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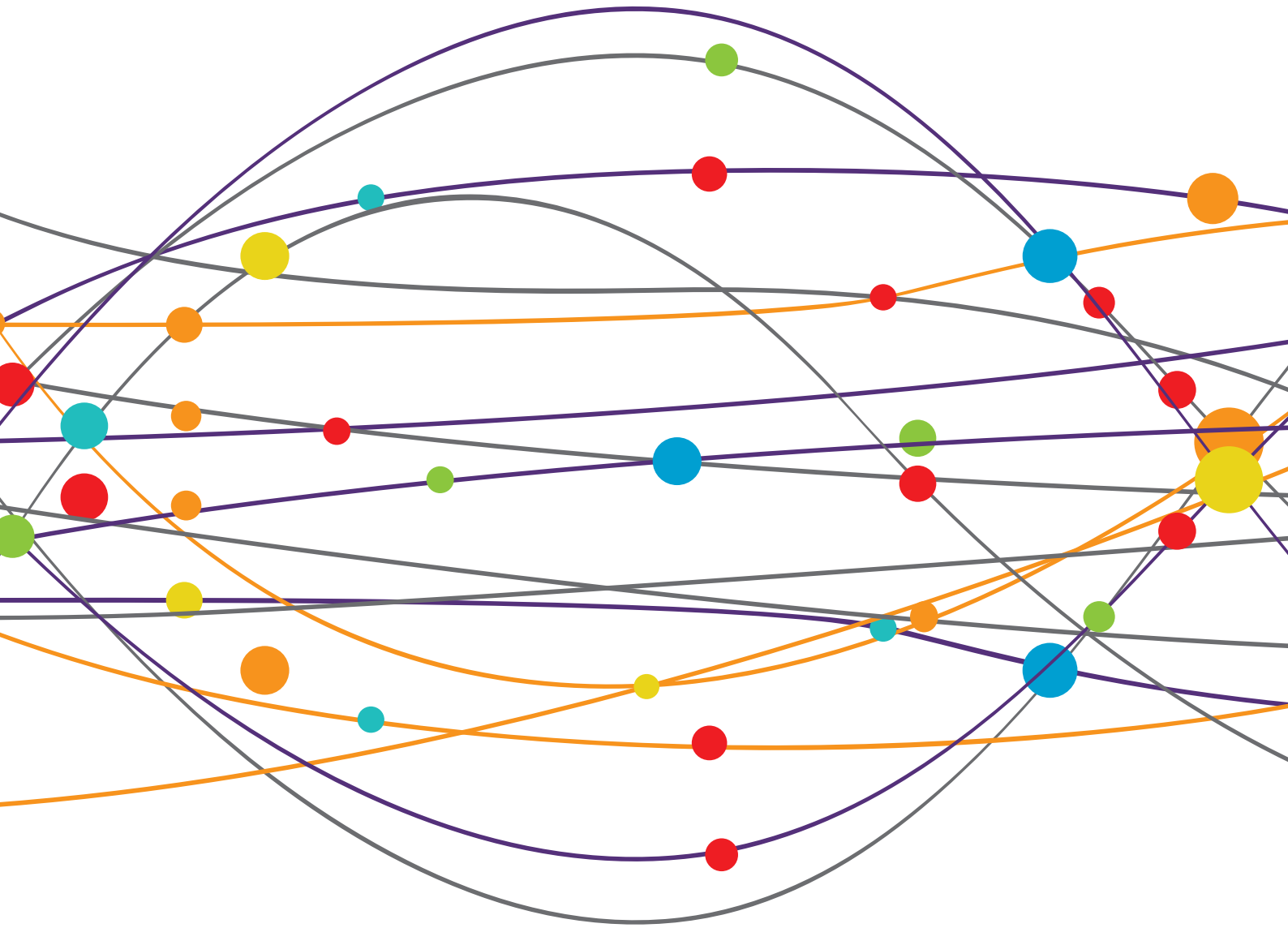

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REVIEW
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Traumatic Spinal Cord Injury: An Overview of Pathophysiology, Models and Acute Injury Mechanisms

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Traumatic spinal cord injury (SCI) is a life changing neurological condition with substantial socioeconomic implications for patients and their care-givers. Recent advances in medical management of SCI has significantly improved diagnosis, stabilization, survival rate and well-being of SCI patients. However, there has been small progress on treatment options for improving the neurological outcomes of SCI patients. This incremental success mainly reflects the complexity of SCI pathophysiology and the diverse biochemical and physiological changes that occur in the injured spinal cord. Therefore, in the past few decades, considerable efforts have been made by SCI researchers to elucidate the pathophysiology of SCI and unravel the underlying cellular and molecular mechanisms of tissue degeneration and repair in the injured spinal cord. To this end, a number of preclinical animal and injury models have been developed to more closely recapitulate the primary and secondary injury processes of SCI. In this review, we will provide a comprehensive overview of the recent advances in our understanding of the pathophysiology of SCI. We will also discuss the neurological outcomes of human SCI and the available experimental model systems that have been employed to identify SCI mechanisms and develop therapeutic strategies for this condition.

