ± 419.1), $P \leq 0.05$). MCP-1/ CCL2 levels showed a non-significant increase from baseline at the post-season time point (mean 164.7 pg/ml (SEM ± 10.09) vs. 165.3 pg/ml (SEM ± 8.623), $P > 0.05$).

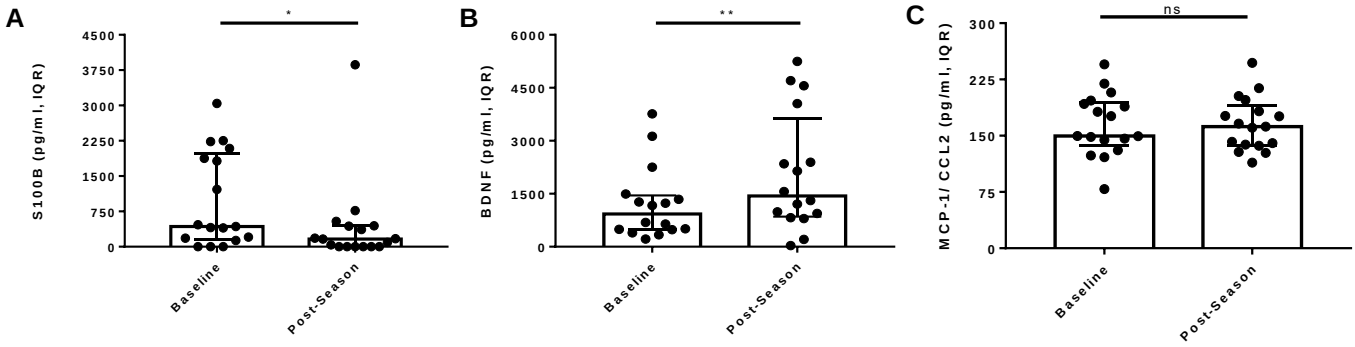


FIGURE 4. 1: CHANGES IN CIRCULATING LEVELS OF POTENTIAL BIOMARKERS FOR TBI FOLLOWING A SEASON OF RUGBY.

Circulating levels of S100β (**A**), BDNF(**B**) and MCP-1/CCL2 (**C**) in plasma of rugby players, as measured by ELISAThe decreases in S100β following a season of play would suggest an absence of BBBD. Similarly, the increase in circulating BDNF levels would suggest some degree of BBBD. However, the half-life of S100β limits the biomarker's utility in detecting BBBD outside of the 12hr post-injury window. Similarly, increases in BDNF could indicate BBBD, but also could be attributed to peripheral sources of BDNF released due to resistance-based exercised. MCP-1/CCL2 did not show any significant changes between time points, suggesting limited utility in long-term assessment of BBB integrity. *$P \leq 0.05$, **$P \leq 0.005$ by paired Wilcoxen test. ns, Not Significant. All data are medians ± IQR. N=17.

4.3.2: Changes immune response to necrotic brain tissue

In addition to plasma collection, PBMCs were isolated from each participant. Cells were seeded in triplicate at a density of $1x10^6$ cells/ml. Cells were primed with LPS-supplements RPMI media for 3 hours, after which, the media was replaced with RPMI media supplement with lysates of necrotic mouse brain tissue. Cells were propagated for 30 minutes in necrotic brain-supplemented media before being spun down and the supernatant was collected for screening of the inflammatory cytokines IL-1β and IL-6 via ELISA. PBMCs collected after a season of rugby produced significantly more IL-1β following stimulation by necrotic brain materials compared matched cells collected at baseline (mean 25.47 pg/ml (SEM ± 9.48) vs. 52.03 pg/ml (SEM ±11.72), $P \leq 0.05$). Post-season PBMCs show no significant difference in the amount of IL-6 produced compared to matched cells collected at baseline (mean 59.61 pg/ml (SEM ± 21.82) vs. 75.01 pg/ml (SEM ±23.53), $P > 0.05$) (**Figure 4.2A** & **B**). IL-1β and IL-6 levels within plasma were also quantified via ELISA. No significant changes were observed in circulating cytokine levels between baseline and post-season samples for IL-1β (mean 50.90 pg/ml (SEM ± 11.51) vs. 46.51 pg/ml (SEM ± 10.11), $P > 0.05$) or IL-6 (mean 33.41 pg/ml (SEM ± 8.313) vs. 26.28 pg/ml (SEM ± 6.755), $P > 0.05$ (Figure 4.2C & D).

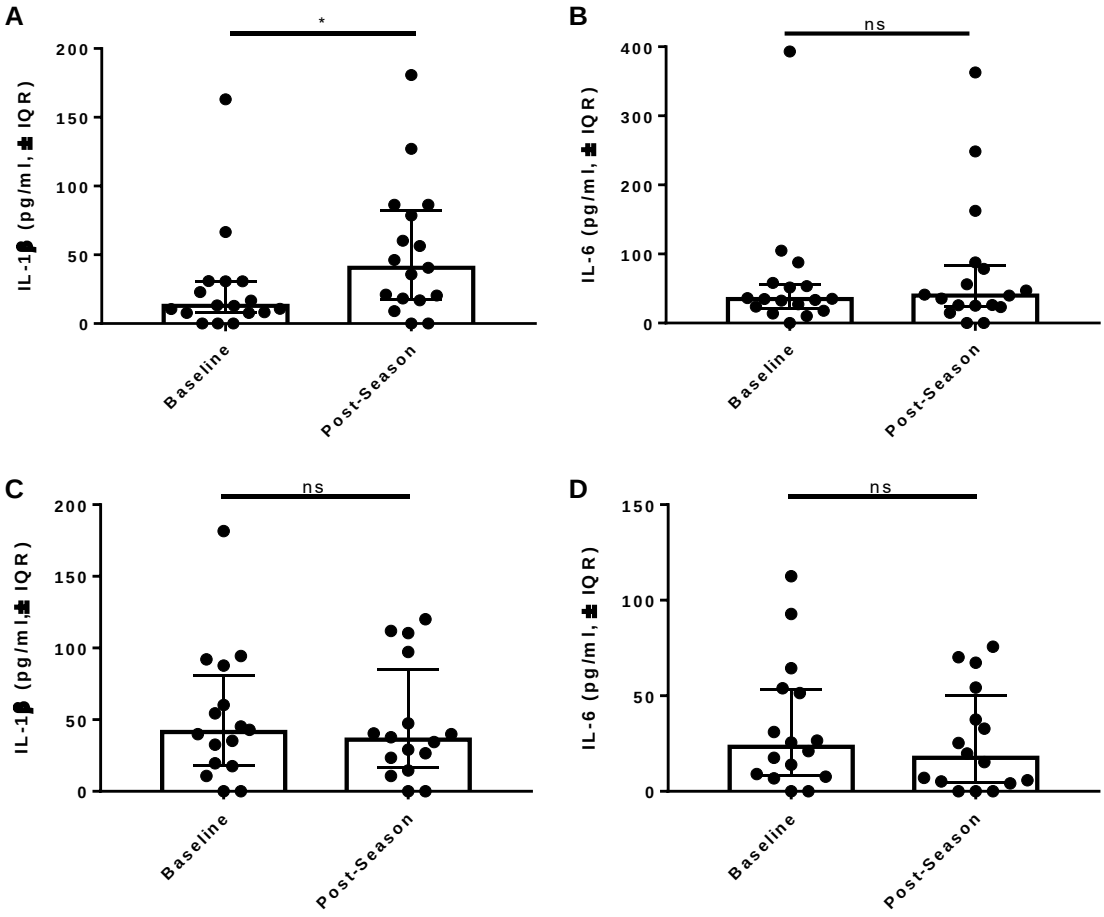


FIGURE 4. 2: CHANGES OF INFLAMMATORY RESPONSE FOLLOWING A SEASON OF RUGBY.

IL-1β and IL-6 production, as measured by ELISA, in PBMCs treated with necrotic mouse brain tissue (**A** & **B**) and plasma (**C** & **D)** (n=17)**.** Increases in the level of IL-1β by post-season PBMCs exposed to necrotic tissue suggests that some degree of trained response to brain tissue has occurred over the course the study period. The absence of change in response in IL-6 indicates that trained response is a "two-hit" DAMP insult. Circulating levels of IL-1β and IL-6 did not show a significant difference between baseline and post-season time points, indicating that increases in cytokine production is a result of the external stimulus of necrotic tissue, rather than underlying variations within individual between timepoints. *$P \leq 0.05$ by paired Wilcoxen test. Ns, Not Significant. All data are medians ± IQR.n=17

4.3.3: Biomarker levels in plasma following a competitive rugby match

In addition to collecting plasma samples at the end of the competitive season, plasma was also collected from a limited number of players within 2 hours of completing a competitive match, the duration of which was 80 mins. Plasma samples were screened for the same biomarkers as those examined at the post-season time point, namely S100β, BDNF and MCP-1/ CCL2. In contrast to post-season findings, significant increases from matched baseline values were observed in both S100β (mean 1703 pg/ml (SEM ± 295.9) vs. 1969 pg/ml (SEM ± 335.1), $P \leq 0.05$) and MCP-1/CCL2 (mean 184 pg//ml (SEM ± 24.81) vs. 254.3 pg/ml (SEM ± 34.1), $P \leq 0.05$). In contrast to post-season observations, BDNF show a non-significant decreasing trend immediately following play (mean 2039 pg/ml (SEM ± 1137) vs. 2339 pg/ml (SEM ± 1841), $P > 0.05$) (**Figure 4.3A-C**)

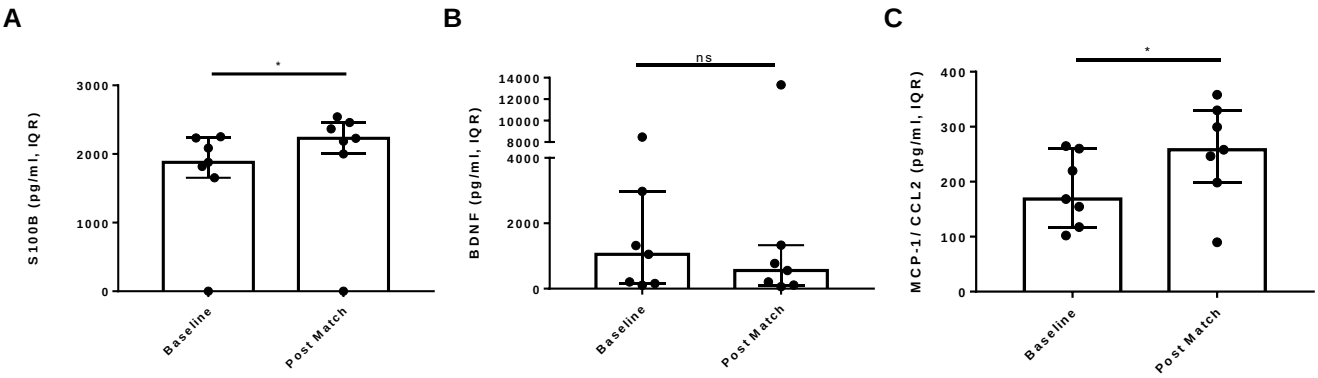


FIGURE 4. 3: CHANGES IN CIRCULATING LEVELS OF POTENTIAL BIOMARKERS FOR TBI IMMEDIATELY FOLLOWING A COMPETITIVE RUGBY MATCH.

Circulating levels of S100β (**A**), BDNF(**B**) and MCP-1/CCL2 (**C**) in plasma of rugby players, as measured by ELISA. Increases in circulating S100β levels may reflect increases in BBB permeability brought about by mechanical forces associated with rugby. Non-significant decreases in BDNF may reflect neuroprotective mechanisms induced by neurotrauma or exercise. Due to the broad role MCP-1/ CCL2 plays in chemotaxis, increases in MCP-1/ CCL2 cannot be attributed head trauma. *$P \leq 0.05$ by paired Wilcoxen test. Ns, Not Significant. All data are medians ± IQR.n=6

4.3.4: PBMC immune response is unaltered following competitive play

Post-match PBMCs were also collected and screened for changes in IL-1β and IL-6 cytokine production via the same means as post-season PBMCs; namely, primed with LPS for 3 hours prior to being propagated in necrotic mouse brain lysate for 30mins. The collected supernatant was screened for IL-1β and IL-6 via ELISA. No significant changes were observed between post-match cytokine production and that of baseline response for IL-1β (mean 17.34 pg/ml (SEM ± 4.81) vs. 33.19 pg/ml (SEM ± 12.6), $P > 0.05$) or IL-6 (mean 77.42 pg/ml (SEM ± 24.48) vs. 180 pg/ml (SEM ± 107.9), $P > 0.05$) (Figure 4.4A & B). Measurement of circulating cytokines was also carried out on samples collected shortly after a match and compared to baseline values. A non-significant increase in IL-1β plasma concentration post-match compared to baseline was present (mean 50.9 pg/ml (SEM ± 11.51) vs. 46.51 pg/ml (SEM ± 10.11), $P > 0.05$). In contrast, IL-6 was found to be significantly lower in post-match samples compared to baseline (mean 33.41 pg/ml (SEM ± 8.313) vs. 26.28 pg/ml (SEM ± 6.755), $P \leq 0.05$) (Figure 4.4C & D).

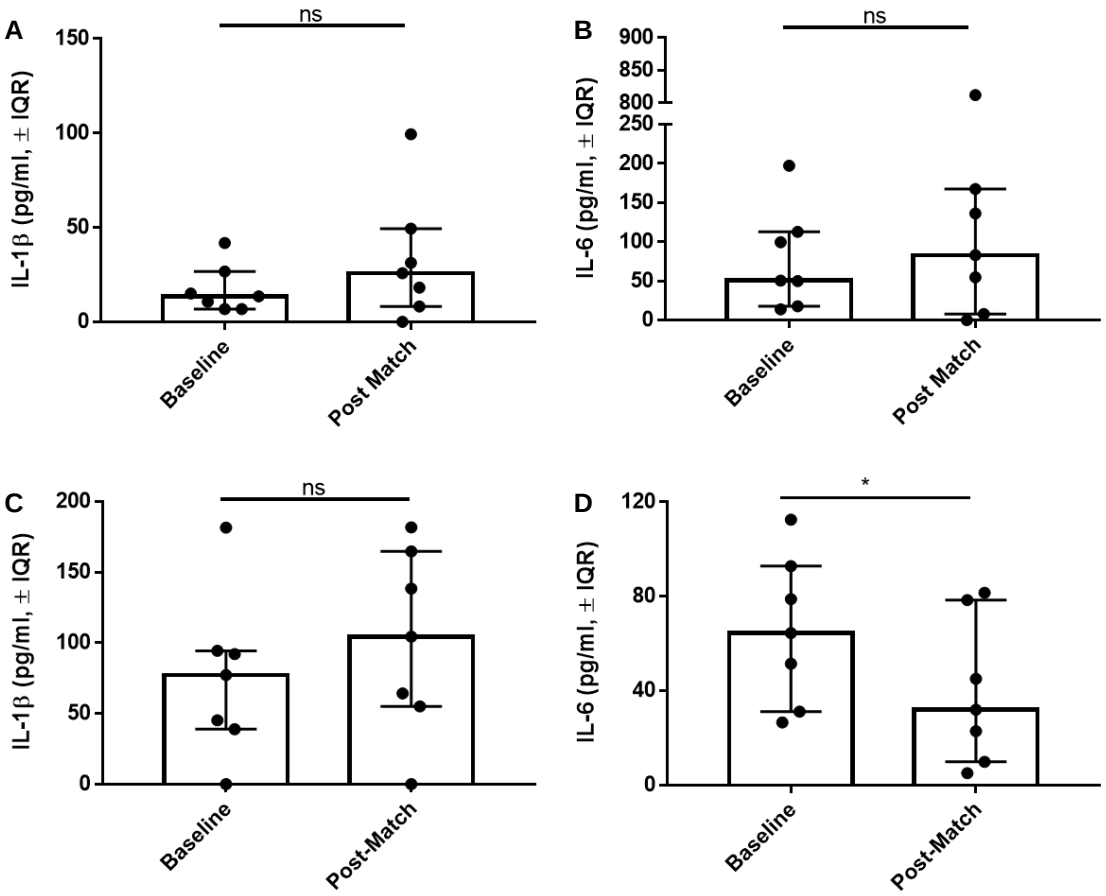


FIGURE 4. 4: CHANGES OF INFLAMMATORY RESPONSE FOLLOWING A COMPETITIVE RUGBY MATCH.

IL-1β and IL-6 production, as measured by ELISA, in PBMCs treated with necrotic mouse brain tissue (**A** & **B**) and plasma (**C** & **D)** (n=6). *$P \leq 0.05$ by paired Wilcox test. All data are median values ± IQR. The non-significant increase in PBMC-produced IL-1β may be due to the small number of participants available to sample at this time point, or a transient point immune response training. A non-significant increase in circulating IL-1β may reflect this trained immune response to circulating neural antigens. Significant decreases IL-6 may reflect initial decreases seen in severe trauma patients following injury. *$P \leq 0.05$ by paired Wilcoxen test. Ns, Not Significant. All data are medians ± IQR.n=6.

4.3.5: Changes in potential biomarkers for TBI following a competitive mixed martial arts bout

Following on from the pilot study in rugby players, a cohort of participants engaged in both professional and semi-professional mixed martial arts (MMA) was recruited as outlined in Chapter 2. The change in sport was decided upon based on the frequency of head injuries in MMA, as well as the greater ease of following up with participants shortly after a competitive event. The results presented both here and in Chapter 5 regarding the MMA cohort represent an ongoing study, and data presented here represents preliminary results.

Plasma samples were collected at a baseline time point and within 48 hours of a competitive MMA bout as detailed in Chapter 2. Plasma samples were screened for the same biomarkers as the rugby cohort; S100β, BDNF and MCP-1/CCL2, using the same protocols as previously described. So far, no significant changes have been observed between baseline and post-fight levels in circulating S100β (mean 455.7 pg/ml (SEM ± 447.7) vs. 20.84 pg/ml (SEM ± 20.84)), BDNF (mean 882.7 pg/ml (SEM ± 193.3) vs. 695.8 pg/ml (SEM ± 131.5)) or MCP-1/CCL2 (mean 126 pg/ml (SEM ± 14.6) vs. 151.2 pg/ml (SEM ± 33.56)) (**Figure 4.5 A-C**). Similarly, no significant changes have been observed in systemic inflammatory states between baseline and post-fight, as measured by circulating levels of IL-1β (mean 5.614 pg/ml (SEM ± 5.614) vs 13.77 pg/ml (SEM ±8.532)) and IL-6 (mean 239.3 pg/ml (SEM ± 184.9) vs. 38.15 pg/ml (SEM ± 18.89)) (**Figure 4.5 D** & **E**)

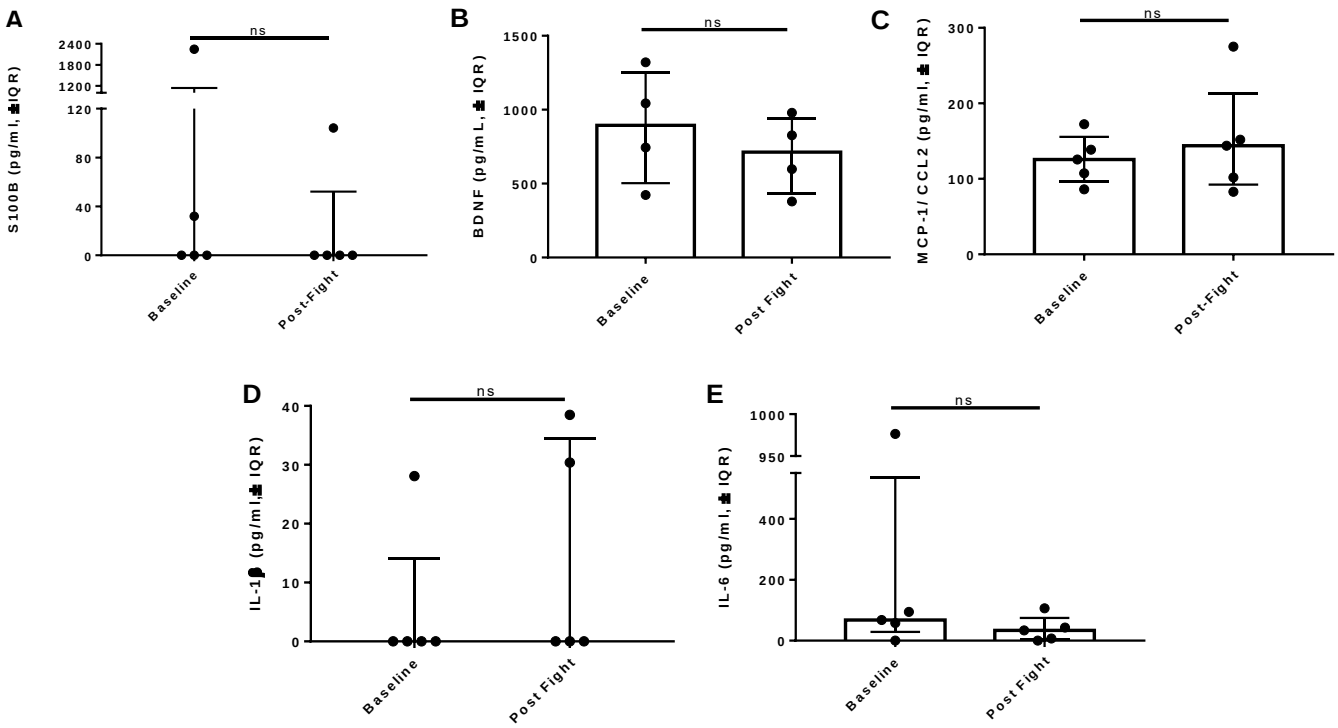


FIGURE 4.5: CHANGES IN CIRCULATING LEVELS OF POTENTIAL BIOMARKERS FOR TBI FOLLOWING AN MMA BOUT.

Circulating levels of S100β (**A**), BDNF(**B**), MCP-1/CCL2 (**C**), IL-1β (**D**) and IL-6 (**E**) in plasma of MMA fighters, as measured by ELISA. Samples collected between 24-48 hours of a bout. All data are medians ± IQR. Levels of S100β were undetectable in post-fight samples, likely due to the short circulating half-life of S100β, and a possible return to baseline for BBB integrity. The absence of significant changes in circulating BDNF, MCP-1/ CCL2, IL-1β and IL-6 also suggest a return to baseline status. The absence of changes could be attributed to an underpowered comparison due to the low number of samples available at the time. $P \leq 0.05$ by paired Wilcoxen test in all cases. All data are medians ± IQR. ns, Not Significant. n=5

4.4: Discussion

Blood-based biomarkers are often hailed as a holy grail of diagnostic tools, especially in kinetic environments or dynamic conditions such as TBI. Recently, the first commercially available panel consisting of glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase (UCH-L1) was approved for diagnostic use in ruling out complicated mTBI (FDA site, news & events). While this panel appears to be an improvement upon S100β's ability to predict complicated mTBI, a biomarker for assessment of mTBI, such as that observed in sport remains to be developed. Presented in this chapter are the changes observed in a range of potential biomarkers, both in the context of the acute scenario following sub-concussive forces, as well as over long-term exposure, as well as purposing a novel means by which to assess an individual's adaptive response to concussive force, namely by measuring peripheral immune cell response to necrotic brain tissue.

Shortly after completion of a competitive rugby match, S100β and MCP-1 were elevated compared to baseline values, and no participant was diagnosed with a mTBI. However, increases in S100β have been reported following exposure to both contact sports and non-contact exercise (Dietrich *et al.,*2003; Marchi *et al.,*2013; Stocchero *et al.,* 2014). MCP-1 has been examined closely in the context of sport-related mTBI, and so changes must be viewed in the context of animal models and observations made as part of exercise studies in healthy individuals. As mentioned previously, MCP-1 appears to play an important early role in mediating the response to injury in animal models of TBI, with increases in chemokine circulation being observed within hours of injury (Dalgard *et al.,*2012; Liu *et al.,*2013). However, the non-specificity of MCP-1 in regard to response to injury makes it impossible to definitively say that this increase is a result of brain trauma. Exercise has a known effect on immune response, and endurance training has been demonstrated to increase serum levels of MCP-1 (Ortega, 2016; Jürimä, Vaiksaar Purge, 2018). The physically demanding nature of contact sport must also be considered. These issues are also highlighted when looking at changes in these proteins over long-term exposure of contact sport. While MCP-1 showed a non-significant increase at the end of the season, S100β was dramatically lower than the values observed at baseline. A recent study in rugby players also found a drop in S100β towards the end of the season, which may reflect changes in play behaviour as the season progresses (O'Connell *et al.,*

2018). Circulating BDNF levels shortly after a match showed a non-significant drop compared to baseline values, while at the post-season timepoint, circulating levels of the neurotrophin were much higher than matched baseline levels. The post-match decrease in BDNF mirrors findings of lower circulating levels of the neurotrophin in severe TBI patients (Failla et al.,2016). In contrast, the post-season increase observed in BDNF may indicate a potential neuroprotective response (Korley et al.,2016). Given the limited context of the study, the implications of these changes could not be assessed, but may represent a gradual change in neural health over time. However, it is also possibility that these increases in BDNF could be derived from endothelial cells, rather than a neuro-adaptive response.

While the number of participants available to be included with the MMA cohort to date is severely limited, it can be noted that the circulating levels of biomarkers are noticeably lower in this cohort compared to the rugby participants, particularly in the cases of S100β and BDNF. While no conclusions can be drawn as the reason for this, as the lower number of MMA samples may be masking the accurate levels of circulating biomarkers, speculation can be made. A possible reason for this discrepancy may be due to the timing of training sessions or other physical activities on part of the participants. Participants from both groups came in roughly at the same time of day, however, the MMA participants would tend to go to training after assessment, on account that they may partake in light sparring sessions during training. Participants from the rugby cohort would often partake in gym sessions almost every morning, as well as physical education activities, which may have contributed to a higher "baseline" value of circulating biomarker, even in the absence of physical contact collisions. This may have also contributed to a static, high level of circulating biomarker at the post-season assessment.

Immune response to necrotic brain tissue demonstrated increases in IL-1β following a season of play, the implications for which remains to be seen. One possibility for the increased release of cytokines following a season of play may be due to some degree of innate immune training. Repeated sub-concussive blows, combined with auto-immune response stimulated by exercise (Ortega, 2016), may provide ample opportunities for the immune training to occur. A recent study in rats suggests that severe TBI can induce inflammatory changes in the periphery within a week of injury, mirroring the changes observed in an inflammatory state of cortical tissue, as well as an impedance to regulatory

T-cell development to an anti-inflammatory profile (Rowe *et al.,*2016). However, the study focused primarily on the influence of TBI on the peripheral immune response to a second, non-TBI injury. The chemokine CCL20 has also been reported to be upregulated following severe TBI in rats, and directing CCR6-expressing cells, such as T-cells, B-cells and dendritic cells, to injured sites of the brain, and as mentioned, neutrophil and monocytes also infiltrate the neural space shortly after injury (Das *et al.,* 2011; Perez-de-Puig *et al.,*2015; McKee & Lukens, 2016). This infiltration of peripheral immune cells has been demonstrated to contribute to the neuroinflammation associated with TBI. The observation of an increased response of peripheral immune cells after a season of play may suggest that upon further TBIs, a heightened immune response to neural tissue may be mounted in the long-term, increasing inflammation-associated damage or prolonging the inflammatory state. The absence of significant changes observed shortly after a match may either reflect a transitory state, in which changes in immune response have not yet manifested or be a result of immune-dampening in response to exercise-induced muscle damage (Ortega, 2016).

Important caveats must be taken into consideration when looking at the results presented here. One of which is the relatively small number of participants. A total of 20 participants were retained over the course of the study, out of a potential 31. Of those recruited to complete a post-match assessment only 7 were retained, and 6 returned for a post-season assessment. In a condition as heterogeneous as mTBI, a small number of participants has a large impact on the interpretation of the results. Also, the changes observed in the biomarkers chosen for this study are those of athletes training and exposed to contact sport at a high level and may not represent the population at large. Also, many of these markers lack the specificity to distinguish neural damage or BBB compromises from that of non-contact, aerobic exercise.

However, the findings here represent a step in a holistic approach to identifying potential markers of mTBI.

Chapter 5: Changes to cerebral vasculature and microstructure associated with contact sports

5.1: Abstract

The previous section examined conventional means by which to identify mild traumatic brain injury (mTBI) in contact sports via serological markers. However, the advent of advanced medical imaging techniques has allowed for non-invasive assessment of changes to the brain and neurovasculature previously undetected by standard medical imaging paradigms. As shown in earlier sections, vascular dysfunction is evident in pathological neurodegenerative conditions associated with repetitive TBI; and may play an inciting role in disease development. Participation in a contact sport may contribute to changes in blood-brain barrier (BBB) integrity, which may contribute to the risk of dementia development later in life. In this section, changes in BBB integrity were visualised using both established and novel dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) analysis tools in participants engaged in rugby and mixed martial arts (MMA). In addition to changes in BBB integrity, changes in the microstructure of neural tissue was assessed using diffusion tensor imaging (DTI) analysis. Using Toft's-extended model to generate K^{trans} measurements to assess BBB integrity at a baseline and post-season time point, a significant increase in barrier permeability was observed following participation in a season of rugby. Measurement of the percentage of BBB disrupted voxels using the novel Linear dynamic model (LDM) showed a non-significant increase in BBB permeability post-season. Post-match assessment showed no changes in BBB permeability as measured by K^{trans} values, and a significant decrease in permeability as measured by suprathreshold voxels. However, a sub-group of individuals were identified to display a significant increase in BBBD as measured by both methods after a season of play. DTI analysis assessing axonal tract integrity at a baseline and post-season time point found significant improvement in fractional anisotropy (FA) within the body of the corpus callosum, as well as improvements in mean diffusivity (MD) and radial diffusivity (RD) within tracts of the anterior limb of the internal capsule (ALIC), the hippocampus and the inferior fronto-occipital fasciculus (IFOF); and significant decreases in axial diffusivity (AxD) within structures of the corpus callosum, hippocampus and IFOF. Also presented here are preliminary findings of BBB state and axonal tract integrity in MMA fighters shortly after a competitive bout.

5.2: Introduction

Given the heterogeneous and complex nature of TBI, the extent of damage can be difficult to assess. While the previous chapter discussed the use of blood-based biomarkers and their potential utility in categorising TBI severity, presented here will be the use of medical imaging techniques, specifically MRI. In most instances of moderate or severe TBI, a non-contrast computed tomography (CT) scan is carried out to identify epidural hematomas, intracranial haemorrhage or contusions (Shetty *et al.,*2016). In the case of mTBI however, CT scans may be indicated only in exceptional circumstances, which some outline as injury with loss of consciousness or posttraumatic amnesia accompanied by coagulopathy or high risk of bleeds, advanced age (>65 years) or focal neurological deficit (Jagoda *et al.,*2009). However, even cases in which abnormal neuroimaging findings are present, these injuries are classified as “complicated mTBI", representing a subset of the mild injury spectrum (Williams *et al.,*1990). Many injuries will not present to medical services for formal evaluation; and often when they do, medical imaging reveals no gross lesions or changes to the brain, and those with prolonged symptoms, sometimes referred to as post-concussion syndrome, have pathologies below current standard imaging techniques (McCrory *et al.,*2017). However, CT scans expose individuals to bouts of radiation, and the use of the imaging modality in both adult and paediatric populations have been increasingly met with concerns over the widespread use of CT imaging (Brenner & Hall, 2007). Repeated exposure of low-dose radiation is also a concern and therefore limits CT imaging as a means of continuous monitoring of evolving processing following mTBI. As discussed in chapter one, the emergence of long-term sequelae of mTBI appears to be a progressive process, potentially evolving over decades after injury. Therefore, the use of advanced MRI techniques, such as DCE-MRI and DTI, have come increasingly to the fore with regards to monitoring the brain following mTBI, as well as detecting findings associated with “complicated mTBI" (Suri & Lipton, 2018).

DCE-MRI is the most well-established means by which to measure BBB integrity, and utilizes a metal-based contrast agent, most commonly gadolinium-based chelates, although iron-based contrast agents are also being investigated for their utility (Bashir *et al.,*2015). Under normal physiological circumstances, the BBB limits and regulates the flux of macromolecule between the brain and circulating blood, confining contrast agent

within the lumen of blood vessels. However, in the presence of a disrupted BBB, the contrast agent can seep into the perivascular space and can be detected using a number of quantification methods (Ingrisch & Sourbron, 2013). In this chapter, BBB integrity was quantified by means of the Toft's Extended model, which measures the BBB integrity via the volume transfer rate, and LDM, which quantifies the extent of BBBD across the scanned area (Sourbron & Buckley, 2011; Chassidim *et al.,*2013). As mentioned, prior, BBB dysfunction is a prominent feature of moderate and severe TBI, as well as being a common element of neurodegenerative conditions associated with a history of TBI. However, disruption of the BBB in mTBI in humans, particularly in the context of contact sports, remains largely understudied. To date, only one study has been published exploring the use of DCE-MRI in measuring BBB changes in contact sport athletes, reporting that several athletes had an increased proportion of voxels displaying BBB disruption (Weissberg *et al.,*2014). Similarly, BBBD has been implicated in individuals experiencing PCS, the umbrella term of concussion symptoms lasting longer than two-to-three weeks (Yoo *et al.,*2019).

DTI is another MRI modality being explored for its use in determining the long-term effects of mTBI on brain health by gauging changes in the microstructure of white matter (WM). DTI detects the direction and magnitude of water movement within the confines of tissue, and within the axonal bundles of WM, water movement is primarily limited along the length of the axon, due to axon's membrane and surrounding myelin sheaths. As DTI can provide a detailed and multifaceted insight into the health of the microstructure of axonal tracts, the imaging modality is held as a potential candidate in identifying the diffuse axonal injury associated with TBI in life (Shenton *et al.,*2013). Changes in DTI parameters have been identified in both the acute stages of TBI, as well as over more prolonged periods after the resolution of the injury, suggesting that the initial injury's insult may trigger a dynamic and gradual process of axonal injury (Arfanakis *et al.,*2002; Van Beek *et al.,* 2015a; Van Beek *et al.,* 2015b). Contact sports, such as American football and ice hockey, have been shown to induce changes in tract integrity over the course of a season, even in the absence of diagnosed mTBIs (Bazarian *et al*., 2012; Davenport *et al.,*014; Bahrami *et al*., 2016). However, to date, there has been little consensuses in the direction of change in DTI indices following injury, as well areas of significant tract changes differing between cohorts. This likely reflects in part the heterogeneity of mTBI, as well as possibly suggesting a different response in axons

depending on the mechanism of injury or whether it was a single insult or several minor events.

5.3: Results

5.3.1: Changes in BBB integrity following a season of competitive rugby

Initially, 30 participants were recruited from a competitive local amateur rugby (aged 17-24 years). Participants underwent a baseline MRI scan, which included a DCE sequence for BBB, at the start of the competitive season. Following the conclusion of the season (lasting roughly 8 months), participants underwent reassessment and MRI scanning, and individual values were compared to matching baseline values. Of the 30 participants recruited to the study, 18 completed baseline and post-season MRI assessment. BBB integrity was measured using two models: Tofts Extended model, which measures the change in volume transfer rate (K^{trans}) between the lumen of the blood vessel and the extravascular space (Sourbron & Buckley, 2011); and LDM, normalising the slope of contrast signal intensity over time to a region of interest (ROI) and highlights voxels with increasing signal intensity, representing accumulating contrast agent and the area of BBBD (Vesker, Shelef & Friedman, 2014). A graphical representation of both models is presented in **Figure 5.1**.

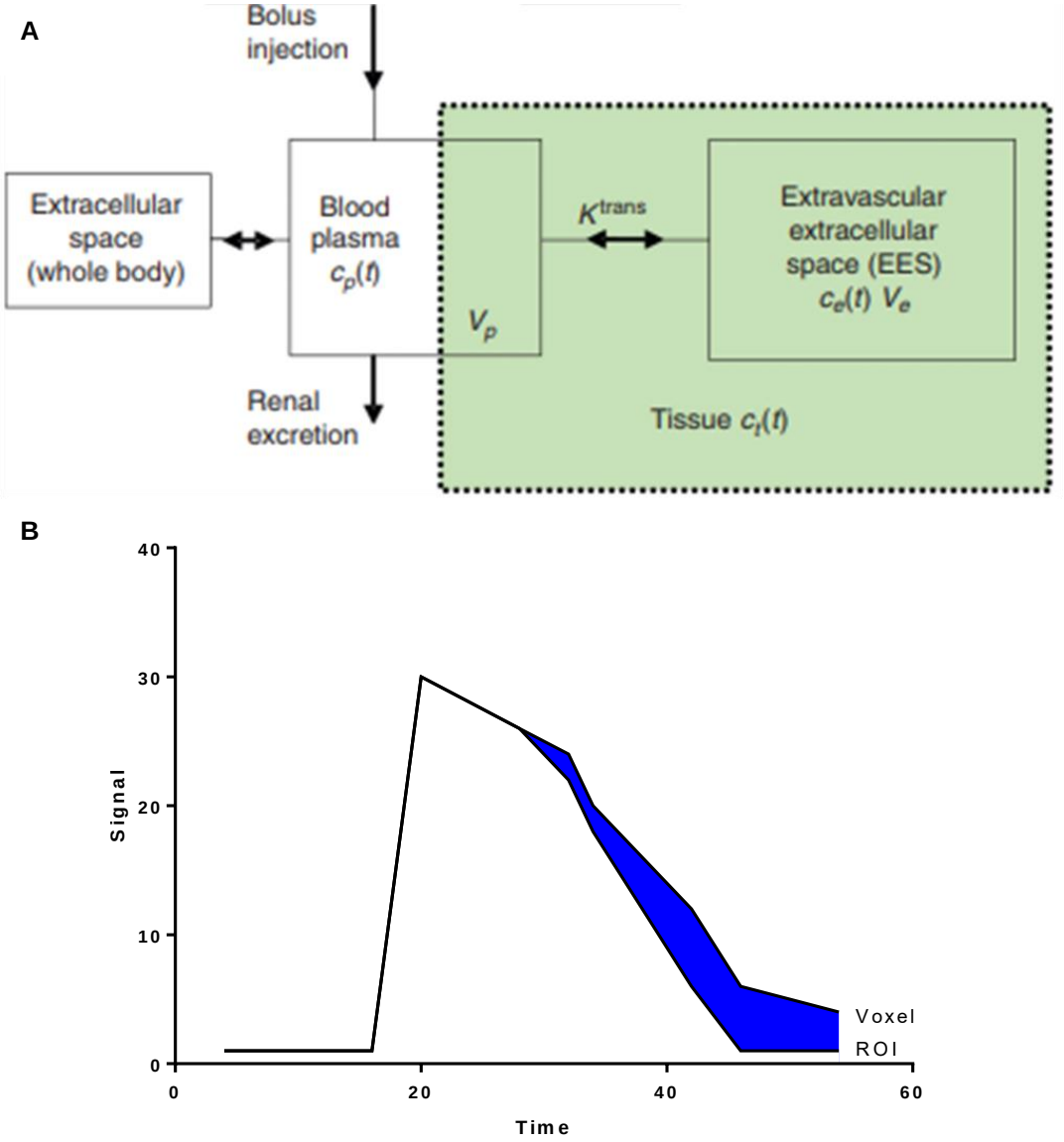


FIGURE 5. 1: SCHEMATIC AND GRAPHIC REPRESENTATIONS OF SIGNAL GENERATION VIA THE TOFT'S EXTENDED MODEL AND THE LINEAR DYNAMIC MODEL

A) A schematic representation of the derivation of primary measurement used from the Toft's Extend Model: K^{tran}. The model requires measurement of the bolus signal as a refence for the concentration of contrast agent within plasma (V_p). This is then used to calculate he concentration of contrast agent within the plasma over time ($c_p(t)$). Extravascular signal is detected during the scan ($V_{e)}$ and used to calculate the extravascular concentration of contrast agent for time ($c_p(t)$). The rate of flux between the plasma concentration and the extravascular concentration represent the degree of integrity within the tissue (K^{trans}). **B**) The Linear Dynamic Model fits the raw signal of contrast agent within a reference area (ROI) to a lineal model over time. The rate of decay of the contrast signal for the reference area is compare with every other voxel within the scanned area. The model assumes that all other voxels will have a signal decay rate equal to or greater than the reference area. Voxels with a slower rate of signal decay (slope) are assumed to be cause by contrast agent retained with extravascular spaces (Blue space). This model cannot measure concentration over time as gadolinium-based contrast agents do not follow a linear rate of decay. Image A) taken from Tofts & Parker, Clinical Perfusion MRI,2013.

Visual comparisons of BBB permeability masks using K^{trans} values displayed no obvious changes in permeability across the scan area; nor was there apparent increases in the number of focal regions displaying high K^{trans} values (**Figure 5.21Aa** & **Ab**). Comparison of post-season median K^{trans} values displayed a significant increase from the values recorded at baseline (median $4.155x10^{-4}$ min^{-1} (IQR $9.604x10^{-5}$ - $1.101x10^{-3}$) vs. $9.198x10^{-4}$ min^{-1} (IQR $5.081x10^{-4}$ - $1.801x10^{-4}$), $*P \leq 0.05$), (**Figure 5.2B**). Association analysis was carried out to determine whether baseline K^{trans} values influenced post-season readouts. Correlation analysis showed no significant association between median K^{trans} measured at baseline and that measured at the post-season timepoint (Spearman r = 0.2529, $P > 0.05$) (**Figure 5.3**). When compared to their baseline values, individuals that showed an increase in K^{trans} measurement showed significant changes from their baseline status (7.309×10^{-4} (SEM ± 3.039×10^{-4}) vs. 2.349×10^{-3} (SEM ± $6.88x10^{-4}$), $**P \leq 0.01$) (**Figure 5.3B**). The same comparison was made within individuals showing a decrease in K^{trans} measurements, and was post-season values were found not to significantly differ from their baseline status ($6.929x\ 10^{-4}$ (SEM ± 2.238×10^{-4}) vs. $4.617x\ 10^{-4}$ (SEM ± 1.529×10^{-4}), $P > 0.05$) (**Figure 5.3C**).

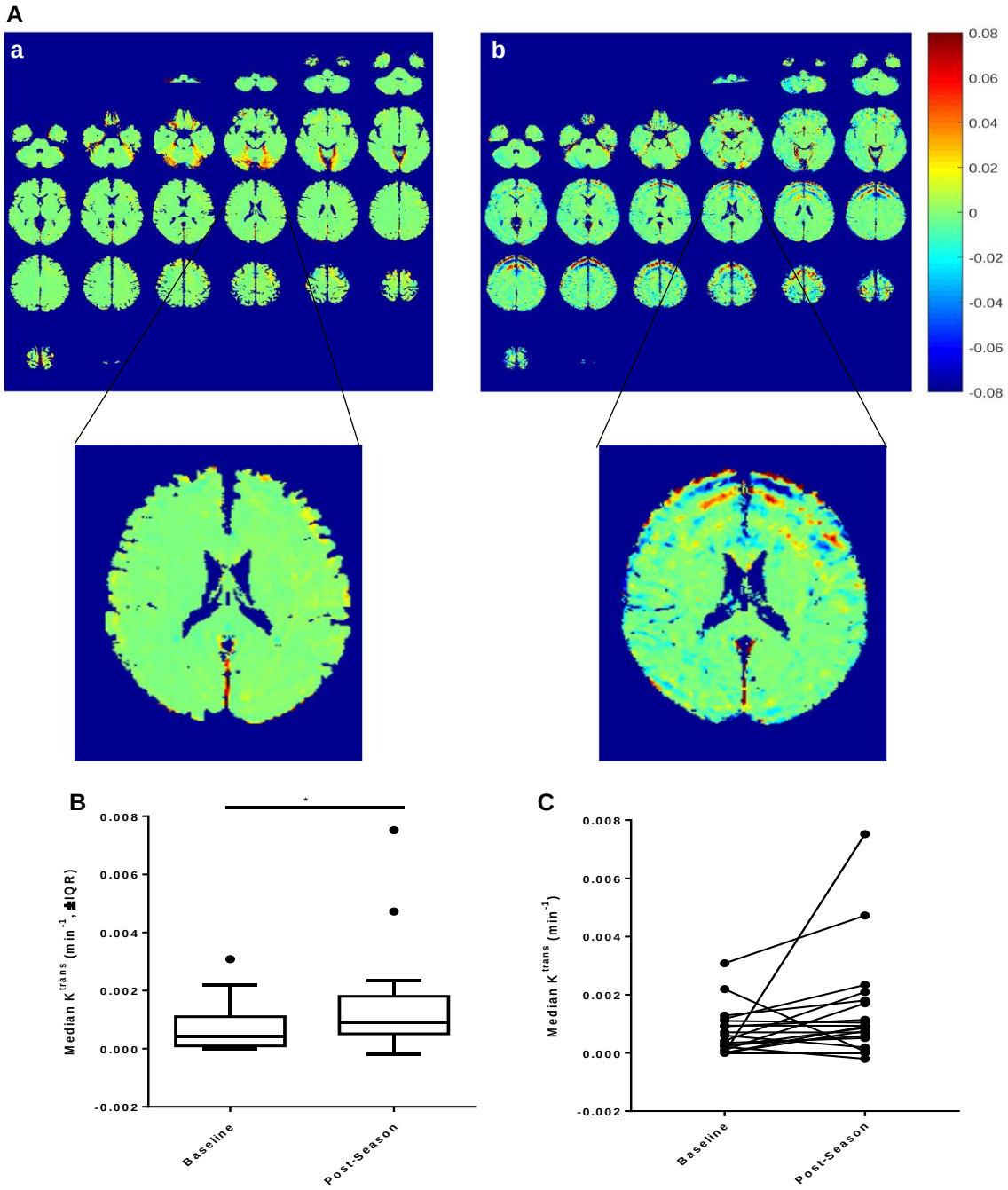


FIGURE 5. 2: CHANGES IN BBB INTEGRITY ARE DETECTABLE USING TOFTS EXTENDED MODEL FOLLOWING A SEASON OF PLAY IN RUGBY.

