

Clinical features, biomechanics and advanced biomarkers across the spectrum of traumatic brain injury.

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Abstract

Long-term outcomes after traumatic brain injury (TBI) are frequently poor, yet hard to predict. Acute clinical symptoms and conventional diagnostics currently used do not necessarily reflect underlying structural damage relevant to outcomes such as diffuse axonal injury (DAI) nor long-term risk of neuropathology. However, advanced fluid and imaging biomarkers, along with impact biomechanics may provide quantitative metrics better able to inform outcomes after a spectrum of TBI.

This thesis uses advanced neuroimaging and fluid biomarkers to assess the extent of DAI after TBI. I assess changes to diffusion tensor imaging (DTI) metrics of DAI and how they relate to fluid biomarkers and clinical outcomes after moderate-severe TBI. I further test whether these DTI measures, along serial structural MRI, also detect evidence of DAI and brain volumetric changes in athletes exposed to head impacts and mild TBIs (mTBI). In American footballers, I analyse impact biomechanics to explore the relationship of brain deformation metrics with acute clinical symptoms and long-term neuropathology.

I show imaging evidence of DAI after moderate-severe TBI, which correlate with neurofilament light concentrations and strongly predict functional outcomes at 12 months post injury. In professional rugby players, I find neuroimaging evidence of DAI irrespective of whether a player has suffered an mTBI recently. I also find abnormal trajectories of white matter volume change. In American footballers, I show that player position influences the magnitude of forces in the brain, and these appear relevant to both acute clinical symptoms and long term neuropathology.

My data highlights the utility of DTI and fluid biomarkers in detecting axonal injury across a spectrum of TBI severity. These approaches can improve current methods for diagnosing and classifying TBI patients. This is particularly relevant to mTBI and participation in contact sports, where risk of acute clinical symptoms doesn't necessarily reflect risk of long term pathology.

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Statement of publications

Portions of this thesis have been submitted for publication. Chapter 3 study protocols have been published in BMJ Open (Graham et al., 2020a). Chapter 3 results has been published (Graham et al., 2021). Chapter 4 has been published (Zimmerman et al., 2021b). Chapter 5 has been published (Zimmerman et al., 2021a). Chapter 6 is currently in preparation for submission. Additionally, parts of Chapters 3-6 have been presented and published in conference proceedings.

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Statement of originality

The data presented in Chapter 3 was collected as part of a European-wide consortium involved in the BIO-AX-TBI study. Imaging and neuropsychological data collected in London was predominantly done by myself and Dr Neil Graham. Neuroimaging pre-processing and quality control checks were done by myself and Dr Federico Moro. Lesion drawings were completed by myself and Dr Neil Graham. I completed the entirety of DTI pre-processing and analysis. Blood biomarker concentrations were assessed at the University College London by Dr Amanda Heslegrave and team, and analysed by Dr Neil Graham. Data collection and pre-processing of imaging, histopathological and biomarker data from the experimental injury model were performed by Dr Cornelius Donat and Dr Maria Yanez Lopez while I performed the statistical analysis of imaging measures. I performed the analysis comparing biomarker levels with DTI results in both human and experimental models of TBI.

The data presented in Chapter 4 was collected as part of a collaboration with University College London. Imaging data for a number of the controls were collected by Dr Maria Yanez-Lopez and Dr Amy Jolly. Neuropsychological data for a number of

controls was collected by Ravjeet Samra, an MRes student under my direct supervision. I completed the majority of neuroimaging collection, and all neuroimaging pre-processing and data analysis.

Video selection, video analysis and physical simulations in Chapter 5 was conducted by Dr Clara Karton at the University of Ottawa as part of other studies. Pre-processing of the simulation data was conducted by Joo Kim, an MRes student under my direct supervision. Analysis pipelines were adapted from work from Helen Lai, an MRes student under my direct supervision, who completed the pilot analysis on ice hockey players with fluid biomarkers. The finite element model was developed by Dr Mazdak Ghajari and analysis scripts adapted from his work.

Video selection, video analysis and physical simulations in Chapter 6 were conducted by Dr Janie Cournoyer at the University of Ottawa as part of other studies.

All other work contained in this thesis is my own and conforms to the rules and guidelines set out for PhD theses by Imperial College London.

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List of abbreviations

ALS	Amyotrophic Lateral Sclerosis
ANOVA	Analysis of Variance
CSF	Cerebral Spinal Fluid
CTE	Chronic Traumatic Encephalopathy
DTI	Diffusion Tensor Imaging
DAI	Diffuse Axonal Injury
DWI	Diffusion Weighted Imaging
FA	Fractional Anisotropy
FDR	False Discovery Rate
FEM	Finite Element Model
FLAIR	Fluid-attenuated Inversion Recovery
FOV	Field of View
FSL	fMRIB Software Library
GCS	Glasgow Coma Scale
GOSE	Glasgow Outcome Scale Extended
ISOVF	Isotropic Volume Fraction
JD	Jacobian Determinant
LOC	Loss of Consciousness
MPRAGE	Magnetisation Prepared Rapid Gradient Echo
MRI	Magnetic Resonance Imaging
NDI	Neurite Density Index
NfL	Neurofilament Light
NODDI	Neurite Orientation Dispersion and Density Imaging
ODI	Orientation Dispersion Index
ROI	Region of Interest
S100B	S100 calcium-binding protein B
SPM	Statistical Parametric Mapping
SWI	Susceptibility Weighted Imaging
TBI	Traumatic Brain Injury
TE	Echo Time
TR	Repetition Time
UCLH-1	Ubiquitin Carboxy-terminal Hydrolase L1
VBM	Voxel Based Morphometry
WM	White Matter

Chapter 1

Introduction

1.1 Overview

Traumatic brain injury (TBI) is common worldwide. In the UK, there are an estimated 1 million emergency department attendances each year due to TBIs, of which 90% are considered mild (Kay and Teasdale, 2001) and costing the economy as much as £5 billion a year (Gustavsson et al., 2011). TBI's occur when an external force causes an alteration in brain function or brain pathology and is most commonly caused by road traffic accidents, falls and violence (Maas et al., 2017). Long-term outcomes after a TBI are often very poor (McMillan et al., 2014) and associated with long-term disabilities such as physical, cognitive, neuropsychiatric and behavioural disorders (McMillan et al., 2012, Ponsford et al., 2008) and increased risk of neurodegenerative diseases (Fleminger et al., 2003, Li et al., 2017, Mortimer et al., 1985).

While advances in monitoring and imaging have led to more targeted treatment during acute care and improved early outcomes after TBI (Stocchetti et al., 2017), long-term outcomes remain difficult to predict. Diffuse axonal injury (DAI) is an important determinant of these clinical outcomes (Smith et al., 2003, Sidaros et al., 2008), however, validated methods to quantify the extent of axonal injury are lacking in a clinical context. Several methods show promise. Diffusion MRI provides a validated and sensitive measure of damage to the white matter (Mac Donald et al., 2007c, Kinnunen et al., 2011a) that helps predict long-term clinical outcome after TBI (Bonnelle et al., 2012, Bonnelle et al., 2011, Hellyer et al., 2012, Sharp et al., 2014, Sidaros et al., 2008) and can be used to assess the presence of axonal injury on an individualised basis (Jolly et al., 2021). Advances in assays of potential fluid biomarkers provide a complementary approach. Cytoskeletal proteins such as neurofilament light (NfL) are released after injury and present in venous blood, with concentrations elevated acutely and sometimes years after an injury (Scott et al., 2018, Shahim et al., 2020a), and show potential as biomarkers for axonal injury following TBI.

Studying impact biomechanics may be another method by which we can predict outcomes after TBI. Axonal injury, or indeed a TBI, can be viewed as an interaction

between physical properties of the brain, and the exogenous forces applied to it during an impact. High levels of rotational acceleration of the head during an impact are thought to produce large magnitude tissue deformation and shearing forces within the brain (Post et al., 2017, Meaney and Smith, 2011), leading to damage to the white matter (Hill et al., 2016). In an experimental injury model, these forces correlate with both MRI and histopathology measures of axonal and glial injuries (Donat et al., 2021). By modelling the effects of the biomechanical forces on the brain's physical structure, we may be able to predict patient outcomes and contribute to the development of protective, preventative and injury management strategies.

In my thesis, I explore each of these methods as tools for investigating the mechanisms of injury and how they relate to patient outcomes after TBI. I investigate the utility of diffusion MRI and blood biomarkers in detecting axonal injuries after moderate-severe TBIs in a large multi-centre study. I then apply the same tools to an elite sporting group to investigate whether elite rugby participation and mild TBIs are associated with the presence of damage to the white matter. Lastly, I investigate whether modelling brain injury biomechanics provides measures useful in relation to both long-term and acute outcomes after sports related TBIs.

1.2 Characterizing traumatic brain injury

TBI is a heterogeneous disease with a spectrum of severity and wide variety of clinical consequences. These can range from momentary loss of consciousness or delayed cognitive problems to coma, long-term disability and death. Initial clinical characteristics of patients are typically used to classify an injury in order to aid acute clinical management and predict long-term patient outcomes. In this thesis, I use the Mayo TBI classification system, which incorporates simple neuroimaging measures of injury severity (Malec et al., 2007) alongside clinical measures of severity such as duration of loss of consciousness (LOC) and lowest Glasgow Coma Scale (GCS). This has the benefit of not relying on a single clinical measure, and is routinely used by our research group and within associated clinical work as a practical way of classifying the injuries. The Mayo TBI classification system produces three levels of injury severity:

symptomatic (possible), mild (probable) and moderate-severe TBI using the following criteria:

A. Classify as Moderate-Severe (Definite) TBI if one or more of the following criteria apply:

- Death due to this TBI
- Loss of consciousness of 30 min or more
- Post-traumatic anterograde amnesia of 24 h or more
- Worst Glasgow Coma Scale full score in first 24 h <13 (unless invalidated upon review e.g., attributable to intoxication, sedation, systemic shock)

One or more of the following present:

- Intracerebral haematoma
- Subdural haematoma
- Epidural haematoma
- Cerebral contusion
- Haemorrhagic contusion
- Penetrating TBI (dura penetrated)
- Subarachnoid haemorrhage
- Brainstem injury

B. If none of Criteria A apply, classify as Mild (Probable) TBI if one or more of the following criteria apply:

- Loss of consciousness momentarily to less than 30 min
- Post-traumatic anterograde amnesia momentarily to less than 24 h
- Depressed, basilar or linear skull fracture (dura intact)

C. If none of Criteria A or B apply, classify as Symptomatic (Possible) TBI if one or more of the following symptoms are present:

- Blurred vision
- Confusion (mental state changes)
- Daze
- Dizziness
- Focal neurological symptoms
- Headache
- Nausea

Following a moderate-severe TBI, long-term outcomes are frequently poor, with close to half of survivors classified as having unfavourable functional outcomes at 12-months post-injury (McCrea et al., 2021). Moderate-severe TBI is also associated with cognitive problems such as impaired processing speed, memory and executive function (Ponsford et al., 2008), along with increased risk of late neurodegenerative

disorders and increased mortality (Maas et al., 2017). In one study of Scottish patients 13 years after TBI, death rates were 6 times higher than in community controls (McMillan et al., 2011). Despite its label, mild TBIs can also lead to poor long-term outcomes, with up to a third of patients reporting symptoms persisting beyond 6 months after injury (Hou et al., 2012). Mild TBIs have also been shown to be associated with a greater risk of death up to 15 years after the initial injury (McMillan et al., 2014). Furthermore, repetitive head-trauma, either those under a symptomatic or mild TBI classification, or even impacts producing no obvious clinical symptoms, have been associated with neurodegenerative diseases such as chronic traumatic encephalopathy (CTE) (Gavett et al., 2010, Guskiewicz et al., 2005, McKee et al., 2009, Mez et al., 2017).

This variability in long-term outcomes despite the initial classifications of injury severity highlights shortcomings in current classification systems. While current methods of characterising an injury are useful for optimising lifesaving acute care, these broad and sweeping classifications do not necessarily map on well to patients' long-term prognosis. This can be problematic for clinicians in providing reliable information to patients and relatives, and for proper allocation of care for patients as they leave intensive care units. Broad classifications also prevent targeting of current and new therapies to patients on an individualised basis, with many clinical trials of interventions failing to show efficacy as a result of not accounting for the heterogeneity of TBI (Maas et al., 2017). A broad selection of better tools that provide quantitative measures of TBI characterization, rather than purely relying on initial clinical symptoms, are therefore needed to match the heterogeneity of TBI and enable therapies to be more carefully matched to patients. A key goal for TBI research remains to develop and validate sensitive and specific measures that help demonstrate the extent of injury and predict its progression and patient outcomes.

1.3 Pathophysiology of TBI and diffuse axonal injury

TBI is a disease with a complex pattern of evolving damage caused by an interaction of both primary and secondary injuries. The primary injury occurs as a result of mechanical force at the time of injury due to either direct physical impact or via

acceleration-deceleration of the head. Direct impacts, for example as a result of objects directly striking the head, or the brain striking within the skull, typically produce focal injuries such as contusions or bleeds within the brain (Maas et al., 2017). however the presence and location of these types of injuries do not correlate well with long-term clinical outcomes (Bigler, 2007).

Acceleration-deceleration type injuries, which can occur as a result of motor vehicle collisions, or from falls or assaults, produce more diffuse injuries such as diffuse axonal and vascular injury as a result of the shear, tensile and compressive strains that long white matter tracts or blood vessels are susceptible to (Hill et al., 2016). Individuals will react differently to similar injuries as a result of the secondary damage initiated by the primary injury that evolves over the hours, days, weeks and even years that follow. These occur as a result of the interaction of metabolic, inflammatory and apoptotic processes from secondary neurochemical cascades and disruption to the blood brain barrier (Werner and Engelhard, 2007, Tagge et al., 2018). Examples of which include in the short-term ischaemia, whereby raised intracranial pressure as a result of swelling leads to impaired blood flow through the brain, or post-acute neuroinflammation and infection (Johnson et al., 2013a).

In contrast to focal injuries, diffuse axonal injury (DAI) is closely related to clinical outcomes (Sidaros et al., 2008), including the type of post-traumatic problems that develop such as cognitive difficulties and poor functional outcomes (Bonnelle et al., 2012, Bonnelle et al., 2011, Hellyer et al., 2012, Sharp et al., 2014). DAI is one of the most common pathologies of TBI (Geddes et al., 1997) and occurs as a result of shear, tensile and compressive forces acting on axons (Eierud et al., 2014). Axons, which are normally compliant when stretched, returning to their original form, become brittle when strain is applied rapidly (Johnson et al., 2013b). This can lead to primary disconnection of axons, however this is relatively rare (Smith et al., 2003). More common is a pathology that develops over time, resulting from the breakage of axonal microtubules which lead to undulation of axons and axon misalignments, which can interrupt axonal transport and induce progressive swelling and degeneration (Tang-Schomer et al., 2010). Axonal swelling and the evolving pathology can lead to

progressive disorganisation of the axonal cytoskeleton and secondary disconnection of the axon, permanently limiting the ability of parent neurons to communicate with its target (Smith et al., 2003). The damage to axons which make up white matter tracts connecting brain regions can then disrupt large-scale networks that support cognition and behaviour (Sharp et al., 2014).

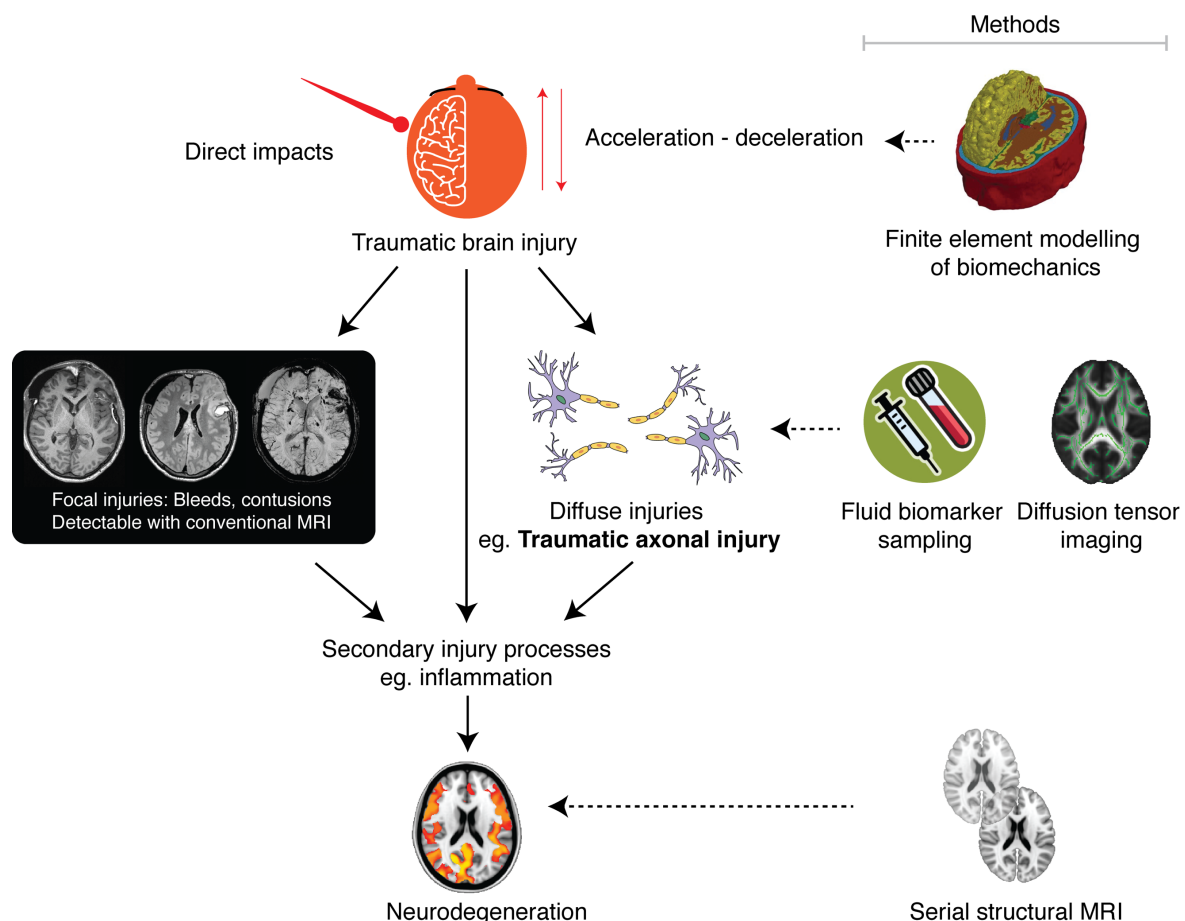


Figure 1. TBI injury processes and analysis methods

Different mechanisms of injury lead to different patterns of injury; direct impacts tend to lead to focal injuries while acceleration-deceleration leads to diffuse injuries. These primary injury processes lead to secondary processes such as inflammation, with neurodegeneration as a possible long-term outcome. These can be measured with different tools (right).

1.4 Measuring diffuse axonal injury

The diagnosis of DAI outside of histopathological examination remains a challenge. Current methods of diagnosis rely on the presentation of symptoms, such as prolonged loss of consciousness, in the absence of observable changes on

conventional CT or MRI imaging (Marshall et al., 1991, Adams et al., 1989). However, these methods are likely to underestimate the presence of DAI due to the low sensitivity of these forms of imaging to microstructural changes in the white matter which therefore can go undetected on routine CT/MRI (Cicuendez et al., 2017, Sharp and Jenkins, 2015, Dodd et al., 2014).

Diffusion tensor imaging

The use of diffusion tensor imaging (DTI), a more advanced MRI technique, can provide information on the structural integrity of white matter tracts. Abnormalities in the directionality of water diffusion, most commonly quantified using changes in fractional anisotropy (FA), provides quantitative information about the integrity of the white matter (Mac Donald et al., 2007a). DTI has been validated as a measure of white matter integrity in animal models of TBI (Mac Donald et al., 2007c) and in humans has shown increased sensitivity to DAI compared to conventional MRI (Kinnunen et al., 2011b). Importantly, DTI also provides information that helps predict long-term clinical outcome after TBI, such as functional outcome as measured by the Glasgow Outcome Scale (Sidaros et al., 2008) and abnormalities in brain function that are present after injury (Sharp et al., 2014).

After a moderate-severe TBI, white matter FA has been shown to be reduced post-acute (greater than 2 weeks after injury) and in more chronic stages (greater than 12 weeks) after injury (Kinnunen et al., 2011a, Sidaros et al., 2008). However, investigations of mild TBI have shown mixed results. Generally, studies tend to report elevated levels of FA in the acute-sub acute phase (less than 2 weeks after injury) which is in contrast to the reduction in FA where patients are scanned 2 weeks after injury (Eierud et al., 2014, Wilde et al., 2008, Croall et al., 2014). These abnormalities appear to be present in large tracts such as the corpus callosum which may be more sensitive to internal shearing forces (Veeramuthu et al., 2015, Zhang et al., 2006, Fakhran et al., 2013). In some studies, no changes in white matter are found acutely (Ilvesmäki et al., 2014). These inconsistencies tend to be present particularly in investigations focusing on sports-related mTBI, where several studies do not find abnormalities in FA (Meier et al., 2016a, Zhang et al., 2010, Strain et al., 2013, Multani

et al., 2016). One potential explanation for these inconsistencies is the possible contribution of repetitive head impacts that do not produce any symptoms, but may lead to alterations in white matter structure. Often these studies use non-symptomatically injured athletes as controls, however these athletes are still likely exposed to repetitive head impacts. Studies that have investigated the effect of participation in contact sports have shown evidence of diffusion abnormalities, possibly due to the repeated head trauma experienced (Shin et al., 2014, Lipton et al., 2013, Schneider et al., 2019).

Fluid biomarkers of axonal injury

Fluid biomarkers of cytoskeletal proteins released from damaged axons can be used to complement DTI measures as markers of axonal injury after both mild and moderate-severe TBI. The most well established fluid biomarker candidates are tau and neurofilament light (NfL) (Zetterberg et al., 2013). NfL is a neurofilament protein, which together with microtubules and microfilaments form the neuronal cytoskeleton. NfL is predominantly expressed in axons, but is also expressed in peripheral nerves (Friede and Samorajski, 1970). Tau is also a structural axonal protein, and is highly expressed in axons where it stabilises microtubule bundles but is also detected in organs such as the liver and kidney. Tau plays a key role in regulating microtubule dynamics by modulating the assembly, behaviour and organisation of the microtubules (Barbier et al., 2019). Both cytoskeletal proteins can be detected in blood using advanced immunoassays such as single molecule arrays (Simoa) which allow ultra-low concentrations to be accurately measured, with concentrations reflecting cerebrospinal fluid (CSF) concentrations of the proteins when released due to mechanical damage to the neuronal cytoskeleton as a result of DAI (Shahim et al., 2020a, Gisslén et al., 2016, Shahim et al., 2016a).

After moderate-severe TBI, concentrations of NfL are elevated acutely in plasma, sometimes remaining raised years after injury (*Scott et al., 2018, Shahim et al., 2020a*). Tau protein levels in blood are also elevated after injury (Thelin et al., 2019), and in some patients remaining raised up to 90 days after injury (Bogoslovsky et al., 2017). Importantly, both proteins have also been indicated as having potential for

predicting adverse clinical outcomes after TBI (Shahim et al., 2016a, Al Nimer et al., 2015b, Thelin et al., 2019, Ost et al., 2006, Magnoni et al., 2012). NfL and tau concentrations are known to also increase after a mild TBI (Zetterberg et al., 2006, Neselius et al., 2012, Shahim et al., 2017, Shahim et al., 2016b) and interestingly NfL concentrations also increased over the course of an American football season, possibly reflecting changes due to accumulated head impacts (Oliver et al., 2016). Both NfL and tau have been shown to correlate with diffusion MRI measures, albeit only with CSF protein levels for tau (Magnoni et al., 2015) and in a very small cohort ($n = 9$) with NfL (Ljungqvist et al., 2017). Examining the time course of these fluid biomarkers and their relationship to imaging measures has great potential for clinical impact. Early evidence suggests that NfL also helps predict the extent of chronic white matter atrophy that can occur progressively after TBI (Scott et al., 2018, Shahim et al., 2020a).

There are several other non-axonal proteins that have shown promise as fluid biomarkers of TBI severity. Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is a deubiquitinase that is highly expressed in neurons, released into the bloodstream as the blood brain barrier is compromised after injury and been shown in mild to moderate severe TBI to associate with GCS, lesions on brain imaging and the need for neurosurgical intervention (Papa et al., 2012). Calcium binding protein S100 B (S100B) and glial fibrillary acidic protein (GFAP) are predominantly expressed in astroglial cells, with concentrations reflecting astroglial injury. In addition to NfL and tau; UCH-L1, GFAP and S100B concentrations are all elevated after TBI and are associated with injury severity, with principle component analyses showing clustering of UCH-L1 and tau as well as GFAP with S100B (Thelin et al., 2019). However, a recent comparison of NfL, tau, GFAP and UCH-L1 highlighted limited diagnostic and prognostic utility of tau, GFAP and UCH-L1 in sub-acute and chronic TBI, with only NfL showing promising results and associations with both TBI severity, functional outcomes and MRI measures of DAI (Shahim et al., 2020b).

1.5 Neurodegeneration after TBI

Neurodegeneration after TBI is well documented, with degeneration particularly prevalent in cerebral white matter tracts (Smith et al., 2013, Cole et al., 2018b). TBI has been linked to risk of neurodegenerative diseases such as Alzheimer's and chronic traumatic encephalopathy (CTE) (Smith et al., 2013, Mayeux et al., 1995), and evidence of atrophy in both the grey and white matter in the chronic phase following a TBI (Cole et al., 2018b). Evidence suggests that TBI triggers atrophy to progress at a faster rate, beyond simply triggering the onset of normal brain volume loss at an earlier age (Cole et al., 2015). High atrophy rates after TBI are clinically relevant and associated with worse clinical outcomes (Brezova et al., 2014, Bendlin et al., 2008, MacKenzie et al., 2002, Trivedi et al., 2007). Evidence of neurodegeneration after mild TBI is less clear. In a study looking at whether a TBI accelerated atrophy rates, cases of single hit mild TBI did not show accelerated rates of atrophy in the white matter compared to controls (Cole et al., 2015). However, repeated exposure to mild TBI has been associated with CTE (McKee et al., 2009).

Chronic traumatic encephalopathy (CTE)

Chronic traumatic encephalopathy (CTE) is a progressive neurodegenerative disorder and form of dementia that is characterised by the accumulation of abnormal tau proteins along small blood vessels and in the depths of the sulci (McKee et al., 2016). CTE has risen to the fore of public health concerns due to the popularity of sports such as soccer, American football and rugby, where head impacts are common aspects of the sport, and the links being made between head impacts and the possible risk of developing CTE presents novel and detrimental risks to participation. Evidence has grown to suggest that even exposure to repeated head impacts (Stein et al., 2015, McKee et al., 2014, Bieniek et al., 2015) or a single severe head injury can also lead to CTE-like pathology (Smith et al., 2013, Johnson et al., 2012). An important question is to what extent different sports accelerate neurodegeneration and increase the risk of dementia. Contact sports with exposures to head impacts see higher risk of neurodegenerative diseases, with risk of mortality with neurodegenerative diseases 2.83 times higher in professional American footballers (Lehman et al., 2012), and 3.45 times higher in soccer players (Mackay et al., 2019). Within these sports, playing

position has been shown to influence dementia risk (Russell et al., 2021, Lehman et al., 2012), possibly as a result of the difference in how frequently, and how forcefully players experience head impacts. While a diagnosis of CTE can only be made at post-mortem, monitoring atrophy rates after repeated head trauma or TBI can help address these questions by studying groups of individuals possibly at risk while alive.

Measuring neurodegeneration

The presence of neurodegeneration can be studied using serial structural MRI. Brain atrophy, which is a common end-point of various types of neurodegeneration, can be sensitively assessed using metrics of volume change over time such as the Jacobian Determinant (JD) (Ashburner and Ridgway, 2012). Atrophy, as measured by serial MRI, has been histologically validated at post-mortem (Bobinski et al., 2000) and associated with poorer outcomes after TBI (Brezova et al., 2014, Bendlin et al., 2008, Cole et al., 2018b). A more negative JD, which indicates more atrophy, was also found in the depths of sulci compared to gyri, corresponding to where tau-pathology is observed in CTE (Cole et al., 2018a). Serial volumetric MRI therefore provides a method of sensitively assessing whether neurodegeneration is taking place and mapping its spatial pattern, which may give an indication of the underlying pathological process.

1.6 Biomechanics of TBI

Investigation of the biomechanics of TBI's can also provide information relevant to patient outcomes. DAI is caused by biomechanical forces that are exerted across white matter tracts at the time of injury (Gennarelli et al., 1982). The magnitude and distribution of these forces at the time of injury are therefore likely to determine whether DAI occurs, its location and possibly where secondary injury processes and neurodegeneration are more likely to occur. These forces can be modelled by using computational methods such as Finite Element Modelling (FEM). FEM models are composed of hexahedral elements that incorporate validated tissue properties into a computational model of the brain. Applying the accelerations at the time of injury to the model, you can simulate the magnitude and spatial location of mechanical forces

such as the stretch (strain) and rate of stretching (strain rate) of elements within the brain during characterised impacts (Zhang et al., 2001).

These forces have been shown on a number of occasions to be relevant to pathology after TBI. Using *in vitro* models of TBI, different levels of biomechanical forces were shown to be associated with cortical cell death and the breakage and buckling of microtubules in axons (Elkin and Morrison, 2007, Morrison et al., 2003, Smith et al., 1999, Tang-Schomer et al., 2010). Biomechanical forces have also been associated with blood-brain barrier injury (Shreiber et al., 1997, Shreiber et al., 1999). *In vivo* models utilising both morphological injury detection using neurofilament staining and functional injury assessment using evoked potentials demonstrated the tolerance to biomechanical forces and subsequent evidence of axonal injury using guinea pig optic nerves (Bain and Meaney, 2000). Our group has recently combined FEM with MRI and quantitative histopathology in a rat model of TBI. A clear dose-dependent relationship was found between the biomechanical forces and post-traumatic brain pathology, including white matter damage as measured by DTI and neuroinflammation (Donat et al., 2021). In humans, simulating the impacts of a small number of cases ($n = 3$), these forces were shown to spatially correlate with where DTI measures of axonal injury are typically observed to be abnormal after TBI (Ghajari et al., 2017). Biomechanical forces are also highest in the depths of the sulci compared to gyri (Ghajari et al., 2017, Ho and Kleiven, 2009), corresponding where increased atrophy is observed after TBI and where tau-pathology is accumulates in CTE (Cole et al., 2018a). However, these results have yet to be replicated in a larger cohort.

1.7 Aims, objectives and outline of thesis

The aim of this thesis is to explore the relationship between impact biomechanics and advanced biomarkers with outcomes after a spectrum of TBI severity. A combination of structural and diffusion MRI, cognitive assessments, fluid biomarker measurement and impact simulations are used.

The objectives are:

- i. To assess the relationship between diffusion MRI and fluid biomarkers of axonal injury, NfL and tau, in moderate-severe TBI patients, and how both measures relate to patient functional outcomes, cognitive impairment and brain atrophy.
- ii. To test if mild TBI, sustained during head impacts in elite sport, is associated with evidence of axonal injury and brain atrophy.
- iii. To investigate the spatial pattern of biomechanical forces produced during head impacts in elite sport, and whether this is related to TBI-related pathology such as CTE or clinical features, such as loss of consciousness.

1.8 Hypothesis

The main hypotheses of the thesis are:

- i. Fractional anisotropy will be reduced, and plasma NfL elevated, in moderate-severe TBI patients.
- ii. Plasma NfL concentrations will be correlated to fractional anisotropy and plasma NfL levels in brain extracellular fluid as measured by cerebral microdialysis.
- iii. Fractional anisotropy and plasma NfL concentrations will be closely related to functional outcomes after moderate-severe TBI as measured by the GOS-E.
- iv. In elite rugby players, DTI metrics will be sensitive to abnormalities as a result of mild TBI.
- v. Elite rugby players will show abnormalities in the trajectory of brain volume changes as measured by analysis of longitudinally collected structural MRI.
- vi. In head impacts of elite athletes, strain and strain rates will be concentrated in the depths of the sulci, and will be closely related to rotational acceleration of the head.
- vii. In impacts leading to mTBI with loss of consciousness, strain and strain rates will spatially correlate with anatomical structures related to maintaining consciousness, such as the brainstem and thalamus.

1.9 Chapter summary

In Chapter 3, I present a multi-centre study of moderate-severe TBI patients in which I investigate the relationship between diffusion MRI measures and fluid biomarkers of DAI. I further investigate if the measures are related to patient outcomes.

In Chapter 4, I present a study of elite rugby players where I investigate if the mild TBI, and/or participation in elite rugby is associated with DAI, measured using both a group wise comparison and individualised assessment of diffusion MRI measures. I additionally investigate whether there is evidence of increased brain atrophy as measured by serial structural MRI.

In Chapter 5, I investigate the relationship between frequency and magnitude of head impacts, and brain biomechanics in elite American football players. I assess the magnitude and spatial location of these forces, and compare the spatial patterns with where CTE and white matter pathology has been previously observed.

In Chapter 6, I present a study in which I investigate whether mTBIs associated with loss of consciousness involve higher level of biomechanical force than impacts that produce no clinical signs. I use a novel application of neuroimaging statistical techniques to test if these forces are constrained to biologically relevant locations such as the brainstem, and assess if the magnitude of biomechanical forces are related to the severity of injuries.

In Chapter 7, I summarise and interpret the findings of the thesis and discuss how they contribute to our understanding of TBI across a spectrum of severities. I discuss the prospects of using diffusion MRI, fluid biomarkers and brain biomechanics as methods to stratify TBI severity and discuss the direction of possible future research.

Chapter 2.

Methods

2.1 MRI acquisition and parameters

All MRI sequences completed in London were acquired on a 3T Siemens Verio (Siemens Healthcare, Erlangen, Germany) scanner using a 32-channel head coil. The parameters used and listed below were used as references when setting up aligned acquisition protocols on scanners from other sites. The relevant sequences acquired across the studies include:

High resolution T1 magnetisation prepared rapid gradient echo (MPRAGE)

The MPRAGE sequence was used to acquire a standard high resolution T1-weighted structural image. This image can be segmented to calculate tissue volumes, and furthermore used longitudinally to assess changes in volume over time. The structural image also aids in the registration of diffusion images and the identification of focal lesions. As a relatively short scan, the images produced are not as sensitive to movement in the scanner and as the scan relies on contrast differences between tissues, data can be analysed across sites with relative ease. The parameters used were: 1mm³ isotropic voxel, 160 slices, repetition time (TR) = 2300 ms, echo time (TE) = 2.98 ms, flip angle = 9 degrees, field of view (FOV) = 256x256 mm, GRAPPA = 2.

T2 fluid-attenuated inversion recovery (FLAIR)

The T2-FLAIR sequence provides a more lesion sensitive contrast than the MPRAGE, and is only used to aid the identification of focal lesions for labelling and manual segmentation. The contrast between grey and white matter is not as high as the MPRAGE sequence and hence it is not used for segmentation and classification of grey and white matter. The parameters used were: 1.0mm³ isotropic voxel, 160 slices, TR= 5000 ms, TE = 395 ms, FOV = 250x250 mm, GRAPPA = 2.

T2 susceptibility weighted imaging (SWI)

An SWI sequence was used to identify microhaemorrhages, which are indicative of diffuse vascular injury. Previously, SWI scans have been quite long and susceptible to movement artefacts, which is problematic when trying to identify microhaemorrhages along tiny vessels. Developments in the SWI sequences have improved the ability to acquire thinner slices and smaller voxel sizes at a faster time,

improving resolution of scans and reducing movement artefacts. The scan is reviewed by a neuroradiologist, who determines if hyperintensities are microhaemorrhages, but not for any quantitative analysis in this thesis. SWI was obtained with the following parameters: 0.8 x 0.6 mm in-plane resolution with 120 1.2-mm-thick transverse slices, TR = 28 ms, TE = 20 ms, flip angle = 15 degrees, FOV = 225 x 225 mm.

Single shell diffusion weighted imaging

Diffusion weighted imaging (DWI) was acquired to assess white matter tract integrity and look for evidence of diffuse axonal injury. Analysis of single shell DWI produces metrics indicative of the motion of water molecules in a particular voxel, which in white matter is attributed to the integrity of white matter tracts (see Section 2.3). The sequences are long, and hence sensitive to movement in the scanner, which needs to be corrected during the analysis stage. The sequence can also be influenced by changes in scanner software, or natural decay of scanner qualities over time, and therefore these factors need to be accounted for in both acquisition and analysis of DWI. A single-shell protocol was acquired using a 64-direction protocol: 2.0 mm³ isotropic voxels, 64 slices, TR = 9500 ms, TE = 103 ms, FOV = 256x256 mm, b-value = 1000mm² s⁻¹ with 4 b-value = 0mm³ s⁻¹ acquired.

Multi-shell diffusion weighted imaging

DWI using a multi-shell protocol was also acquired which allows for a more flexible analysis using the Neurite Orientation Dispersion and Density Imaging (NODDI) models. Multi-shell DWI has similar limitations to single shell DWI, however provides more information on the underlying causes of changes in the diffusion of water (see Section 2.3). The multi-shell sequence was acquired with the following protocol: 2.0 mm³ isotropic voxels, 66 slices, TR = 5000 ms, TE = 85 ms, FOV = 256x256 mm, GRAPPA = 2, b-value 1 = 700 mm² s⁻¹ (30 directions), b-value 2 = 2000 mm² s⁻¹ (60 directions) and 6 b-value = 0 mm² s⁻¹ acquired with 6 reversed phase encoding images without diffusion weighting.

2.2 Structural imaging

Structural imaging preprocessing

T1-weighted structural data was preprocessed using standard approaches (Cole et al., 2018a). SPM12 (Statistical Parametric Mapping software - UCL), a standard neuroimaging processing tool, was used to segment structural MRI data and calculate the probability that a particular voxel was grey matter, white matter or CSF. Volumes of these tissue classes were then calculated for baseline and (if available) follow up scans using a threshold for classification. To facilitate the comparison of these images, a study template is generated using SPM DARTEL non-linear registration (Ashburner, 2007) before affine registration of segmented images to MNI152 space, with normalisation of volume and smoothing using an 8mm kernel. Imaging data can then be compared on a voxelwise basis using voxel-based morphometry (VBM), or sampling tissue classification probabilities from a particular region of interest (ROI).

For longitudinal comparisons investigating change in volumes over time, pre-processing is performed using the SPM12 longitudinal registration tool. Baseline and follow-up T1 images are iteratively co-registered to produce a temporal average image, with the transformation required to move from the baseline to follow up image encoded as the Jacobian determinant (JD) statistic. This captures the expansion/contraction between scans at a voxelwise level (Ashburner and Ridgway, 2012). The JD is then weighted by the interval between baseline and follow up to give an annualised rate of change and is further weighted by tissue class. A similar process of image registration to a standard space is used to allow for voxelwise comparisons of JD.

Interpreting the Jacobian determinant

The JD statistic, when weighted by the time interval between the two scans used to calculate it, provides an annualised rate of change on a voxelwise basis. Each voxel carries a value in percentage form; a positive JD of 0.05 refers to an annualised expansion of 5% that the voxel underwent during the study period, whereas a negative JD of 0.05 refers to a contraction of 5%. The JD therefore reflect the changes in brain volume in a sensitive, voxelwise manner, providing an informative way to determine

the long-term neurodegenerative effects after an injury (Cole et al., 2018b). However, in group comparisons a significantly reduced JD does not automatically mean that there is evidence of atrophy and may be either caused by a less positive JD (less expansion than expected) or more negative JD (more contraction than expected). Reporting the JD values, rather than simply whether group differences are present, are therefore important in the interpretation of the findings.

Focal lesion identification and drawing

All T1-structural, T2-FLAIR and SWI images are examined by a consultant neuroradiologist for the presence of focal lesions or microbleeds. In instances where focal lesions are identified, lesions (not including microbleeds on SWI) are manually segmented using an in-house image segmentation tool developed at Imperial College London (ImSeg). These lesion masks are produced in the same orientation space as the T1-image the drawing is based on, and is therefore registered to the required space before being used to exclude lesioned voxels from analysis. This is used on an individualised-subject level for diffusion analyses and structural analyses to minimise the effect of focal lesions on either diffusion metrics or volumetric changes.

2.3 Diffusion tensor imaging

Diffusion tensor imaging metrics are based on the assessment of the motion of water molecules within the brain. These water molecules have different patterns of movement depending on their environment. For example, within white matter tracts, water molecules have directionally restricted movement (anisotropic movement) whereas in the cerebrospinal fluid or grey matter, fluid motion is more free and random (isotropic movement). This motion can be modelled along the axis of 3 directions, with an eigenvalue associated with the extent of diffusion along each axis (Alexander et al., 2011). The fractional anisotropy (FA) represents the extent of diffusion in the longitudinal direction (Figure 2), whereby mean diffusivity (MD) reflects the mean of all 3 eigenvalues.

FA values are indicative of the microstructural integrity of white matter. After injury, FA values reducing reflect water molecule movement becoming more isotropic, and less

constrained by intact white matter. FA has been demonstrated to be a valid measure of white matter integrity after TBI in animals models of TBI (Mac Donald et al., 2007c) and shown increased sensitivity to diffuse axonal injury (DAI) compared to conventional MRI (Kinnunen et al., 2011b). However, FA values are not only influenced by axonal injury, and has been shown to be affected by factors such as intelligence (Schmithorst et al., 2005, Yu et al., 2008), normal aging (Pfefferbaum et al., 2000) or even training (Moore et al., 2017). FA values can also reflect axonal count, myelin content and fibre orientation (Winston, 2012), such as where there are areas of crossing fibres where FA may be calculated as relatively low. These issues arise due to the limitations of resolving only a single fibre orientation per voxel, and can be resolved or complemented by using Neurite Orientation Dispersion and Density Index (NODDI) modelling of multi-shell DWI.

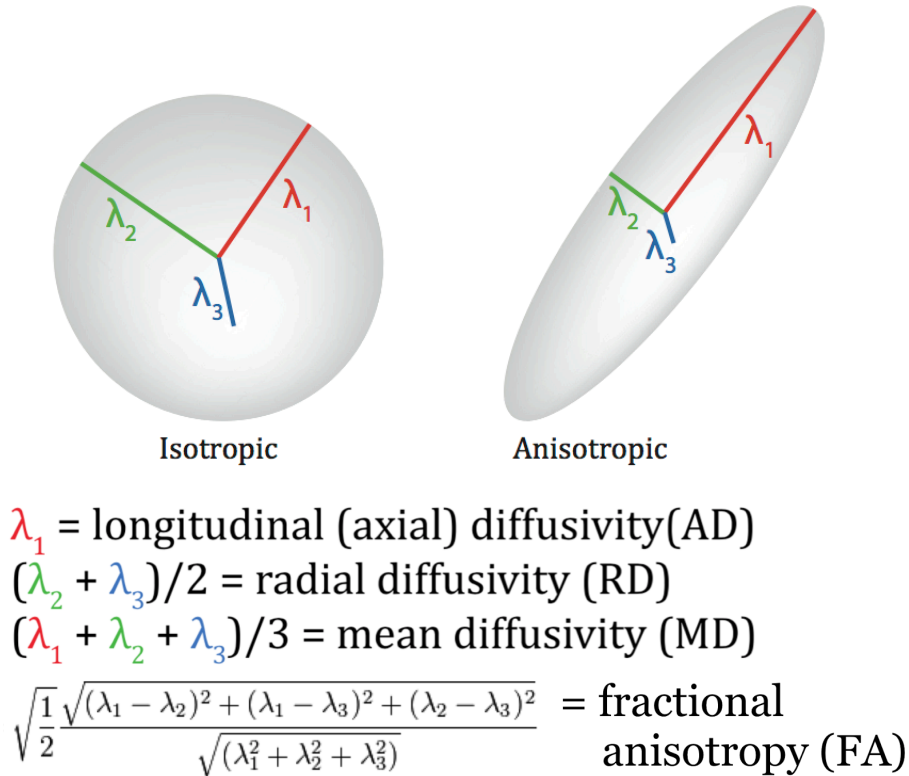


Figure 2. Diffusion tensor imaging measures of white matter integrity

Diffusion tensor imaging (DTI) metrics are based on the motion of water molecules. Random, unrestricted, isotropic motion is more common in the cerebral spinal fluid or grey matter, while restricted, anisotropic motion is more common in the white matter.