Keywords: spinal cord injury, secondary injury mechanisms, clinical classifications and demography, animal models, glial and immune response, glial scar, chondroitin sulfate proteoglycans (CSPGs), cell death

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INTRODUCTION

Spinal cord injury (SCI) is a debilitating neurological condition with tremendous socioeconomic impact on affected individuals and the health care system. According to the National Spinal Cord Injury Statistical Center, there are 12,500 new cases of SCI each year in North America (1). Etiologically, more than 90% of SCI cases are traumatic and caused by incidences such as traffic accidents, violence, sports or falls (2). There is a reported male-to-female ratio of 2:1 for SCI, which happens more frequently in adults compared to children (2). Demographically, men are mostly affected during their early and late adulthood (3rd and 8th decades of life) (2), while women are at higher risk during their adolescence (15–19 years) and 7th decade of their lives (2). The age distribution is bimodal, with a first peak involving young adults and a second peak involving adults over the age of 60 (3). Adults older than 60 years of age whom suffer SCI have considerably worse outcomes than younger patients, and their injuries usually result from falls and age-related bony changes (1).

The clinical outcomes of SCI depend on the severity and location of the lesion and may include partial or complete loss of sensory and/or motor function below the level of injury. Lower thoracic lesions can cause paraplegia while lesions at cervical level are associated with quadriplegia (4). SCI typically affects the cervical level of the spinal cord (50%) with the single most common level affected being C5 (1). Other injuries include the thoracic level (35%) and lumbar region (11%). With recent advancements in medical procedures and patient care, SCI patients often survive these traumatic injuries and live for decades after the initial injury (5). Reports on the clinical outcomes of patients who suffered SCI between 1955 and 2006 in Australia demonstrated that survival rates for those suffering from tetraplegia and paraplegia is 91.2 and 95.9%, respectively (5). The 40-year survival rate of these individuals was 47 and 62% for persons with tetraplegia and paraplegia, respectively (5). The life expectancy of SCI patients highly depends on the level of injury and preserved functions. For instance, patients with ASIA Impairment Scale (AIS) grade D who require a wheelchair for daily activities have an estimated 75% of a normal life expectancy, while patients who do not require wheelchair and catheterization can have a higher life expectancy up to 90% of a normal individual (6). Today, the estimated life-time cost of a SCI patient is $2.35 million per patient (1). Therefore, it is critical to unravel the cellular and molecular mechanisms of SCI and develop new effective treatments for this devastating condition. Over the past decades, a wealth of research has been conducted in preclinical and clinical SCI with the hope to find new therapeutic targets for traumatic SCI.

An Overview of Primary Injury

SCI commonly results from a sudden, traumatic impact on the spine that fractures or dislocates vertebrae. The initial mechanical forces delivered to the spinal cord at the time of injury is known as primary injury where "displaced bone fragments, disc materials, and/or ligaments bruise or tear into the spinal cord tissue" (7–9). Notably, most injuries do not completely sever the spinal cord (10). Four main characteristic mechanisms of primary injury have been identified that include: (1) Impact plus persistent compression; (2) Impact alone with transient compression; (3) Distraction; (4) Laceration/transection (8, 11). The most common form of primary injury is impact plus persistent compression, which typically occurs through burst fractures with bone fragments compressing the spinal cord or through fracture-dislocation injuries (8, 12, 13). Impact alone with transient compression is observed less frequently but most commonly in hyperextension injuries (8). Distraction injuries occur when two adjacent vertebrae are pulled apart causing the spinal column to stretch and tear in the axial plane (8, 12). Lastly, laceration and transection injuries can occur through missile injuries, severe dislocations, or sharp bone fragment dislocations and can vary greatly from minor injuries to complete transection (8). There are also distinct differences between the outcomes of SCI in military and civilian cases. Compared to civilian SCI, blast injury is the common cause of SCI in battlefield that usually involves multiple segments of the spinal cord (14). Blast SCI also results in higher severity scores and is associated with longer hospital stays (15). A study on American military personnel, who sustained SCI in a combat zone from 2001 to 2009, showed increased severity and poorer neurological recovery compared to civilian SCI (15). Moreover, lower lumbar burst fractures and lumbosacral dissociation happen more frequently in combat injuries (1). Regardless of the form of primary injury, these forces directly damage ascending and descending pathways in the spinal cord and disrupt blood vessels and cell membranes (11, 16) causing spinal shock, systemic hypotension, vasospasm, ischemia, ionic imbalance, and neurotransmitter accumulation (17). To date, the most effective clinical treatment to limit tissue damage following primary injury is the early surgical decompression (<24 h post-injury) of the injured spinal cord (18, 19). Overall, the extent of the primary injury determines the severity and outcome of SCI (20, 21).

An Overview on Clinical Classification Systems for Spinal Cord Injury

Functional classification of SCI has been developed to establish reproducible scoring systems by which the severity of SCI could be measured, compared, and correlated with the clinical outcomes (20). Generally, SCI can be classified as either complete or incomplete. In complete SCI, neurological assessments show no spared motor or sensory function below the level of injury (4). In the past decades, several scoring systems have been employed for clinical classification of neurological deficits following SCI. The first classification system, "Frankel Grade," was developed by Frankel and colleagues in 1969 (22). They assessed the severity and prognosis of SCI using numerical sensory and motor scales (22). This was a 5-grade system in which Grade A was the most severe SCI with complete loss of sensory and motor function below the level of injury. Grade B represented complete motor loss with preserved sensory function and sacral sparing. Patients in Grade C and D had different degrees of motor function preservation and Grade E represented normal sensory and motor function. The "Frankel Grade" was widely utilized after its publication due to its ease of use. However, lack of clear distinction between Grades C and D and inaccurate categorization of motor improvements in patients over time, led to its replacement by other scoring systems (20).