A) Representative of brain mask of brain sections along the inferior-superior axis generated using K^{trans} values of a player at baseline (**a**) and post-season (**b**). K^{trans} value for each voxel is denoted using a psudo-colour chart, ranging from blue (flow in the direction of the blood vessel lumen) to red (flow in the direction of the perivascular space). Qualitative assessment of the sections suggests on large changes in K^{trans} values over the course of the study period. **B)** Groups analysis of median K^{trans} value at baseline and post-season time points. The significant increase in median K^{trans} suggest that changes in BBB integrity come about as result of non-concussive forces associated with normal play. $*P \leq 0.05$ by paired Wilcoxen test. **C)** Before-and-after plot of median K^{trans} values, with lines pairing baseline and post-season values**.** A number of participants (n =7) showed little change from their baseline value, sub-groups showed an increase (n=8) and decreases from baseline (n=3), suggesting a possible increased vulnerability in the BBB to mechanical force in a limited population.

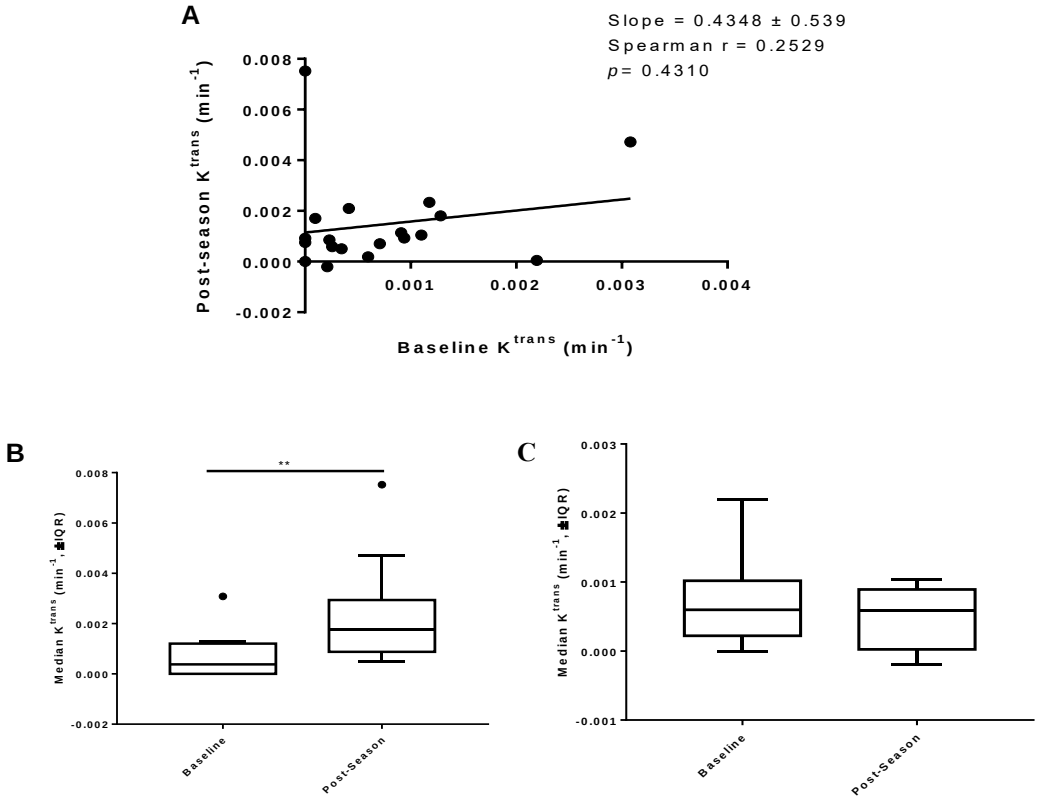


FIGURE 5. 3: BASELINE K^{TRANS} VALUES DO NOT CORRELATE WITH POST-SEASON READOUTS.

A) Linear regression analysis comparing baseline median K^{trans} values with post-season median K^{trans} values. The trend line represents a non-significant correlation between higher baseline values and higher post-season values, suggesting that higher baseline values are not assoicated with a higher K^{trans} value following a season of rugby. **B**) Paired analysis of median K^{trans} values in individuals showing an increase in BBBD signal at post-season (n=8). Changes in K^{trans} values observed within this sub-set of individuals represented a significant change in their BBB integrity over the course of the season, even in the absence of injury. **$P \leq 0.005$ by paired Wilcoxen test. **C)** Paired analysis of median K^{trans} values in individuals showing a decrease in BBBD signal at post-season (n=3). $P > 0.05$ by paired Wilcoxen test

BBBD using the LDM was quantified by the percentage of voxels showing an increasing slope, set to a previously established threshold in non-contact athletes (Weissberg *et al.,* 2014). Visualisation of BBBD using the LDM showed widespread changes in the area displaying BBBD from baseline in several participants, as well as an increase in the signal within these regions (**Figure 5.4Aa** & **Ab**). Comparison of the percentage of disrupted voxels at post-season showed a non-significant increase in the volume of BBBD compared to that measured at baseline (median 22.28% (interquartile range (IQR) 15.38-27.25) vs 24.99% (IQR 17.99- 30.27), $P > 0.05$) (**Figure 5.4B**). Similar to K^{trans} measurements, the percentage of suprathreshold voxels at baseline did not indicate the percentage of suprathreshold voxels measured at the post-season timepoint (Spearman r = 0.04211, $P > 0.05$) (**Figure 5.5A**). Comparison of post-season values to the paired baseline status of individuals that displayed an increase in the percentage of suprathreshold voxels indicated that the measured increases differed significantly from baseline (19.38% (SEM ± 2.523) vs. 29.02% (SEM ± 1.977), $*P \leq 0.01$) (**Figure 5.5B**). However, a significant decrease in the number of suprathreshold voxels was observed in individuals that showed a decrease from their baseline evaluation (27.12% (SEM ± 2.45) vs. 17.06% (SEM ± 3.265), $*P \leq 0.01$) (**Figure 5.5C**). This is likely why the comparison of baseline and post-season suprathreshold voxels only trended towards an increase.

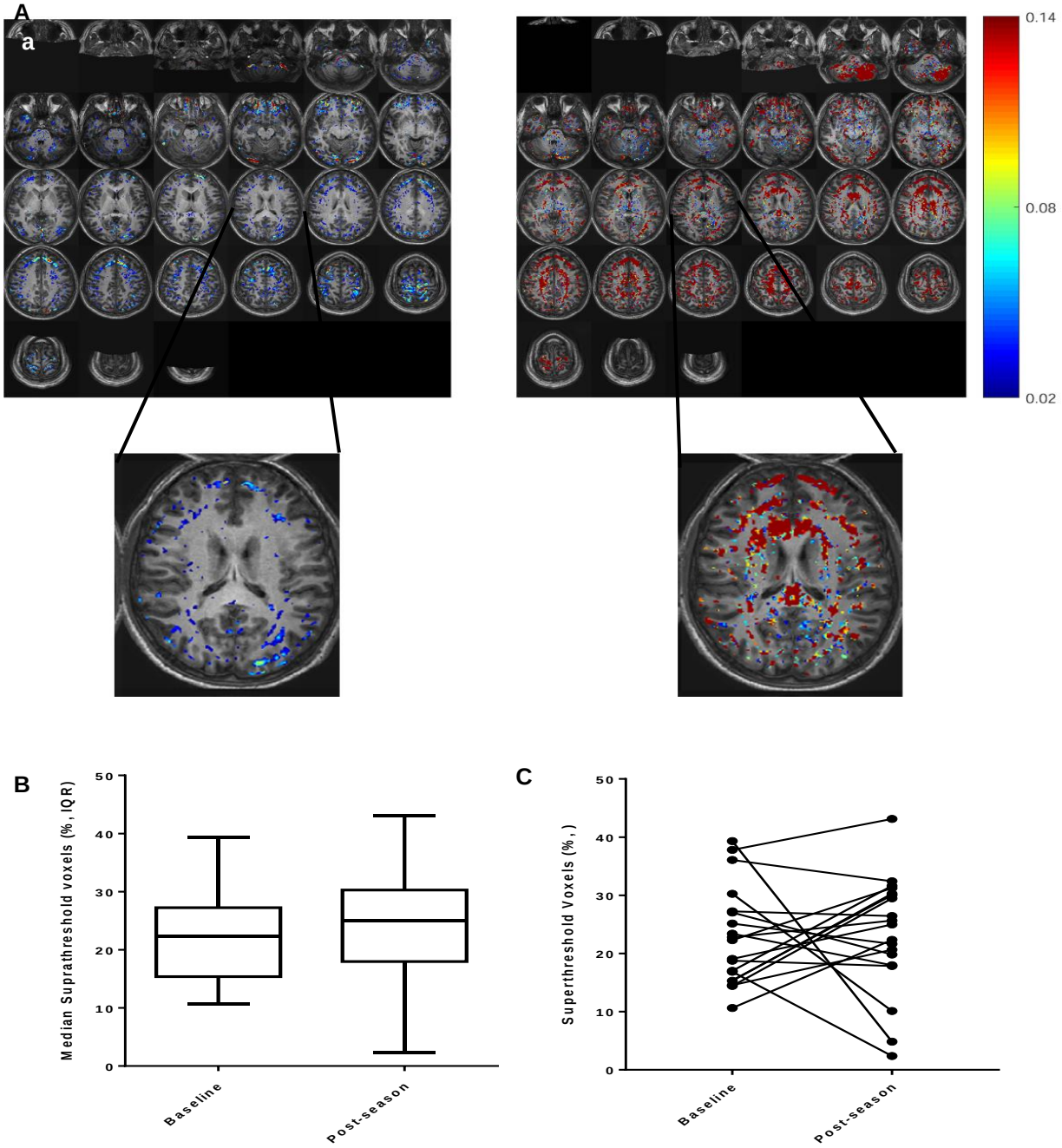


FIGURE 5. 4: CHANGES IN BBB INTEGRITY ARE NOT OBSERVE ON A GROUP'S BASIS FOLLOWING A SEASON OF RUGBY USING LINEAR DYNAMIC MODELLING.

A) Representative of brain mask of brain sections along the inferior-superior axis generated using normalised slope values over T1 images of a baseline (**a**) and post-season (**b**). Voxels with positive normalised slope values are denoted with pseudo-colour values, ranging from minimum threshold value set in blue (0.02) to the maximum threshold value in red (0.14 **B)** Groups analysis of percentage of suprathreshold voxels at baseline and post-season time points. The range in the percentage voxel showing BBBD were greater following a season of rugby compared to those recorded at bassline. However, the upward trend in the percentage of suprathreshold voxels did not significantly changed from base line values. $P > 0.05$ by paired Wilcoxen test. **C)** Before and after plot of percentage of suprathreshold voxels, with lines pairing baseline and post-season values. A sub-set of participants showed increases (n=7) and decreases (n=9) in the percentage of disrupted voxels, likely contributing non-significant observation when comparing baseline and post-season values. As a sub-set of participants did display increases in BBB permeability following a season of play in the absence of injury, they may represent a cohort with increased vulnerability to mechanical forces.

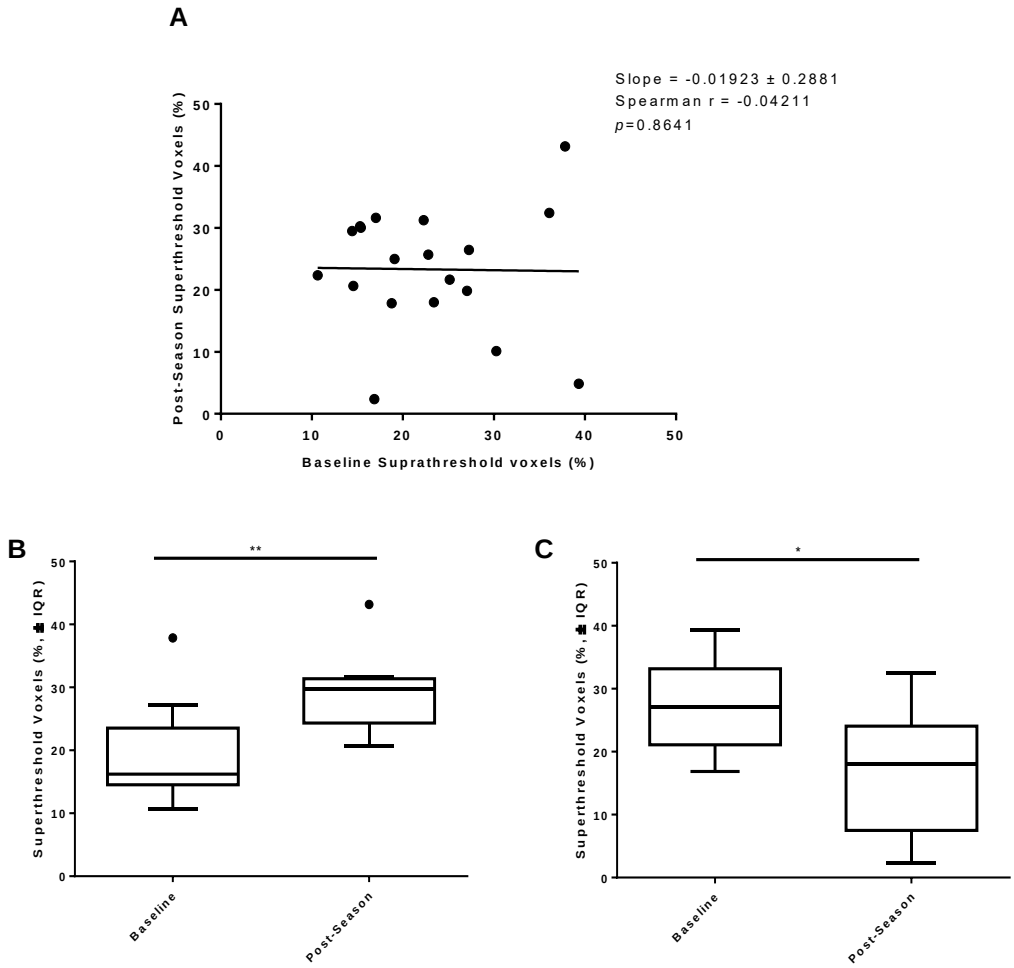


FIGURE 5.5: ANALYSIS OF TRENDS IN VOXELS DISPLAYING BBBD IN RUGBY PLAYERS FOLLOWING A SEASON OF PLAY.

A) Linear regression analysis comparing baseline percentage suprathreshold voxels with post-season percentage suprathreshold voxels. No correlation was observed between baseline suprathreshold voxels and post-season values. **B)** Paired analysis of percentage of suprathreshold voxels in individuals showing an increase in BBBD signal at post-season. **P ≤ 0.01 by paired Wilcoxen test (n=7). **C)** Before and after plot of percentage of suprathreshold voxels, with lines Paired analysis of percentage of suprathreshold voxels in individuals showing a decrease in BBBD signal at post-season. *P ≤ 0.05 by paired Wilcoxen test (n=9).

5.3.2: Acute post-match assessment of BBB integrity

A small number of participants (n=8) underwent MRI scanning within two hours of completion of a full competitive rugby match.

Visual comparison of K^{trans} value brain masks showed no obvious changes in BBB permeability from that of baseline assessment (**Figure 5.6A**). Median K^{trans} values did not differ significantly from matched baseline values (median $6.785x10^{-4}$ min^{-1} (IQR $2.203x10^{-4}$ - $1.269x10^{-4}$) vs. $2.467x10^{-3}$ min^{-1} (IQR $-3.748x10^{-4}$ - $1.844x10^{-4}$), $P > 0.05$) (**Figure 5.6B**). No association was found between baseline K^{trans} values and values measured shortly after a match (Spearman r = -0.381, $P > 0.05$) (**Figure 5.6C**).

Brain masks of suprathreshold voxels over T1 images showed substantially less BBBD voxels post-match in all but one player, who is discussed in the next section (**Figure 5.7A**) In contrast to post-season findings using the LDM, which suggested increases in the volume of BBBD, the percentage of suprathreshold voxels was significantly decreased within the acute phase following a match (median 18.58% (IQR 13.63 - 22.56) vs. 14.45% (IQR 9.995 - 16.38), $P = 0.0052$) (**Figure 5.7B**). In contrast to K^{trans}, there was a weak association between the percentage of suprathreshold voxels measured at baseline and those measured shortly after a match. However, this association proved non-significant in the limited cohort available. (Spearman r = 0.4286, $P > 0.05$) (**Figure 5.7C**).

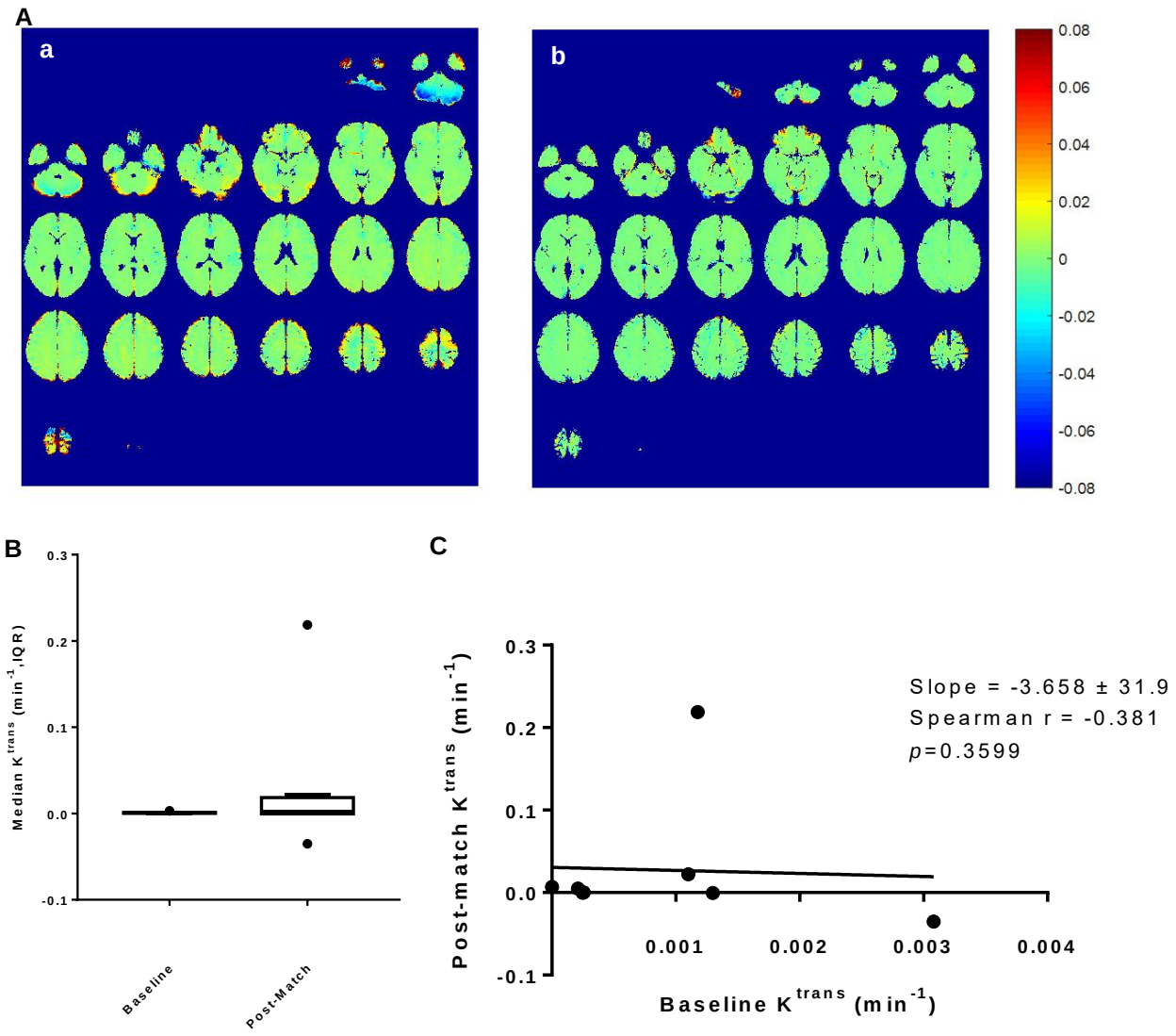


FIGURE 5. 6: ASSESSMENT OF BBB PERMEABILITY IN RUGBY PLAYERS FOLLOWING A COMPETITIVE RUGBY MATCH, USING TOFTS-EXTENDED MODEL

A) Representative of baseline (a) and post-match (b) brain masks of K^{trans} values. **B)** Groups analysis of median $K^{trans\ at}$ baseline and post-match time points (n=8). $P > 0.05$ by paired Wilcoxen test. **C)** Linear regression analysis comparing baseline median K^{trans} values with post-season median K^{trans} values. No correlation was observed between baseline values and post-season values.

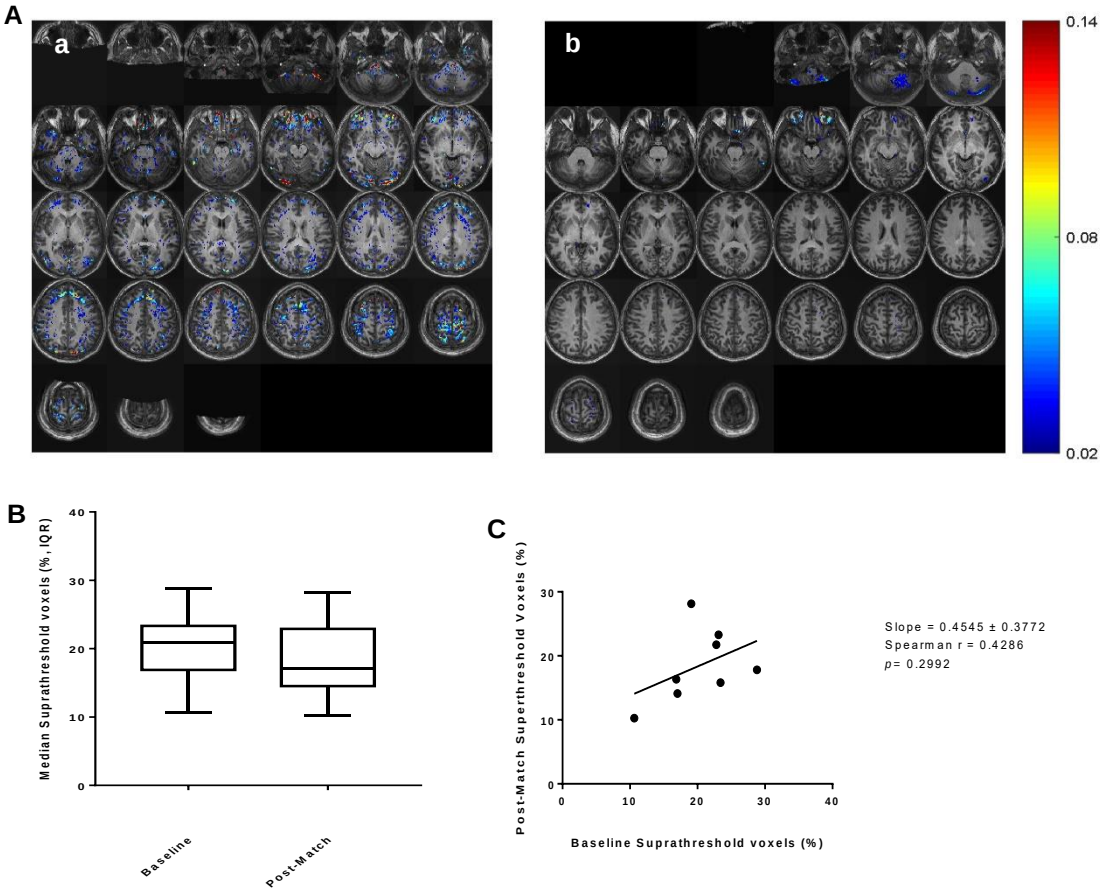


FIGURE 5. 7: ASSESSMENT OF BBB PERMEABILITY IN RUGBY PLAYERS FOLLOWING A COMPETITIVE RUGBY MATCH, USING THE LINEAR DYNAMIC MODEL

A) Representative of baseline (a) and post-match (b) brain masks of suprathreshold voxels over T1 images. **B)** Groups analysis of suprathreshold voxels [at] baseline and post-match time points (n=8). $P > 0.05$ by paired Wilcoxen test. **C)** Linear regression analysis comparing baseline percentage of suprathreshold voxels with post-season values. The trend line represents a moderate, non-significant correlation between higher baseline values and values measured after a rugby match.

5.3.3: BBB changes in an injured player

While no rugby player was diagnosed with a mTBI over the course of the study, one player did receive a heavy injury as a result of a tackle early in a game, injuring his hip and sustaining a possible blow to the head in the subsequent fall. The player was assessed by on-site medical personnel and cleared of mTBI. However, he subsequently sat out the remainder of the game and agreed to post-match MRI assessment. As this individual was the only participant to sustain a recognised injury over the study period, his BBB status was followed and compared to other participants who had completed both post-match and post-season assessments (n=6). Brain masks of K^{trans} values showed no substantial focal increases when compared to baseline (**Figure 5.8A**). However, the signal generated using the LDM showed large, unilateral increases in signal compared to baseline (**Figure 5.8B**). Post-season BBB assessment showed no large changes in K^{trans} values across the brain (**Figure 5.9Aiii**). Signal generated using the LDM remained high and overlap with some regions of high signal observed at the post-match assessment (Figure 5.8 Aiii). While no focal changes in K^{trans} were apparent, median K^{trans} values for the injured participant was substantially higher than others who underwent post-match assessment in the absence of injury (**Figure 5.8B**), the individual is highlighted in red). However, the percentage of suprathreshold voxels as measured after a match was not noticeably different from those figures from the rest of the cohort (**Figure 5.9B**). The individual's median K^{trans} values and the percentage of suprathreshold voxels did not differ substantially from the rest of the cohort at the post-season assessment.

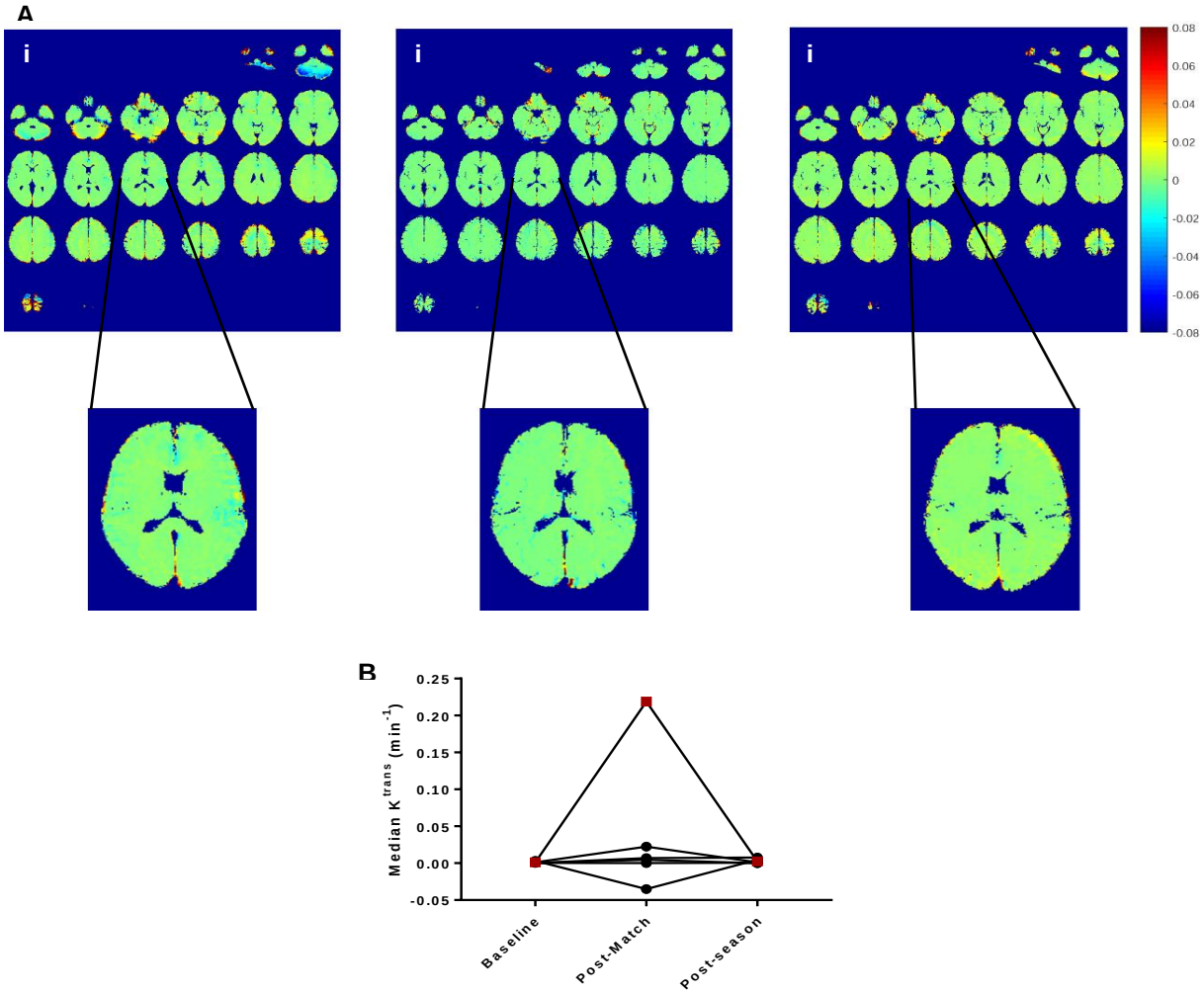


FIGURE 5. 305: BBB PERMEABILITY IN AN INJURED PLAYER OVER THE COURSE OF A SEASON, EVALUATED USING TOFTS EXTENDED MODEL.

A) BBB permeability mask generated using K^{trans} values for the same player who sustained a non-head injury and was removed from play at **i**) baseline, **ii**) post-match and **iii**) post-season. **B**) Comparison of median K^{trans} value for all players who completed a post-match and post-season MRI scan. Changes in BBB integrity as measured by the Tofts extended model did not differ much from baseline and post-season values observed for the same player. The same player who sustained a non-head injury depicted in (**A**) is denoted with a square symbol and red colouration. The injured player illustrates a potential temporal confounder with the use of the Tofts extended model in the context of post-exertion assessment, with the player's median K^{trans} value being noticeable higher than his peers.

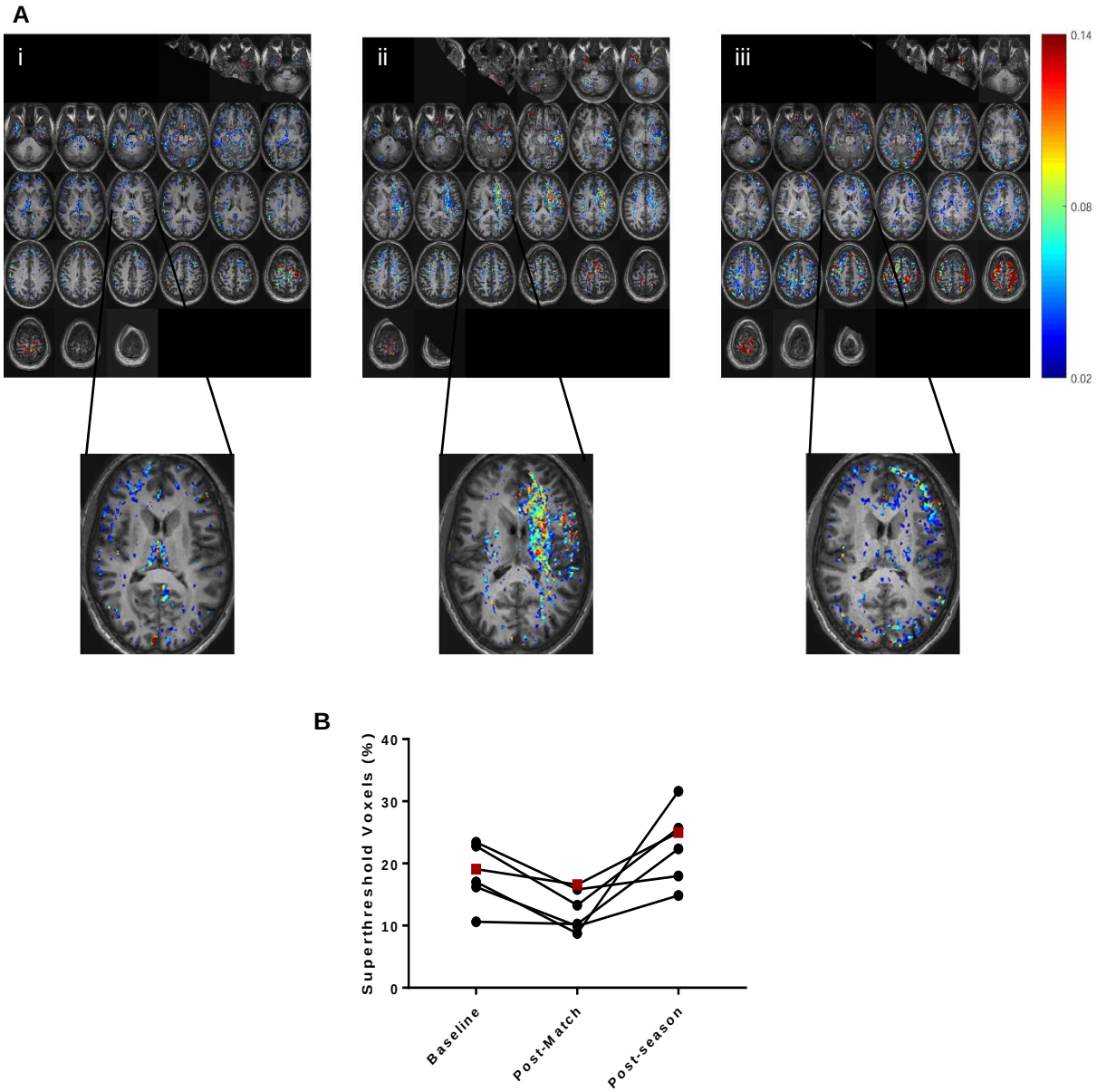


FIGURE 5. 9: BBB PERMEABILITY IN AN INJURED PLAYER OVER THE COURSE OF A SEASON, EVALUATED USING THE LINEAR DYNAMIC MODEL.

A) BBB permeability mask generated using suprathreshold voxels over T1 images for the same player who sustained a non-head injury and was removed from play at **i**) baseline, **ii**) post-match and **iii**) post-season. **B)** Comparison of percentage of suprathreshold voxels for all players who completed a post-match and post-season MRI scan. Changes in BBB integrity as measured by the LDM did not appear to mirror the change observed for the same player at the send of the season. The same player who sustained a non-head injury depicted in (**A**) is denoted with a square symbol and red colouration. The player illustrates a potential temporal confounder with the use of the LDM in the context of post-exertion assessment, with visual signal being noticeable higher than his peers. This was not reflected in the percentage of disrupted voxels measured by the LDM (**B**, red square).

5.3.4: Changes in neural tissue microstructure following a season of competitive rugby

MRI assessment also included a 32-direction DTI sequence in order to assess changes in the microstructure of WM neural fibres over the course of a season of play. The four parameters measured included FA, a general measure of microstructural integrity, MD, an inverse measure of axonal membrane density, AxD, a measurement of water movement along axonal fibres and axonal health, and RD, a measure of water movement perpendicular to the fibre track and changes in myelination of neural fibres. Ten regions, examined on both hemispheres, were chosen for examination due to historic findings of changes in tract integrity following mTBI and concussion. These are listed in **Table 5.1** and were examined in all participants that underwent pre-and post-season assessment (n=18).

FA was found to be significantly increased compared to baseline in the tracts of the Corpus Callosum body (0.5229 (standard deviation (SD) ±0.0153) vs. 0.5310 (SD ±0.0139, $*P \leq 0.05$). All other regions did not significantly differ from matched baseline measurements.

Significant decreases in MD were observed in the Corpus Callosum body (8.4732×10^{-3} mm^2/s (SD ± 0.8562) vs 8.1679×10^{-3} mm^2/s (SD ± 0.4225), $** P \leq 0.01$), the left anterior limb of the internal Capsule (7.6816×10^{-3} mm^2/s (SD ± 0.2120) vs. 7.5305×10^{-3} mm^2/s (SD ± 0.2041), $*P \leq 0.05$), left hemispherical hippocampal tracks (8.2426×10^{-3} mm^2/s (SD ± 0.2130) vs. 8.0715×10^{-3} mm^2/s (SD ± 0.2790), $*P \leq 0.05$), and the left inferior fronto-occipital fasciculus (8.1184×10^{-3} mm^2/s (SD ± 0.2303) vs. 7.9953×10^{-3} mm^2/s (SD ± 0.1525), $* P \leq 0.05$).

Similar changes were made in AxD upon comparison of post-season values to baseline, and in overlapping regions. AxD was significantly decreased from baseline values in the corpus callosum body (13.8084×10^{-3} mm^2/s (SD ± 0.5147) vs 13.5228×10^{-3} mm^2/s (SD ± 0.4023), $*P \leq 0.05$), the splenium of the corpus callosum (13.2284×10^{-3} mm^2/s (SD ± 0.3896) vs. 13.1521×10^{-3} mm^2/s (SD ± 0.6116), $**P \leq 0.01$), left hemispherical hippocampal tracks (13.9374×10^{-3} mm^2/s (SD ± 0.6226) vs. 13.5837×10^{-3} mm^2/s (SD ± 0.8035), $*P \leq 0.05$), and the left inferior fronto-occipital fasciculus (13.1989×10^{-3} mm^2/s (SD ± 0.4431) vs. 13.0095×10^{-3} mm^2/s (SD ± 0.3001), $*P \leq 0.05$).

Finally, RD values were also observed to change significantly from those measured at baseline. Namely, the corpus callosum body (5.6292 x 10^{-3} mm^2/s (SD ± 0.3832) vs. 5.3668 x 10^{-3} mm^2/s (SD ± 0.2106), $^{**}P \leq 0.01$), the splenium of the corpus callosum (5.3989 x 10^{-3} mm^2/s (SD ± 0.2269) vs. 5.3105 x 10^{-3} mm^2/s (SD ± 0.1626), $^{*}P \leq 0.05$), the left anterior limb of the internal Capsule (5.3158 x 10^{-3} mm^2/s (SD ± 0.1549) vs. 5.2045 x 10^{-3} mm^2/s (SD ± 0.1532), $^{*}P \leq 0.05$), left hemispherical hippocampal tracks (5.1379 x 10^{-3} mm^2/s (SD ± 0.1385) vs. 5.0526 x 10^{-3} mm^2/s (SD ± 0.1336), $^{*}P \leq 0.05$), and the left inferior fronto-occipital fasciculus (5.6480 x 10^{-3} mm^2/s (SD ± 0.1837) vs. 5.6141 (SD ± 0.1669), $^{*}P \leq 0.05$) all displayed significant decreases from matched values taken at baseline.

	FA						MD (x10*-3) (mm^2/s)						AD (x10*-3)(mm^2/s)						RD (x10*-3)(mm^2/s)					
	Baseline		Post-Season			*P*-value	Baseline		Post-Season			*P*-value	Baseline		Post-Season			*P*-value	Baseline		Post-Season			*P*-value
	Mean	SD	Mean	SD	Change		Mean	SD	Mean	SD	Change		Mean	SD	Mean	SD	Change		Mean	SD	Mean	SD	Change	
Corpus Callosum, Body	0.523	0.015	0.531	0.014	↑	*	8.473	0.856	8.168	0.422	↓	**	13.808	0.515	13.523	0.402	↓	*	5.629	0.383	5.367	0.211	↓	**
Corpus Callosum, Genu	0.494	0.037	0.510	0.021	-	n.s.	8.039	0.308	7.923	0.434	-	n.s.	14.742	0.534	14.224	0.612	-	n.s.	5.451	0.266	5.337	0.237	-	n.s.
Corpus Callosum, Splenium	0.563	0.017	0.555	0.028	-	n.s.	8.514	0.266	8.380	0.264	-	n.s.	13.228	0.390	13.152	0.612	↓	**	5.399	0.227	5.311	0.163	↓	*
ALIC, Left	0.495	0.010	0.501	0.014	-	n.s.	7.682	0.212	7.531	0.204	↓	*	12.275	0.436	12.182	0.403	-	n.s.	5.316	0.155	5.204	0.153	↓	*
ALIC, Right	0.486	0.008	0.485	0.018	-	n.s.	7.569	0.236	7.545	0.202	-	n.s.	12.066	0.380	12.022	0.412	-	n.s.	5.318	0.180	5.306	0.168	-	n.s.
PLIC, Left	0.521	0.009	0.525	0.011	-	n.s.	7.565	0.214	7.452	0.225	-	n.s.	12.462	0.370	12.333	0.365	-	n.s.	5.118	0.155	5.066	0.272	-	n.s.
PLIC, Right	0.515	0.009	0.517	0.013	-	n.s.	7.546	0.175	7.453	0.144	-	n.s.	12.358	0.298	12.254	0.282	-	n.s.	5.138	0.139	5.053	0.134	↓	*
Cingulum (Hippocampus), Left	0.439	0.029	0.451	0.041	-	n.s.	8.243	0.213	8.071	0.279	↓	*	13.937	0.623	13.584	0.803	↓	*	5.437	0.292	5.353	0.238	-	n.s.
Cingulum (Hippocampus), Right	0.443	0.033	0.438	0.028	-	n.s.	8.238	0.377	8.175	0.354	-	n.s.	13.827	0.519	13.551	0.688	-	n.s.	5.489	0.392	5.463	0.326	-	n.s.
External Capsule, Left	0.483	0.013	0.484	0.017	-	n.s.	7.882	0.220	7.822	0.187	-	n.s.	12.548	0.418	12.467	0.444	-	n.s.	5.551	0.165	5.499	0.127	-	n.s.
External Capsule, Right	0.469	0.016	0.470	0.020	-	n.s.	7.891	0.233	7.852	0.176	-	n.s.	12.376	0.495	12.327	0.385	-	n.s.	5.648	0.157	5.614	0.167	-	n.s.
IFOF, Left	0.503	0.012	0.504	0.010	-	n.s.	8.118	0.230	7.995	0.153	↓	*	13.199	0.443	13.009	0.300	↓	*	5.648	0.157	5.614	0.167	↓	*
IFOF, Right	0.487	0.012	0.489	0.013	-	n.s.	8.050	0.228	7.994	0.138	-	n.s.	12.858	0.392	12.725	0.298	-	n.s.	5.646	0.184	5.588	0.121	-	n.s.
SFOF, Left	0.517	0.021	0.510	0.027	-	n.s.	7.577	0.315	7.525	0.337	-	n.s.	12.391	0.498	12.405	0.737	-	n.s.	5.149	0.316	5.137	0.325	-	n.s.
SFOF, Right	0.477	0.027	0.470	0.026	-	n.s.	7.513	0.293	7.546	0.365	-	n.s.	11.957	0.482	12.181	0.932	-	n.s.	5.303	0.296	5.387	0.317	-	n.s.