NODDI is a biophysical model of diffusion MRI, and is a three-compartment model, separating the diffusion signal into signals from three different tissue compartments: intra-neurite (restricted diffusion), extra-neurite (hindered diffusion) and cerebrospinal fluid (free diffusion). The diffusion signals are described by three corresponding metrics; a neurite density index (NDI), orientation dispersion index (ODI) and isotropic volume fraction (ISOVF) (Zhang et al., 2012) (Figure 3). NDI represents axons and dendrite processes, with changes in diffusion signals from these compartments representing changes in axonal density in the white matter. ODI encompasses all everything except neurites and free water, such as cell bodies or astrocytes, and is higher in loosely organised white matter and lower in tracts with parallel fibre bundles such as the corpus callosum. ISOVF refers to the unrestricted diffusion of water molecules found for example in cerebrospinal fluid or extracellular oedema. These measures can provide complementary metrics that provide sensitive and specific quantification of white matter pathology.

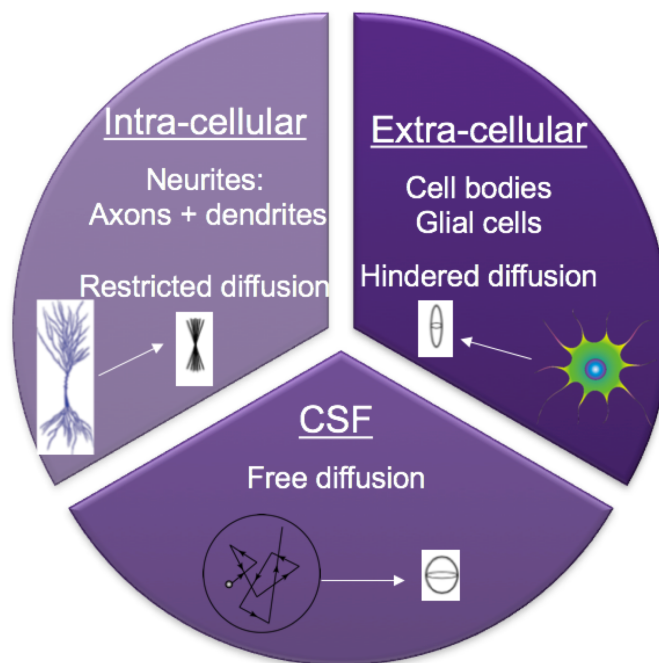


Figure 3. NODDI modelling of diffusion MRI

NODDI models three compartments characterised by distinct diffusion properties. These are labelled as neurite density index (NDI) – restricted diffusion, orientation dispersion index (ODI) – hindered diffusion, and isotropic volume fraction (ISOVF) – free diffusion.

Pre-processing – diffusion tensor imaging

Both single shell and multi-shell diffusion data are preprocessed using FSL and DTI-TK (Zhang et al., 2007) to generate DTI metrics such as FA and MD. DTI data is preprocessed following the standard TBSS pipeline in the FMRIB Software Library (version 5.0.8) (Smith et al., 2006), including correcting for head motion, susceptibility and eddy current induced distortions (Andersson and Sotiropoulos, 2016) and diffusion tensor fitting. Tensor-based registration was performed using DTI-TK and involves the creation of a group template and registration of participant diffusion imaging to said template. Images are warped to 1mm isotropic space and the mean FA map produced is thresholded at 0.2 to produce a white matter skeleton. Subject FA and MD data is projected onto the mean FA skeleton and tract level data generated using the Johns Hopkins University (JHU) white matter atlas.

Pre-processing – NODDI modelling

Multi-shell diffusion data is preprocessed using the Accelerated Microstructure Imaging via Convex Optimization (AMICO) framework (Daducci et al., 2015). This method produces NDI, ODI and ISOVF image maps which can be manipulated in a similar fashion to FA and MD images. These images were first registered to DTITK-space using the transformation matrix generated during the DTITK pipeline, before being used in ROI and voxelwise analyses.

Individualised assessment

DTI has not typically been used clinically because of a lack of a robust method of analysis to diagnose axonal injury at an individual level. Recently, our research group has developed and utilised a method to accurately assess the presence of axonal injury in large white matter tracts in individuals (Jolly et al., 2021). Mean FA is calculated for a select number of large white matter tracts selected on the basis of tract size, reproducibility and sensitivity. These include: the body of the corpus callosum body, genu of the corpus callosum, splenium of the corpus callosum, right and left corticospinal tracts, right and left inferior longitudinal fasciculus, right and left corona radiata and the middle cerebellar peduncle. The mean and standard deviation

of FA for each tract ROI across a control cohort are then used to calculate subject Z-scores for each ROI. Z-scores are converted to P-values (two-tailed, with 95% confidence interval) and corrected for multiple comparisons across all tract ROIs using false discovery rate (FDR) correction. Abnormalities in subjects' FA are determined by tracts that have significantly lower or higher FA in the subject compared to the control group, $p < 0.05$, FDR corrected. An individual is classified as having abnormalities in DTI if a single tract ROI is abnormal. This method of classification has been shown to be relevant to cognitive and functional outcomes (Jolly et al., 2021).

2.4 Blood biomarker assessment

Fluid biomarkers can provide a complementary and less expensive approach to assessing axonal injury. However, due to the low concentrations of potential blood biomarkers, previous work has been limited by the analytic sensitivity of the methods used to assess them. Recent advances have drastically improved the analytic sensitivity, by using a “digital ELISA” technique where the detection reaction is compartmentalised in a classical sandwich ELISA format into 50 femtolitre microwells, allowing for the counting of single target molecules (Shahim et al., 2014, Gisslen et al., 2016). Subtle changes in protein concentration in the plasma can therefore be detected. All fluid biomarker assays are work completed by the biomarker lab at the UK Dementia Research Institute at UCL, led by Dr Amanda Heslegrave and Professor Henrik Zetterberg, and the methods are reported below for reproducibility.

Venous blood is sampled using ethylenediaminetetraacetic acid (EDTA) coated tubes and clot-activator for plasma and serum samples respectively. After 30 minutes at room temperature, samples are centrifuged at 2000-2500g at 4°C, transferred into 1.4ml aliquots and frozen at -80°C. NfL, total tau, GFAP and UCH-L1 concentrations are assessed in plasma using a Neurology 4-plex B assay on the Simoa-HD1 platform, following the manufacturer's protocol with lower limit of quantifications of 0.5, 0.125, 9.38 and 9.38 pg/ml respectively (Quanterix Corp, Boston, MA). Serum S100B was measured using a Millipore ELISA kit (Merck Millipore, Darmstadt, Germany) with a lower limit of quantification of 2.7 pg/ml. Samples are thawed on wet ice, centrifuged at 500 × g for 5 minutes at 4°C. Calibrators (neat) and samples (1:4 dilution) were

measured in duplicates. A four-parameter logistic curve fit data reduction method was used to generate a calibration curve. Two control samples of known concentrations of the protein of interest (high-control and low-control) were included as quality control. Cerebral microdialysis samples are analysed using the Neurology 4-plex B assay on the Quanterix Simoa-HD1 platform, as per plasma biomarkers, but using a 20:1 dilution. Samples are run in duplicate, with the mean of both samples used for further analyses.

2.5 Impact simulations & finite element modelling

Simulating impacts are a method of investigating the impact biomechanics of injuries, and can help develop an understanding of the relationship between biomechanics and pathology. This requires two steps. Firstly, the impact needs to be reconstructed, in order to provide estimations of head movement at the time of impact. These can come in the form of physical or computational reconstructions. This produces head kinematic metrics including linear and rotational acceleration and velocities of the head during an impact. These capture the motion in the x, y and z plane of direction, but can also be combined into a single measure termed the peak resultant linear/rotational acceleration via the formula: $\text{resultant} = \sqrt{x^2 + y^2 + z^2}$. These metrics alone are informative in predicting the degree of tissue deformation and shearing forces within the brain (Post et al., 2017, Meaney and Smith, 2011). The second step involves applying the head kinematics to a model to calculate brain specific metrics, which can range from either a general brain injury score, or to higher resolution metrics such as metrics of tissue deformation in specific areas of the brain.

Historically, the second step of impact simulations have come in the form of using physical models of skulls/brains and animal models of TBI (Meaney et al., 1995, Bain and Meaney, 2000). However, these models are obviously limited by the differences, such as structural differences, between the model and human brains in producing simulations with high fidelity to what was occurring at the time of injury. Computational modelling, in the form of finite element models (FEM), can help overcome these differences. Finite element modelling enables the brain's complex geometry to be modelled, and incorporates mechanical tissue properties and responses (Zhang et al.,

2001). Information on the motion of the head can be fed into the model, and the response of the mechanical response of various elements recorded.

Early versions of FEMs were simplistic in structure, with large elements representing whole regions of the brain and little interaction between different tissue types. However, as computational capabilities have advanced, these models have become more complex and aligned with the brains anatomy. Within this thesis, I use a high-fidelity 3D FEM of the human head developed by Dr Mazdak Ghajari (Figure 4). The model was developed using high resolution magnetic resonance images of a healthy 34-year-old male subject and consists of nearly one million hexahedral elements and a quarter of a million quadrilateral elements. To generate the model, the T1-weighted image of the subject was segmented into skull, CSF, grey matter, white matter, ventricles and brainstem using Freesurfer (v6.0) and FSL (v5.0.10) (Dale et al., 1999, Fischl et al., 2004). Each voxel of the image was replaced with a hexahedral element (1.5mm edge length) using an in-house code. The falx and tentorium were added between the hemispheres and cerebrum/cerebellum respectively using shell elements. A layer of shell elements was added on the grey matter to represent the pia matter. The mesh at the interface between CSF/skull and grey matter/CSF was smoothed, but the mesh at the interface between grey matter and white matter was not smoothed as they had the same material properties. The final model had an average element size of 1.5 mm, with 0.1mm for the smallest element, representing 11 tissues, including the scalp, skull, brain, meninges, subarachnoid space and ventricles, as well as anatomical features such as sulci. For validation, the model predictions of brain displacement were compared to recently published experimental data from well-documented and open-access post-mortem human subject (PMHS) experiments (Alshareef et al., 2018, Alshareef et al., 2020). Further details on the model's material characteristics, development details and validation are described in published work (Ghajari et al., 2017).

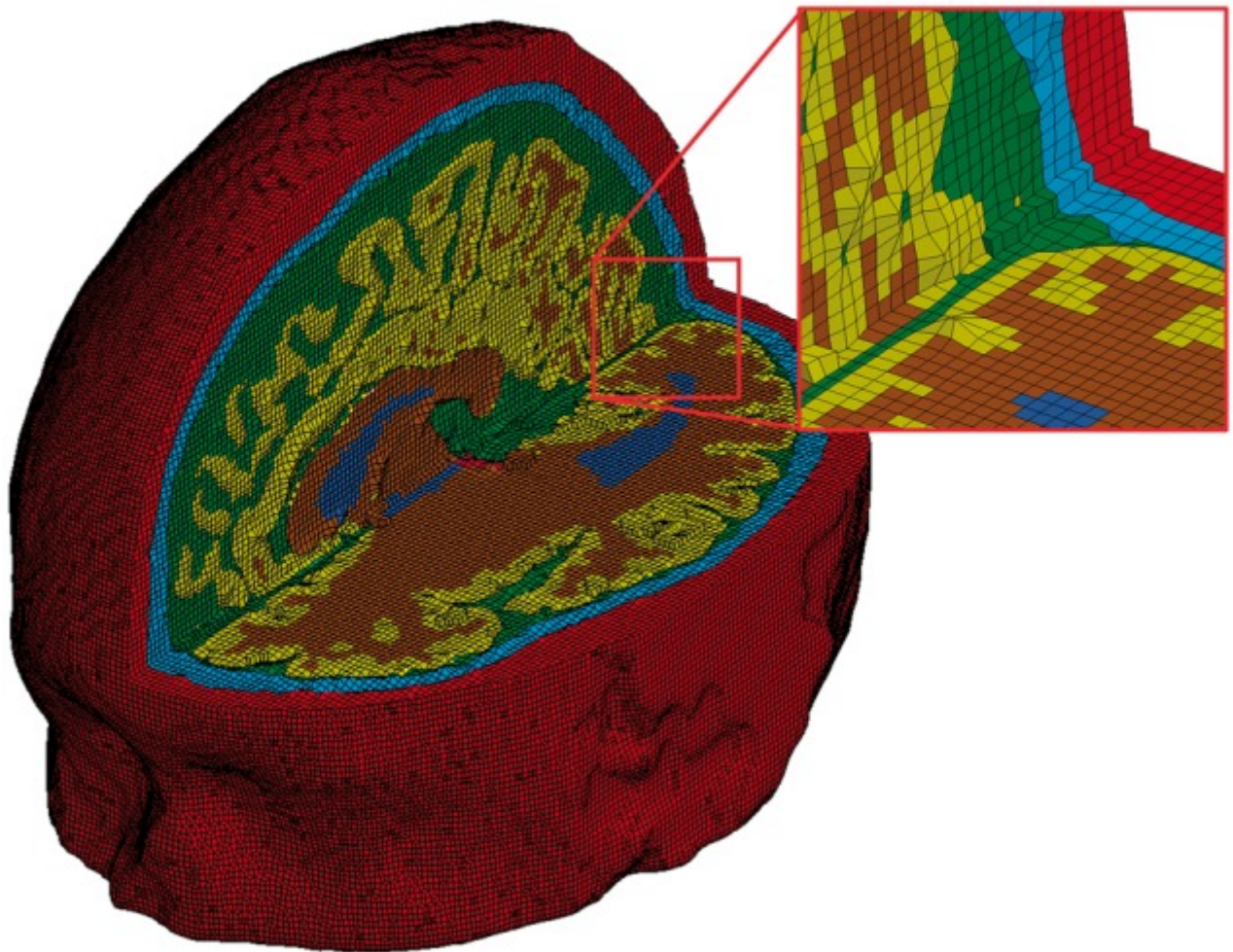


Figure 4. Finite element modelling of TBI biomechanics

The finite element model of the head and brain is composed of over a million elements. These elements are colour coded to represent different tissue classes in the figure and are allocated different mechanical properties based on cadaver experiments. The model is then loaded with a pattern of motion/acceleration for a particular impact, and the stretch of each elements calculated over the duration of impact. Red = skin, light blue = skull, green = CSF, yellow = grey matter, brown = white matter and dark blue = ventricles.

Using this FEM, I can calculate brain injury metrics in each element of the model. I investigate two metrics: strain and strain rate. Strain is defined as the value of the Green-Lagrange strain tensor (EE), a measure of how much the element has been stretched with respect to its original shape, while strain rate is defined as the total time derivative of the Green-Lagrange strain tensor that the element experienced during the simulation, which is a measure of the maximum time rate of stretch within each element (Holzapfel, 2000). For the thesis, I calculate the maximum principal values for

strain and strain rate reached during the impact. These are encoded in an voxelwise basis and saved as a NIFTI (Neuroimaging Informatics Technology Initiative) file.

Neuroimaging applications in impact reconstructions

To fully exploit the benefits of using the high spatial resolution of the FEM, I use neuroimaging statistical tools. These provide validated statistical methods to investigate group differences in brain biomechanics with high spatial and temporal resolution. This is in contrast to methods used currently in the biomechanics field, which typically involve the generation of a generalized metric, such as maximum principle stress, to summarize the forces across the whole brain and relate it to injury risk (Kleiven, 2007, Knowles and Dennison, 2017). These oversimplified metrics ignore the implication of where forces are greatest at the time of injury, or indeed temporal patterns of change in deformation measures during an impact. However, despite the established use and potential benefits of neuroimaging statistical tools, as far as I have observed there has been no previous application of these in research into brain biomechanics.

The application of neuroimaging statistical tools in a biomechanics framework is relatively easy to implement. In standard neuroimaging analyses, a value of interest is assigned to a voxel within an image, either stored as a 3D image (spatial information only) or 4D image (spatial and over a time series, for example with temporal information such as BOLD signal over a set time with functional MRI data). The same can be done with outputs from modelling impacts, whereby strain and strain rate can be saved as a 3D image (maximum strain achieved over the impact duration) or a 4D image (strain at each voxel, with a value per time point simulated). Hereby in this thesis I showcase, for the first time, the application of standard neuroimaging analysis techniques to perform voxelwise comparisons of 3D images of strain and strain rate, investigating where strain and strain rates may be higher or lower between groups of interest. This work lays out a framework for future studies interested in investigating the spatial and temporal significance of brain biomechanics in relation to the pathology that is produced.

Chapter 3

Axonal injury and outcomes after TBI

A study of diffusion MRI and blood biomarkers as measures of axonal injury after brain injury and their relationship with long-term patient outcomes.

In this chapter, I investigate the relationship between imaging and potential fluid biomarkers of axonal injury in a moderate-severe TBI cohort and test how well these biomarkers help predict patient outcomes.

3.1 Abstract

Background

Axonal injury is a key determinant of long-term outcomes after traumatic brain injury (TBI) but has been difficult to measure other than through histopathological examination. Advances in MRI now appear to allow axonal injury to be sensitively detected after TBI. New fluid biomarker assays, which sensitively quantify neuronal proteins in blood, allow for the identification of complementary blood biomarkers of axonal injury such as neurofilament light (NfL) and tau. Using data collected from the multi-centre BIO-AX-TBI study of moderate-severe TBI, I investigate the relationship between advanced neuroimaging, fluid biomarkers and clinical outcomes.

Methods

In 197 moderate-severe TBI patients recruited after a recent injury from hospitals across Europe, diffusion MRI and blood biomarker sampling is used to estimate axonal injury, and compared with patient outcomes at 6 and 12 months post injury. Blood biomarker concentrations are compared with concentrations in brain extracellular fluid obtained using cerebral microdialysis. An experimental injury model is then used to validate biomarkers against histopathology.

Results

A diffusion MRI measure of axonal injury, fractional anisotropy, was abnormally reduced in the TBI group at all time points after injury compared to controls, with the degree of abnormality increasing sub-acutely to 6 months after injury. Plasma NfL increased after TBI, peaking at 10 days to 6 weeks after injury, but remaining abnormal one year post-injury. These concentrations were markedly higher after TBI than in patients with extracranial injuries alone. NfL concentrations correlated with diffusion MRI measures of axonal injury and was the strongest predictor of functional outcomes one year post-injury. NfL levels in plasma and brain extracellular fluid sampled from cerebral microdialysis were highly correlated. An experimental injury model replicated a dose response relationship of plasma NfL to histopathologically defined measures of axonal injury.

Conclusions

These findings suggest that diffusion MRI and plasma NfL are complementary measures of axonal injury that can predict functional outcomes up to a year after injury.

3.2 Introduction

Long-term outcomes of traumatic brain injury (TBI) are frequently poor (Maas et al., 2017). Diffuse axonal injury (DAI) is a key determinant of these clinical outcomes (Smith et al., 2003, Sidaros et al., 2008). However, validated methods to quantify the extent of axonal injury are lacking in a clinical context. Diffusion tensor imaging (DTI) provides a sensitive and robust method of assessing axonal injury within the brain. Fractional anisotropy (FA), a DTI metric, is widely used as a measure of axonal injury that correlates with histopathological evidence of DAI (Mac Donald et al., 2007b) and provides prognostic information about long-term clinical outcomes (Bonnelle et al., 2012, Bonnelle et al., 2011, Hellyer et al., 2012, Sharp et al., 2014).

Fluid biomarkers could provide a complementary measure, as cytoskeletal proteins are released from damaged axons. Cytoskeletal proteins NfL and microtubule associated protein tau (tau) have been of particular interest. NfL has shown diagnostic, monitoring and prognostic utility across several neurological diseases (Gaetani et al., 2019). Using advanced immunoassays such as single molecule array (Simoa), NfL and tau can be sampled at ultra-low concentrations in venous blood, with levels reflecting cerebrospinal fluid concentrations (Shahim et al., 2020a, Gisslén et al., 2016, Shahim et al., 2016a). After moderate-severe TBI, NfL is acutely elevated in plasma, sometimes remaining increased years after injury (Scott et al., 2018, Shahim et al., 2020a). In contrast to other blood biomarkers which typically peak within days after injury, plasma levels continue to rise (Thelin et al., 2017, Shahim et al., 2016a). Initial work shows that blood levels of NfL can predict late clinical outcomes, although it is uncertain how much prognostic information is provided when compared to other biomarkers such as the widely expressed neuronal ubiquitinating enzyme ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) or predominantly astroglial expressed proteins such as glial fibrillary acidic protein (GFAP) and calcium binding protein S100 B (S100B) (Thelin et al., 2019, Shahim et al., 2016a, Al Nimer et al., 2015a, Shahim et al., 2020a).

There are several hurdles these potential blood biomarkers face as biomarkers of axonal injury. The optimal time to sample NfL after TBI is unclear as the dynamics of its fluctuation over time have not been characterised in detail. Furthermore, the source of plasma NfL after TBI is also unclear, which limits its diagnostic value. NfL is present in high quantities within long myelinated axons in the cerebral deep white matter that are susceptible to DAI (Donat et al., 2021, Schlaepfer and Lynch, 1977). However, plasma NfL could also originate outside the brain and it remains unclear how specific increased levels are to TBI. Blood NfL levels can be elevated in both chronic (Sandelius et al., 2018) and acute peripheral neuropathies such as Charcot-Marie-Tooth disease (Martín-Aguilar et al., 2021). This is important as TBI often occurs alongside peripheral nerve injury, which can potentially increase NfL concentrations in blood. This study aims to address these hurdles in order to validate the use of NfL as a clinically useful biomarker of DAI in TBI.

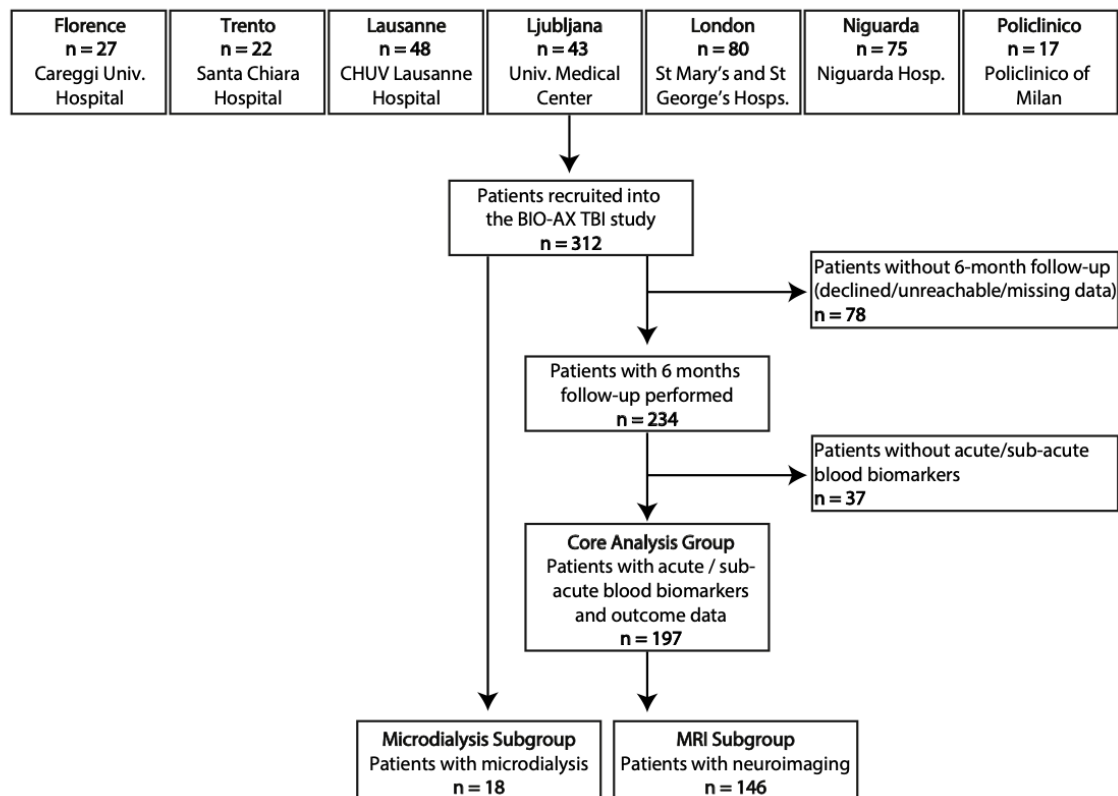
One way to assess NfL specificity to axonal injury is to directly measure levels from extracellular fluid collected from damaged white matter. This has not been done previously but can be achieved using invasive cerebral microdialysis with catheters permeable to neuronal breakdown products. High levels of tau in brain extracellular fluid early post-injury have been demonstrated using this method, with levels correlating with imaging evidence of axonal injury (Magnoni et al., 2015). Preliminary microdialysis work with NfL has shown increased levels in peri-contusional and apparently normal-appearing white matter regions that correlate with extracellular tau levels (Magnoni, Esparza et al. 2012). Plasma NfL can also be compared with diffusion imaging measures of DAI in the brain, as levels of plasma NfL should correlate if it originates from damaged axons. We can also investigate NfL levels in non-TBI trauma patients compared to TBI patients to further our understanding of the specificity of NfL as a measure of DAI.

Using an experimental injury models of TBI is another method of investigating the specificity of FA and NfL concentrations to axonal injury. Experimental injury models can be used to test the relationship between blood biomarkers and axonal injuries,

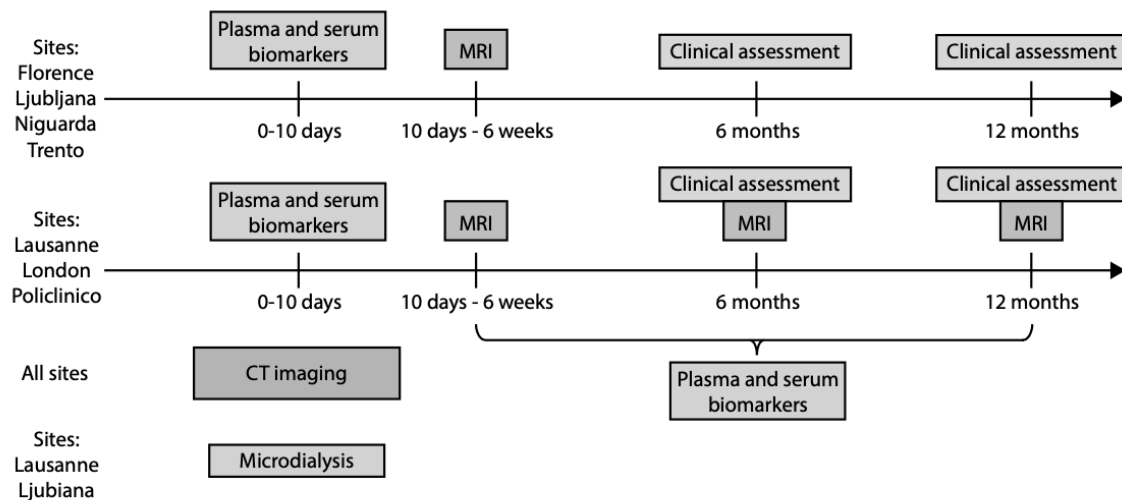
providing ground truth validation. The controlled cortical impact (CCI) model provides a reproducible means to control the extent and distribution of injury, and to examine the associated histopathological effects (Donat et al., 2017, Donat et al., 2021, Edward Dixon et al., 1991). The histopathological effects of injury can therefore be compared with neuroimaging measures of axonal injury and blood biomarker levels of NfL in an aligned fashion to human studies. Previous experimental work on blood NfL in TBI is limited but suggests a relationship between plasma NfL levels and neurofilament-immunoreactive axonal swellings indicative of injury (Cheng et al., 2018, Bashir et al., 2020). A dose-response relationship of NfL to injury has not previously been directly established.

Here, I analyse data from the multicentre BIO-AX-TBI study (Graham et al., 2020a) and aligned experimental injury model study (Donat et al., 2021) to: (a) assess how diffusion MRI measures and fluid biomarker assessments of axonal injury change over time after a moderate-severe TBI; (b) assess how these measures are related to one another, and to clinical outcomes; (c) assess the specificity of these measures by comparing them to fluid biomarker levels in brain extracellular fluid in humans; and (d) to histopathology measures in an experimental injury model (Figure 5). I test the following specific hypotheses in relation to FA and NfL: (a) FA will be reduced after TBI; (b) NfL levels will increase after TBI and will be detectable in plasma; (c) specific changes in NfL will be seen after TBI when compared to patients with extracranial injuries but no TBI; (d) plasma NfL will correlate with diffusion MRI measures of axonal injury, specifically reduced FA; (e) FA and plasma NfL will correlate with levels in extracellular fluid on cerebral microdialysis; (f) FA and plasma NfL concentrations will relate to histopathological evidence of axonal injury in the CCI model; (g) plasma NfL will predict clinical outcomes at six and twelve months post-injury.

A TBI patient recruitment and study flow



B Patient assessment protocol



C Experimental injury protocol

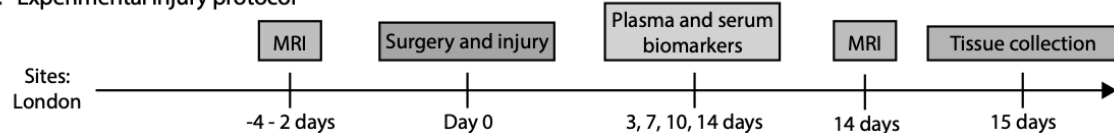


Figure 5. Study overview and design.

The BIO-AX-TBI study is a multicentre study designed to identify the most informative plasma biomarkers of the severity of axonal injury in moderate-severe patients with TBI. The study started recruitment in 30 November 2017 and aimed to recruit 250 patients in total. Excess recruitment was conducted to meet targets of follow-up and microdialysis. (A) BIO-AX-TBI patient recruitment and analysis groups

from Florence (Italy), Trento (Italy), Lausanne (Switzerland), Ljubljana (Slovenia), London (UK), Niguarda, (Italy) and Policlinico (Italy). **(B)** Patient assessments protocols and timings. The core assessment pathway (top), with some centres performing additional neuroimaging and blood biomarkers (middle). CT is performed in hospital at all sites during patient stay, and invasive cerebral microdialysis is performed at two sites. **(C)** The experimental injury model timeframe.

3.3 Methods

Cohort and study overview

Patients with moderate-severe TBI, defined by the Mayo classification (Malec et al., 2007), were recruited from eight trauma centres (Figure 5A). Patients unable to provide consent underwent an assent process and were re-consented if they subsequently regained capacity. Inclusion criteria were age between 18 to 80 at time of injury and a diagnosis of moderate-severe TBI. Exclusion criteria were pre-existing neurological disease, previous TBI requiring hospitalisation, significant drug or alcohol abuse, pregnancy and for the MRI component, the presence of typical contraindications such as ferrous implants. A total of 312 patients were included in the study, of which 78 patients did not complete a 6-month follow-up and further 37 patients did not have biomarker assessments in the first 10 days and hence were not included in the study analysis.

Clinical and demographic information were collected electronically using TBI common data elements. Blood biomarkers were assessed twice in the first ten days following injury. Patients were followed up at a subacute timepoint ten days to six weeks post-injury. Follow-up visits included blood, MRI and clinical assessment (Figure 5B). Additional visits were performed at six and twelve months' post-injury with a functional assessment including the GOSE (Wilson et al., 1998). In a subset of sites (Figure 5B), six and twelve month assessments included blood and neuroimaging. Cerebral microdialysis was performed acutely at two sites, and in patients undergoing cerebral microdialysis, plasma biomarker assessment was performed up to twice per 24 hours acutely.

Healthy controls were recruited at each study site according to the same exclusion criteria. Controls underwent a single MRI and blood biomarker assessment. A

separate group of longitudinally-scanned controls were included, comprising healthy volunteers who had participated in more than one study on the same scanner system with an aligned acquisition protocol (Cole et al., 2018a).

Non-TBI trauma patients were recruited in London and underwent blood biomarker assessment on two occasions in the first ten days following injury. Inclusion criteria were the extracranial injuries requiring hospitalisation. As with the main patient group, exclusions included premorbid significant neurological disease in addition to any suspicion of TBI (i.e. symptomatic possible injuries), or a diagnosis of spinal cord injury, specific to this group.

Neuroimaging - Human

Human neuroimaging acquisition

Participants in all sites had a high resolution structural T1 MPRAGE, T2 fluid-attenuated inversion recovery (FLAIR), susceptibility-weighted imaging (SWI) and diffusion tensor imaging (DTI). DTI sequences were aligned at each site and acquired in 64 directions with an isotropic voxel size of 2mm^3 , $b = 1000 \text{ s/mm}^3$, four images with $b = 0 \text{ s/mm}^2$ except in a single site (Santa Chiara Hospital – ‘Trento’) where only 60 directions were acquired and with a $1\text{mm} \times 2\text{mm} \times 2\text{mm}$ voxelwise (Table 1). Two healthy volunteers were scanned at each site and oversaw standardisation of scanning protocols. DTI scans from the travelling volunteers were analysed and FA measures in 10 white matter regions of interests analysed. All regions were under 5% coefficient of variation, which is used as an acceptable threshold for variation (Palacios et al., 2017). A physical N-tridecane phantom was scanned repeatedly at each site to ensure acquisition quality throughout the study. Diffusion derived analysis values were compared across the duration of the study and compared with temperature dependent thresholds (Tofts et al., 2000). Further details for scanners and acquisition parameters can be found in the study protocol (Graham et al., 2020a). Patients and controls were scanned using the same protocols within each centre and this protocol was kept consistent for the duration of the study. Structural MRI scans from all study sites were reviewed by a team of neuroradiologists in London and reported using electronic case report forms adapted from the National Institute of Neurological Disorders and Stoke

(NINDS) TBI Imaging CDEs, providing a gold standard readout of imaging abnormalities over time across the group.

Table 1. Imaging acquisition parameters and scanner details.

MPRAGE: Magnetization Prepared Rapid Gradient Echo; DTI: diffusion tensor imaging; FLAIR: FLuid Attenuated Inversion Recovery; SWI: Susceptibility Weighted Imaging.

	Research Site						
	Florence	Lausanne	Ljubljana	London	Niguarda	Policlinico	Trento
Scanner							
manufacturer	Siemens	Siemens	Siemens	Siemens	Philips	Philips	GE
model	Aera	Skyra Fit	Trio Tim	Verio	Achieva	Achieva	Optima
field strength	1.5 Tesla	3 Tesla	3 Tesla	3 Tesla	1.5 Tesla	3 Tesla	1.5 Tesla
software	VE11a	MR B19	MR B19	MR B17	Rel. 5 Neuroradio	Rel. 5 Neuroradio	DV25.1 _R03_1 802.a
Voxelsize (mm)							
MPRAGE	1x1x1	1x1x1	1x1x1	1x1x1	1x1x1	1x1x1	0.5x0.5x1
DTI	2x2x2	2x2x2	2x2x2	2x2x2	2x2x2	2x2x2	1x2x2
FLAIR	0.5x0.5x1	0.5x0.5x1	1x1x1	1x1x1	1.2x1.2x0.7	1x1x1	1.2x0.5x0.5
SWI	0.8x0.8x0.8	0.3x0.3x1.6	0.6x0.5x1.2	0.6x0.5x1.2	1x1x1	0.5x0.5x1.2	0.5x0.5x1
DTI parameters							
receiver coil	20	64	32	32	8	32	8
channels							
directions	64	64	64	64	64	64	60
b-value	1000	1000	1000	1000	1000	1000	1000

Human MRI analysis - lesions

Focal lesions identified were manually segmented in native T1 space and scan/session-specific lesion masks were registered to MNI152 standard space and DTITK standard space using affine linear registration in FSL (Figure 6). An individual's

lesions were incorporated into the voxelwise analysis by including the lesioned voxels as voxelwise regressors in the general linear model.

Human MRI analysis – diffusion MRI including z-score normalisation across centres

Diffusion-weighted images were visually inspected before processing. Scans with uncorrectable artefacts or errors within acquisition were removed at this stage ($n = 6$). Scans were then processed following the standard tract based spatial statistics (TBSS) pipeline in the FMRIB Software Library (version 5.0.8) (Smith et al., 2006), including correcting susceptibility and eddy current induced distortions and diffusion tensor fitting. Brain extraction was performed using HD-BET (Heidelberg University Hospital & Division of Medical Image Computing, German Cancer Research Centre) (Isensee et al., 2019). Tensor-based registration was performed using DTI-TK (Zhang et al., 2007) involving the creation of a group template using affine and non-linear diffeomorphic registrations followed by registration of participant diffusion imaging to the template. Images were warped to 1mm isotropic space and the mean FA map produced was thresholded at 0.2 to produce a white matter skeleton. Subject FA data were projected onto the mean FA skeleton, with corpus callosum region of interest data generated using the Johns Hopkins University (JHU) white matter atlas. Images were repeatedly inspected at the brain extraction, eddy current correction and tensor registration stage. The mean Pearson correlation coefficient of the neighbouring diffusion weighted images and slice-wise signal dropout for each slice was calculated for quality control checks. All data passed the criteria of baseline and follow-up having no difference in mean Pearson correlation coefficient greater than 0.1 while two scans from the same participant were removed due to signal dropout being higher than 0.1% of the total slice number (Yeh et al., 2019). In total twelve diffusion scans were removed due to quality control issues.

Images were normalised across different centres to account for the inter-site scanner differences and enable groupwise comparisons in FA, with a z-scoring approach. In each site, raw FA maps in patients were normalised in a voxelwise manner to the local site specific healthy control FA maps, who were age/sex matched to TBI patients across the group. The following equation was used at each voxel:

$$zFA_{\text{patient}} = (\text{raw } FA_{\text{patient}} - \text{mean } FA_{\text{control group}}) / \text{standard deviation } FA_{\text{control group}}$$

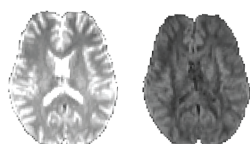
This generates an individualised, normalised z-scored FA map for each patient which can then be compared between different sites. Mean z-scored FA was calculated for the whole white matter skeleton, and to account for any potential issues with scan quality diminishing at the boundaries, we also sampled zFA within the corpus callosum, a large, robust, central tract, while excluding sampling in areas with lesions.

Voxelwise analysis of zFA was performed using threshold free cluster enhancement (TFCE) multiple comparisons correction and a reported at a threshold significance level of $P < 0.05$. Longitudinal and cross sectional analysis of diffusion metrics was conducted using the general linear model with non-parametric permutation testing (10,000) in FSL Randomise (Winkler et al., 2014), with age and gender included as a nuisance covariates in cross sectional analyses, and individualised lesion masking.

In patients with microdialysis, the catheter tip was identified on CT imaging. CT scans were registered to DTITK space and a 10mm radius spherical region of interest was generated. The spherical region of interest was overlaid on the white matter skeleton and FA sampled within the overlapping voxels.

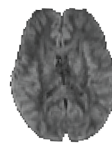
Input data and groups

Diffusion weighted images
b=0 b=1000



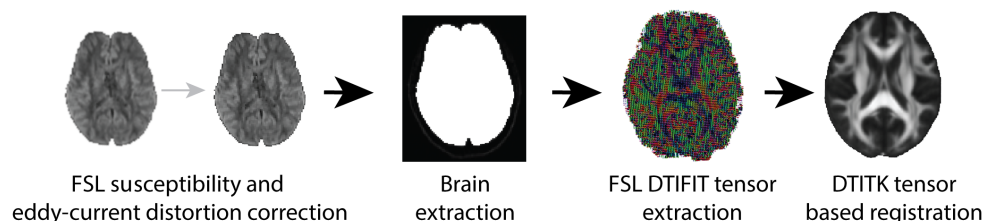
TBI patients n = 131
Healthy controls n = 121

Repeated diffusion imaging



TBI patients n = 53

A Diffusion weighted image processing



B T1-MRI cross sectional structural image processing



Manual lesion masking

C Statistical analysis

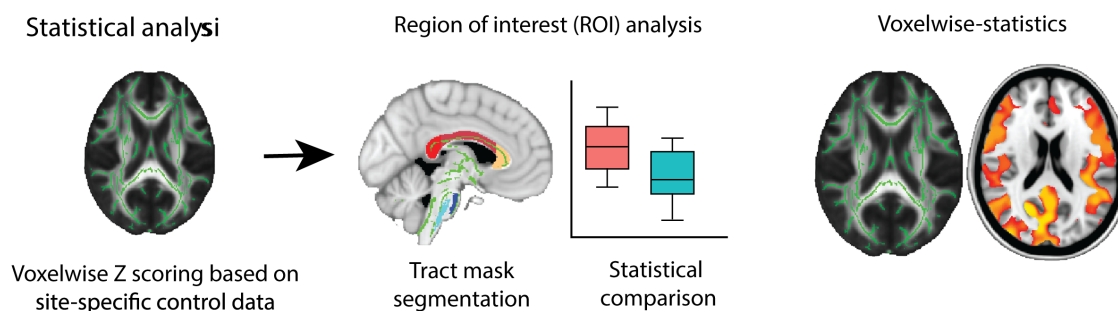


Figure 6. Neuroimaging acquisition and analysis pipelines.

MRI data including diffusion weighted imaging (DWI) and T1-volumetric structural images were processed using the following approaches: (A) DWI images are eddy-corrected, undergo brain extraction, FSL DTIFIT extraction of tensors and DTITK registration. (B) Custom lesion masks are created per subject in native space to be incorporated into statistical analysis (C) Statistical comparisons are undertaken using summary measures, region of interest approaches or voxelwise analyses.

Fluid biomarker assessment – Human

Human blood biomarker assessment

Venous blood was sampled peripherally or by central access line if available, using ethylenediaminetetraacetic acid (EDTA) coated tubes and a clot-activator for plasma and serum samples respectively. After 30 minutes at room temperature, samples were centrifuged at 2000-2500g at 4°C, transferred into 1.4ml aliquots and frozen at -80°C. NfL, total tau, GFAP and UCH-L1 concentrations were assessed in plasma using a Neurology 4-plex B assay on the Simoa-HD1 platform, following the manufacturer's protocol (Quanterix Corp, Boston, MA). Serum S100B was measured using a Millipore ELISA kit (Merck Millipore, Darmstadt, Germany) with a lower limit of quantification of 2.7pg/ml. Samples were thawed on wet ice, centrifuged at 500 × g for 5 minutes at 4°C. Calibrators (neat) and samples (1:4 dilution) were measured in duplicates. A four-parameter logistic curve fit data reduction method was used to generate a calibration curve. Two control samples of known concentrations of the protein of interest (high-control and low-control) were included as quality control. Samples were run in duplicate. The mean coefficient of variation across the study was 4.0% for NfL, 7.4% for total tau, 16.5% for UCH-L1, 4.7% for GFAP, and 14% for S100B. The mean of both samples was used for further analyses. Any result falling below the lower limit of quantification (LLoQ) for a given biomarker was re-recorded as 50% of the LLoQ (GFAP: 9.38 pg/ml; NfL 0.5 pg/ml; S100B 2.7 pg/ml; tau 0.125 pg/ml; UCH-L1 9.38 pg/ml). All results were included, with no removal of 'outliers' at lower or upper limits. Assays of blood biomarker concentrations were conducted at the biomarker lab at the UK Dementia Research Institute at UCL. Peak biomarker concentrations within an individual were defined as the highest concentration over 6 weeks post-injury.