Other classification methods followed Frankel's system. In 1987, Bracken et al. at Yale University School of Medicine classified motor and sensory functions separately in a 5 and 7-scale systems, respectively (23). However, this scoring system failed to account for sacral function (20). Moreover, integration of motor and sensory classifications was impossible in this system and it was abandoned due to complexity and impracticality in clinical settings (20). Several other scoring systems were developed in 1970' and 1980's by different groups such as Lucas and Ducker at the Maryland Institute for Emergency Medical Services in late 1970's (24), Klose and colleagues at the University of Miami Neuro-spinal Index (UMNI) in early 1980s (25) and Chehrazi and colleagues (Yale Scale) in 1981 (26). These scoring systems also became obsolete due to their disadvantage in evaluation of sacral functions, difficulty of use or discrepancies between their motor and sensory scoring sub-systems (20).

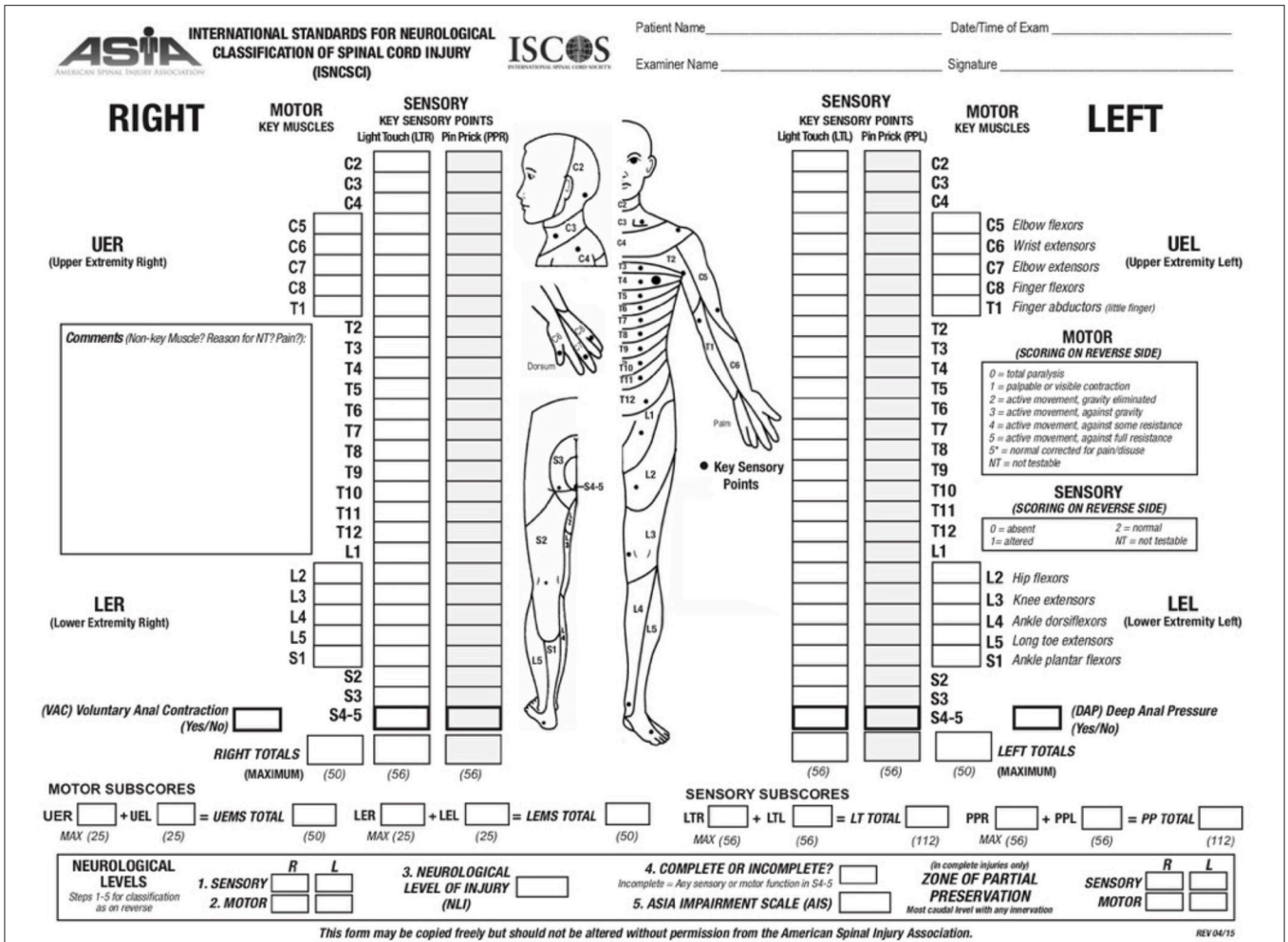

FIGURE 1 | ASIA scoring for the neurological classification of the SCI. A sample scoring sheet used for ASIA scoring in clinical setting is provided (adopted from: http://asia-spinalinjury.org).