TABLE 5. 81: CHANGES IN MEASURES OF AXONAL INTEGRITY IN RUGBY PLAYERS FOLLOWING A SEASON OF PLAY.

Changes in measures of axonal integrity using tractography analysis via ExploreDTI suite. Tract structures were generated using the ICBM brain atlas template. * $P \leq 0.05$, * $P \leq 0.01$ by paired Wilcoxon test. Significant improvements were observed in FA values of the body of the corpus callous, possibly driven by improvements in MD and RD values within this structure. Improvements in MD and RD, suggesting increases axonal membrane density and myelination respectively, were also observed in the left ALIC and left IFOF. MD values also improved in the left cingulum (hippocampus). Improvements in RD were observed in the splenium of the corpus callosum and right PLIC. However, these were not reflected in improvements in FA within these structures. Decreases in AD values, reflecting axonal shortening, was observed in body of the corpus callosum, the splenium of the corpus callosum, the left cingulum (hippocampus) and the left IFOF. While significant, these changes were not sufficient to detract from FA values. ALIC: anterior limb of the internal capsule. PLIC: posterior limb of the internal capsule. IFOF: inferior fronto-occipital fasciculus. SFOF: superior fronto-occipital fasciculus.

5.3.5: Correlation of BBB permeability measurements with relative changes in serological markers of mTBI

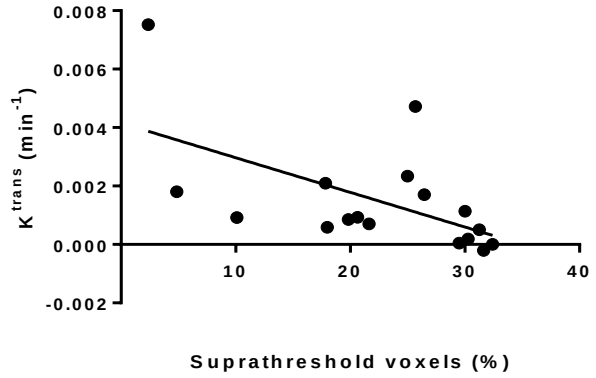
Linking phenotype with objectively measurable markers is a requirement for any successful biomarker. To that end, it was investigated whether relative changes from baseline in serological markers, discussed in Chapter 4, held any association with the measures of the integrity of the BBB at the end of a season of rugby. While the values of both the Toft's model and the LDM are not directly comparable, the relationship between post-season values of K^{trans} and percentage of suprathreshold voxels as also investigated. A correlation matrix was produced based on these parameters, with association strength being determined by Spearman r (**Figure 5.10A**). Association strength was set at 0-0.39 for no association, 0.4-.0.59 for a weak association, 0.6-0.79 for a moderate association and 0.8-1 for a strong association. Correlation analysis showed a moderate inverse association between the percentage of suprathreshold voxels and median K^{trans} values (Spearman r = -0.637, $^{**}P \leq 0.01$) (**Figure 5.10B**). A weak, non-significant inverse association was also identified between the percentage of suprathreshold voxels and relative changes in circulating S100β levels (Spearman r = -0.417, $P > 0.05$) (**Figure 5.10C**). No association was found linking any other variable (Spearman r < 0.4).

A

Variable	% voxels	K^{trans}	S100β	BDNF	MCP-1	IL-1β PBMCs	IL-6 PBMCs	IL-1β Plasma	IL-6 Plasma
% voxels									
K^{trans}	-0.637								
S100β	-0.417	0.060							
BDNF	-0.057	-0.088	0.320						
MCP-1	-0.213	0.091	0.249	0.356					
IL-1β PBMCs	-0.026	-0.284	0.393	0.109	-0.171				
IL-6 PBMCs	-0.041	-0.079	-0.275	0.173	0.215	-0.200			
IL-1β Plasma	0.396	0.090	-0.316	0.052	-0.156	-0.398	0.316		
IL-6 Plasma	-0.118	0.088	0.100	0.011	-0.215	-0.147	0.049	0.061	

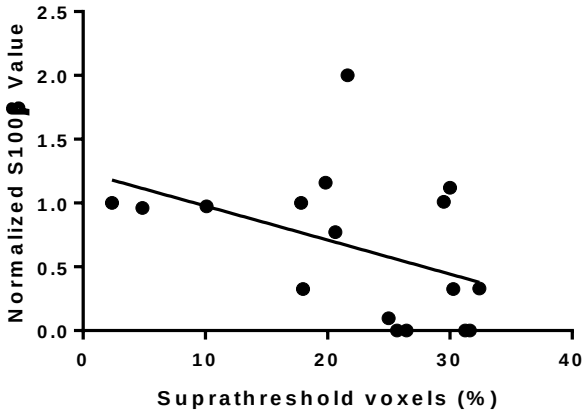
±0.0-0.39	No assoication
±0.4-0.59	weak assoication
±0.6-0.79	moderate assoication
±0.8-1.0	Strong assoication

B



Slope =-0.0001186 ± 4.455e-005
Spearman's r = -0.6372
P= 0.007

C



Slope =-0.02669 ± 0.01429
Spearman's r =-0.4172
P= 0.096

FIGURE 5. 10: CORRELATION ANALYSIS OF POTENTIAL MARKERS OF MTBI IN RUGBY PLAYERS

A) Correlation matrix of Spearman's r value of all measured outputs from the cohort of rugby players at post-season. % Voxel and K^{trans} measurements were post-season values. Biomarker data was normalised to find individual relative changes in biomarker levels. An inverse correlation between K^{trans} values and % voxels measured at the post-season time point. It should be noted that the two measurements examine different aspects of BBB integrity. K^{trans} measures the overall rate of flow between the luminal and perivascular spaces, while the % voxel values measure the of volume of BBB within the measured area. As such a negative correlation is possible. **B)** Linear regression of post-season percentage of suprathreshold voxels vs. median K^{trans} values. $^{**}P \leq 0.01$ by Spearman correlation. **C).** Linear regression of post-season percentage of suprathreshold voxels vs. relative changes in S100β. $P > 0.05$ by Spearman correlation.

5.3.6: BBB changes following competitive a mixed martial arts bout

As part of an ongoing project, 17 MMA fighters have been recruited in order to link BBB phenotype with the magnitude of the force applied to the head during a competitive fight. Similar to the rugby cohort, participants undergo a baseline scan and a post-fight scan within 48 hours of a competitive fight. To date, 5 participants have completed baseline and post-fight assessment. The results presented represent an ongoing study.

BBB integrity as measured by Tofts extended model displayed few focal areas of high K^{trans} measurement either at baseline or with the 48hrs after a fight (**Figure 5.11Aa** & **Ab**). Groups analysis of median K^{trans} values showed a greater range after a fight, but no significant changes were observed between baseline and post-fight K^{trans} measurements (median 2 x 10^{-3} min^{-1} (IQR 4.704 x 10^{-4} - 4.903 x 10^{-3}) vs 5.28 x 10^{-5} min^{-1} (IQR -2.625 x 10^{-4} – 1.274 x 10^{-2}), $P< 0.05$) (**Figure 5.11B**). A significantly strong correlation was observed between baseline median K^{trans} and K^{trans} values measured after a fight (Spearman r = 1, $*P \leq 0.05$) (**Figure 5.11C**) However, the study remains in its infancy and this correlation may be lost with the addition of more participants.

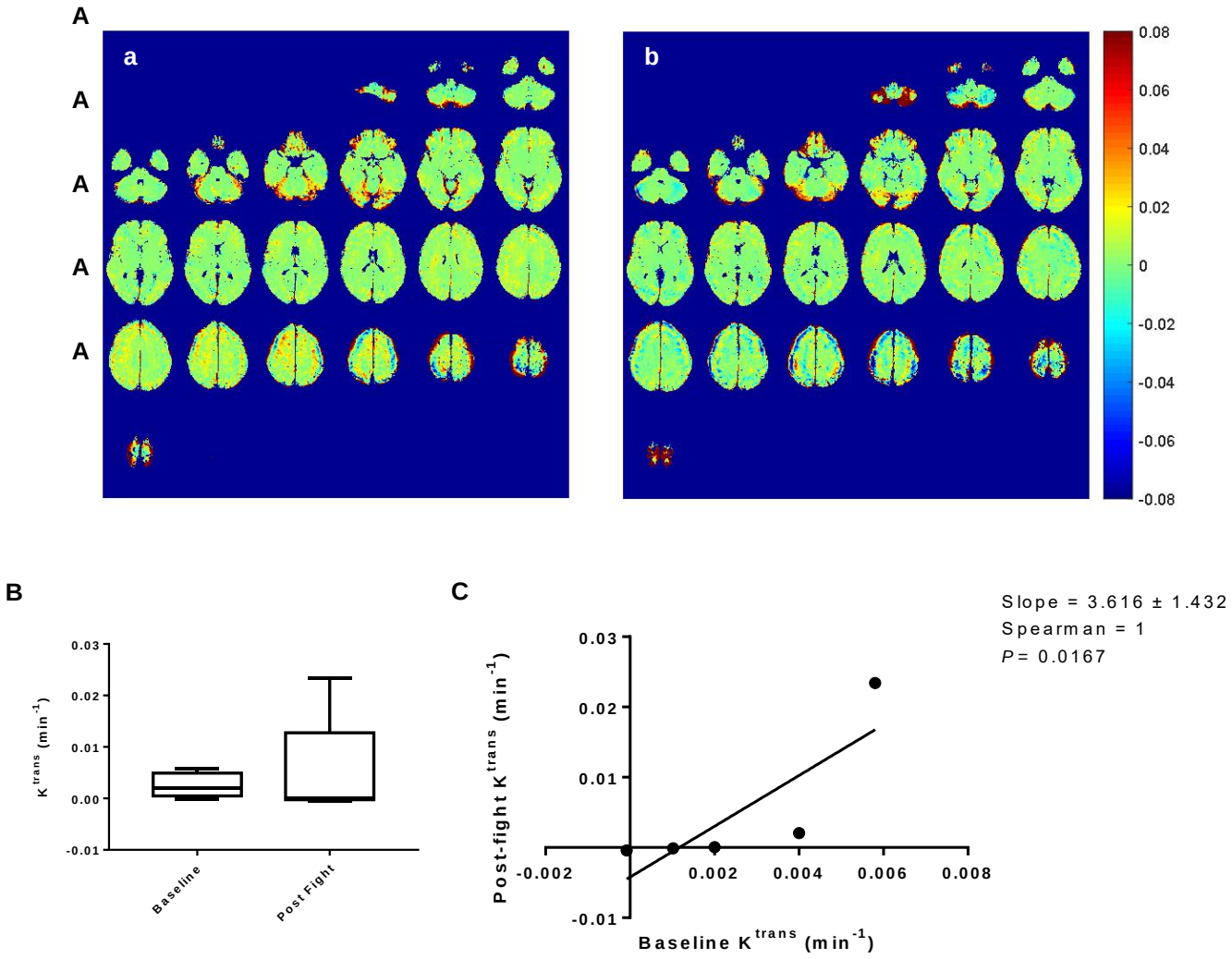


FIGURE 5. 11: ASSESSMENT OF BBB PERMEABILITY IN MIXED MARTIAL ARTS FIGHTERS FOLLOWING A COMPETITIVE BOUT USING TOFTS-EXTENDED MODEL.

A) Representative of baseline (**a**) and post-fight (**b**) brain masks of K^{trans} values for MMA fighters assessed within 48hrs post-bout. Visual inspection of K^{trans} brain masks showed no obvious lesions or changes in BBB integrity. **B)** Groups analysis of median $K^{trans\ at}$ baseline and post-fight time points (n=5). $P > 0.05$ by paired Wilcoxen test. Signficant changes from baseline values were not observed within the limited cohort available. **C)** Linear regression analysis of post-match (blue) and post-season (red) median K^{trans} values. The trend line indicates a strong correlation between baseline and post-fight K^{trans} values in the limited cohort. However, this is likely driven by a single individual who had a high baseline and post-bout K^{trans} values and therefore cannot be deemed an accurate indication of the correlation between baseline and post-fight BBB integrity.

BBB assessment by the LDM showed much more widespread barrier disruption following a fight when compared to the same regions at baseline (**Figure 5.12 Aa** & **Ab**. However, no significant differences were observed upon group analysis of baseline and post-fight percentage of suprathreshold voxels (median 28.32% (IQR 18.04 – 31.09) vs. 31.26% (IQR 21.45 – 33.6), $P > 0.05$) (**Figure 5.12 B**). No association was observed between the percentage of suprathreshold voxel at baseline vs the percentage observed after a fight (Spearman r = 0.1, $P > 0.05$) (**Figure 5.12 C**).

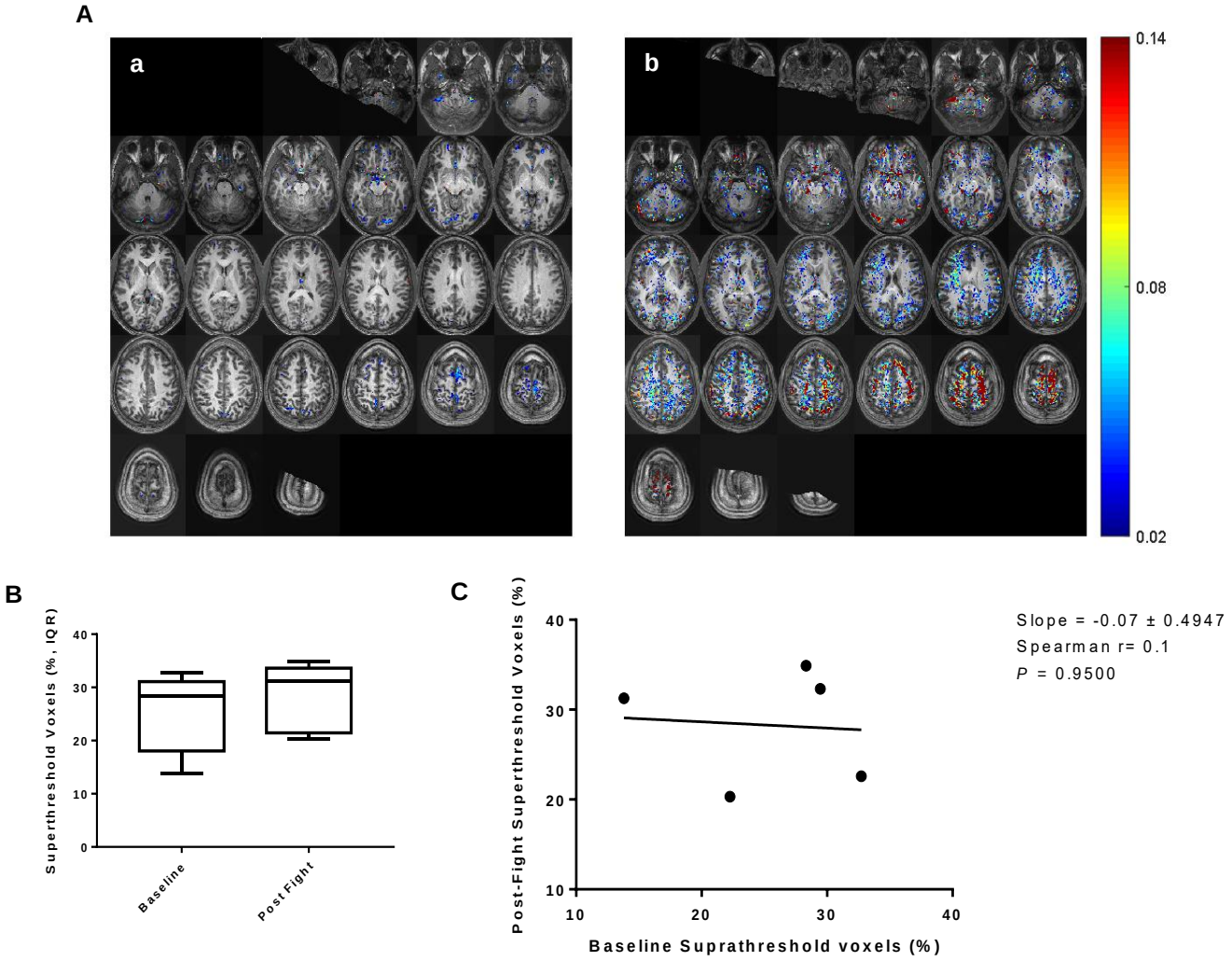


FIGURE 5. 12: ASSESSMENT OF BLOOD BRAIN BARRIER PERMEABILITY IN MIXED MARTIAL ARTS FIGHTERS FOLLOWING A COMPETITIVE BOUT USING THE LINEAR DYNAMIC MODEL.

A) Representative of baseline (**a**) and post-fight (**b**) brain masks suprathreshold voxels over T1 images for MMA fighters assessed within 48hrs post-bout. Visual inspection of the brain masks showed an increase in suprathreshold signal in all participants post-bout**. B)** Groups analysis of the % suprathreshold voxel baseline and post-fight time points (n=5). $P > 0.05$ by paired Wilcoxen test. There was no significant difference in the % of suprathreshold voxels observed between baseline and post-bout assessment it the limited cohort available. However, a small increase in % of suprathreshold voxels was observed at the post-bout time point. **C)** Linear regression analysis of post-fight percentage of suprathreshold voxels. The trend line indicates a no correlation between baseline and post-fight the percentage of suprathreshold voxels.

5.3.7: *Changes in neural tissue microstructure following a season of MMA bout*

Using the same DTI sequence and parameters used to assess axonal tract variables within the cohort of rugby players, changes in the microstructure of neural tissue were measured within the cohort of MMA fighters.

Across all structures and measures, no changes in axonal tract integrity were observed when participants underwent scanning within 48 hours of a bout (**Table 5.2**).

	FA						MD (x10*3) (mm^2/s)						AD (x10*3)(mm^2/s)						RD (x10*3)(mm^2/s)					
	Baseline		Post-fight			*P*-value	Baseline		Post-fight			*P*-value	Baseline		Post-fight			*P*-value	Baseline		Post-fight			*P*-value
	Mean	SD	Mean	SD	Change		Mean	SD	Mean	SD	Change		Mean	SD	Mean	SD	Change		Mean	SD	Mean	SD	Change	
Corpus Callosum, Body	0.507	0.021	0.507	0.021	-	n.s.	8.596	0.496	8.516	0.224	-	n.s.	14.036	0.521	13.934	0.284	-	n.s.	5.874	0.485	5.806	0.204	-	n.s.
Corpus Callosum, Genu	0.494	0.018	0.493	0.012	-	n.s.	8.262	0.398	8.206	0.229	-	n.s.	14.864	0.544	14.858	0.316	-	n.s.	5.758	0.374	5.798	0.253	-	n.s.
Corpus Callosum, Splenium	0.542	0.014	0.539	0.011	-	n.s.	8.794	0.429	8.818	0.273	-	n.s.	13.392	0.426	13.262	0.355	-	n.s.	5.696	0.401	5.676	0.195	-	n.s.
ALIC, Left	0.483	0.012	0.487	0.014	-	n.s.	7.706	0.293	7.722	0.265	-	n.s.	12.276	0.511	12.254	0.527	-	n.s.	5.442	0.189	5.458	0.186	-	n.s.
ALIC, Right	0.475	0.006	0.478	0.017	-	n.s.	7.664	0.252	7.750	0.335	-	n.s.	12.092	0.361	12.264	0.595	-	n.s.	5.450	0.203	5.494	0.260	-	n.s.
PLIC, Left	0.500	0.011	0.504	0.016	-	n.s.	7.714	0.253	6.382	2.793	-	n.s.	13.592	0.870	14.234	0.292	-	n.s.	5.780	0.368	5.938	0.352	-	n.s.
PLIC, Right	0.504	0.014	0.507	0.012	-	n.s.	7.688	0.240	7.664	0.185	-	n.s.	13.004	1.000	13.316	0.799	-	n.s.	5.612	0.156	5.814	0.166	-	n.s.
Cingulum (Hippocampus), Left	0.444	0.078	0.463	0.046	-	n.s.	8.490	0.387	8.832	0.412	-	n.s.	12.508	0.384	12.538	0.386	-	n.s.	5.320	0.224	5.290	0.157	-	n.s.
Cingulum (Hippocampus), Right	0.438	0.068	0.448	0.044	-	n.s.	8.082	0.414	8.358	0.282	-	n.s.	12.440	0.307	12.408	0.318	-	n.s.	5.308	0.213	5.292	0.173	-	n.s.
External Capsule, Left	0.472	0.018	0.472	0.015	-	n.s.	7.714	0.253	6.382	2.793	-	n.s.	12.706	0.217	12.766	0.240	-	n.s.	5.768	0.254	5.792	0.251	-	n.s.
External Capsule, Right	0.475	0.019	0.467	0.015	-	n.s.	7.688	0.240	7.664	0.185	-	n.s.	12.824	0.290	12.834	0.381	-	n.s.	5.766	0.281	5.834	0.214	-	n.s.
IFOF, Left	0.492	0.014	0.489	0.008	-	n.s.	8.372	0.240	8.438	0.203	-	n.s.	13.458	0.203	13.522	0.220	-	n.s.	5.830	0.262	5.894	0.200	-	n.s.
IFOF, Right	0.481	0.013	0.476	0.012	-	n.s.	8.308	0.354	8.308	0.243	-	n.s.	13.210	0.446	13.156	0.365	-	n.s.	5.858	0.316	5.884	0.208	-	n.s.
SFOF, Left	0.471	0.038	0.471	0.025	-	n.s.	7.738	0.357	7.774	0.413	-	n.s.	12.162	0.763	12.282	0.799	-	n.s.	5.530	0.331	5.556	0.295	-	n.s.
SFOF, Right	0.438	0.019	0.443	0.037	-	n.s.	7.812	0.317	7.814	0.437	-	n.s.	11.866	0.399	11.906	0.573	-	n.s.	5.788	0.319	5.764	0.495	-	n.s.

TABLE 5. 82: TABLE: CHANGES IN MEASURES OF AXONAL INTEGRITY IN MMA FIGHTER MEASURED WITHIN 48 HOURS A COMPETITIVE BOUT.

Changes in measures of axonal integrity using tractography analysis via the ExploreDTI software suite. Tract structures were generated using the ICBM brain atlas template. n.s signifies $P > 0.05$ by paired Wilcoxon test. No significant changes were observed for any of the parameters measured in any of the structures investigated. This may be due to the limited number of particpants available for analysis at this time. It may also be due to a temporal factor, in that axonal changes may not have taken effect within the 48hrs after a bout. ALIC: anterior limb of the internal capsule. PLIC: posterior limb of the internal capsule. IFOF: inferior fronto-occipital fasciculus. SFOF: superior fronto-occipital fasciculus.

5.4: Discussion

The rotational and translational forces involved in TBI subjects the brain and its vasculature to substantial shear stress. Medical imaging already plays a role in determining the severity of moderate and severe TBI, as well as confirming the presence of complicated mTBI. However, conventional imaging techniques have yet to identify changes in neural anatomy associated with mTBI. As a result, advanced medical imaging modalities, such as DTI and DCE-MRI are used in order to identify markers of mTBI-induced changes to the neural structure and the implications these changes may have on long-term brain health. Presented in this chapter are the results of assessing changes in the integrity of the BBB over the course of a competitive season rugby, as well as changes in the microstructure of neural tissue. In addition, primarily data regarding changes to BBB integrity and axonal integrity following competitive MMA bouts is also presented.

The novel LDM has recently been shown to detect BBB disruption *in vivo* in mouse models of mTBI, with regions of high disrupted voxels overlapping with the regions exposed to the TBI force and Evans blue extravasation (Tagge *et al.,* 2018). In addition to being shown to detect mTBI-induce changes, the LDM has also been shown to match up with regions of high K^{trans} values in cases of brain tumours and ischemic stroke, indicating that the method is capable of detecting areas of increased barrier permeability (Veksler, Shelef & Friedman, 2014). Measurement of BBB permeability by both Tofts Extended Model and the LDM displayed that a subset of individuals experienced an increase in barrier permeability to the contrast agent after a full season of play. On a group basis, BBB permeability was increased when measuring the volume transfer coefficient at post-season, while minor, non-significant changes were observed in the percentage of disrupted voxels, suggesting that repeated exposure to the forces involved in competitive rugby are sufficient enough to influence the BBB. An interesting finding was the significant decrease in the number of disrupted voxels immediately following a match, despite little change observed in K^{trans} values. Currently, the reason for this remains to be determined, as this study is the first use of the LDM in this context. A possible explanation may be due to the LDM being more sensitive to changes in cerebral blood

flow. Moderate exercise can increase cerebral blood flow by 10-30%, increasing with exercise intensity up to roughly 60% of maximal oxygen uptake (Smith & Ainslie, 2017). As the LDM relies on the venous output function as the reference measure of outflow from the brain, increased arterial pressure and mean systemic pressure may affect the result. As increases in arterial and systemic blood pressure are associated with exercise, these changes could have contributed to a lower venous return within the sagittal sinus, resulting in a slower outflow rate compared to that of the rest of the cerebral vasculature (Young, Control of Cardiac Output, 2010; Vilcant & Zeltser, Treadmill Stress Testing, 2018).

Another point to note is that the number of disrupted voxels measured by the LDM is much higher in our cohort than in the previously studied cohort by Weissberg *et al.* (2014). The "pathological" group identified in that study had an average of 16.29% of suprathreshold voxels. In contrast, our cohort had a median value of 22.28% at baseline. A possible explanation for this may be due to different contrast agents used, or the nature of the sports involved. In many instances, rugby players will engage in play for the length of a match, while American football involves many player changes depending on the tactics employed. This may reduce an individual's lifetime exposure to sub-concussive forces and may be reflected in differences in the number of suprathreshold voxels.

A finding of note among the correlation analysis was the inverse association between the percentage of suprathreshold voxels and median K^{trans} values. As outlined through this chapter, the outputs of the Tofts extended model and the LDM show contrasting results at both the post-match and post-season measurement points. While median K^{trans} appears comparable to baseline values shortly following a rugby match, the percentage of suprathreshold voxels dramatically drops. However, after a season of play, median K^{trans} increases, in the absence of focal areas of high permeability, while suprathreshold voxels visually appear to increase in focal regions, but when quantified fails to demonstrate significant changes from values measured at baseline. Both methods have been used to measure BBB permeability in conditions such as glioblastoma and ischemic stroke, and have shown similar findings (Veskler, Shelef & Friedman, 2014). However, as outlined in Chapter 1, few studies have examined how K^{trans} values preform in mTBI, and while the LDM has primarily been used to investigate mTBI in humans, only a limited number

of studies using the method have been published to date, and only one with a positive control for head trauma (Tagge *et al.,* 2018). As well as that, to our knowledge, no study to date has compared how both models perform in mTBI. Therefore, caution must be exercised in the interpretation of both models in this context when determining if BBBD is a result of contact sports. However, while K^{trans} and suprathreshold voxels are not directly comparable, the lack of a correlation between the two remains an interesting finding. This finding, that K^{trans} increases in the absence of significant changes to BBBD volume, suggests that regions of compromised BBB integrity may be getting worse, rather than a spread of dysfunction to additional areas.

Comparison of microstructure parameters via DTI analysis showed changes in MD, AxD and RD in several structures of the corpus callosum, however, only the corpus callosum body showed significant changes in FA. The corpus callosum is one of the largest axon fibre bundles in the human brain and links motor, perceptual and cognitive input between hemispheres (Glickstein & Berlucchi, 2008). The corpus callosum is also one of the most frequently reported regions to experience axonal injury following TBI, and finite element modelling suggests that it is a region that experiences the greatest strain during an injury (Bigler & Maxwell, 2011). The observed increase in FA, and by extension decrease in MD, is likely driven by reduced RD, as AxD is also reduced at the post-season timepoint. This suggests that despite reduced diffusion along the axonal tract, the reduced diffusion away from the length of the axon is a net positive in diffusion directionality. However, the reduction in AxD is interesting to note, as it may indicate that axonal damage, or at least axonal pruning, has occurred during the intervening times between scans (Alexander *et al.,* 2011). Several other regions also demonstrated decreased AxD, such as the cingulum (hippocampus) and the inferior fronto-occipital fasciculus (IFOF). The cingulum runs around the corpus callosum and issues projections in the frontal, parietal, occipital and temporal lobe, integrating connections from these regions to the hippocampus (Catani & Thiebaut de Schotten, 2008). Through these connections, the structure plays a role in attention and memory functions, as well as emotional response. The IFOF connects the occipital lobe with the posterior temporal lobe and orbtio-frontal cortex, however, its function is not yet fully defined. Due to its proximal overlap with the inferior longitudinal fasciculus, it is thought to play roles in facial recognition, visual memory and language functions (Catani & Thiebaut de Schotten, 2008). Both regions have been reported as injury sites

following TBI previously, while emotional imbalance, lack of attention and memory complaints are common symptoms of PCS and were therefore investigated in this study. However, aside from decreases in AxD, these regions showed decreases in both MD and RD, suggesting improving tract integrity, although this was not represented in changes to FA.

These findings contrast with previous studies looking at medium-term changes in axonal microstructure, even when participants incurred non-concussive blows (McAllister *et al.,* 2014; Bahrami *et al.,* 2016). It is possible that the changes seen in our cohort could reflect the maturation of the brain during adolescence, as numerous cross-sectional studies have demonstrated increases in FA, as well as decreases in MD and RD, during maturation (Tamnes *et al.,* 2018). It may be the case that these changes are less than those undergone by their peers who have not experienced mTBI or sub-concussive blows, such as was demonstrated by Van Beek and colleagues (2015b) However, in the absence of a control cohort, this remains speculation. It can be noted, however, that many of the changes appeared unilaterally, similar to a finding present in young American football players (Bahrami *et al.,* 2016). While IFOF tracks are known to be asymmetrical, which may generate different shear forces on tracks, this does not account for unilateral changes observed in other structures. Unilateral changes may, therefore, be a product of the sport itself and a function of the position of the players and the direction of incoming blows.

Chapter 6: General discussion

General discussion

Traumatic brain injury (TBI) is thought to be one of the most prevalent forms of trauma globally, often dubbed as a "silent epidemic" in both public and scientific media. The incidence rates of TBI are estimated to range from 7.3 per 100,000 people in Western Europe to 811 per 100, 000 people in New Zealand, with mild TBI (mTBI) making up between 15-97% of cases depending on the country (Faul & Coronado, 2015; Li *et al.,* 2016). However, many epidemiology studies acknowledge that these rates may greatly underestimate the number of TBIs that occur each year, as such studies rely on hospital visitation figures; often mTBI cases, such as those incurred during sport, are deemed unwarranted of hospitalisation, As such, a recent meta-analysis places the incidence of mTBI alone at roughly 224 per 100,000 people (Nguyen *et al.,* 2016). With currently available data, however, disability as a result of TBI already places substantial burdens on both the individual affected, and on the economic costs associated with their care and management. In 2010, it was estimated that TBI-related disability cost the US economy alone roughly $76.5 billion (CDC, 2010). In addition, while moderate and severe TBIs have been indicated as a risk factor for the development of neurodegenerative conditions, mTBI's influence in long term neural health is continuing to be explored (Nordström *et al.,* 2012; Godbolt *et al.,* 2012; Gardner *et al.,* 2014). Should mTBI be found to contribute to neurodegenerative disease progression, the cost associated with seemingly inconsequential injuries may be much greater than previously estimated.

Vascular pathology has been implicated in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD), with recent studies showing that BBBD may be an early inciting event in disease progression (Desai *et al*., 2007; Drouin-Ouellet *et al.,* 2015; Nation *et al.,* 2018). Both animal models and human studies of TBI at all severities have suggested that such injuries can lead to temporary disruption of the BBB, which could provide an early seed for the development of neurological conditions later in life (Tagge *et al.,* 2017; Johnson *et al.,* 2018). However, as discussed previously, the connection between mTBI and neurological disease progression remains to be firmly established. This may be in part due to the difficulty in stratifying mTBI from more severe injuries, such as complicated mTBI, in addition to issues such as relying on recalled injuries and associated bias, low recruitment numbers and lack of long term follow up. Some of these shortcomings have been

attempted to be circumvented by studies involving athletes competing in contact sports, where head trauma and mTBI may be better documented than those experienced by the public. However, while such studies offer promising insight into the risks posed by mTBI, they remain to demonstrate established evidence of dementia as a result of repetitive mTBI. For example, the incidence rates of mTBI in sports such as American football, ice hockey and wrestling are estimated to be roughly 6.71, 7.91 and 10.92 injuries per 10, 000 competitive events, respectively (Zuckerman *et al.,* 2015). Currently, however, participation in contact sports only tentatively suggests a predisposition in individuals for early onset of dementia compared to the general population (Karantzoulis & Randolph, 2013). In parallel to these long-term studies, much research has been committed to finding a measurable marker of mTBI that may suggest long-term neural damage that remains after resolution of transient neurological impairment. To that end, the main objective of this doctoral work was to determine whether participation in contact sports such as rugby or mixed martial arts (MMA) resulted in detectable changes in BBB permeability, as well as to investigate the utility of novel markers of mechanisms involved in neuronal damage following TBI. While definitive evidence for or against this hypothesis could not be achieved in this study, the work presented provides important establishing work to further the understanding of the long-term consequences of mTBI for neural structures.

The case studies demonstrate a possible mechanistic link between TBI and the development of dementia later in life. Case 1 represents an intersection between paranoid schizophrenia, chronic traumatic encephalopathy (CTE) and BBB dysfunction. Both CTE and schizophrenia are conditions that have been associated with changes in the BBB, but in Case 1, given the context of a history of mTBI, both conditions may share the common origin of in reductions in tight junction (TJ) components stemming from repetitive head trauma. Similarly, in Case 2, BBB dysfunction was observed in a dementia case where the patient was known for having a long history of participation in rugby and incurring multiple mTBIs throughout their career. In both case reports, dense regions of phospho-tau were closely associated with reductions in TJ protein, suggestive of BBBD and. Potential mechanisms leading to this pathology may involve axonal shearing as a result of mechanical forces involved in TBI, leading to changes in phosphorylation states of tau, which subsequently reduces TJ levels. Alternatively, shear stress on endothelial cells may disrupt TJ complexes, which lead to increased permeability to blood-based agents that

trigger changes in tau phosphorylation states. Exact mechanisms for how the mechanical force associated with TBI produces molecular changes within the brain remain to be elucidated. The third case report suggests the possibility that a TBI of sufficient severity may contribute to detrimental effects in the long-term. The manifestation of Diffuse Lewy body disease (DLBD), frontotemporal dementia (FTD) and AD within an individual may suggest that a common factor, such as BBB dysfunction, may have contributed to their manifestation. As mentioned previously, multiple pathologies have been previously reported in individuals with a history of TBI (Ling *et al.,* 2017). However, with all cases presented here, only tentative conclusions may be drawn. As these reports detail case studies, they may only offer suggestive paths of investigation, and further studies on a larger scale are required.

Analysis of the biomarkers available over the course of this work showed that S100β, one of the most heavily investigated biomarkers for mTBI, did not correspond with changes in the BBB over the course of a season of rugby. Comparison of post-season values to baseline values demonstrated a drop in circulating levels of the predominantly astrocytic protein, which if taking S100β as a biomarker of BBBD, would suggest that barrier integrity improved over the course of the study period. This observation is surmised in the non-significant, inverse association between the percentage of suprathreshold voxels, measured using the LDM of BBB permeability measurement, and the relative changes in circulating S100β levels, while no association was found between changes in S100β levels and K^{trans} measurements, which displayed a significant increase at the end of the season compared to baseline. However, this finding at least follows a previous study that found no correlation between S100β and K^{trans} in mild and moderate TBI cases (Winter *et al.,* 2015). While S100β was increased shortly after a match, suggesting increased permeability, this was not reflected in an increase in either MRI-based measurement of BBB permeability. Increases in S100β following exercise in the absence of mTBI have already been discussed in both contact and non-contact sports. However, what may be considered is whether these increases are comparable to increases observed following mTBI. Another consideration is whether the increases in circulating S100β levels reflect protein released from astrocytic sources, and therefore reflect some change in BBB permeability, or whether such increases stem from peripheral sources such as adipocytes, which may suggest that peripheral sources of S100β contribute to post-injury levels more than previously thought. Increases in BDNF following a season of play may suggest some

degree of neurotrophic response to the forces sustained over the course of the season, but the protein's presence within peripheral tissue such a neuromuscular junction limits its utility as a specific marker of BBB integrity. In addition to this, BDNF lacked an association with either MRI-based measure of BBB integrity. However, a non-significant decrease in BDNF was observed shortly following match play, a pattern mirrored in studies of severe TBI (Failla *et al.,* 2016; Korley *et al.,* 2016). While conclusions cannot be drawn from the findings presented here, it may be worth exploring longitudinal changes in BDNF levels in contact sports and corresponding neuropsychological scores in memory and learning.

Changes in inflammatory responses, while non-specific in terms of source, could contribute to changes in BBB integrity and in the propagation of secondary injury mechanisms associated with TBI if occurring within the neural space. Examination of inflammatory cytokines IL-1β and IL-6 did not show significant changes when examined after a season of rugby, nor did the chemokine (MCP-1)/ 2 (CCL2), indicating that the homeostatic status present at the start of the season is not greatly affected following a season of play. However, upon exposure to lysates of necrotic brain tissue, PBMCs collected after a season of rugby mounted a greater IL-1 response compared to PBMCs collected at baseline. This change in peripheral immune cell behaviour may reflect adaptive changes in response to neural tissue. Such adaptive changes in immune status may lead to an exaggerated response to neural antigens by infiltrating immune cells in the event of BBBD. Post-match PBMC immune responses showed a non-significant increase in IL-1β production to the same stimuli, which may suggest that such changes in immune responses can occur rapidly after the commencement of exercise, although this aspect of the work was limited by numbers, which may obscure small effects.

In addition to measurements of serological markers, a unique aspect of this work was the measurement of BBB permeability by DCE-MRI in healthy athletes. To date, only a limited number of studies have utilised this imaging modality in the context of non-pathological conditions, and so there is little knowledge surrounding how output parameters, such as the volume transfer coefficient (K^{trans}), changes in regard to mild injuries. As K^{trans} is primarily used to measure the extent of BBBD in brain tumours and ischemic stroke, its ability to detect minor changes, such as those resulting mTBI, remain largely understudied. In addition to the use of the Tofts extended model to measure BBB

dysfunction, the recently developed LDM, which quantifies the percentage of white and grey matter voxels showing increasing contrast signal relative to the outflow rate from the brain, was also available for use as part of this study. The model has been demonstrated to detect a comparable extent of BBBD to K^{trans} in stroke and glioblastoma cases in a limited number of studies; been used to separate American football players into groups of high and low BBBD, as well as being able to detect barrier dysfunction in a mouse model of mTBI (Chassidim *et al.,* 2013; Weissberg *et al.,* 2014; Tagge *et al.,* 2017). In the cohort of rugby players participating in a season of rugby, BBBD was indicated by measurement of K^{trans} values and suggested by increases in the number of disrupted voxels measured by the LDM. This finding suggests that changes in BBB permeability may occur after participation in contact sports and is capable of being measured in life. However, several caveats are worth noting from these observations, Analysis of post-match BBB integrity demonstrates a potential nuance of the LDM, specifically that it may be sensitive to changes in cerebral blood flow. As this is the first time the model has been used in this context, and blood pressure measurements were not collected at the time of scanning, it not possible to comment on whether this is the case. However, based on the finding of reduction from baseline of disrupted voxels, in comparison to a largely stationary K^{trans} measurement, the LDM may not be suitable for immediate post-exercise assessment.

Also measured over the course of a competitive season were changes in axonal tract parameters as measured by Diffusion Tensor Imaging (DTI). In almost all structures previously demonstrated to show changes following mTBI, no gross changes were observed in any of the parameters measured. However, overall tract integrity, measured by fractional anisotropy (FA) was increased from baseline in the body of the corpus callosum, a structure thought to be subjected to high shear force as a result of mTBI. This increase in FA was likely driven by improvements in radial diffusivity (RD) and subsequently mean diffusivity (MD). However, also observed was a decrease in axial diffusivity (AxD), suggesting axonal tract shortening. While this may indicate axonal damage, any potential deleterious effects seem to be offset by improvements in RD. A potential reason for an overall improvement in axonal tract integrity may be a result of ongoing brain development in this adolescent cohort, as well as continued engagement in formal education and training. The improvement in axonal tract integrity also coincides with the observation of increased levels of circulating neurotrophic factor, BDNF, which

may be a result of continued participation in sport and contribute to improvement in the brain's microstructure.

Within this study, the lack of controls limits the information gleaned from all aspects of characterising changes in BBB permeability as a result of participation in rugby. While changes were observed in parameters of BBB permeability, they lack the context of whether these changes are significant amongst the general population, or even among those participating in non-contact sports. Many of the participants have participated in rugby for many years prior to the start of the study, and the barrier phenotype observed at baseline may reflect changes that have occurred years prior. It is worth noting, that while not directly comparable, the values of percentage disrupted voxels at baseline of our rugby cohort are generally higher than those observed by Weissberg *et al.* (2014) in both their contact and non-contact cohorts, which suggests that the cohort of rugby players presented here may already have a greater extent of BBB dysfunction at baseline compared to other cohorts. Compounding this issue is the variability in MRI scanning time both prior to the commencement of the competitive season and following its conclusion. While all scans were completed at baseline prior to the start of the season, training had already commenced prior to the start of the study. These may have contributed to higher baseline signals and therefore reduced the extent of the effect size. Another consideration of importance, and the impetus to change the focus of study to a different sport was the lack of recognised mTBIs incurred by participants over the course of the study. The lack of injuries prevents tying changes in BBB phenotype observed in this cohort with mTBI history; but allows for speculation that participation in contact sports may contribute to changes in the BBB.

Findings from MMA fighters represent part of an ongoing study, and only a limited number of participants from this cohort were available for assessment at the time of writing However, so far, all participants have been observed to have received numerous head impacts during bout, as recorded by video recordings by medical research personnel. Available data suggests a trend towards an increased number of voxels showing BBB dysfunction using the LDM, while low numbers may mask changes K^{trans} values. Lack of changes in DTI parameters observed so far may be a result of the limited power available currently in the study due to numbers, or it may be too early within the study period to observe noticeable changes. Follow-up of participants at a later date for reassessment may

provide more information on the changes to axonal tract integrity as a result of repetitive head trauma associated with MMA. Plans for this study are to correlate the degree of force applied to the head during a bout, and correlate the associated strain placed on regions of the brain with changes in BBB phenotype. This is part of an ongoing collaboration the Camerillo lab of Stanford University, California, USA, who have developed the mouthguard-based accelerometers as well as providing expertise and analysis of force data and finte-element modelling, and the Dempsey lab of Instiute of Technology Tallaght, Dublin, Ireland, who capture the mouthguard accelerometer data as well as conducting data analysis on the captured acceleration data.

In conclusion, this study has piloted the used of DCE-MRI as a tool to assess BBB integrity in contact sports athletes, investigated the utility of potential blood-based biomarkers of BBB dysfunction and characterised TJ and BBB integrity in diagnosed cases of CTE.

Reference List.

Abbott, N. Joan, Lars Rönnbäck, and Elisabeth Hansson. 2006. "Astrocyte–endothelial Interactions at the Blood–brain Barrier." *Nature Reviews Neuroscience* 7 (1): 41–53. doi:10.1038/nrn1824.