Human invasive cerebral microdialysis protocol and analysis

Microdialysis was initiated when clinically indicated and discontinued when no longer needed, typically five to seven days post-injury. 100kD cut-off MDialysis AB microdialysis catheters were surgically implanted through a triple lumen bolt cranial device into white matter regions that were not affected by gross focal pathologies such as contusions or haemorrhage on CT. This was typically in right, non-dominant frontal

lobe. In patients with frontal contusions, catheters were placed distant to intra-cerebral lesions. The placement was confirmed using a standard 2mm non-contrast CT head. Standardised commercially-available 3% Dextran 500 (M Dialysis, Stockholm, Sweden) solution was used to increase protein recovery, as indicated by the manufacturer (Giorgi-Coll et al., 2020). A standard flow rate of 0.3 mL/min was used in all patients, allowing hourly recovery of brain extracellular fluid (about 20 mL). Vials taken four hourly were immediately frozen and stored after collection and used exclusively for the measurement of biomarker concentrations. Samples were analysed using the Neurology 4-plex B assay on the Quanterix Simoa-HD1 platform, as per blood biomarkers, but using a 20:1 dilution.

The relationships between biomarker concentrations in blood and cerebral microdialysate were assessed by Dr Neil Graham. A linear mixed effects model was used, requiring complete data sampled in plasma/brain at aligned timepoints. Two values were identified missing in two patients (a GFAP result and UCH-L1 result). Using the markers surrounding the concentration and the time since injury as features vectors, they were imputed independently using a sklearn distance-based k nearest neighbours imputer (k=2). To account for temporal misalignment between brain and blood markers, we first upsampled the brain markers using a time weighted interpolation model. We then removed any missing paired observations in either the beginning or the end of the matching sequence. Finally, redundant brain observations were omitted to match the sampling frequency in the blood. In the model for each biomarker, subject was a fixed effect, with main effects of brain concentration, and an interaction of log-transformed brain concentration and time since injury, to predict log-transformed plasma concentration.

Experimental injury model overview

The experimental injury data was collected from experiments completed by Dr Cornelius Donat (Donat et al., 2021). All procedures were conducted under a Home Office license and were in keeping with the Animal [Scientific Procedures] Act 1986 and EU legislation. Thirty-four Male Sprague Dawley rats (~8-9 weeks old) were housed in standard conditions. MRI was performed in 18 animals prior to group

assignment. Animals were then randomised to sham-operation (n=4), mild/1 mm CCI (n=15) and moderate/2 mm CCI (n=15). Surgery and CCI were performed as previously described (Donat et al., 2017) and are detailed in brief below. Sham-operated animals were subjected to all drugs and surgical procedures except craniotomy and impact. Blood was taken at baseline (two to four days prior to surgery), 3, 7, 10 and 14 days' post-injury (Figure 5). Fourteen days' post-impact, animals underwent a second MRI. The next day, the rats underwent terminal anaesthesia followed by transcardial perfusion and tissue harvesting. Ten additional naïve animals served as histology controls and were only subjected to anaesthesia prior to transcardial perfusion.

Experimental injury model surgery and controlled cortical impact

Anaesthesia was induced at least 30 min before the first incision and body temperature was adjusted and maintained at 37°C. Surgery and Controlled Cortical Impact were performed as previously published (Donat et al., 2017). A ~6 mm unilateral rectangular craniotomy, -0.5mm to -6.5 mm posterior and + 3.5 mm lateral to Bregma, was performed with a high-speed microdrill. The bone flap was stored in sterile saline. Immediately thereafter, CCI was induced using the 5 mm flat electromagnetically driven steel impactor (23° angle) performed at ~4 m/s for 100 milliseconds with an impact depth of either 1 or 2 mm. The dura was then inspected for signs of rupture, which occurred in one animal, in which the craniotomy was covered with absorbable gelatine sponges. The bone flap was reimplanted and the craniotomy was sealed with a nontoxic light curing resin. After thorough irrigation and cleaning, the subcutaneous tissue and scalp were sutured and anaesthesia stopped. After surgery, all animals were single housed and checked for signs of pain and discomfort every 30 minutes for the following 4 hours. For the next three days, all animals were weighed and inspected twice daily and thereafter every morning. All animals were then housed with the same cage mate as before. Full details can be found in a previous report (Donat et al., 2021).

Experimental injury model MRI acquisition

MRI scanning was performed in a 9.4T Bruker BioSpec scanner, equipped with a 4-channel phase array receiver coil and Paravision 6.1 software. Briefly, after localiser scans, high-resolution structural imaging was acquired with the following parameters: 3D T1 (TE=5 ms, TR=60 ms, $0.2 \times 0.2 \times 0.2 \text{ mm}^3$ resolution, 12 min acquisition) and 2D T2 (TE=33 ms, TR=5.5 s, RARE factor=8, $0.2 \times 0.2 \times 0.5 \text{ mm}^3$ resolution, 20 slices, acquisition time approximately 3 min). For diffusion, the multi-shell protocol included one shell with 40 gradient directions and $b = 1000 \text{ s/mm}^2$ and another with 40 directions and $b = 2000 \text{ s/mm}^2$. The protocol also contained four images without diffusion weighting ($b = 0 \text{ s/mm}^2$) and a single reversed-phase encoding image without diffusion weighting. The EPI readout (TE = 21 ms, TR = 4 s) had a resolution of $0.25 \times 0.25 \times 0.40 \text{ mm}^3$. A total of 34 contiguous slices were acquired for whole brain coverage.

Experimental injury model MRI analysis – diffusion MRI

Standard postprocessing steps were undertaken using FSL and FA extracted from the corrected multi shell diffusion data. The corpus callosum was selected as a region of interest and segmented into 5 equal sections using MATLAB (Figure 7). Segments closest to the midline were labelled as segment 1. CC region of interest z-FA scores were generated at 14 days post injury using the mean and standard deviation of baseline scans of all animals as a ‘healthy control’ reference, to facilitate comparisons with the human neuroimaging data.

Experimental injury model blood biomarker assessment

Rats were inserted into a restrainer, the tail cleaned and approximately 200 μL of whole blood withdrawn into EDTA-coated tubes. After centrifugation (10 min, 2000g), plasma was collected and immediately frozen. A single-plex Simoa NfL assay (Quanterix) was used to quantify rat plasma and CSF NfL and analysis conducted at the biomarker lab at the UK Dementia Research Institute at UCL using the same protocol as for human samples.

Histology data from the experimental injury model were collected as part of a previously reported study (Donat et al., 2021).

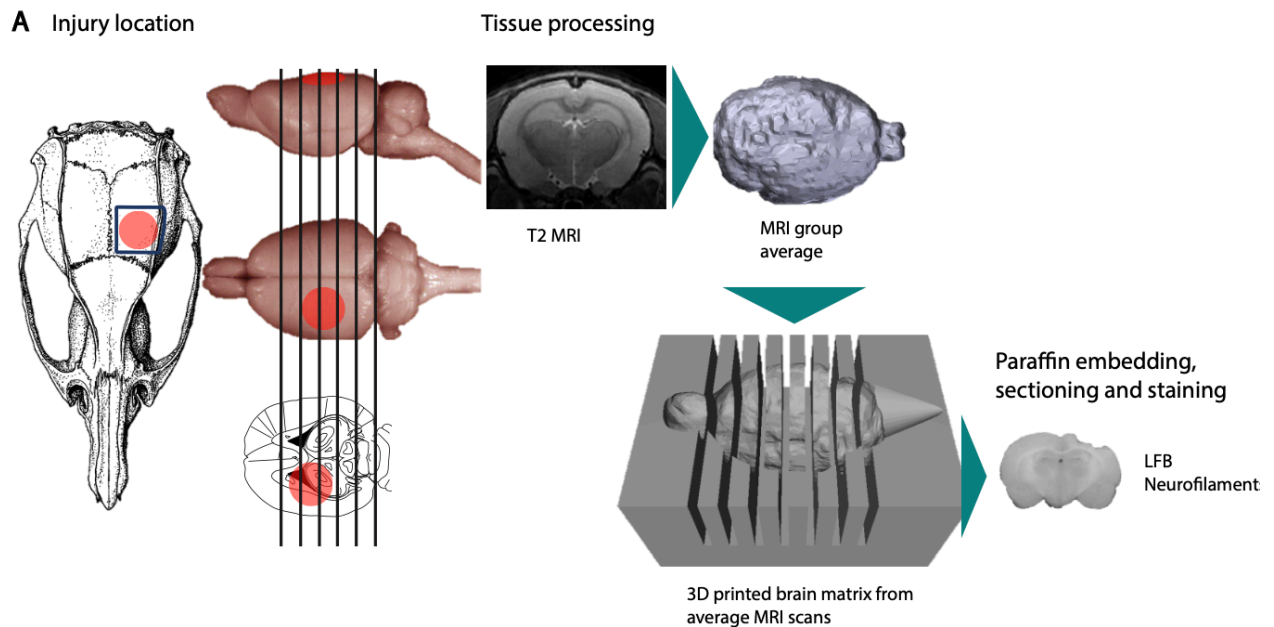


Figure 7. Controlled cortical injury methods.

Adapted from (Donat et al., 2021) (A) The red circle indicates the location of the craniotomy and injury. From T2 MRI scans, a 3D model is printed with brain matrix. In vivo DTI-MRI data was acquired and sections were stained for white matter integrity (Luxol Fast Blue).

Statistical analysis

An a priori level for statistical significance was set at 0.05 for two sided tests. Standard t-tests were used for comparisons of normally distributed continuous data, and the non-parametric Wilcoxon test for non-normally distributed continuous variables. Paired t-tests, repeated measures ANOVA designs or linear mixed effects models, were used for longitudinal within-subject data, as appropriate. Owing to the data distribution, linear regressions on fluid biomarker concentrations were performed on $\log_{10}$ transformed data. Bonferroni multiple comparison corrections were performed to correct for the number of timepoints and biomarkers/groups assessed, other than for NfL where a clear a priori hypothesis existed. Hence. NfL comparisons were corrected for the groups/timepoints only. In exploratory analysis of other biomarker levels and within the experimental injury model, a correction for the number of biomarkers was

conducted. Unless otherwise stated, all boxplots all show median, hinges at 25th and 75th centiles, and whiskers extending to the largest value less than or equal to 1.5 times the interquartile range from the hinge. To assess the relationships between biomarker concentrations in blood and cerebral microdialysate, a linear mixed effects model was used. Missing data was imputed independently for the model to facilitate analysis of plasma/brain at aligned timepoints. For the experimental injury model, a one-way ANOVA was used to test differences in plasma NfL levels at baseline and CSF NfL concentrations. A mixed-effects model, with time and injury as fixed effects, were used to test differences in plasma NfL levels across different animal injuries and time since injury. CSF NfL concentrations were not significantly different between naïve animals and shams, and hence these groups were combined as controls. DTI changes were analysed using a mixed effects model with injury and segment as fixed effects.

3.4 Results

Demographics, clinical characteristics and outcomes of patients and healthy controls

One hundred and ninety-seven patients were recruited after moderate-severe TBI from eight major trauma centres (Figure 5) (Table 2). The median age at injury was 47 years (IQR 30) with 77% male. 91% of injuries were accidental, with the highest proportion being road traffic related (47%) (Table 3). The cohort included a range of severities within the moderate-severe classification, with pre-hospital Glasgow Coma Scale (GCS) 3 to 8 in 45% (Table 4). A range of findings were reported on acute CT imaging, including diffuse axonal injury in 18% (Table 5). Neurosurgery was performed in 35% of patients (defined as any neurosurgical intervention), and during the study period, 7% of patients died, at a median of 11 days post-injury. Functional outcomes were assessed over a year and all patients had a known outcome at either 6 or 12 months post injury, with the Glasgow Outcome Scale Extended (GOSE) available in 94% at 6 months and 82% at 12 months. At 6 months, 29% of patients made a good recovery, defined as as a GOSE score of 7 or 8. Six individuals were in a vegetative state (GOSE 2) at 6 months, and were unchanged at 12 months. By 12 months post-injury 32% of patients made a good recovery. Further breakdowns in clinical characteristics and outcomes of TBI patients are available in Table 3-5.

MRI was performed on 74% of patients with a median age of 44 (IQR 30) and were 80% male (Table 2). Patients with MRIs did not significantly differ in demographics or in clinical measures of severity such as lowest initial GCS, percentage with CT evidence of DAI or percentage with neurosurgery compared to patients who did not undergo sub-acute MRI imaging. Fifty-eight patients had a scan at 6 months post injury, and 39 at 12 months post injury. Cerebral microdialysis and simultaneous plasma sampling was performed in 9% of patients with a median age of 34 (IQR 27.5) and 78% male. Patients who underwent cerebral microdialysis were not significantly different in demographics or injury severity as measured by initial GCS or presence of CT evidence of DAI, however neurosurgical intervention was higher in this subgroup (100% compared to 35%).

Healthy volunteers were assessed across all the sites, comprising of 128 individuals with a median age of 39 (IQR 27), with 78% male. 95% underwent MRI and 75% had blood biomarker assessment. Controls were younger ($W = 10784$, $P=0.028$) and more were female ($X^2 9.10$, $df = 1$, $P=0.003$) compared to TBI patients, however there was no significant difference in age between patients and healthy controls who underwent MRI. To assess the blood biomarker specificity, 25 patients hospitalised with non-TBI traumatic (NTT) extracranial injuries were recruited. These patients, who had no head injury history, had a median age at injury of 41 (IQR 29) of whom 92% were male. NTT patients had fluid biomarker assessment twice in the first ten days post-injury, aligned to the TBI group and did not differ significantly in age or sex to the TBI patients.

Table 2. Participant demographics.

Five microdialysis patients are also in the main group ('All'). NTT = non-TBI trauma patients

	TBI			NTT	Healthy controls
	All	Imaging	Microdialysis	All	All
Total, N	197	146	18	25	128
Age, median (IQR)	47 (30)	44 (29.5)	34 (27.5)	41 (29)	39 (27)
Male, N (%)	152 (77.2)	116 (79.5)	14 (77.8)	23 (92.0)	78 (61)
GCS at admission, median (IQR)	9 (8)	9 (7)	8 (8)	NA	NA
CT evidence of DAI (% with)	35 (17.8)	29 (19.7)	7 (38.9)	NA	NA
Neurosurgical intervention (% with)	70 (35.5)	58 (39.5)	18 (100)	NA	NA
Centre, N					
Florence	13	11	0	0	13
Lausanne	22	19	14	0	15
Ljubljana	32	19	4	0	15
London	54	39	0	25	39
Niguarda	40	25	0	0	15
Policlinico	14	13	0	0	16
Trento	22	20	0	0	15

Table 3. Demographics and clinical characteristics of TBI patients.

All values are N (%) unless otherwise stated.

Demographics		Injury details	
Premorbid employment		Biophysics	
Unfit for work	16 (8.1)	High energy TBI	110 (55.8)
Home-maker	2 (1.0)	Low energy TBI	68 (34.5)
Retired	2 (1.0)	Unknown	19 (9.6)
Student	11 (5.6)	Injury cause	
Jobseeker	31 (15.7)	Accidental	180 (91.4)
Unknown	36 (18.3)	Violence/assault	13 (6.6)
Worker	99 (50.3)	Self-harm	1 (0.5)
Ethnicity		Other/unknown, N (%)	3 (1.5)
Asian	12 (6.0)	Occupational injury	22 (11%)
Black/African/Caribbean	5 (2.5)	Road traffic accident	93 (47)
Other	1 (0.5)	Bicycle	22
Unknown	15 (7.6)	Car/Van	23
White	164 (83.2)	Motorbike	23
Education		Other	2
University	36 (18.3)	Pedestrian	23
Secondary education	30 (15.2)	Blunt weapon	4 (2.0)
Primary education	65 (33.0)	Gunshot	1 (0.5)
Unknown	65 (33.0)	Crush	2 (1.0)

No formal education	1 (0.5)		
Clinical Characteristics		Retrograde amnesia	
Initial GCS		definite	57 (28.9)
3 to 8	89 (45.2)	suspected	63 (32.0)
9 to 13	55 (27.9)	absent	19 (9.6)
14 to 15	51 (25.9)	unknown	58 (29.4)
unknown	2 (1.0)	Post traumatic amnesia	
Lowest GCS first 24h, median (IQR)	7.5 (8)	definite/suspected	136 (69.0)
Pupils in ED		duration 1-30 mins	3 (2.2)
bilaterally reactive	138 (70.1)	duration 30-60 mins	4 (2.9)
unilaterally dilated/non-reactive	25 (12.7)	duration 1-24 hours	10 (7.4)
bilaterally dilated/nonreactive	1 (0.5)	duration 1-7 days	20 (14.7)
unknown	34 (17.3)	duration > 7 days	23 (16.9)
Loss of consciousness		duration unknown	76 (55.9)
definite	121 (61.4)	absent	14 (7.1)
suspected	26 (13.2)	unknown	61 (31.0)
absent	27 (13.7)		
unknown	23 (11.7)		

Table 4. Management and outcomes of TBI patients.

All values are N (%) unless otherwise stated.

Management and clinical course		Neurosurgery	
Intubated & ventilated	135 (68.5)	evac of EDH	18 (33.5)
Intracranial hypertension	58 (29.4)	evac of SDH	31 (15.7)
persistent	31 (15.7)	evac of parenchymal haematoma	8 (4.1)
		secondary decompression	8 (4.1)
Outcomes		GOSE, 12 months	
GOSE, 6 months		GOSE, 12 months	
dead (1)	13 (6.6)	dead (1)	13 (6.6)
vegetative state (2)	6 (3.0)	vegetative state (2)	6 (3.0)
severe disability (3,4)	48 (24.4)	severe disability (3,4)	28 (14.2)
moderate disability (5,6)	60 (30.5)	moderate disability (5,6)	52 (26.4)
good recovery (7,8)	58 (29.4)	good recovery (7,8)	62 (31.5)
unknown	12 (6.1)	unknown	36 (18.3)

Table 5. Neuroimaging findings of TBI patients.

All values are N (%) unless otherwise stated.

Neuroimaging reports		MRI performed (10 days - 6 weeks), N	140 (71.1)
CT (acute), N	197	Subdural - acute	88 (62.9)
DAI	35 (17.8)	Subdural - subacute	7 (5.0)
Extradural	44 (22.3)	Contusion	119 (85.0)
SDH	113 (57.4)	Intracerebral haemorrhage	8 (5.7)
SAH	118 (59.9)	Intraventricular haemorrhage	39 (27.9)
Contusion/ICH	130 (66.0)	SAH	30 (21.4)
Midline shift	41 (20.8)	Extradural	6 (4.3)
CT Marshall classification (acute)		Microhaemorrhages	97 (69.3)
I (DI, no vis. Pathology)	23 (12.0)	Oedema	38 (27.1)
II (DI)	85 (44.5)	Midline shift	30 (21.4)
III (DI, oedema)	12 (6.3)	Cisternal compression	2 (1.4)
IV (DI, shift > 5mm)	6 (3.1)	Venous sinus injury	2 (1.4)
V (EML)	51 (26.7)	Brain atrophy	3 (2.1)
VI (NEML)	14 (7.3)	Ischaemia	1 (0.7)

Imaging evidence of diffuse axonal injury

Diffusion MRI showed evidence of extensive DAI (Figure 8A). Individual z-score maps of fractional anisotropy (FA) were generated by comparing patients with local controls at each scanning site to account for scanner specific site differences. Significant reductions in FA were present in a large number of white matter tracts at the subacute scanning visit, 6 and 12 months post-injury (Figure 8A, B). We examined the whole white matter skeleton and corpus callosum as specific region of interests (ROI). Both ROIs showed significant reductions in FA at all time points (all $P < 0.001$, Figure 8A).

Longitudinal analysis showed that in patients with serial imaging, FA declined over time between the acute and six month assessments in a large number of white matter tracts including the whole white matter and corpus callosum ROIs ($n=49$, $V=975$, $p < 0.001$) (Figure 8C, D). Further reductions were present from 6-12 months but were not significant ($n=36$, $V=209$, $P=0.052$). Patients with serial diffusion imaging ($n=56$) were well matched in age (serial imaging mean 47.6 (sd = 15.8) vs no serial imaging mean 46.2 (sd = 18.5)), and gender (serial imaging 21.1% female vs no serial imaging

23.6% female) to those without serial diffusion imaging. However, patients with serial diffusion imaging had significantly higher initial GCS ($W=668$, $P<0.0001$) and GOSE scores at 12 months ($t=4.2$, $p<0.0001$), less negative zFA at the subacute scanning visit ($W=1843$, $P=0.0012$) and reduced peak biomarker concentrations for NfL ($W=3457$, $P<0.001$), tau ($W=4284$, $P<0.001$), UCH-L1 ($W=4272$, $P<0.001$), GFAP ($W=3804$, $P<0.001$) and S100B ($W=4993$, $P<0.001$). In all analyses, focal lesions were excluded from the calculation of FA using hand drawn lesion maps (Figure 8E). A sensitivity analysis of patients without focal lesion pathology ($n = 22$) show reductions in whole white matter (mean zFA -0.39, IQR= 0.57) and corpus callosum ROIs (mean zFA -0.56, IQR= 0.5) were still present compared to controls ($W=1118$, $p=0.003$ and $W=1211$, $p=0.003$ respectively).

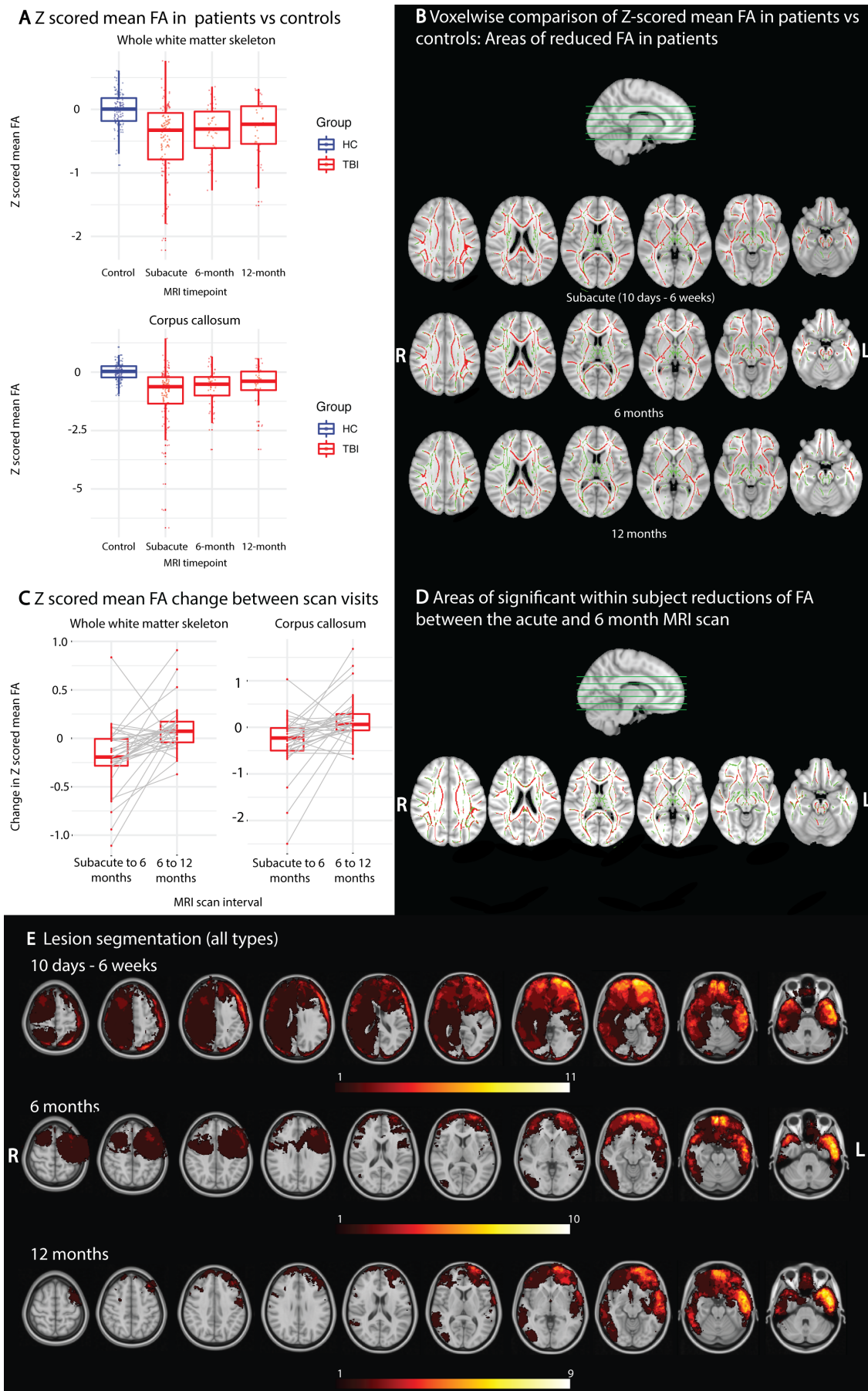


Figure 8. Axonal injury and lesion location after moderate-severe TBI.

(A) Fractional anisotropy (FA) in patients z-scored against site-specific healthy controls in the whole white matter (WM) and the corpus callosum (CC), for controls (blue) and subacute, 6 and 12 month visits and patients (red). (B) Voxelwise comparison of zFA in patients compared to controls with areas of significant group differences in red ($p < 0.05$), overlaid on the WM skeleton in green. (C) Longitudinal change in FA measures between subacute and six month, and six to twelve month visits in WM and CC regions in TBI patients. (D) Voxelwise map of regions with significant within-subject FA reductions between subacute and 6 months visits in red ($p < 0.05$). (E) Spatial representation of hand drawn lesion segmentation maps at all visits. *R* = right, *L* = left

Blood biomarker profiles after traumatic brain injury

This analysis was led by Dr Neil Graham. The data was jointly collected and are presented to contextualise the imaging and larger study findings.

Injury markers including plasma NfL were serially assessed on two occasions within ten days of injury ($n = 167$), subacutely at ten days to six weeks ($n = 141$), six ($n = 53$) and twelve months ($n = 25$) (Figure 9). Plasma NfL was significantly elevated post injury (Figure 9A, B), increasing over time and peaking subacutely, around 20 days post-injury (median peak NfL 507.3 pg/ml, IQR 728.2). NfL remained elevated at six and twelve months' post-injury compared with healthy controls (both $P < 0.001$, $W = 387/431$, respectively, Figure 9C). At 6 months post injury, 85% of TBI patients had NfL levels greater than 2 standard deviations from the healthy control mean and 52% had levels that remained elevated at 12 months' post-injury (Figure 9C).

TBI patients also had significant elevations of plasma total tau, serum S100B, plasma UCH-L1 and GFAP compared with healthy controls. Levels peaked within the first day of injury (median peak for tau 7.0pg/ml; S100B 215.1pg/ml; UCH-L1 97.6pg/ml; GFAP 12,590.5 pg/ml). These biomarkers normalised following the subacute visit except for plasma GFAP, which remained chronically raised at 12 months ($W = 691$, $P = 0.041$). 34% (18/53) of patients had raised plasma GFAP at 6, as did 32% (8/25) at 12 months. Age at injury modestly contributed to GFAP levels at 6 (adjusted R^2 0.27, $P < 0.001$) but not 12 months post injury. Patients with raised plasma GFAP at 6 months were significantly older than those with normal levels ($W = 134$, $P = 0.001$).

Patients with non-TBI extracranial injuries (n=25) showed lower concentrations of all blood biomarkers than the TBI group (all P values <0.001). Increased plasma NfL and UCH-L1 were evident in NTT patients compared to healthy controls, but there were no differences in tau, GFAP or S100B (Figure 9). As the groups were unbalanced for gender, I analysed if there was a significant effect of gender on biomarker concentrations. There was no effect of gender on peak NfL concentrations (mean 678pg/ml female, 562pg/ml male), peak tau concentrations (mean 23.9pg/ml female, 23.7pg/ml male), peak UCH-L1 concentrations (mean 146pg/ml female, 103pg/ml male), peak S100B concentrations (mean 378pg/ml female, 266pg/ml male) or peak GFAP concentrations (mean 23500 pg/ml female, 16600pg/ml male).

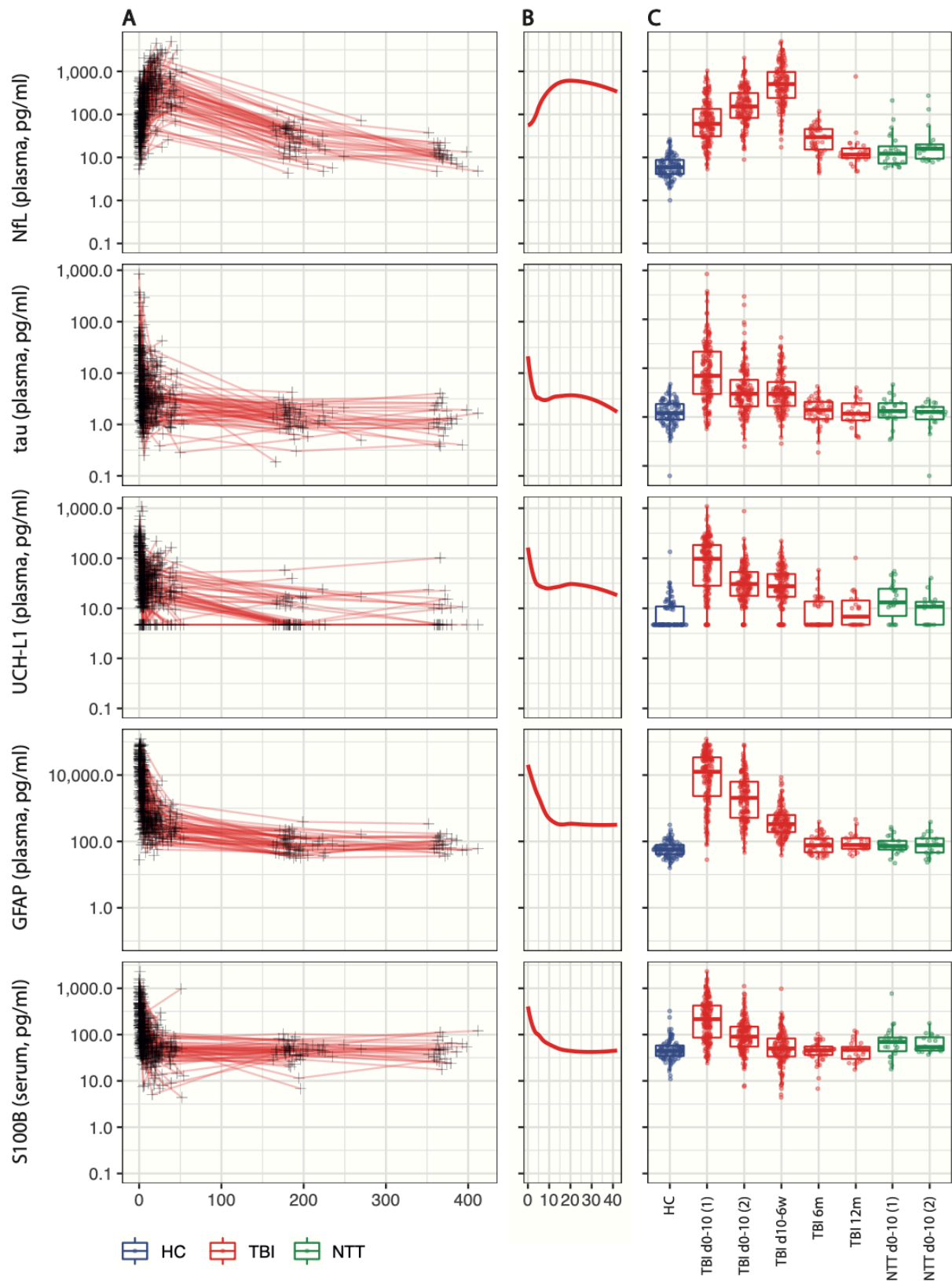


Figure 9. Blood biomarker trends in TBI, non-TBI trauma and healthy controls.

(A) Trends of blood NfL, tau, UCH-L1, GFAP and S100B concentrations after TBI. (B) Regression curves modelling concentrations in the first 40 days post-TBI. (C) Group comparisons of biomarkers in healthy controls (HC blue, TBI patients red and patients

with non-TBI traumatic injuries green) at different time points (d0-10 (1)= first sample within days 0-10 after injury, d0-10 (2) = second sample within days 0-10 after injury, d10-6w = sample at 10 days to 6 weeks after injury, 6m = sample at 6 months after injury and 12m = sample at 12 months after injury). TBI = TBI patients, NTT = Non-TBI trauma controls.

Neurosurgery subgroup analysis

Neurosurgery was performed in 38% (n=75) of patients, hence we assessed whether neurosurgical interventions influenced peak fluid biomarker concentrations or zFA in the first 6 weeks following injury. Peak biomarker and mean skeleton zFA values were compared between the surgery and nonsurgery groups. The surgical group had significantly higher peak NfL after surgery (W=2920), TAU (W=2960), UCH-L1 (W=3141), GFAP (W=3125), S100B concentrations (W=3111) and mean skeleton zFA values (W=2765, all P values < 0.002, Table 6). The neurosurgical group likely had more severe injuries than the non-neurosurgical group. Hence the difference in biomarker concentrations may not be the result of neurosurgery. To investigate this issue, a sensitivity analysis was performed taking into account the white matter FA, in patients for which these data were available (n=131). Of these, 37% (n=49) underwent surgery. For each biomarker, linear regression was performed between peak plasma concentrations and FA. Group means on the residuals from this regression were then compared for each biomarker, between patients with and without surgery, as above. Accounting for severity in this way, we continued to detect a significant neurosurgery-associated increase in peak NfL concentration (P=0.042, W=1581) but not the other fluid biomarkers assessed (Table 7).

Table 6. Blood and imaging biomarker concentrations: neurosurgery analysis.

Analysis of TBI patients with and without neurosurgery, comparing peak biomarker concentrations and mean skeleton zFA in the first six weeks post-injury.

	Intervention		Statistical comparison	
	No surgery	Surgery	W	P
NfL				
N	122	73		
median	290.2	574.9	2920	<0.001
TAU				
N	122	73		
median	5	13.5	2960	<0.001
UCH-L1				
N	122	73		
median	58.4	123.2	3140.5	0.0023
GFAP				
N	122	72		
median	6411.8	18402.5	3125	0.0032
S100B				
N	121	75		
median	144.2	281.4	3110.5	<0.001
Mean Skeleton zFA				
N	-0.325	-0.697		
median	83	49	2765	<0.001

Table 7. Blood biomarker concentrations: neurosurgery analysis accounting for white matter FA.

Analysis of TBI patients with and without neurosurgery, comparing peak biomarker concentrations in the first six weeks post-injury while accounting for injury severity as measured by white matter FA.

	Biomarker concentration		Statistical comparison	
	No surgery (n=82)	Surgery (n=49)	W	P
NfL				
peak in 6 weeks (pg/ml)	372.6	759.8	1168	<0.001
residuals	-196.6	22.9	1168	<0.001
TAU				
peak in 6 weeks (pg/ml)	3.8	9.8	1192	<0.001
residuals	-14.3	-12.7	1738	0.792
UCH-L1				
peak in 6 weeks (pg/ml)	45.9	114	1137	0.011
residuals	-58.2	-10.9	1627	0.28
GFAP				
peak in 6 weeks (pg/ml)	6016.4	17435.1	1361	0.0083
residuals	-10068.4	-1994.4	1705	0.6
S100B				
peak in 6 weeks (pg/ml)	131.9	235.4	1416	0.019
residuals	-106.7	-43.1	1812	1

Plasma NfL and zFA were highly predictive of long-term functional outcomes

We assessed the relationship of blood and imaging biomarkers of axonal injury with functional outcomes as measured by the GOSE. GOSE scores of greater or equal to 6 were classified as favourable outcomes, while scores below 6 were defined as unfavourable. Peak plasma NfL concentration in the first 6 weeks post-injury, during the subacute time period, had the highest pseudo R^2 value in predicting the GOSE at 6 and 12 months post-injury (McFadden's R^2 , 0.24, 0.48, respectively) (Table 8). Z-scored FA in the corpus callosum had the second highest pseudo R^2 , for both six and twelve month GOSE scores. Weaker but significant other predictors of outcome included peak plasma UCH-L1, plasma GFAP and serum S100B, but not plasma tau.

Table 8. Relationship of blood biomarkers and long-term outcomes after TBI.

Relationships of peak blood biomarkers sampled in first six weeks after injury and outcome measures

Outcome and Timepoint		Biomarker Predictors						
		Fluid markers					DTI MRI markers	
		NfL (peak)	Tau (peak)	UCH-L1 (peak)	GFAP (peak)	S100B (peak)	zFA WM all (subacute)	zFA CC (subacute)
Function: GOSE								
6m (n=126)	R ² *	0.24	0.04	0.12	0.12	0.16	0.11	0.18
	AUC	0.83	0.77	0.77	0.73	0.81	0.69	0.76
	95% CI	0.75-0.91	0.68-0.87	0.68-0.86	0.63-0.82	0.72-0.89	0.59-0.80	0.66-0.85
	P	<0.001	0.13	<0.001	<0.001	<0.001	0.001	<0.001
12m (n=108)	R ² *	0.28	0.05	0.12	0.13	0.16	0.22	0.25
	AUC	0.85	0.85	0.81	0.78	0.83	0.79	0.81
	95% CI	0.77-0.94	0.78-0.92	0.72-0.89	0.69-0.86	0.74-0.91	0.68-0.91	0.70-0.92
	P	<0.001	0.17	0.002	0.001	<0.001	<0.001	<0.001

Plasma NfL correlates with diffusion MRI measures of axonal injury

I tested the specificity of blood biomarkers to axonal injury by correlating the peak concentrations with diffusion measures of axonal injury. Plasma NfL was negatively correlated with FA ($R^2=-0.28$, $P<0.001$) along with plasma S100B ($R^2=-0.071$, $P=0.005$), which while significant had a very weak correlation with mean skeletal zFA (Figure 10). Peak tau, UCH-L1 and GFAP were not significantly correlated to FA. A factor analysis of the peak blood biomarker levels and the corpus callosum FA was also performed (Graham et al., 2021). Three factors explained 71% of the variance in the data. Peak NfL and subacute FA in the corpus callosum both loaded heavily on the same factor, suggesting a close association of these measures (loading strengths 0.56 and -0.99, respectively). The other two factors were loaded variably by the other blood biomarkers, with little contribution of corpus callosum FA and lower NfL loading.

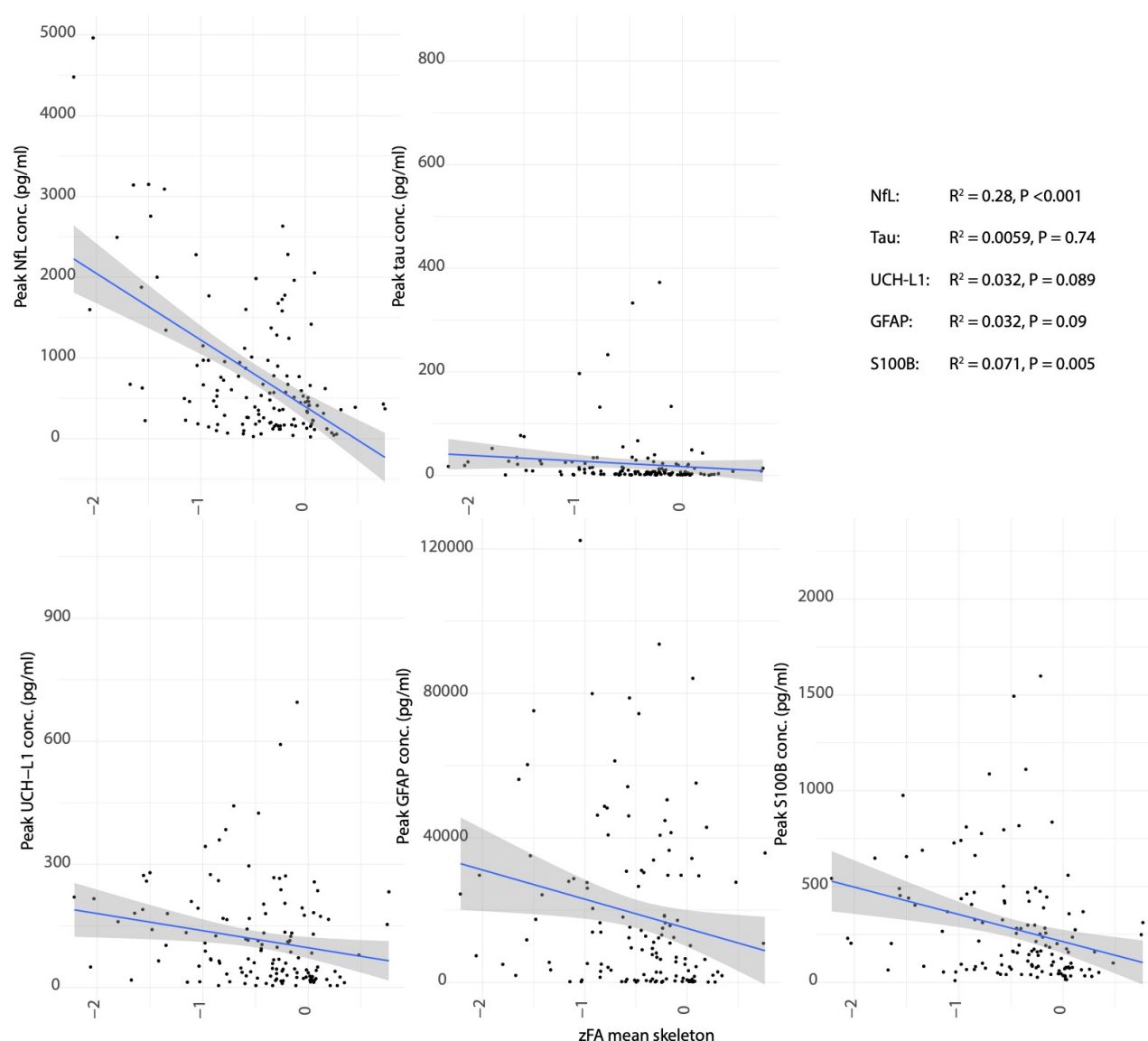


Figure 10. Correlations of blood and neuroimaging biomarkers after TBI.

Relationships of peak axonal injury plasma biomarker concentrations and subacute z-scored fractional anisotropy (zFA) in the mean skeleton of TBI patients. $N=117$ (NfL), $n=118$ (tau), $n=118$ (UCH-L1), $n=117$ (GFAP), $n=120$ (S100B).