American Spinal Injury Association (ASIA) Scoring System

The ASIA scoring system is currently the most widely accepted and employed clinical scoring system for SCI. ASIA was developed in 1984 by the American Spinal Cord Injury Association and has been updated over time to improve its reliability (**Figure 1**). In this system, sensory function is scored from 0–2 and motor function from 0 to 5 (20). The ASIA impairment score (AIS) ranges from complete loss of sensation and movement (AIS = A) to normal neurological function (AIS = E). The first step in ASIA system is to identify the neurological level of injury (NLI). In this assessment, except upper cervical vertebrae that closely overlay the underlying spinal cord segments, the anatomical relationship between the spinal cord segments and their corresponding vertebra is not reciprocally aligned along the adult spinal cord (20). At thoracic and lumbar levels, each vertebra overlays a spinal cord segment one or two levels below and as the result, a T11 vertebral burst fracture results in neurological deficit at and below L1 spinal cord segment. Hence, the neurological level of injury (NLI) is defined as "the most caudal neurological level at which all sensory and motor functions are normal" (20). Upon identifying the NLI, if the injury is complete (AIS = A), "zone of partial preservation" (ZPP) is determined (20). ZPP is defined as all the segments below the NLI that have some preserved sensory or motor function. A precise record of ZPP enables the examiners to distinguish spontaneous from treatment-induced functional recovery, thus, essential for evaluating the therapeutic efficacy of treatments (20). Complete loss of motor and preservation of some sensory functions below the neurological level of the injury is categorized as AIS B (20). If motor function is also partially spared below the level of the injury, AIS score can be C or D (20). The AIS is scored D when the majority of the muscle groups below the level of the injury exhibit strength level of 3 or higher (for more details see **Figure 1**). ASIA classification combines the assessments of motor, sensory and sacral functions, thus addressing the shortcomings of previous scoring systems (20). The validity and reproducibility of ASIA system combined

with its accuracy in prediction of patients' outcome have made it the most accepted and reliable clinical scoring system utilized for neurological classification of SCI (20).

Neurological Outcomes of Spinal Cord Injury

In clinical management of SCI, neurological outcomes are generally determined at 72 h after injury using ASIA scoring system (20, 27). This time-point has shown to provide a more precise assessment of neurological impairments after SCI (28). One important predictor of functional recovery is to determine whether the injury was incomplete or complete. As time passes, SCI patients experience some spontaneous recovery of motor and sensory functions. Most of the functional recovery occurs during the first 3 months and in most cases reaches a plateau by 9 months after injury (20). However, additional recovery may occur up to 12–18 months post-injury (20). Long term outcomes of SCI are closely related to the level of the injury, the severity of the primary injury and progression of secondary injury, which will be discussed in this review.

Depending on the level of SCI, patients experience paraplegia or tetraplegia. Paraplegia is defined as the impairment of sensory or motor function in lower extremities (27, 28). Patients with incomplete paraplegia generally have a good prognosis in regaining locomotor ability (∼76% of patients) within a year (27). Complete paraplegic patients, however, experience limited recovery of lower limb function if their NLI is above T9 (29). An NLI below T9 is associated with 38% chance of regaining some lower extremity function (29). In patients with complete paraplegia, the chance of recovery to an incomplete status is only 4% with only half of these patients regaining bladder and bowel control (29). Tetraplegia is defined as partial or total loss of sensory or motor function in all four limbs. Patients with incomplete tetraplegia will gain better recovery than complete tetra- and paraplegia (30). Unlike complete SCI, recovery from incomplete tetraplegia usually happens at multiple levels below the NLI (20). Patients generally reach a plateau of recovery within 9–12 months after injury (20). Regaining some motor function within the first month after the injury is associated with a better neurological outcome (20). Moreover, appearance of muscle flicker (a series of local involuntary muscle contractions) in the lower extremities is highly associated with recovery of function (31). Patients with complete tetraplegia, often (66–90%) regain function at one level below the injury (28, 30). Importantly, initial muscle strength is an important predictor of functional recovery in these patients (20). Complete tetraplegic patients with cervical SCI can regain antigravity muscle function in 27% of the cases when their initial muscle strength is 0 on a 5-point scale (32). However, the rate of regaining antigravity muscle strength at one caudal level below the injury increases to 97% when the patients have initial muscle strength of 1–2 on a 5-point scale (33).

An association between sensory and motor recovery has been demonstrated in SCI where spontaneous sensory recovery usually follows the pattern of motor recovery (20, 34). Maintenance of pinprick sensation at the zone of partial preservation or in sacral segments has been shown as a reliable predictor of motor recovery (35). One proposed reason for this association is that pinprick fibers in lateral spinothalamic tract travel in proximity of motor fibers in the lateral corticospinal tract, and thus, preservation of sensory fibers can be an indicator of the integrity of motor fiber (20). Diagnosis of an incomplete injury is of great importance and failure to detect sensory preservation at sacral segments results in an inaccurate assessment of prognosis (20).