Alexander, Andrew L., Samuel A. Hurley, Alexey A. Samsonov, Nagesh Adluru, Ameer Pasha Hosseinbor, Pouria Mossahebi, Do P.M. Tromp, Elizabeth Zakszewski, and Aaron S. Field. 2011. "Characterization of Cerebral White Matter Properties Using Quantitative Magnetic Resonance Imaging Stains." *Brain Connectivity* 1 (6): 423–46. doi:10.1089/brain.2011.0071.

Alexander, Andrew L., Jee Eun Lee, Mariana Lazar, and Aaron S. Field. 2007. "Diffusion Tensor Imaging of the Brain." *Neurotherapeutics* 4 (3): 316–29. doi:10.1016/j.nurt.2007.05.011.

Alluri, Himakarnika, Chinchusha Anasooya Shaji, Katie Wiggins-Dohlvik, Ryan Oakley, Hayden Stagg, Matthew Davis, and Binu Tharakan. 2014. "Interleukin-1β Induces Brain Microvascular Endothelial Cell Hyperpermeability through Matrix Metalloproteinase-9 (678.17)." *The FASEB Journal* 28 (1_supplement). Federation of American Societies for Experimental Biology: 678.17. doi:10.1096/fasebj.28.1_supplement.678.17.

Alluri, Himakarnika, Marcene Grimsley, Chinchusha Anasooya Shaji, Kevin Paul Varghese, Shenyuan L. Zhang, Chander Peddaboina, Bobby Robinson, Madhava R. Beeram, Jason H. Huang, and Binu Tharakan. 2016. "Attenuation of Blood-Brain Barrier Breakdown and Hyperpermeability by Calpain Inhibition." *Journal of Biological Chemistry* 291 (53): 26958–69. doi:10.1074/jbc.M116.735365.

Alluri, Himakarnika, Rickesha L. Wilson, Chinchusha Anasooya Shaji, Katie Wiggins-Dohlvik, Savan Patel, Yang Liu, Xu Peng, et al. 2016. "Melatonin Preserves Blood-Brain Barrier Integrity and Permeability via Matrix Metalloproteinase-9 Inhibition." Edited by Bobby Thomas. *PLOS ONE* 11 (5): e0154427. doi:10.1371/journal.pone.0154427.

Alosco, Michael L., Jesse Mez, Yorghos Tripodis, Patrick T. Kiernan, Bobak Abdolmohammadi, Lauren Murphy, Neil W. Kowall, et al. 2018. "Age of First Exposure to Tackle Football and Chronic Traumatic Encephalopathy." *Annals of Neurology* 83 (5): 886–901. doi:10.1002/ana.25245.

Alvarez, Jorge Ivan, A. Dodelet-Devillers, Hania Kebir, Igal Ifergan, Pierre J Fabre, Simone Terouz, Mike Sabbagh, et al. 2011. "The Hedgehog Pathway Promotes Blood-Brain Barrier Integrity and CNS Immune Quiescence." *Science* 334 (6063): 1727–31. doi:10.1126/science.1206936.

Alvarez, Jorge Ivan, Takahiro Katayama, and Alexandre Prat. 2013. "Glial Influence on the Blood Brain Barrier." *Glia* 61 (12): 1939–58. doi:10.1002/glia.22575.

Andersson, P. B., V H Perry, and S Gordon. 1992. “Intracerebral Injection of Proinflammatory Cytokines or Leukocyte Chemotaxins Induces Minimal Myelomonocytic Cell Recruitment to the Parenchyma of the Central Nervous System.” *The Journal of Experimental Medicine* 176 (1): 255–59. doi:10.1084/jem.176.1.255.

Arfanakis, Konstantinos, Victor M Haughton, John D Carew, Baxter P Rogers, Robert J Dempsey, and M Elizabeth Meyerand. 2002. “Diffusion Tensor MR Imaging in Diffuse Axonal Injury.” *AJNR. American Journal of Neuroradiology* 23 (5): 794–802. http://www.ncbi.nlm.nih.gov/pubmed/11237964.

Ariza, Mar, Josep M. Serra-Grabulosa, Carme Junqué, Blanca Ramírez, Maria Mataró, Antonia Poca, Nuria Bargalló, and Juan Sahuquillo. 2006. “Hippocampal Head Atrophy after Traumatic Brain Injury.” *Neuropsychologia* 44 (10): 1956–61. doi:10.1016/j.neuropsychologia.2005.11.007.

Arts, R. J. W., Joosten, L. A. B., & Netea, M. G. (2016). Immunometabolic circuits in trained immunity. *Seminars in Immunology*, *28*(5), 425–430. https://doi.org/10.1016/j.smim.2016.09.002

Balança, Baptiste, Lionel Bapteste, Thomas Lieutaud, Denis Ressnikoff, Rainui Guy, Laurent Bezin, and Stéphane Marinesco. 2016. “Neuronal Loss as Evidenced by Automated Quantification of Neuronal Density Following Moderate and Severe Traumatic Brain Injury in Rats.” *Journal of Neuroscience Research* 94 (1): 39–49. doi:10.1002/jnr.23676.

Balda, Maria S, J Andrew Whitney, Catalina Flores, S González, M Cereijido, and K Matter. 1996. “Functional Dissociation of Paracellular Permeability and Transepithelial Electrical Resistance and Disruption of the Apical-Basolateral Intramembrane Diffusion Barrier by Expression of a Mutant Tight Junction Membrane Protein.” *The Journal of Cell Biology* 134 (4): 1031–49. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2120963/pdf/jc13441031.pdf.

Baratz, Renana, David Tweedie, Jia Yi Wang, Vardit Rubovitch, Weiming Luo, Barry J Hoffer, Nigel H Greig, and Chaim G Pick. 2015. “Transiently Lowering Tumor Necrosis Factor-Aα Synthesis Ameliorates Neuronal Cell Loss and Cognitive Impairments Induced by Minimal Traumatic Brain Injury in Mice.” *Journal of Neuroinflammation* 12 (1): 1–14. doi:10.1186/s12974-015-0237-4.

Bashir, Mustafa R., Lubna Bhatti, Daniele Marin, and Rendon C. Nelson. 2015. “Emerging Applications for Ferumoxytol as a Contrast Agent in MRI.” *Journal of Magnetic Resonance Imaging* 41 (4): 884–98. doi:10.1002/jmri.24691.

Başkaya, M. K., Muralikrishna Rao, A., Doğan, A., Donaldson, D., & Dempsey, R. J. (1997). The biphasic opening of the blood–brain barrier in the cortex and hippocampus after traumatic brain injury in rats. *Neuroscience Letters*, *226*(1), 33–36. https://doi.org/10.1016/S0304-3940(97)00239-5

Bauer, H., J. Zweimueller-Mayer, P. Steinbacher, A. Lametschwandtner, and H. C. Bauer. 2010. “The Dual Role of Zonula Occludens (ZO) Proteins.” *Journal of Biomedicine and Biotechnology* 2010 (Figure 1): 1–11. doi:10.1155/2010/402593.

Bazarian, Jeffrey J, Frank P Zemlan, Sohug Mookerjee, and Torgney Stigbrand. 2006. “Serum S-100B and Cleaved-Tau Are Poor Predictors of Long-Term Outcome after Mild Traumatic Brain Injury.” *Brain Injury* 20 (7): 759–65. doi:10.1080/02699050500488207.

Bekkering, S., Blok, B. A., Joosten, L. A. B., Riksen, N. P., van Crevel, R., & Netea, M. G. (2016). In Vitro Experimental Model of Trained Innate Immunity in Human Primary Monocytes. *Clinical and Vaccine Immunology*, *23*(12), 926–933. https://doi.org/10.1128/CVI.00349-16

Bell, Robert D, and Berislav V. Zlokovic. 2009. “Neurovascular Mechanisms and Blood–brain Barrier Disorder in Alzheimer’s Disease.” *Acta Neuropathologica* 118 (1): 103–13. doi:10.1007/s00401-009-0522-3.

Bennett, G, S. Al-Rashed, S. J.R. Hoult, and D. S. Brain. 1998. “Nerve Growth Factor Induced Hyperalgesia in the Rat Hind Paw Is Dependent on Circulating Neutrophils.” *Pain* 77 (3): 315–22. doi:10.1016/S0304-3959(98)00114-6.

Ben-Yosef, Tamar. 2003. “Claudin 14 Knockout Mice, a Model for Autosomal Recessive Deafness DFNB29, Are Deaf Due to Cochlear Hair Cell Degeneration.” *Human Molecular Genetics* 12 (16): 2049–61. doi:10.1093/hmg/ddg210.

Bishop, P., Rocca, D., & Henley, J. M. (2016). Ubiquitin C-terminal hydrolase L1 (UCH-L1): structure, distribution and roles in brain function and dysfunction. *Biochemical Journal*, *473*(16), 2453–2462. https://doi.org/10.1042/BCJ20160082

Block, Michelle L, Luigi Zecca, and Jau-Shyong Hong. 2007. “Microglia-Mediated Neurotoxicity: Uncovering the Molecular Mechanisms.” *Nature Reviews Neuroscience* 8 (1): 57–69. doi:10.1038/nrn2038.

Blumbergs, P.C., G Scott, J Manavis, H Wainwright, D.A. Simpson, and A.J. McLean. 1994. “Stalning Af Amyloid Percursor Protein to Study Axonal Damage in Mild Head Injury.” *The Lancet* 344 (8929): 1055–56. doi:10.1016/S0140-6736(94)91712-4.

Boutin, H, R A LeFeuvre, R Horai, M Asano, Y Iwakura, and N J Rothwell. 2001. “Role of IL-1alpha and IL-1beta in Ischemic Brain Damage.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 21 (15): 5528–34. http://www.ncbi.nlm.nih.gov/pubmed/11466424.

Bouvier, Damien, Thomas Duret, Mathieu Abbot, Thibault Stiernon, Bruno Pereira, Aurélien Coste, Jean Chazal, and Vincent Sapin. 2017. “Utility of S100B Serum Level for the Determination of Concussion in Male Rugby Players.” *Sports Medicine* 47 (4): 781–89. doi:10.1007/s40279-016-0579-9.

Brenner, David J., and Eric J. Hall. 2007. “Computed Tomography — An Increasing Source of Radiation Exposure.” *New England Journal of Medicine* 357 (22): 2277–84. doi:10.1056/NEJMra072149.

Bryan-Hancock, C., and J. Harrison. 2010. “The Global Burden of Traumatic Brain Injury: Preliminary Results from the Global Burden of Disease Project.” *Injury Prevention* 16 (Supplement 1): A17–A17. doi:10.1136/ip.2010.029215.61.

Buonora, John E, Michael Mousseau, David M Jacobowitz, Rachel C Lazarus, Angela M Yarnell, Cara H Olsen, Harvey B Pollard, Ramon Diaz-Arrastia, Lawrence Latour, and Gregory P Mueller. 2015. “Autoimmune Profiling Reveals Peroxiredoxin 6 as a Candidate Traumatic Brain Injury Biomarker.” *Journal of Neurotrauma* 32 (22). Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD, United States: 1805–14. doi:10.1089/neu.2014.3736.

Cai, W., Liu, H., Zhao, J., Chen, L. Y., Chen, J., Lu, Z., & Hu, X. (2017). Pericytes in Brain Injury and Repair After Ischemic Stroke. *Translational Stroke Research*, *8*(2), 107–121. https://doi.org/10.1007/s12975-016-0504-4

Campbell, Matthew, Anna-Sophia Kiang, Paul F. Kenna, Christian Kerskens, Christoph Blau, Laurence O’Dwyer, Amanda Tivnan, et al. 2008. “RNAi-Mediated Reversible Opening of the Blood-Brain Barrier.” *The Journal of Gene Medicine* 10 (8): 930–47. doi:10.1002/jgm.1211.

Carpentier, Pamela A, Wendy Smith Begolka, Julie K Olson, Adam Elhofy, William J Karpus, and Stephen D Miller. 2005. “Differential Activation of Astrocytes by Innate and Adaptive Immune Stimuli.” *GLIA* 49 (3): 360–74. doi:10.1002/glia.20117.

Cassidy, J David, Linda J Carroll, Paul M Peloso, Jörgen Borg, Hans von Holst, Lena Holm, Jess Kraus, Victor G Coronado, and WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. 2004. “Incidence, Risk Factors and Prevention of Mild Traumatic Brain Injury: Results of the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury.” *Journal of Rehabilitation Medicine*, no. 43 Suppl (February). Sweden: 28–60. http://www.ncbi.nlm.nih.gov/pubmed/15083870.

Castellani, Rudy J., and George Perry. 2017. “Dementia Pugilistica Revisited.” Edited by Massimo Tabaton. *Journal of Alzheimer’s Disease* 60 (4): 1209–21. doi:10.3233/JAD-170669.

Catafau, Ana M, and Eduardo Tolosa. 2004. “Impact of Dopamine Transporter SPECT Using123I-Ioflupane on Diagnosis and Management of Patients with Clinically Uncertain Parkinsonian Syndromes.” *Movement Disorders* 19 (10): 1175–82. doi:10.1002/mds.20112.

Catani, Marco, and Michel Thiebaut de Schotten. 2008. “A Diffusion Tensor Imaging Tractography Atlas for Virtual in Vivo Dissections.” *Cortex; a Journal Devoted to the Study of the Nervous System and Behavior* 44 (8): 1105–32. doi:10.1016/j.cortex.2008.05.004.

CDC Injury Center. 2018. “Rates of TBI-Related Emergency Department Visits, Hospitalizations, and Deaths — United States, 2001–2010.” https://www.cdc.gov/traumaticbraininjury/data/rates.html.

Černý, J., & Stříž, I. (2019). Adaptive innate immunity or innate adaptive immunity? *Clinical Science*, *133*(14), 1549–1565. https://doi.org/10.1042/CS20180548

Chen, Jieli, Chunling Zhang, Hao Jiang, Yi Li, Lijie Zhang, Adam Robin, Mark Katakowski, Mei Lu, and Michael Chopp. 2005. “Atorvastatin Induction of VEGF and BDNF Promotes Brain Plasticity after Stroke in Mice.” *Journal of Cerebral Blood Flow & Metabolism* 25 (2): 281–90. doi:10.1038/sj.jcbfm.9600034.

Ciaramella, Antonio, Cecilia Della Vedova, Francesca Salani, Mara Viganotti, Mariagrazia D’Ippolito, Carlo Caltagirone, Rita Formisano, Umberto Sabatini, and Paola Bossù. 2014. “Increased Levels of Serum IL-18 Are Associated with the Long-Term Outcome of Severe Traumatic Brain Injury.” *Neuroimmunomodulation* 21 (1): 8–12. doi:10.1159/000354764.

Clausen, Fredrik, Anders Hånell, Maria Björk, Lars Hillered, Anis K. Mir, Hermann Gram, and Niklas Marklund. 2009. “Neutralization of Interleukin-1β Modifies the Inflammatory Response and Improves Histological and Cognitive Outcome Following Traumatic Brain Injury in Mice.” *European Journal of Neuroscience* 30 (3): 385–96. doi:10.1111/j.1460-9568.2009.06820.x.

Clausen, Fredrik, Anders Hånell, Charlotte Israelsson, Johanna Hedin, Ted Ebendal, Anis K Mir, Hermann Gram, and Niklas Marklund. 2011. “Neutralization of Interleukin-1β Reduces Cerebral Edema and Tissue Loss and Improves Late Cognitive Outcome Following Traumatic Brain Injury in Mice.” *European Journal of Neuroscience* 34 (1): 110–23. doi:10.1111/j.1460-9568.2011.07723.x.

Clausen, Fredrik, Tomas Lorant, Anders Lewén, and Lars Hillered. 2007. “T Lymphocyte Trafficking: A Novel Target for Neuroprotection in Traumatic Brain Injury.” *Journal of Neurotrauma* 24 (8): 1295–1307. doi:10.1089/neu.2006.0258.

Czigner, A, A. Mihály, O Farkas, A. Büki, B. Krisztin-Péva, E. Dobó, and P. Barzó. 2007. “Kinetics of the Cellular Immune Response Following Closed Head Injury.” *Acta Neurochirurgica* 149 (3): 281–89. doi:10.1007/s00701-006-1095-8.

Rosa, Maria Inês da, Carla Simon, Antonio Jose Grande, Tatiana Barichello, Jean Pierre Oses, and João Quevedo. 2016. “Serum S100B in Manic Bipolar Disorder Patients: Systematic Review and Meta-Analysis.” *Journal of Affective Disorders* 206 (December). Elsevier: 210–15. doi:10.1016/j.jad.2016.07.030.

Dallérac, Glenn, and Nathalie Rouach. 2016. “Astrocytes as New Targets to Improve Cognitive Functions.” *Progress in Neurobiology* 144: 48–67. doi:10.1016/j.pneurobio.2016.01.003.

Dams-O’Connor, Kristen, Laura E Gibbons, James D Bowen, Susan M. McCurry, Eric B Larson, and Paul K Crane. 2013. “Risk for Late-Life Re-Injury, Dementia and Death among Individuals with Traumatic Brain Injury: A Population-Based Study.” *Journal of Neurology, Neurosurgery & Psychiatry* 84 (2): 177–82. doi:10.1136/jnnp-2012-303938.

Daneman, Richard, Lu Zhou, Dritan Agalliu, John D. Cahoy, Amit Kaushal, and Ben A. Barres. 2010. “The Mouse Blood-Brain Barrier Transcriptome: A New Resource for Understanding the Development and Function of Brain Endothelial Cells.” *PLoS ONE* 5 (10): 1–16. doi:10.1371/journal.pone.0013741.

Daneman, Richard, Lu Zhou, Amanuel Kebede, and Ben Barres. 2011. “Pericytes Are Required for Blood–brain Barrier Integrity during Embryogenesis.” *Nature* 468 (7323): 562–66. doi:10.1038/nature09513.Pericytes.

Das, Mahasweta, Christopher C. Leonardo, Saniya Rangooni, Shyam S Mohapatra, Subhra Mohapatra, and Keith R. Pennypacker. 2011. “Lateral Fluid Percussion Injury of the Brain Induces CCL20 Inflammatory Chemokine Expression in Rats.” *Journal of Neuroinflammation* 8 (1). BioMed Central Ltd: 148. doi:10.1186/1742-2094-8-148.

Davenport, Elizabeth M., Christopher T. Whitlow, Jillian E. Urban, Mark A. Espeland, Youngkyoo Jung, Daryl A. Rosenbaum, Gerard A. Gioia, Alexander K. Powers, Joel D. Stitzel, and Joseph A. Maldjian. 2014. “Abnormal White Matter Integrity Related to Head Impact Exposure in a Season of High School Varsity Football.” *Journal of Neurotrauma* 31 (19): 1617–24. doi:10.1089/neu.2013.3233.

Kruijk, J R de, P Leffers, P P Menheere, S Meerhoff, and A Twijnstra. 2001. “S-100B and Neuron-Specific Enolase in Serum of Mild Traumatic Brain Injury Patients. A Comparison with Health Controls.” *Acta Neurologica Scandinavica* 103 (3): 175–79. http://www.ncbi.nlm.nih.gov/pubmed/11240565.

Desai Bradaric, Brinda, Aditiben Patel, Julie A. Schneider, Paul M. Carvey, and Bill Hendey. 2012. “Evidence for Angiogenesis in Parkinson’s Disease, Incidental Lewy Body Disease, and Progressive Supranuclear Palsy.” *Journal of Neural Transmission* 119 (1): 59–71. doi:10.1007/s00702-011-0684-8.

Desai, Brinda S., Angela J. Monahan, Paul M. Carvey, and Bill Hendey. 2007. “Blood–Brain Barrier Pathology in Alzheimer’s and Parkinson’s Disease: Implications for Drug Therapy.” *Cell Transplantation* 16 (3): 285–99. doi:10.3727/000000007783464731.

Battista, Alex P. Di, Shawn G. Rhind, Michael G. Hutchison, Syed Hassan, Maria Y. Shiu, Kenji Inaba, Jane Topolovec-Vranic, Antonio Capone Neto, Sandro B. Rizoli, and Andrew J. Baker. 2016. "Inflammatory Cytokine and Chemokine Profiles Are Associated with Patient Outcome and the Hyperadrenergic State Following Acute Brain Injury." *Journal of Neuroinflammation* 13 (1). Journal of Neuroinflammation: 40. doi:10.1186/s12974-016-0500-3.

Dietrich, Marcelo O., Adriano B. Tort, Débora V. Schaf, Marcelo Farina, Carlos A. Gonçalves, Diogo O. Souza, and Luis V. Portela. 2003. "Increase in Serum S100B Protein Level After a Swimming Race." *Canadian Journal of Applied Physiology* 28 (5): 710–16. doi:10.1139/h03-054.

Doherty, Colin P, Eoin O'Keefe, Eugene Wallace, Teresa Loftus, James Keaney, John Kealy, Marian M Humphries, et al. 2016. "Blood–Brain Barrier Dysfunction as a Hallmark Pathology in Chronic Traumatic Encephalopathy." *Journal of Neuropathology & Experimental Neurology* 75 (7): 656–62. doi:10.1093/jnen/nlw036.

Donato, R, B.R. Cannon, G Sorci, F Riuzzi, K Hsu, D.J. Weber, and C.L. Geczy. 2013. "Functions of S100 Proteins." *Current Molecular Medicine* 13 (1): 24–57.

Dore-Duffy, Paula, Cheri Owen, Roumen Balabanov, Sharon Murphy, Thomas Beaumont, and José A. Rafols. 2000. "Pericyte Migration from the Vascular Wall in Response to Traumatic Brain Injury." *Microvascular Research* 60 (1): 55–69. doi:10.1006/mvre.2000.2244.

Drouin-Ouellet, Janelle, Stephen J. Sawiak, Giulia Cisbani, Marie Lagacé, Wei-Li Kuan, Martine Saint-Pierre, Richard J. Dury, et al. 2015. "Cerebrovascular and Blood-Brain Barrier Impairments in Huntington's Disease: Potential Implications for Its Pathophysiology." *Annals of Neurology* 78 (2): 160–77. doi:10.1002/ana.24406.

Dyck, Allison C. F., and Tammy L. Ivanco. 2018. "BDNF Expression Increases without Changes in Play Behavior Following Concussion in Juvenile Rats (Rattus Norvegicus)." *Developmental Neurorehabilitation* 21 (7). Taylor & Francis: 475–79. doi:10.1080/17518423.2018.1460878.

Ebnet, K. 2003. "The Junctional Adhesion Molecule (JAM) Family Members JAM-2 and JAM-3 Associate with the Cell Polarity Protein PAR-3: A Possible Role for JAMs in Endothelial Cell Polarity." *Journal of Cell Science* 116 (19): 3879–91. doi:10.1242/jcs.00704.

Ebnet, K., Atsushi Suzuki, Shigeo Ohno, and Dietmar Vestweber. 2004. "Junctional Adhesion Molecules (JAMs): More Molecules with Dual Functions?" *Journal of Cell Science* 117 (1): 19–29. doi:10.1242/jcs.00930.

Egea-Guerrero, J. J., J. Revuelto-Rey, F. Murillo-Cabezas, M. A. Muñoz-Sánchez, A. Vilches-Arenas, P. Sánchez-Linares, J. M. Domínguez-Roldán, and J. León-Carrión. 2012. "Accuracy of the S100 β Protein as a Marker of Brain Damage in Traumatic Brain Injury." *Brain Injury* 26 (1): 76–82. doi:10.3109/02699052.2011.635360.

Enwright III, J F, Z Huo, D Arion, J P Corradi, George Tseng, and David A Lewis. 2018. "Transcriptome Alterations of Prefrontal Cortical Parvalbumin Neurons in Schizophrenia." *Molecular Psychiatry* 23 (7): 1606–13. doi:10.1038/mp.2017.216.

Erlanger, D. M. (2015). Exposure to sub-concussive head injury in boxing and other sports. *Brain Injury*, *29*(2), 171–174. https://doi.org/10.3109/02699052.2014.965211

Feddermann-Demont, Nina, Ruben J. Echemendia, Kathryn J. Schneider, Gary S. Solomon, K. Alix Hayden, Michael Turner, Jiří Dvořák, Dominik Straumann, and Alexander A. Tarnutzer. 2017. "What Domains of Clinical Function Should Be Assessed after Sport-Related Concussion? A Systematic Review." *British Journal of Sports Medicine* 51 (11): 903–18. doi:10.1136/bjsports-2016-097403.

Fee, Dominic, Amanda Crumbaugh, Thomas Jacques, Benjamin Herdrich, Diane Sewell, David Auerbach, Shari Piaskowski, Michael N Hart, Matyas Sandor, and Zsuzsa Fabry. 2003. "Activated/Effector CD4+ T Cells Exacerbate Acute Damage in the Central Nervous System Following Traumatic Injury." *Journal of Neuroimmunology* 136 (1–2): 54–66. doi:10.1016/S0165-5728(03)00008-0.

Fenn, Ashley M, John C Gensel, Yan Huang, Phillip G Popovich, Jonathan Lifshitz, and Jonathan P Godbout. 2014. "Immune Activation Promotes Depression 1 Month After Diffuse Brain Injury: A Role for Primed Microglia." *Biological Psychiatry* 76 (7). Elsevier: 575–84. doi:10.1016/j.biopsych.2013.10.014.

Filley, Christopher M., and James P. Kelly. 2018. "White Matter and Cognition in Traumatic Brain Injury." *Journal of Alzheimer's Disease* 65 (2): 345–62. doi:10.3233/JAD-180287.

Fleminger, S. 2003. "Head Injury as a Risk Factor for Alzheimer's Disease: The Evidence 10 Years on; a Partial Replication." *Journal of Neurology, Neurosurgery & Psychiatry* 74 (7): 857–62. doi:10.1136/jnnp.74.7.857.

Furuse, M., T Hirase, M Itoh, A Nagafuchi, S Yonemura, S Tsukita, and S Tsukita. 1993. "Occludin: A Novel Integral Membrane Protein Localizing at Tight Junctions." *The Journal of Cell Biology* 123 (6 Pt 2): 1777–88. doi:10.1083/jcb.123.6.1777.

Furuse, Mikio, Masahiko Itoh, Tetsuaki Hirase, Akira Nagafuchi, Shigenobu Yonemura, Sachiko Tsukita, and Shoichiro Tsukita. 1994. "Direct Association of Occludin with ZO-1 and Its Possible Involvement in the Localization of Occludin at Tight Junctions." *The Journal of Cell Biology* 127 (6): 1617–26.

Gao, Xiang, and Jinhui Chen. 2011. "Mild Traumatic Brain Injury Results in Extensive Neuronal Degeneration in the Cerebral Cortex." *Journal of Neuropathology & Experimental Neurology* 70 (3): 183–91. doi:10.1097/NEN.0b013e31820c6878.

Gardner, Raquel C, James F Burke, Jasmine Nettiksimmons, Allison Kaup, Deborah E Barnes, and Kristine Yaffe. 2014. "Dementia Risk After Traumatic Brain Injury vs Nonbrain Trauma." *JAMA Neurology* 71 (12): 1490. doi:10.1001/jamaneurol.2014.2668.

Gavett, Brandon E., Robert A. Stern, and Ann C. McKee. 2011. “Chronic Traumatic Encephalopathy: A Potential Late Effect of Sport-Related Concussive and Subconcussive Head Trauma.” *Clinics in Sports Medicine* 30 (1): 179–88. doi:10.1016/j.csm.2010.09.007.

Gentleman, S. M., G. W. Roberts, T. A. Gennarelli, W. L. Maxwell, J. H. Adams, S. Kerr, and D. I. Graham. 1995. “Axonal Injury: A Universal Consequence of Fatal Closed Head Injury?” *Acta Neuropathologica* 89 (6): 537–43. doi:10.1007/BF00571509.

GLICKSTEIN, M, and G BERLUCCHI. 2008. “Classical Disconnection Studies of the Corpus Callosum.” *Cortex* 44 (8): 914–27. doi:10.1016/j.cortex.2008.04.001.

Godbolt, Alison K., Carol Cancelliere, Cesar A. Hincapié, Connie Marras, Eleanor Boyle, Vicki L. Kristman, Victor G. Coronado, and J. David Cassidy. 2014. “Systematic Review of the Risk of Dementia and Chronic Cognitive Impairment After Mild Traumatic Brain Injury: Results of the International Collaboration on Mild Traumatic Brain Injury Prognosis.” *Archives of Physical Medicine and Rehabilitation* 95 (3): S245–56. doi:10.1016/j.apmr.2013.06.036.

Gow, Alexander. 2004. “Deafness in Claudin 11-Null Mice Reveals the Critical Contribution of Basal Cell Tight Junctions to Stria Vascularis Function.” *Journal of Neuroscience* 24 (32): 7051–62. doi:10.1523/JNEUROSCI.1640-04.2004.

Graham, M.R., T Myers, P Evans, B Davies, S.M. Cooper, K Bhattacharya, F.M. Grace, and J.S. Baker. 2011. “Direct Hits to the Head during Amateur Boxing Is Associated with a Rise in Serum Biomarkers for Brain Injury.” *International Journal of Immunopathology and Pharmacology* 24 (1): 119–25. doi:10.1177/039463201102400114.

Greene, C, J Kealy, M M Humphries, Y Gong, J Hou, N Hudson, L M Cassidy, et al. 2018. “Dose-Dependent Expression of Claudin-5 Is a Modifying Factor in Schizophrenia.” *Molecular Psychiatry* 23 (11). Nature Publishing Group: 2156–66. doi:10.1038/mp.2017.156.

Gyoneva, Stefka, and Richard M. Ransohoff. 2015. “Inflammatory Reaction after Traumatic Brain Injury: Therapeutic Potential of Targeting Cell–cell Communication by Chemokines.” *Trends in Pharmacological Sciences* 36 (7): 471–80. doi:10.1016/j.tips.2015.04.003.

Halliday, Matthew R., Sanket V. Rege, Qingyi Ma, Zhen Zhao, Carol A. Miller, Ethan A. Winkler, and Berislav V. Zlokovic. 2016. “Accelerated Pericyte Degeneration and Blood–brain Barrier Breakdown in Apolipoprotein E4 Carriers with Alzheimer’s Disease.” *Journal of Cerebral Blood Flow & Metabolism* 36 (1): 216–27. doi:10.1038/jcbfm.2015.44.

Hamada, A., Torre, C., Drancourt, M., & Ghigo, E. (2019). Trained Immunity Carried by Non-immune Cells. *Frontiers in Microbiology*, *9*(JAN), 1–8.https://doi.org/10.3389/fmicb.2018.03225

Hasselblatt, M, F C Mooren, N von Ahsen, K Keyvani, A Fromme, K Schwarze-Eicker, V Senner, and W Paulus. 2004. “Serum S100β Increases in Marathon Runners Reflect Extracranial Release Rather than Glial Damage.” *Neurology* 62 (9): 1634–36. doi:10.1212/01.WNL.0000123092.97047.B1.

Hay, Jennifer R., Victoria E. Johnson, Adam M.H. Young, Douglas H. Smith, and William Stewart. 2015. “Blood-Brain Barrier Disruption Is an Early Event That May Persist for Many Years After Traumatic Brain Injury in Humans.” *Journal of Neuropathology & Experimental Neurology* 74 (12): 1147–57. doi:10.1097/NEN.0000000000000261.

Helmy, Adel, Keri L.H. Carpenter, David K Menon, John D Pickard, and Peter J.A. Hutchinson. 2011. “The Cytokine Response to Human Traumatic Brain Injury: Temporal Profiles and Evidence for Cerebral Parenchymal Production.” *Journal of Cerebral Blood Flow & Metabolism* 31 (2): 658–70. doi:10.1038/jcbfm.2010.142.

HICKS, R.R., D.H. SMITH, D.H. LOWENSTEIN, R. SAINT MARIE, and T.K. McINTOSH. 1993. “Mild Experimental Brain Injury in the Rat Induces Cognitive Deficits Associated with Regional Neuronal Loss in the Hippocampus.” *Journal of Neurotrauma* 10 (4): 405–14. doi:10.1089/neu.1993.10.405.

Hope, Michael D., Thomas A. Hope, Chengcheng Zhu, Farshid Faraji, Henrik Haraldsson, Karen G. Ordovas, and David Saloner. 2015. “Vascular Imaging With Ferumoxytol as a Contrast Agent.” *American Journal of Roentgenology* 205 (3): W366–73. doi:10.2214/AJR.15.14534.

Hori, Satoko, Sumio Ohtsuki, Ken-ichi Hosoya, Emi Nakashima, and Tetsuya Terasaki. 2004. “A Pericyte-Derived Angiopoietin-1 Multimeric Complex Induces Occludin Gene Expression in Brain Capillary Endothelial Cells through Tie-2 Activation in Vitro.” *Journal of Neurochemistry* 89 (2): 503–13. doi:10.1111/j.1471-4159.2004.02343.x.

Hou, Jianghui, Qixian Shan, Tong Wang, Antonio S Gomes, Qingshang Yan, David L Paul, Markus Bleich, and Daniel A Goodenough. 2007. “Transgenic RNAi Depletion of Claudin-16 and the Renal Handling of Magnesium.” *Journal of Biological Chemistry* 282 (23): 17114–22. doi:10.1074/jbc.M700632200.

Huang, Chi-Hsien, Chi-Wei Lin, Yi-Che Lee, Chih-Yuan Huang, Ru-Yi Huang, Yi-Cheng Tai, Kuo-Wei Wang, San-Nan Yang, Yuan-Ting Sun, and Hao-kuang Wang. 2018. “Is Traumatic Brain Injury a Risk Factor for Neurodegeneration? A Meta-Analysis of Population-Based Studies.” *BMC Neurology* 18 (1). BMC Neurology: 184. doi:10.1186/s12883-018-1187-0.

Igarashi, Yo, Hiroyuki Utsumi, Hideki Chiba, Yumiko Yamada-Sasamori, Hirotoshi Tobioka, Yasuhiro Kamimura, Ken Furuuchi, et al. 1999. “Glial Cell Line-Derived Neurotrophic Factor Induces Barrier Function of Endothelial Cells Forming the Blood–Brain Barrier.” *Biochemical and Biophysical Research Communications* 261 (1): 108–12. doi:10.1006/bbrc.1999.0992.

Inglese, Matilde, Sachin Makani, Glyn Johnson, Benjamin a Cohen, Jonathan a Silver, Oded Gonen, and Robert I Grossman. 2005. "Diffuse Axonal Injury in Mild Traumatic Brain Injury: A Diffusion Tensor Imaging Study." *Journal of Neurosurgery* 103 (2): 298–303. doi:10.3171/jns.2005.103.2.0298.

Ingrisch, Michael, and Steven Sourbron. 2013. "Tracer-Kinetic Modeling of Dynamic Contrast-Enhanced MRI and CT: A Primer." *Journal of Pharmacokinetics and Pharmacodynamics* 40 (3): 281–300. doi:10.1007/s10928-013-9315-3.

Isgrò, Maria Antonietta, Patrizia Bottoni, and Roberto Scatena. 2015. "Neuron-Specific Enolase as a Biomarker: Biochemical and Clinical Aspects." In *Advances in Cancer Biomarkers*, edited by Roberto Scatena, 867:125–43. Advances in Experimental Medicine and Biology. Dordrecht: Springer Netherlands. doi:10.1007/978-94-017-7215-0_9.

Jaerve, Anne, and Hans Werner Müller. 2012. "Chemokines in CNS Injury and Repair." *Cell and Tissue Research* 349 (1): 229–48. doi:10.1007/s00441-012-1427-3.

Jagoda, Andy S., Jeffrey J. Bazarian, John J. Bruns, Stephen V. Cantrill, Alisa D. Gean, Patricia Kunz Howard, Jamshid Ghajar, et al. 2008. "Clinical Policy: Neuroimaging and Decisionmaking in Adult Mild Traumatic Brain Injury in the Acute Setting." *Annals of Emergency Medicine* 52 (6): 714–48. doi:10.1016/j.annemergmed.2008.08.021.

Jellinger, K. A., W. Paulus, C. Wrocklage, and I. Litvan. 2001. "Effects of Closed Traumatic Brain Injury and Genetic Factors on the Development of Alzheimer's Disease." *European Journal of Neurology* 8 (6): 707–10. doi:10.1046/j.1468-1331.2001.00322.x.

Johnson, Victoria E, William Stewart, and Douglas H Smith. 2010. "Traumatic Brain Injury and Amyloid-β Pathology: A Link to Alzheimer's Disease?" *Nature Reviews Neuroscience* 11 (5): 361–70. doi:10.1038/nrn2808.

Jones, Emma V, and David S Bouvier. 2014. "Astrocyte-Secreted Matricellular Proteins in CNS Remodelling during Development and Disease." *Neural Plasticity* 2014. Hindawi Publishing Corporation: 1–12. doi:10.1155/2014/321209.

Jordan, B. D. (2000). Chronic Traumatic Brain Injury Associated with Boxing. *Seminars in Neurology*, *20*(02), 179–186. https://doi.org/10.1055/s-2000-9826

Joseph, J. R., Swallow, J. S., Willsey, K., Lapointe, A. P., Khalatbari, S., Korley, F. K., … Broglio, S. P. (2019). Elevated markers of brain injury as a result of clinically asymptomatic high-acceleration head impacts in high-school football athletes. *Journal of Neurosurgery*, *130*(5), 1642–1648. https://doi.org/10.3171/2017.12.JNS172386

Jürimäe, Jaak, Sille Vaiksaar, and Priit Purge. 2018. "Circulating Inflammatory Cytokine Responses to Endurance Exercise in Female Rowers." *International Journal of Sports Medicine* 39 (14): 1041–48. doi:10.1055/a-0723-4421.

K. Verger, C. Junqué, H. S. Levin. 2001. “Correlation of Atrophy Measures on MRI with Neuropsychological Sequelae in Children and Adolescents with Traumatic Brain Injury.” *Brain Injury* 15 (3): 211–21. doi:10.1080/02699050010004059.

K. Verger, C. Junqué, M. A. Jurado. 2000. “Age Effects on Long-Term Neuropsychological Outcome in Paediatric Traumatic Brain Injury.” *Brain Injury* 14 (6): 495–503. doi:10.1080/026990500120411.

Kang, Wenfei, Francesca Balordi, Nan Su, Lin Chen, Gordon Fishell, and J. M. Hebert. 2014. “Astrocyte Activation Is Suppressed in Both Normal and Injured Brain by FGF Signaling.” *Proceedings of the National Academy of Sciences* 111 (29): E2987–95. doi:10.1073/pnas.1320401111.

Karantzoulis, Stella, and Christopher Randolph. 2013. “Modern Chronic Traumatic Encephalopathy in Retired Athletes: What Is the Evidence?” *Neuropsychology Review* 23 (4): 350–60. doi:10.1007/s11065-013-9243-4.

Katsuno, Tatsuya, Kazuaki Umeda, Takeshi Matsui, Masaki Hata, Atsushi Tamura, Masahiko Itoh, Kosei Takeuchi, et al. 2008. “Deficiency of Zonula Occludens-1 Causes Embryonic Lethal Phenotype Associated with Defected Yolk Sac Angiogenesis and Apoptosis of Embryonic Cells.” Edited by Keith Mostov. *Molecular Biology of the Cell* 19 (6): 2465–75. doi:10.1091/mbc.e07-12-1215.

Kavalci, Cemil, Murat Pekdemir, Polat Durukan, Necip Ilhan, Mustafa Yildiz, Selami Serhatlioglu, and Dilara Seckin. 2007. “The Value of Serum Tau Protein for the Diagnosis of Intracranial Injury in Minor Head Trauma.” *The American Journal of Emergency Medicine* 25 (4): 391–95. doi:10.1016/j.ajem.2006.10.008.

Kelly, Daniel F., Rouzbeh K. Kordestani, Neil A. Martin, Tien Nguyen, David A. Hovda, Marvin Bergsneider, David L. McArthur, and Donald P. Becker. 1996. “Hyperemia Following Traumatic Brain Injury: Relationship to Intracranial Hypertension and Outcome.” *Journal of Neurosurgery* 85 (5): 762–71. doi:10.3171/jns.1996.85.5.0762.

Kelly, Daniel F., Neil A. Martin, Rouzbeh Kordestani, George Counelis, David A. Hovda, Marvin Bergsneider, Duncan Q. McBride, Ehud Shalmon, Dena Herman, and Donald P. Becker. 1997. “Cerebral Blood Flow as a Predictor of Outcome Following Traumatic Brain Injury.” *Journal of Neurosurgery* 86 (4): 633–41. doi:10.3171/jns.1997.86.4.0633.

Kenne, Ellinor, Anna Erlandsson, Lennart Lindbom, Lars Hillered, and Fredrik Clausen. 2012. “Neutrophil Depletion Reduces Edema Formation and Tissue Loss Following Traumatic Brain Injury in Mice.” *Journal of Neuroinflammation* 9 (1): 512. doi:10.1186/1742-2094-9-17.

Kiechle, Karin, Jeffrey J Bazarian, Kian Merchant-Borna, Veit Stoecklein, Eric Rozen, Brian Blyth, Jason H Huang, Samantha Dayawansa, Karl Kanz, and Peter Biberthaler. 2014. “Subject-Specific Increases in Serum S-100B Distinguish Sports-

Related Concussion from Sports-Related Exertion." Edited by Charles C. Caldwell. *PLoS ONE* 9 (1): e84977. doi:10.1371/journal.pone.0084977.

Kim, H.J., H.L. Fillmore, T.M. Reeves, and L.L. Phillips. 2005. "Elevation of Hippocampal MMP-3 Expression and Activity during Trauma-Induced Synaptogenesis." *Experimental Neurology* 192 (1): 60–72. doi:10.1016/j.expneurol.2004.10.014.

Kim, Jeong Hun, Jin Hyoung Kim, Young Suk Yu, Dong Hun Kim, and Kyu-Won Kim. 2009. "Recruitment of Pericytes and Astrocytes Is Closely Related to the Formation of Tight Junction in Developing Retinal Vessels." *Journal of Neuroscience Research* 87 (3): 653–59. doi:10.1002/jnr.21884.

Kim, Jong-hyeon, Kyoung-Jin Min, Wongi Seol, Ilo Jou, and Eun-hye Joe. 2010. "Astrocytes in Injury States Rapidly Produce Anti-Inflammatory Factors and Attenuate Microglial Inflammatory Responses." *Journal of Neurochemistry* 115 (5): 1161–71. doi:10.1111/j.1471-4159.2010.07004.x.

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L. A. B., Ifrim, D. C., Saeed, S., … Netea, M. G. (2012). Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proceedings of the National Academy of Sciences*, *109*(43), 17537–17542. https://doi.org/10.1073/pnas.1202870109

Koga, Shunsuke, Dennis W. Dickson, and Kevin F. Bieniek. 2016. "Chronic Traumatic Encephalopathy Pathology in Multiple System Atrophy." *Journal of Neuropathology & Experimental Neurology* 75 (10): 963–70. doi:10.1093/jnen/nlw073.

Kolaczkowska, Elzbieta, and Paul Kubes. 2013. "Neutrophil Recruitment and Function in Health and Inflammation." *Nature Reviews Immunology* 13 (3). Nature Publishing Group: 159–75. doi:10.1038/nri3399.

Konrad, Martin, Jianghui Hou, Stefanie Weber, Jörg Dötsch, Jameela A. Kari, Tomas Seeman, Eberhard Kuwertz-Bröking, et al. 2008. "CLDN16 Genotype Predicts Renal Decline in Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis." *Journal of the American Society of Nephrology* 19 (1): 171–81. doi:10.1681/ASN.2007060709.

Korley, Frederick K, Ramon Diaz-Arrastia, Alan H B Wu, John K Yue, Geoffrey T Manley, Haris I Sair, Jennifer Van Eyk, et al. 2016. "Circulating Brain-Derived Neurotrophic Factor Has Diagnostic and Prognostic Value in Traumatic Brain Injury." *Journal of Neurotrauma* 33 (2): 215–25. doi:10.1089/neu.2015.3949.

Kossmann, Thomas, Volkmar Hans, Hans-Georg Imhof, Otmar Trentz, and Maria Cristina Morganti-Kossmann. 1996. "Interleukin-6 Released in Human Cerebrospinal Fluid Following Traumatic Brain Injury May Trigger Nerve Growth Factor Production in Astrocytes." *Brain Research* 713 (1–2): 143–52. doi:10.1016/0006-8993(95)01501-9.