I then compared blood biomarker levels in patients with or without pathology on CT or MRI. Similar plasma levels of NfL and tau were seen in patients with and without visible pathology on CT (Table 9). There were no significant differences in peak NfL or tau when comparing Marshall grade I patients ($n=23$, no lesions on scan) with Marshall grades II-VI ($n=168$, encompassing all diffuse / mass lesions visible). In contrast, in the other biomarkers, higher peak concentrations were seen in patients with Marshall grades II-VI versus grade I (UCH-L1 ($W=910, P=0.033$), S100B ($W=776, P=0.0027$)).

and GFAP ($W=779$, $P=0.005$)). Similarly, there were no significant differences in peak NfL between patients with any evidence of lesions on MRI ($n=125$) compared to those without lesions ($n=18$). Higher concentrations of all other biomarkers were observed in patients with lesions on MRI (tau ($W=1518$, $P<0.001$), UCH-L1 ($W=1428$, $P<0.001$), S100B ($W=1469$, $P<0.001$) and GFAP ($W=1484$, $P<0.001$)). Within patient who had lesions present on MRI, the lesion volume was significantly correlated with peak concentrations in blood of NfL, tau, UCH-L1, S100B and GFAP (all $P<0.001$, $\rho=0.50$, 0.52, 0.52, 0.53, 0.45 respectively).

Table 9. Blood biomarker concentrations: lesion related analysis.

Analysis of TBI patients either in Marshall Grade I compared to Marshall Grade II-VI on CT, or presence of lesions on MRI to no presence of lesion on MRI. Conc. = concentration.

	Blood biomarker (peak within 6 weeks of injury)				
	NfL	TAU	UCH-L1	GFAP	S100B
Marshall Grade I					
N	19	19	19	19	19
conc, median (IQR)	53.2 (134)	3.57 (7.99)	111 (145)	1980 (10600)	79.6 (98.3)
Marshall Grade II-VI					
N	154	155	155	153	159
conc, median (IQR)	147 (243)	8.42 (22.0)	35.8 (73.4)	15200 (31800)	257 (357)
W	1321	976	910	779	776
P	0.084	0.083	0.033	0.005	0.0027
Lesions present on MRI					
N	112	113	113	112	115
conc, median (IQR)	153 (257)	7.52 (19.9)	108 (150)	15800 (29300)	252 (343)
No lesions present					
N	16	16	16	16	16
conc, median (IQR)	46.2 (147)	1.19 (2.89)	22.9 (37.1)	1710 (2200)	65.3 (112)
W	1547	1518	1427.5	1484	1469
P	0.052	<0.001	<0.001	<0.001	<0.001
Correlation with lesion volume on MRI					
ρ	0.5	0.52	0.52	0.53	0.45
P	<0.001	<0.001	<0.001	<0.001	<0.001

NfL, tau and UCH-L1 levels are highly correlated between plasma and brain microdialysate

This cerebral microdialysis and blood biomarker analysis was led by Dr Neil Graham. The data is presented to contextualise the blood biomarker and imaging findings. I led the analysis looking at correlations with DTI.

We performed additional work to assess the specificity of NfL to damage to the white matter. Microdialysis was initiated a median of 21 hours' post-injury (IQR 25), continuing for a median of 6 days in total (IQR 4.75). We assessed concentrations in blood levels and compared them with concentrations in the brain extracellular fluid sampled from white matter potentially affected by diffuse axonal injury. Frontal white matter without evidence of focal brain injury was sampled (Figure 11A), with 83% of patients having the catheter inserted in the right hemisphere and 17% in the left. NfL, tau, UCH-L1 and GFAP concentrations were significantly higher in brain extracellular fluid than plasma (all $P < 0.001$ Figure 11B). Concentrations of NfL, tau and UCH-L1 levels were around 100 times higher in microdialysate than plasma, whereas GFAP was around four times higher. Linear mixed effects modelling accounting for time since injury and patient variability showed a strong relationship between blood and brain extracellular fluid concentrations for NfL ($P = 0.001$, $t = 3.3$, $AIC = -128.4$), tau ($P = 0.003$, $t = 2.9$, $AIC = 57.8$) and UCH-L1 ($P < 0.001$, $t = 4.8$, $AIC = 19.7$) but not GFAP ($P = 0.76$).

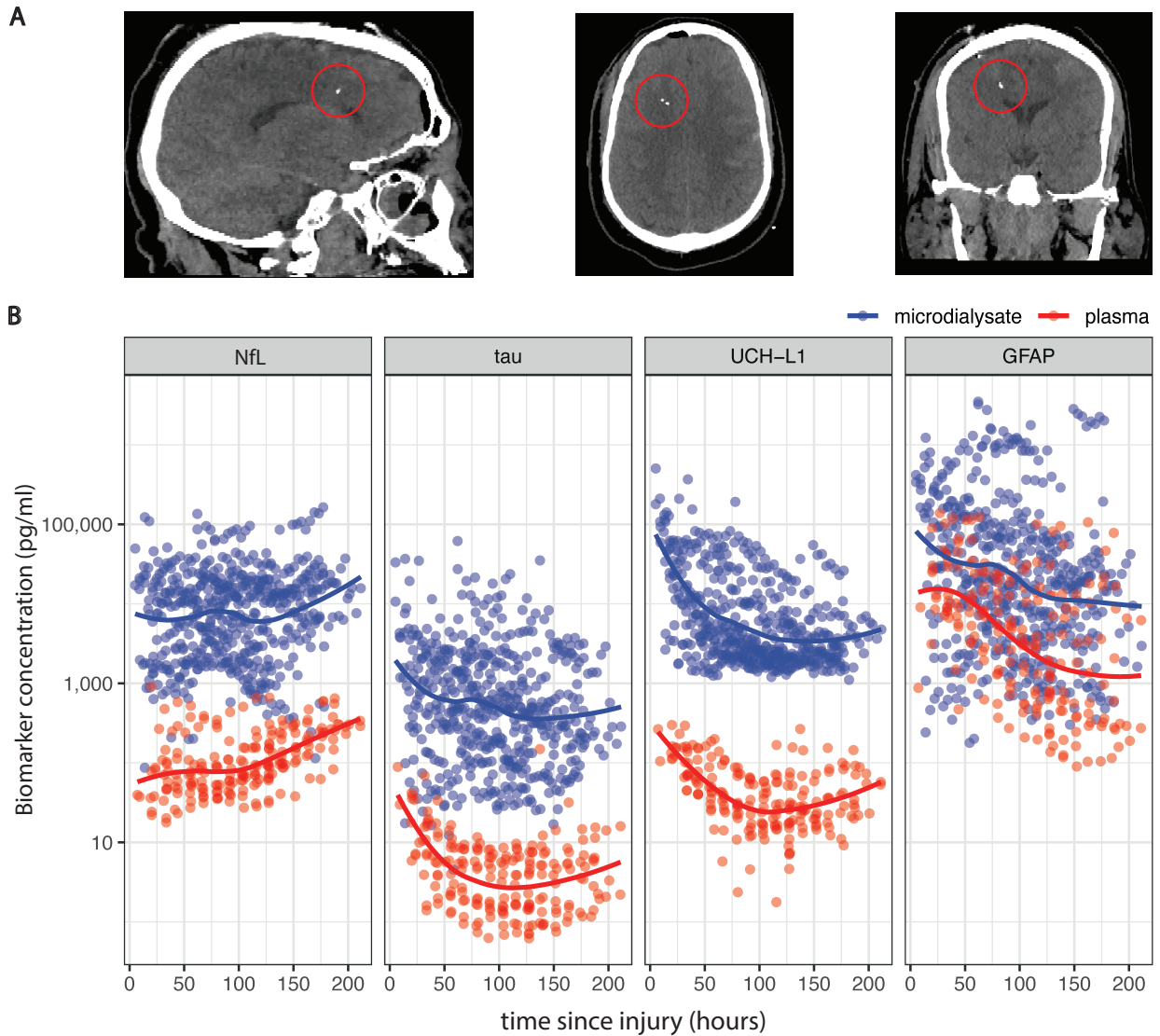


Figure 11. Cerebral microdialysis evidence of axonal injury acutely following TBI.

(A) CT images showing indicative placement of cerebral microdialysis catheter in deep white matter (red circle) **(B)** Longitudinal trajectories NfL, tau and UCH-L1 and GFAP in brain extracellular fluid (blue) compared to plasma (red) over the first 10 days post-TBI.

Diffusion tensor MRI was also performed in 12 of 18 patients who had undergone microdialysis (mean 25 days after injury; SD 12). Across the group, zFA was reduced relative to controls ($n=116$) in the whole white matter (-0.9 (1.15) vs 0.004 (0.365), $W=339$, $P<0.001$). More specifically, FA from a spherical region of interest around the catheter tip insertion site was also reduced (mean zFA= -1.5 (sd = 1.23), $V=0$, $P=0.003$). However, none of the microdialysate biomarker concentrations showed a significant correlation to zFA at the catheter site or whole white matter.

Validation of axonal injury markers in an experimental injury model

Trends over time in fluid biomarkers and histopathological evidence of axonal injury were analysed by Dr Cornelius Donat. I performed the statistical analysis of the diffusion data and subsequent correlations to fluid biomarker concentrations and histopathological measures.

We also assessed the specificity of NfL to axonal injury by comparing plasma NfL concentrations against neuroimaging and histopathological evidence of axonal injury in a controlled cortical impact (CCI) model. Other fluid biomarkers were not assessed in the rodent work. Thirty-four rats underwent either sham operation (n=4), 1mm CCI (n=15, 'mild injury') or 2mm CCI (n=15, 'moderate-severe injury'). Ten naïve animals served as histological controls. Plasma NfL increased over two weeks following 1mm or 2mm injury. Mixed effects modelling with time and injury as fixed effects showed a significant interaction between time and injury severity ($F(4,90) = 14.9, P < 0.001$). This was the result of similar plasma NfL concentrations at baseline, but higher plasma NfL at all post-injury timepoints in 1mm and 2mm CCI animals (all $P < 0.001$, Figure 12A). Plasma NfL levels in sham/naïve animals did not change over time. Cerebrospinal fluid was also sampled 15 days after CCI. CSF NfL was increased ($F(2,27) = 24.2, P < 0.001$) with higher levels in the 2 mm group than the naïve/sham ($t=6.9, P < 0.001$) and 1mm CCI animals ($t=5.0, P < 0.001$).

White matter (WM) integrity was assessed in the corpus callosum (CC) using DTI at 14 days' post-injury. zFA scores were produced by comparing post-injury FA to baseline FA across all animals. FA was reduced after injury (Fig. 12C, D) with a main effect of injury severity ($F(1,100) = 28.3, P < 0.001$) due to significantly lower FA associated with a 2 mm impact. In a related study using the same animals, axonal integrity was also assessed using fluorescent neurofilament staining. 2 mm, but not 1 mm or sham injury was associated with disorganised swollen axons and axonal bundles, and there was a significant reduction in NF staining in segments 2-5 in 2 mm-CCI animals (Donat et al., 2021). WM tissue density in the corpus callosum was also reduced. Following a 2 mm impact, the WM in the corpus callosum was markedly

thinned proximally to the injury site (segments 2-4), but unchanged following 1 mm or sham injury (Donat et al., 2021).

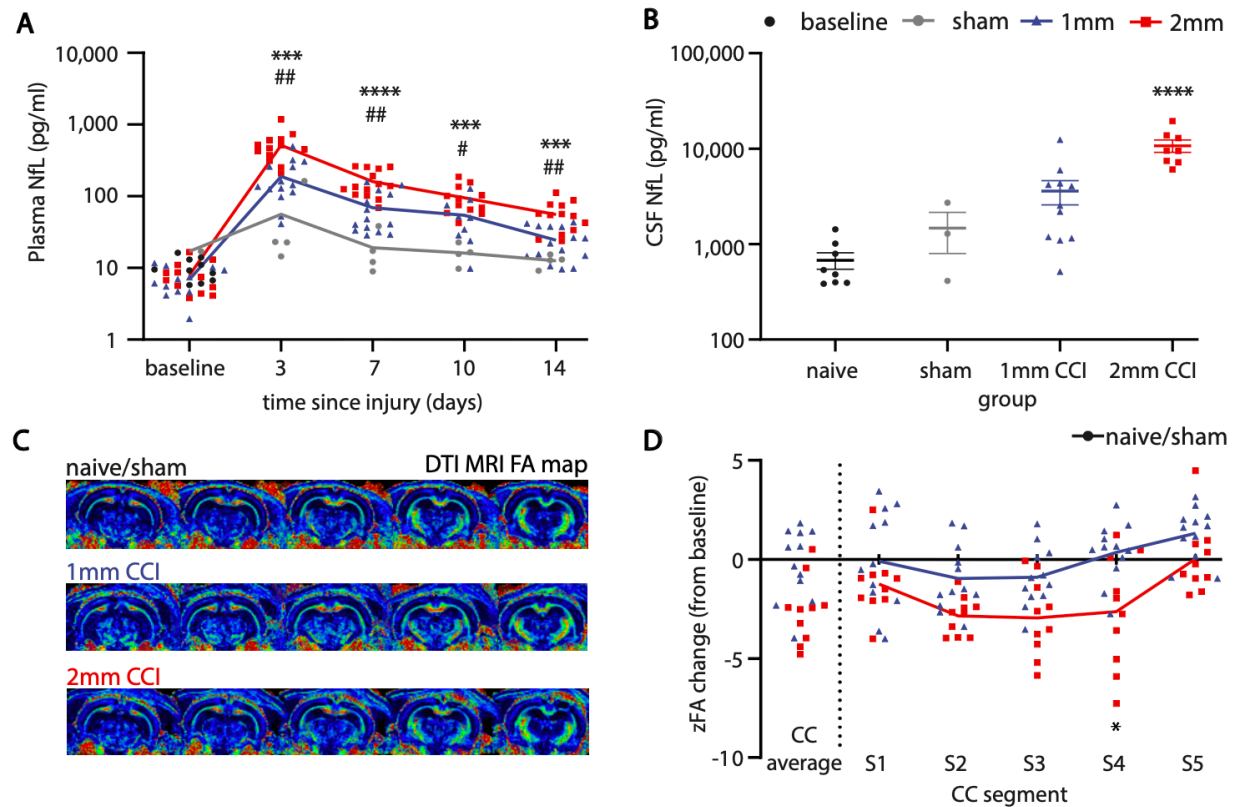


Figure 12. Axonal injury markers including NfL and DTI MRI in a controlled cortical impact model of TBI.

(A) NfL concentration in plasma at baseline, 3, 7, 10, 14 days' post-injury in naive, sham (blue) and after 1mm and 2mm CCI (red). (B) CSF NfL 15 days post-injury (mean $\pm$ standard error of the mean) after different injury conditions. (C) DTI-MRI images (D) zFA after 1mm / 2mm CCI calculated from baseline FA values. CC = Corpus Callosum, CCI = Controlled Cortical Impact.

I assessed the relationship between the fluid biomarkers, imaging and histopathological measures of axonal injury in the experimental injury model. Peak plasma NfL concentrations correlated with CSF NfL ($P=0.003$, $\rho=0.52$), but were not significantly correlated with zFA. However, peak plasma NfL concentrations were negatively correlated with neurofilament stain intensity ($\rho=-0.48$, $P=0.042$) and mean CC thickness ($\rho=-0.61$, $P=0.02$). FA was not significantly correlated with neurofilament stain intensity, but was positively correlated with mean CC thickness ($r=0.67$, $P=0.008$) (Figure 13).

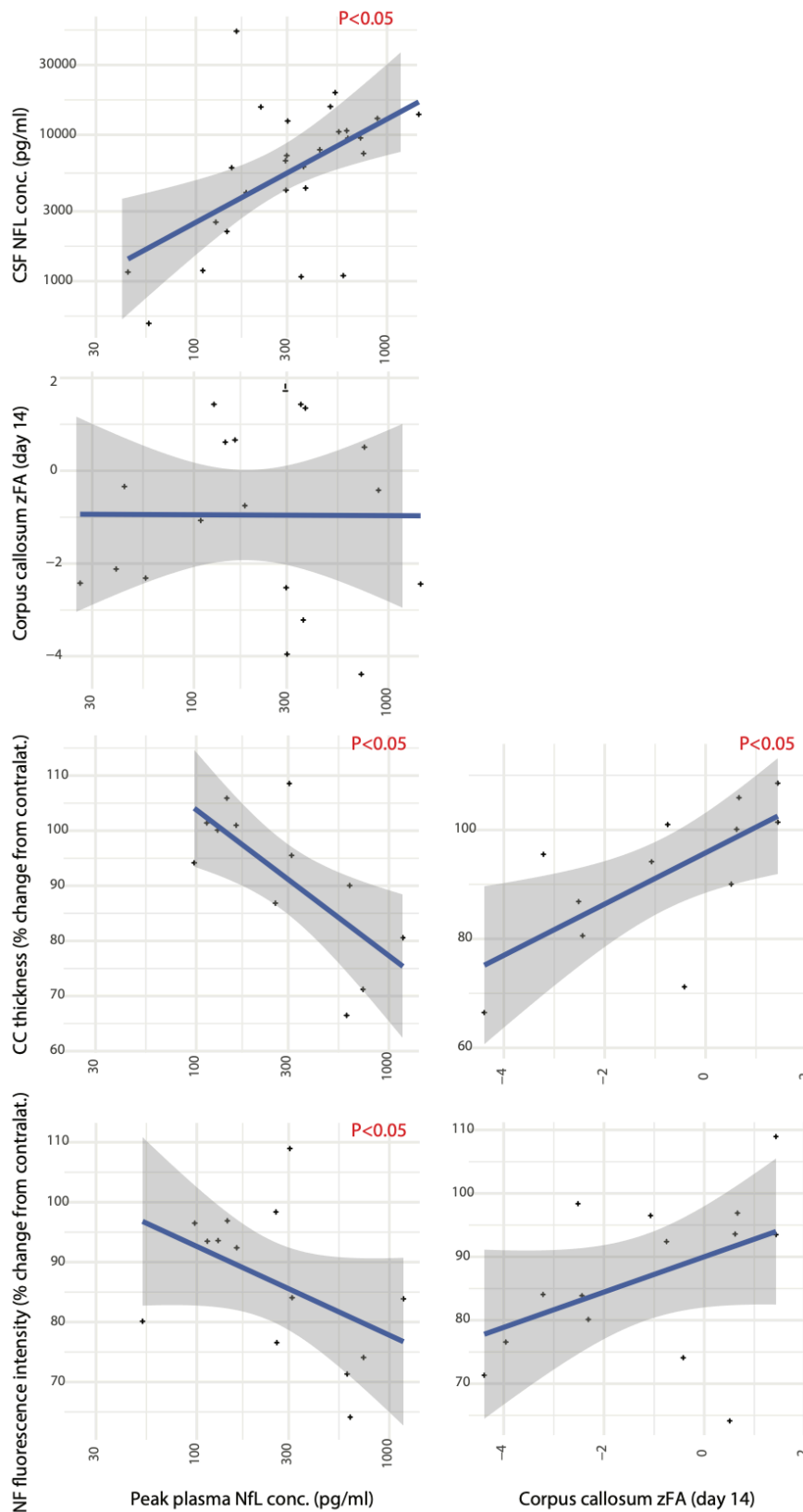


Figure 13. Experimental injury model fluid biomarker, neuroimaging and histology relationships.

Correlations between plasma NfL, CSF NfL, zFA from DTI and histopathological results. Asterisk denotes significant result at $P < 0.05$.

3.5 Discussion

I present the findings of a collaborative project investigating neuroimaging and fluid biomarkers of axonal injury after moderate-severe TBI. We show that after TBI, there are widespread reductions in white matter integrity as measured by diffusion MRI which progresses from the subacute time period after injury to 6 months after injury. We also show increases in blood concentrations of NfL, tau, UCH-L1, S100B and GFAP after injury. We tested the specificity of these blood biomarkers to axonal injury using a variety of methods which highlighted NfL in particular as a promising marker of axonal damage. Plasma NfL concentrations correlated with the extent of axonal damage seen on diffusion MRI and the variability in NfL levels were not explained by the presence of CT findings or lesions on MRI, contrasting with the other proteins. NfL and diffusion MRI measures also best predicted late functional outcomes, known to be related to axonal injury. We directly measured NfL concentrations from damaged white matter using cerebral microdialysis and showed that plasma NfL levels reflected concentrations of NfL within the damaged white matter in the brain. Furthermore, we show using an experimental injury model an increase in NfL and reduction in diffusion MRI metrics relating to injury severity which also correlate with histopathologically-defined axonal injury within the white matter. The results show that diffusion MRI and plasma NfL provide complementary and sensitive measures of axonal injury relevant to patient outcomes when measured subacutely after TBI.

Diffusion MRI metrics, such as fractional anisotropy (FA), provide a sensitive measure of axonal damage (Mac Donald et al., 2007c, Donat et al., 2021) which can often be missed on CT or standard MRI (Jolly et al., 2021). Similar to previous work in moderate-severe TBI patients, we observed widespread reductions in FA, indicative of diffuse axonal injury (Kinnunen et al., 2011a, Sidaros et al., 2008). These abnormalities in FA were observed in a large number of white matter tracts, including the corpus callosum. We replicated these findings using an experimental injury model, whereby we observed an injury severity dependent reduction in FA in the corpus callosum. Our results further support the utility of diffusion MRI as a measure of axonal injury.

In TBI patients, we observed a reduction in FA over time from the subacute scan to 6 month scan, and a trend of reduction between 6 to 12 month scans. Patients who had serial diffusion MRI at these time points had less severe injuries and better outcomes compared to patients who were unable, or unwilling to attend follow-up scanning visits. It is possible that in the more severe patients, the effect size of FA reductions between scanning visits could be larger. Indeed, patients with neurosurgery, and therefore likely a more severe injury, saw greater reductions in FA compared to controls at the 10 day to 6 week period. The reduction in FA could reflect ongoing secondary injury processes and reflect ongoing degeneration of the white matter, a process known to progress for years after injury (Schlaepfer, 1974, Vargas and Barres, 2007). We have previously shown that FA abnormalities predicts the degree of neurodegeneration chronically post-injury (Graham et al., 2020b). Similarly, within the patients in this study, FA measured subacutely predicted spatial patterns of late white matter atrophy (Graham et al., 2021). Subacutely measured FA could therefore be a marker of whether an individual is at higher risk of neurodegenerative processes or poor cognitive outcomes.

Our work provides evidence that plasma NfL is an informative biomarker of DAI. We highlight a novel proposed optimal time window for assessing NfL concentrations, with concentrations rising in blood after injury, peaking subacutely at around 20 days post-injury. Our results replicated previous findings whereby plasma and cerebrospinal fluid NfL increased after various types of TBI (Thelin et al., 2019, Shahim et al., 2016a, Al Nimer et al., 2015a, Shahim et al., 2020a) including after mild TBI (Zetterberg et al., 2006, Neselius et al., 2012). For the first time, ultrasensitive analysis of microdialysate and plasma was carried out on simultaneously acquired samples, showing that concentrations in blood were highly correlated with concentrations of NfL in brain extracellular fluid sampled directly from damaged white matter. We also show that plasma NfL is closely related to FA, replicating similar findings in a previous investigation of serum NfL (Shahim et al., 2020a). This was supplemented by a factor analysis, providing further evidence that plasma NfL and FA are closely related in

quantifying underlying axonal injury (Graham et al., 2021). Plasma NfL and FA also best predicted patient outcomes at 6 and 12 months post injury, outperforming the other biomarkers. These results indicate that plasma NfL concentrations provide information relevant to patients prognosis, potentially as a result of reflecting post-traumatic changes in damaged white matter.

NfL constitutes a core part of the axonal backbone and is highly expressed within the long myelinated axons of the cerebral deep white matter (Schlaepfer and Lynch, 1977) which are susceptible to DAI that arises from shearing forces of rotational acceleration in head injuries (Donat et al., 2021). However, blood NfL levels are also elevated in both chronic (Sandelius et al., 2018) and acute peripheral neuropathies (Martín-Aguilar et al., 2021). We tested if elevations in plasma NfL are influenced by non-axonal specific trauma. Increased plasma NfL levels were seen in trauma patients without TBI, although these concentrations were still 5 to 15 times lower than concentrations in the TBI group, with a larger difference developing with time since injury increasing over the first two weeks. These increases could be from possible spinal cord injuries from patients who had vertebral fractures, although we did not observe evidence of spinal cord injury in any of the cases (Kuhle et al., 2015). Specificity to intracranial injuries were also briefly examined, and high levels of NfL were seen in patients irrespective of CT or MRI evidence of focal brain injury. Although the extent of lesion volumes did correlate with plasma NfL concentrations in patients with lesions, large lesions were likely to overlap with white matter and therefore contribute to NfL release from damaged axons.

We assessed the specificity of NfL to axonal injury using data from an experimental model of TBI. Results from the model show that plasma and CSF NfL levels increase, and FA decreases with more severe injuries. These changes were accompanied by histopathological evidence of axonal injury (Donat et al., 2021). These measures were assessed over two weeks post-injury, which was in parallel with the timing of data collection in the human study. My results indicate that NfL levels in CSF and plasma correlate with extent of axonal damage quantitatively, as calculated by reduced white

matter thickness and reduced neurofilament immunofluorescence staining in the callosum. While we cannot exclude the contribution of the larger contusion produced by a more severe impact in this CCI model, the relationship between these measures further strengthens the association between plasma NfL and axonal damage. However, we did not find a significant relationship between plasma NfL with corpus callosum zFA in the CCI model, despite respective elevations and reductions which were in line with results from human studies. There are several possible reasons why this might have been the case beyond the low number of animals studied. DTI images were not skeletonised when sampling CCI model zFA, which can lead to partial volume effects reducing the sensitivity of the DTI comparison detecting changes. Furthermore, the contribution of large contusions to elevated NfL concentrations would further reduce the sensitivity to zFA changes.

There were several potential limitations of the study. One concern is the potential influence of focal post-traumatic lesions on the calculation of brain atrophy rates and diffusion MRI measures. We minimised this effect by assessing each MRI for the presence of focal pathologies and lesions, with lesioned areas excluded in a voxelwise manner from subsequent analyses. FA measures of axonal integrity were therefore sampled only from non-lesioned voxels. A sensitivity analysis showed zFA reductions persisted in non-focal lesioned TBI patients. We did not collect information on ischemia and therefore cannot discount the effect of ischemia on diffusion MRI measures, however this should be addressed in future work. A further concern is the multi-centre nature of the study, particularly the effect of MRI scanner variability. We carefully aligned all data collection and analysis protocols as much as possible. We addressed scanner differences by producing normalised FA maps (z-scored FA) for each patient relative to healthy age and sex-matched controls at each study site at a voxelwise level. This minimises issues with scanner variability and facilitates group comparisons across sites. In relation to the analysis of fluid biomarkers, it has previously been recognised that there can be some variability in the assays used between different reagents. To minimise the chance of introducing noise into the comparison of biomarkers in blood and cerebral microdialysate, these tests were run in single batch with a one set of reagents. We also used the same platform to assess plasma NfL and

CSF NfL in the experimental injury model. It is also possible that the insertion of microdialysis catheter influenced NfL levels, but we are not currently able to address this question as the majority of patients with microdialysis have not had their blood samples beyond 7 days post-injury analysed. We found that higher concentrations of peak plasma NfL were associated with the occurrence of neurosurgery, even accounting for greater injury severity in the surgical group, measured using FA. Future work assessing plasma NfL in the context of elective surgeries unrelated to trauma will help to validate this finding and facilitate interpretation in TBI. However, we were unable to differentiate the effect of injury severity from neurosurgical intervention on FA. NTT controls were considerably more male in proportion than healthy controls, however we believe this is likely to have a minimal effect as there is limited evidence of gender differences in baseline fluid biomarker levels and an analysis conducted showed no differences in peak biomarker concentrations by gender. Limitations of the CCI model have been discussed in depth (Donat et al., 2021), and while the CCI model usually causes less primary axonal injury, it provides excellent biomechanical control and reproducibility, which were requirements of the original study in investigating the relationship between biomechanical forces, imaging and histopathology.

3.6 Conclusions

In summary, I demonstrate the utility of advanced fluid and imaging markers in diagnosing axonal injury after moderate-severe TBI. Additionally, by combining human data with experimental modelling, I show that FA and NfL levels sampled from blood change proportionately to injury severity and are related to histopathological measures of axonal damage. NfL is also highly correlated with FA, and predicts at 6 and 12 months post-injury. Both measures therefore reflect axonal injury within the brain and are clinically relevant to patient prognosis.

In the next chapter of this thesis, I investigate if mild TBI, or participation in elite sports are also associated with diffuse axonal injury as measured by both plasma NfL and diffusion MRI.

Chapter 4.

Assessing white matter damage in collision sports

A study of diffusion MRI and longitudinal structural MRI in elite rugby players.

In this chapter, I investigate whether mild TBI or participation in professional rugby is associated with damage to white matter using advanced MRI techniques.

4.1 Abstract

Background

Head injuries are the most commonly reported injury in professional rugby. However, it is unclear how the severity and number of injuries sustained relate to brain injuries such as diffuse axonal injury, diffuse vascular injury and progressive neurodegeneration. Advances in neuroimaging techniques enable the investigation of neuropathologies associated with the acute and long-term effects of injury. Here I present a longitudinal observational study investigating the relationship between rugby participation and acute head injuries in professional adult male and female rugby players using advanced MRI.

Methods

Diffusion tensor imaging and susceptibility weighted imaging was used to assess white matter structure and evidence of axonal and diffuse vascular injury. I also studied changes in brain structure over time using Jacobian Determinant statistics extracted from serial volumetric imaging. I tested 41 male and 3 female adult elite rugby players, of whom 21 attended study visits after a head injury, alongside 32 non-sporting controls, 15 non-collision sport athletic controls and 16 longitudinally assessed controls. 18 rugby players participated in the longitudinal part of the study, with a second visit at least 6 months after their first scan.

Results

An individualised neuroimaging analysis revealed evidence of diffuse axonal injury in 17% (10/44) of players. Abnormalities of fractional anisotropy and other diffusion measures were seen in both rugby players with and without an acute injury. In contrast, non-collision sport athletic controls were not classified as showing abnormalities. A group level contrast also showed evidence of acute injury using diffusion tensor imaging in rugby players. Examination of longitudinal imaging revealed unexpected reductions in white matter volume in around 50% of the elite rugby players studied. These changes were not related to self-reported head injury history or neuropsychological test scores and might indicate excess neurodegeneration in white matter tracts affected by injury.

Conclusions

These findings suggest an association of participation in elite adult rugby with changes in brain structure, with undefined clinical significance.

4.2 Introduction

While moderate-severe TBIs produce obvious and potentially fatal pathologies and are the focus of acute clinical care teams, mild traumatic brain injuries (mTBIs) are much more common, accounting for 90% of all TBI incidences (Kay and Teasdale, 2001). Globally, there are 45 to 54 million mTBI's a year (Maas et al., 2017). Sport related mTBIs account for 15% to 20% of these injuries requiring treatment in the USA (Centers for Disease and Prevention, 2007, Faul et al., 2010). Mild TBIs were long considered relatively harmless due to the transient nature of the injury. However, there is a growing body of evidence associating mTBIs with long-term effects such as persistent neurological deficits (Hou et al., 2012, Sharp and Jenkins, 2015, Guskiewicz et al., 2007) and the onset of neurodegenerative diseases such as chronic traumatic encephalopathy (CTE) (Gavett et al., 2010, Guskiewicz et al., 2005, McKee et al., 2009, Mez et al., 2017) even after injuries that appear relatively trivial. In the previous chapter I demonstrated the utility of diffusion MRI and blood biomarkers in detecting axonal injury and predicting patient outcomes. In this chapter, I investigate if these measures can detect evidence of damage to white matter in a cohort exposed to sports related mild TBI's, where neuropathologies such as diffuse axonal injury may be missed by conventional diagnostic investigations.

Diffuse axonal injury (DAI) can produce neurological deficits after a mTBI, occurring when the brain is exposed to rapid deformations as a result of trauma (Smith et al., 2003). The biomechanical stress can result in damage to white matter tracts connecting brain regions, leading to the disruption of large-scale networks that support cognition and behaviour (Sharp et al., 2014). However, DAI is often under-recognised by conventional diagnostic imaging that can appear normal after mTBI (Sharp and Jenkins, 2015). As demonstrated, advanced MRI techniques such as diffusion MRI can be used to sensitively identify the presence of white matter injuries (Kinnunen et al., 2011b, Mac Donald et al., 2007a, Bonnelle et al., 2012). Diffusion MRI allows for the quantification of abnormalities in the directionality of water diffusion, most commonly quantified using changes in fractional anisotropy (FA). FA is sensitive to microstructural abnormalities and provides information about the location and severity of diffuse axonal injury. It has been validated as a measure of white matter integrity

after TBI in animals models of TBI (Mac Donald et al., 2007c), has shown increased sensitivity to DAI compared to conventional MRI (Kinnunen et al., 2011b), and provides information that helps predict long-term clinical outcome after TBI (Sidaros et al., 2008), including the type of post-traumatic problems that develop (Bonnelle et al., 2012, Bonnelle et al., 2011, Hellyer et al., 2012, Sharp et al., 2014).

Diffusion MRI has been shown to be informative in the context of sports related mTBI, however the direction of change in FA is inconsistent. In mixed sport cohorts, FA has been shown to increase acutely after a sports related mTBI (Henry et al., 2011, Jing et al., 2015, Meier et al., 2016b, Manning et al., 2017). This elevation has been attributed to increased anisotropy caused by cerebral oedema (Eierud et al., 2014, Asken et al., 2018). In several studies, these changes persist in the chronic stage (Meier et al., 2016b, Churchill et al., 2017a, Sasaki et al., 2014), particularly in cohorts of younger athletes. In others, FA is observed to be decreased relative to controls in the chronic stages after a sports mTBI (Churchill et al., 2019, Tremblay et al., 2014, Chappell et al., 2006, Fakhraan et al., 2013), similar to changes observed in non-sport mTBI and moderate-severe TBI. Several studies do not find abnormalities in FA as a result of sports-related mTBI (Meier et al., 2016a, Zhang et al., 2010, Strain et al., 2013, Multani et al., 2016). Further complexities arise from the contribution of repetitive head impacts outside of a symptom inducing injury, which have also been shown to influence white matter integrity (Shin et al., 2014, Chappell et al., 2008, Lipton et al., 2013, Schneider et al., 2019).

The simplistic nature of traditional DTI metrics such as FA may limit the interpretation of differences in these studies, making it difficult to attribute changes to changes in cell morphology, density, fibre orientation or partial volume contribution of free water. These limitations can be addressed by using advanced multi-shell diffusion-MRI technique called Neurite Orientation Dispersion and Density Imaging (NODDI). NODDI separates the diffusion signal from three different tissue compartments: intra-neurite (restricted diffusion), extra-neurite (hindered diffusion) and cerebrospinal fluid (free diffusion) to extract a neurite density index (NDI), orientation dispersion index (ODI) and isotropic volume fraction (ISOVF) (Zhang et al., 2012). Acutely after a mild

TBI, studies have observed reduced NDI (Wu et al., 2018), which has correlated with reductions in FA and increased symptom load (Churchill et al., 2019), with changes persisting up to 6 months after the injury (Palacios et al., 2018). In athletes not acutely injured, but with a history of injury, studies have found both increases and decreases in FA which were negatively correlated to ODI (Churchill et al., 2017a, Mayer et al., 2017). Results from NODDI models can be informative to contextualise DTI findings; changes in DTI metrics without changes in ISOVF suggest that DTI changes are not primarily driven by variations in free water volumes, while NDI reductions are suggestive of degeneration of the white matter.

In addition to the acute effects of sport mTBI, repeated head impacts can lead to progressive neurodegeneration (Smith et al., 2013, Tagge et al., 2018, McKee et al., 2009). In sports such as American football and boxing, a risk of chronic traumatic encephalopathy (CTE) has been demonstrated (Omalu et al., 2005, Mez et al., 2017, McKee et al., 2009, Stewart et al., 2016). However, the neurodegenerative risks and the prevalence of sports related dementia such as CTE across contact-sports remain unclear, as is the dose of head injury exposure required to accelerate neurodegeneration. Recent work has demonstrated a surprisingly large increase in the risk of dementia, in particular Alzheimer's disease, in deceased football players (Mackay et al., 2019). Therefore, an important question is to what extent different sports accelerate and increase the risk of neurodegeneration.

In vivo detection of the evidence of neurodegeneration can be studied using longitudinal volumetric MRI. Brain atrophy, which is a common end point of various types of neurodegeneration, can be sensitively measured using serial structural brain imaging. Serial structural imaging can be used to generate metrics of volume change over time such as the Jacobian Determinant (JD) (Ashburner and Ridgway, 2012). In the TBI population, a more negative JD, implying elevated rates of atrophy, has been observed and associated with poorer outcomes (Brezova et al., 2014, Bendlin et al., 2008, MacKenzie et al., 2002, Trivedi et al., 2007). Atrophy is also increased after single moderate-severe TBI (Cole et al., 2018b), is more prominent in the depths of sulci where CTE pathology occurs (Ghajari et al., 2017). In other neurodegenerative

diseases, atrophy measured by MRI has been histologically validated at post-mortem (Bobinski et al., 2000). Hence, serial structural MRI provides a method of sensitively assessing whether neurodegeneration is taking place and mapping its spatial pattern, which may give an indication of the underlying pathological process.

Rugby is a high intensity collision team sport played in over 120 countries by over 8 million people (World Rugby, 2017). In professional rugby, mTBI is the most common reported match injury (~20% of injuries) with an incidence of 20.4 per 1000 hours (West et al., 2020). There has been relatively little research investigating the long-term effects of rugby participation. Previous work in retired rugby players has found evidence of a limited effect of higher self-reported rates of depression and mild cognitive impairment compared to controls (Decq et al., 2016, McMillan et al., 2017, Gallo et al., 2020). However, rates of dementia in the retired rugby player population are not necessarily different from control levels (Davies et al., 2017). CTE has recently been reported in a small number of ex-players: two from rugby league (Buckland et al., 2019) and five from rugby union (Stewart et al., 2016, Lee et al., 2019), but the prevalence and consequences of neurodegenerative pathology in rugby players remain unclear. Additionally, only a few studies have investigated solely a population of rugby players with diffusion MRI, with evidence of reduced FA as well as elevated diffusivity in 73 uninjured female varsity (Manning et al., 2020) and 11 retired rugby league players (Wright et al., 2020). Further research is needed to understand whether long-term rugby participation and head injuries experienced during a rugby career are sufficient to lead to lasting brain injury.

Here, I report the findings from a longitudinal observational study of 41 male and 3 female elite rugby players using repeated diffusion, susceptibility weighted and volumetric MRI to measure diffuse axonal injury, diffuse vascular injury and brain atrophy respectively. Players were recruited with or without a recent history of mTBI and compared against sporting and non-sporting controls to investigate whether imaging abnormalities were associated with participation in high intensity sporting activity. I test the hypotheses that participation in elite rugby will be associated with: a) evidence of diffuse axonal injury as measured by diffusion MRI; b) evidence of

diffuse vascular injury as measured by SWI; and c) evidence of increased brain atrophy, as measured by longitudinal changes in brain volume.

Study populations and design

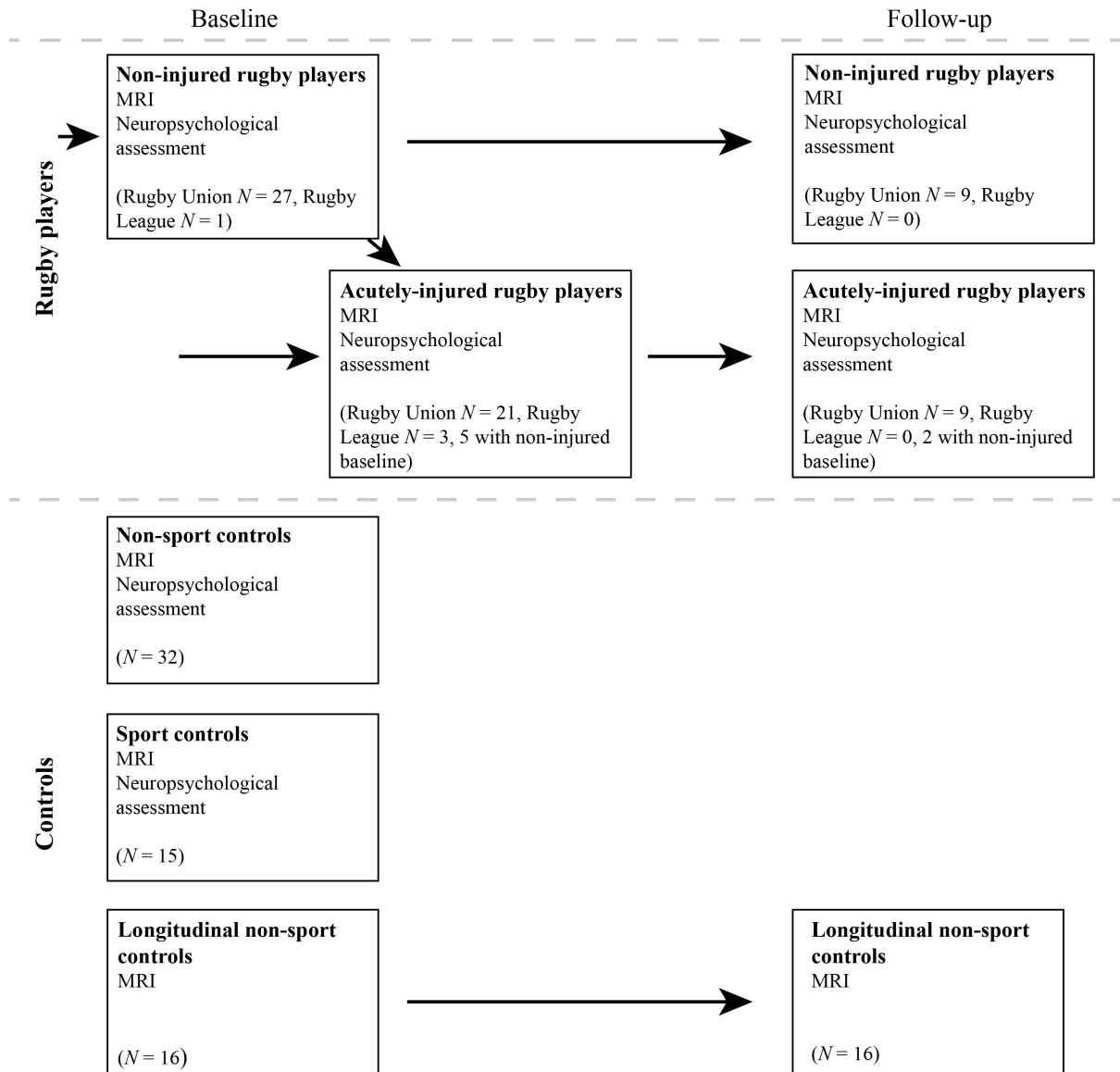


Figure 14. Flow diagram outlining player and control recruitment into the study.

Details of players and controls recruited and those who returned for a follow-up assessment. MRI and neuropsychological tests were consistent across baseline and follow-up visits and across the groups except for longitudinal non-sport controls (referred to as Longitudinal Controls) who only had structural imaging.