EXPERIMENTAL MODELS OF SPINAL CORD INJURY

An Overview of Available Animal Models

In the past few decades, various animal models have been developed to allow understanding the complex biomedical mechanisms of SCI and to develop therapeutic strategies for this condition. An ideal animal model should have several characteristics including its relevance to the pathophysiology of human SCI, reproducibility, availability, and its potential to generate various severities of injury (36).

Small rodents are the most frequently employed animals in SCI studies due to their availability, ease of use and cost-effectiveness compared to primates and larger non-primate models of SCI (36, 37). Among rodents, rats more closely mimic pathophysiological, electrophysiological, functional, and morphological features of non-primate and human SCI (38). In rat (39), cat (40), monkey (41), and human SCI (17), a cystic cavity forms in the center of the spinal cord, which is a surrounded by a rim of anatomically preserved white matter. A study by Metz and colleagues compared the functional and anatomical outcomes of rat contusive injuries and human chronic SCI (42). High resolution MRI assessments identified that SCI-induced neuroanatomical changes such as spinal cord atrophy and size of the lesion were significantly correlated with the electrophysiological and functional outcomes in both rat and human contusive injuries (42). Histological assessments in rats also showed a close correlation between the spared white matter and functional preservation following injury (42). These studies provide evidence that rat models of contusive SCI could serve as an adequate model to develop and evaluate the structural and functional benefits of therapeutic strategies for SCI (42).

Mice show different histopathology than human SCI in which the lesion site is filled with dense fibrous connective-like tissue (43–46). Mouse SCI studies show the presence of fibroblast-like cells expressing fibronectin, collagen, CD11b, CD34, CD13, and CD45 within the lesion core of chronic SCI, while it is absent in the injured spinal cord of rats (47). Another key difference between rat and mice SCI is the time-point of inflammatory cell infiltration. While microglia/macrophage infiltration is relatively consistent between rat and mouse models of SCI (47), there is a temporal difference in infiltration of neutrophils and T cells between the two species (47, 48). In SCI rats, infiltration of neutrophils, the first responders, peaks at 6 h post injury, followed by a significant decline at 24–48 h after SCI (48). Similarly, in mouse SCI, neutrophil infiltration occurs within 6 h following injury; however, their numbers continue to rise and do not peak

until 3–14 days post injury (49). T cell infiltration also varies between rat and mouse SCI models (50). In rats, T cell infiltration occurs between 3 and 7 days post injury and declines by 50% in the following 2 weeks (47), whereas in mice, T cell infiltration is not detected until 14 days post injury and their number doubles between 2 and 6 weeks post injury (47). Regardless of their pathophysiological relevance, mice have been used extensively in SCI studies primarily due to the availability of transgenic and mutant mouse models that have allowed uncovering molecular and cellular mechanisms of SCI (38).

In recent years, there has been emerging interest in employment of non-human primates and other larger animals such as pig, dog and cat as intermediate pre-clinical models (51–53) to allow more effective translation of promising treatments from rodent models to human clinical trials (50). Although rodents have served as invaluable models for studying SCI mechanisms and therapeutic development, larger mammals, in particular non-human primates, share a closer size, neuroanatomy, and physiology to humans. Importantly, their larger size provides a more relevant platform for drug development, bioengineering inventions, and electrophysiological and rehabilitation studies. Nonetheless, both small and large animal models of SCI have limitations in their ability to predict the outcome in human SCI. One important factor is high degree of variability in the nature of SCI incidence, severity and location of the injury in human SCI, while in laboratory animal models, these variabilities are less (36). Values acquired by clinical scoring systems such as ASIA or Frankel scoring systems lack the consistency of the data acquired from laboratory settings, which makes the translation of therapeutic interventions from experimental to clinical settings challenging (36). A significant effect from an experimental treatment in consistent laboratory settings may not be reproducible in clinical settings due to high variability and heterogeneity in human populations and their injuries (36). To date, several pharmacological and cellular preclinical discoveries have led to human clinical trials based on their efficacy in improving the outcomes of SCI in small animal models. However, the majority of these trials failed to reproduce the same efficacy in human SCI. Thus, in pre-clinical studies, animal models, and study designs should be carefully chosen to reflect the reality of clinical setting as closely as possible (36). Larger animals provide the opportunity to refine promising therapeutic strategies prior to testing in human SCI; however, their higher cost, need for specialized facilities and small subject (sample) size have limited their use in SCI research (50). Thus, rodents are currently the most commonly employed models for preclinical discoveries and therapeutic development, while the use of larger animals is normally pursued for late stage therapies that have shown efficacy and promise in small animal models. **Table 1** provides a summary of available SCI models.

An Overview of Experimental Models of Spinal Cord Injury

Animal models are also classified based on the type of SCI. The following sections will provide an overview on the available SCI models that are developed based on injury mechanisms, their specifications and relevance to human SCI (**Table 1**).