Kovacs, Gabor G., and Ellen Gelpi. 2012. “Clinical Neuropathology Practice News 3-2012: The ‘ABC’ in AD-Revised and Updated Guideline for the Neuropathologic Assessment of Alzheimer’s Disease.” *Clinical Neuropathology* 31 (3): 116–18. doi:10.5414/NP300512.

Krause, Gerd, Lars Winkler, Sebastian L Mueller, Reiner F Haseloff, Jörg Piontek, and Ingolf E Blasig. 2008. “Structure and Function of Claudins.” *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1778 (3): 631–45. doi:10.1016/j.bbamem.2007.10.018.

Kristman, Vicki L, Jörgen Borg, Alison K Godbolt, L Rachid Salmi, Carol Cancelliere, Linda J Carroll, Lena W Holm, et al. 2014. “Methodological Issues and Research Recommendations for Prognosis After Mild Traumatic Brain Injury: Results of the International Collaboration on Mild Traumatic Brain Injury Prognosis.” *Archives of Physical Medicine and Rehabilitation* 95 (3): S265–77. doi:10.1016/j.apmr.2013.04.026.

Lee, Sae-Won, Woo Jean Kim, Yoon Kyung Choi, Hyun Seok Song, Myung Jin Son, Irwin H Gelman, Yung-Jin Kim, and Kyu-Won Kim. 2003. “SSeCKS Regulates Angiogenesis and Tight Junction Formation in Blood-Brain Barrier.” *Nature Medicine* 9 (7): 900–906. doi:10.1038/nm889.

Ley, Eric J, Morgan A Clond, Matt B Singer, Daniel Shouhed, and Ali Salim. 2011. “IL6 Deficiency Affects Function After Traumatic Brain Injury.” *Journal of Surgical Research* 170 (2): 253–56. doi:10.1016/j.jss.2011.03.006.

Li, Wei, Justin Alexander Long, Lora Talley Watts, Zhao Jiang, Qiang Shen, Yunxia Li, and Timothy Q Duong. 2014. “A Quantitative MRI Method for Imaging Blood-Brain Barrier Leakage in Experimental Traumatic Brain Injury.” *PLoS ONE* 9 (12). Public Library of Science: e114173. http://dx.doi.org/10.1371%2Fjournal.pone.0114173.

Li, Yanjun, Yongming Li, Xiaotao Li, Shuang Zhang, Jincheng Zhao, Xiaofeng Zhu, and Guozhong Tian. 2017. “Head Injury as a Risk Factor for Dementia and Alzheimer’s Disease: A Systematic Review and Meta-Analysis of 32 Observational Studies.” Edited by Jonathan Lifshitz. *PLOS ONE* 12 (1): e0169650. doi:10.1371/journal.pone.0169650.

Li, Yue-Hua, Jian-Bo Wang, Ming-Hua Li, Wen-Bin Li, and Dan Wang. 2012. “Quantification of Brain Edema and Hemorrhage by MRI after Experimental Traumatic Brain Injury in Rabbits Predicts Subsequent Functional Outcome.” *Neurological Sciences* 33 (4): 731–40. doi:10.1007/s10072-011-0768-0.

Liliang, Po-Chou, Cheng-Loong Liang, Kang Lu, Kuo-Wei Wang, Hui-Ching Weng, Ching-Hua Hsieh, Yu-Duan Tsai, and Han-Jung Chen. 2010. “Relationship between Injury Severity and Serum Tau Protein Levels in Traumatic Brain Injured Rats.” *Resuscitation* 81 (9). Elsevier Ireland Ltd: 1205–8. doi:10.1016/j.resuscitation.2010.05.016.

Lin, M.T., T.Y. Kao, Y.T. Jin, and C.F. Chen. 1995. “Interleukin-1 Receptor Antagonist Attenuates the Heat Stroke-Induced Neuronal Damage by Reducing the Cerebral Ischemia in Rats.” *Brain Research Bulletin* 37 (6): 595–98. doi:10.1016/0361-9230(95)00046-H.

Ling, Helen, Huw R. Morris, James W. Neal, Andrew J. Lees, John Hardy, Janice L. Holton, Tamas Revesz, and David D. R. Williams. 2017. “Mixed Pathologies Including Chronic Traumatic Encephalopathy Account for Dementia in Retired Association Football (Soccer) Players.” *Acta Neuropathologica* 133 (3). Springer Berlin Heidelberg: 337–52. doi:10.1007/s00401-017-1680-3.

List, Jonathan, Stefanie Ott, Martin Bukowski, Robert Lindenberg, and Agnes Flöel. 2015. “Cognitive Function and Brain Structure after Recurrent Mild Traumatic Brain Injuries in Young-to-Middle-Aged Adults.” *Frontiers in Human Neuroscience* 9 (May): 1–9. doi:10.3389/fnhum.2015.00228.

Little, Deborah M., Elizabeth K. Geary, Michael Moynihan, Aristides Alexander, Michelle Pennington, Patrick Glang, Evan T. Schulze, et al. 2014. “Imaging Chronic Traumatic Brain Injury as a Risk Factor for Neurodegeneration.” *Alzheimer’s & Dementia* 10 (3). Elsevier Ltd: S188–95. doi:10.1016/j.jalz.2014.04.002.

Liu, Su, Lixia Zhang, Qinfeng Wu, Qinfeng Wu, and Tong Wang. 2013. “Chemokine CCL2 Induces Apoptosis in Cortex Following Traumatic Brain Injury.” *Journal of Molecular Neuroscience* 51 (3): 1021–29. doi:10.1007/s12031-013-0091-8.

Lozano, Diego, Gabriel S Gonzales-Portillo, Sandra Acosta, Ike de la Pena, Naoki Tajiri, Yuji Kaneko, and Cesar V Borlongan. 2015. “Neuroinflammatory Responses to Traumatic Brain Injury: Etiology, Clinical Consequences, and Therapeutic Opportunities.” *Neuropsychiatric Disease and Treatment* 11 (January): 97–106. doi:10.2147/NDT.S65815.

Donald, Christine L. Mac, Ann M Johnson, Dana Cooper, Elliot C Nelson, Nicole J Werner, Joshua S Shimony, Abraham Z Snyder, et al. 2011. “Detection of Blast-Related Traumatic Brain Injury in U.S. Military Personnel.” *New England Journal of Medicine* 364 (22): 2091–2100. doi:10.1056/NEJMoa1008069.

Mahmood, Asim, Anton Goussev, Humaira Kazmi, Changsheng Qu, Dunyue Lu, and Michael Chopp. 2009. “LONG-TERM BENEFITS AFTER TREATMENT OF TRAUMATIC BRAIN INJURY WITH SIMVASTATIN IN RATS.” *Neurosurgery* 65 (1): 187–92. doi:10.1227/01.NEU.0000343540.24780.D6.

Manley, Geoffrey T, Miki Fujimura, Tonghui Ma, Nobuo Noshita, Ferda Filiz, Andrew W Bollen, Pak Chan, and A.S. Verkman. 2000. “Aquaporin-4 Deletion in Mice Reduces Brain Edema after Acute Water Intoxication and Ischemic Stroke.” *Nature Medicine* 6 (2): 159–63. doi:10.1038/72256.

Mannix, Rebekah, Matthew Eisenberg, Mark Berry, William P. Meehan, and Ronald L Hayes. 2014. “Serum Biomarkers Predict Acute Symptom Burden in Children after

Concussion: A Preliminary Study." *Journal of Neurotrauma* 31 (11): 1072–75. doi:10.1089/neu.2013.3265.

Marchi, Nicola, Jeffrey J Bazarian, Vikram Puvenna, Mattia Janigro, Chaitali Ghosh, Jianhui Zhong, Tong Zhu, et al. 2013. "Consequences of Repeated Blood-Brain Barrier Disruption in Football Players." *PLoS ONE* 8 (3). Public Library of Science: e56805. http://dx.doi.org/10.1371%2Fjournal.pone.0056805.

Mariano, Cibelle, Hiroyuki Sasaki, Dora Brites, and Maria Alexandra Brito. 2011. "A Look at Tricellulin and Its Role in Tight Junction Formation and Maintenance." *European Journal of Cell Biology* 90 (10). Elsevier GmbH.: 787–96. doi:10.1016/j.ejcb.2011.06.005.

Mazaud-Guittot, S., B. Le Magueresse-Battistoni, E Meugnier, A Gow, X Wu, S Pesenti, and H Vidal. 2009. "Claudin 11 Deficiency in Mice Results in Loss of the Sertoli Cell Epithelial Phenotype in the Testis1." *Biology of Reproduction* 82 (1): 202–13. doi:10.1095/biolreprod.109.078907.

McCrory, Paul, Nina Feddermann-Demont, Jiří Dvořák, J David Cassidy, Andrew McIntosh, Pieter E Vos, Ruben J Echemendia, Willem Meeuwisse, and Alexander A Tarnutzer. 2017. "What Is the Definition of Sports-Related Concussion: A Systematic Review." *British Journal of Sports Medicine* 51 (11): 877–87. doi:10.1136/bjsports-2016-097393.

McCrory, Paul, Willem H. Meeuwisse, Mark Aubry, Robert C. Cantu, Jiři Dvořák, Ruben J. Echemendia, Lars Engebretsen, et al. 2013. "Consensus Statement on Concussion in Sport: The 4th International Conference on Concussion in Sport, Zurich, November 2012." *Journal of Athletic Training* 48 (4): 554–75. doi:10.4085/1062-6050-48.4.05.

McKee, Ann C., Nigel J. Cairns, Dennis W. Dickson, Rebecca D. Folkerth, C. Dirk Keene, Irene Litvan, Daniel P. Perl, et al. 2016. "The First NINDS/NIBIB Consensus Meeting to Define Neuropathological Criteria for the Diagnosis of Chronic Traumatic Encephalopathy." *Acta Neuropathologica* 131 (1). Springer Berlin Heidelberg: 75–86. doi:10.1007/s00401-015-1515-z.

McKee, Celia A., and John R Lukens. 2016. "Emerging Roles for the Immune System in Traumatic Brain Injury." *Frontiers in Immunology* 7 (DEC): 1–17. doi:10.3389/fimmu.2016.00556.

Metting, Z, N Wilczak, L A Rodiger, J. M. Schaaf, and J. van der Naalt. 2012. "GFAP and S100B in the Acute Phase of Mild Traumatic Brain Injury." *Neurology* 78 (18): 1428–33. doi:10.1212/WNL.0b013e318253d5c7.

Meythaler, Jay M., Jean D. Peduzzi, Evangelos Eleftheriou, and Thomas A. Novack. 2001. "Current Concepts: Diffuse Axonal Injury–associated Traumatic Brain Injury." *Archives of Physical Medicine and Rehabilitation* 82 (10): 1461–71. doi:10.1053/apmr.2001.25137.

Mez, Jesse, Daniel H Daneshvar, Patrick T Kiernan, Bobak Abdolmohammadi, Victor E Alvarez, Bertrand R Huber, Michael L Alosco, et al. 2017. “Clinicopathological Evaluation of Chronic Traumatic Encephalopathy in Players of American Football.” *JAMA* 318 (4): 360. doi:10.1001/jama.2017.8334.

Michael, Alex P., Jeffrey Stout, P. Tyler Roskos, Jacob Bolzenius, Jeffrey Gfeller, David Mogul, and Richard Bucholz. 2015. “Evaluation of Cortical Thickness after Traumatic Brain Injury in Military Veterans.” *Journal of Neurotrauma* 32 (22): 1751–58. doi:10.1089/neu.2015.3918.

Montenigro, Philip H, Charles Bernick, and Robert C Cantu. 2015. “Clinical Features of Repetitive Traumatic Brain Injury and Chronic Traumatic Encephalopathy.” *Brain Pathology* 25 (3): 304–17. doi:10.1111/bpa.12250.

Moorlag, S. J. C. F. M., Röring, R. J., Joosten, L. A. B., & Netea, M. G. (2018). The role of the interleukin-1 family in trained immunity. *Immunological Reviews*, *281*(1), 28–39. https://doi.org/10.1111/imr.12617

Morganti, Josh M, Timothy D Jopson, Sharon Liu, L.-K. Riparip, Cristian K Guandique, N. Gupta, Adam R Ferguson, and Susanna Rosi. 2015. “CCR2 Antagonism Alters Brain Macrophage Polarization and Ameliorates Cognitive Dysfunction Induced by Traumatic Brain Injury.” *Journal of Neuroscience* 35 (2): 748–60. doi:10.1523/JNEUROSCI.2405-14.2015.

Mortimer, J A, C M Van Duijn, V Chandra, L Fratiglioni, A B Graves, A Heyman, A F Jorm, et al. 1991. “Head Trauma as a Risk Factor for Alzheimer’s Disease: A Collaborative Re-Analysis of Case-Control Studies.” *International Journal of Epidemiology* 20 (Supplement 2): S28–35. doi:10.1093/ije/20.Supplement_2.S28.

Morris, M. C., Bercz, A., Niziolek, G. M., Kassam, F., Veile, R., Friend, L. A., … Goodman, M. D. (2019). UCH-L1 is a Poor Serum Biomarker of Murine Traumatic Brain Injury After Polytrauma. *Journal of Surgical Research*, *244*, 63–68. https://doi.org/10.1016/j.jss.2019.06.023

Murata, Masaki, Takashi Kojima, Toshinobu Yamamoto, Mitsuru Go, Ken-ichi Takano, Makoto Osanai, Hideki Chiba, and Norimasa Sawada. 2005. “Down-Regulation of Survival Signaling through MAPK and Akt in Occludin-Deficient Mouse Hepatocytes in Vitro.” *Experimental Cell Research* 310 (1): 140–51. doi:10.1016/j.yexcr.2005.07.017.

Naeem, Muhammad, Sofia Hussain, and Naureen Akhtar. 2011. “Mutation in the Tight-Junction Gene Claudin 19 *(CLDN19)* and Familial Hypomagnesemia, Hypercalciuria, Nephrocalcinosis (FHHNC) and Severe Ocular Disease.” *American Journal of Nephrology* 34 (3): 241–48. doi:10.1159/000330854.

Naeimi, Zahra S, Alexandra Weinhofer, Kambiz Sarahrudi, Thomas Heinz, and Vilmos Vécsei. 2006. “Predictive Value of S-100B Protein and Neuron Specific-Enolase as Markers of Traumatic Brain Damage in Clinical Use.” *Brain Injury* 20 (5): 463–68. doi:10.1080/02699050600664418.

Nag, Sukriti, Roopa Venugopalan, and Duncan J. Stewart. 2007. “Increased Caveolin-1 Expression Precedes Decreased Expression of Occludin and Claudin-5 during Blood–brain Barrier Breakdown.” *Acta Neuropathologica* 114 (5): 459–69. doi:10.1007/s00401-007-0274-x.

Nation, Daniel A., Melanie D. Sweeney, Axel Montagne, Abhay P. Sagare, Lina M. D’Orazio, Maricarmen Pachicano, Farshid Sepehrband, et al. 2019. “Blood–brain Barrier Breakdown Is an Early Biomarker of Human Cognitive Dysfunction.” *Nature Medicine* 25 (2). Springer US: 270–76. doi:10.1038/s41591-018-0297-y.

Ndode-Ekane, Xavier Ekolle, Liz Matthiesen, Ivette Bañuelos-Cabrera, Cátia Alexandra Pêgas Palminha, and Asla Pitkänen. 2018. “T-Cell Infiltration into the Perilesional Cortex Is Long-Lasting and Associates with Poor Somatomotor Recovery after Experimental Traumatic Brain Injury.” *Restorative Neurology and Neuroscience* 36 (4): 485–501. doi:10.3233/RNN-170811.

Neselius, Sanna, Henrik Zetterberg, Kaj Blennow, Jeffrey Randall, David Wilson, Jan Marcusson, and Helena Brisby. 2013. “Olympic Boxing Is Associated with Elevated Levels of the Neuronal Protein Tau in Plasma.” *Brain Injury* 27 (4): 425–33. doi:10.3109/02699052.2012.750752.

Nguyen, Rita, Kirsten M. Fiest, Jane McChesney, Churl-Su Kwon, Nathalie Jette, Alexandra D. Frolkis, Callie Atta, et al. 2016. “The International Incidence of Traumatic Brain Injury: A Systematic Review and Meta-Analysis.” *Canadian Journal of Neurological Sciences / Journal Canadien Des Sciences Neurologiques* 43 (06): 774–85. doi:10.1017/cjn.2016.290.

O’Connell, Brendan, Áine M. Kelly, David Mockler, Matej Orešič, Karl Denvir, Garreth Farrell, Damir Janigro, and Fiona Wilson. 2018. “Use of Blood Biomarkers in the Assessment of Sports-Related Concussion—A Systematic Review in the Context of Their Biological Significance.” *Clinical Journal of Sport Medicine* 28 (6): 561–71. doi:10.1097/JSM.0000000000000478.

Ohtsuki, Sumio, Hirofumi Yamaguchi, Yuki Katsukura, Tomoko Asashima, and Tetsuya Terasaki. 2007. “MRNA Expression Levels of Tight Junction Protein Genes in Mouse Brain Capillary Endothelial Cells Highly Purified by Magnetic Cell Sorting.” *Journal of Neurochemistry*, October, 071106220454002–??? doi:10.1111/j.1471-4159.2007.05008.x.

O’Leary, Ruth A, and Alistair D. Nichol. 2018. “Pathophysiology of Severe Traumatic Brain Injury.” *Journal of Neurosurgical Sciences* 62 (5): 542–48. doi:10.23736/S0390-5616.18.04501-0.

Olsson, Bob, Henrik Zetterberg, Harald Hampel, and Kaj Blennow. 2011. “Biomarker-Based Dissection of Neurodegenerative Diseases.” *Progress in Neurobiology* 95 (4): 520–34. doi:10.1016/j.pneurobio.2011.04.006.

Omalu, Bennet I., Robert P. Fitzsimmons, Jennifer Hammers, and Julian Bailes. 2010. "Chronic Traumatic Encephalopathy in a Professional American Wrestler." *Journal of Forensic Nursing* 6 (3): 130–36. doi:10.1111/j.1939-3938.2010.01078.x.

Omalu, Bennet I., Ronald L. Hamilton, Ilyas M. Kamboh, Steven T. DeKosky, and Julian Bailes. 2010. "Chronic Traumatic Encephalopathy (CTE) in a National Football League Player." *Journal of Forensic Nursing* 6 (1): 40–46. doi:10.1111/j.1939-3938.2009.01064.x.

Oppenheimer, D R. 1968. "Microscopic Lesions in the Brain Following Head Injury." *Journal of Neurology, Neurosurgery, and Psychiatry* 31 (4): 299–306. http://www.ncbi.nlm.nih.gov/pubmed/4176429.

Ortega, Eduardo. 2016. "The 'Bioregulatory Effect of Exercise' on the Innate/Inflammatory Responses." *Journal of Physiology and Biochemistry* 72 (2). Journal of Physiology and Biochemistry: 361–69. doi:10.1007/s13105-016-0478-4.

Ozaki, Ema, Matthew Campbell, and Sarah Doyle. 2015. "Targeting the NLRP3 Inflammasome in Chronic Inflammatory Diseases: Current Perspectives." *Journal of Inflammation Research* 8 (January): 15. doi:10.2147/JIR.S51250.

Padhani, Anwar R., and Andy Dzik-Jurasz. 2004. "Perfusion MR Imaging of Extracranial Tumor Angiogenesis." *Topics in Magnetic Resonance Imaging* 15 (1): 41–57. doi:10.1097/00002142-200402000-00005.

Papa, Linda, Gretchen M Brophy, Robert D Welch, Lawrence M Lewis, Carolina F Braga, Ciara N Tan, Neema J Ameli, et al. 2016. "Time Course and Diagnostic Accuracy of Glial and Neuronal Blood Biomarkers GFAP and UCH-L1 in a Large Cohort of Trauma Patients With and Without Mild Traumatic Brain Injury." *JAMA Neurology* 73 (5): 551. doi:10.1001/jamaneurol.2016.0039.

Papa, Linda, Manoj K Mittal, Jose Ramirez, Michelle Ramia, Sara Kirby, Salvatore Silvestri, Philip Giordano, et al. 2016. "In Children and Youth with Mild and Moderate Traumatic Brain Injury, Glial Fibrillary Acidic Protein Out-Performs S100β in Detecting Traumatic Intracranial Lesions on Computed Tomography." *Journal of Neurotrauma* 33 (1): 58–64. doi:10.1089/neu.2015.3869.

Papa, L., Akinyi, L., Liu, M. C., Pineda, J. A., Tepas, J. J., Oli, M. W., … Wang, K. K. W. (2010). Ubiquitin C-terminal hydrolase is a novel biomarker in humans for severe traumatic brain injury*. *Critical Care Medicine*, *38*(1), 138–144. https://doi.org/10.1097/CCM.0b013e3181b788ab

Papa, L., Zonfrillo, M. R., Welch, R. D., Lewis, L. M., Braga, C. F., Tan, C. N., … Mittal, M. K. (2019). Evaluating glial and neuronal blood biomarkers GFAP and UCH-L1 as gradients of brain injury in concussive, subconcussive and non-concussive trauma: a prospective cohort study. *BMJ Paediatrics Open*, *3*(1), e000473. https://doi.org/10.1136/bmjpo-2019-000473

Papa, Linda, Michelle M. Ramia, Damyan Edwards, Brian D. Johnson, and Semyon M. Slobounov. 2015. "Systematic Review of Clinical Studies Examining Biomarkers of Brain Injury in Athletes after Sports-Related Concussion." *Journal of Neurotrauma* 32 (10): 661–73. doi:10.1089/neu.2014.3655.

Papa, Linda, Salvatore Silvestri, Gretchen M Brophy, Philip Giordano, Jay L Falk, Carolina F Braga, Ciara N Tan, et al. 2014. "GFAP Out-Performs S100β in Detecting Traumatic Intracranial Lesions on Computed Tomography in Trauma Patients with Mild Traumatic Brain Injury and Those with Extracranial Lesions." *Journal of Neurotrauma* 31 (22): 1815–22. doi:10.1089/neu.2013.3245.

Parker, Harry L. 1934. "Traumatic Encephalopathy (`Punch Drunk') of Professional Pugilists." *Journal of Neurology, Neurosurgery & Psychiatry* s1-15 (57): 20–28. doi:10.1136/jnnp.s1-15.57.20.

Penkowa, Milena, Torben Moos, Javier Carrasco, Hanne Hadberg, Amalia Molinero, Horst Bluethmann, and Juan Hidalgo. 1999. "Strongly Compromised Inflammatory Response to Brain Injury in Interleukin-6-Deficient Mice." *Glia* 25 (4): 343–57. doi:10.1002/(SICI)1098-1136(19990215)25:4<343::AID-GLIA4>3.0.CO;2-V.

Perez-de-Puig, Isabel, Francesc Miró-Mur, Maura Ferrer-Ferrer, Ellen Gelpi, Jordi Pedragosa, Carles Justicia, Xabier Urra, Angel Chamorro, and Anna M Planas. 2015. "Neutrophil Recruitment to the Brain in Mouse and Human Ischemic Stroke." *Acta Neuropathologica* 129 (2): 239–57. doi:10.1007/s00401-014-1381-0.

Perígolo-Vicente, Rafael, Karen Ritt, Mariana Rodrigues Pereira, Patrícia Maria Mendonça Torres, Roberto Paes-de-Carvalho, and Elizabeth Giestal-de-Araujo. 2013. "IL-6 Treatment Increases the Survival of Retinal Ganglion Cells in Vitro: The Role of Adenosine A1 Receptor." *Biochemical and Biophysical Research Communications* 430 (2): 512–18. doi:10.1016/j.bbrc.2012.12.004.

Perry, David C., Virginia E. Sturm, Matthew J. Peterson, Carl F. Pieper, Thomas Bullock, Bradley F. Boeve, Bruce L. Miller, et al. 2016. "Association of Traumatic Brain Injury with Subsequent Neurological and Psychiatric Disease: A Meta-Analysis." *Journal of Neurosurgery* 124 (2): 511–26. doi:10.3171/2015.2.JNS14503.

Pienaar, Ilse S., Cecilia Heyne Lee, Joanna L. Elson, Louisa McGuinness, Stephen M. Gentleman, Raj N. Kalaria, and David T. Dexter. 2015. "Deep-Brain Stimulation Associates with Improved Microvascular Integrity in the Subthalamic Nucleus in Parkinson's Disease." *Neurobiology of Disease* 74 (1). Elsevier Inc.: 392–405. doi:10.1016/j.nbd.2014.12.006.

Pinter, Nandor K, Joshua P Klein, and Laszlo L. Mechtler. 2016. "Potential Safety Issues Related to the Use of Gadolinium-Based Contrast Agents." *CONTINUUM: Lifelong Learning in Neurology* 22 (5): 1678–84. doi:10.1212/CON.0000000000000378.

Piontek, Jörg, Lars Winkler, Hartwig Wolburg, Sebastian L. Müller, Nikolaj Zuleger, Christian Piehl, Burkhard Wiesner, Gerd Krause, and Ingolf E Blasig. 2008. "Formation of Tight Junction: Determinants of Homophilic Interaction between Classic Claudins." *The FASEB Journal* 22 (1): 146–58. doi:10.1096/fj.07-8319com.

Poole, V. N., Breedlove, E. L., Shenk, T. E., Abbas, K., Robinson, M. E., Leverenz, L. J., … Talavage, T. M. (2015). Sub-Concussive Hit Characteristics Predict Deviant Brain Metabolism in Football Athletes. *Developmental Neuropsychology*, *40*(1), 12–17. https://doi.org/10.1080/87565641.2014.984810

Puvenna, V., Brennan, C., Shaw, G., Yang, C., Marchi, N., Bazarian, J. J., … Janigro, D. (2014). Significance of Ubiquitin Carboxy-Terminal Hydrolase L1 Elevations in Athletes after Sub-Concussive Head Hits. *PLoS ONE*, *9*(5), e96296. https://doi.org/10.1371/journal.pone.0096296

Prince, Martin, Renata Bryce, Emiliano Albanese, Anders Wimo, Wagner Ribeiro, and Cleusa P. Ferri. 2013. "The Global Prevalence of Dementia: A Systematic Review and Metaanalysis." *Alzheimer's & Dementia* 9 (1). Elsevier Ltd: 63–75.e2. doi:10.1016/j.jalz.2012.11.007.

Qian, Huihui, Qinghe Li, and Woda Shi. 2017. "Hyperbaric Oxygen Alleviates the Activation of NLRP-3-Inflammasomes in Traumatic Brain Injury." *Molecular Medicine Reports* 16 (4): 3922–28. doi:10.3892/mmr.2017.7079.

Raleigh, David R, Amanda M Marchiando, Yong Zhang, Le Shen, Hiroyuki Sasaki, Yingmin Wang, Manyuan Long, and Jerrold R Turner. 2010. "Tight Junction-Associated MARVEL Proteins Marveld3, Tricellulin, and Occludin Have Distinct but Overlapping Functions." *Molecular Biology of the Cell* 21 (7): 1200–1213. doi:10.1091/mbc.e09-08-0734.

Ramalho, Joana, and Mauricio Castillo. 2015. "Dementia Resulting from Traumatic Brain Injury." *Dementia & Neuropsychologia* 9 (4): 356–68. doi:10.1590/1980-57642015DN94000356.

Ramlackhansingh, Anil F., David J. Brooks, Richard J. Greenwood, Subrata K. Bose, Federico E. Turkheimer, Kirsi M. Kinnunen, Steve Gentleman, et al. 2011. "Inflammation after Trauma: Microglial Activation and Traumatic Brain Injury." *Annals of Neurology* 70 (3): 374–83. doi:10.1002/ana.22455.

Ren, Zeguang, Jeffrey J Iliff, Lijun Yang, Jiankai Yang, Xiaolin Chen, Michael J Chen, Rebecca N Giese, Baozhi Wang, Xuefang Shi, and Maiken Nedergaard. 2013. "'Hit & Run' Model of Closed-Skull Traumatic Brain Injury (TBI) Reveals Complex Patterns of Post-Traumatic AQP4 Dysregulation." *Journal of Cerebral Blood Flow & Metabolism* 33 (6). Nature Publishing Group: 834–45. doi:10.1038/jcbfm.2013.30.

Reuss, Bernhard, Rosanna Dono, and Klaus Unsicker. 2003. “Functions of Fibroblast Growth Factor (FGF)-2 and FGF-5 in Astroglial Differentiation and Blood-Brain Barrier Permeability: Evidence from Mouse Mutants.” *The Journal of Neuroscience* 23 (16): 6404–12. doi:10.1523/JNEUROSCI.23-16-06404.2003.

Rhodes, Jonathan, John Sharkey, and Peter Andrews. 2009. “Serum IL-8 and MCP-1 Concentration Do Not Identify Patients With Enlarging Contusions After Traumatic Brain Injury.” *The Journal of Trauma: Injury, Infection, and Critical Care* 66 (6): 1591–98. doi:10.1097/TA.0b013e31819a0344.

Rogatzki, Matthew J., Sydney A. Keuler, Abigail E. Harris, Scott W. Ringgenberg, Ryanne E. Breckenridge, Jeffrey L. White, and Julien S. Baker. 2018. “Response of Protein S100B to Playing American Football, Lifting Weights, and Treadmill Running.” *Scandinavian Journal of Medicine & Science in Sports* 28 (12): 2505–14. doi:10.1111/sms.13297.

Rogatzki, Matthew J., Scott E. Soja, Colleen A. McCabe, Ryanne E. Breckenridge, Jeffrey L. White, and Julien S. Baker. 2016. “Biomarkers of Brain Injury Following an American Football Game: A Pilot Study.” *International Journal of Immunopathology and Pharmacology* 29 (3): 450–57. doi:10.1177/0394632016657091.

Roth, A., Gustafson, P., Nhaga, A., Djana, Q., Poulsen, A., Garly, M.-L., … Aaby, P. (2005). BCG vaccination scar associated with better childhood survival in Guinea-Bissau. *International Journal of Epidemiology*, *34*(3), 540–547. https://doi.org/10.1093/ije/dyh392

Roth, Theodore L, Debasis Nayak, Tatjana Atanasijevic, Alan P Koretsky, Lawrence L Latour, and Dorian B. McGavern. 2014. “Transcranial Amelioration of Inflammation and Cell Death after Brain Injury.” *Nature* 505 (7482). Nature Publishing Group: 223–28. doi:10.1038/nature12808.

Rowe, Rachel K., Gavin I. Ellis, Jordan L. Harrison, Adam D. Bachstetter, Gregory F. Corder, Linda J. Van Eldik, Bradley K. Taylor, Francesc Marti, and Jonathan Lifshitz. 2016. “Diffuse Traumatic Brain Injury Induces Prolonged Immune Dysregulation and Potentiates Hyperalgesia Following a Peripheral Immune Challenge.” *Molecular Pain* 12 (January): 174480691664705. doi:10.1177/1744806916647055.

Saitou, Mitinori, Mikio Furuse, Hiroyuki Sasaki, Jörg-Dieter Schulzke, Michael Fromm, Hiroshi Takano, Tetsuo Noda, and Shoichiro Tsukita. 2000. “Complex Phenotype of Mice Lacking Occludin, a Component of Tight Junction Strands.” Edited by W. James Nelson. *Molecular Biology of the Cell* 11 (12): 4131–42. doi:10.1091/mbc.11.12.4131.

Sandvik, U., Koskinen, L.-O., Lundquist, A., & Blomstedt, P. (2012). Thalamic and Subthalamic Deep Brain Stimulation for Essential Tremor. *Neurosurgery*, *70*(4), 840–846. https://doi.org/10.1227/NEU.0b013e318236a809

Saruta, Juri, Kazuhiro Fujino, Masahiro To, and Keiichi Tsukinoki. 2012. “Expression and Localization of Brain-Derived Neurotrophic Factor (BDNF) MRNA and Protein in Human Submandibular Gland.” *ACTA HISTOCHEMICA ET CYTOCHEMICA* 45 (4): 211–18. doi:10.1267/ahc.12005.

Schroeter, M. 2003. “Serum S100B Is Increased during Early Treatment with Antipsychotics and in Deficit Schizophrenia.” *Schizophrenia Research* 62 (3): 231–36. doi:10.1016/S0920-9964(02)00383-3.

Schulzke, J.D., A.H. Gitter, J Mankertz, S Spiegel, U Seidler, S Amasheh, M. Saitou, S. Tsukita, and M. Fromm. 2005. “Epithelial Transport and Barrier Function in Occludin-Deficient Mice.” *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1669 (1): 34–42. doi:10.1016/j.bbamem.2005.01.008.

Semple, Bridgette D, Nicole Bye, Mario Rancan, Jenna M Ziebell, and M. Cristina Morganti-Kossmann. 2010. “Role of CCL2 (MCP-1) in Traumatic Brain Injury (TBI): Evidence from Severe TBI Patients and CCL2−/− Mice.” *Journal of Cerebral Blood Flow & Metabolism* 30 (4). Nature Publishing Group: 769–82. doi:10.1038/jcbfm.2009.262.

Semple, Bridgette D, Nicole Bye, Jenna M Ziebell, and M. Cristina Morganti-Kossmann. 2010. “Deficiency of the Chemokine Receptor CXCR2 Attenuates Neutrophil Infiltration and Cortical Damage Following Closed Head Injury.” *Neurobiology of Disease* 40 (2). Elsevier B.V.: 394–403. doi:10.1016/j.nbd.2010.06.015.

Semple, Bridgette D, Alpa Trivedi, Kayleen Gimlin, and Linda J. Noble-Haeusslein. 2015. “Neutrophil Elastase Mediates Acute Pathogenesis and Is a Determinant of Long-Term Behavioral Recovery after Traumatic Injury to the Immature Brain.” *Neurobiology of Disease* 74 (February). Elsevier Inc.: 263–80. doi:10.1016/j.nbd.2014.12.003.

Sengillo, Jesse D., Ethan A. Winkler, Corey T. Walker, John S. Sullivan, Mahlon Johnson, and Berislav V. Zlokovic. 2013. “Deficiency in Mural Vascular Cells Coincides with Blood-Brain Barrier Disruption in Alzheimer’s Disease.” *Brain Pathology* 23 (3): 303–10. doi:10.1111/bpa.12004.

Shaftel, Solomon S, Thaddeus J Carlson, John A Olschowka, Stephanos Kyrkanides, Sarah B Matousek, and M. K. O’Banion. 2007. “Chronic Interleukin-1 Expression in Mouse Brain Leads to Leukocyte Infiltration and Neutrophil-Independent Blood Brain Barrier Permeability without Overt Neurodegeneration.” *Journal of Neuroscience* 27 (35): 9301–9. doi:10.1523/JNEUROSCI.1418-07.2007.

Shahim, Pashtun, Yelverton Tegner, David H. Wilson, Jeffrey Randall, Tobias Skillbäck, David Pazooki, Birgitta Kallberg, Kaj Blennow, and Henrik Zetterberg. 2014. “Blood Biomarkers for Brain Injury in Concussed Professional Ice Hockey Players.” *JAMA Neurology* 71 (6): 684. doi:10.1001/jamaneurol.2014.367.

Shahjouei, S., Sadeghi-Naini, M., Yang, Z., Kobeissy, F., Rathore, D., Shokraneh, F., … Wang, K. K. W. (2018). The diagnostic values of UCH-L1 in traumatic brain injury: A meta-analysis. *Brain Injury*, *32*(1), 1–17. https://doi.org/10.1080/02699052.2017.1382717

Shen, Fanxia, Espen J Walker, Lidan Jiang, Vincent Degos, Jianping Li, Baoliang Sun, Fransisca Heriyanto, William L Young, and Hua Su. 2011. "Coexpression of Angiopoietin-1 with VEGF Increases the Structural Integrity of the Blood–Brain Barrier and Reduces Atrophy Volume." *Journal of Cerebral Blood Flow & Metabolism* 31 (12). Nature Publishing Group: 2343–51. doi:10.1038/jcbfm.2011.97.

Shenton, M E, H M Hamoda, J S Schneiderman, S Bouix, O Pasternak, Y Rathi, M-A Vu, et al. 2012. "A Review of Magnetic Resonance Imaging and Diffusion Tensor Imaging Findings in Mild Traumatic Brain Injury." *Brain Imaging and Behavior* 6 (2): 137–92. doi:10.1007/s11682-012-9156-5.

Shetty, Vilaas S., Martin N. Reis, Joseph M. Aulino, Kevin L. Berger, Joshua Broder, Asim F. Choudhri, A. Tuba Kendi, et al. 2016. "ACR Appropriateness Criteria Head Trauma." *Journal of the American College of Radiology* 13 (6). Elsevier Inc: 668–79. doi:10.1016/j.jacr.2016.02.023.

Shi, Junhe, May Barakat, Dandan Chen, and Lin Chen. 2018. "Bicellular Tight Junctions and Wound Healing." *International Journal of Molecular Sciences* 19 (12): 3862. doi:10.3390/ijms19123862.

Shimizu, Fumitaka, Yasuteru Sano, Kazuyuki Saito, Masa-aki Abe, Toshihiko Maeda, Hiroyo Haruki, and Takashi Kanda. 2012. "Pericyte-Derived Glial Cell Line-Derived Neurotrophic Factor Increase the Expression of Claudin-5 in the Blood–brain Barrier and the Blood-Nerve Barrier." *Neurochemical Research* 37 (2): 401–9. doi:10.1007/s11064-011-0626-8.

Simon, Daniel, Joice Evaldt, Débora Dreher Nabinger, Mariano Feraboli Fontana, Mirelli Gabardo Klein, Julia do Amaral Gomes, and Andrea Regner. 2017. "Plasma Matrix Metalloproteinase-9 Levels Predict Intensive Care Unit Mortality Early after Severe Traumatic Brain Injury." *Brain Injury* 31 (3). Taylor & Francis: 390–95. doi:10.1080/02699052.2016.1259501.

SMITH, DOUGLAS H., XIAO-HAN CHEN, JEAN E. S. PIERCE, JOHN A. WOLF, JOHN Q. TROJANOWSKI, DAVID I. GRAHAM, and TRACY K. MCINTOSH. 1997. "Progressive Atrophy and Neuron Death for One Year Following Brain Trauma in the Rat." *Journal of Neurotrauma* 14 (10): 715–27. doi:10.1089/neu.1997.14.715.

Smith, Douglas H, and David F Meaney. 2000. "Axonal Damage in Traumatic Brain Injury." *The Neuroscientist* 6 (6): 483–95. doi:10.1177/107385840000600611.

Smith, Kurt J, and Philip N Ainslie. 2017. “Regulation of Cerebral Blood Flow and Metabolism during Exercise.” *Experimental Physiology* 102 (11): 1356–71. doi:10.1113/EP086249.

Soares, H D, R R Hicks, D Smith, and T K McIntosh. 1995. “Inflammatory Leukocytic Recruitment and Diffuse Neuronal Degeneration Are Separate Pathological Processes Resulting from Traumatic Brain Injury.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 15 (12): 8223–33. http://www.ncbi.nlm.nih.gov/pubmed/8613756.

Sourbron, Steven P., and David L. Buckley. 2011. “On the Scope and Interpretation of the Tofts Models for DCE-MRI.” *Magnetic Resonance in Medicine* 66 (3): 735–45. doi:10.1002/mrm.22861.

Stålnacke, Britt-Marie, and Peter Sojka. 2008. “Repeatedly Heading a Soccer Ball Does Not Increase Serum Levels of S-100B, a Biochemical Marker of Brain Tissue Damage: An Experimental Study.” *Biomarker Insights* 3 (3): 87–91. http://www.ncbi.nlm.nih.gov/pubmed/19578497.

Stålnacke, Britt-Marie, Yelverton Tegner, and Peter Sojka. 2004. “Playing Soccer Increases Serum Concentrations of the Biochemical Markers of Brain Damage S-100B and Neuron-Specific Enolase in Elite Players: A Pilot Study.” *Brain Injury* 18 (9): 899–909. doi:10.1080/02699050410001671865.

Stålnacke, Britt-Marie, Yelverton Tegner, and Peter Sojka. 2003. “Playing Ice Hockey and Basketball Increases Serum Levels of S-100B in Elite Players.” *Clinical Journal of Sport Medicine* 13 (5): 292–302. doi:10.1097/00042752-200309000-00004.

Starr, John M., Andrew J. Farrall, Paul Armitage, Brian McGurn, and Joanna Wardlaw. 2009. “Blood–brain Barrier Permeability in Alzheimer’s Disease: A Case–control MRI Study.” *Psychiatry Research: Neuroimaging* 171 (3). Elsevier Ireland Ltd: 232–41. doi:10.1016/j.pscychresns.2008.04.003.

Stevenson, B R, J D Siliciano, M S Mooseker, and D A Goodenough. 1986. “Identification of ZO-1: A High Molecular Weight Polypeptide Associated with the Tight Junction (Zonula Occludens) in a Variety of Epithelia.” *The Journal of Cell Biology* 103 (3): 755–66. doi:10.1083/jcb.103.3.755.

Stocchero, Cintia Mussi Alvim, Jean Pierre Oses, Giovani Santos Cunha, Jocelito Bijoldo Martins, Liz Marina Brum, Eduardo Rigon Zimmer, Diogo Onofre Souza, Luis Valmor Portela, and Alvaro Reischak-Oliveira. 2014. “Serum S100B Level Increases after Running but Not Cycling Exercise.” *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition et Metabolisme* 39 (3): 340–44. doi:10.1139/apnm-2013-0308.

Stolp, H B, and K M Dziegielewska. 2009. “Review: Role of Developmental Inflammation and Blood-Brain Barrier Dysfunction in Neurodevelopmental and Neurodegenerative Diseases.” *Neuropathology and Applied Neurobiology* 35 (2): 132–46. doi:10.1111/j.1365-2990.2008.01005.x.

Suri, Asif K, and Michael L Lipton. 2018. “Neuroimaging of Brain Trauma in Sports.” In *Sports Neurology*, edited by Brian Hainline and Robert A B T - Handbook of Clinical Neurology Stern, 158:205–16. Elsevier. doi:10.1016/B978-0-444-63954-7.00021-5.

Swartz, Karin R, Frances Liu, Diane Sewell, Terri Schochet, Iain Campbell, Matyas Sandor, and Zsuzsanna Fabry. 2001. “Interleukin-6 Promotes Post-Traumatic Healing in the Central Nervous System.” *Brain Research* 896 (1–2): 86–95. doi:10.1016/S0006-8993(01)02013-3.

Sweeney, Melanie D, Abhay P Sagare, and Berislav V Zlokovic. 2018. “Blood–brain Barrier Breakdown in Alzheimer Disease and Other Neurodegenerative Disorders.” *Nature Reviews Neurology* 14 (3). Nature Publishing Group: 133–50. doi:10.1038/nrneurol.2017.188.

Tagge, Chad A., Andrew M. Fisher, Olga V. Minaeva, Amanda Gaudreau-Balderrama, Juliet A. Moncaster, Xiao-Lei Zhang, Mark W. Wojnarowicz, et al. 2018. “Concussion, Microvascular Injury, and Early Tauopathy in Young Athletes after Impact Head Injury and an Impact Concussion Mouse Model.” *Brain* 141 (2): 422–58. doi:10.1093/brain/awx350.

Taheri, Saeid, Charles Gasparovic, Nadim Jon Shah, and Gary A. Rosenberg. 2011. “Quantitative Measurement of Blood-Brain Barrier Permeability in Human Using Dynamic Contrast-Enhanced MRI with Fast T1 Mapping.” *Magnetic Resonance in Medicine* 65 (4): 1036–42. doi:10.1002/mrm.22686.

Tamnes, Christian K., David R. Roalf, Anne-Lise Goddings, and Catherine Lebel. 2018. “Diffusion MRI of White Matter Microstructure Development in Childhood and Adolescence: Methods, Challenges and Progress.” *Developmental Cognitive Neuroscience* 33 (December 2017): 161–75. doi:10.1016/j.dcn.2017.12.002.

Tartaglia, Maria Carmela, Lili-Naz Hazrati, Karen D. Davis, Robin E. A. Green, Richard Wennberg, David Mikulis, Leo J. Ezerins, Michelle Keightley, and Charles Tator. 2014. “Chronic Traumatic Encephalopathy and Other Neurodegenerative Proteinopathies.” *Frontiers in Human Neuroscience* 8 (January): 1–6. doi:10.3389/fnhum.2014.00030.