4.3 Methods

Subjects

The study was designed to recruit 20 rugby players with an acute injury, and 20 without, with an undefined number of players to have both a baseline visit and acute injury visit, and a target of following-up at least 20 players. Originally designed as a single-club study, in order to recruit enough players the study expanded to recruit from several teams across both Rugby League and Rugby Union. In total thirty-seven elite male players, 3 female rugby union players, and 4 male rugby league players were recruited from professional rugby clubs in England (mean age 25.2, standard deviation (SD) = 3.5, 41 males, 3 females). Twenty-eight players had baseline study visits without an acute injury, while 21 players attended study visits shortly after a head injury as determined by medical professionals in line with Rugby Union and Rugby League guidelines. A mismatched number of baseline, to acute injury visits, were reached through an attempt to recruit as many players as possible to follow-up, and covers players who has both a baseline and acute injury visit. All head injuries met the classification of mild - probable TBI (Malec *et al.*, 2007). Post injury study visits for acutely injured players were performed a maximum of 7 days after injury [mean days 4.7, SD = 1.2]. All study visits included an MRI assessment and battery of neuropsychological tests. Five players had both a baseline non-injury study visit and a study visit following an acute head injury (Figure 14). 18 rugby players attended a second assessment a mean of 12.1 months later. Inclusion criteria were age 16-45, active rugby players with exclusion criteria comprising other major neurological or psychiatric issues (e.g. substance abuse, previous moderate-severe TBI) or contraindication to MRI.

Injury characteristics, including total numbers of symptoms after injury and symptomatic days as well as the duration the player remained in the return to play (RTP) protocol were recorded. Acutely injured players completed the SCAT-5 which includes a cognitive and neurological screen, and a symptom evaluation (symptom severity score out of 132). For all players, head injury history and length of professional

career were collected from the professional sporting body, and where gaps existed, from players and the club.

Healthy controls were screened according to the same exclusion criteria and were required to additionally have no self-reported history of mTBI. Three control groups were recruited to the study, including a Non-Sport and Sport Control group for cross-sectional comparison, and a separate group of Longitudinal Controls for longitudinal comparison. The Non-Sport Control group were a cohort of controls not playing sports at university or professional competition level. The Sport Controls group were a cohort of non-collision sport athletes recruited from university sport clubs. Inclusion criteria for the Sport Controls were aged 16-45, active athletes with at least 6 hours of exercise per week with an additional exclusion criteria of a history of playing collision sports competitively. This included representing their high school competitively in rugby or soccer. Sport Controls comprised of 3 swimmers, 1 water polo player, 9 rowers, 1 cyclist and 1 weightlifter, with a median hours of exercise per week of 12 (IQR=6.9). The Longitudinal Control group were the third cohort of controls and comprised of individuals who had participated in a longitudinal imaging study of outcomes after TBI which used aligned imaging and exclusion protocols (Cole et al., 2018b). Longitudinal Controls only completed the structural imaging components of the study.

Recruitment was coordinated by local research facilities at Hammersmith Hospital. All study participants provided written informed consent in accordance with the Declaration of Helsinki. Ethical approval was approved by the University College London research ethics committee (7385/001).

Neuropsychological testing

Established paper neuropsychological tests sensitive to impairments after TBI (Ramlackhansingh et al., 2011) were administered during study visits including the Test of Premorbid Functioning (TOPF) – pre-morbid intelligence, Trail Making Test (TMT) - processing speed, Delis-Kaplan Executive Function System (D-KEFS Stroop) – executive function, Hopkins Verbal Learning-Test (HVLT) and the Brief Visuospatial Memory Test-Revised (BVMt) – verbal and spatial working memory and recall.

Participants also completed a battery of cognitive tasks on an iPad as part of a subset of previously studied and described tasks (Hampshire et al., 2012, Daws and Hampshire, 2017). These included: Visuospatial Working Memory –working memory; Paired Associates –working memory and Self-Ordered Search – working memory; Feature Match –processing speed; Odd One Out - intelligence; and Spatial Planning - intelligence. A visuospatial memory *Fractals* task on a touchscreen computer was also completed following a previously described protocol (Pertzov et al., 2013).

MRI acquisition

Participants were scanned with the following sequences: resolution structural T1-weighted MPRAGE with voxel dimensions of 1mm^3 (160 slices, matrix = 208×208 , repetition time = 2300ms, echo time = 2.98ms, flip angle = 9 degrees, FOV = $256 \times 256\text{mm}$, GRAPPA=2), T2 FLAIR (Fluid attenuated inversion recovery), T2 susceptibility weighted imaging (SWI; 120 1.2 mm -thick transverse slices, TR = 28 ms, TE = 20 ms, FA = 15° , in-plane resolution = $0.8 \times 0.6\text{ mm}$, field of view = $225 \times 225\text{ mm}$) and diffusion tensor imaging using a multishell 99 direction protocol (66 slices, isotropic 2 mm^3 voxels, field of view = $25.6\text{ cm} \times 25.6\text{ cm}$, TR= 5000 ms, TE = 85 ms, b-value1= 700 s/mm^2 with 30 directions, b-value2= 2000 s/mm^2 with 60 directions, 6 direction b-value= 0 s/mm^2 and 6 reverse phase encoding direction b-value= 0 s/mm^2 volumes).

Longitudinal controls were scanned with the same structural sequences. Participants were all scanned on a Siemens 3T Verio (Siemens Healthcare) scanner using a 32-channel head coil. All scans were examined by a consultant neuroradiologist for the presence of focal lesions or microbleeds.

Neuroimaging analysis

Diffusion data processing

Diffusion weighted images were processed following the standard TBSS pipeline in the FMRIB Software Library (version 5.0.8) (Smith et al., 2006), including correcting susceptibility and eddy current induced distortions (Andersson and Sotiropoulos, 2016) and diffusion tensor fitting (Figure 15). Freesurfer (Dale et al., 1999) was used to skull strip brains and remove non-brain voxels from the field map. Tensor-based

registration was performed using DTI-TK (Zhang et al., 2007) involving the creation of a group template using affine and non-linear diffeomorphic registrations followed by registration of participant diffusion imaging to the template. Images were warped to 1mm isotropic space and the mean fractional anisotropy (FA) map produced was thresholded at 0.2 to produce a white matter skeleton. Subject FA and mean diffusivity (MD) data was projected onto the mean FA skeleton and tract level data generated using the Johns Hopkins University (JHU) white matter atlas. Neurite density index (NDI), orientation dispersion index (ODI) and isotropic volume fraction (ISOVF) maps were generated using NODDI modelling using the Accelerated Microstructure Imaging via Convex Optimization (AMICO) framework (Daducci et al., 2015). NDI, ODI and ISOVF maps were registered to DTITK space using the transformation matrix generated during the DTITK pipeline. Images were visually inspected at the brain extraction, eddy current correction and tensor registration stage. Subjects with poor quality data or non-correctable artefacts were removed.

Subject level diffusion analysis

Mean FA was calculated for selected large white matter tracts based on tract size, reproducibility and sensitivity (Jolly et al., 2021). The ROI's selected were: the body of the corpus callosum body, genu of the corpus callosum, splenium of the corpus callosum, right and left corticospinal tracts, right and left inferior longitudinal fasciculus, right and left corona radiata, the middle cerebellar peduncle and whole white matter skeleton. The mean and standard deviation of FA for each tract region of interest (ROI) across the Non-Sport Control cohort was then used to calculate subject Z-scores for each ROI. Z-scores were converted to P-values (two-tailed, with 95% confidence interval) and corrected for multiple comparisons across all tract ROI's using FDR. Abnormalities in subjects' FA were determined by tracts that have significantly lower or higher FA in the subject compared to the control group, $p < 0.05$, FDR corrected. Subject-level and ROI analysis was only conducted for FA and not for other diffusion metrics.

Voxelwise diffusion analysis

Voxelwise analysis of skeletonised diffusion metrics were conducted using the general linear model with non-parametric permutation testing (5,000) in FSL Randomise (Winkler et al., 2014). Diffusion weighted voxelwise results reported are corrected for multiple comparisons at a threshold of $p < 0.05$ with age and gender included as a nuisance covariate.

Cross-sectional structural data processing and analysis

Volumetric data was analysed using standard approaches (Cole et al., 2018a) (Fig. 1). SPM12 (UCL) was used to segment T1 images into grey matter, white matter and CSF, and to calculate volumes of these tissue classes at baseline and follow-up. Cross-sectional analysis involved the generation of a study template using SPM DARTEL non-linear registration (Ashburner, 2007) before affine registration of segmented images to MNI152 space, with normalisation of volume and smoothing using an 8 mm kernel. Tract level data was generated using eroded JHU white matter atlas masks to sample tissue volumes. Cross-sectional voxelwise analyses were conducted using non-parametric permutation testing (5,000) with age, gender and intracranial volume included as nuisance covariates.

Longitudinal structural data processing and analysis

Longitudinal volumetric analysis was performed using the SPM12 longitudinal registration tool. Baseline and follow-up T1 images were iteratively co-registered to produce a temporal average image, with the transformation required to move from the baseline to follow-up image encoded in the Jacobian determinant (JD) of the expansion/contraction at a voxel-wise level (Ashburner and Ridgway, 2012). JD was then weighted by the interval between baseline and follow-up to give an annualised rate of change and further weighted by tissue class. A study specific template was generated using SPM DARTEL's non-linear registration and tissue specific JD images normalised to group space before affine registration to MNI152 space with normalisation of volume and smoothing using an 8 mm kernel. Tract level data was generated using skeletonised JHU white matter atlas masks in MNI152 space. Voxelwise analysis was conducted using non-parametric permutation testing (5,000)

with age included as a nuisance covariate and results reported are corrected for multiple comparisons at a threshold of $p < 0.1$. Voxelwise correlation between JD and DTI metrics were conducted after registration of DTI images to MNI152 space, using non-parametric permutation testing (5,000).

Input data and groups

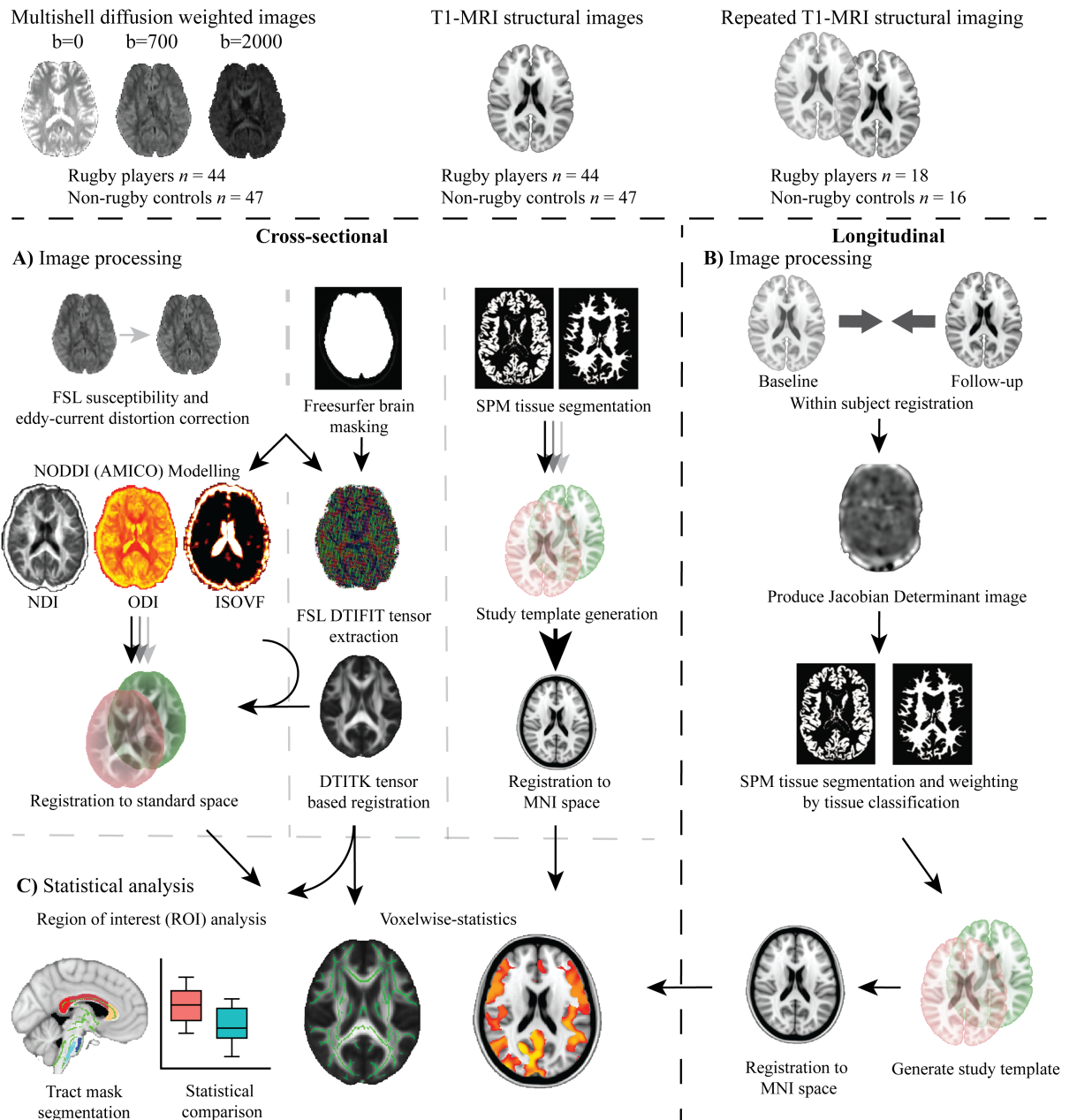


Figure 15 Overview of neuroimaging methods.

(A) Image processing of diffusion data followed the standard TBSS pipeline with tensor-based registration performed using DTI-TK. Neurite density index (NDI), orientation dispersion index (ODI) and isotropic volume fraction (ISOVF) maps were

generated using NODDI modelling using the Accelerated Microstructure Imaging via Convex Optimization (AMICO) framework. Image processing of cross-sectional structural data used SPM to segment T1 images into tissue probability maps. **(B)** Processing of longitudinal structural images involved within subject registration of baseline and follow-up images and generation of a within subject temporal average image and Jacobian determinant image. **(C)** Statistical analysis was carried out in both a region of interest and voxelwise manner. Region of interest analysis involved sampling of mean diffusion and structural metrics of interest within pre-defined regions of interest in spatially standardised images.

Statistical analysis

An a priori level for statistical significance was set at 0.05 for two sided tests. Whole brain summary and voxelwise measures were calculated by adding grey and white matter tissue data. Summary measures of brain volume were normalised for participants' head size by dividing into total intracranial volume (grey matter + white matter + CSF). Statistical tests were completed in the open source software package R (R Core Team, 2018). Group differences in neuropsychological performances were investigated using either T-tests or analyses of variance with Tukey multiple post-hoc comparisons of means where appropriate. FDR correction for multiple comparisons was used. Categorical variables were compared using the Chi-squared test. Pearson's correlation was used where appropriate. In cases where the normality assumption was violated, the Wilcoxon test was used.

4.4 Results

Subject demographics

From 26 July 2017 to 1 March 2019, 44 elite level rugby players were recruited into the study. Twenty-one players were assessed shortly after a mild TBI that was sustained whilst playing professional rugby. On average, these players were 4.7 days post-injury. Acutely injured players presented with a mean symptom severity score of 10.3 on the SCAT-5 during their pitch side assessment at the time of injury and had an average return to play duration of 7.2 days (Table 10). Demographic and physical characteristics were well matched between acutely injured and non-acutely injured

rugby players. Eighteen rugby players attended a second assessment a mean of 12.1 months later and players who returned for a follow-up were not significantly different in demographics, baseline neuropsychological test performance or any imaging measures compared to players who did not return for follow-up.

Rugby players were compared with three control groups. A non-collision sport cross-sectional (Sport Controls), a non-sport cross-sectional (Non-Sport Controls) and non-sport longitudinal control group (Longitudinal Controls). Sport Controls were slightly younger than rugby players ($t = -3.57$, $P=0.0017$) with no difference in proportion of males to females. Non-Sport Controls were well matched for age, but had a lower proportion of males to female ($X^2= 12.4$, $p<0.001$) than the rugby players. Longitudinal Controls were older than the rugby players who returned for their longitudinal assessment ($t = 6.28$, $p<0.001$) but were matched for gender. Alcohol intake was not significantly different between rugby players and controls.

Table 10. Participant demographics

* Significance at $P < 0.05$ in block 1 are for comparisons either between Rugby players and Non-Rugby Controls (independent t-tests or Chi-Squared tests). Block 2 comparisons are a comparison of means in acutely injured rugby players, non-acutely injured rugby players, Non-Sport Controls, and Sport Controls using an ANOVA. * indicates significance at $P < 0.05$ for Tukey post hoc tests relative to Non-Sport Controls.

	Rugby players ($n = 44$)	Non-Rugby Controls ($n = 47$)	Non-acutely injured rugby players ($n = 28$)	Acutely injured rugby players ($n = 21$)	Non-Sport Controls ($n = 32$)	Sport Controls ($n = 15$)	Longitudinal non-sport controls ($n = 16$)
Age, years	25.2 $\pm$ 3.5	24.3 $\pm$ 3.9	25.8 $\pm$ 3.3	25.0 $\pm$ 3.8	25.7 $\pm$ 3.3	21.5 $\pm$ 3.6*	33.3 $\pm$ 4.0
Male sex, n (%)	41 (93)	34 (72)*	25 (89)*	21 (100)*	19 (59)	15 (100)*	9 (56)
Career details							
Length of professional career, years	5.6 $\pm$ 3.6	NA	6.1 $\pm$ 3.3	5.3 $\pm$ 3.8	NA	NA	NA
Previous head injuries, n	2.6 $\pm$ 2.9	NA	2.5 $\pm$ 2.7	3.0 $\pm$ 3.3	NA	NA	NA
Physical characteristics							
Weight, kg			99 $\pm$ 15	102 $\pm$ 11	NA	80 $\pm$ 7	NA
Height, cm			184 $\pm$ 9	185 $\pm$ 8	NA	184 $\pm$ 9	NA
Head injury details							
Time since injury, days		NA	NA	4.7 $\pm$ 1.2	NA	NA	NA
Symptom Severity Score at time of injury	NA	NA	NA	10.3 $\pm$ 5.3	NA	NA	NA
Return to play duration, days	NA	NA	NA	7.2 $\pm$ 1.5	NA	NA	NA
Baseline MRI characteristics							
DTI abnormalities, n (%)	7 (17)	1 (2)	5 (19)	2 (10)	1 (3)	0 (0)	NA
Microhaemorrhages, n (%)	3 (7)	1 (2)	1 (4)	2 (10)	0 (0)	1 (7)	NA

Rugby players showed lower neuropsychological test performance at baseline

Rugby players as a group showed lower visual memory immediate recall ($t=3.64$, $P=0.0083$) and lower premorbid intelligence scores (FSIQ; $t=3.2$, $P=0.030$) compared to Non-Sport and Sport controls as a single group (Non-Rugby controls) (Table 11, Figure 16). Rugby players also showed trends towards slower performance on two tests of processing speed (Trails B, Trails B minus A) and poorer performance on verbal memory immediate recall, however these did not survive correction for multiple comparisons. There were no differences between players and controls in computerised testing performance or on measures of executive function.

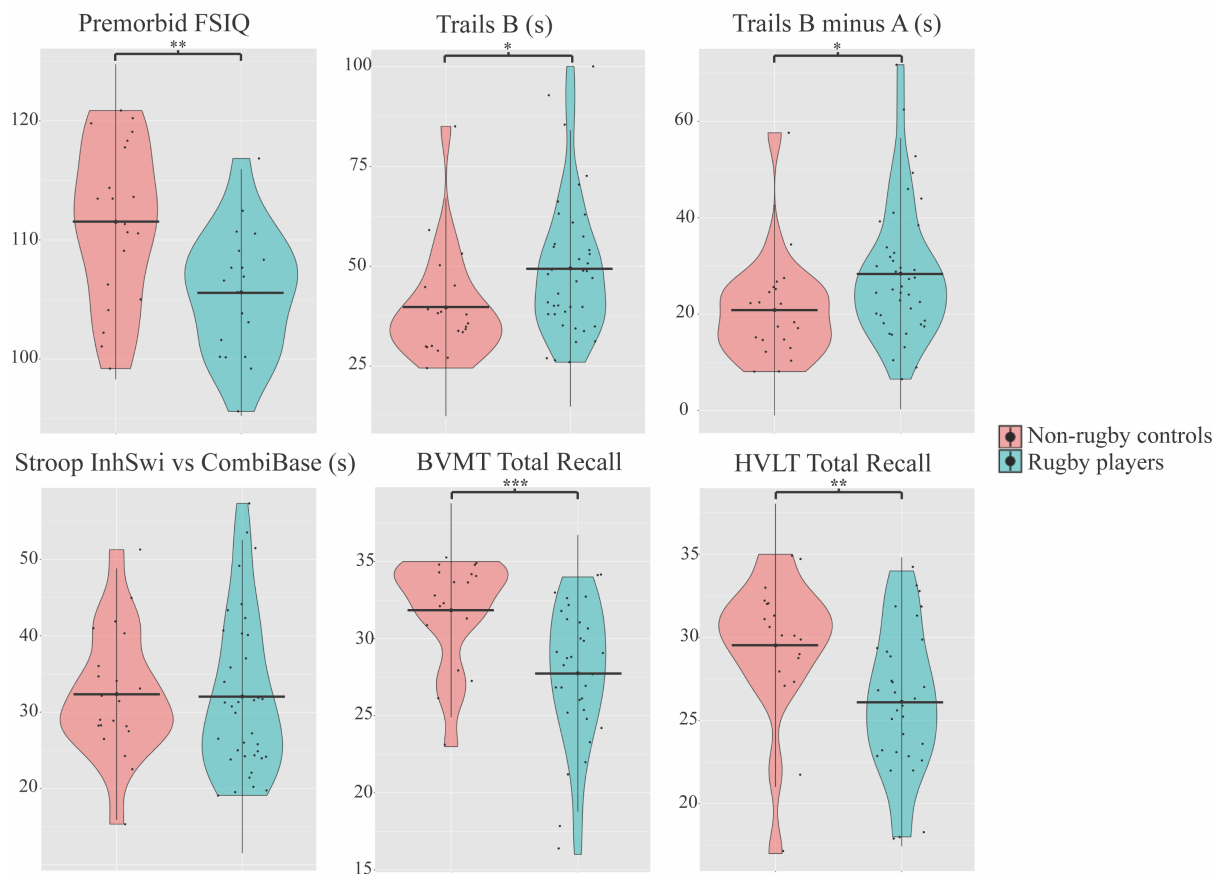


Figure 16. Neuropsychological performance in all controls and rugby players.

Grouped violin plots of rugby player and non-rugby control performance in select neuropsychological tests. The width of the coloured area represents the distribution of scores, with the mean defined by the black horizontal lines. Independent samples t-tests were conducted, with * = uncorrected $P < 0.05$, ** = uncorrected $P < 0.01$, *** = uncorrected $P < 0.001$.

Neuropsychological test performance in rugby players was not influenced by sub-acute injury

To assess if these differences were present in both sub-acutely injured players and non-acutely injured players, and if there was an effect of sport participation in controls, I compared baseline performance between the 4 groups. A significant effect of group was found for premorbid intelligence scores (FSIQ; $F_{3,37} = 5.74$, $P = 0.03$). Post hoc tests showed that these effects were driven by lower FSIQ scores in both sub-acutely injured rugby players ($P = 0.0035$) and non-acutely injured players ($P = 0.0095$) when compared to Non-Sport Controls. There were trends towards effects of group on visual memory immediate recall and verbal memory immediate recall, however these did not survive correction for multiple comparisons (Table 11). There were no differences between neuropsychological test scores of non-acutely and sub-acutely injured rugby players at baseline, nor any differences between baseline and follow-up scores within players who returned for a second visit.

Table 11. Participant neuropsychological test performance.

* Significance at $P < 0.05$ in block 1 are for comparisons either between Rugby players and Non-Rugby Controls (independent t-tests or Chi-Squared tests). Block 2 comparisons are a comparison of means in acutely injured rugby players, non-acutely injured rugby players, Non-Sport Controls, and Sport Controls using an ANOVA. * indicates significance at $P < 0.05$ for Tukey post hoc tests relative to Non-Sport Controls, not corrected for multiple comparisons. FSIQ = Full Scale Intelligence Quotient, BVMT = Brief Visuospatial Memory Test, HVLT = Hopkins Verbal Learning Test. Data presented as mean (sd) unless otherwise stated.

	Rugby players ($n = 44$)	Non-Rugby Controls ($n = 47$)	Non-acutely injured rugby players ($n = 28$)	Acutely injured rugby players ($n = 21$)	Non-Sport Controls ($n = 32$)	Sport Controls ($n = 15$)	Longitudinal non-sport controls ($n = 16$)
Baseline paper cognitive examination							
Premorbid FSIQ	105.6 $\pm$ 5.2*	111.5 $\pm$ 6.6	106.5 $\pm$ 5.0*	102.9 $\pm$ 5.1*	115.7 $\pm$ 4.4	109.9 $\pm$ 6.7	NA
Trail Making Test A, s	21.1 $\pm$ 6.9	18.9 $\pm$ 5.3	21.8 $\pm$ 7.9	20.4 $\pm$ 4.2	17.2 $\pm$ 3.4	19.5 $\pm$ 5.9	NA
Trail Making Test B, s	49.5 $\pm$ 17.3*	39.7 $\pm$ 13.7	48.7 $\pm$ 18.0	48.9 $\pm$ 14.9	37.2 $\pm$ 11.9	40.7 $\pm$ 14.6	NA
Trail Making Test B-A, s	28.4 $\pm$ 14.1*	20.8 $\pm$ 10.9	27.0 $\pm$ 12.7	28.5 $\pm$ 16.2	20.0 $\pm$ 9.4	21.2 $\pm$ 11.8	NA
Stroop Colour Naming and Word Reading Composite Score, s	23.4 $\pm$ 3.1	23.5 $\pm$ 3.8	23.4 $\pm$ 3.4	23.1 $\pm$ 2.4	22.5 $\pm$ 3.5	23.6 $\pm$ 3.9	NA
Stroop Inhibition, s	46.4 $\pm$ 8.6	46.9 $\pm$ 17.6	46.6 $\pm$ 9.2	45.3 $\pm$ 7.3	42.4 $\pm$ 6.2	51.4 $\pm$ 20.1	NA
Stroop Inhibition-Switching, s	55.5 $\pm$ 11.7	55.6 $\pm$ 10.2	54.6 $\pm$ 12.1	56.6 $\pm$ 11.3	56.0 $\pm$ 6.4	55.5 $\pm$ 11.6	NA
Stroop Inhibition-Switching versus Baseline Contrast, s	32.0 $\pm$ 10.3	32.4 $\pm$ 8.2	31.2 $\pm$ 10.1	33.5 $\pm$ 10.6	33.5 $\pm$ 4.8	31.9 $\pm$ 9.4	NA
BVMT Total Recall	27.8 $\pm$ 4.5*	31.8 $\pm$ 3.5	27.3 $\pm$ 4.6*	28.4 $\pm$ 4.9	29.8 $\pm$ 4.8	32.6 $\pm$ 2.7	NA
BVMT Recognition Discrimination Index	5.8 $\pm$ 0.4	5.9 $\pm$ 0.2	5.8 $\pm$ 0.4	5.9 $\pm$ 0.3	5.8 $\pm$ 0.4	6.0 $\pm$ 0.0	NA
HVLT Total Recall	26.1 $\pm$ 4.3*	29.5 $\pm$ 4.3	26.5 $\pm$ 4.4	24.3 $\pm$ 3.8*	31.0 $\pm$ 1.0	29.0 $\pm$ 4.9	NA
HVLT Recognition Discrimination Index	10.6 $\pm$ 1.3	11.2 $\pm$ 1.1	10.6 $\pm$ 1.4	10.5 $\pm$ 0.7	11.6 $\pm$ 0.5	11.0 $\pm$ 1.2	NA

Baseline computerised cognitive examination							
Visuospatial Working Memory	7.8 ± 1.1	7.9 ± 1.1	7.7 ± 1.0	8.0 ± 0.8	8.0 ± 1.3	7.8 ± 0.9	NA
Paired Associates	5.3 ± 1.1	5.2 ± 1.2	5.3 ± 1.1	5.3 ± 0.9	5.1 ± 1.3	5.3 ± 1.0	NA
Self-Ordered Search	8.8 ± 1.4	8.6 ± 1.9	8.7 ± 1.5	8.5 ± 1.3	8.4 ± 2.1	8.9 ± 1.7	NA
Feature Match	118.0 ± 23.4	119.7 ± 34.6	118.8 ± 24.2	110.2 ± 25.1	121.6 ± 37.1	116.9 ± 31.6	NA
Odd One Out	14.8 ± 2.0	15.4 ± 2.0	14.7 ± 2.1	15.3 ± 1.9	15.9 ± 1.8	14.8 ± 2.1	NA
Spatial Planning	16.9 ± 5.3	18.3 ± 6.1	16.9 ± 5.7	16.8 ± 4.6	19.7 ± 6.6	16.3 ± 4.6	NA
<i>Fractals task</i>							
Identification error - 1 item	99.2 ± 2.7	99.7 ± 1.7	99.2 ± 2.7	99.4 ± 2.5	100.0 ± 0.0	99.3 ± 2.6	NA
Identification error - 3 item	91.2 ± 5.3	93.2 ± 5.0	91.1 ± 5.2	91.4 ± 5.5	92.5 ± 5.4	94.1 ± 4.3	NA
Localisation error - 3 item	6.6 ± 1.7	6.2 ± 2.2	6.6 ± 1.8	6.7 ± 1.5	6.5 ± 2.8	5.9 ± 1.2	NA
Swap error (4s delay)	13.3 ± 9.3	14.9 ± 9.3	13.2 ± 8.9	12.2 ± 9.5	15.6 ± 9.6	14.0 ± 9.1	NA

Abnormal white matter integrity in rugby players

White matter structure was assessed using diffusion, volumetric and susceptibility weighted MRI scans. Three baseline DTI scans of rugby players and 2 Sport Control DTI scans were discarded after quality control checks. I investigated white matter structure at the individual level by calculating the mean fractional anisotropy (FA) from a number of large white matter tracts and the whole white matter skeleton and comparing results to control ranges. This approach is illustrated in the case study presented in Figure 17. Conventional MRI of this rugby player with an acute injury showed no evidence of abnormalities. In contrast, FA averaged across specific white matter tracts showed abnormalities in the genu of the corpus callosum, and left and right corticospinal tract, with low FA characteristic of white matter abnormality.

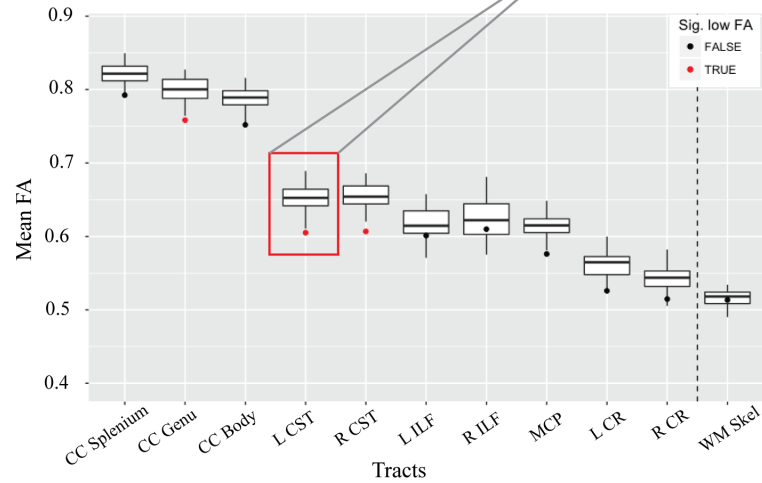
Across the whole cohort, there was a higher proportion of rugby players with DTI abnormalities defined in this way than controls ($\chi^2 = 5.43$, $P = 0.0198$). Seventeen percent of rugby players (7/42) had abnormalities in mean FA in at least one of the sampled tracts, compared to 3% of Non-Sport Controls (1/32) (Fig 17B). Sport

Controls showed no abnormality in FA. The most common tracts affected were the corpus callosum and the corticospinal tracts. The majority of rugby players with diffusion abnormalities showed reduced FA within the corticospinal tracts (5/7, 71%). Sport Controls showed no abnormality in FA. There were no differences in age (24.1 (sd = 3.62) vs 25.5 (sd = 3.58) years old, gender (100% male vs 92% male) or alcohol intake (4.7 vs 4.6 average weekly units) between players with diffusion abnormalities and those without.

A) Case study 1



ii) Region of interest analysis - Tract DTI



B) Region of interest analysis - comparison of individual classifications across the groups

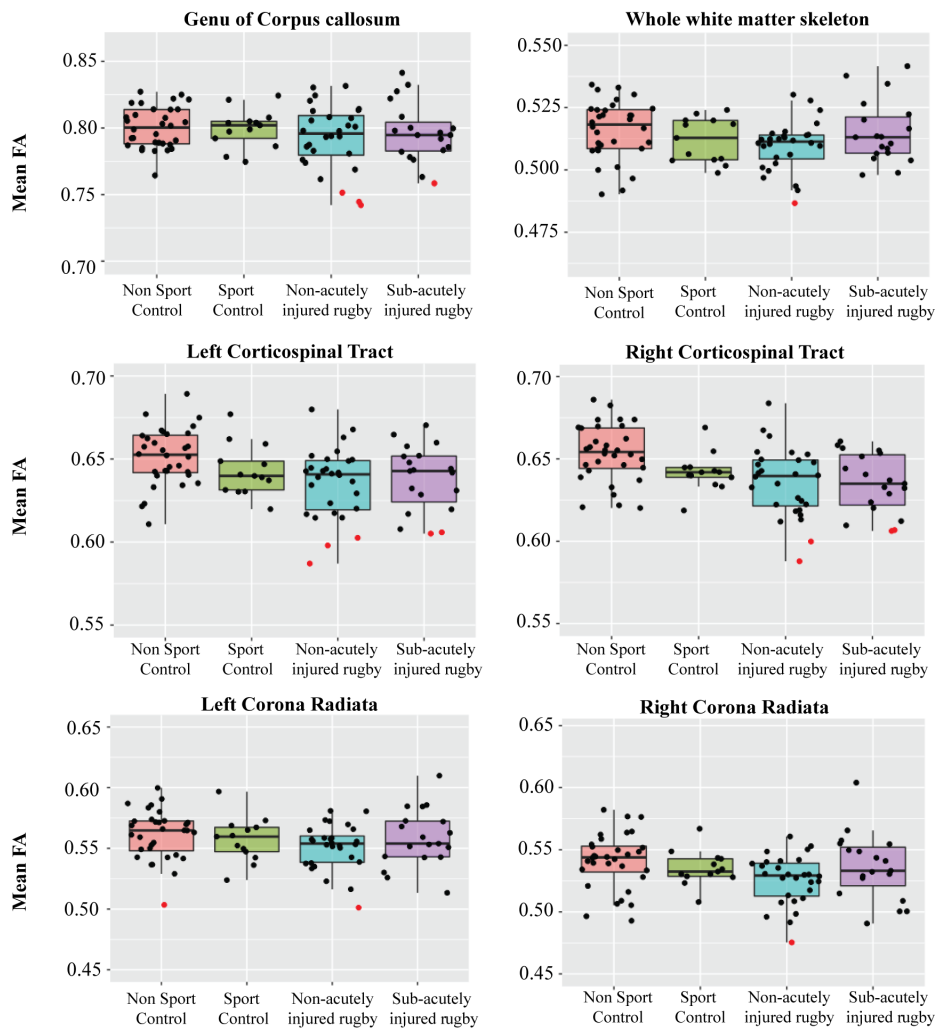


Figure 17. Subject level analysis of fractional anisotropy.

(A) (i) Routine MRI scans including T1-MPRAGE, SWI and FLAIR of the player showed no abnormalities. (ii) An individualised approach to DTI analysis. Mean FAs from specific white matter tracts are plotted; highlighted in red and inset above are the areas sampled corresponding to the left corticospinal tract. The boxplots represent the values found using the Non-Sport Control cohort (n=32). Single points on the plot represent the individual's mean FA value within the specific tract. Black dots represent where a value is not abnormal, while red points indicate where an individual has a significantly abnormal FA compared to the control cohort, $p < 0.05$, 95% confidence interval, FDR corrected. (B) Group comparisons of the number of individuals with abnormalities in mean FA of specific tracts showed a higher proportion of rugby players with abnormalities compared to controls. CC = Corpus Callosum, CST = Corticospinal Tract, ILF = Inferior longitudinal fasciculus, MCP = Middle cerebellar peduncle, CR = Corona Radiata, WM Skel = Whole white matter skeleton.

Voxelwise group comparisons of diffusion MRI also provided evidence for altered white matter integrity in rugby players. Voxelwise cross-sectional comparison of all rugby players to all controls (Non-Sport and Sport Controls combined) showed reductions in FA in the posterior thalamic radiation, left posterior corona radiata, left posterior limb of internal capsule and the superior cerebellar peduncle bilaterally (Figure 18A). No significant differences were observed for the other DTI metrics investigated, which were: mean diffusivity (MD); neurite density index (NDI); orientation dispersion index (ODI); and isotropic volume fraction (ISOVF).

Abnormalities in white matter integrity present in primarily in non-acutely injured players.

To disentangle the effects of a sub-acute injury I investigated diffusion changes in sub-acute and non-acutely injured players separately. Out of the 7 players with DTI abnormalities, 5 were non-acutely injured and 2 were scanned sub-acutely after injury. Voxelwise analysis showed that imaging abnormalities were most prominent in non-acutely injured players. Compared to all controls, reductions in FA in the non-acutely injured group were seen in the corona radiata and sagittal stratum, as well as elevations of MD in the genu of the corpus callosum, corona radiata, posterior thalamic radiation, retrolenticular part of internal capsule and sagittal stratum. Furthermore, lower NDI was seen in widespread white matter regions in the non-acutely injured

players (Figure 18B). In contrast, sub-acutely injured rugby players showed no difference in diffusion metrics when compared to all controls.

I then compared the sub-acutely injured players with non-acutely injured rugby players. MD was reduced in sub-acutely injured players compared to non-acutely injured players within the genu of the corpus callosum, retrolenticular part of internal capsule, corona radiata and external capsule (Figure 18C). There were no differences in the other DTI metrics.

I then split the control groups to investigate the effect of sporting activity on diffusion measures. There were no differences between observed in any of the DTI metrics between the Non-Sport Controls and Sport Controls. I investigated if the diffusion differences were still present using only Sport or Non-Sport Controls. Reductions in FA were still observed in non-acutely injured rugby players when compared to only Non-Sport Controls in the corona radiata, posterior thalamic radiation, sagittal stratum and corticospinal tract (Figure 18D). There were no reductions in other DTI metrics, differences between sub-acutely injured players and Non-Sport Controls nor differences when comparing the Sports Control group with either rugby group.

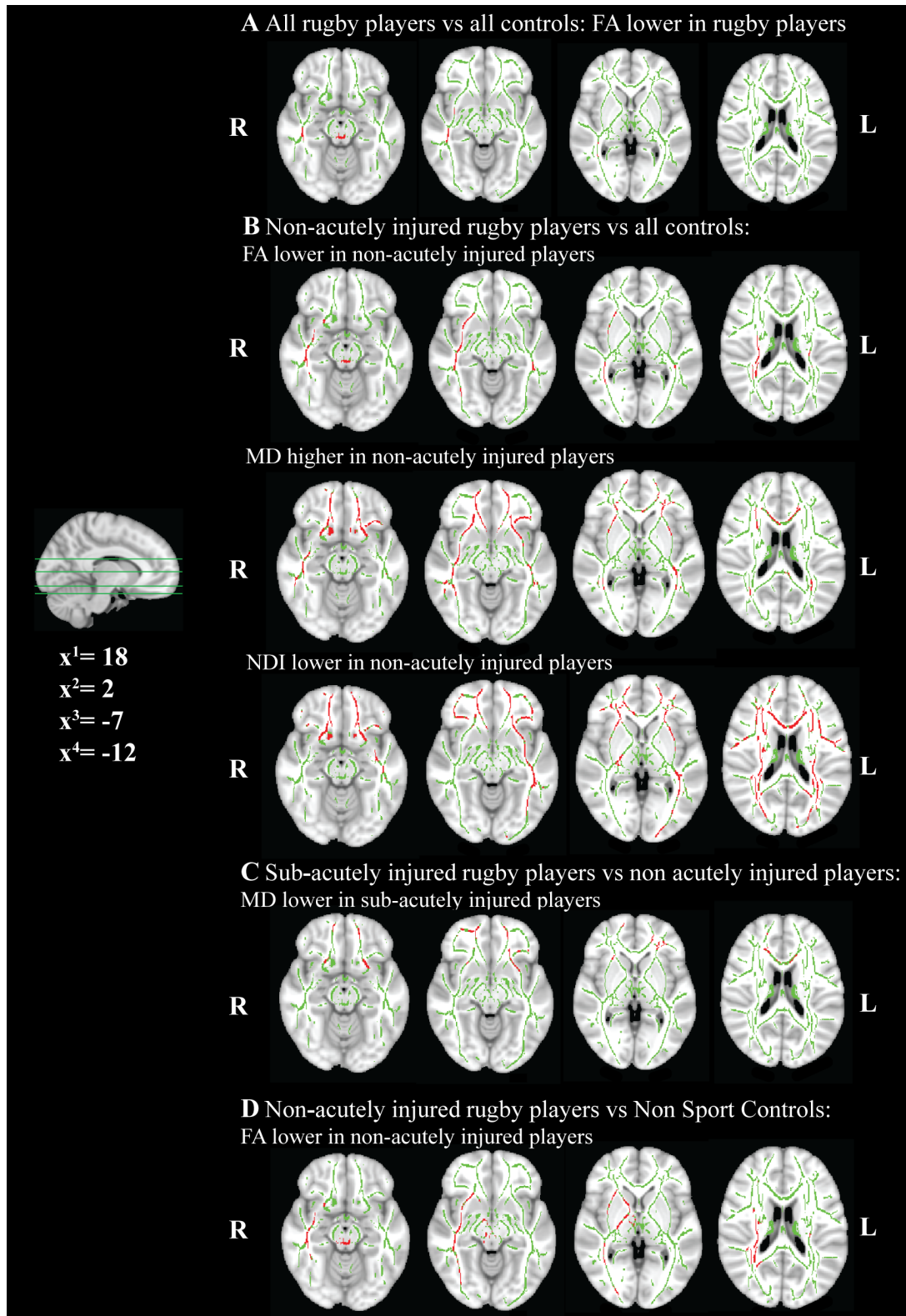


Figure 18. Voxelwise comparison of diffusion metrics between groups.

Voxelwise comparisons were conducted using FSL Randomise with age and gender added as covariates. Green denotes the white matter skeleton and areas in red indicate a result where corrected $P < 0.05$. **(A)** Comparisons of fractional anisotropy (FA) between all rugby players ($n = 42$) and all controls ($n = 45$) showed regions of lower FA in rugby players compared to controls. No differences were seen in other

diffusion metrics. **(B)** Regions of lower FA, higher mean diffusivity (MD), and lower neurite density index (NDI) were observed in non-acutely injured rugby players ($n = 27$) when compared to all controls. **(C)** Elevated FA was observed in acutely injured players ($n = 19$) when compared to non-acutely injured players ($n = 24$) excluding multiple observations of the same player. No differences were seen in other DTI metrics. **(D)** When splitting the control group, non-acutely injured rugby players still showed areas of reduced FA relative to Non-Sport Controls ($n = 32$). No differences were observed in other DTI metrics, nor between Non-Sport Controls and acutely injured players or between Sport Controls ($n = 13$) and either rugby group. *X coordinates are given in Montreal Neurological Institute (MNI) space. R = right, L = left.*

Rugby players show abnormal white matter volume change over time

I assessed volumetric differences between the rugby players and controls. Cross-sectional comparisons were made between the groups at baseline. A voxelwise analysis of grey and white matter volumes showed no significant differences at baseline between players and all controls. There were no differences between the control groups, or between non-acutely injured and sub-acutely injured rugby players.