Transection Models

A complete transection model of SCI is relatively easy to reproduce (51). However, this model is less relevant to human SCI as a complete transection of the spinal cord rarely happens (51). While they do not represent clinical reality of SCI, transection models are specifically suitable for studying axonal regeneration or developing biomaterial scaffolds to bridge the gap between proximal and distal stamps of the severed spinal cord (51). Due to complete disconnection from higher motor centers, this model is also suitable for studying the role of propriospinal motor and sensory circuits in recovery of locomotion following SCI (51, 80). Partial transection models including hemi-section, unilateral transection and dorsal column lesions are other variants of transection models (51). Partial transection models are valuable for investigation of nerve grafting, plasticity and where a comparison between injured and non-injured pathways is needed in the same animal (51). However, these models lead to a less severe injury and higher magnitude of spontaneous recovery rendering them less suitable for development and evaluation of new therapies (51).

Contusive Models

Contusion is caused by a transient physical impact to the spinal cord and is clinically-relevant. There are currently three types of devices that can produce contusion injury in animal models: weight-drop apparatus, electromagnetic impactor, and a recently introduced air gun device (51). The impactor model was first introduced by Gruner at New York University (NYU) in 1992 (81). The original NYU impactor included a metal rod of specific weight (10 g) that could be dropped on the exposed spinal cord from a specific height to induce SCI (51). This model allowed induction of a defined severity of SCI by adjusting the height, which the rod fell on the spinal cord (81). Parameters such as time, velocity at impact and biomechanical response of the tissue can be recorded for analysis and verification (51). The NYU impactor was later renamed to Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor, and conditions surrounding the study and use of the MASCIS impactor were standardized (51). Since its introduction, the MASCIS impactor has been updated twice. The most recent version, MACIS III, was introduced in 2012 and included both electromagnetic control and digital recording of the impact parameters (51). However, inability to control duration of impact and "weight bounce," that could cause multiple impacts, have been known limitations of MASCIS impactors (51).

The Infinite Horizon (IH) impactor is another type of impactor that utilizes a stepping motor to generate force-controlled impact in contrast to free fall in the MASICS impactor (51). This feature allows for better control over the force of impact and prevents "weight bounce" as the computer-controlled metal impounder can be immediately retracted upon transmitting a desired force to the spinal cord (51). IH impactor can be set to different force levels to provide mild, moderate and severe SCI in rats (ex. 100, 150, and 200 kdyn) (51). A limitation

TABLE 1 | Summary of SCI models.

References	Model	Species	Year developed	Mechanism	Pros	Cons
Beattie et al. (54) Constantini et al. (55) Basso et al. (56)	MASCIS	Rodents	Early 1990s	Weight drop (10 g), contusion	Most widely used, impact velocity, compression distance, time, and rate are measurable	Bouncing effect causing double impact, inconsistent results
Scheff et al. (57)	IH Impactor	Rodents	Early 2000s	Controlled contusive impact	No bouncing, Graded injury severity	Learning curve
Stokes (58) Somerson and Stokes (59) Stokes and Somerson (60)	OSU/ESCID*	Rodents	Late 1980s/ Late 1990s	Controlled rapid contusive impact using an electromagnetic vibrator	Controlled displacement, reproducible, no bouncing, more similar to clinical SCI impact, precise, low variability	Complicated device setup, requires testing components, limited technical assistance
Rivlin and Tator (61) Joshi and Fehlings (62)	Clip compression	Rodents	Late 1970s	Modified aneurysm clip compression, compressive and contusive injury	Inexpensive, availability, simplicity, stabilization of spinal cord is not required, can inflict both contusion and compression injuries, different injury severity, clinically relevant	Need for calibration due to the loss of force after repeated use, difficult to reproduce consistent results between different operators, impact parameters not recordable
Marcol et al. (63)	Air-gun impactor	Rats	Early 2010s	Air pressure mediated contusion	Less invasive, no contact	Inconsistency, not validated, unable to produce graded injury severity
Blight (64) Plemel et al. (65)	Forceps compression	Guinea pig, rodents	Early 1990s	Compressive injury by a calibrated forceps	Bilateral compression, simple, inexpensive	Lack of accuracy, lack of contusion and compression, impact parameters not recordable not recordable
Tarlov and Klinger (66) Bao and Liu (67)	Balloon compression	Dogs, rats, primates, rabbits	Early 1950s	Compressive and contusive injury	Easy to perform	Inconsistency, impact parameters not recordable, lacks acute impact
da Costa et al. (68)	Spinal cord strapping	Rats	Late 2000s	Compressive injury using SC-strapper	Non-invasive, does not require laminectomy, graded injury possible, 100% survival rate	Inconsistency, not reproducible, not recordable
Choo et al. (12) Dabney et al. (69) Seifert et al. (70)	Harrington, UBC and UTA distractors	Rats	Early 2000s	Distraction	Resemblance to clinical scenarios	Inconsistency and complexity, not validated
Choo et al. (12) Fiford et al. (71)	Dislocation model	Rats	Early 2000s	Spinal dislocation	Resembles the clinical scenarios, no need for complex surgical procedures	Not validated, inconsistent
Kwon et al. (37) Heimburger (72)	Complete transection	A wide variety of small and large animals	1990s	Complete transection	Reproducible, consistent, easy to perform, useful for studying regeneration	Not clinically relevant
Dyer et al. (73) Seitz et al. (74) Inman et al. (75)	Partial transection	Same as above	1990s	Partial transection	Easier postoperative animal care compared to above, ideal for studying contra and ipsilateral lesions and plasticity	Inconsistency, not precise
Hall and Gregson (76) Dubois-Dalcq et al. (77) Matsushima and Morell (78) Woodruff and Franklin (79)	Chemical models	Rodents	Early 1970s onwards	Reagents such as ethidium bromide, lysolecithin, murine hepatitis virus, cuprizone, myelin specific antibodies and complement	Simple, allows for studying demyelination and remyelination	Inconsistency

ESCID, Electromagnetic SCI Device.

with IH impactors is unreliability of their clamps in holding the spinal column firmly during the impact that can cause inconsistent parenchymal injury and neurological deficits (51).