Tasçı, Alptekin, Önder Okay, Ali Rıza Gezici, Rüçhan Ergün, and Fikret Ergüngör. 2003. “Prognostic Value of Interleukin-1 Beta Levels after Acute Brain Injury.” *Neurological Research* 25 (8): 871–74. doi:10.1179/016164103771953998.

Thelin, Eric Peter, Frederick Adam Zeiler, Ari Ercole, Stefania Mondello, András Büki, Bo-Michael Bellander, Adel Helmy, David K. Menon, and David W. Nelson. 2017. “Serial Sampling of Serum Protein Biomarkers for Monitoring Human Traumatic Brain Injury Dynamics: A Systematic Review.” *Frontiers in Neurology* 8 (July): 1–23. doi:10.3389/fneur.2017.00300.

Tobin, Richard P, Sanjib Mukherjee, Jessica M Kain, Susannah K Rogers, Stephanie K Henderson, Heather L Motal, M Karen Newell Rogers, and Lee A Shapiro. 2014. "Traumatic Brain Injury Causes Selective, CD74-Dependent Peripheral Lymphocyte Activation That Exacerbates Neurodegeneration." *Acta Neuropathologica Communications* 2 (1): 143. doi:10.1186/s40478-014-0143-5.

Tomkins, O., I. Shelef, I. Kaizerman, A. Eliushin, Z. Afawi, A. Misk, M. Gidon, A. Cohen, D. Zumsteg, and Alon Friedman. 2008. "Blood-Brain Barrier Disruption in Post-Traumatic Epilepsy." *Journal of Neurology, Neurosurgery & Psychiatry* 79 (7): 774–77. doi:10.1136/jnnp.2007.126425.

Townend, Will, and Tor Ingebrigtsen. 2006. "Head Injury Outcome Prediction: A Role for Protein S-100B?" *Injury* 37 (12): 1098–1108. doi:10.1016/j.injury.2006.07.014.

Trotter, Benjamin B., Meghan E. Robinson, William P. Milberg, Regina E. McGlinchey, and David H. Salat. 2015. "Military Blast Exposure, Ageing and White Matter Integrity." *Brain* 138 (8): 2278–92. doi:10.1093/brain/awv139.

Tsukita, Shoichiro, Mikio Furuse, and Masahiko Itoh. 2001. "Multifunctional Strands in Tight Junctions." *Nature Reviews Molecular Cell Biology* 2 (4): 285–93. doi:10.1038/35067088.

Undén, Johan, Tor Ingebrigtsen, and Bertil Romner. 2013. "Scandinavian Guidelines for Initial Management of Minimal, Mild and Moderate Head Injuries in Adults: An Evidence and Consensus-Based Update." *BMC Medicine* 11 (1): 50. doi:10.1186/1741-7015-11-50.

Urra, Xabier, Neus Villamor, Sergio Amaro, Manuel Gómez-Choco, Víctor Obach, Laura Oleaga, Anna M Planas, and Angel Chamorro. 2009. "Monocyte Subtypes Predict Clinical Course and Prognosis in Human Stroke." *Journal of Cerebral Blood Flow & Metabolism* 29 (5): 994–1002. doi:10.1038/jcbfm.2009.25.

Beek, Leen Van, Pol Ghesquière, Lieven Lagae, and Bert De Smedt. 2015. "Mathematical Difficulties and White Matter Abnormalities in Subacute Pediatric Mild Traumatic Brain Injury." *Journal of Neurotrauma* 32 (20). Mary Ann Liebert, Inc., publishers: 1567–78. doi:10.1089/neu.2014.3809.

Beek, Leen Van, Jolijn Vanderauwera, Pol Ghesquière, Lieven Lagae, and Bert De Smedt. 2015. "Longitudinal Changes in Mathematical Abilities and White Matter Following Paediatric Mild Traumatic Brain Injury." *Brain Injury* 29 (13–14): 1701–10. doi:10.3109/02699052.2015.1075172.

Itallie, C. M. Van, O R Colegio, and J M Anderson. 2004. "The Cytoplasmic Tails of Claudins Can Influence Tight Junction Barrier Properties through Effects on Protein Stability." *Journal of Membrane Biology* 199 (1): 29–38. doi:10.1007/s00232-004-0673-z.

Itallie, Christina M. Van, and James Melvin Anderson. 2004. “The Molecular Physiology of Tight Junction Pores.” *Physiology* 19 (6): 331–38. doi:10.1152/physiol.00027.2004.

Veksler, Ronel, Ilan Shelef, and Alon Friedman. 2014. “Blood–Brain Barrier Imaging in Human Neuropathologies.” *Archives of Medical Research* 45 (8). Elsevier Inc: 646–52. doi:10.1016/j.arcmed.2014.11.016.

Verkman, A.S., Devin K Binder, Orin Bloch, Kurtis Auguste, and Marios C Papadopoulos. 2006. “Three Distinct Roles of Aquaporin-4 in Brain Function Revealed by Knockout Mice.” *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1758 (8): 1085–93. doi:10.1016/j.bbamem.2006.02.018.

Vezzani, A, D Moneta, M Conti, C Richichi, T Ravizza, A. De Luigi, M. G. De Simoni, et al. 2000. “Powerful Anticonvulsant Action of IL-1 Receptor Antagonist on Intracerebral Injection and Astrocytic Overexpression in Mice.” *Proceedings of the National Academy of Sciences* 97 (21): 11534–39. doi:10.1073/pnas.190206797.

Vilcant, Viliane, and Roman Zeltser. 2019. *Treadmill Stress Testing*. *StatPearls*. United States: StatPearls Publishing. http://www.ncbi.nlm.nih.gov/pubmed/29763078.

Villapol, Sonia, Kimberly R Byrnes, and Aviva J Symes. 2014. “Temporal Dynamics of Cerebral Blood Flow, Cortical Damage, Apoptosis, Astrocyte–vasculature Interaction and Astrogliosis in the Pericontusional Region after Traumatic Brain Injury.” *Frontiers in Neurology* 5 (June): 1–9. doi:10.3389/fneur.2014.00082.

Walcheck, Bruce, Kevin L. Moore, Rodger P. McEver, and Takashi Kei Kishimoto. 1996. “Neutrophil-Neutrophil Interactions under Hydrodynamic Shear Stress Involve L-Selectin and PSGL-1. A Mechanism That Amplifies Initial Leukocyte Accumulation of P-Selectin in Vitro.” *Journal of Clinical Investigation* 98 (5): 1081–87. doi:10.1172/JCI118888.

Wang, Ying, Yanling Wei, Samuel Oguntayo, Donna Wilder, Lawrence Tong, Yan Su, Irene Gist, Peethambaran Arun, and Joseph B. Long. 2017. “Cerebrospinal Fluid Chemokine (C-C Motif) Ligand 2 Is an Early-Response Biomarker for Blast-Overpressure-Wave–Induced Neurotrauma in Rats.” *Journal of Neurotrauma* 34 (4): 952–62. doi:10.1089/neu.2016.4465.

Watson, Phillip, Katherine E Black, Susan C Clark, and Ronald J Maughan. 2006. “Exercise in the Heat: Effect of Fluid Ingestion on Blood-Brain Barrier Permeability.” *Medicine and Science in Sports and Exercise* 38 (12): 2118–24. doi:10.1249/01.mss.0000235356.31932.0a.

Weckbach, Sebastian, Miriam Neher, Justin T. Losacco, Ashley L. Bolden, Liudmila Kulik, Michael A. Flierl, Scott E. Bell, V. Michael Holers, and Philip F. Stahel. 2012. “Challenging the Role of Adaptive Immunity in Neurotrauma: Rag1 −/− Mice Lacking Mature B and T Cells Do Not Show Neuroprotection after Closed Head Injury.” *Journal of Neurotrauma* 29 (6): 1233–42. doi:10.1089/neu.2011.2169.

Wei, Xiao-Er, Yue-Hua Li, Hui Zhao, Ming-Hua Li, Min Fu, and Wen-Bin Li. 2014. "Quantitative Evaluation of Hyperbaric Oxygen Efficacy in Experimental Traumatic Brain Injury: An MRI Study." *Neurological Sciences* 35 (2): 295–302. doi:10.1007/s10072-013-1514-6.

Wei, Xiao-Er, Dan Wang, Ming-Hua Li, Yu-Zhen Zhang, Yue-Hua Li, and Wen-Bin Li. 2011. "A Useful Tool for the Initial Assessment of Blood–Brain Barrier Permeability After Traumatic Brain Injury in Rabbits: Dynamic Contrast-Enhanced Magnetic Resonance Imaging." *The Journal of Trauma: Injury, Infection, and Critical Care* 71 (6): 1645–51. doi:10.1097/TA.0b013e31823498eb.

Weintraub, Daniel, Nicole Dietz, John E. Duda, David A. Wolk, Jimit Doshi, Sharon X. Xie, Christos Davatzikos, Christopher M. Clark, and Andrew Siderowf. 2012. "Alzheimer's Disease Pattern of Brain Atrophy Predicts Cognitive Decline in Parkinson's Disease." *Brain* 135 (1): 170–80. doi:10.1093/brain/awr277.

Weissberg, Itai, Ronel Veksler, Lyn Kamintsky, Rotem Saar-Ashkenazy, Dan Z. Milikovsky, Ilan Shelef, and Alon Friedman. 2014. "Imaging Blood-Brain Barrier Dysfunction in Football Players." *JAMA Neurology* 71 (11): 1453. doi:10.1001/jamaneurol.2014.2682.

Whalen, M J, T M Carlos, P M Kochanek, R S Clark, S Heineman, J K Schiding, D Franicola, et al. 1999. "Neutrophils Do Not Mediate Blood-Brain Barrier Permeability Early after Controlled Cortical Impact in Rats." *Journal of Neurotrauma* 16 (7). Mary Ann Liebert, Inc., publishers: 583–94. doi:10.1089/neu.1999.16.583.

Wilcox, Edward R, Quianna L Burton, Sadaf Naz, Saima Riazuddin, Tenesha N Smith, Barbara Ploplis, Inna Belyantseva, et al. 2001. "Mutations in the Gene Encoding Tight Junction Claudin-14 Cause Autosomal Recessive Deafness DFNB29." *Cell* 104 (1): 165–72. doi:10.1016/S0092-8674(01)00200-8.

Williams, D H, H S Levin, and H M Eisenberg. 1990. "Mild Head Injury Classification." *Neurosurgery* 27 (3). United States: 422–28. http://www.ncbi.nlm.nih.gov/pubmed/2234336.

Williams, David H., Harvey S. Levin, and Howard M. Eisenberg. 1990. "Mild Head Injury Classification." *Neurosurgery* 27 (3): 422–28. doi:10.1227/00006123-199009000-00014.

Wilson, Emma H., Wolfgang Weninger, and Christopher A. Hunter. 2010. "Trafficking of Immune Cells in the Central Nervous System." *Journal of Clinical Investigation* 120 (5): 1368–79. doi:10.1172/JCI41911.

Winkler, Ethan A, Jesse D Sengillo, Robert D Bell, Joseph Wang, and Berislav V. Zlokovic. 2012. "Blood–Spinal Cord Barrier Pericyte Reductions Contribute to Increased Capillary Permeability." *Journal of Cerebral Blood Flow & Metabolism* 32 (10). Nature Publishing Group: 1841–52. doi:10.1038/jcbfm.2012.113.

Winter, Craig, Christopher Bell, Timothy Whyte, John Cardinal, David Macfarlane, and Stephen Rose. 2015. “Blood–brain Barrier Dysfunction Following Traumatic Brain Injury: Correlation of K Trans (DCE-MRI) and SUVR (99mTc-DTPA SPECT) but Not Serum S100B.” *Neurological Research* 37 (7): 599–606. doi:10.1179/1743132815Y.0000000018.

Xu, Xin, Dongpei Yin, Honglei Ren, Weiwei Gao, Fei Li, Dongdong Sun, Yingang Wu, et al. 2018. “Selective NLRP3 Inflammasome Inhibitor Reduces Neuroinflammation and Improves Long-Term Neurological Outcomes in a Murine Model of Traumatic Brain Injury.” *Neurobiology of Disease* 117 (May). Elsevier: 15–27. doi:10.1016/j.nbd.2018.05.016.

Yang, Sung H, Matt Gangidine, Timothy A Pritts, Michael D Goodman, and Alex B Lentsch. 2013. “Interleukin 6 Mediates Neuroinflammation and Motor Coordination Deficits After Mild Traumatic Brain Injury and Brief Hypoxia in Mice.” *Shock* 40 (6): 471–75. doi:10.1097/SHK.0000000000000037.

Yoo, Roh-Eul, Seung Hong Choi, Byung-Mo Oh, Sang Do Shin, Eun Jung Lee, Dong Jae Shin, Sang Won Jo, et al. 2019. “Quantitative Dynamic Contrast-Enhanced MR Imaging Shows Widespread Blood-Brain Barrier Disruption in Mild Traumatic Brain Injury Patients with Post-Concussion Syndrome.” *European Radiology* 29 (3). European Radiology: 1308–17. doi:10.1007/s00330-018-5656-z.

Yu, Hao, Changliang Wang, Xiaolong Wang, Hongbo Wang, Chunan Zhang, Jiabin You, Pengfei Wang, et al. 2017. “Long-term Exposure to Ethanol Downregulates Tight Junction Proteins through the Protein Kinase Cα Signaling Pathway in Human Cerebral Microvascular Endothelial Cells.” *Experimental and Therapeutic Medicine*, September, 4789–96. doi:10.3892/etm.2017.5180.

Zagorchev, Lyubomir, Carsten Meyer, Thomas Stehle, Fabian Wenzel, Stewart Young, Jochen Peters, Juergen Weese, et al. 2016. “Differences in Regional Brain Volumes Two Months and One Year after Mild Traumatic Brain Injury.” *Journal of Neurotrauma* 33 (1): 29–34. doi:10.1089/neu.2014.3831.

Zaigham, Mehreen, Fredrik Lundberg, and Per Olofsson. 2017. “Protein S100B in Umbilical Cord Blood as a Potential Biomarker of Hypoxic-Ischemic Encephalopathy in Asphyxiated Newborns.” *Early Human Development* 112 (July). Elsevier: 48–53. doi:10.1016/j.earlhumdev.2017.07.015.

Zeighami, Yashar, Miguel Ulla, Yasser Iturria-Medina, Mahsa Dadar, Yu Zhang, Kevin Michel-Herve Larcher, Vladimir Fonov, Alan C Evans, D Louis Collins, and Alain Dagher. 2015. “Network Structure of Brain Atrophy in de Novo Parkinson’s Disease.” *ELife* 4 (September): 1–20. doi:10.7554/eLife.08440.

Zetterberg, Henrik, Michael Jonsson, Abdullah Rasulzada, Cornel Popa, Ewa Styrud, M. A. Hietala, Lars Rosengren, Anders Wallin, and Kaj Blennow. 2007. “No Neurochemical Evidence for Brain Injury Caused by Heading in Soccer.” *British Journal of Sports Medicine* 41 (9): 574–77. doi:10.1136/bjsm.2007.037143.

Zetterberg, Henrik, Fatih Tanriverdi, Kursad Unluhizarci, Ahmet Selcuklu, Fahrettin Kelestimur, and Kaj Blennow. 2009. “Sustained Release of Neuron-Specific Enolase to Serum in Amateur Boxers.” *Brain Injury* 23 (9): 723–26. doi:10.1080/02699050903120399.

Zuckerman, Scott L., Zachary Y. Kerr, Aaron Yengo-Kahn, Erin Wasserman, Tracey Covassin, and Gary S. Solomon. 2015. “Epidemiology of Sports-Related Concussion in NCAA Athletes From 2009-2010 to 2013-2014.” *The American Journal of Sports Medicine* 43 (11): 2654–62. doi:10.1177/0363546515599634

Appendix I

clinical neuropathology

Blood-brain barrier dysfunction in a boxer with chronic traumatic encephalopathy and schizophrenia

Michael Farrell[1], Susan Aherne[2], Sean O'Riordan[3,4], Eoin O'Keeffe[5], Chris Greene[5], and Matthew Campbell[5]

[1]Department of Neuropathology, Beaumont Hospital, [2]Department of Pathology, St. Columcille's Hospital, Loughlinstown, [3]Department of Neurology, St. Vincent's University Hospital, [4]School of Medicine and Medical Sciences, University College Dublin, and [5]Smurfit Institute of Genetics, Trinity College Dublin, Dublin, Ireland

ISSN 0722-5091

DOI 10.5414/NP301130
e-pub: ■■month ■■day, ■■year

Key words
chronic traumatic encephalopathy (CTE) – blood-brain barrier (BBB) – schizophrenia – claudin-5

Abstract. Chronic traumatic encephalopathy (CTE) is a neurodegenerative condition characterized by the perivascular deposition of phosphorylated τ (p-τ) protein aggregates resulting from repetitive mild traumatic brain injury (rTBI). Advances in the have revealed the of repetitive head trauma in the pathogenesis of CTE in contact sports as well as military veterans. In this study we provide evidence of blood-brain barrier (BBB) disruption in regions of intense perivascular p-τ deposition in a former profession boxer diagnosed with CTE and schizophrenia. P-τ deposition was associated with loss of the tight junction protein claudin-5 and enhanced extravasation of endogenous blood components - gen and IgG. We also provide evidence of tight junction disruption in individuals with schizophrenia, with discontinuous claudin-5 immunoreactivity in the parietal cortex. This data highlights a common phenotype of a dysfunctional BBB in individuals with CTE and schizophrenia and may represent a novel correlate of neural dysfunction in individuals at risk of developing CTE and schizophrenia.

Introduction

Chronic traumatic encephalopathy (CTE) is a neurological disorder resulting from repetitive concussive and sub-concussive blows to the head sustained in contact sports such as boxing, American football, and martial arts. CTE is characterized neuropathologically by the accumulation of hyperphosphorylated τ (p-τ) protein throughout the brain [1]. Clinically, CTE manifests behaviorally as in cognition (memory and executive function), mood (aggression, depression, and suicidality), or motor function (parkinsonism, dysarthria, and dysphagia) [2]. At present, CTE can only be diagnosed post mortem by immunohistochemical examination of brain tissue and as such, there are no treatment options available. Furthermore, there are no criteria in place to diagnose individuals "in life" although groups are striving towards neuropathological criteria to diagnose the condition [1]. A clinical diagnosis of CTE is as the symptoms of CTE share much in common with those of other neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease, and frontotemporal dementia [3].

The hallmark pathology of CTE is the perivascular deposition of p-τ especially in sulcal depths. The abnormally phosphorylated-τ is present as - lary tangles (NFT) and as dystrophic neurites. Depending on the disease stage the abnormal τ is distributed in the frontal and temporal lobes including the hippocampus, amygdala, and entorhinal cortex. Additionally, there is a paucity of β-amyloid neuritic plaques in individuals with CTE [4].

First recognized in association with boxing, as either "punch drunk syndrome" or dementia pugilistica [5], CTE has since been linked with various contact sports including combat sports, Association Football, and American football as well as blast exposed military veterans such that all neuropathologically cases of CTE have a history of repetitive traumatic brain injury (rTBI) [6, 7, 8, 9, 10, 11]. Interestingly, many CTE cases with a history of rTBI do not have a history of concussion, emphasizing the role of sub-concussive blows or mild rTBI in the

Received
July 11, 2018;
accepted in revised form
September 8, 2018

Correspondence to
Matthew Campbee, PhD

Genetics, Trinity College Dublin, Trinity Place Gate, Dublin 2, Ireland
canpbem2@tcd.ie

pathogenesis of the disease [12]. While rTBI is a requisite for the pathological cascade of neurodegeneration leading to CTE, a majority of individuals who have suffered from rTBI have not developed CTE, pointing to as yet unknown risk factors including genetics.

Given the unique and pathognomonic perivascular distribution of p-τ in CTE there is considerable interest in perturbations of the blood-brain barrier (BBB) as a possible forerunner to accumulation of p-τ in CTE. Recently, various species of τ were found to readily diffuse across the BBB bidirectionally, and dysregulation of the BBB may contribute to disease progression [13]. The vasculature of the brain is extensive, selective, and tightly regulated owing to the need to maintain homeostasis of the neural microenvironment for synaptic signaling. Lining blood vessels of the CNS are endothelial cells that are linked together by tight junction protein complexes and contain a variety of polarized receptors and transport proteins to selectively permit entry of essential nutrients, carbohydrates, and proteins [14]. Recently, we revealed extensive BBB dysfunction in a deceased rugby union player with a post-mortem diagnosis of CTE with notable loss of the BBB-enriched tight junction protein claudin-5 in regions of perivascular p-τ deposition with associated microvascular leakage of endogenous immuno-

In this study, we performed histological analysis on the brain of a former professional boxer diagnosed with CTE and with a comorbidity of schizophrenia to determine the extent of tight junction disruption and BBB dysfunction. In addition, we analyzed the brains of individuals with schizophrenia to identify evidence of barrier dysfunction.

Materials and methods

Immunohistochemistry

blocks of brain tissue from individuals diagnosed with CTE and age-matched controls were provided by the Dublin Brain Bank. blocks were sectioned (8 μm), in 3 changes of xylene (2 minutes each), and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) in a microwave (2 × 5 minutes). Sections were blocked/permeabilized in blocking buffer (5% normal goat serum, 0.5% Triton X-100) for 1 hour at room temperature followed by overnight incubation at 4 °C with primary antibodies: rabbit anti-claudin-5 (1 : 500), rabbit anti-ZO-1 (1 : 500), and mouse anti-p-τ (1 : 500). Following incubation, sections were washed in PBS (3 × 5 minutes) followed by incubation for 2 hours at room temperature with secondary antibodies: goat anti-rabbit cy3 (1 : 1,000) and goat anti-mouse Alexa Fluor 488 (1 : 1,000). Following incubation, sections were washed in PBS (4 × 5 minutes) and counterstained with Hoechst staining solution (1 : 10,000) for 30 seconds at room temperature. For analysis of BBB extravasation of endogenous molecules, sections were incubated for 2 hours at room temperature with rabbit anti-human -brinogen (1 : 500) and rabbit anti-human IgG (1 : 500). Slides were imaged with a Zeiss LSM-710 laser scanning microscope with 10× and 40× objectives.

Additionally, 24 schizophrenia and 24 age-matched control, 60-μm-thick brain sections from the inferior parietal lobe were acquired from the Stanley Medical Research Institute and stained for claudin-5 as above. All patients were diagnosed according to the DSM-IV (Diagnostic and Statistical Manual of Mental Health Disorders, 4th Edition) criteria.

Results

Clinical history and diagnosis

A 64-year-old man presented to neurology services in 2009 with short-term memory loss, slurring of speech/dysarthria, swallowing problems, gait disturbance (dragging one lower limb and unsteady gait), tremor in the right hand, and rigidity of the upper limbs. Up until 1 year prior to presentation he had been well and had been attending the gym regularly. Over the course of 8 years, the patient developed worsening bradykinesia, rigidity, increasing falls, abnormal gait (shuf- gait), dysarthria, cognitive impairment

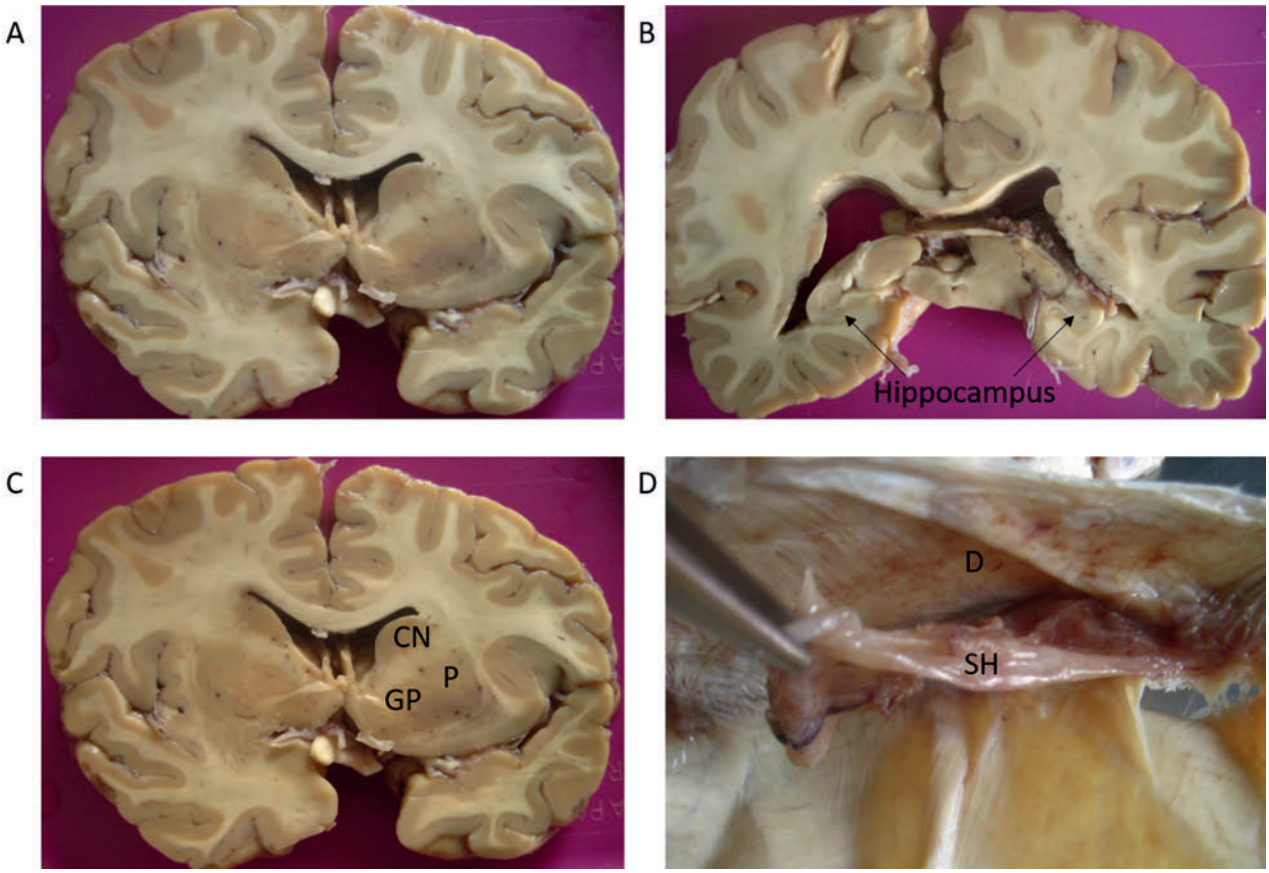


Figure 1. Macroscopic pathology in the brain of a former professional boxer diagnosed with chronic traumatic encephalopathy. There were no signs of atrophy in the (A) temporal lobes, (B) hippocampus and (C) caudate, putamen, and global pallidus. (D) There was evidence of prior brain trauma with a chronic subdural hematoma membrane. CN = caudate nucleus; GP = globus pallidus; P = putamen; D = dura; SH = subdural hematoma.

with poor short-term memory, and emotional lability. Overall assessment was suggestive of a progressive neurodegenerative disorder with elements of cognitive impairment and parkinsonism.

Needle electromyography detected evidence of scattered minimal recent motor axonal loss in each limb. Single-photon emission computed tomography (spect) imaging of the brain displayed scintographic [■■■ scintigraphic?] features in keeping with Parkinson's disease with Catafau - cation 1. Involutional change prominent for the subject's age were apparent on CT brain scan, brain MRI showed mild periventricular and left frontal subcortical deep white matter ischemia. The subject had a medical history of excessive alcohol consumption from the 1980's onwards, type 2 diabetes mellitus, and hypercholesterolemia. The indications of mental problems occurred in 1989 when the subject displayed psychiatric symptoms including anger, impulsive behavior, and paranoia. He was subsequently diagnosed with paranoid schizophrenia (herein referred to as schizophrenia) in 1991.

He was also an ex-professional boxer with a 19-year history of competitive professional boxing between the ages of 16 and 35. The subject was also a physical trainer in the Air Force in the Middle East. The subject presented to the acute services of the hospital having suffered a fatal fall at home. Post-mortem showed severe acute traumatic subarachnoid hemorrhage. There were no signs of atrophy in the temporal lobes, hippocampus, caudate, putamen, or global pallidus (Figure 1).

Immunohistochemistry for p-τ revealed NFTs in the pyramidal layer and dentate fascia of the hippocampus along with globose NFTs in the substantia nigra. Perivascular astrocytic p-τ was detected in the neocortex and at the sulcal depths of the neocortex

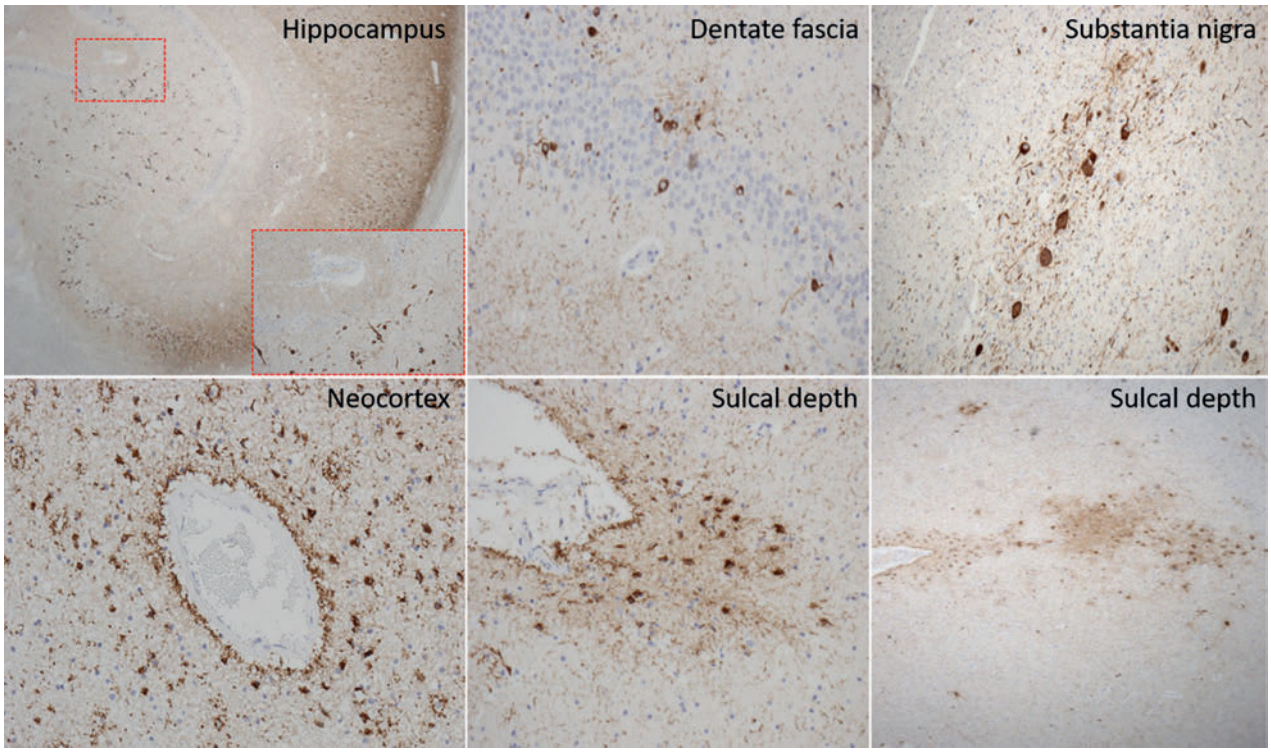


Figure 2. p-τ deposition in multiple brain regions. Photomicrographs of brain sections immunostained with a polyclonal p-τ antibody. P-τ deposition was evident in the hippocampus, pyramidal layer of the dentate fascia of the hippocampus and substantia nigra. Perivascular p-τ deposition was evident in the sulcal depths of the neocortex.

(Figure 2). Amyloid-β was not present in any brain region, and coupled with the previous history of rTBI and perivascular deposition of p-τ, a diagnosis of CTE was made.

Tight junction disruption and BBB dysfunction

Immunohistochemical examination of the tight junction protein claudin-5 revealed discontinuous and complete absence of claudin-5 in regions of intense perivascular accumulation of p-τ. Regions devoid of p-τ immunoreactivity displayed normal and continuous claudin-5 patterns of expression (Figure 3). To determine the extent of BBB dysfunction, sections of brain were immunostained for the endogenous blood components (340 kDa) and IgG (150 kDa). Fibrinogen and IgG extravasation was evident in larger vessels with minimal extravasation apparent in capillaries (Figure 3). As the subject was diagnosed "in life" with schizophrenia, we next examined brain sections received from the Stanley Medical Research Institute of individuals with schizophrenia and age-matched controls to identify evidence of BBB dysfunction and tight junction disruption. Immunohistochemical analysis of claudin-5 revealed large regions of discontinuous claudin-5 immunoreactivity in 15/24 cases with schizophrenia. 3D reconstructions of z-stack images revealed continuous claudin-5 staining in age-matched control brains with disrupted tight junction staining and absence of claudin-5 immunoreactivity along vessels of the brain of an individual with schizophrenia (Figure 4).

Discussion

In this study, we present for the time, a characterization of the BBB in a former professional boxer with CTE and schizophrenia.

A commonality of TBI is vascular impairment resulting from the shearing of small vessels [16]. Furthermore, TBI leads to a biphasic opening of the BBB with disruption of tight junctions and subsequent extravasation

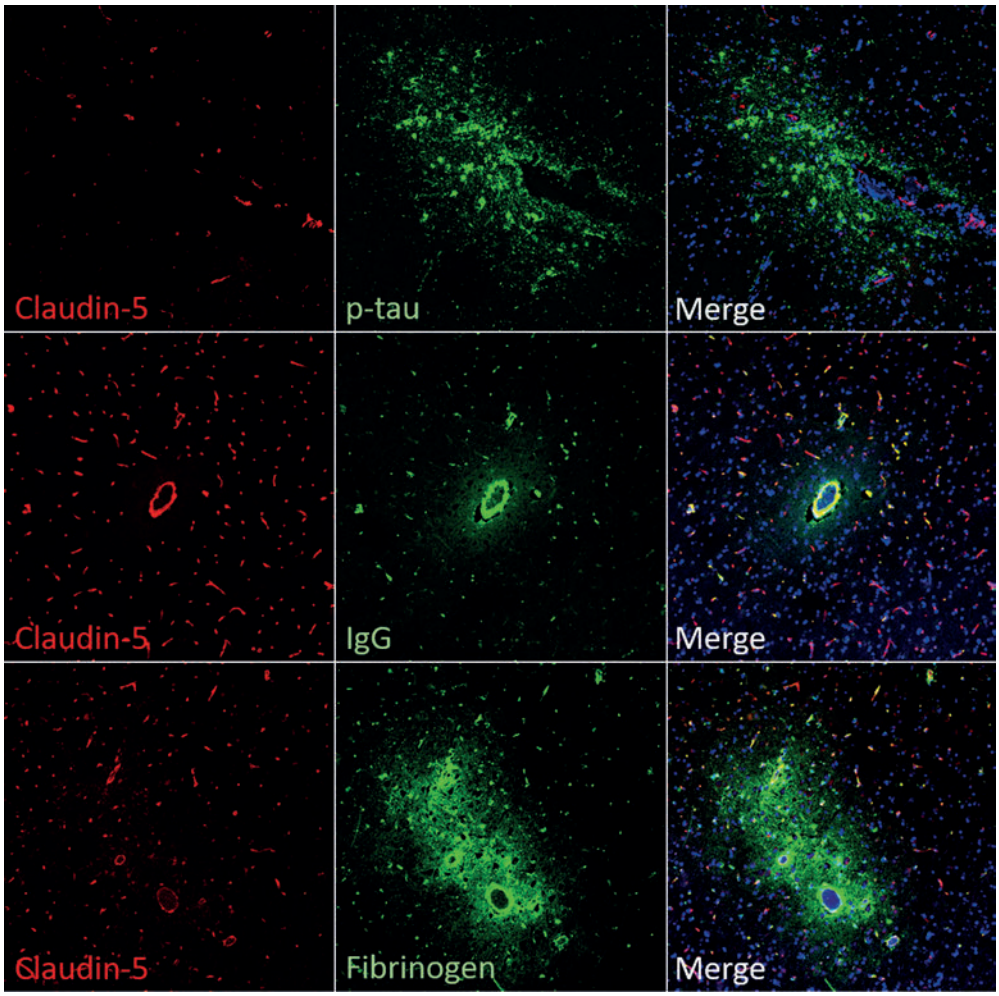


Figure 3. Tight junction protein and p-τ expression at the blood-brain barrier of a former boxer diagnosed with chronic traumatic encephalopathy (CTE). Top: Claudin-5 expression (red) and p-τ expression (green) in CTE brain. Discontinuous and loss of claudin-5 staining co-localized with p-τ deposition in perivascular regions. Mid: Claudin-5 expression (red) and leakage of IgG (green) in CTE brain. IgG leakage was prominent in large vessels with no IgG leakage evident in nearby capillaries. Bottom: Claudin-5 expression (red) and leakage of (green) in CTE brain. Fibrinogen leakage was prominent in larger vessels and some nearby small vessels.

of endogenous blood components [17]. The importance of the BBB is highlighted by the severe pathologies of diseases in which the BBB is compromised. Many of the symptoms of stroke, brain trauma, and edema are due to a breakdown of the BBB following the primary insult [18]. Additionally, recent studies have highlighted the neurobehavioral complications that can arise from BBB dysfunction. Targeted suppression of claudin-5 in the nucleus accumbens can lead to social in a mouse model of depression [19]. Further to this, knockdown of claudin-5 in mice manifests as a range of phenotypic correlates of schizophrenia and other psychiatric disorders [20]. This case is of particular interest given the diagnosis of paranoid schizophrenia prior to the post-mortem diagnosis of CTE. It is possible the subject presented with CTE at the time of diagnosis of schizophrenia as many clinical features of schizophrenia are also associated with CTE. Incidence of paranoia in individuals with CTE is high [8], and coupled with the history of professional boxing, schizophrenia may have been mistaken for CTE. Post-mortem analysis of brain sections from the subject revealed disruption of the BBB localized to regions of perivascular deposition of p-τ with notable loss of the tight junction protein claudin-5. Indeed, there is accumulating evidence suggesting that anomalies of the microvasculature and BBB are involved in the pathogenesis of schizophrenia [21, 22]. In

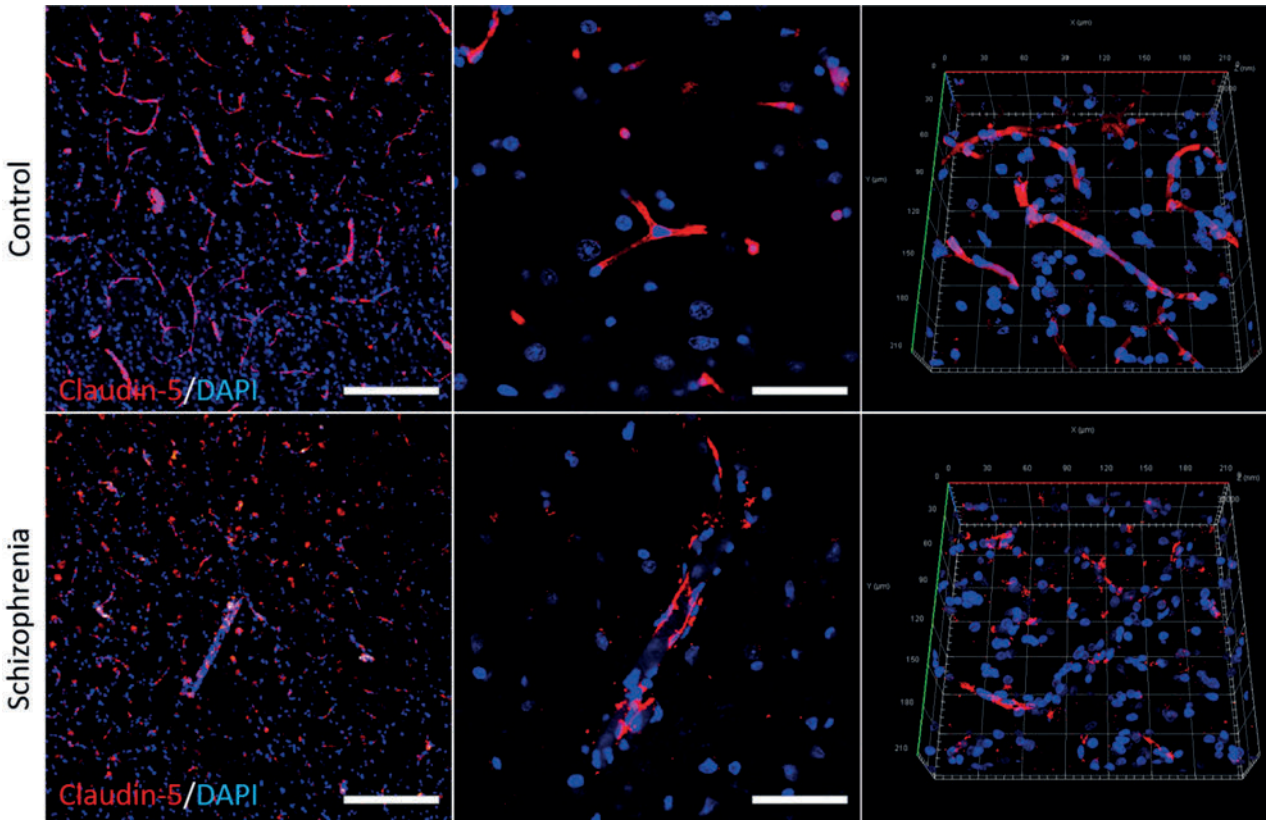


Figure 4. Claudin-5 expression in the parietal cortex in the brain of an individual with schizophrenia and an age-matched control. Normal claudin-5 immunoreactivity was evident in control brain tissue (n = 24), while discontinuous claudin-5 immunoreactivity was apparent in schizophrenic brains (n = 24). 3D-reconstructed images of z-stack series reveal discontinuous claudin-5 immunoreactivity in the schizophrenic brain. Scale bar: 200 μm (left), 50 μm (middle).

particular, alterations in expression of tight junction components have been observed in schizophrenia patients compared to controls with 12/21 tight junction related transcripts reduced [23]. Additionally, similar to our here, expression of claudin-5 protein is decreased in the prefrontal cortex of schizophrenia patients [24]. Further evidence for the involvement of the BBB in schizophrenia comes from cerebrospinal (CSF) and serum studies of markers of BBB dysfunction. Notably, S100β is increased in the blood, brain, and serum of individuals with schizophrenia [25-27]. Likewise, CSF levels of albumin are increased in schizophrenia patients [28]. Claudin-5 is vital to BBB function with loss of claudin-5 lethal in mice [20]. As claudin-5 regulates paracellular movement of material, loss of this protein may facilitate entry of low molecular weight blood components, thus impacting homeostasis of the neural microenvironment. In addition, mutations in the claudin-5 gene have been associated with schizophrenia [29].

The pattern of expression of claudin-5 in the brain of a boxer with CTE and schizophrenia in regions of perivascular p-τ deposition highlights the potential role of a dysfunctional BBB in tauopathies. Indeed, BBB dysfunction may result in abnormal clearance of τ from the brain leading to aggregation and deposition of this neurotoxic protein. Given these and underscoring the crucial role of the BBB in maintaining normal neurological function, examining BBB function in individuals involved in contact sports may be paramount to identifying individuals at risk of developing CTE.

Funding

This work was supported by grants from Science Foundation Ireland (SFI), (12/YI/B2614; 08/IN.I/B2033), The Health Research Board of Ireland (HRB), the Bright Focus Foundation and St James' Hospital Research Foundation.

Conflict of interest

References

[1] *McKee AC, Cairns NJ, Dickson DW, Folkerth RD, Keene CD, Litvan I, Perl DP, Stein TD, Vonsattel JP, Stewart W, Tripodis Y, Crary JF, Bieniek KF, Dams-O'Connor K, Alvarez VE, Gordon WA*; *TBI/CTE group*. The NINDS/NIBIB consensus meeting to neuropathological criteria for the diagnosis of chronic traumatic encephalopathy. Acta Neuropathol. 2016; *131:* 75-86.