I then investigated whether there was any evidence of abnormal brain volume changes in rugby players using a sensitive measure of brain volume change over time (Jacobian Determinant, 'JD'). Voxelwise comparison of JD maps between rugby players who attended 2 visits and the Longitudinal Control group showed lower white matter JD in rugby players, specifically within the lateral occipital cortex and post central gyri (Figure 19A). A lower JD suggests that either the rate of volume expansion was lower in rugby players than controls, or players were losing volume at a higher rate than controls. Longitudinal Controls were slightly older than the rugby players (mean age in years 33.3 vs 24.8, $t = 6.28$, $p < 0.001$), and so would be expected to have lower JD values than the rugby players. However, the reverse was true. Sampling the mean JD values from these regions revealed that 50% of players (9/18) had mean JD values below zero (indicating brain volume loss) compared to 25% of controls (4/16) (Figure 19B). These results suggest that the rugby players had an abnormal trajectory of white matter volume change for individuals of that age (Figure 19C). Mean JD within the grey, white matter or whole brain did not correlate significantly with head injury history.

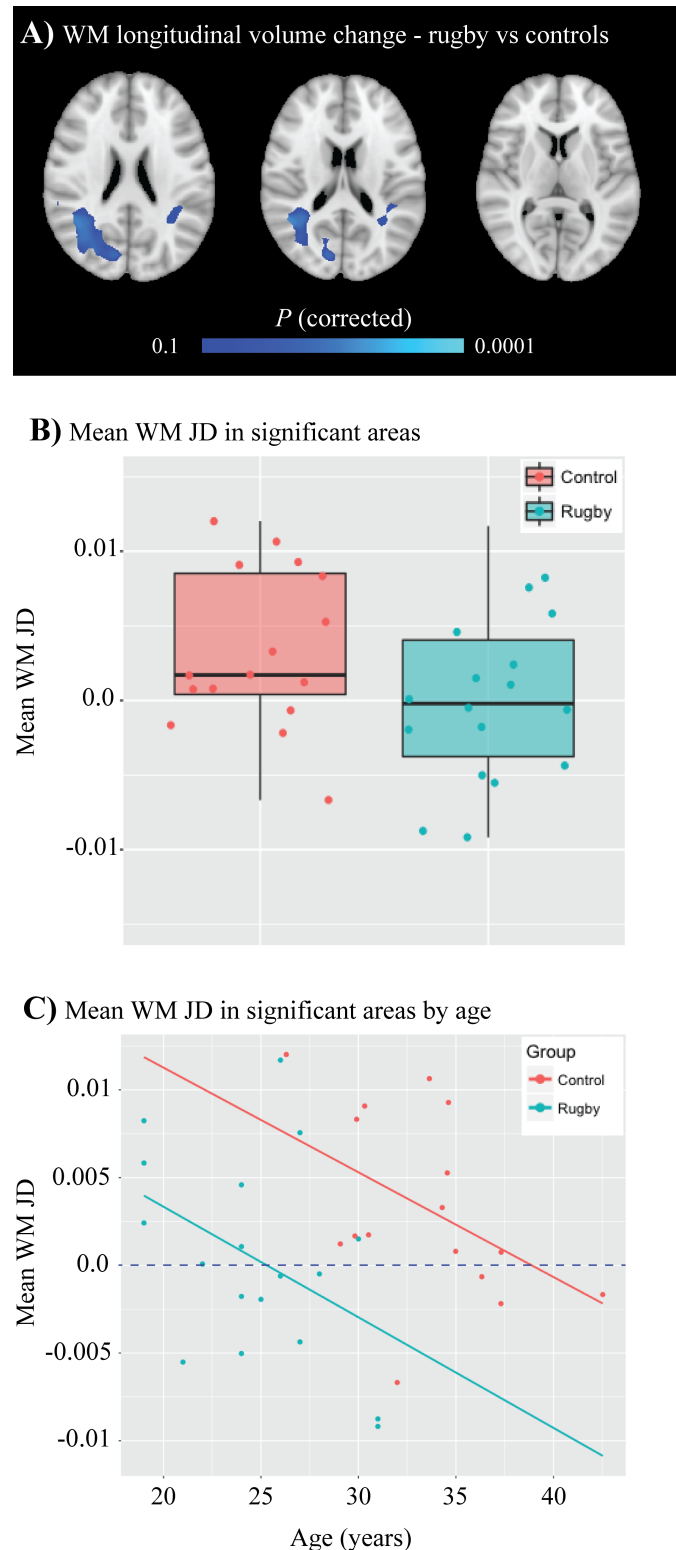


Figure 19. Longitudinal changes in volume in rugby players.

Voxelwise comparisons were conducted using FSL Randomise with age as a covariate and results shown for areas where corrected $P < 0.1$ (one tailed t-test). **(A)** Lower annualised Jacobian Determinant (JD) in rugby players ($n = 18$) compared to Longitudinal Controls ($n = 16$) was observed in the white matter. **(B)** When sampling

the JD values within the significant areas, values between rugby players (blue) and Longitudinal Controls (red) look similar. (C) However, accounting for age, a proportion of rugby players have JD values that are lower than what would be expected for individuals of that age, suggesting there are altered trajectories for white matter volume change in rugby players.

White matter integrity and longitudinal volume change in rugby players

I tested whether areas of the white matter showing evidence of abnormal volume changes over time (low JD) in rugby players are associated with abnormalities in white matter integrity (low FA). I overlaid the white matter skeleton with the area where there was significantly reduced JD, and sampled the diffusion metrics and JD values in the overlapping region. I found no correlation between baseline FA, MD or NDI with JD in these areas. Additionally, there were no significant correlations when restricting analysis only to non-acutely injured rugby players.

White matter volume changes, white matter integrity and neuropsychological performance

I also explored the relationship between atrophy rates, white matter integrity and cognitive function. Investigating tasks which showed differences at baseline, no correlations were found between mean FA, MD or NDI in the whole white matter skeleton ROI and neuropsychological test performance (Table 12). Rugby players who showed abnormalities in at least one white matter tract ROIs ($n = 7$) did not perform significantly differently in neuropsychological tests, including premorbid intelligence scores, compared to players not classified as abnormal on their individualised DTI. No correlations were found either with mean JD in grey, white matter or whole brain and neuropsychological test scores. I investigated if premorbid intelligence influenced the DTI results. I removed 3 non-sport controls at the higher range of FSIQ scores so that the non-sport controls ($n=29$) and rugby players ($n=42$) were matched for premorbid intelligence. Running the individualised DTI assessment with FSIQ matched controls, players previously identified as abnormal on DTI remained abnormal. Furthermore, FSIQ scores were not correlated with mean FA within rugby players or controls.

Table 12. Correlations between neuropsychological tests and DTI metrics in the white matter skeleton.

Neuropsychological tests	Whole white matter skeleton		
	FA (<i>n</i> =39)	MD (<i>n</i> =38)	NDI (<i>n</i> =38)
TOPF	p=0.329	p=0.682	p=0.764
Visual Memory Immediate Recall	p=0.531	p=0.804	p=0.770
Verbal Memory Immediate Recall	p=0.646	p=0.457	p=0.633
Trails B	p=0.423	p=0.923	p=0.950
Trails B minus A	p=0.422	p=0.806	p=0.748

4.5 Discussion

I present the findings of a longitudinal observational study investigating the presence of abnormalities in brain structure associated with elite rugby participation. I use diffusion tensor imaging (DTI) to assess white matter structure in an individualised manner and found evidence of axonal injury in several of the players studied. Assessing the rugby players as a group, abnormalities of fractional anisotropy (FA) and other diffusion measures were seen. In contrast, non-collision sport playing controls were not classified as showing abnormalities of FA. I also studied changes in brain structure over time, finding evidence of abnormal volume change in the white matter. These results suggest an association between participation in elite adult rugby and abnormalities in the white matter.

I assessed FA, a sensitive marker of axonal injury (Chapter 3), but used an individualised approach to DTI analysis (Jolly et al., 2021). This ensured that the analysis was well positioned to detect abnormalities in DTI for each individual player within the cohort. This process includes correction for multiple comparisons across each region of interest investigated, and facilitates the investigation of the structure of a number of large white matter tracts in a focused way. Seventeen percent of the rugby players showed abnormalities of FA, which could be as a result of diffuse axonal injury. In contrast, non-collision sport athletic controls did not show any abnormalities in FA. These abnormalities were present in both sub-acutely injury, and in non-acutely

injured players, indicating that the damage to the white matter was not specific to the presence of a recent symptomatic injury.

Assessing the players as a group, I observed reductions in FA, neurite density and increased mean diffusivity in rugby players. These abnormalities replicated changes seen in studies of mild TBI patients scanned in the chronic stage after an injury (Fakhran et al., 2013, Veeramuthu et al., 2015, Churchill et al., 2017a) and longitudinally (Wu et al., 2020). These differences persisted in non-acutely injured players, but were less prevalent in sub-acutely injured players. This is likely due to an effect of acute injury which we saw evidence of. In acutely injured rugby players, reduced mean diffusivity (opposite direction to findings of players versus controls) was seen compared to non-acutely injured players. This was in keeping with several previous studies of sport mTBI, which additionally showed elevated FA in acutely injured players alongside reduced diffusivity (Churchill et al., 2019, Virji-Babul et al., 2013, Churchill et al., 2017b). Hence, the absence of evidence for diffusion changes in acutely injured players may be due to the acute effects being occluded by longer term reductions in FA, as observed in the context of reduced FA in our non-acutely injured players.

Multi-shell diffusion-MRI was acquired. This allowed for a more advanced technique, NODDI, to be applied in separating the diffusion signal that may provide more specific diffusion metrics of white matter pathology. I observed widespread reductions of NDI in our non-acutely injured rugby players, but not in the acutely injured players, with no differences in ODI or ISOVF. These findings replicated results from studies using NODDI in patients with mild TBIs (Wu et al., 2018, Churchill et al., 2019). These results suggest that the differences in FA between rugby players may be due to lower white matter tissue density and not primarily driven by variations in free water volume.

This hypothesis is compatible with my longitudinal structural imaging findings. Abnormal white matter volume changes were observed in the rugby group. Reductions in white matter volume were seen in around 50% of the rugby players studied and lower white matter volume was seen across the group when compared to a control

group who were slightly older. White matter volume changes are highly dependent on age. White matter volume is expected to increase until the age of around 40 (Sowell et al., 2003, Bartzokis et al., 2001), and this pattern was seen in our control group who had longitudinal imaging. However, this was not the case in the rugby players longitudinally scanned, who had an average age of 25 years old, with the oldest being 31 years old. In this age range, the reductions in white matter volume are abnormal and may be an early sign of an active neurodegenerative process that might increase the risk of neurodegenerative disease in later life.

The clinical implications of my findings are unclear. Abnormalities of white matter structure, measured by JD or FA, were not associated with any clinical measures used in this study. Although the rugby players we studied did show poorer performance on a number of cognitive tests, players with white matter abnormalities did not perform significantly worse than those classified as normal. Rather than the effect of rugby participation or acute injury, the difference in performance observed across a select number of neuropsychological tests may have been related to group differences in IQ and appears unrelated to injury or imaging abnormalities within this study. Furthermore, while the majority of the players with evidence of DAI showed abnormalities within the corticospinal tract, which in other contexts are associated with abnormalities of motor function (Caeyenberghs et al., 2011, Ressel et al., 2017), the players did not show any evident abnormalities of motor function and all were successfully playing rugby at an elite level.

However, retired rugby players have been shown to display reduced fine motor control ability (McMillan et al., 2017, Pearce et al., 2018). Therefore, it is possible that reductions of FA within the corticospinal tract might be significant in terms of future motor function. Several studies have reported a connection between amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disease primarily of the motor system, and contact sports participation and related head injuries (Woods, 1911, Chio et al., 2005, Chio et al., 2009, Armon, 2007, Lehman et al., 2012, Mackay et al., 2019). Specifically, reduced corticospinal tract FA has also been associated with ALS (Blain et al., 2011, Turner et al., 2012).

The pathological correlates of abnormal white matter volume may also be associated with other neurodegenerative diseases. Evidence of CTE in retired professional rugby players has been previously observed (Stewart et al., 2016, Lee et al., 2019, Buckland et al., 2019), and in deceased professional Scottish football players where acute head injury risk is lower, but exposure to heading the football is seen as a unique sport specific risk factor, a five-fold increased risk of developing Alzheimer's disease was identified (Mackay et al., 2019). Animal studies show that early markers of neurodegeneration such as tau pathology, vascular damage and neuroinflammation can be produced by mild injuries of the type frequently experienced by professional rugby players (Tagge et al., 2018, Laitinen et al., 2015, Glushakova et al., 2014, Kane et al., 2012, Hamberger et al., 2009). Importantly, this pathology can be seen in the absence of concussive symptoms. Impacts of a sufficient biomechanical threshold to result in subsequent development of neurodegenerative pathology, but not acute symptoms, are not routinely measured over a player's career. Further studies are needed to better the mechanisms of developing late neurodegenerative pathology, and investigate whether these subthreshold impacts, as well as reported mTBIs, are associated with evidence of brain injury.

There are some limitations to our study. The longitudinal control group were non-sport controls, meaning that the effect of exercise was not controlled for in our longitudinal analyses. However, I do not believe this likely to have a significant effect on the comparison, as studies suggest exercise should lead to higher grey and white matter volume (Erickson et al., 2014, Wittfeld et al., 2020, Sexton et al., 2016). A further limitation of this study was that there were gender and age differences in our control groups when compared to the rugby players. I minimised this effect by modelling age in all analyses, and gender where appropriate. One potential limitation is that diffusion metrics such as FA have been shown to be related to premorbid intelligence scores (Schmithorst et al., 2005, Yu et al., 2008), which were higher in our control group. This did not appear to be a major confound for our results, as a sensitivity analysis showed no difference in classification of DTI abnormalities in rugby players when the groups were matched for FSIQ, and intelligence scores were not correlated to FA. The non-

collision sport control group were also not professional athletes and not matched for BMI to the rugby group. This limits my ability to exclude the effect of high level sporting participation, or for the influence of BMI on DTI or WM volume, which has been shown to correlate negatively with FA in a study of normal weight, overweight and obese individuals (Xu et al., 2013). I only had a small sample of players who had a follow-up visit, meaning I was not well powered to investigate the effect of an acute injury on longitudinal volume change, which may be influenced by the resolution of oedema or inflammation. Furthermore, it is important to note that these results in adult professional rugby union and league players are not directly comparable to play at the community or youth levels.

4.6 Conclusions

In summary, I present evidence that participation in rugby at an elite level is associated with diffusion and brain volume abnormalities. These results are in keeping with the presence of axonal injury, potentially related to repeated head impacts. There were unexpected patterns of white matter volume change in players scanned longitudinally which may indicate excess neurodegeneration in white matter tracts affected by injury. Strikingly, these changes appear to be irrespective of the presence of a sub-acute injury. Further research is needed to understand the mechanisms behind neuropathology following repeated sports related head impacts and head injuries.

In the following chapter, I tackle this question and investigate the biomechanics associated with head impacts, and how they relate to neuropathology.

Chapter 5.

Impact biomechanics in collision sports

A study of impact biomechanics in American football players.

In this chapter, I investigate what the spatial location and magnitude of biomechanical forces produced by head impacts appear like in a large cohort of professional American football players and whether this is influenced by the magnitude and frequency of the impacts they are exposed to.

5.1 Abstract

Background

Professional collision sport athletes, such as those in rugby and American football, are frequently exposed to head impacts which can cause mild traumatic brain injuries and may lead to neurodegenerative diseases such as chronic traumatic encephalopathy (CTE). There have been limited investigations into the possible biomechanical mechanisms for these pathologies and the features of the injuries that convey the highest risk of developing them. Here I present a study testing whether impact profiles, which relate to a player's position, influences the magnitude of biomechanical forces in the brain. Specifically, I test if brain deformation, a possible biomechanical trigger for pathology, is higher within the sulci compared to gyri in professional American football players.

Methods

I assessed 148 head impacts from professional American football games. These impacts were separated into 3 different profiles based on the position of the players involved, which were grouped based on the magnitude and frequency of impacts players in this position would typically experience. Using video footage, the impacts were physically reconstructed and a detailed finite element model of TBI then used to predict brain deformation metrics, strain and strain rate, across the brain and within sulci. I compared the magnitude of these metrics across all players, and compared them between position profiles.

Results

I found that across all players, the magnitude of deformation metrics was highest in the sulci, which spatially corresponds to where CTE pathology is observed. In positions where players are typically exposed to large magnitude and low frequency impacts, strain and strain rate across the brain and in sulci were also highest. I also found that rotational head motion is a key determinant in producing large strains and strain rates in the sulci.

Conclusions

These findings provide a biomechanical basis for why abnormal pathology appears in the depths of sulci. The results suggest that the type of impacts typically experienced by players in particular positions have a significant effect on accelerations to the head, influencing the magnitude of deformations within sulci.

5.2 Introduction

Athletes participating in collision sports, such as rugby and American football, are frequently exposed to head impacts and are at risk of suffering mild traumatic brain injuries (TBI). Head injuries are associated with a risk of developing Chronic Traumatic Encephalopathy (CTE), a neurodegenerative disease (Mez et al., 2017, McKee et al., 2013). However, there is little consensus as to the frequency or severity of injuries required to produce CTE pathology. Previous studies have shown that CTE-like pathology can be produced from a single severe head injury (Smith et al., 2013, Johnson et al., 2012). However, the majority of cases, particularly in a sporting context, have been in relation to repeated mild TBIs (McKee et al., 2009) or exposure to “sub-threshold” head impacts that produce no immediate symptoms (Stein et al., 2015, McKee et al., 2014, Bieniek et al., 2015). It is therefore likely that there is an interaction between impact or injury severity and impact frequency in the risks associated with developing CTE.

The severity of a head impact can be defined by the biomechanics of the head and brain at the time of the injury. This can be achieved by using computational modelling, which allows for the quantification of brain deformation metrics such as Green-Lagrange strain and strain rate during the short time of impact (Zhang et al., 2001, Kleiven and Hardy, 2002, Ghajari et al., 2017). The distribution and magnitude of strain and strain rate have been informative in predicting post-traumatic pathology such as axonal injury and permanent tissue damage (Bain and Meaney, 2000, Morrison et al., 2003, Tagge et al., 2018, Donat et al., 2021), and are likely to be informative in predicting the onset of CTE.

Our group has previously investigated where within the brain these brain deformation metrics are highest. We used a Finite Element (FE) model of the brain with detailed anatomical features and predicted distribution of Green-Lagrange strain and strain rate within the brain during an American football head collision, a fall and a motorcycle accident (Ghajari et al., 2017). In all cases, the model predicted high levels of strain within the depths of sulci, where the CTE pathology has been observed (McKee et al., 2016), and converged with where abnormalities in MRI metrics of axonal injury were

observed in a large cohort of TBI patients. These results highlighted an interesting relationship that needed to be clarified and replicated in a larger cohort of impacts.

Playing in different positions has been shown to influence risk of mTBI in American footballers (Nathanson et al., 2016, Dick et al., 2007, Pellman et al., 2004). This is likely due to the fact that head kinematics, such as linear and rotational accelerations of the head, and brain deformation vary depending on the position of the player (Karton et al., 2020, Funk et al., 2012). Brain deformation has been shown to be particularly sensitive to rotational acceleration (Post and Blaine Hoshizaki, 2015, Holbourn, 1943, Gennarelli et al., 1972, Gennarelli et al., 1987, Margulies et al., 1990, Takhounts et al., 2013, Kleiven, 2007). Player positions where there is high risk of head impacts with rapid rotational acceleration may therefore contribute to the increased risk of mTBI. While it is unclear if a similar relationship exists with the risk of developing CTE pathology, a relationship does appear present when investigating dementia risk. A study of professional American footballers showed players in speed positions had higher neurodegenerative mortality compared to non-speed (Lehman et al., 2012), and in deceased Scottish soccer players, neurodegenerative disease risk was higher in nongoalkeeper positions, suggesting an association with exposure to head impacts (Russell et al., 2021). Further suggestions lie with a recent study using a novel repetitive head impact exposure tool, which quantifies impact frequency, impact magnitude and time interval, and split players into 3 clear head impact exposure (HIE) profiles which coincidentally aligned with their playing positions (Karton et al., 2020). The three profiles correspond to Profile 1: Quarterbacks, Wide receivers and Defensive backs; Profile 2: Running backs, linebackers and tight ends and; Profile 3: offensive and defensive linesmen. These player position profiles may have different inherent risks of developing CTE based on the pattern of deformation across the brain and particularly in sulcal depths where CTE pathology is seen.

In this chapter, I present a study of American football impacts investigating the spatial pattern and magnitude of strain and strain rate during typical impacts. Using the profiles assigned by the novel classification tool (Karton et al., 2020), I use my groups' previously developed FE model to simulate 148 impacts in professional American

football, representing a range of ‘typical impacts’ experienced by the 3 profiles of HIE. I assess: (a) differences in the magnitude of strain and strain rate across the whole brain, and within gyri and sulci, for impacts of different head impact profiles; (b) differences in the magnitude of strain and strain rate between gyri and sulci; and (c) the relationship between acceleration and velocity measures, and strain and strain rate. I test the following hypothesis: that a) strain and strain rate will be higher in sulci compared to gyri; b) impact profiles associated with larger rotational impacts will have the highest strain and strain rate in the sulci and; c) rotational head kinematics can predict the sulcal strain and strain rate across all collisions.

5.3 Methods

Case selection and video analysis

Videos of 32 American football games were analysed by colleagues at the University of Ottawa as part of previous investigations (Karton et al., 2020). As part of this investigation, playing positions were grouped into 3 different impact profiles based on the frequency of impacts received in typical play, the typical magnitude and time interval between impacts (Figure 20A). Recorded video was used to document all head contact and estimate impact velocity, location/orientation and event type using Kinovea software (version 0.8.20) (Figure 20B). This method has been previously validated using real world ice hockey collisions. The average error reported in the estimated collision speed and angle was approximately 10% (Post et al., 2018). Measurements from this analysis are likely to have a lower error due to the availability of camera placements in professional American football, which are typically perpendicular to the field, thus increasing the field of view and accuracy.

Of the 3,439 documented head impacts, 166 exemplar events were selected by researchers in Ottawa independent to this analysis to represent the trauma profile of the 3 distinct groups. Exemplar events were selected at random for each permutation of player position, head impact location (helmet, shoulder, hip and ground to helmet) and velocity level (Collisions: very low = <2.0 m/s, low = $2.1\text{--}4.5$ m/s, moderate = $4.6\text{--}7.0$ m/s, high = $7.1\text{--}9.5$ m/s, very high = $9.6+$ m/s; falls: very low = <2.0 m/s, low = $2.1\text{--}4.0$ m/s, moderate = $4.1\text{--}6.0$ m/s, high = $6.1\text{--}8.0$ m/s, very high = $8.1+$ m/s) to

build a pool of impact events representing all impact conditions. The current analysis excluded events that represented redundant conditions in the previous analysis by (Karton et al., 2020). Exemplar events were not selected on the basis of a head injury occurring but represented a range of impacts experienced by players in typical play.

Laboratory reconstructions

Parameters obtained from video analysis of the selected exemplar events were used to guide physical reconstructions of the head impact events using a Hybrid III 50th-percentile adult male headform and neutral unbiased neckform (Walsh et al., 2018). These were mounted with accelerometers to capture the linear and rotational acceleration of the headform during an impact (Figure 20C).

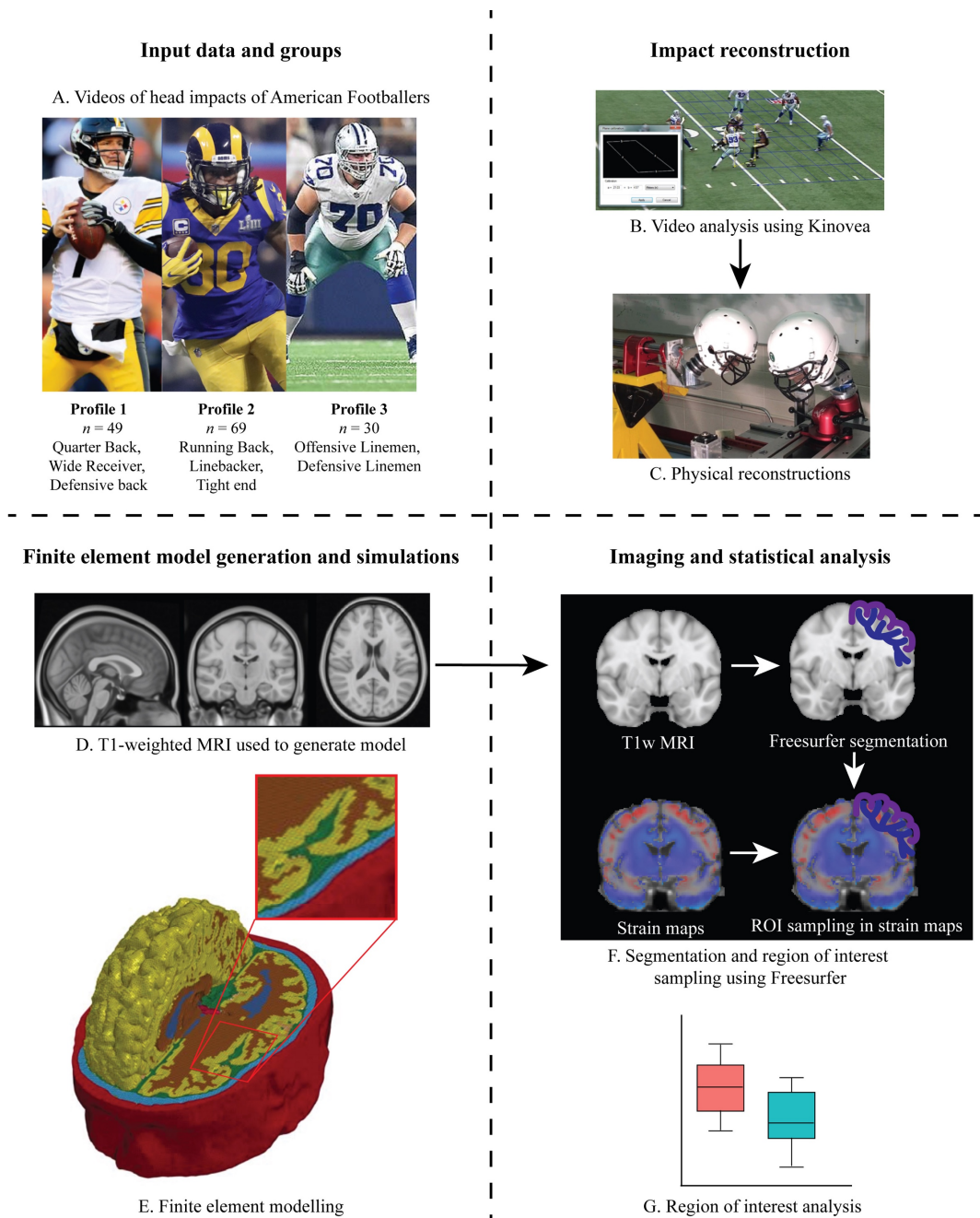


Figure 20. Analysis and study overview

(A) Player positions and number of videos after quality control checks in each profile, (B) Snapshot of the video analysis software, Kinovea, used to determine the speed and angle of colliding players, (C) Lab set-up for physical reconstruction of the collisions utilising a Hybrid-III head and unbiased neckform, (D, E) the finite element model of TBI based on a T1-weighted MRI scan from a 34 year old male, is loaded by the head accelerations recorded in the laboratory reconstructions, (F) Freesurfer segmentation of the sulcal and gyral regions are overlaid onto images of the

computational predictions of strain and strain rate for sampling within those regions, (G) statistical analysis of strain and strain rate distribution in sulci and gyri.

Finite element model and injury simulations

A 3D finite element model of the human head developed at Imperial College London was used to calculate brain deformation from each head impact. The model was developed using a high resolution T1-weighted magnetic resonance image of a healthy 34-year-old male participant and consists of nearly one million hexahedral elements and a quarter of a million quadrilateral elements, representing 11 tissues, including the scalp, skull, brain, meninges, subarachnoid space and ventricles, as well as anatomical features such as sulci. Model material characteristics, development and validation details are described in the methods (Chapter 2) and in previous published work (Ghajari et al., 2017, Zimmerman et al., 2021a). Eighteen events were discarded at the simulation stage due to incorrect coding of velocity leaving 148 impacts.

The head model was loaded by the linear and rotational accelerations obtained from physical reconstructions of the impacts. For each element of the model, I determined what is called the strain hereafter, which is the maximum principal value of the Green-Lagrange strain tensor and a measure of how much the element has been stretched with respect to its original shape. In addition, I determined the strain rate, which is the highest value of the total time derivative of the Green-Lagrange strain tensor that the element experienced during the simulation and is a measure of the maximum time rate of stretch within each element (Holzapfel, 2000). Values for strain and strain rate were encoded in an element-by-element basis and saved as a NIFTI (Neuroimaging Informatics Technology Initiative) file for neuroimaging analysis.

Injury model image processing and analysis

Strain and strain rate data for each impact were analysed using fMRIB Software Library (FSL) v6.0.2. The 90th percentile maximum principal strain and strain rate across the whole brain was calculated per impact to account for possible abnormally large strains in poorly defined elements and used to represent the peak strain and strain rate values reached across the whole brain during and impact. The T1-weighted magnetic resonance image used to generate the computational model was segmented using Freesurfer (Fischl et al., 2004). This process resulted in an accurate reconstruction of the pial surface and white matter/grey matter boundary as a 3D surface for each of the left and right hemispheres within Freesurfer space (Desikan et al., 2006). The Freesurfer surface was then subdivided into regions of interest based on the Destrieux Atlas (Fischl et al., 2004) and anatomical labelling resulting in regions of interest for 30 gyri and 33 sulci in each hemisphere (Ghajari et al., 2017). The NIFTI images containing strain and strain rate values were registered to Freesurfer space using a standard affine transformation. The mean value of the maximum principal strain and strain rates were then calculated for areas within each region of interest mask generated from the Freesurfer segmentation. These values were further averaged to calculate a mean strain and strain rate for gyral and sulcal regions for each impact. To investigate the relationship between impact kinematics and predicted brain deformation as modelled, we compared the peak resultant linear and rotational velocity and accelerations of the head in each impact with the 90th percentile strain and strain rate across the whole brain.

Statistical analysis

An a priori level for statistical significance was set at 0.05 for two sided tests. Statistical tests were completed with the open source software package R (R Core Team, 2018). Two-tailed paired T-test was performed to compare whether mean strain and strain rate was higher in gyri or sulci and Cohen's d reported as a measure of the effect size. In cases where the normality assumption was violated, the Wilcoxon test was used. A one-way ANOVA reported as F-value (degrees of freedom of independent variables, residuals), p-value, was used to assess the effect of player position profile (one level, three factors) on peak linear and rotational acceleration and velocity, 90th percentile

strain across the whole brain and 90th percentile strain rate across the whole brain. A two way repeated measures ANOVA using player position profile (one level, three factors) and brain structure as a within subject factor (one level, two factors: gyri and sulci) was conducted comparing the profiles to explore profile wise differences in strain and strain rate in sulci and gyri. Values for strain were root or log transformed to fit assumptions for normality. Pearson correlation was used to determine the relationship between sulcal strain and strain rate with peak resultant linear and rotational velocity/acceleration. Multiple linear regression analysis was used using peak linear and rotational acceleration and velocity as predictor variables to test the contributions of the predictor variables in predicting 90th percentile strain and strain rate in the sulci.

5.4 Results

Differences in impact biomechanics between players in different HIE profiles

Comparing whole brain 90th percentile strain and strain rates across the different head impact exposure (HIE) profiles, a one-way ANOVA indicated there was a significant effect of player position profile on both strain ($F_{2,145} = 4.401$, $P=0.014$) and strain rate ($F_{2,145} = 4.672$, $P = 0.011$). Post-hoc Tukey HSD tests showed that these effects were driven by significant differences between profile 1 and profile 3 for strain ($P = 0.016$) and strain rate ($P = 0.012$) (Table 13).

I then investigated if there were differences in head impact kinematics between HIE profiles. A one way ANOVA showed a significant effect of HIE profile on peak resultant linear acceleration ($F_{2,145} = 3.887$, $P = 0.023$), linear velocity ($F_{2,145} = 4.241$, $P = 0.016$), rotational acceleration ($F_{2,145} = 4.583$, $P = 0.012$) and rotational velocity ($F_{2,145} = 4.574$, $P = 0.012$) (Table 13). Post-hoc Tukey HSD tests showed that these effects were driven by higher values in linear acceleration ($P = 0.026$), linear velocity ($P=0.023$), rotational acceleration ($P = 0.028$) and rotational velocity ($P = 0.022$) in profile 1 impacts compared to profile 3. Profile 1 impacts additionally had higher rotational acceleration ($P=0.028$) and rotational velocity ($P = 0.035$) when compared to profile 2 impacts. All other comparisons were not significant.

Table 13. Mean kinematics and brain deformation per player position profile.

Data presented as mean (sd) unless otherwise stated. One-way ANOVA statistics investigating the effect of Profile on measure provided. Post hoc Tukey HSD tests are provided show significance compared to Profile 1 values.

Kinematics and brain deformation measures	Profile 1 <i>n</i> = 49	Profile 2 <i>n</i> = 69	Profile 3 <i>n</i> = 30
90 th Percentile Strain $F_{2,145} = 4.401$, $P = 0.014$ Post hoc <i>P</i> -values	0.164 (0.096) NA	0.129 (0.088) n.s	0.109 (0.053) 0.016
90 th Percentile Strain rate (s^{-1}) $F_{2,145} = 4.672$, $P = 0.011$ Post hoc <i>P</i> -values	35.889 (27.584) NA	25.939 (25.001) n.s	19.704 (12.967) 0.012
Linear Velocity (mm/ms) $F_{2,145} = 4.241$, $P = 0.016$ Post hoc <i>P</i> -values	3.955 (2.201) NA	3.153 (1.741) n.s	2.809 (1.465) 0.023
Linear Acceleration (mm/ms ²) $F_{2,145} = 3.887$, $P = 0.023$ Post hoc <i>P</i> -values	0.428 (0.295) NA	0.329 (0.250) n.s	0.273 (0.196) 0.026
Rotational Velocity (rad/ms) $F_{2,145} = 4.574$, $P = 0.012$ Post hoc <i>P</i> -values	0.025 (0.012) NA	0.019 (0.012) 0.035	0.018 (0.009) 0.022
Rotational Acceleration (rad/ms ²) $F_{2,145} = 4.583$, $P = 0.012$ Post hoc <i>P</i> -values	0.003 (0.0019) NA	0.002 (0.0016) 0.028	0.002 (0.0012) 0.028

Larger strain is present in sulcal regions of all players

Large strain and strain rate were confined to the depths of sulci in a symmetrical distribution, with lower strain and strain rate seen at the cortical surface and gyral regions and noticeably in sub-cortical structures (Figure 21A). These results were similar to the distinction between the maximal strain and strain rate in the sulci compared to gyri present at the grey/white matter interface (Figure 21B). I further analysed this data by determining strain and strain rate in sulcal and gyral regions of interest across all impacts and comparing them (Figure 21C). I found significantly higher strain (0.094 ± 0.062 vs 0.077 ± 0.049 , $V = 3$, $P < 0.001$, $d = 1.31$) and strain rate ($18.99 \pm 16.20/s$ vs $15.98 \pm 13.09/s$, $V = 84$, $P < 0.001$, $d = 0.89$) in the sulcal regions compared to gyral regions (Figure 21D).

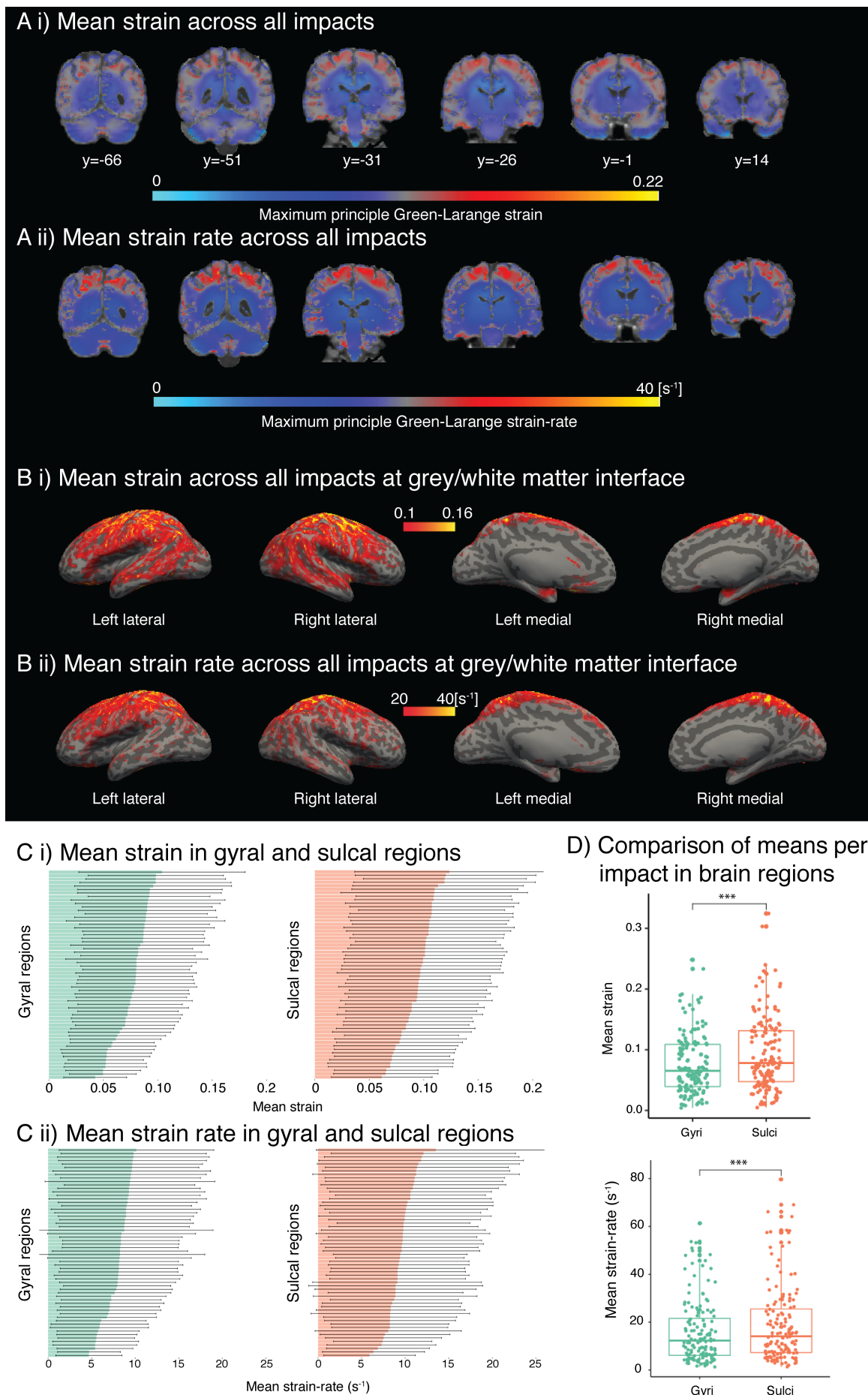


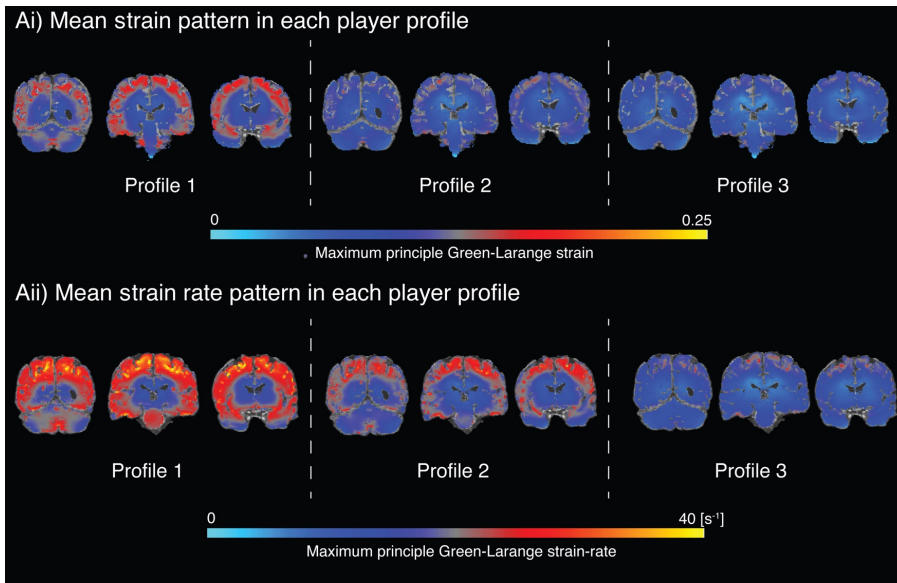
Figure 21. Computational results for all players.

(A) Whole brain mean value of strain and strain rate (areas in yellow-red indicate higher values and light blue-blue lower values), (B) Mean value of strain and strain rate at grey/white matter interface (sulci: light grey, gyri: dark grey, yellow areas indicate higher values), (C) Mean value and standard deviation of strain and strain rate in each sulcal and gyral region, and (D) mean values collapsed to two anatomical regions, gyri and sulci. *Y coordinates are given in Montreal Neurological Institute (MNI) space.*

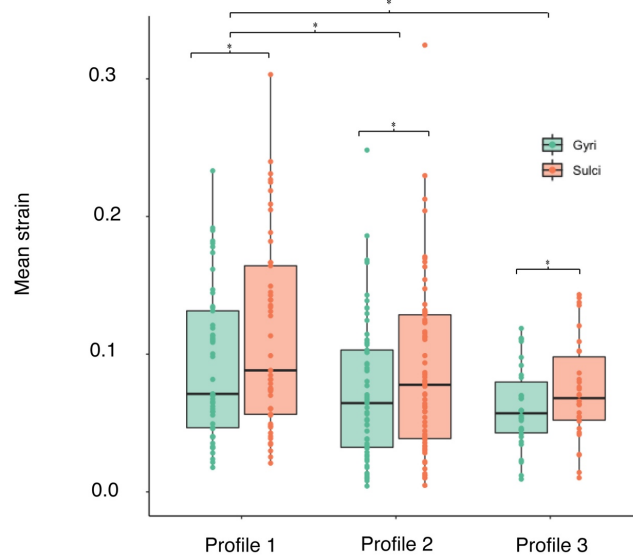
Higher strain and strain rate is present in sulci than gyri in all players

I investigated if the sulcal and gyral differences present across all impacts were preserved between HIE profiles and whether the profiles had an effect on increasing sulcal strain and strain rate. Brain contours of strain showed that strains and strain rates are larger in the brain of players at HIE profile 1 compared to the other two HIE profiles and concentrated in sulcal regions (Figure 22A). A repeated measures ANOVA showed that there was a significant effect of HIE profile ($F_{2,145} = 4.26$, $P = 0.016$) and brain region (sulcal vs gyral, $F_{1,145} = 718$, $P < 0.001$) on strain in sulcal and gyral regions. Furthermore, there was an interaction between HIE profile and brain region on strain ($F = 3.63$, $P = 0.029$). Post-hoc Tukey HSD tests showed that these effects were driven by higher strain in HIE profile 1 compared to profiles 2 and 3 in both gyri (profile 1 vs 2: $P = 0.047$, profile 1 vs 3: $P = 0.027$) and sulci (profile 1 vs 2: $P = 0.003$, profile 1 vs 3: $P < 0.001$) with a more significant effect of player position profile on strain in sulci than gyri (Figure 22Bi).