Ohio State University (OSU) impactor is a computer controlled electromagnetic impactor that was originally invented in 1987 and refined in 1992 to improve reliability (58). As the OSU impactor is electromagnetically controlled, multiple strikes are avoided (51). Subsequently, a modified version of the OSU impactor was developed in 2000 for use in mice (43). However, the OSU impactor is limited by its inability to determine the precise initial contact point with the spinal cord due to displacement of CSF upon loading the device (51). To date, MASCIS, IH and OSU impactor devices have been employed extensively and successfully to induce SCI. These impactor devices are available for small and large animals such as mice, rats, marmosets, cats, and pigs (51, 82).

Compressive Models

Compressive models of SCI have been also employed for several decades (61). While contusion injury is achieved by applying a force for a very brief period (milliseconds), the compression injury consists of an initial contusion for milliseconds followed by a prolonged compression through force application for a longer duration (seconds to minutes) (51). Thus, compression injury can be categorized as contusive-compressive models (51). Various models of compressive SCI are available.

Clip compression is the most commonly used compression model of SCI in rat and mice (51, 61, 62, 83). It was first introduced by Rivlin and Tator in 1978 (61). In this model, following laminectomy, a modified aneurism clip with a calibrated closing force is applied to the spinal cord for a specific duration of time (usually 1 min) to induce a contusive-compressive injury (51). The severity of injury can be calibrated and modified by adjusting the force of the clip and the duration of compression (51). For example, applying a 50 g clip for 1 min typically produces a severe SCI, while a 35 g clip creates a moderate to severe injury with the same duration (83). Aneurysm clips were originally designed for use in rat SCI, however, in recent years smaller and larger clips have been developed to accommodate its use in mice (62) and pig models (52). The clip compression model has several advantages compared to contusion models. This method is less expensive and easier to perform (51). Importantly, in contrast to the impactor injury that contusion is only applied dorsally to the spinal cord, the clip compression model provides contusion and compression simultaneously both dorsally and ventrally. Hence, clip compression model more closely mimics the most common form of human SCI, which is primarily caused by dislocation and burst compression fractures (83). Despite its advantages, clip compression model can create variabilities such as the velocity of closing and actual delivered force that cannot be measured precisely at the time of application (51).

Calibrated forceps compression has been also employed to induce SCI in rodents. This simple and inexpensive compressive model was first utilized in 1991 for induction of SCI in guinea pigs (64). In this method, a calibrated forceps with a spacer is used to compress the spinal cord bilaterally (51). This model lacks the initial impact and contusive injury, which is associated with most cases of human traumatic SCI. Accordingly, this model is not a clinically relevant model for reproducing human SCI pathology and therapeutic development (51).

Balloon Compression model has been also utilized extensively in primates and larger animals such as dogs and cats (84–86). In this model, a catheter with an inflatable balloon is inserted in the epidural or subdural space. The inflation of the balloon with air or saline for a specific duration of time provides the force for induction of SCI (51). Generally, all compression models (clip, forceps, and balloon) have the same limitation as the velocity and amount of force are unmeasurable (51).

In conclusion, while existing animal models do not recapitulate all clinical aspects of human SCI, the compression and contusion models are considered to be the most relevant and commonly employed methods for understanding the secondary injury mechanisms and therapeutic development for SCI.

Overview of Secondary Mechanisms of Spinal Cord Injury

Secondary injury begins within minutes following the initial primary injury and continues for weeks or months causing progressive damage of spinal cord tissue surrounding the lesion site (7). The concept of secondary SCI was first introduced by Allen in 1911 (87). While studying SCI in dogs, he observed that removal of the post traumatic hematomyelia improved neurological outcome. He hypothesized that presence of some "biochemical factors" in the necrotic hemorrhagic lesion causes further damage to the spinal cord (87). The term of secondary injury is still being used in the field and is referred to a series of cellular, molecular and biochemical phenomena that continue to self-destruct spinal cord tissue and impede neurological recovery following SCI (**Figure 2**) (20).

Secondary injury can be temporally divided into acute, sub-acute, and chronic phases. The acute phase begins immediately following SCI and includes vascular damage, ionic imbalance, neurotransmitter accumulation (excitotoxicity), free radical formation, calcium influx, lipid peroxidation, inflammation, edema, and necrotic cell death (7, 20, 88). As the injury progresses, the sub-acute phase of injury begins which involves apoptosis, demyelination of surviving axons, Wallerian degeneration, axonal dieback, matrix remodeling, and evolution of a glial scar around the injury site (**Figure 3**). Further changes occur in the chronic phase of injury including the formation of a cystic cavity, progressive axonal die-back, and maturation of the glial scar (7, 89–92). Here, we will review the key components of acute secondary injury that contribute to the pathophysiology of SCI (**Figures 2**, **3**).