[2] *Stern RA, Daneshvar DH, Baugh CM, Seichepine DR, Montenigro PH, Riley DO, Fritts NG, Stamm JM, Robbins CA, McHale L, Simkin I, Stein TD, Alvarez VE, Goldstein LE, Budson AE, Kowall NW, Nowinski CJ, Cantu RC, McKee AC*. Clinical presentation of chronic traumatic encephalopathy. Neurology. 2013; *81:* 1122-1129.

[3] *Gardner RC*, *Yaffe K*. Epidemiology of mild traumatic brain injury and neurodegenerative disease. Mol Cell Neurosci. 2015; *66:* 75-80.

[4] *Mez J, Stern RA, McKee AC*. Chronic traumatic encephalopathy: where are we and where are we going? Curr Neurol Neurosci Rep. 2013; *13:* 407-407.

[5] *Roberts GW, Allsop D, Bruton C*. The occult aftermath of boxing. J Neurol Neurosurg Psychiatry. 1990; *53:* 373-378.

[6] *Goldstein LE, Fisher AM, Tagge CA, et al*. Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model. Sci Transl Med. 2012; *4:* 134ra60.

[7] *Ling H, Morris HR, Neal JW, Lees AJ, Hardy J, Holton JL, Revesz T, Williams DD*. Mixed pathologies including chronic traumatic encephalopathy account for dementia in retired association football (soccer) players. Acta Neuropathol. 2017; *133:* 337-352.

[8] *McKee AC, Cantu RC, Nowinski CJ, Hedley-Whyte ET, Gavett BE, Budson AE, Santini VE, Lee HS, Kubilus CA, Stern RA*. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol. 2009; *68:* 709-735.

[9] *Omalu B, Hammers JL, Bailes J, Hamilton RL, Kamboh MI, Webster G, Fitzsimmons RP*. Chronic traumatic encephalopathy in an Iraqi war veteran with posttraumatic stress disorder who committed suicide. Neurosurg Focus. 2011; *31:* E3.

[10] *Omalu BI, DeKosky ST, Minster RL, Kamboh MI, Hamilton RL, Wecht CH*. Chronic traumatic encephalopathy in a National Football League player. Neurosurgery. 2005; *57:* 128-134, discussion 128-134.

[11] *Tagge CA, Fisher AM, Minaeva OV, Gaudreau-Balderrama A, Moncaster JA, Zhang XL, Wojnarowicz MW, Casey N, Lu H, Kokiko-Cochran ON, Saman S, Ericsson M, Onos KD, Veksler R, Senatorov VV Jr, Kondo A, Zhou XZ, Miry O, Vose LR, Gopaul KR, et al*. Concussion, microvascular injury, and early tauopathy in young athletes after impact head injury and an impact concussion mouse model. Brain. 2018; *141:* 422-458.

[12] *Stein TD, Alvarez VE, McKee AC*. Concussion in chronic traumatic encephalopathy. Curr Pain Headache Rep. 2015; *19:* 47.

[13] *Banks WA, Kovac A, Majerova P, Bullock KM, Shi M, Zhang J*. Tau proteins cross the blood-brain barrier. J Alzheimers Dis. 2017; *55:* 411-419.

[14] *Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ*. Structure and function of the blood-brain barrier. Neurobiol Dis. 2010; *37:* 13-25.

[15] *Doherty CP, O'Keefe E, Wallace E, Loftus T, Keaney J, Kealy J, Humphries MM, Molloy MG, Meaney JF, Farrell M, Campbell M*. Blood-brain barrier dysfunction as a Hallmark Pathology in chronic traumatic encephalopathy. J Neuropathol Exp Neurol. 2016; *75:* 656-662.

[16] *Shenton ME, Hamoda HM, Schneiderman JS, Bouix S, Pasternak O, Rathi Y, Vu MA, Purohit MP, Helmer K, Koerte I, Lin AP, Westin CF, Kikinis R, Kubicki M, Stern RA, Zafonte R*. A review of magnetic resonance imaging and diffusion tensor imaging in mild traumatic brain injury. Brain Imaging Behav. 2012; *6:* 137-192.

[17] *Başkaya MK, Rao AM, Doğan A, Donaldson D, Dempsey RJ*. The biphasic opening of the blood-brain barrier in the cortex and hippocampus after traumatic brain injury in rats. Neurosci Lett. 1997; *226:* 33-36.

[18] *Weiss N, Miller F, Cazaubon S, Couraud PO*. The blood-brain barrier in brain homeostasis and neurological diseases. Biochim Biophys Acta. 2009; *1788:* 842-857.

[19] *Menard C, Pfau ML, Hodes GE, Kana V, Wang VX, Bouchard S, Takahashi A, Flanigan ME, Aleyasin H, LeClair KB, Janssen WG, Labonté B, Parise EM, Lorsch ZS, Golden SA, Heshmati M, Tamminga C, Turecki G, Campbell M, Fayad ZA, et al*. Social stress induces neurovascular pathology promoting depression. Nat Neurosci. 2017; *20:* 1752-1760.

[20] *Greene C, Kealy J, Humphries MM, Gong Y, Hou J, Hudson N, Cassidy LM, Martiniano R, Shashi V, Hooper SR, Grant GA, Kenna PF, Norris K, Callaghan CK, Islam MD, O'Mara SM, Najda Z, Campbell SG, Pachter JS, Thomas J, et al*. Dose-dependent expression of claudin-5 is a modifying factor in schizophrenia. Mol Psychiatry. 2017; doi: 10.1038/mp.2017. epub ahead of print.

[21] *Najjar S, Pahlajani S, De Sanctis V, Stern JNH, Najjar A, Chong D*. Neurovascular unit dysfunction and blood-brain barrier hyperpermeability contribute to schizophrenia neurobiology: A theoretical integration of clinical and experimental evidence. Front Psychiatry. 2017; *8:* 83.

[22] *Najjar S, Pearlman DM, Alper K, Najjar A, Devinsky O*. and psychiatric *10:* 43.

[23] *Enwright Iii JF, Huo Z, Arion D, Corradi JP, Tseng G, Lewis DA*. Transcriptome alterations of prefrontal cortical parvalbumin neurons in schizophrenia. Mol Psychiatry. 2017; doi: 10.1038/mp.2017.216.

[24] *Nishiura K, Ichikawa-Tomikawa N, Sugimoto K, Kunii Y, Kashiwagi K, Tanaka M, Yokoyama Y, Hino M, Sugino T, Yabe H, Takahashi H, Kakita A, Imura T, Chiba H*. PKA activation and endothelial claudin-5 breakdown in the schizophrenic prefrontal cortex. Oncotarget. 2017; *8:* 93382-93391.

[25] *Lara DR, Gama CS, Belmonte-de-Abreu P, Portela LV, Gonçalves CA, Fonseca M, Hauck S, Souza DO*. Increased serum S100B protein in schizophrenia: a study in medication-free patients. J Psychiatr Res. 2001; *35:* 11-14.

[26] *Rothermundt M, Missler U, Arolt V, Peters M, Leadbeater J, Wiesmann M, Rudolf S, Wandinger KP, Kirchner H*. Increased S100B blood levels in unmedicated and treated schizophrenic patients are correlated with negative symptomatology. Mol Psychiatry. 2001; *6:* 445-449.

[27] *Schroeter ML, Abdul-Khaliq H, Frühauf S, Höhne R, Schick G, Diefenbacher A, Blasig IE*. Serum S100B is increased during early treatment with antipsychotics and in schizophrenia. Schizophr Res. 2003; *62:* 231-236.

[28] *Bechter K, Reiber H, Herzog S, Fuchs D, Tumani H, Maxeiner HG*. Cerebrospinal analysis in affective and schizophrenic spectrum disorders: of subgroups with immune responses and blood-CSF barrier dysfunction. J Psychiatr Res. 2010; *44:* 321-330.

[29] *Sun ZY, Wei J, Xie L, Shen Y, Liu SZ, Ju GZ, Shi JP, Yu YQ, Zhang X, Xu Q, Hemmings GP*. The CLDN5 locus may be involved in the vulnerability to schizophrenia. Eur Psychiatry. 2004; *19:* 354-357.

Appendix II

J Neuropathol Exp Neurol
Vol. 75, No. 7, July 2016, pp. 656–662
doi: 10.1093/jnen/nlw036

OXFORD

ORIGINAL ARTICLE

Blood–Brain Barrier Dysfunction as a Hallmark Pathology in Chronic Traumatic Encephalopathy

Colin P. Doherty, MD, FRCPI, Eoin O'Keefe, MSc, Eugene Wallace, MB BCh, MRCPI, Teresa Loftus, BSc, James Keaney, PhD, John Kealy, PhD, Marian M. Humphries, MSc, PhD, Michael G. Molloy, FRCP, FRCPI, James F. Meaney, FRCR, FFRRCSI, FRCP, Michael Farrell, FRCPI, FRCPC, FRCPath, and Matthew Campbell, PhD

Abstract

Chronic traumatic encephalopathy (CTE) is a neurodegenerative condition associated with repetitive mild traumatic brain injury. In recent years, attention has focused on emerging evidence linking the development of CTE to concussive injuries in athletes and military personnel; however, the underlying molecular pathobiology of CTE remains unclear. Here, we provide evidence that the blood–brain barrier (BBB) is disrupted in regions of dense perivascular p-Tau accumulation in a case of CTE. Immunoreactivity patterns of the BBB-associated tight junction components claudin-5 and zonula occludens-1 were markedly discontinuous or absent in regions of perivascular p-Tau deposition; there was also immunohistochemical evidence of a BBB in these foci. Because the patient was diagnosed premortem clinically as having progressive supranuclear palsy (PSP), we also compromised that the CTE alterations appear to be distinct from those in the brain of a patient with PSP. This report represents the first description of BBB dysfunction in a pathologically proven CTE case and suggests a vascular component in the postconcussion cascade of events that may ultimately lead to development of a progressive degenerative disorder. BBB dysfunction may represent a correlate of neural dysfunction in live subjects suspected of being at risk for development of CTE.

Key Words: Blood–brain barrier, Chronic traumatic encephalopathy, Claudin-5, Tight junctions.

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death in children and young adults globally (1). Malignant brain swelling and a breakdown in the integrity of the blood–brain barrier (BBB) are central features of the pathophysiology that evolves after severe TBI (2–4). While major brain injury is a risk in modern contact sports, the number of deaths and legacy of continuing disability emanating from sports-related head injury appear at first glance to be small. A far greater challenge is the occurrence of concussive and subconcussive injuries, which fall within the spectrum of mild TBI. Indeed, in recent years, chronic traumatic encephalopathy (CTE) has come to the fore as a neurologic condition associated with repetitive mild TBI in military personnel and athletes (5). The underlying molecular pathobiology associated with CTE is, however, poorly understood and numerous critiques have highlighted the lack of distinct "in-life" diagnostic criteria for CTE (6, 7). Postmortem microscopic diagnosis of CTE is almost exclusively limited to the use of antibodies directed against phosphorylated tau protein (p-Tau), with p-Tau immunoreactivity observed in a perivascular pattern and at the depths of the sulci in addition to p-Tau immunopositive glial and neuronal profiles in subpial regions and astrocytic p-Tau positive plaques (8). Given the relatively distinctive perivascular nature of p-Tau accumulation in CTE and its association with repetitive mild TBI, it can be hypothesized that the BBB may be involved in some capacity, in the development of CTE.

The BBB plays a critical role in maintaining CNS homeostasis. It has been estimated that a neuron is never more than approximately 20 μm from a capillary. Cerebral endothelial cells are distinguished from systemic endothelial cells by high electrical resistance tight junctions that limit paracellular transport between adjacent cells. Inter-endothelial cell tight junctions are comprised of a series of up to 30 interacting proteins that seal gaps between endothelial cells in the neural microvasculature. Tight junction proteins, both intracellular and membrane-bound, include junctional adhesion molecule-1 (JAM-1); claudins-3, -5, and -12; occludin; tricellulin; zonula occludens (ZO)-1, -2, and -3; and the recently discovered lipolysis-stimulated lipoprotein receptor (LSR). Together they constitute, as the name implies, a tight and highly regulated

From the Department of Neurology, Health Care Centre, Hospital 5, St James's Hospital, Dublin, Ireland (CPD, EW); Smurfit Institute of Genetics, Trinity College Dublin, University of Dublin, Dublin, Ireland (EO, JK, JoK, MMH, MC); Department of Neuropathology, Beaumont Hospital, Dublin, Ireland (TL, MF); University School of Veterinary Medicine, Basseterre, St. Kitts, West Indies (JK); Department of Medicine, University College Cork, Cork, Ireland (MGM); and Department of Radiology, St James's Hospital, Dublin, Ireland (JFM).

Send correspondence to: Matthew Campbell, PhD, Smurfit Institute of Genetics, Trinity College Dublin, University of Dublin, Lincoln Place Gate, Dublin 2, Ireland; E-mail: matthew.campbell@tcd.ie

Colin P. Doherty and Eoin O'Keefe contributed equally to this work.

This work was supported by grants from Science Foundation Ireland (12/YI/B2614; 08/IN.I/B2033), The Health Research Board of Ireland, the Bright Focus Foundation and St James' Hospital Research Foundation.

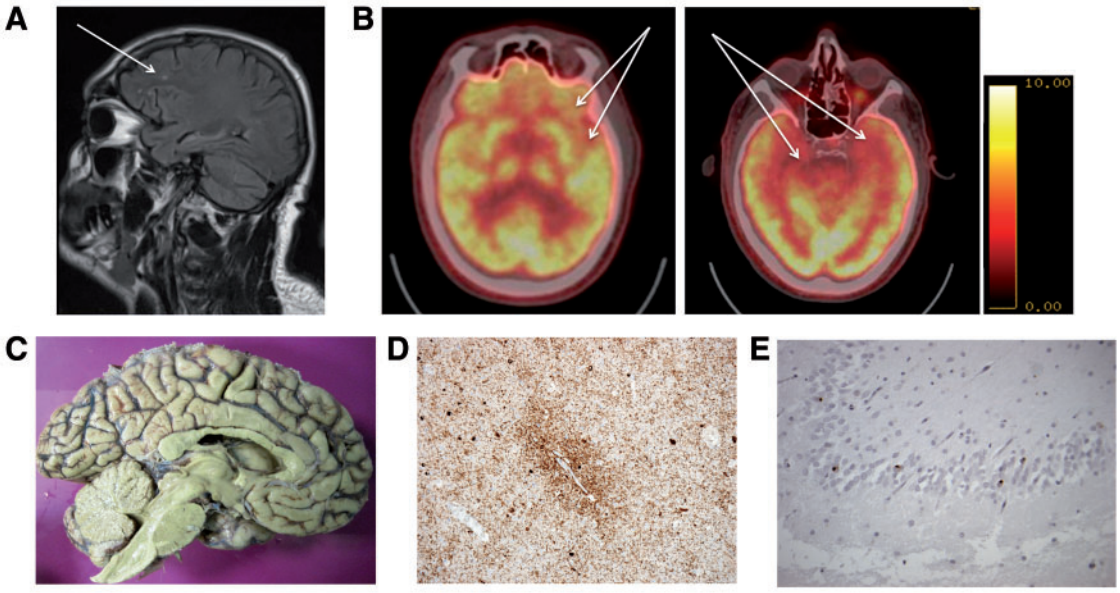


FIGURE 1. MRI and PET imaging. **(A)** Fluid attenuated inversion recovery sequence, sagittal orientation. Arrow indicates white matter abnormalities in an area of tissue sampling (left frontal). **(B)** Fludeoxyglucose (18F), FDG-PET imaging. Arrows indicate areas of hypometabolism in the left inferior frontal and anterior temporal (left) and bilateral medial temporal areas (right). **(C)** Medial view of the left cerebral hemisphere showing minimal anterior frontal atrophy. **(D)** Perivascular phospho-Tau deposition. **(E)** Cytoplasmic TDP-43 accumulation in dentate fascia neurons.

barrier, even to very small molecules. In addition to regulating the exchange of ions and macromolecules between the blood and the delicate neural microenvironment, cerebral endothelial cells protect the brain by restricting entry of potentially damaging blood–borne agents, such as neurotoxic chemicals, antibodies, pathogens, immune cells, and anaphylatoxins (9). Claudin-5 is the most enriched tight junction protein and is likely involved in mediating size-selective passive diffusion of material at the BBB. In addition, claudin-5 levels have been shown to be dysregulated in many neurological conditions (10).

We sought to examine the pattern of expression of claudin-5 in relation to p-Tau in a confirmed case of CTE. Here, we report evidence of localized BBB dysfunction in an individual originally diagnosed clinically with progressive supranuclear palsy (PSP) and in whom CTE was confirmed at postmortem brain examination.

MATERIALS AND METHODS

Clinical History and Diagnosis

Briefly, the clinical details are as follows and are provided in more detail in a related manuscript (11). A 56-year-old man presented for the first time in 2011 after an episode of acute confusion. He had a 5-year history of cognitive dysfunction ranging from memory and organizational problems to difficulty managing his day-to-day working life. He was a former rugby union player with a very long career that began in his teens and extended to the age of 50. As noted in the clinical details in another manuscript, the subject experienced numerous head injuries during his playing career that would likely have amounted to a clinical diagnosis of concussion (11). While it is acknowledged that the retrospective history of concussion is fraught, in this case, the history was very clear and the individual's on-field behavior was noted for its physicality as was reported by numerous playing colleagues as well as by family members.

There was no history of participation in other sports such as boxing or martial arts. Notably, the subject was diagnosed with PSP in life based on the cognitive profile and diagnostic motor features such as vertical gaze palsy and axial rigidity with falls. His clinical deterioration continued and after a period of hospitalization, he died 1 year later of hypostatic pneumonia. Neuropathology (as reported elsewhere), and outlined here in Figure 1, confirmed the diagnosis of CTE (11).

Immunofluorescence

We sought to explore the hypothesis that the diagnostic perivascular distribution of p-Tau was related to a compromise of the BBB. To explore this hypothesis, we examined the integrity of the BBB using previously described techniques pioneered in our laboratory.

Slides of sectioned brain tissue (8 μm) from individuals diagnosed with PSP and CTE, and sectioned tissue from a control brain, were provided by the Dublin Brain Bank. Tissue sections were permeabilized using 0.5% Triton-X (Cat. #T8787-100ML, Sigma, St. Louis, MO) for 20 minutes followed by blocking in 5% Normal Goat Serum (Cat. #G9023-10ML, Sigma) for 40 minutes. Double-labeled immunofluorescence was carried out on the sections with overnight incubations at 4°C in primary antibodies: rabbit anti-claudin-5 (1:250 dilution; Cat. #34-1600, Source Bioscience, Nottingham, UK) and mouse anti-phospho-Tau (1:250 dilution; Cat. #90206, Innogenetics, Ghent, Belgium). After incubation,

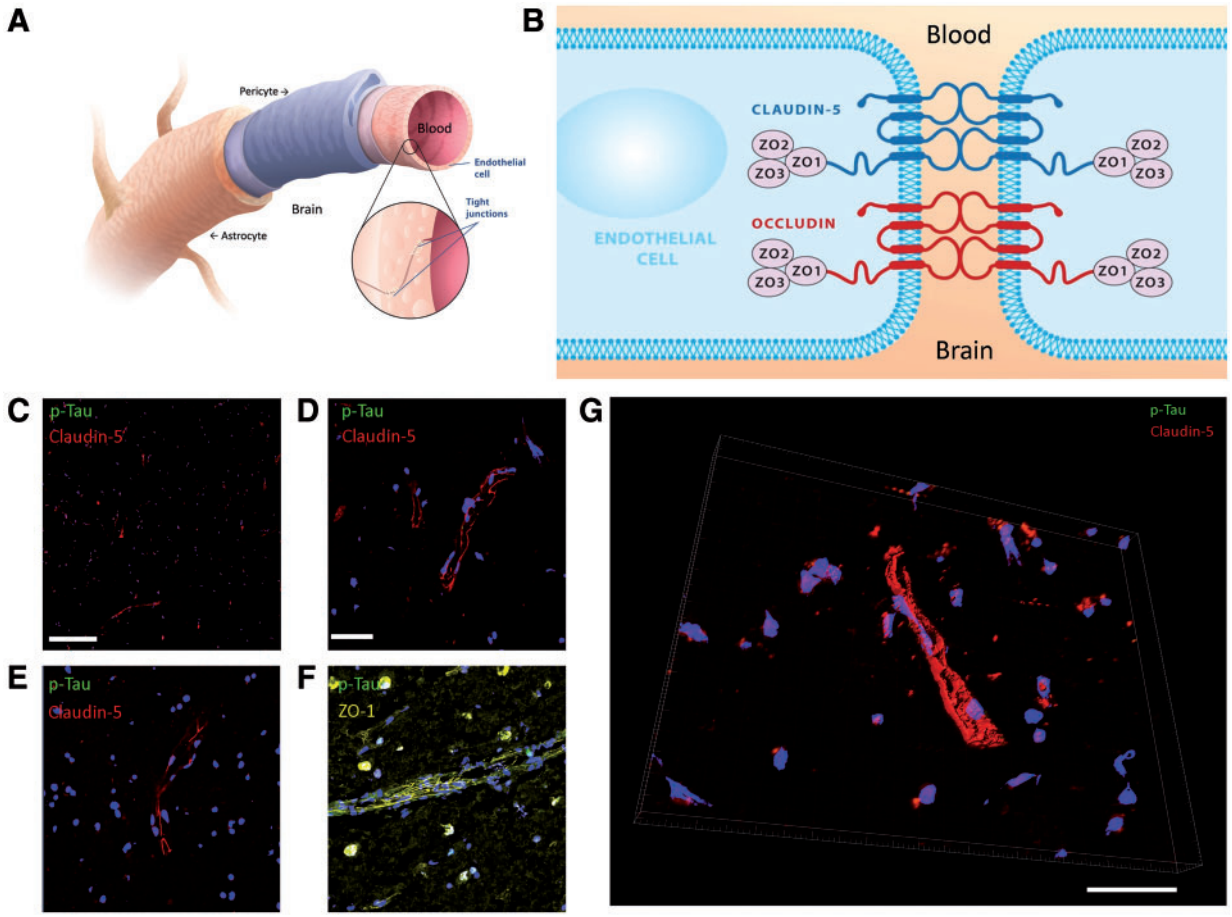


FIGURE 2. Tight junction expression at the blood–brain barrier (BBB) in a normal human control brain sample. **(A)** Schematic representation of the human BBB. **(B)** Schematic representation of tight junction molecules associated with the BBB. **(C)** Claudin-5 expression (red) and p-Tau (green) in normal control brain (10X objective; scale bar: 100 μm). **(D, E)** Claudin-5 (red) and p-Tau (green) expression in normal control brain sample (40X objective; scale bar: 20 μm [applies to **E–G**]). **(F)** Zonula occludens-1 (yellow) and p-Tau (green) expression. **(G)** Three-dimensional rendered image of claudin-5 (red) and p-Tau (green) expression at the BBB.

sections were washed in PBS followed by incubation in fluorescently conjugated secondary antibodies: goat anti-rabbit Cy3 conjugate (1:1000; Cat. #ab6939, Abcam, Cambridge, UK) and goat anti-mouse Alexa Fluor 488 conjugate (1:1000; Cat. #A-11001, Source Bioscience), for 3 hours at room temperature. Sections were washed with PBS and counterstained with Hoechst staining solution (Sigma; B2261-25MG) at a dilution of 1:5000 for 30 seconds. Labeling of tissues for fibrinogen and human IgG used rabbit antihuman fibrinogen antibodies (Dako, Glostrup, Denmark) and rabbit antihuman IgG (Abcam). Imaging of stained sections was carried out at room temperature using a Zeiss LSM-710 confocal microscope and a Zeiss T-PMT camera using 10X and 40X objectives.

Neuroimaging and Postmortem Review

We retrospectively examined brain MRI and PET scans obtained during life. Fluid attenuated inversion recovery (FLAIR) sequences indicated global atrophy and white matter abnormalities from the area of postmortem tissue sampling; however, these white matter changes were in keeping with a standard pattern of involutional age-related change that might be expected in an individual of similar age to this patient (Fig. 1A). We subsequently reexamined FDG-PET scans from this patient and identified evidence of very severe hypometabolism in the region of tissue sampling (left inferior and anterior temporal lobes) (Fig. 1B). A medial view of the left cerebral hemisphere at autopsy showed minimal anterior frontal atrophy (Fig. 1C). Characteristic perivascular p-Tau accumulation was evident and led to a diagnosis of CTE (Fig. 1D). Additionally, cytoplasmic TDP-43 accumulation in the cytoplasm of the dentate fascia neurons was evident, adding to the CTE diagnosis (12).

RESULTS

The cellular and molecular architecture of brain microvascular tight junctions is depicted diagrammatically in Figure 2A, B. Claudin-5 immunoreactivity was clearly visible, and it was expressed exclusively in the vasculature of a normal-aged matched healthy control brain section; there was minimal de-

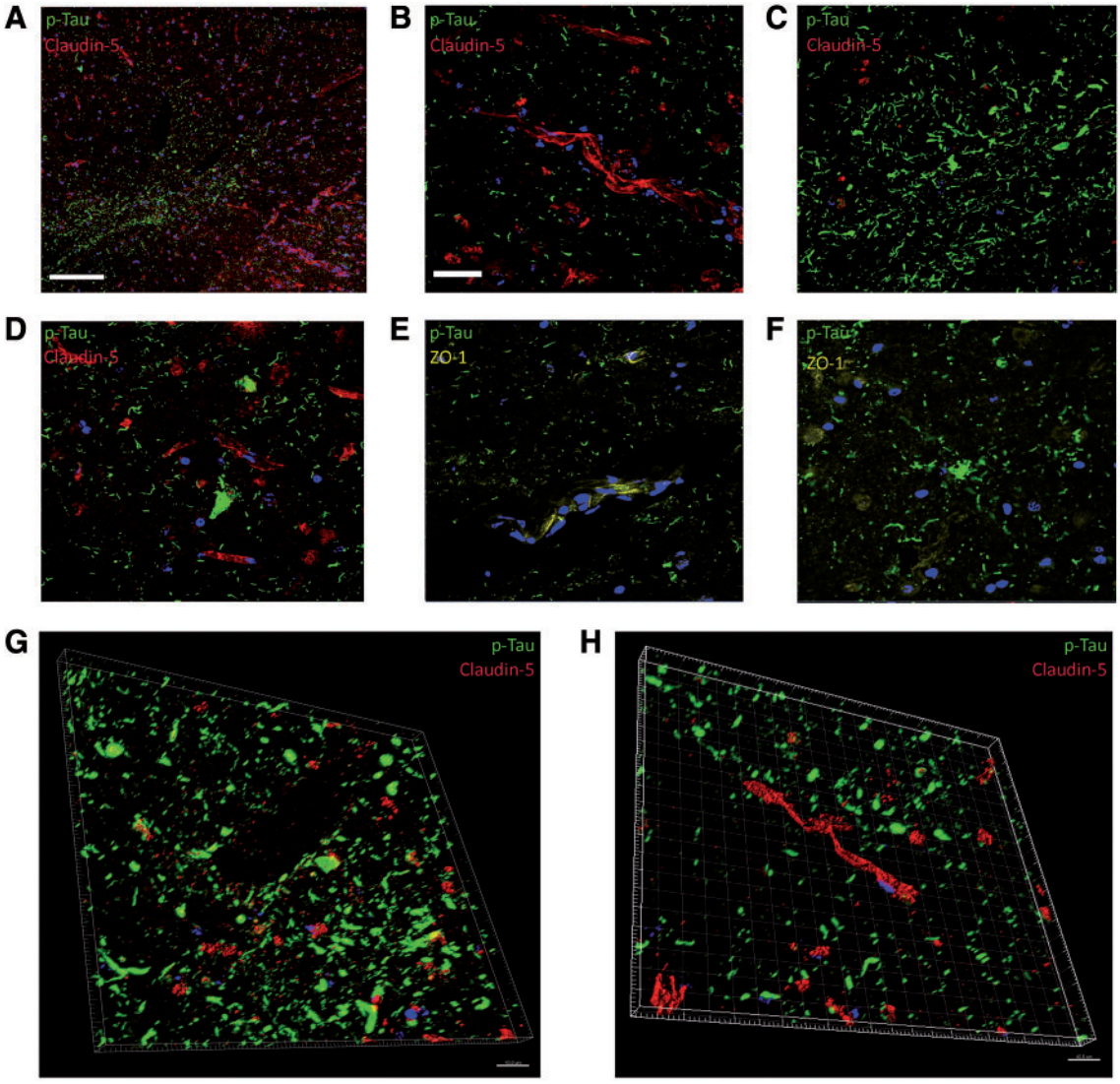


FIGURE 3. Blood–brain barrier (BBB) tight junction molecule and p-Tau immunoreactivity in patient with chronic traumatic encephalopathy (CTE). **(A)** Claudin-5 expression (red) and p-Tau (green) (10X objective; scale bar: 100 μm). **(B)** Claudin-5 (red) and p-Tau (green) expression in CTE brain (40X objective; scale bar: 20 μm, applies to **C–F**). **(C)** Aberrant claudin-5 (red) expression and p-Tau (green) (40X objective). **(D)** Claudin-5 (red) and p-Tau (green) expression in astrocytic plaque in human CTE brain. **(E, F)** Zonula occludens-1 (yellow) and p-Tau (green) expression. **(G, H)** Three-dimensional rendered image of claudin-5 (red) and p-Tau (green) expression at the human BBB in CTE brain. Scale bars: 20 μm.

tection of p-Tau in the normal human brain (Fig. 2C–F). Zonula occludens-1 (ZO-1) immunoreactivity was similarly equally abundant in the normal brain microvasculature (Fig. 2F). Three-dimensional reconstruction of confocal Z-stack images highlighted the spatial pattern of claudin-5 expression in the brain (Fig. 2G).

Detailed analysis of sections of the left medial frontal lobe of the CTE case showed an aberrant immunoreactivity pattern of claudin-5 in relation to p-Tau. In regions of dense perivascular p-Tau expression, claudin-5 was almost exclusively absent or expression was nonlinear and not localized to the tight junction (Fig. 3A). However, this pattern of claudin-5 expression was not pervasive throughout the section, and in regions displaying p-Tau tangles similar to those observed in Alzheimer disease, claudin-5 staining was identical to that observed in the normal control brain sample (Fig. 3B). Areas of dense perivascular p-Tau associated with larger vessels showed little or no positive immunoreactivity for claudin-5 (Fig. 3C). Similarly, in regions displaying dense p-Tau positive astrocytic plaques, claudin-5 staining was absent (Fig. 3D). The pattern of ZO-1 immunostaining in the CTE brain recapitulated that observed of claudin-5 (Fig. 3E, F). Three-dimensional rendered images derived from confocal z-stack imaging of claudin-5 and p-Tau show the spatial pattern of perivascular p-Tau in relation to claudin-5 (Fig. 3G, H).

In order to evaluate the integrity of the BBB, we stained sections for the blood component fibrinogen and human IgG as has previously been reported in postmortem samples of TBI

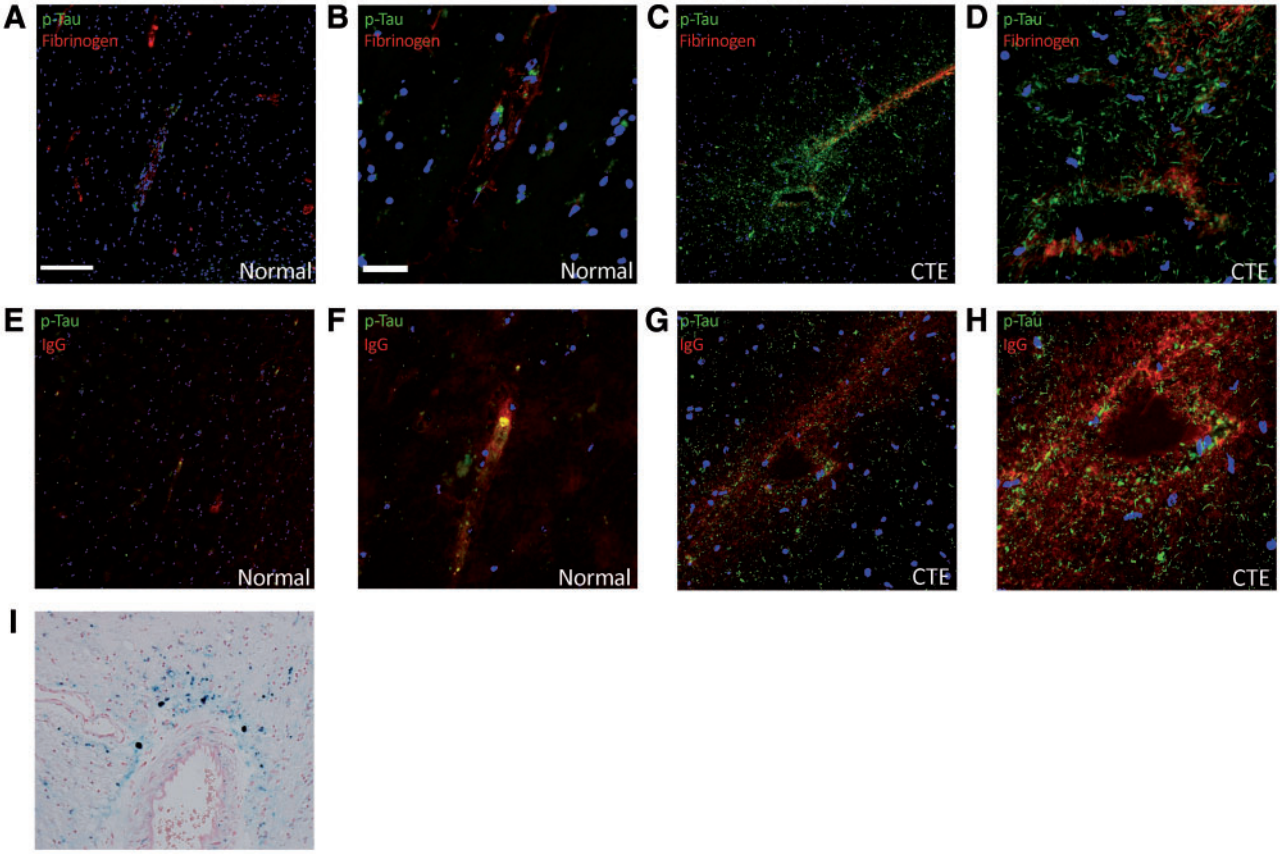


FIGURE 4. Fibrinogen and IgG extravasation in normal brain **(A, B, E, F)** and in the brain of a patient with chronic traumatic encephalopathy (CTE) **(C, D, G–I)**. **(A–D)** Fibrinogen (red) and p-Tau (green) expression in normal human brain **(A, B)** and CTE **(C, D)** samples. **(E–H)** Human IgG (red) and p-Tau (green) staining in normal human brain **(E, F)** and CTE brain **(G, H)** samples. **(I)** White matter perivascular hemosiderin deposition in the CTE patient brain demonstrated with Perl's stain. **(A, E, G, I)** 10X objective; scale bar: 100 μm; **(B, D, F, H)** 40X objective, scale bar: 20 μm.

(13). Extravasation of these systemic components was evident in regions of perivascular p-Tau deposition in the CTE patient brain (Fig. 4C, D, G, H), but not in the normal control brain sample (Fig. 4A, B, E, F). Additionally, white matter perivascular hemosiderin deposition as depicted by Perls Prussian blue stain was evident in this CTE case, adding to the evidence of BBB dysfunction (Fig. 4I).

The CTE patient was originally clinically diagnosed as having PSP, and areas of the brain in this patient not displaying perivascular p-Tau showed p-Tau-positive tangles similar to those observed in PSP. Therefore, we performed a similar analysis on the brain of a patient with PSP. In that patient, areas of p-Tau immunoreactivity did not necessarily correlate with aberrant claudin-5 or ZO-1 staining (Fig. 5A–H). These observations bolster the concept that BBB dysfunction may represent a distinct pathological feature of the tauopathy observed in CTE; furthermore, the globose tangles, typical of PSP, were not observed in the CTE patient.

DISCUSSION

Currently, there is a paucity of information on the underlying mechanism(s) that lead to the distinctive pattern of perivascular Tau phosphorylation characteristic of CTE. Here, we show BBB dysfunction in a patient with CTE that may shed light on these mechanisms. We demonstrate for the first time that the signature perivascular accumulation of p-Tau is associated with an identifiable compromise in the integrity of components and the function of the BBB.

BBB dysfunction transcends a range of neurological conditions, none so evidently as the malignant cerebral edema associated with severe TBI (13). The pathology of TBI has a very clear vascular component, most often manifested through shearing of small vessels (14). The degree to which these traumatically sheared vessels undergo repair and reconstitution of their endothelial lining (the functional BBB) is unclear. We show here that the BBB is compromised through claudin-5 and ZO-1 loss many years after repetitive TBI. Additionally, the BBB compromise observed is distinctly associated with perivascular p-Tau accumulation. Previously, it has been shown that cerebral vascular malformations, which are devoid of a responsive functioning BBB, are also associated with perivascular p-Tau deposition (15).

Claudin-5 is the most enriched tight junction protein at the BBB, and studies in rodents show that deletion of this gene is embryonically lethal, suggesting dosage sensitivity of the protein product. In addition, claudin-5 has 2 extracellular do-

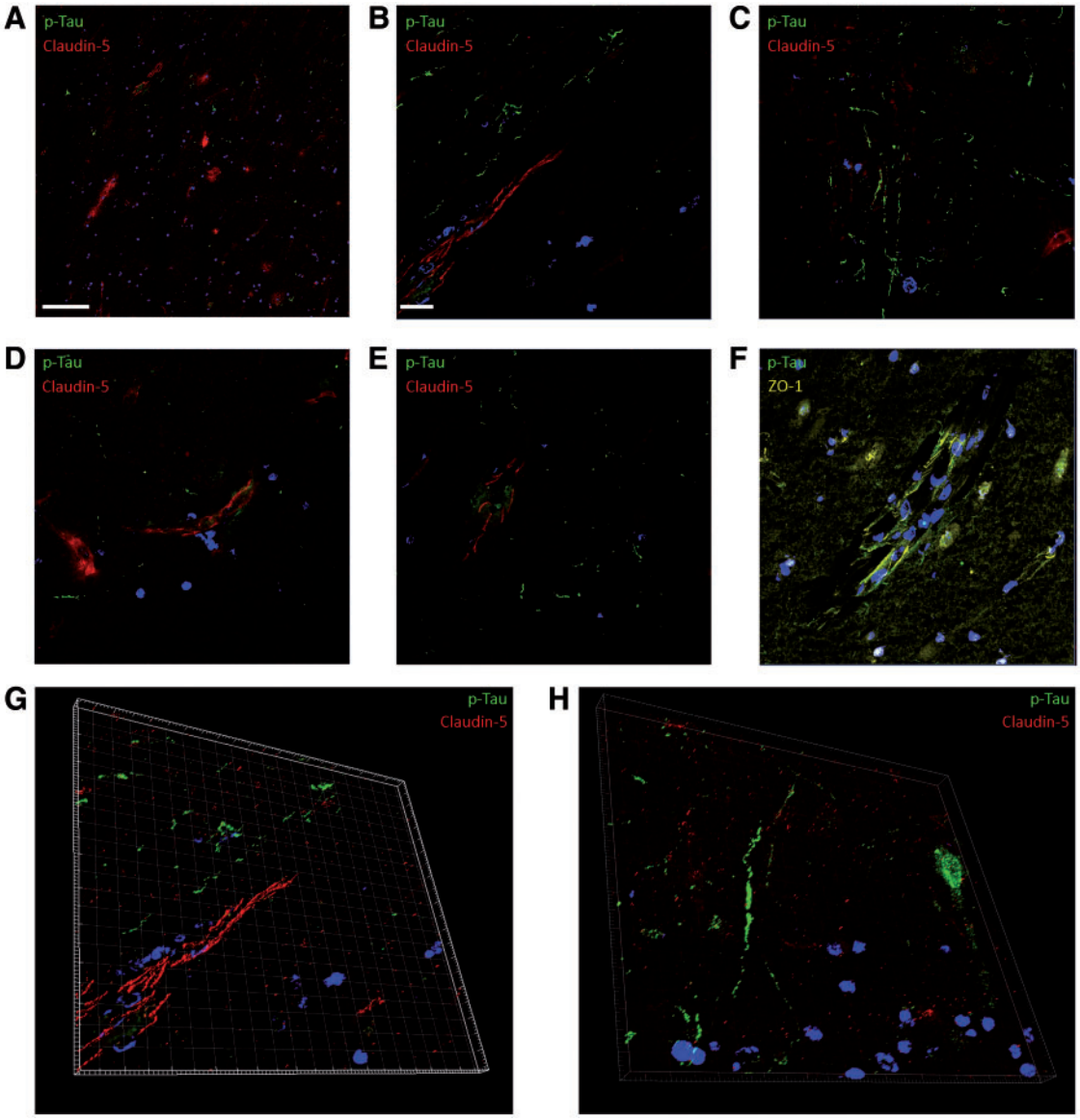


FIGURE 5. Endothelial cell tight junction immunoreactivity in the brain of a patient with PSP. **(A, B)** Normal claudin-5 expression (red) and p-Tau (green) (10X objective; scale bar: 100 μm **(A)**; (40X objective; scale bar: 20 μm) **(B)**. **(C)** Aberrant claudin-5 (red) expression and p-Tau (green) in PSP brain (40X objective; scale bar: 20 μm). **(D, E)** Linear claudin-5 (red) and p-Tau (green) expression in PSP brain. **(F)** Zonula occludens-1 (yellow) and p-Tau (green) expression (scale bar [as in **b**: 20 μm). **(G, H)** Three-dimensional rendered image of claudin-5 (red) and p-Tau (green) expression at the BBB.

mains that interact homotypically with claudin-5 molecules on adjacent endothelial cells (Fig. 2B). These domains are susceptible to environmental stimuli, and when certain residues are phosphorylated, claudin-5 localization can be discontinuous at the tight junction (16).

The patterns of claudin-5 and ZO-1 expression observed in regions of perivascular p-Tau raise interesting questions. For example, CTE may be triggered by a trauma-induced primary vascular insult (which may be repeated) before a perturbed BBB has reconstituted. A clinical correlate of a compromised BBB may be observed in the so-called "second impact syndrome"; indeed, the posttraumatic malignant brain swelling of childhood often is attributed to a perceived immaturity of the BBB. Alternatively, the primary TBI-related stretch insult to axons may impair the BBB, which in turn, could disrupt the balance between tau phosphorylation and kinase activity.

Given the importance of the BBB in maintaining neural homeostasis and taking the present findings of BBB disruption into consideration, we suggest that it may now be time to examine BBB integrity as a correlate of cognitive dysfunction in individuals suspected of being at risk for CTE.