A similar effect was found when investigating strain rate, with a significant effect of HIE profile ($F_{2,145} = 4.45$, $P = 0.013$) and brain region (sulcal vs gyral, $F_{1,145} = 1109.82$, $P < 0.001$). There was no interaction between player position profile and brain region on strain rate. Post-hoc tests showed that these effects were driven by higher strain rates in HIE profile 1 compared to profiles 2 and 3 in both gyri (profile 1 vs 2: $P = 0.039$, profile 1 vs 3: $P = 0.026$) and sulci (profile 1 vs 2: $P = 0.0016$, profile 1 vs 3: $P < 0.001$) (Figure 22Bii).



Bi) Mean strain comparison in gyral and sulcal regions per player profile



Bii) Mean strain rate comparison in gyral and sulcal regions per player profile

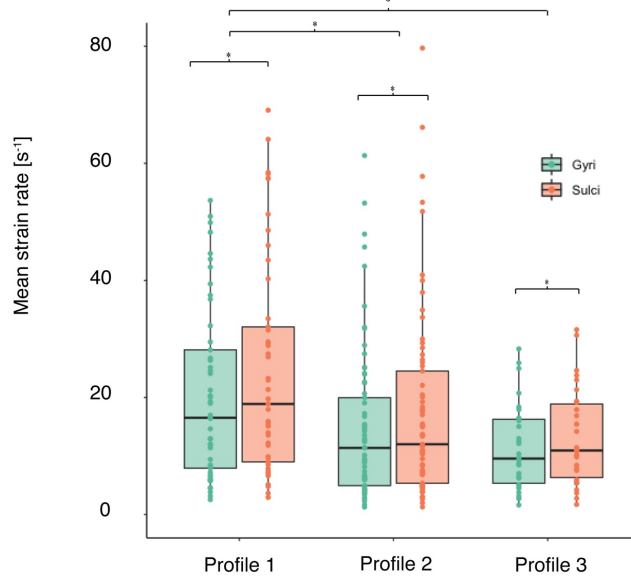


Figure 22. Predicted patterns of cortical strain in players at different positions.

(Ai) Mean value of strain and (Aii) strain rate across the whole brain, (Bi) comparison between mean strain and (Bii) strain rate across profiles and sulci/gyri.

Correlation between head rotational velocity, acceleration, and strain and strain rate in sulci

I investigated whether there is a correlation between measures of head kinematics (peak values of linear velocity, linear acceleration, rotational velocity and rotational acceleration) and strain and strain rate in sulci. I found significant positive correlations between all head kinematics measures and strain and strain rate in sulci ($p < 0.001$) (Table 14). R^2 values were higher for rotational acceleration and velocity than linear acceleration and velocity. Regression analysis incorporating linear and rotational velocity and acceleration indicated rotational velocity ($P < 0.001$, $\beta = 3.03$, 95% CI 2.39 – 3.65) and acceleration ($P < 0.001$, $\beta = 22.26$, 95% CI 17.02 – 27.50) were both significant independent predictors of strain in sulci while linear velocity and acceleration were not. In predicting strain rate in sulci, rotational velocity ($P = 0.01$, $\beta = 206.77$, 95% CI 46.65 – 366.89) and rotational acceleration ($P < 0.001$, $\beta = 8160.87$, 95% CI 6828.41 – 9493.32) were significant independent predictors while all other factors were not significant.

Table 14. Pearson correlation between mean sulcal strain and kinematics measurements of head collisions

	Linear Velocity	Linear Acceleration	Rotational Velocity	Rotational Acceleration
Strain (r^2 , 95% CI)	0.69 (0.59- 0.77)	0.70 (0.60- 0.77)	0.89 (0.86- 0.92)	0.88 (0.84- 0.91)
Strain Rate (r^2 , 95% CI)	0.75 (0.67- 0.81)	0.78 (0.71- 0.84)	0.83 (0.77- 0.87)	0.93 (0.91- 0.95)

5.5 Discussion

I present the findings of investigations into the biomechanical profiles of head impacts in American football. I show that strain and strain rate are higher in the sulci compared to gyri as a feature across all impacts, replicating findings from previous investigations but with a substantially larger cohort (Ghajari et al., 2017). I find that head impact exposure profiles, which align with playing positions, have a significant effect on strain and strain rate. Furthermore, I replicate findings in the literature and show a strong correlation between rotational motion of the head and sulcal strain and strain rate.

I found that strain and strain rates were higher sulci compared to gyri, a spatial pattern that was preserved across all HIE profiles. These findings replicate pilot results from our groups' previous study, whereby in 3 cases, including one American football impact, higher strains were found in the sulci compared to gyri (Ghajari et al., 2017). Similar observations were made in an earlier study (Ho and Kleiven, 2009) when introducing sulci into a finite element model, suggesting these results are not artefacts specific to this computational model, but a consequence of introducing inhomogeneity in brain structure (Cloots et al., 2008). Other structures that share these features, such as the grey/white matter interface and microvascular/brain parenchyma junctures are also areas that show the most TBI and CTE-related pathology (Holleran et al., 2017, McKee et al., 2016, Ghajari et al., 2017). However, other areas of the brain, such as the hippocampus and medial temporal lobe, which also show CTE pathology in later stages, appear to experience relatively low levels of strain and strain rate. The relatively low level of forces in these areas may partly explain why pathology is only present in more severe and later stages of CTE (McKee et al., 2016), however the spatial presentation of CTE pathology is also likely due to vasculature and tissue and cellular composition.

The mechanistic link between brain strain and pathology is unclear. Mechanical strain can cause blood/brain barrier injury (Shreiber et al., 1997, Shreiber et al., 1999), which may lead to the typical pattern of tau pathology observed in the sulci along small blood vessels. Sulcal strain also corresponds with where disruption to white matter axons have been observed in cases of CTE (McKee et al., 2013, Holleran et al., 2017). In a

recent study that combined finite element modelling with MRI and quantitative histopathology in a rat model of TBI, strain and strain rate correlated spatially and in magnitude with MRI and histopathology measures of diffuse axonal and glial injuries 14 days after the impact (Donat et al., 2021). Microglial response and axonal injury often co-localise in animals and humans and can still be detected several years after an injury (Scott et al., 2015, Csuka et al., 2000, Lafrenaye et al., 2015), possibly contributing to tau accumulation in cases of CTE (Cherry et al., 2016). These findings suggest that initial impact biomechanics can be informative in predicting long term clinically relevant pathology.

I found that strain and strain rate were highest in sulci for players in HIE profile 1. These players were exposed to the largest linear and rotational accelerations and velocities, but at a low frequency, and typically report the highest incidence rates of mTBI (Karton et al., 2020, Dick et al., 2007, Nathanson et al., 2016, Horstemeyer et al., 2019). Conversely, players in HIE profile 3, who experience head impacts with low magnitude but at high frequency, had the lowest magnitude of strain within the sulci. These results would suggest that players in HIE profile 1 may be at higher risk of developing CTE. However, a recent histopathological study of deceased footballers did not find an association between player position and risk of developing CTE (Mez et al., 2020), and sub-dividing incidences reported in the histopathological study with the HIE profiles show no increased risk between HIE profiles. This suggests there may be different mechanisms in player groups who are exposed to either low frequency, higher amplitude impacts or high frequency, lower amplitude impacts, as previously suggested by (Horstemeyer et al., 2019). These results are also in keeping with previous animal studies which demonstrated that while high strain in the cortex is required to produce concussion-like symptoms (i.e. HIE profile 1, high strain, high risk of mTBI), CTE like pathology can be triggered by much lower magnitude impacts (Tagge et al., 2018).

I found that linear and rotational measures of head kinematics during an impact were highly correlated with the strain and strain rate in sulci. However, when all of these kinematics measures were incorporated into a model, only rotational kinematics

predicted strain and strain rate. This relationship is not unexpected. Kinematic magnitudes are closely related to brain deformations (Post and Blaine Hoshizaki, 2015), and previous studies have indicated that rotational acceleration plays a key role in producing high tissue deformation and shearing within the brain (Meaney and Smith, 2011, Post et al., 2017). This has been demonstrated consistently across a variety of sports (Post and Blaine Hoshizaki, 2015, Rowson and Duma, 2013, Forero Rueda et al., 2011). In this study, I was able to show the link between rotational kinematics of the head and larger forces specifically in the sulci. These results suggest that putting in place measures to mitigate exposure to large rotational forces, via modulated helmet designs or rule changes, are likely to reduce the effects of head collisions on producing brain injuries.

There are several limitations to this investigation. The bio-fidelity of the video analysis and physical reconstructions methods have yet to be precisely quantified. However, these methods still remain one of the key approaches to determining the kinematics of the head during sports collisions. An unbiased neckform was used for physical reconstructions which has been shown to be repeatable and typically only vary by small standard deviations on series of impacts to various locations (Chen et al., 2020, de Grau et al., 2020, Post et al., 2020). The unbiased neck has been shown to perform similarly to the Hybrid-III neck (Walsh et al., 2018) which performs within the wide corridor of responses in terms of stiffness observed for the human neck. There are known limitations, such as its stiffness in axial loading (Yoganandan et al., 1989), however previous investigations have found minimal influences of neck stiffness on brain strains in helmeted impacts (Rousseau et al., 2010). These limitations have yet to be addressed in available neckforms, and may be addressed in the future when an improved neck is available. The accuracy of the finite element model, as with any other models, depends on a number of factors including the mesh density and quality, fidelity of the anatomy and fidelity of material models and properties. Presently these have been optimised based on the availability of experimental data, and with the availability of more higher quality experimental data in the future, the accuracy of the finite element model can be improved. I was limited to the selection of only a single exemplar event per condition. Future work should be extended to using multiple events

per condition to generate a more reliable database of impacts representing the exposure of players during normal play.

5.6 Conclusions

In summary, I find that strain and strain rate is higher in the sulci compared to gyri. Furthermore, playing position, which influences a player's impact profile, also influences the magnitude of strain and strain rate across the brain and particularly in sulci. This may explain why there is a higher rate of mTBI in certain positions in American football. However, it is likely that the impact frequency is a key factor in producing the neurodegenerative processes, which may occur at lower strains than those required to produce acute symptoms. I also found that rotational head motion is a key determinant in producing large strains in the sulci. These results highlight the utility of studying impact biomechanics in relation to brain pathology, and can contribute to the development of protective, preventative and injury management strategies aimed at reducing both acute and long-term effects of head collisions in sports.

In the next chapter of this thesis, I investigate if impact biomechanics are informative in the context of acute symptoms of mild TBI, and whether acute symptoms such as loss of consciousness are produced by impacts with particular characteristic patterns of strain.

Chapter 6.

Injury biomechanics and relationships to clinical TBI features

An investigation of American footballers using neuroimaging statistical techniques applied to biomechanical data.

In this chapter, I investigate if acute symptoms such as loss of consciousness are associated with particular characteristic patterns of biomechanical forces during injury, and what this may mean in terms of predicting patient outcomes after sports-related mTBIs.

6.1 Abstract

Background

Sports related mild traumatic brain injuries (mTBI) can be associated with transient neurological signs such as loss of consciousness (LOC) and dystonic posturing. However, it is unknown why impacts that may appear similar can produce a range of acute neurological effects, and whether neurological signs such as LOC indicate if an injury is more severe. The thalamus is thought to be an important part of maintaining consciousness, while the motor cortex is known to be critical for motor control. I investigate whether impacts that produce LOC are associated with a higher magnitude of biomechanical forces, and whether these forces are highest in the thalamus and brainstem, and whether forces are highest in areas of the motor cortex in impacts leading to dystonic posturing.

Methods

Using 81 videos of impacts in professional American football, I investigate brain injury biomechanics in 20 impacts that lead to LOC, 20 that lead to posturing and 41 impacts that lead to no neurological signs ('No Signs'). I use a computational model of the brain to calculate biomechanical metrics, strain and strain rate, and compare the magnitude and spatial location of maximal forces across the groups using neuroimaging techniques novel to this field.

Results

Impacts leading to LOC involved significantly higher acceleration and velocities of the head, and produced larger brain deformation compared to No Signs impacts. A voxelwise analysis demonstrated that higher strain and strain rates were present across the whole brain in impacts leading to LOC. Impacts leading to dystonic posturing also showed regions of significantly higher strain and these areas were constrained to the cortical regions including the motor cortex. Correcting for the magnitude of strain and strain rates, regions of disproportionately higher strain rates, but not strain, were present in the brainstem and cerebellum in impacts leading to LOC compared to No Signs and Posturing.

Conclusions

These results provide evidence that loss of consciousness is produced by head impacts that produce disproportionately high strain rates in the cerebellum and brainstem and indicates a more severe injury.

6.2 Introduction

Mild traumatic brain injuries (mTBI) are common, with 45 to 54 million injuries per year globally (Maas et al., 2017) and can occur after impacts that appear relatively trivial. TBIs can be associated with loss of consciousness (LOC) or an abnormal motor response called posturing (Malec et al., 2007, Pellman et al., 2004, Hosseini and Lifshitz, 2009, McCrory and Berkovic, 2000). It is unclear why impacts that appear similar produce different neurological events. Studying brain injury biomechanics can provide a useful way of quantifying the severity of the initial injury. The areas of the brain most affected by biomechanical forces may contribute to the symptoms that are being produced. In this chapter, I investigate if impacts that produce LOC or posturing are associated with higher magnitude of biomechanical forces, and whether these forces are highest in specific areas of the brain.

Loss of consciousness is characterised by complete loss of motor tone and is easy to identify on video or via pitch-side surveillance (Cournoyer and Hoshizaki, 2019b, Cournoyer and Hoshizaki, 2019a). Because of this, LOC is one of the most recognisable features of mTBI to the public (and often accompanied by a misconception that mTBI occurs only with LOC), and for ‘concussion spotters’ in place for sports such as professional American football or rugby union who decide if athletes should be removed from play (Fuller et al., 2017). In the moderate-severe TBI setting, the evaluation of consciousness using the Glasgow Coma Scale (GCS) (Teasdale and Jennett, 1974) and duration of LOC are used to classify the severity of an injury and are associated with poorer outcomes (Malec et al., 2007). In milder forms of TBI, LOC, even of just a few seconds, has been associated with longer recovery times and symptom severity in collegiate and professional sports (Benson et al., 2011, McCrea et al., 2013). However, this relationship is not well established, and several studies have failed to replicate this (Erlanger et al., 2003, Morgan et al., 2015, Meehan et al., 2013).

Mechanistically, LOC from trauma is thought to be due to disruptions to the circuitry involved in maintaining consciousness, including via damage to regions such as the

caudate, thalamus or areas of the brainstem including the pontine tegmentum or midbrain (Fischer et al., 2016, Parvizi and Damasio, 2003, Rohaut et al., 2019). This is supported by imaging studies finding white matter damage predominantly localises in the brainstem and thalamus of TBI patients with disorders of consciousness (Gentry et al., 1988, Moe et al., 2018, Delano-Wood et al., 2015, Sandhu et al., 2019), with the degree of damage correlating with the severity of impairment in consciousness (Jang and Kwon, 2020). LOC is also associated with impacts with high rotational accelerations (Gennarelli et al., 1972), which produce high levels of tissue deformation and shearing forces within the brain (Post et al., 2017, Meaney and Smith, 2011), however it is currently unclear whether these forces are localised in the brainstem or thalamus where pathology is observed.

Posturing is a second neurological event easily identifiable after an impact indicative of mTBI. This abnormal motor response, often characterised by a 'fencing' response, is where flexor and/or extensor behaviour is observed in the upper limbs either unilaterally or bilaterally. Posturing is relatively uncommon, has not been associated with any long-term imaging or cognitive impairments and is thought to be relatively benign (Kuhl et al., 2018, McCrory and Berkovic, 2000, McCrory et al., 1997). However, in an animal model of TBI, posturing was only produced when inducing a more severe injury (Hosseini and Lifshitz, 2009). Posturing can occur distinctly without LOC and is not epileptic in origin, with an unknown mechanism that is proposed to involve a loss of cortical inhibition and release of brainstem activity (McCrory and Berkovic, 1998). Lesions in the motor cortex and corticospinal tract after stroke have been shown to lead to reduced cortical inhibitory properties (Liepert et al., 2005). Posturing may therefore be associated with impacts involving disproportionately high biomechanical forces in regions such as the cortical spinal tracts or motor cortex compared to impacts that produce different neurological events such as LOC.

The relationship between clinical features of mTBI and the distribution of forces in the brain during an injury can be studied using finite element (FE) models of TBI. FE models incorporate mechanical tissue properties (Zhang et al., 2001) and are

validated against cadaver experiments to accurately reflect simulate brain motion following an impact (Alshareef et al., 2020). By incorporating information about the rotational and linear acceleration of the head at the time of injury, FE models can be used to predict the patterns of force distribution and deformation within the brain. These forces have been shown to be predictive of acute and longer term pathology within the brain. Green-Lagrange strain and strain rate, which are measures of local deformation and the rate of deformation, have been shown in a FE model of a rat TBI to co-localise and correlate in magnitude with MRI and histopathology measures of diffuse axonal and glial injuries (Donat et al., 2021).

In humans, it has been demonstrated that high levels of brain deformation during sporting impacts converge with where abnormalities in MRI metrics of axonal injury are observed after TBI, and within the depths of sulci, where neurodegenerative pathology develops (Ghajari et al., 2017, Zimmerman et al., 2021a). FE modelling has been employed in previous investigations of the clinical features of mTBI, finding that measures of brain deformation are higher in impacts that produce visible signs, and that LOC is associated with higher strain rates than posturing cases in the white matter (Cournoyer and Hoshizaki, 2019b, Cournoyer and Hoshizaki, 2019a).

Here, for the first time, I apply neuroimaging statistical techniques to investigate the biomechanical correlates of clinical features of mTBI using a highly anatomically detailed 3D model of TBI biomechanics. This allows for the comparison of strain and strain rate magnitude and distribution differences between groups in a voxelwise manner, providing results with greater spatial resolution. Using videos of 81 impacts leading to a mTBI in professional American football, I investigate differences in the magnitude and distribution of brain deformation from impacts that lead to either LOC, posturing or produce no visible neurological signs. I test the hypotheses that: a) impacts leading to LOC will be associated with higher strains and strain rates across the brain; b) these forces will be particular high within the brainstem and thalamus in impacts leading to LOC; c) impacts leading to posturing will be associated with high

strain in the motor cortex and/or corticospinal tracts and; d) impacts leading to LOC will be associated with higher rotational acceleration than those that did not.

6.3 Methods

Case selection and video analysis.

Case selection, video analysis and physical reconstructions were undertaken by collaborators at the University of Ottawa as part of a previous study (Cournoyer and Hoshizaki, 2019b). 1,280 elite American football games were reviewed to identify cases with mTBI and LOC, posturing or no visible signs (Figure 23A). Cases were verified by associated press releases from by the team's medical personnel to confirm the player involved had a mild TBI. LOC was defined as impacts leading to a player displaying a complete loss of motor tone or a failure to protect themselves during the ensuing fall. Posturing was defined as impacts leading to flexion or extension in the upper or lower extremities in any combination. In cases where the events could not be confirmed, cases were excluded from the study. Control impacts were defined as mTBI without producing any visible signs on video including ataxia. An event matched (e.g., a fall, or shoulder/helmet to helmet collision) control impact was selected at random per LOC and posturing case from a subset of event matched control cases. In total, 20 LOC cases, 21 posturing cases and 41 impacts leading to no visible signs (No Signs cases) were included in this study. Return to play duration was defined as when the players were returned to a game, or were allowed full contact as stated in a press release on the internet. Players were grouped into either fewer than 14 days required for return to play, or 14 days or more for subgroup analysis, in line with definitions for persistent post-traumatic symptoms in adults (McCrory et al., 2017).

Laboratory reconstructions

The methodology involved in video analysis and laboratory reconstructions of head impacts has been previously described in detail (Karton et al., 2020, Cournoyer and Hoshizaki, 2019a) and within Chapter 5. In brief, videos were screened for suitability of physical reconstruction, requiring a clear view of the impact and distinguishable pitch markings to allow for calibration of distances on video to known pitch dimensions.

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Kinovea (version 0.8.20) was used to calculate impact velocity by determining the distance between the injured players head and impacting surface 0.04-0.2 seconds prior to impact (Figure 23B). Head location/orientation and event type was also recorded from video footage.

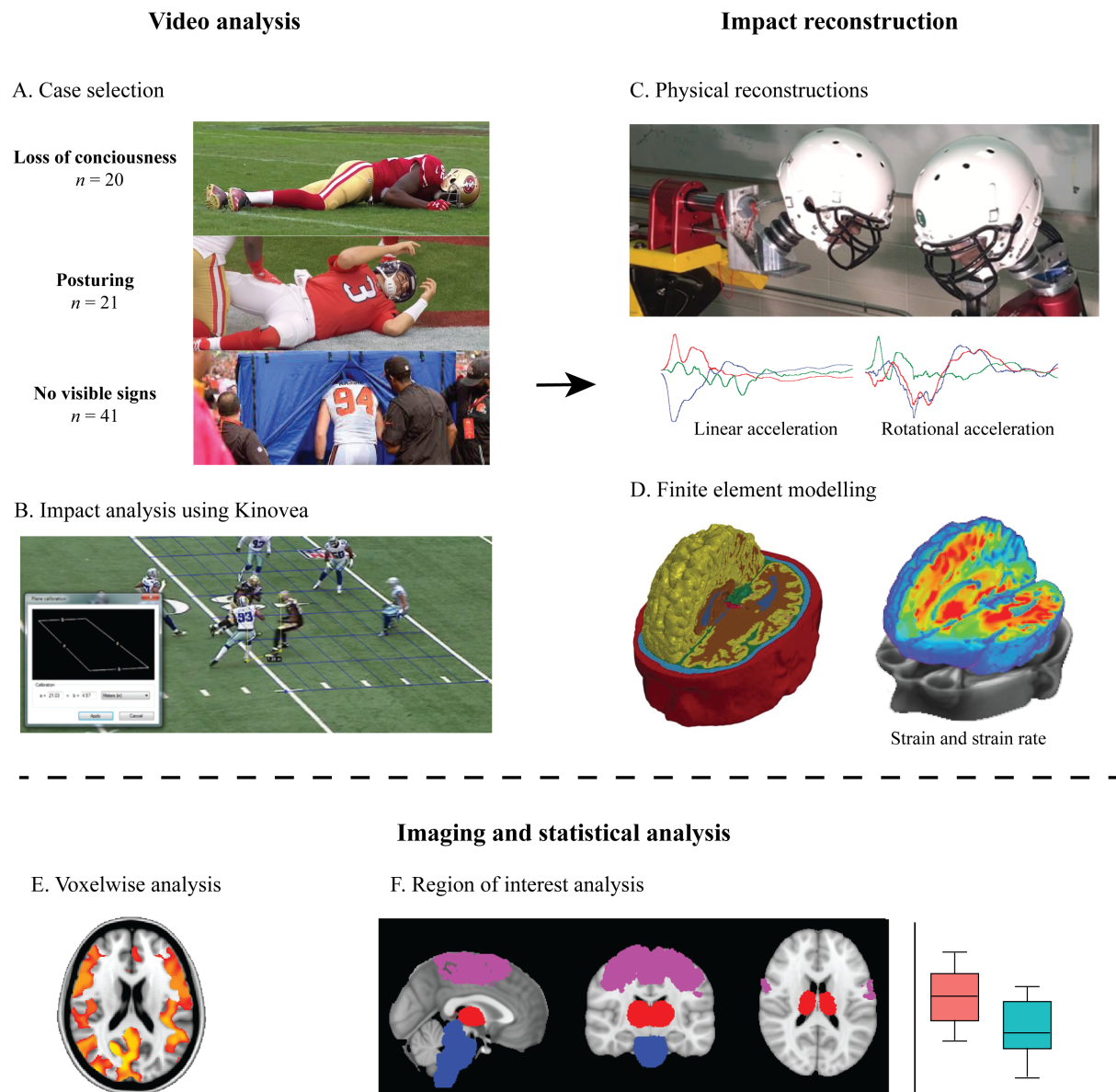


Figure 23. Methods and workflow.

Videos of professional American football games were analysed to select cases of (A) Players who lost consciousness, experienced dystonic posturing or did not show any visible signs of injury but was later diagnosed by a team's medical professional. (B) Impacts were analysed using Kinovea and (C) physically reconstructed in a lab to estimate the acceleration of the head during the impact. (D) Information on head acceleration is then used as input for Finite Element Modelling of the biomechanics

of the injury, producing metrics such as strain and strain rate. (E) Biomechanical metrics were then analysed using neuroimaging techniques such as voxelwise analyses and (F) region of interest analysis, focusing on the brainstem, thalamus and motor cortex.

These parameters were used to guide physical reconstructions of the head impact events using a Hybrid III 50th percentile adult male headform and neutral unbiased neckform (Walsh et al., 2018) (Figure 23C). Mounted accelerometers were used to capture linear and rotational acceleration of the head during an impact. The headform was equipped with an American football helmet with matching liners based on helmets worn at the time of impact. Helmet to helmet collisions were simulated using a 15.9 kg impacting arm wearing an American football helmet. In cases of shoulder to helmet impacts, a 13 kg impact arm with an American football shoulder pad was used. Ascertainment of head kinematics were performed by researchers blinded to the neurological events sustained during impact.

Finite element model and injury simulations

A 3D finite element model of the human head developed at Imperial College London was used to calculate brain deformations from each head impact. This model has been previously described and characterised in Chapter 5.3, with material characteristics, development details and validation described in previous work (Ghajari et al., 2017, Zimmerman et al., 2021a). The finite element head model was loaded by linear and rotational accelerations obtained from the physical reconstructions of the impacts (Figure 23D). For each element of the model, I determined the maximum principal value of the Green-Lagrange strain tensor (EE), a measure of how much the element has been stretched with respect to its original shape (hereafter labelled as strain). I also calculate the highest value of the total time derivative of the Green-Lagrange strain tensor that the element experienced during the simulation, which is a measure of the maximum time rate of stretch within each element (hereafter labelled as the strain rate) (Holzapfel, 2000). Values for strain and strain rate were encoded in an voxelwise basis and saved as a NIFTI (Neuroimaging Informatics Technology

Initiative) file for neuroimaging analysis. One posturing impact was discarded due to simulation errors.

Injury model image processing and analysis

Strain and strain rate data for each impact were analysed using fMRIB Software Library (FSL) v6.0.2. Whole brain measures of strain and strain rates, called 90th Percentile Strain and Strain Rate, were produced using the 90th percentile value of strain and strain rate of all voxels across the brain. This was done to account for possible abnormally large strains in poorly defined elements, and provides a summary measure of the peak strain and strain rate values reached across the whole brain during an impact. Strain and strain rate images were merged and means calculated at each voxel across all impacts per neurological event type. Voxelwise analysis was conducted using the general linear model with non-parametric permutation testing (10,000) in FSL Randomise (Winkler et al., 2014), and results reported are corrected for multiple comparisons at a threshold of $p < 0.05$ (Figure 23E). A secondary voxelwise analysis was repeated but with 90th percentile strain and strain rates used as covariates to investigate proportional spatial differences. Region of interest analysis was conducted after registering strain and strain rate images to MNI152 space, and sampling from within masks of the thalamus, brainstem, motor cortex (Figure 23F) and within white matter tracts such as the corpus callosum and corticospinal tracts. Thalamus and brainstem masks were derived from the Harvard-Oxford brain atlas (Frazier et al., 2005) while the motor cortex atlas was derived from the primary and pre-motor masks from the Jülich histological atlas (Eickhoff et al., 2007).

Statistical analysis

Statistical tests were completed in the open source software package R. Peak resultant linear and rotational acceleration and velocities were calculated from accelerations and velocities in the x, y and z plane. Head kinematics, whole brain deformation measures and region of interest analysis of strain and strain rates were compared using ANOVAs (one level, three factors) with Tukey post-hoc t-tests. Voxelwise imaging statistical analysis to test whether there were differences between

impacts leading to different neurological events was used as described above. Group differences in impact kinematics and brain deformation between players with return to play durations greater or less than 14 days long were investigated using T-tests. The influence of head impact location and posturing lateralization was compared using a Chi-squared test.

6.4 Results

Study participants and classification

Eighty-one impacts were reconstructed and simulated. Twenty of which were classified as causing LOC, 20 as causing dystonic posturing and 41 with no visible neurological signs produced (Table 15). All impacts were reported to have caused a mild TBI as declared by their team's medical officials. Physical reconstructions of the impacts produced a number of head kinematic measurements which were combined to produce peak resultant linear and rotational acceleration. Kinematics were used as input for the FEM of TBI to produce brain deformation metrics including strain and strain rate. Across the group, all impact categories were well matched for impact direction, with the highest number of impacts occurring to the left side of the head. In impacts that led to posturing, the majority of posturing observed was bilateral ($n = 21$) compared to unilateral ($n = 8$), and more impacts were from the left side ($n = 12$) compared to right ($n = 6$). There was no relationship between the side of the head impacted (left versus right) and the lateralisation of the upper limb posturing response (unilateral on the left versus unilateral on the right). There were no differences in the proportion of individuals who had a return to play duration less than 14 days or greater than 14 days between the groups.

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Table 15. Subject demographics and injury metrics

Data are presented as mean, (sd) unless stated otherwise. One-way ANOVAs have been reported testing the effect of neurological event on metric. Post-hoc t-tests are provided, with comparisons made between LOC and Posturing with No visible signs.

	Loss of consciousness, mTBI (n = 20)	Posturing, mTBI (n = 21)	No visible signs, mTBI (n = 41)
Kinematic description			
Peak Resultant Linear Acceleration	0.80 (0.39)	0.62 (0.29)	0.47 (0.21)
F = 9.1, P<0.001			
Post hoc t-test	P<0.001	ns	na
Peak Resultant Rotational Acceleration	0.0059 (0.0024)	0.0049 (0.0020)	0.0035 (0.0016)
F = 11.2, P<0.001			
Post hoc t-test	P<0.001	P = 0.045	na
Brain deformation			
90 th Percentile Strain	0.11 (0.076)	0.084 (0.068)	0.053 (0.034)
F = 7.1, P = 0.0014			
Post hoc t-test	P = 0.0013	ns	na
90 th Percentile Strain Rate	34 (12)	31 (10)	24 (10)
F = 6.9, P = 0.0018			
Post hoc t-test	P = 0.0026	P = 0.046	na
Impact location (N)			
Back	0	1	2
Front	3	1	5
Left	9	12	19
Right	8	6	15
Posturing Lateralisation (N)			
Right	NA	3	NA
Left	NA	5	NA
Bilateral	NA	21	NA
Return to play duration (N)			
=> 14 days	5	6	19
< 14 days	10	8	21

Examples of case reconstructions and measures can be found in Figure 24. I investigated the thalamus and brainstem as regions of interest, along with areas of the motor cortex responsible for upper limb function, supplementary motor area, and white matter tracts such as the corticospinal tracts, thalamic radiation and corpus callosum. Case study 1 (Figure 24A) is a player who was struck towards the front of the head, around 15-50 degrees off centre on the right, in a head to head impact and lost consciousness. This is reflected in the acceleration profile of the impact, with high negative linear acceleration in the x-axis (forward and back) and high levels of strain, with strain exceeding 1.5 times the group IQRs in all but two regions of interests. Case study 2 (Figure 24B) was a player who was struck on the left side of the head in a head to head impact and displayed dystonic posturing. This is reflected in the negative linear acceleration in the y-axis (right to left) which resulted in strain above the IQR relative to the other impacts in all areas. Lastly, case study 3 focuses on a player who sustained a mild TBI during a head to head impact on the left side of the head, but did not display any visible signs at the time. This is again reflected by the negative linear acceleration in the y-axis. The player experienced average to below average strains during the impact in all areas assessed.

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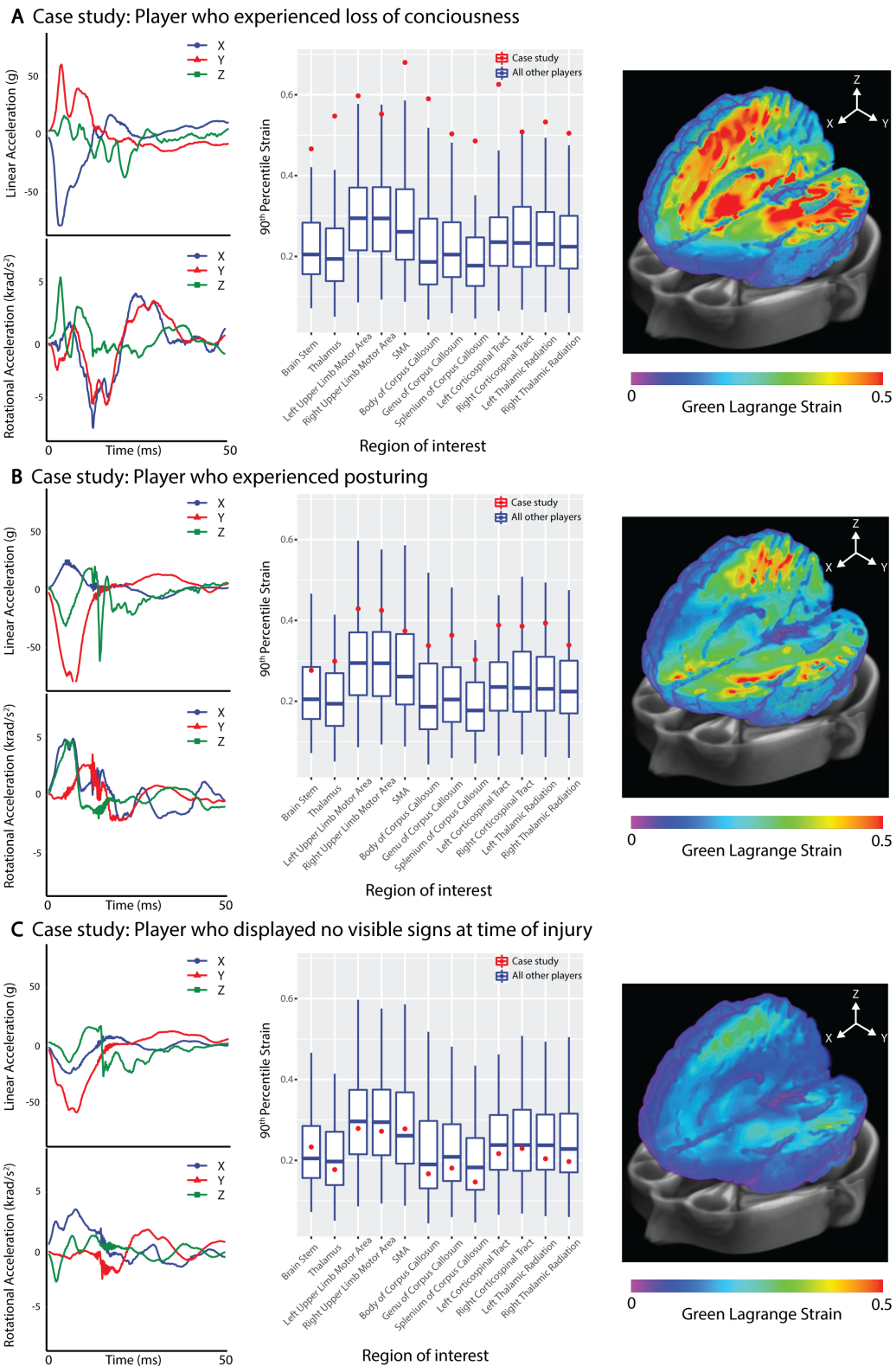


Figure 24. Case studies of injuries with different clinical features

Case studies are presented with kinematics (linear and rotational acceleration – left), 90th percentile strain within regions of interest (middle) and strain distribution (right). Presented are (A) A player who lost consciousness, (B) experienced posturing and

(C) did not show any visible signs of injury but was later diagnosed by a team's medical professional. The individual is the red dot, and plotted against the values in all other players. The solid lines within the boxes are the median, with the borders indicating the IQR and whiskers extending to 1.5 times the IQR. *SMA = Supplementary motor area*

Head kinematics is highest in impacts leading to loss of consciousness

I assessed if there were differences in the head kinematic metrics (rotational and linear acceleration and velocity) during impacts between the player groups. Using a one way ANOVA with three levels (per neurological event), I found a significant effect of neurological event on peak resultant linear ($F = 9.1$, $P < 0.001$), peak resultant rotational acceleration ($F = 11.2$, $P < 0.001$), peak resultant linear velocity ($F_{2,78} = 4.5$, $P = 0.014$) and peak resultant rotational velocity ($F_{2,78} = 4.4$, $P = 0.015$) (Figure 25A). These effects were primarily driven by differences between the LOC group and no visible signs group, with impacts leading to LOC having higher linear acceleration ($P < 0.001$), rotational acceleration ($P < 0.001$), linear velocity ($P = 0.0113$) and rotational velocity ($P = 0.0183$). Impacts leading to dystonic posturing also had higher rotational acceleration than impacts leading to no visible signs ($P = 0.045$).

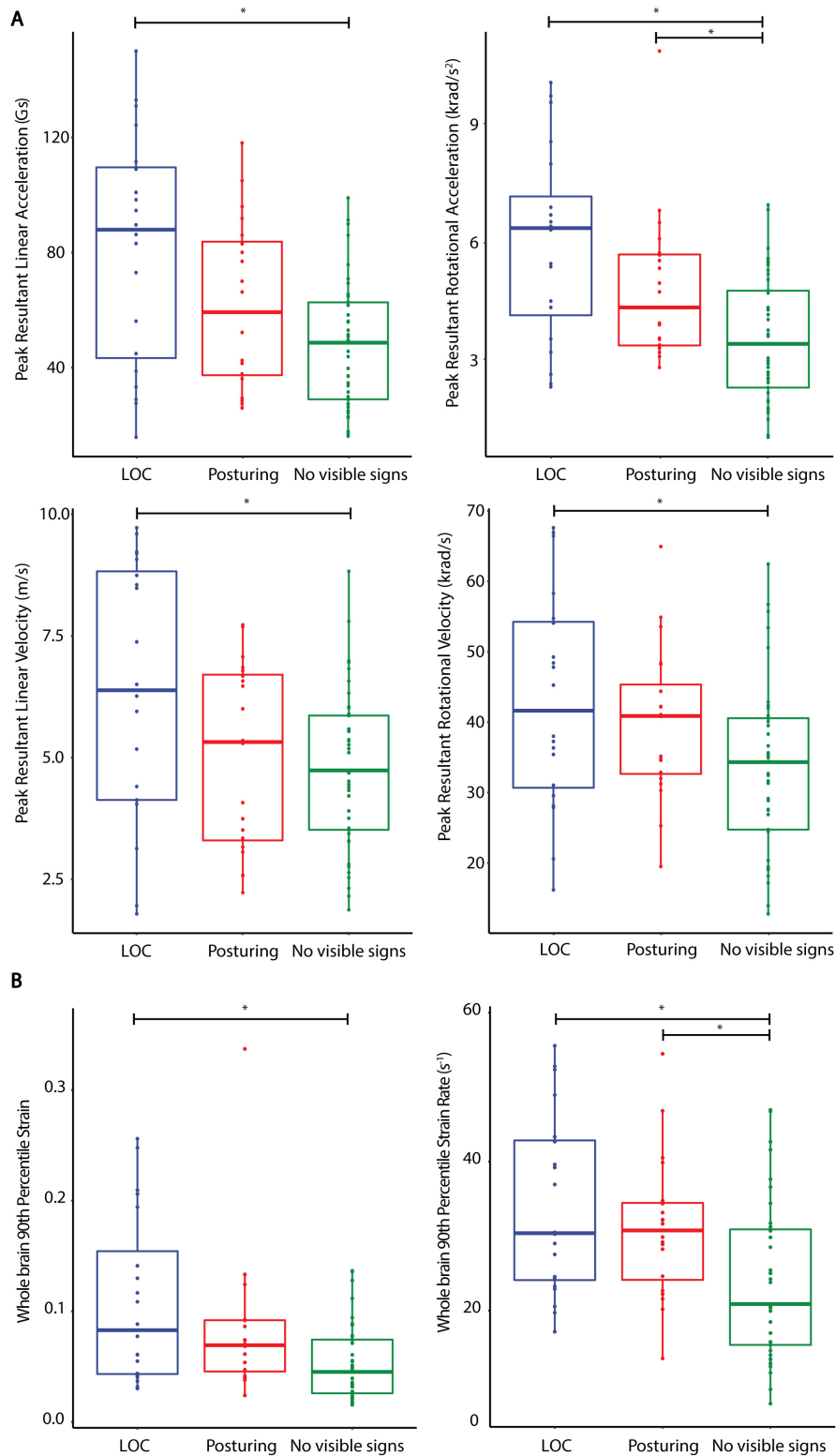


Figure 25. Kinematics and biomechanics of impacts leading to different clinical features

(A) Plots of rotational and linear acceleration and velocity, (B) strain and strain rates for impacts leading to LOC (blue), posturing (red) or no visible signs (green). The solid lines within the boxes are the median, with the borders indicating the IQR and whiskers extending to 1.5 times the IQR.

Brain strain and strain rate are also highest in impacts leading to loss of consciousness

Kinematics were input into the FEM to estimate brain deformation metrics during the impact. Whole brain deformation was assessed using the 90th percentile strains and strain rates across the whole brain. In a similar fashion to head kinematics, there was a significant effect of clinical presentation on 90th percentile strain ($F = 7.1$, $P = 0.0014$) and 90th percentile strain rate ($F = 6.9$, $P = 0.0018$) (Figure 25B). Impacts leading to LOC had significantly higher 90th percentile strain ($P = 0.0013$) and strain rate ($P = 0.0026$) than impacts with no visible signs. Strain rates were also higher in the Posturing group compared to the no visible signs group ($P = 0.046$).

These effects were also present upon visual inspection of mean strain and strain rate maps highlighting the distribution of strain and strain rate across the brain. Higher mean strains (Figure 26A) and strain rates (Figure 26B) across the group are observable for head injury events with loss of consciousness compared to events where there were no visible clinical features produced. High strain is concentrated within the depths of the sulci for all three event types in a roughly symmetrical fashion. In contrast, the sulcal concentration is less obvious for strain rate, and a lateralised effect on strain rate can be seen in posturing cases (Figure 26Bii).

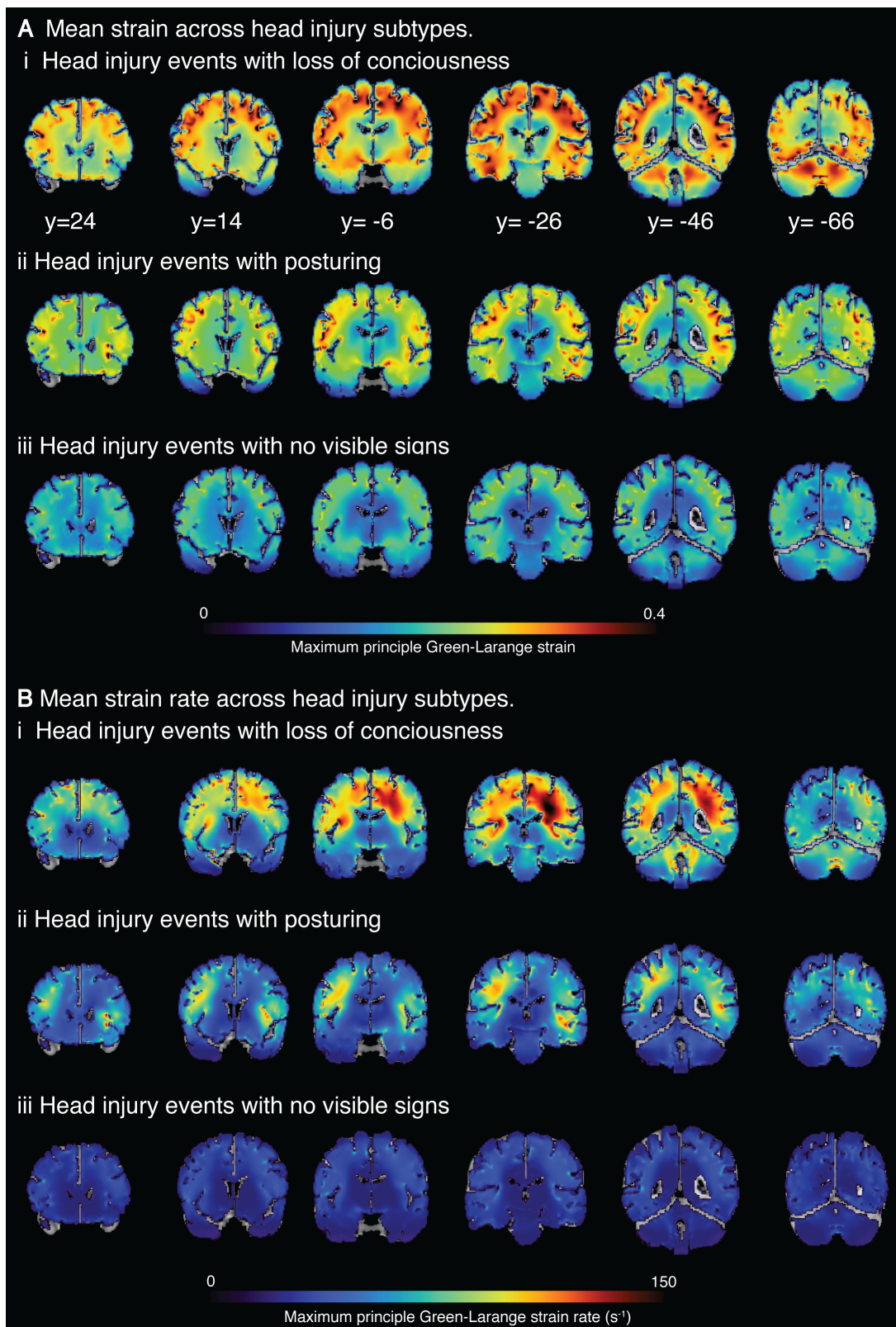


Figure 26. Spatial distribution of strain, strain rate across head injury subtypes.

(**A**) Mean strain and (**B**) strain rates, calculated per voxel, for each head injury subtype. Y coordinates are given in Montreal Neurological Institute (MNI) space.

Voxelwise analysis shows higher strain in impacts leading to LOC and posturing.

I assessed strain in a voxelwise manner to investigate if differences in whole brain strain were due to peaks in specific areas of the brain. A voxelwise comparison demonstrated that the higher strain in impacts leading to LOC compared to those with no visible signs (No Signs) were present across large portions of the brain, including the brainstem, cerebellum and cortical regions, but not in the frontal lobe (Figure 27A). This was consistent with a region of interest analysis, where significant effects of group on strain were found in the brainstem ($F = 7.6$, $P = 0.001$), motor cortex ($F = 7.7$, $P < 0.001$) and thalamus ($F = 8.5$, $P < 0.001$) which were driven by higher strain in impacts leading to LOC compared to No Signs (brainstem, $P < 0.001$; motor cortex, $P < 0.001$; thalamus, $P < 0.001$, Table 16). In contrast, voxelwise analysis revealed that in impacts leading to posturing, areas of higher strain compared to players with No Signs were restricted to the left temporal lobe and right motor cortical regions, with no differences in strain in the cerebellum or brainstem (Figure 27B). There were no regions of the brain where impacts leading to LOC had higher strain than impacts leading to posturing, or vice versa.

Table 16. Region of interest analysis split by neurological event

	Region of interest - strain (mean, sd)			Region of interest - strain rate (mean, sd)		
	Brainstem	Motor cortex	Thalamus	Brainstem	Motor cortex	Thalamus
LOC	0.176 (0.063)	0.256 (0.092)	0.209 (0.097)	5.54 (3.51)	7.8 (4.77)	5.45 (4.19)
Posturing	0.150 (0.039)	0.222 (0.061)	0.170 (0.061)	3.64 (1.61)	5.42 (2.95)	3.85 (2.21)
No visible Signs	0.122 (0.051)	0.177 (0.075)	0.128 (0.065)	2.58 (1.44)	3.75 (2.21)	2.63 (1.73)

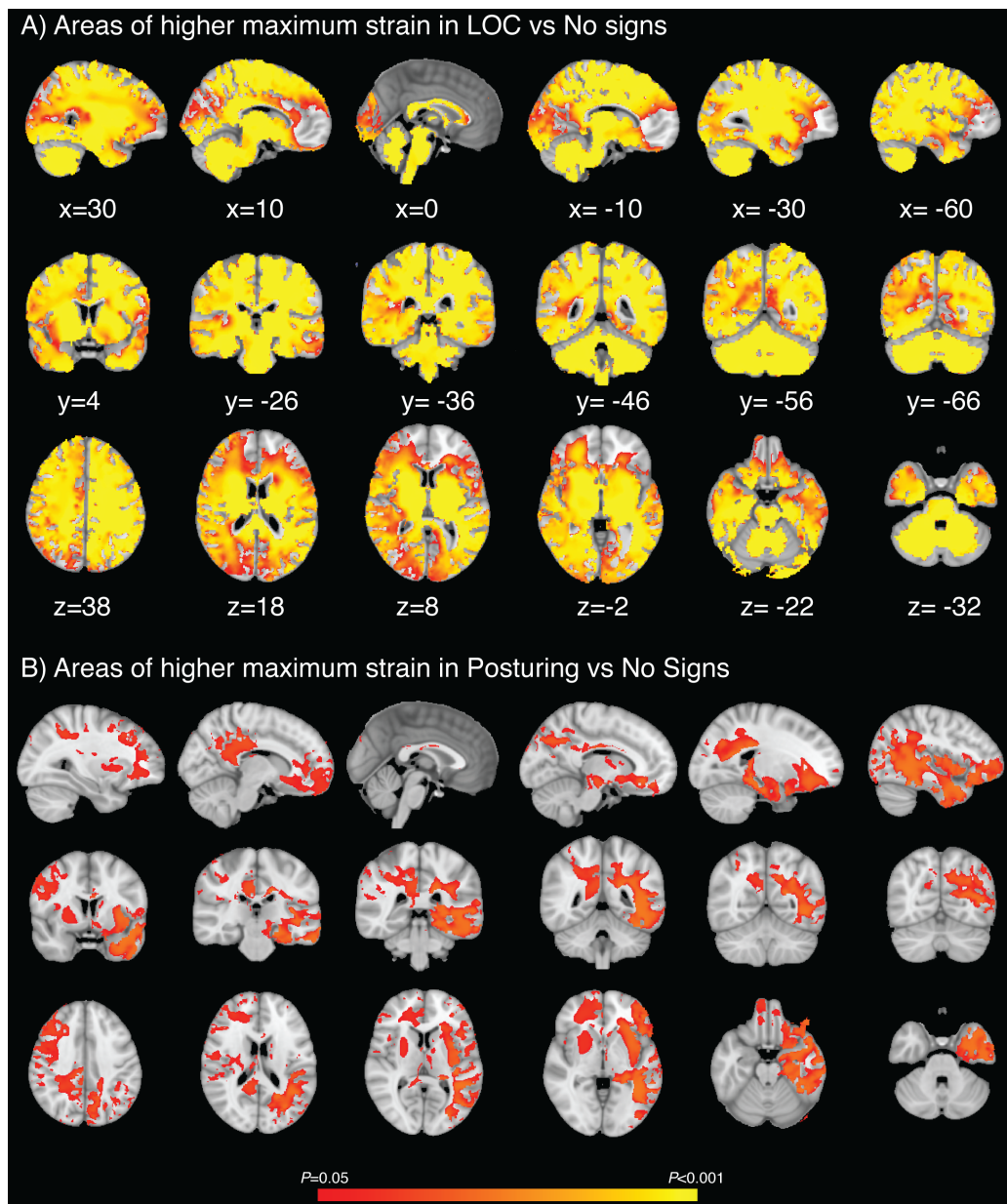


Figure 27. Voxelwise analysis of strain between injury subtypes.

Areas of significantly higher strain present in impacts leading to (A) LOC compared to impacts leading to no visible signs and (B) posturing compared to impacts leading to no visible signs. There were no areas where strain was lower in LOC, Posturing compared to No Signs or areas of significant difference between LOC and Posturing. X, Y & Z coordinates are given in Montreal Neurological Institute (MNI) space.

Voxelwise analysis shows higher strain rates in cerebellum for impacts leading to LOC compared to posturing.

Strain rates were also assessed in a voxelwise manner. Higher strain rates in impacts leading to LOC compared to those with no visible signs (No Signs) were present in

similar areas of the brain where higher strain was observed, also excluding the frontal lobe (Figure 28A). Strain rates were also higher in impacts leading to posturing compared to players with No Signs, but in a more widespread manner than for strain. Regions with higher strain rates included the left temporal lobe and cerebellum, parts of the brainstem, parietal lobes bilaterally and right frontal lobe (Figure 28B). Additionally, impacts leading to LOC also had higher strain rates in the cerebellar white matter and periaqueductal grey compared with impacts leading to Posturing, but not in areas of the brainstem or cortical regions (Figure 28C).

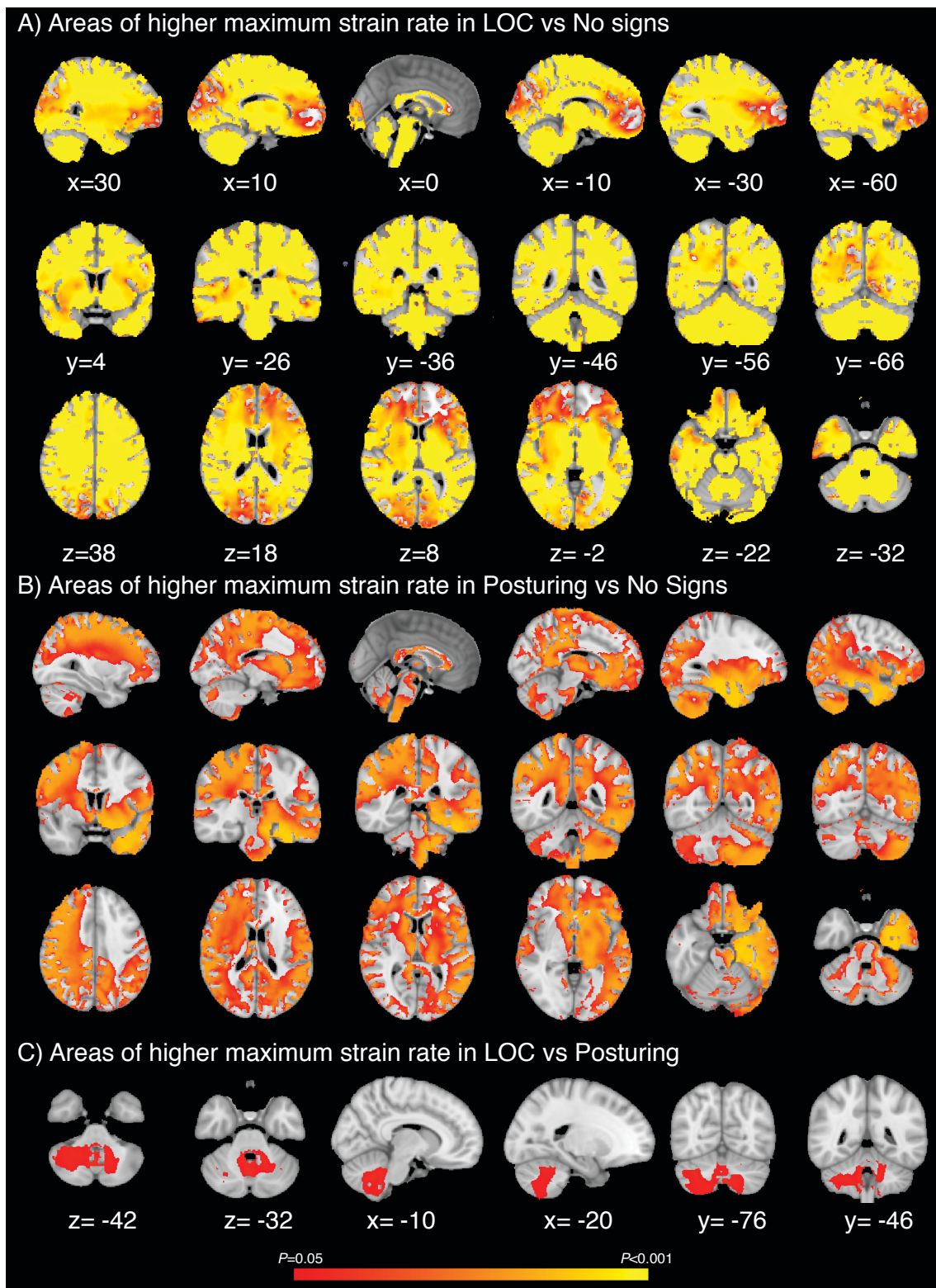


Figure 28. Voxelwise analysis of strain rate between injury subtypes.

Areas of significantly higher strain rate present in impacts leading to (A) LOC compared to impacts leading to no visible signs and (B) posturing compared to impacts leading to no visible signs. (C) Higher strain rates are present in impacts leading LOC compared to posturing. X, Y & Z coordinates are given in Montreal Neurological Institute (MNI) space.

Impacts leading to LOC had disproportionally high strain rates in the brainstem and cerebellum compared to impacts with no signs.

The spatial strain and strain rate differences observed may only be due to the differences in impact severity. For example, it is possible that the differences in strain between impacts leading to LOC or 'No Signs' are only a reflection of more biomechanically severe impacts, rather than a specific phenomenon associated with a particular neurological event. Therefore, to account for the effects of biomechanical impact severity, I included whole brain 90th percentile strain as a covariate. This allowed investigation of spatial differences where high strain was located regardless of the overall magnitude of strain.

In impacts leading to LOC, proportionally higher strain rates were located in the cerebellum, pons and medulla of the brainstem and small areas of the motor cortex compared to impacts with no signs (Figure 29A). Proportionally higher strain rates were also observed in impacts leading to LOC compared to impacts leading to Posturing, primarily in the same areas of cerebellum and brainstem, including a small area of the midbrain, along with the left motor cortex and right temporal lobe (Figure 29B). There were large regions of overlap in the cerebellum where strain rate was higher in impacts leading to LOC compared to both impacts leading to Posturing and No Signs, whereas the areas of higher strain rates were only present in large areas of the motor cortex for LOC compared to Posturing impacts (Figure 29C). There were no differences in proportional strain between any of the groups, or differences in strain rates observed between impacts leading to Posturing and No signs. There were no regions where strain rate was disproportionally higher in impacts leading to No signs, or for Posturing impacts compared to LOC.

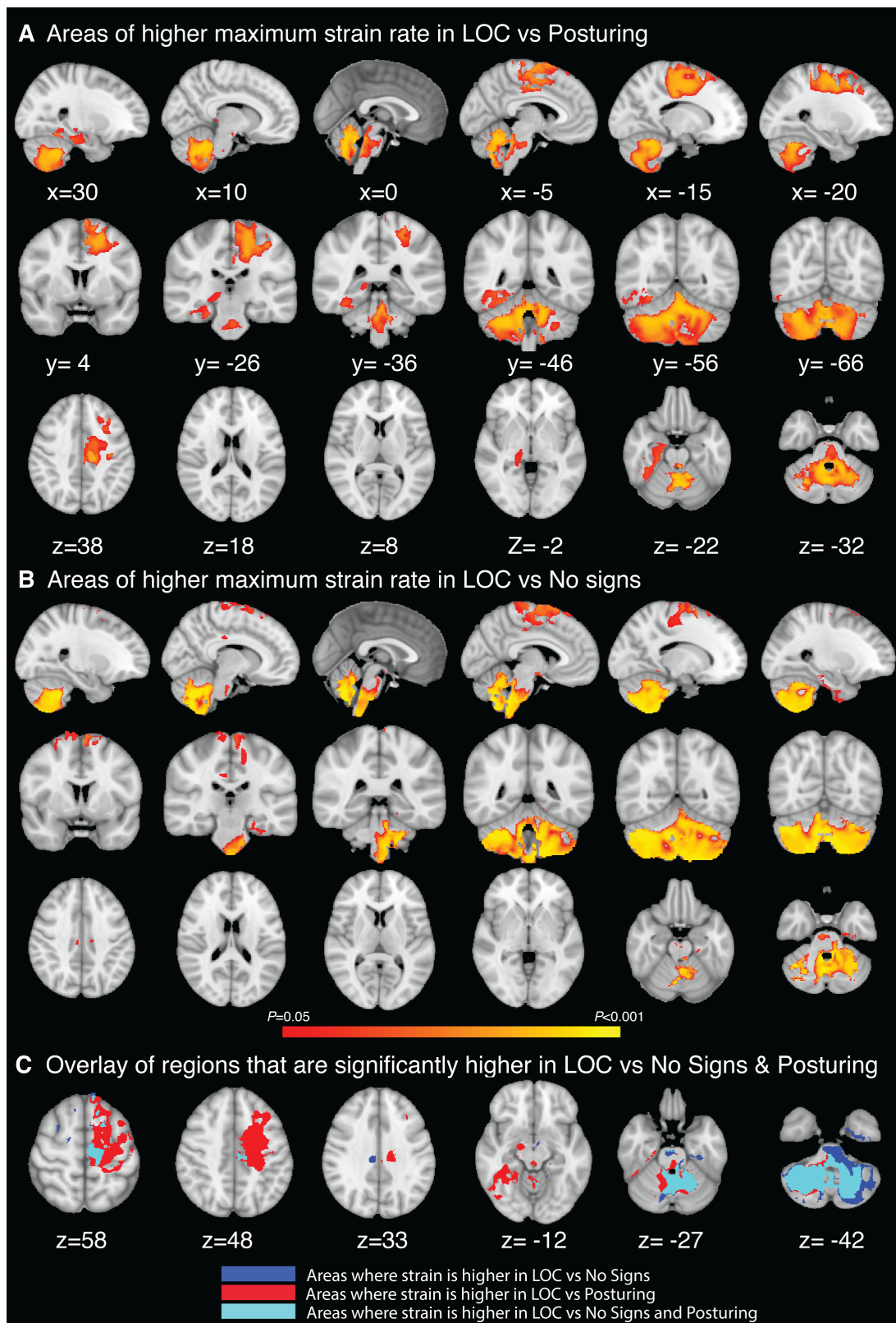


Figure 29. Areas of disproportionately high strain rates across injury subtypes.

A voxelwise analysis taking into account 90th percentile whole brain strain rate shows areas of disproportionately higher strain rate in impacts leading to (A) LOC compared to impacts leading to no visible signs and (B) LOC compared to impacts

leading to posturing. (C) An overlay of the regions show substantial overlap. X, Y & Z coordinates are given in Montreal Neurological Institute (MNI) space.

Brain deformation and clinical measures

I assessed if brain deformation metrics were related to return to play duration, which was the only clinical measures we had available. Comparing peak resultant rotational and linear acceleration, and whole brain 90th percentile strain and strain rates, there were no significant differences between players who had a return to play duration greater than 14 days ($n = 30$) and less than 14 days ($n = 29$) in all measures tested (Table 17).

Table 17. Return to play duration: kinematic and brain biomechanical measures

Results are in mean (sd). RTP = return to play duration

	Peak resultant linear acceleration	Peak resultant rotational acceleration	Whole brain 90th percentile strain	Whole brain 90th percentile strain rate
RTP less than 14 days	0.603 (0.308)	0.0046 (0.0022)	0.0695 (0.053)	26.14 (11.7)
RTP greater than 14 days	0.551 (0.311)	0.0042 (0.0022)	0.0723 (0.066)	27.7 (11.5)

6.5 Discussion

I present my findings investigating impacts leading to an mTBI. I show that there are differences in the magnitude and distribution of forces within the brain depending on the neurological events occurring at the time of injury. I use FE modelling to investigate the strain and strain rates within the brain a short time after impact. Higher strain and strain rates were observed across large areas of the brain in players who lost consciousness compared to players who showed no visible signs. When accounting for the higher brain wide strain and strain rate in players with LOC, strain rates, but not strain, were higher in the cerebellum, brainstem and small areas of the motor

cortex in impacts leading to LOC compared to impacts leading to mTBI with no visible signs. Strain rates were also disproportionately higher in impacts leading to LOC compared to those leading to Posturing in the same areas of cerebellum and brainstem, along with the left motor cortex and right temporal lobe. These results suggest that areas of brainstem, and possibly cerebellum, are structures involved in maintaining consciousness.

Measures of whole brain strain and strain rates were highest in impacts leading to LOC. Using a voxelwise analysis, I show that the higher strain and strain rates were not restricted to a single area but appeared to be an effect across large areas of the brain, excluding the frontal lobe. These findings replicate previous results using a different FE model, where strain was higher in all regions of interest tested for LOC cases (Cournoyer and Hoshizaki, 2019b). I also found evidence of higher strain rates for impacts leading to LOC in the cerebellar white matter and periaqueductal grey compared to impacts that led to Posturing, also replicating findings using a different FEM (Cournoyer and Hoshizaki, 2019a). Cerebellar structures are not closely implicated in maintaining consciousness (Lemon and Edgley, 2010), although the periaqueductal grey is thought to coordinate states of tonic immobility in response to trauma that are associated with altered states of consciousness (Terpou et al., 2019).

These spatial results may have only been features of more biomechanically severe impacts, of which a majority lead to LOC, rather than features specific to the neurological event itself. To account for this, and test whether strains and strain rates were proportionally higher in certain areas of the brain in impacts leading to different neurological events, I used the 90th percentile strain/strain rate as a covariate in the voxelwise statistical model. This allowed me to compare strain/strain rates across the groups while accounting for individual differences in maximum strain/strain rates within an individual. The results, therefore reflect spatially where strain/strain rate is higher regardless of the overall magnitude of strain/strain rates. I show that in LOC cases, strain rates were disproportionately higher in the pons, medulla and cerebellum compared to both impacts leading to no visible signs and posturing. These regions,

which are largely overlapping when comparing LOC to either the No Signs or Posturing impacts, may be key to maintaining consciousness. Lesions to the pons, and specifically the pontine tegmentum, have been shown to cause coma even in absence of damage to the midbrain or thalamus (Parvizi and Damasio, 2003, Rohaut et al., 2019, Sandhu et al., 2019, Hindman et al., 2018, Fischer et al., 2016). High strain rates in this area may therefore induce temporary disruption to neural activity key to maintaining arousal. Furthermore, these results further implicate the cerebellar structures highlighted when whole brain strain rates were not accounted for.

In comparison to strain rate, proportional strain was not significantly different between the groups. Furthermore, uncorrected strain rates, but not strain, were significantly higher in the cerebellum for impacts leading to LOC compared to those leading to no visible signs. It is unclear why the differences in strain rates are not replicated by strain. Both strain and strain rates are required to be above certain thresholds to induce cell death (Morrison et al., 2003, Elkin and Morrison, 2007) and both biomechanical measures correlate with imaging and histopathological measures of axonal injury (Donat et al., 2021). My results suggest that strain rates, rather than strain, is more critical to disrupting function on a temporary basis, with unknown significance in relation to longer term damage. The differences between the measures and the implication on pathology should be explored further, for example by using techniques employed in fMRI studies to delineate the time component of strain distribution, and by considering the interaction of both strain and strain rates in producing pathology.

I also assessed cases which led to posturing, an abnormal motor response characterised by 'fencing', where flexor and/or extensor behaviour is observed in the upper limbs. Cases with posturing also produced higher strains and strain rates compared to impacts producing no signs. Voxelwise analysis showed that strain and strain rates were higher in the temporal lobe and parts of the cerebral cortex in a lateralised fashion, overlapping the motor cortex. This likely contributes to the production of a 'fencing' response, as high levels of strain within the motor cortex and other cortical regions can cause a dissociation in cortical and brainstem activity

(McCrory and Berkovic, 1998), and specifically motor dysfunction by disrupting activity in the motor cortex. The lateralisation was also present when comparing the impacts leading to LOC and Posturing, whereby the areas of higher biomechanical forces present in Posturing cases compared to No Signs were not significantly different to the distribution of forces during impacts that led to LOC. The lateralisation of strain and strain rates observed was likely due to the larger proportion of impacts coming from the left (66%) than right (33%), although this was not explicitly tested. The location of impacts did not appear to influence whether posturing was unilateral on the left or right, however this analysis was limited by the small number of unilateral posturing cases as most displayed forms of posturing in both upper limbs.

Higher strain and strain rates are likely a result of the higher accelerations and velocities of impacts leading to LOC, which I also observed, in particular rotational kinematics which are known to be highly correlated to whole brain measures of strain and strain rates (Zimmerman et al., 2021a, Gabler et al., 2016, Knowles and Dennison, 2017). These results therefore support suggestions that impacts leading to LOC may be more severe. High strain and strain rates have been related to both grey and white matter injuries (Bain and Meaney, 2000, Donat et al., 2021, Elkin and Morrison, 2007), with white matter tracts particularly vulnerable to mechanical loading (Hill et al., 2016). In cases of mild TBI, LOC has also been shown to be related to injury imaging measures of white matter damage (Wilde et al., 2016). LOC at the time of injury may therefore reflect a higher risk of white matter damage, although in this study the majority of impacts leading to LOC remained below the defined optimal strain threshold of 0.21 for axonal injury (Bain and Meaney, 2000). Future work should be done to assess the predictive value of rotational acceleration in predicting LOC and posturing in a larger sample size.

I visually inspected the distribution of the mean strain and strain rates for the 3 subgroups across the brain. In keeping with findings from Chapter 5, strains and strain rates appeared to concentrate in the depths of sulci near the cortical surface. Interestingly, strain and strain rates appear lowest in midline structures, including the

corpus callosum, despite these being sites of pathology (Johnson et al., 2013b) and imaging abnormalities (see Chapters 3 & 4). This contradiction may have several causes. Firstly, impacts are limited to 50ms in duration, which may limit the propagation of forces inside the model to midline structures. Additionally, directionality of the white matter tracts are not included in the model, with grey and white matter being given the same properties, therefore affecting how strain and strain rates are modelled in these midline white matter tracts. Future analysis should investigate the white matter in isolation, assessing if midline structures sustain higher strain and strain rates than peripheral white matter tracts, in order to assess the validity of the modelling by comparing strain and strain rates to real-world pathology.

There are some limitations to our study. I have previously described the limitations of the video analysis and impact reconstruction methods in Chapter 5.5 and in other publications (Ghajari et al., 2017, Karton et al., 2020, Zimmerman et al., 2021a, Cournoyer and Hoshizaki, 2019a). The classification of players having experienced a mild TBI was based on press releases from the teams' medical officials. While guidance is available, the diagnosis is entirely dependent on the interpretation of the medical professionals involved, and therefore the severity of injury may vary across players. The sample size limited my ability to look at posturing lateralisation, which could be expanded by investigating if unilateral posturing cases, for example of primarily limbs on the left, involved strain/strain rates confined to the left/right side of the brain. I was also limited in accessing further clinical data, which would have allowed for further investigation into whether cases with higher strain or strain rates produced more symptoms and could be defined as more 'severe'. The physical reconstruction methods and computational modelling did not take into account physiological differences that may influence kinematics and brain deformations. Future studies should take into account the possibility of using the pipeline for generating the FE model on an individualised basis from subject MRI scans, which could be combined with advanced MRI such as diffusion tensor imaging of subjects to look at measures of white matter injury (Jolly et al., 2021).

6.6 Conclusions

In summary, I present results showing that impacts leading to LOC involve higher accelerations and velocities of the head, which in turn lead to higher levels of strain and strain rates within the brain. These forces appear to be widespread, but are disproportionately higher in areas of the cerebellum, brainstem and thalamus compared to cases where there are no visible signs of injury. This is in contrast to impacts leading to posturing, which involved higher strain and strain rates in cortical regions including the motor cortex. These results show that brain biomechanics may be informative in predicting acute outcomes after head injury, and provide evidence that loss of consciousness may indicate a more severe injury. This work also presents a novel way of investigating brain biomechanics by using conventional neuroimaging statistical tools, the combination of which should be exploited further in future research.

Chapter 7.

Discussion

7.1 Summary of findings

The aim of this thesis was to explore various techniques that could be used to assess the severity of a TBI and predict patient outcomes. I used a combination of advanced MRI, fluid biomarker sampling and computational modelling of brain biomechanics to produce measures that appeared relevant to TBI related pathology and long-term outcomes. I focused on mild TBIs and sports related head injuries in the later chapters, due to the particular problems faced in predicting outcomes after mTBI and where the question of the risks involved from repetitive head impacts are uncertain.

Axonal injury is closely related to clinical outcomes (Sidaros et al., 2008). However, validated methods to quantify axonal injury are lacking in a clinical context. In Chapter 3, I assessed the diffusion MRI metric, FA, and plasma NfL as measures of diffuse axonal injury (DAI) in a cohort of moderate-severe TBI patients. I found that FA is reduced, and is correlated with plasma NfL concentrations which are elevated after injury. I show that plasma NfL is closely related to NfL concentrations sampled from brain extracellular fluid directly from damaged white matter. Furthermore, both FA and plasma NfL were correlated with histopathological measures of axonal injury in an experimental model of injury. Both measures predicted patient outcomes at 6 and 12 months' post-injury. This work supports the hypothesis that FA and plasma NfL can accurately reflect the presence of axonal injury in a clinically relevant fashion.

Head injuries in the context of participation in elite collision sports are a growing concern due to an increasing body of evidence associating mTBI and repetitive head impacts with persistent neurological deficits. In Chapter 4, I assess if participation in elite rugby is associated with DAI and brain atrophy. I used the MRI techniques shown to be sensitive to DAI from Chapter 3, and additionally more advanced diffusion MRI modelling and volumetric analysis to assess brain structure. Applying an individualised diffusion MRI analysis technique, I show that there is evidence of DAI in elite rugby players in both those with and without a sub-acute injury. Unexpectedly, I also found evidence of an abnormal trajectory for white matter volume change, with half of the players studied showing evidence of white matter volume reduction. Alongside recent animal modelling studies (Tagge et al., 2018), these results provide further evidence

that neuropathology can occur after exposure to head impacts without producing obvious symptoms.

I then sought to study if the biomechanics of head impacts could prove to be informative in predicting neuropathology. In Chapter 5, I investigated the biomechanical pattern of impacts in American football irrespective of a diagnosis of mild TBI. Using a computational model of the brain, I simulated a pool of representative impacts that players of different positions would experience during typical play. I show that in all cases, the biomechanical forces were higher in the sulci, compared to gyri, co-localising with where CTE pathology is observed and replicating our previous pilot results (Ghajari et al., 2017). I also show that player positions, which influence the type of repetitive head impact profile a player would fall under, have an effect on the biomechanics of impacts experienced. Importantly, players exposed to impacts with high rotational accelerations have a higher magnitude of biomechanical forces within the brain and sulci.

In Chapter 6, I investigate if head impact biomechanics are related to acute symptoms that can present after a mild TBI. I studied professional American footballers who had a mild TBI captured on video, with players either losing consciousness, displaying a posturing response or displayed no visible signs of injury but were later diagnosed. I show this using similar impact reconstruction techniques used in Chapter 5, but with a novel application of neuroimaging statistical analysis methods, namely, that during these impacts, strain rates are particularly high in the brainstem and cerebellum compared to impacts that led to either posturing or no visible signs. Furthermore, impacts that cause a loss of consciousness were associated with higher biomechanical forces across the whole brain. This data supported the hypothesis that the presence of loss of consciousness after a mild TBI may indicate a more severe injury and provides further evidence that biomechanics can be informative in predicting early and late clinical features of TBI.

7.2 Interpretation, implications and limitations

These findings support the breadth of literature suggesting axonal injury is a key determinant of patient long-term outcomes after TBI, and suggests that we can measure this sensitively in different ways. Plasma NfL and individualised diffusion MRI analysis both appear useful as diagnostic tools in a clinical setting, however further investigation is needed to determine the optimal conditions for using either tool and if both measures are required.

Our data indicates that plasma NfL appears to be a relatively early biomarker of axonal damage, peaking at around 20 days post injury in moderate-severe TBI patients. While this has not yet been validated in studies of mild TBI, which either stop sampling before or skip over this time period (Neselius et al., 2013, Shahim et al., 2018, Laverse et al., 2020), assessing plasma NfL at this time can provide greater utility in detecting the presence of axonal injury over navigating unspecific symptoms. Elevations in NfL appear to be a specific increase related to head trauma, as while patients with non-TBI trauma also show increased concentrations, this was still markedly lower than TBI patients. Furthermore, plasma sampling appears to reflect concentrations from extracellular fluid from damaged white matter, alleviating the need for more invasive investigations such as lumbar puncture.

In contrast, diffusion MRI appears to be more sensitive to axonal injury in more chronic stages after trauma. An individualised analyses of diffusion MRI from a sub-sample of the patients scanned subacutely from Chapter 3 highlighted increased sensitivity to axonal injury at 6 months compared to data from their subacute scan (Jolly et al., 2021). White matter FA has been shown to increase acutely and subsequently reduce in the presence of longer-term damage (Eierud et al., 2014, Wilde et al., 2008, Croall et al., 2014). This can mean individuals with injuries in the presence of additional pre-existing longer-term damage may present as ‘normal’ on subacute diffusion imaging, perhaps explaining why we did not observe differences between controls and subacutely injured rugby players (Chapter 4). Diffusion MRI appeared to be most sensitive to the accumulation of axonal injury over a career. Therefore, diffusion MRI as an axonal injury test may be more appropriate at more chronic stages after injury.

A further consideration is whether there is redundancy in undertaking both these investigations. Plasma NfL concentrations and white matter FA are correlated, and both show the highest predictive power in determining patient outcomes at 6 and 12 months. However, it has yet to be shown whether using both biomarkers adds to a model's ability to predict patient outcomes, or if they outperform current models such as the IMPACT model (Steyerberg et al., 2008). This remains an aim of the BIO-AX-TBI study from which Chapter 3's data is derived from (Graham et al., 2020a). The outcome of these investigations should recommend what combination of measures, assessed at what times, highlight patients who will need additional resources for long-term care and/or would be ideal targets for interventions.

Plasma NfL and white matter FA also appear to be informative in predicting neurodegeneration after TBI. Patterns of axonal injury measured by FA predict neurodegeneration in the chronic phase post-injury (Graham et al., 2020b), a relationship we replicated in moderate-severe TBI patients (Graham et al., 2021), but not in the elite rugby players. We also show in the same study of moderate-severe TBI patients that peak plasma NfL predicted late white matter neurodegeneration 6 to 12 months post-injury (Graham et al., 2021). This is not entirely surprising as axonal injury has been shown to lead to neuronal loss over time (Povlishock and Katz, 2005) and trauma to axons can help contribute to the development of proteinopathies such as hyperphosphorylated tau (Tagge et al., 2018). NfL is already widely used as a biomarker in neurodegenerative disease (Gaetani et al., 2019) and has been shown to correlate with atrophy rates in a spectrum of diseases (Johnson et al., 2018, Rohrer et al., 2016, Barro et al., 2018). Hence, chronically raised NfL or a reduction in FA over time could function as an early warning sign that an individual may be at higher risk of developing a neurodegenerative disease.

These investigations would be best placed within a framework of longitudinal assessments available for individuals or athletes who are identified as being at risk of neurodegenerative diseases. This framework would also facilitate the analysis of brain volume changes over time. I highlighted the utility of quantifying atrophy in elite rugby

players, and found unexpected abnormalities in the trajectory of white matter volume in some players. Early detection of abnormal trajectories in volume change using repeated structural MRI may help detect an increased risk of neurodegeneration, although we have yet to quantifying this relationship on an individualised level. This can be useful clinically. For example, CTE, a form of dementia currently with one of the highest profile in the sporting community, can only be diagnosed at post-mortem (McKee et al., 2016). The unknown risk can perpetuate anxiety over post-traumatic symptoms that may be unrelated or treatable, but are not properly investigated (Sharp and Jenkins, 2015). Incorporating established measures such as plasma NfL sampling and structural MRI in a framework of longitudinal monitoring can help with the precise diagnosis of the underlying cause of post-traumatic symptoms and future disease risk.

Moving away from relying on initial injury classification and clinical symptoms is particularly relevant to the impact of repetitive head impacts on brain health. It is becoming increasingly clear that head impacts, including those that do not produce detectable clinical symptoms, and of an unknown frequency, can produce neuropathology (Smith et al., 2013, Tagge et al., 2018, McKee et al., 2009). My work supports this; I show that participation in elite rugby, regardless of 'diagnosed' injuries, is associated with evidence of axonal injury and abnormal white matter volume changes. These signs of underlying damage are present regardless of the risk of producing initial clinical symptoms, and impact events that don't produce clinical symptoms may still be relevant to long term pathology. While we currently rely on the presence of clinical signs or abnormalities on conventional CT/MRI for a diagnosis of TBI, establishing and using more sensitive measures of underlying damage, such as quantitative fluid biomarkers or advanced imaging, can help quantify the damage caused by head impacts. This could be implemented for example over the course of a season of sports participation and change the conception of what defines an injury. Whether these measures are currently suitable and sensitive enough to the types of damage relevant to longer-term neuropathology has yet to be established.

Another approach to quantifying injury severity is to study the forces at the time of impact in the brain. Impact biomechanics provides a quantitative measure of impact

severity which appears to be sensitive to the types of clinical symptoms appearing at the time of injury (Chapter 6), and to immediate primary injury pathology (Donat et al., 2021). Understanding the biomechanical thresholds for injury would be an important development in both identification and prevention of TBI, particularly in a sporting context. I show that American footballers in positions most at risk of an mTBI, as defined using clinical symptoms, are exposed to impacts with a higher biomechanical load (Chapter 5). While players in these positions have not been shown to have an increased risk of CTE (Mez et al., 2020), evidence suggests there are effects of position in sport on dementia risk (Lehman et al., 2012, Russell et al., 2021), likely via positional head impact exposure differences. These findings mirror results from an experimental injury study indicating that high biomechanical forces are related to the production of mTBI-related clinical symptoms, aptly termed ‘concussion-like deficits’, but were not required to produce CTE-like brain pathology. This would indicate that while measures of the impact biomechanics may be useful in predicting the onset of acute clinical symptoms in isolation, these measures do not necessarily map onto the risk of long-term neuropathology alone.

These points highlight two key issues. Firstly, the limitations and problems of current methods of classifying and diagnosing TBI inherently limit our ability to predict or calculate thresholds for TBI. Studies of relationships between biomechanics and TBI are inherently reliant on the ‘correct’ diagnosis of injury. These have been more successful at predicting pathology relevant to immediate mortality and have driven the success of helmet designs. However, current biomechanics literature that focuses on preventing milder TBIs tends to rely on a diagnosis of concussion/mTBI made by medical staff based on a clinical assessment of whether symptoms are present (Rowson and Duma, 2013, Takhounts et al., 2013, Zhang et al., 2004). This means that current thresholds in place, for example for assessing helmet safety, are designed on the basis of preventing head injuries that produce immediate symptoms, but are not optimally designed or tested for their ability to prevent axonal injury or reduce the risks of longer term pathology.

Secondly, the relationship between biomechanics and pathology is complex and likely to be influenced by factors specific to the individual which are not accounted for. Within this thesis, I replicate previous findings (Ghajari et al., 2017) that biomechanical forces are higher in the sulci and where white matter injuries are prevalent in a TBI population. The behaviour of biomechanical forces within the brain during impact may therefore provide insight into why we observe accumulation of tau pathology perivascularly and around the base of the sulci. However, biomechanics alone are likely not to be able to predict the development of long-term neuropathology on an individualised basis. Genetic risk is likely to be important; for example, individuals with a genetic predisposition to Alzheimer's disease suffer poorer outcomes after mild TBI (Hayes et al., 2017). Furthermore, individual factors, alongside an interaction with exposure to biomechanical forces over time, are also likely to influence the ability to clear abnormal proteins from the brain. Incorporating genome sequencing to assess polymorphisms across genes of interest, and studying how these factors, alongside impact frequency and magnitudes, are likely to provide a clearer picture of how biomechanics can influence long-term neuropathology.

Future directions

I have highlighted the benefits of incorporating quantitative measures such as plasma NfL and DTI FA in the stratification of patients after TBI. There is an opportunity to use these measures as specific outcomes to test early interventions such as in clinical trials or in the development of helmet testing thresholds. Future investigations should aim to better our understanding of how impact biomechanics are related to clinically relevant pathology such as axonal injury as measured by plasma NfL or DTI FA. I initially planned to do this, but was limited by the number of cases where high quality videos and either plasma sampling or detailed MRI follow-ups were available. However, the landscape is changing rapidly, and it is only a matter of time before this type of analysis is possible. The availability of high quality video footage of road traffic accidents, or sports related injuries are increasing year on year, making it easier to reconstruct impacts where more clinical information, fluid sampling and neuroimaging will be available.

This thesis presents a multi-disciplinary approach to investigating TBI, involving collaborations across clinicians and neurologists with engineers. This collaborative approach benefits both the clinical and biomechanical disciplines and presents opportunities in both fields. In particular, the framework of neuroimaging analysis pipelines being applied to both the creation and analysis of finite element models can facilitate developing models based on an individual level, if scans are available, where we can account for age, gender and size differences. Furthermore, the novel application of neuroimaging statistical analysis methods presented in Chapter 6 highlights a possible method for investigating how the temporal pattern of both the magnitude and spatial location of biomechanical forces affect pathology.

Concluding remarks

Traumatic brain injury is a complex, heterogeneous disease with dynamic processes that can have implications for health over a lifetime. The stratification of patients who present with a TBI in acute care settings is essential for providing optimal care, however the current labels provide unrealistic and unhelpful expectations of longer term prospects. Current classifications can be improved upon by incorporating quantitative measures of injury severity that are relevant to longer-term clinical outcomes, for example diffusion MRI and fluid biomarkers.

In professional sports, where athletes are exposed to repetitive head impacts organising bodies have a duty of care to players to inform them of the risks of participating. It is increasingly evident that impacts that don't produce immediate clinical symptoms are not necessarily harmless. A framework of longitudinal assessments of player health should be considered to provide players with an informed perspective of their current and future brain health, alongside further research to understand the implications of repetitive head impacts. At the amateur or youth level, the health benefits of participating in collision sports need to be weighed up against the risk of long-term brain damage, which have yet to be studied in detail.

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Appendix

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