REFERENCES

1. Hardman JM, Manoukian A. Pathology of head trauma. Neuroimaging Clin N Am 2002;12:175–87
2. Berg J, Tagliaferri F, Servadei F. Cost of trauma in Europe. Eur J Neurol 2005;12(suppl 1):85–90

3. Cassidy JD, Carroll LJ, Peloso PM, et al. Incidence, risk factors and prevention of mild traumatic brain injury: Results of the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. J Rehabil Med 2004;(suppl 43):28–60
4. Ukaszewicz AC, Soyer B, Payen D. Water, water, everywhere: Sodium and water balance and the injured brain. Curr Opin Anaesthesiol 2011; 24:138–43
5. Goldstein LE, Fisher AM, Tagge CA, et al. Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model. Sci Transl Med 2012;4(134):134ra60
6. Mez J, Solomon TM, Daneshvar DH, et al. Assessing clinicopathological correlation in chronic traumatic encephalopathy: Rationale and methods for the UNITE study. Alzheimers Res Ther 2015;7:62
7. Sundman M, Doraiswamy PM, Morey RA. Neuroimaging assessment of early and late neurobiological sequelae of traumatic brain injury: Implications for CTE. Front Neurosci 2015;9:334
8. Omalu B. Chronic traumatic encephalopathy. Prog Neurol Surg 2014;28: 38–49
9. Daneman R. The blood–brain barrier in health and disease. Ann Neurol 2012;72:648–72
10. Keaney J, Campbell M. The dynamic blood-brain barrier. FEBS J 2015; 282:4067–79
11. Stewart W, McNamara PH, Lawlor B, et al. Chronic traumatic encephalopathy: A potential late and under recognized consequence of rugby union? QJM 2016;109:11–5
12. McKee AC, Cairns NJ, Dickson DW, et al. The first NINDS/NIBIB consensus meeting to define neuropathological criteria for the diagnosis of chronic traumatic encephalopathy. Acta Neuropathol 2016;131: 75–86
13. Hay JR, Johnson VE, Young AM, et al. Blood-brain barrier disruption is an early event that may persist for many years after traumatic brain injury in humans. J Neuropathol Exp Neurol 2015;74:1147–57
14. Shenton ME, Hamoda HM, Schneiderman JS, et al. A review of magnetic resonance imaging and diffusion tensor imaging findings in mild traumatic brain injury. Brain Imaging Behav 2012;6:137–92
15. Kon T, Mori F, Tanji K, et al. Abnormal tau deposition in neurons, but not in glial cells in the cerebral tissue surrounding arteriovenous malformation. Neuropathology 2012;32:267–71
16. Nitta T, Hata M, Gotoh S, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 2003;161:653–60

Appendix III

clinical neuropathology

Neuropolypathology as a result of severe traumatic brain injury?

Colin P. Doherty[1], Eoin O'Keefe[2], James Keaney[2], Brian Lawlor[3], Robert F. Coen[3], Michael Farrell[4], and Matthew Campbell[2]

[1]Department of Neurology, Health Care Centre, Hospital 5, St James's Hospital, [2]Smurfit Institute of Genetics, Trinity College Dublin, [3]Mercer's Institute for Research on Ageing, St James's Hospital, and [4]Department of Neuropathology, Beaumont Hospital, Dublin, Ireland

ISSN 0722-5091

DOI 10.5414/NP301131
e-pub: ■■month ■■day, ■■year

Key words
blood-brain barrier (BBB) – polypathology – claudin-5

Abstract. A history of brain trauma has long been acknowledged as increasing an individual's risk of developing dementia in later life. The underlying mechanisms that belie this pre-disposition are, however, very poorly understood. Here, we report a clinical-neuropathological correlation of a man who presented at the age of 66 with a progressive complex atypical dementia with early and prominent neurobehavioral symptoms. His neurological condition continued to decline up to his death at the age of 74. During the compilation of his clinical history, it was established that the subject had experienced a single severe traumatic brain injury (TBI) aged 12 years in 1954 resulting in loss of consciousness, hospitalization, and coma for a number of days after which he was deemed to have recovered. Following post-mortem neuropathological analysis, numerous distinct neuropathologies were observed in various brain regions and these included i) widespread Braak stage VI neurofibrillary tangle formation, ii) widespread α-synuclein positive Lewy bodies and Lewy neurites and iii) diffuse amyloid plaques and severe cerebral amyloid angiopathy (CAA). Added to this, a comprehensive analysis of blood-brain barrier (BBB) integrity, known to be disrupted during and after TBI, showed iv) distinct BBB breakdown with extravasated IgG and activated microglia present. This report represents an interesting documented case of neuropolypathology that may be associated with prior history of severe TBI. We propose one testable theory that a history of brain trauma may be a potential trigger for late onset dementia due to damage and unresolved functioning of the cerebral microvasculature.

Received
July 12, 2018;
accepted in revised form
August 3, 2018

Correspondence to
Matthew Campbell,
■■■MD, …?

Genetics, Trinity College
Dublin, Dublin 2, Ireland
matthew.campbell@tcd.ie

Introduction

Malignant cerebral edema as a result of traumatic brain injury (TBI) is the single greatest cause of mortality in children and young adults globally [1, 2]. However, the long-term sequelae in those individuals who survive a moderate or severe TBI are less well and a molecular understanding of clinical prognosis is almost completely absent. A clinical history of TBI has previously been reported to increase the risk of Alzheimer's disease (AD) by up to 82% in men [3, 4]. Additionally, exposure to moderate or severe TBI has also been shown to increase the risk of non-AD dementia up to 30% and Parkinson's disease by up to 44% [5, 6, 7, 8]. It is now clear that damage to the integrity of the blood-brain barrier (BBB) is one of the earliest and potentially most cerebral insults that mediates the progression of TBI-associated neuropathology [9, 10].

In the context of dementia, -lary tangle formation manifested by the accumulation of phosphorylated τ (p-τ) protein is evident across multiple forms of brain disease. For example, p-τ tangles appearing in a diffuse pattern in the neocortex and hippocampus are classic hallmarks of AD and more diffusely throughout frontal and temporal lobes in frontotemporal Dementia (FTD). Conversely, p-τ immunoreactivity in a perivascular pattern at the depths of the cortical sulci is a hallmark pathology of chronic traumatic encephalopathy (CTE), a condition classically associated with repetitive head trauma [11]. Diffuse amyloid plaques are also a feature of AD and other dementias, with up to 80% of AD cases showing perivascular amyloid accumulation and evidence of cerebral amyloid angiopathy (CAA) [12]. Abnormal α-synuclein accumulation inside neurons, manifesting what are termed "Lewy bodies" is seen in one of the parkinsonian dementias – diffuse Lewy body dementia (DLBD) – and occasionally these bodies are seen in AD cas-

es. It is, however, exceedingly uncommon to observe all of these pathological hallmarks in a single individual, where a diagnosis could be given for DLBD, FTD, and AD in the same subject [13, 14].

Here, we report evidence of polypathology observed in a subject with progressive cognitive decline. We suggest the possibility that a history of TBI can lead to a plethora of neurological sequelae.

Clinical history and diagnosis

A 66-year-old man presented for the time in 2009, 3 years after a house move. The subject was experiencing repetitive and unexplained bouts of anger and frustration. He had memory and concentration problems related to words and names while also displaying story repetition. He did not however appear disorientated. He complained of and was treated for severe depression in reaction to the death of a friend who had been unwell. His medication consisted of clopidogril [**■■■ clopidogrel?**] and escitalopram. He had a family history of cancer and heart disease and his aunt had experienced memory prior to her death in her 80's.

On neurological examination, cranial nerves, limbs, gait, and balance were all normal.

On cognitive assessment using the Montreal Cognitive Assessment (MOCA) the subject displayed mild agitation but a good recall of events and no speech or naming problems. He scored 25 out of a possible 30 points losing marks in executive function (placing hands on clock (Figure 1g), delayed recall (2/5), and orientation (5/6). The impairment on this occasion was considered mild and the focus was on his depressive behavioral problems.

He proceeded to more formal neuropsychological assessment at the end of 2010. National Adult Reading Test (NART2) estimated at least high average intellect or higher. Global scores included the Cambridge Cognitive Examination (CAMCOG) = 94/107 and Mini-Mental State Examination (MMSE) = 27/30. Again, on this occasion, his number placement on clock drawing was normal but his time conceptualization was inaccurate. **Copy of the Rey Osterreith Complex Figure was normal.** -sive language including the Boston Naming Test (BNT) 28/30 (65th percentile), but -ency was reduced. Performance was normal on brief tests of calculation and perception. The neuropsychologist's impression was that the patient did not appear to be amnestic although his memory was occasionally patchy (encoding and retrieval). Wechsler Memory Scale (WMS-III) Immediate Auditory Index = 105 (63rd percentile) and Delayed Auditory Index = 117 (87th percenile). **Recall of the Rey Osterreith Complex Figure was normal [■■■ Test done twice?]**. There was no evidence of the rapid forgetting typical of Alzheimer's disease. There was evidence of mild executive function primarily on tests of verbal with interference/intrusion and reduced mental causing at times, and performance was slow on both trail making part A and part B. On occasion, he had moving from one task to the next with previous task intruding into the next one causing interference or loss of train of thought. Wechsler Adult Intelligence Scale (WAIS-III) similarities (verbal abstract reasoning) was 95th percentile.

An MRI was performed (Figure 1a, b, c) showing mild generalized atrophy without obvious focal atrophy and normal white mater appearance for his age. There was no clear evidence on either clinical assessment, neuropsychological assessment, nor radiologically of a neurodegenerative disease. He was seen by psychiatry and treatment increased for his mood disorder. Positron emission tomography (PET) however, showed widespread cortical hypo-metabolism suggestive of reduced function and more than might be expected with a pure psychiatric illness (Figure 1d, e, f)

During a return visit in 2011, it was noted that there was progression in symptoms with the main experienced by the subject being psychiatric and then cognitive. The subject displayed agitation, extreme stress reactions, depression, and anxiety and reported one paranoid psychotic episode. Burgeoning cognitive problems included memory and executive function Repeat MOCA 2 years after the test gave a score of 16/30 (Figure 1h).

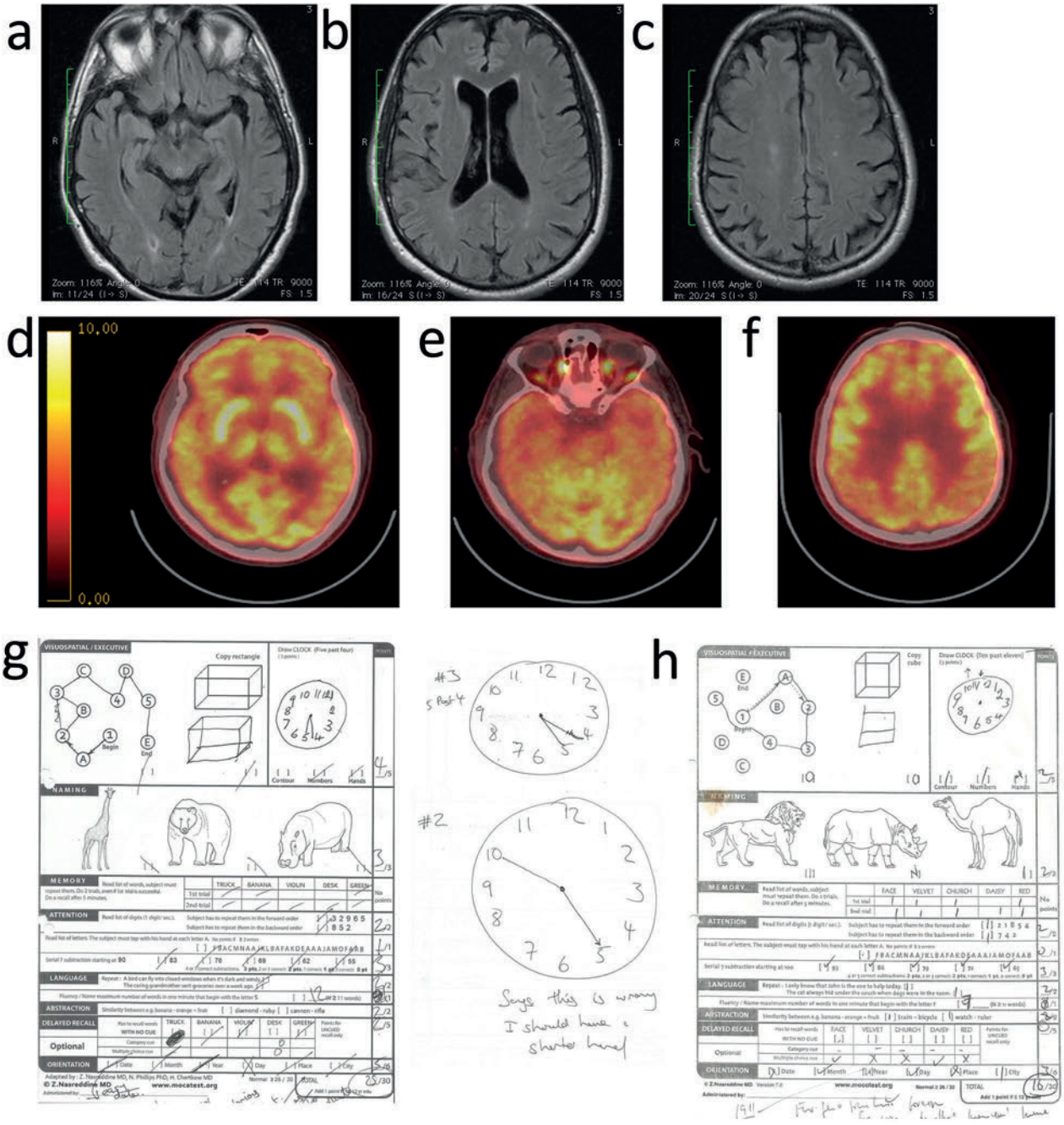


Figure 1. MRI and PET imaging. a, b, c: Axial MRI (FLAIR) images though the temporal lobes (a), the lateral ventricles (b) and the centrum semi-ovale (c). The images show mild generalized atrophy without focal lobar, cortical, or subcortical atrophy and normal white matter for his age. d, e, f: Positron emission tomography (PET) using radiolabeled though the caudate (d), the temporal lobes (e), and the centrum semiovale (f) showing generalized cortical hypometabolism. g: Montreal Cognitive Assessment (MOCA) exam 2009 with abnormal hand placement on several attempts at the clock. h: MOCA exam 2011.

There were widespread es in executive function (2/5), language (naming, 2/3 and letter , 7 in 1 minute) delayed recall (0/5), and orientation (3/6). Within a year, he was admitted to psychiatric care with paranoid delusions, low level agitation, word problems, and disorientation. He never experienced any motor or visual-spatial s. The working diagnosis in-life was of atypical Alzheimer's disease even though several typical features were missing.

His health steadily declined until his death from hypostatic pneumonia in 2016. He and his family had generously agreed to brain donation to the Dublin Brain bank in the event of his death.

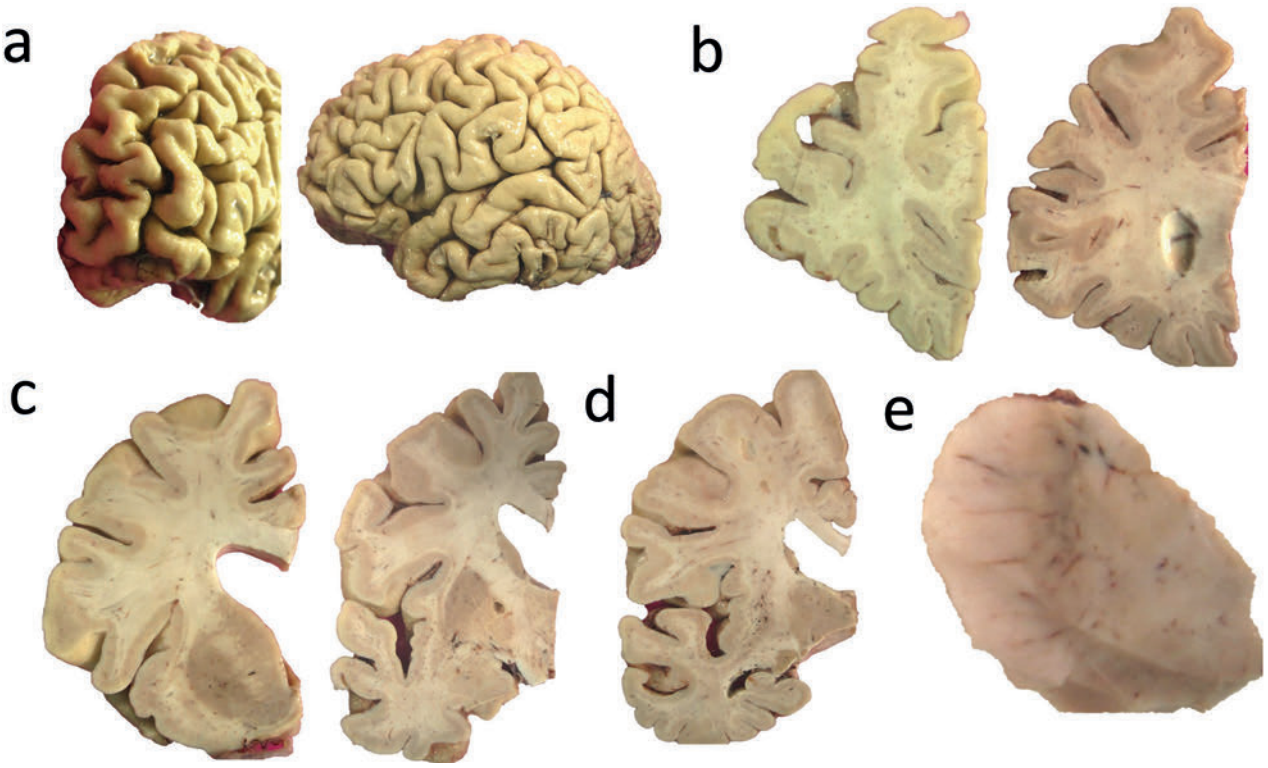


Figure 2. Macroscopic post-mortem images. a: Frontal atrophy section and frontal atrophy whole brain. b: Frontal atrophy sections. c: Ventriculomegaly and enlarged Sylvian d: Hippocampal atrophy, not present. e: Pallor of the substantia nigra.

Materials and methods

Immunohistochemistry

Slides of sectioned brain tissue (8 µm) were provided by the Dublin Brain Bank. Tissue sections were permeabilized using 0.5% Triton-X (Sigma, ■■■ **City?, Country?**, Cat. #T8787-100ML) for 20 minutes, followed by blocking in 5% normal goat serum (Sigma, Cat. #G9023-10ML) for 40 minutes. Double-labeled - cence was carried out on the sections with overnight incubations at 4 °C in primary antibodies: rabbit anti-claudin-5 (1 : 250 dilution; Source Biosciences, ■■■ **City?, Country?**, Cat. #34-1600) and mouse anti-phospho-τ (1 : 250 dilution; Innogenetics, ■■■ **City?, Country?**, Cat. #90206), (as described previously, [15]). After incubation, sections were washed in PBS followed by incubation in conjugated secondary antibodies: goat anti-rabbit Cy3 conjugate (1 : 1,000; Abcam, ■■■ **City?, Country?**, Cat. #ab6939) and goat anti-mouse Alexa Fluor 488 conjugate (1 : 1,000; Source Bioscience, Cat. #A-11001), for 3 hours at room temperature. Sections were washed with PBS and counterstained with Hoechst staining solution (Sigma; B2261-25MG) at a dilution of 1 : 5,000 for 30 seconds. Labelling of tissues for human IgG used rabbit anti-human IgG (Abcam). Labeling of tissue for human CD163 (1 : 250, Novo Castra, ■■■ **City?, Country?**, Ncl163). Imaging of stained sections was carried out at room temperature using a Zeiss LSM-710 confocal microscope and a Zeiss T-PMT camera, using 10× and 40× objectives. For the routine diagnostic neuropathology, anti-τ (AT8, 1 : 100 Innogenetics, ■■■ **City?, Country?**), anti-BA4 (6F/3D 1 : 100, Dako, ■■■ **City?, Country?**), anti-α-synuclein (LB509 1 : 1,500 Covance, ■■■ **City?, Country?**), and anti-TDP43 (1 : 1,500, Proteintech, ■■■ **City?, Country?**) were used.

Neuroimaging and post-mortem review

We examined brain MRI and PET scans obtained during the subject's life. Fluid attenuated inversion recovery sequences

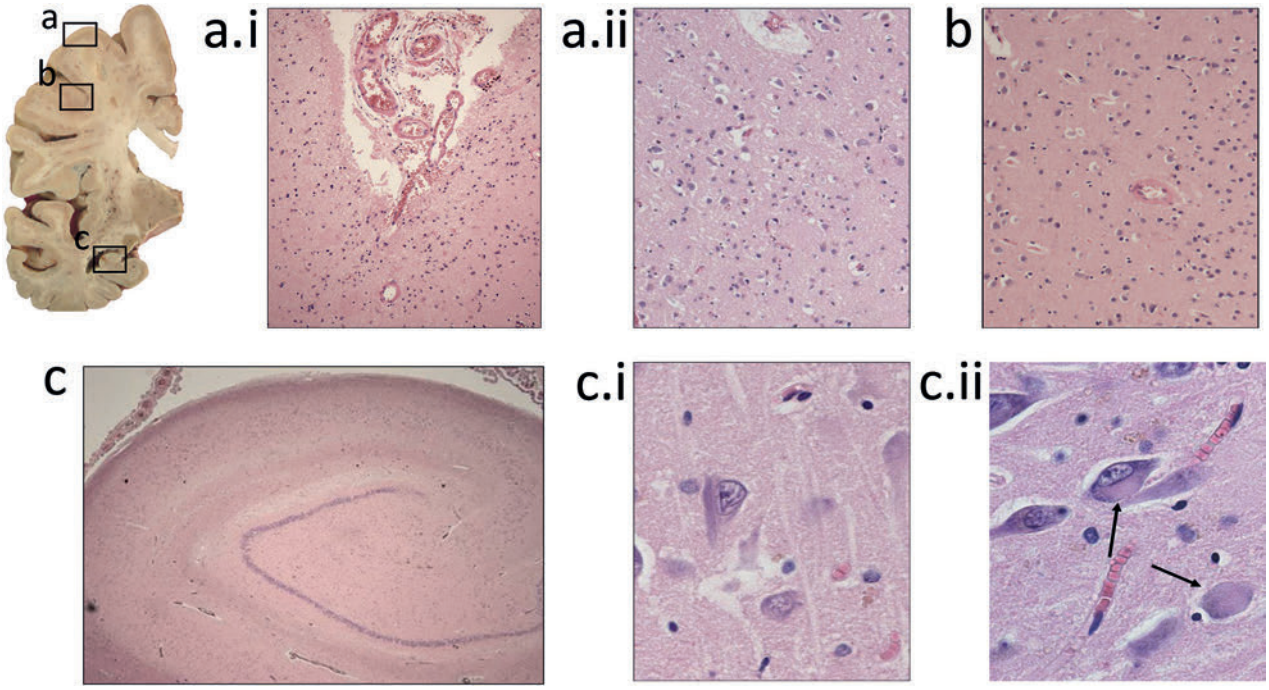


Figure 3. Hyaline changes and hippocampal tangles. a: i) Hyaline change. ii) spongiosis. b: Hyaline change in parenchyma. c: Hippocampal atrophy not present. i) Hippocampus tangles frequent. ii) Hippocampal pyramidal inclusions present.

(FLAIR) indicated a degree of atrophy and numerous abnormalities in white matter that are not uncommon with aging (Figure 1a, b, c). FDG-PET scans from the subject showed evidence of severe hypometabolism in regions of tissue sampling (Figure 1d, e, f).

Results

Post-mortem analysis of brain tissues showed frontal atrophy that was evident in intact and sectioned tissues (Figure 2a, b). Venticulomegaly and enlarged Sylvian - sure were observed (Figure 2c); however, hippocampal atrophy was not present (Figure 2d). Pallor of the substantia nigra was observed (Figure 2e). A post-traumatic cicatrix was present in the inferior frontal regions which caused the arachnoid to adhere to the dura, but contusions were not evident.

Distinctive hyaline changes were observed in meningeal vessels (Figure 3a.i, a.ii) and this also manifested in parenchymal vessels (Figure 3b). Intriguingly, while hippocampal atrophy was not present (Figure 3c), tangles (Figure 3c.i) and pyramidal inclusions (Figure 3c.ii) were observed; with the tangles appearing to be tangles (NFTs). Severe CAA was evident in neocortical regions with diffuse non-neuritic amyloid-β plaques observed (Figure 4a.i). Frequent p-τ-positive NFTs were also evident (Figure 4a.ii) in these neocortical regions with dystrophic neurites also present (Figure 4b, c, d). Sulcal exaggeration of p-τ was not evident though astrocytic perivascular p-τ was evident in the subcortical white matter (Figure 4e).

Examination of the superior temporal gyrus (STG) showed Lewy bodies and Lewy neurites (Figure 5a) with similar α-synuclein staining apparent in the lateral frontal lobe (Figure 5b). The amygdala also showed Lewy bodies (Figure 5c) as did the substantia nigra (Figure 5d). The ABC Score was A3, B3, C2. TDP43-positive inclusions were not found. Neocortical Lewy bodies were readily ident Hippocampal sclerosis was not evident. The p-τ- and BA4-related pathology was not more advanced in the inferior frontal regions next to the post-traumatic cicatrix.

Given the presence of multiple pathologies in this case, we processed tissues to examine the integrity of the BBB. BBB dysfunction has been strongly associated with acute TBI, and indeed a disrupted BBB can manifest for many years after the initial in-

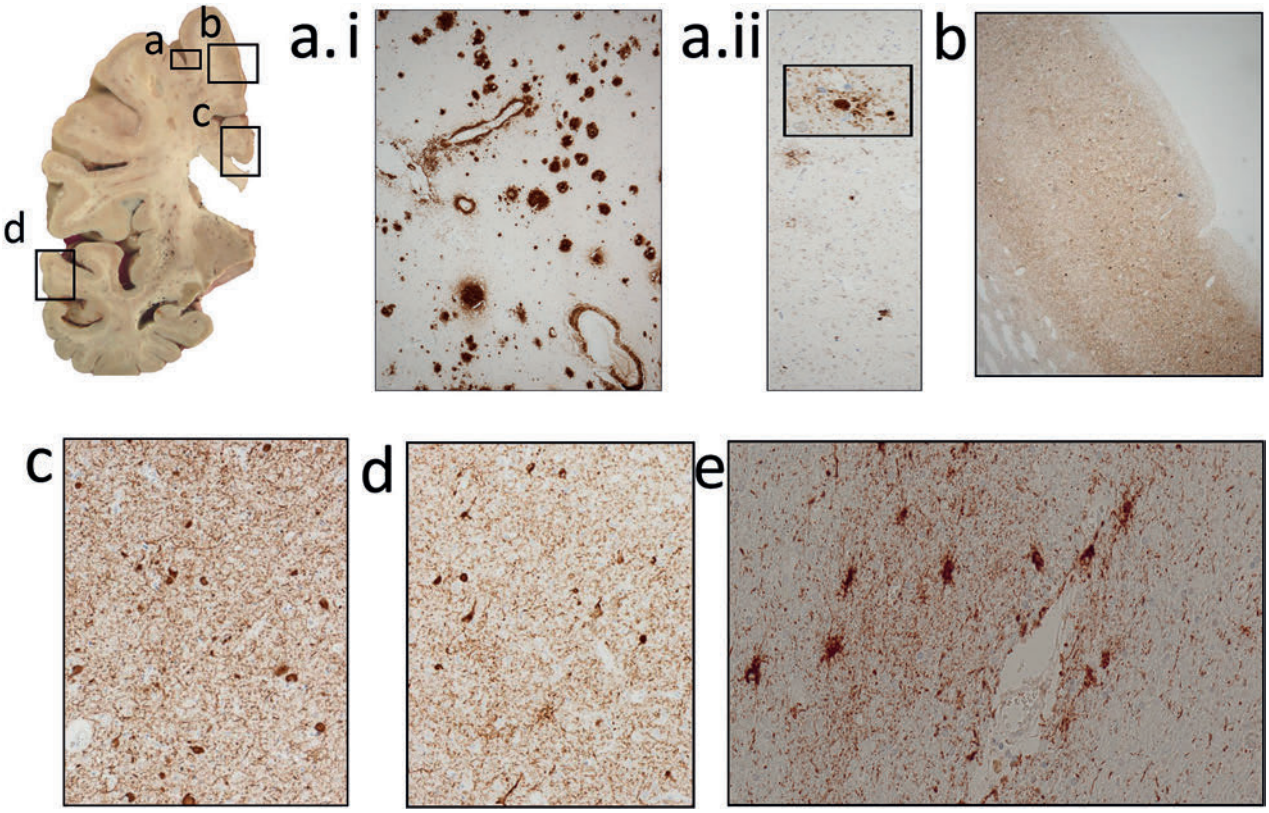


Figure 4. Amyloid-β and p-τ staining. a: i) Amyloid-β staining, severe cerebral amyloid angiopathy (CAA). ii) p-τ staining, diffuse plaques not neuritic. b: p-τ staining, tangles (NFTs), and dystrophic neurites. c, d: p-τ staining in NFTs and dystrophic neurites. e: Astrocytic perivascular p-τ was evident in the subcortical white matter.

sult [16]. Positive immunoreactivity for claudin-5, a tight junction protein enriched at the BBB, was evident in sections; however in regions of diffuse IgG extravasation, claudin-5 was absent suggestive of a disrupted BBB (Figure 6a, b, c). This also appeared to occur in regions of dense p-τ staining, where claudin-5 levels were decreased or indeed completely absent (Figure 6d, e). Interestingly, regions lacking an intact BBB showed evidence of activated microglia as manifested by positive immunoreactivity to CD163 (Figure 6f, g).

Discussion

This case report highlights the occurrence of multiple neuropathologies that may be related to a single severe TBI in a subject presenting with late-onset complex atypical dementia. Indeed, the variable nature of TBI may represent an unrecognized pre-disposing factor to late-onset dementia in the general population.

TBI is well recognized as the leading cause of death in children and young adults globally, and accounts for ~ 20% of deaths in people aged 5 – 35 years old, and accounts for 1% of all adult mortality [1, 2]. Malignant brain swelling and a breakdown in the integrity of the BBB is a central feature of the pathology that evolves after severe TBI. TBI induces an initial rapid increase in BBB permeability that appears to return to baseline levels within hours, and this loss of integrity is largely due to TBI-induced decreases in tight junctional components such as claudin-5. More detailed analyses have shown that BBB permeability can persist and remain higher for up to 5 days following TBI with the BBB allowing molecules up to 10,000 Da to access the cerebral parenchyma [17, 18, 19]. Additionally, a recent study showed that BBB integrity was still compromised in human brain samples examined many years after moderate or severe TBI [16].

The intracranial compartment consists of brain, cerebrospinal (CSF), and blood.

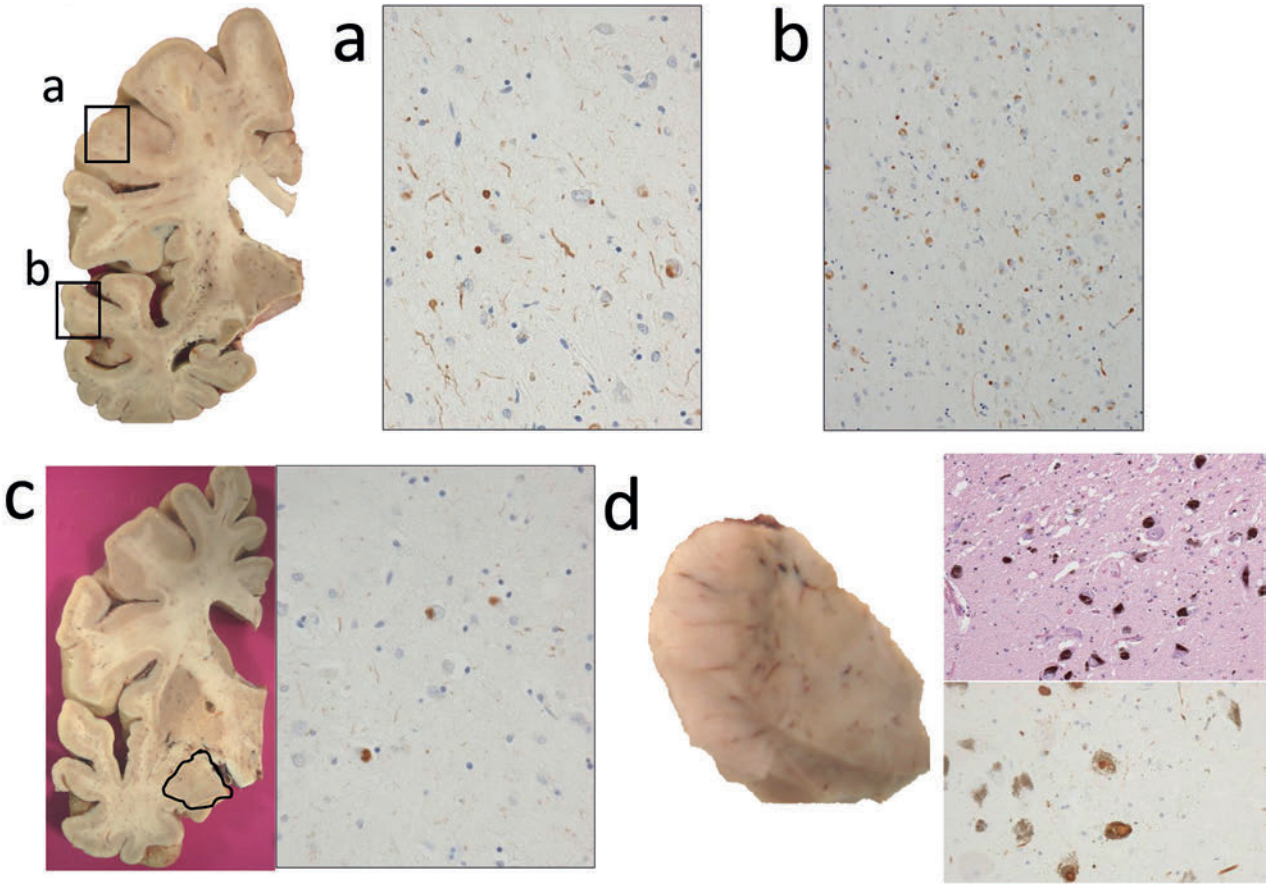


Figure 5. α-synuclein-positive Lewy body Lewy bodies and neurites present in the a) superior temporal gyrus (STG) and b) lateral frontal lobe. c: Lewy bodies in the amygdala. d: Pallor of the substantia nigra and presence of Lewy bodies.

These factors all contribute to the intra-cranial pressure (ICP). However in the case of TBI, pathological masses will exert extra pressure within the of the skull. One of the key mediators of increased ICP is a dramatic shift in water diffusion across the BBB. A build-up of water within the brain will exert rises in ICP leading to increased risks of secondary complications impacting on the nature and degree of cerebral edema [20]. It is these initial acute stresses to neural tissue architecture that may ultimately lead to the polypathology that can manifest post TBI and ultimately lead to late-onset dementia.

In this case, an isolated post-mortem diagnosis of DLBD, FTD, or AD could have been made; however, all 3 conditions were apparent in the same subject. This observation, while limited to a single case report is important as it suggests that each of these clinically independent conditions may have a common pre-disposing factor, that being TBI. The evidence of BBB dysfunction further adds to this hypothesis as it is well described in pre-clinical animal models that the BBB becomes disrupted across a range of neural injury models. Added to this, the endothelial cells of the cerebral microvasculature maintain mitotic division capacity which allows them to respond and react to neural tissue injury. It is this response in the acute and long-term setting that may be a major pre-disposing factor to eventual dementia onset given the strong cerebrovascular component to dementia progression.

Late-onset dementias are potentially linked to a prior history of moderate or severe TBI, and understanding the underlying molecular hallmarks of pathology will shed light on these devastating conditions.

Funding

This work was supported by grants from Science Foundation Ireland (SFI), (12/YI/

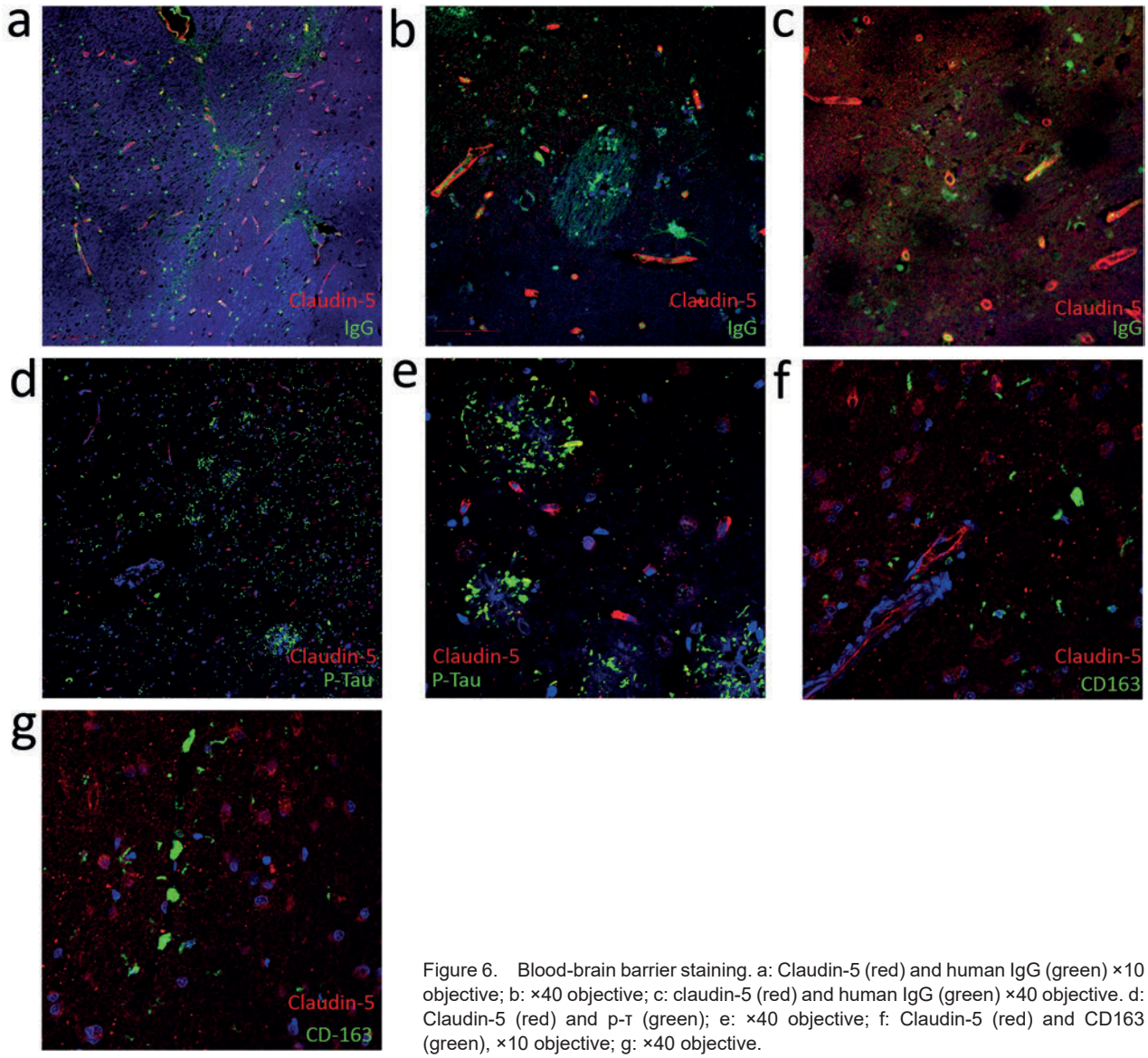


Figure 6. Blood-brain barrier staining. a: Claudin-5 (red) and human IgG (green) ×10 objective; b: ×40 objective; c: claudin-5 (red) and human IgG (green) ×40 objective. d: Claudin-5 (red) and p-т (green); e: ×40 objective; f: Claudin-5 (red) and CD163 (green), ×10 objective; g: ×40 objective.

B2614; 08/IN.I/B2033), The Health Research Board of Ireland (HRB), the Bright Focus Foundation and St James' Hospital Research Foundation.

Conflicts of interest

References

[1] *Hardman JM, Manoukian A*. Pathology of head trauma. Neuroimaging Clin N Am. 2002; *12:* 175-187.

[2] *Berg J, Tagliaferri F, Servadei F*. Cost of trauma in Europe. Eur J Neurol. 2005; *12 (Suppl 1):* 85-90.

[3] *Mortimer JA, van Duijn CM, Chandra V, Fratiglioni L, Graves AB, Heyman A, Jorm AF, Kokmen E, Kondo K, Rocca WA, Shalat SL, Soininen H*; *EURODEM Risk Factors Research Group*. Head trauma as a risk factor for Alzheimer's disease: a collaborative re-analysis of case-control studies. Int J Epidemiol. 1991; *20 (Suppl 2):* S28-S35.

[4] *Fleminger S, Oliver DL, Lovestone S, Rabe-Hesketh S, Giora A*. Head injury as a risk factor for Alzheimer's disease: the evidence 10 years on; a partial replication. J Neurol Neurosurg Psychiatry. 2003; *74:* 857-862.

[5] *Gardner RC, Burke JF, Nettiksimmons J, Kaup A, Barnes DE, Yaffe K*. Dementia risk after traumatic brain injury vs nonbrain trauma: the role of age and severity. JAMA Neurol. 2014; *71:* 1490-1497.

[6] *Rosso SM, Landweer EJ, Houterman M, Donker Kaat L, van Duijn CM, van Swieten JC*. Medical and environmental risk factors for sporadic frontotemporal dementia: a retrospective case-control study. J Neurol Neurosurg Psychiatry. 2003; *74:* 1574-1576.

[7] *Kalkonde YV, Jawaid A, Qureshi SU, Shirani P, Wheaton M, Pinto-Patarroyo GP, Schulz PE*. Medical and environmental risk factors associated with frontotemporal dementia: a case-control study in a veteran population. Alzheimers Dement. 2012; *8:* 204-210.

[8] *Wang HK, Lee YC, Huang CY, Liliang PC, Lu K, Chen HJ, Li YC, Tsai KJ*. Traumatic brain injury causes frontotemporal dementia and TDP-43 proteolysis. Neuroscience. 2015; *300:* 94-103.

[9] *Keaney J, Campbell M*. The dynamic blood-brain barrier. FEBS J. 2015; *282:* 4067-4079.

[10] *Prakash R, Carmichael ST*. Blood-brain barrier breakdown and neovascularization processes after stroke and traumatic brain injury. Curr Opin Neurol. 2015; *28:* 556-564.

[11] *McKee AC, Cantu RC, Nowinski CJ, Hedley-Whyte ET, Gavett BE, Budson AE, Santini VE, Lee HS, Kubilus CA, Stern RA*. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol. 2009; *68:* 709-735.

[12] *Yamada M*. Cerebral amyloid angiopathy: emerging concepts. J Stroke. 2015; *17:* 17-30.

[13] *Graff-Radford J, Aakre J, Savica R, Boeve B, Kremers WK, Ferman TJ, Jones DT, Kantarci K, Knopman DS, Dickson DW, Kukull WA, Petersen RC*. Duration and Pathologic Correlates of Lewy Body Disease. JAMA Neurol. 2017; *74:* 310-315.

[14] *Marin R, Fabelo N, Martín V, Garcia-Esparcia P, Ferrer I, Quinto-Alemany D, Díaz M*. Anomalies occurring in lipid and protein distribution in frontal cortex lipid rafts in dementia with Lewy bodies disclose neurochemical traits partially shared by Alzheimer's and Parkinson's diseases. Neurobiol Aging. 2017; *49:* 52-59.

[15] *Doherty CP, O'Keefe E, Wallace E, Loftus T, Keaney J, Kealy J, Humphries MM, Molloy MG, Meaney JF, Farrell M, Campbell M*. Blood-brain barrier dysfunction as a hallmark pathology in chronic traumatic encephalopathy. J Neuropathol Exp Neurol. 2016; *75:* 656-662.

[16] *Hay JR, Johnson VE, Young AM, Smith DH, Stewart W*. Blood-brain barrier disruption is an early event that may persist for many years after traumatic brain injury in humans. J Neuropathol Exp Neurol. 2015; *74:* 1147-1157.

[17] *Nag S, Venugopalan R, Stewart DJ*. Increased caveolin-1 expression precedes decreased expression of occludin and claudin-5 during blood-brain barrier breakdown. Acta Neuropathol. 2007; *114:* 459-469.

[18] *Barzó P, Marmarou A, Fatouros P, Corwin F, Dunbar J*. Magnetic resonance imaging-monitored acute blood-brain barrier changes in experimental traumatic brain injury. J Neurosurg. 1996; *85:* 1113-1121.

[19] *Habgood MD, Bye N, Dziegielewska KM, Ek CJ, Lane MA, Potter A, Morganti-Kossmann C, Saunders NR*. Changes in blood-brain barrier permeability to large and small molecules following traumatic brain injury in mice. Eur J Neurosci. 2007; *25:* 231-238.

[20] *Helmy A, Vizcaychipi M, Gupta AK*. Traumatic brain injury: intensive care management. Br J Anaesth. 2007; *99:* 32-42.