Vascular Injury, Ischemia and Hypoxia

Disruption of spinal cord vascular supply and hypo-perfusion is one of the early consequences of primary injury (93). Hypovolemia and hemodynamic shock in SCI patients due to excessive bleeding and neurogenic shock result in compromised spinal cord perfusion and ischemia (93). Larger vessels such as anterior spinal artery usually remain intact (94, 95), while rupture of smaller intramedullary vessels and capillaries that

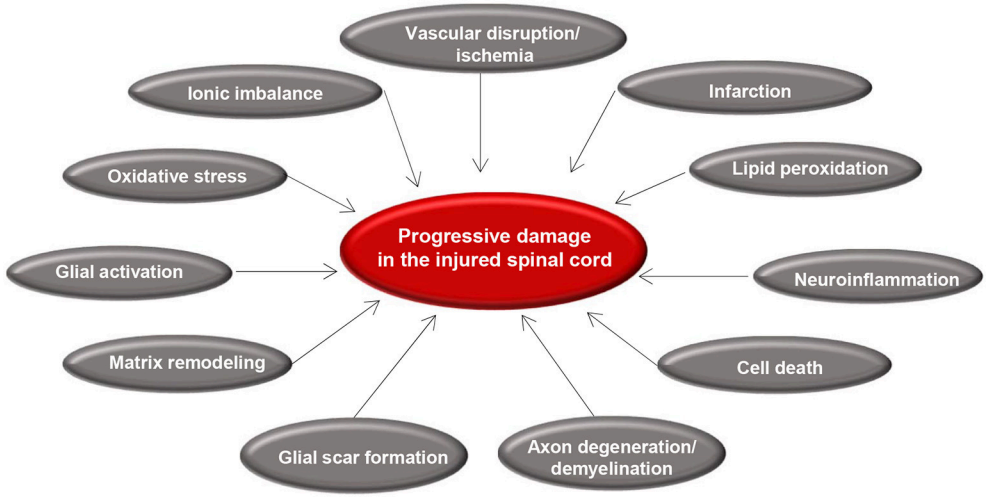

FIGURE 2 | Summary of secondary injury processes following traumatic spinal cord injury. Diagram shows the key pathophysiological events that occur after primary injury and lead to progressive tissue degeneration. Vascular disruption and ischemia occur immediately after primary injury that initiate glial activation, neuroinflammation, and oxidative stress. These acute changes results in cell death, axonal injury, matrix remodeling, and formation of a glial scar.

are susceptible to traumatic damage leads to extravasation of leukocytes and red blood cells (93). Increased tissue pressure in edematous injured spinal cord and hemorrhage-induced vasospasm in intact vessels further disrupts blood flow to the spinal cord (93, 95). In rat and monkey models of SCI, there is a progressive reduction in blood flow at the lesion epicenter within the first few hours after injury which remains low for up to 24 h (96). The gray matter is more prone to ischemic damage compared to the white matter as it has a 5-fold higher density of capillary beds and contains neurons with high metabolic demand (95, 97, 98). After injury, white matter blood flow typically returns to normal levels within 15 min post injury, whereas there are multiple hemorrhages in the gray matter and as a result, re-perfusion usually does not occur for the first 24 h (9, 99, 100). Vascular insult, hemorrhage and ischemia ultimately lead to cell death and tissue destruction through multiple mechanisms, including oxygen deprivation, loss of adenosine triphosphate (ATP), excitotoxicity, ionic imbalance, free radical formation, and necrotic cell death. Cellular necrosis and release of cytoplasmic content increase the extracellular level of glutamate causing glutamate excitotoxicity (93, 101). Moreover, re-establishment of blood flow in ischemic tissue leads to further damage through generating free radicals and eliciting an inflammatory response (93, 102) that will be discussed in this review.

Ionic Imbalance, Excitotoxicity and Oxidative Damage

Within few minutes after primary SCI, the combination of direct cellular damage and ischemia/hypoxia triggers a significant rise of extracellular glutamate, the main excitatory neurotransmitter in the CNS (7). Glutamate binds to ionotropic (NMDA, AMPA, and Kainate receptors) as well as metabotropic receptors resulting in calcium influx inside the cells (103–105) (93). The effect of glutamate is not restricted to neurons as its receptors are vastly expressed on the surface of all glia and endothelial cells (103–106). Astrocytes can also release excess glutamate extracellularly upon elevation of their intracellular Ca^{2+} levels. Reduced ability of activated astrocytes for glutamate re-uptake from the interstitial space due to lipid peroxidation results in further accumulation of glutamate in the SCI milieu (93). Using microdialysis, elevated levels of glutamate have been detected in the white matter in the acute stage of injury (107). Based on a study by Panter and colleagues, glutamate increase is detected during the first 20–30 min post SCI and returns to the basal levels after 60 min (108).

Under normal condition, concentration of free Ca^{2+} can considerably vary in different parts of the cell (109). In the cytosol, Ca^{2+} ranges from 50–100 nM while it approaches 0.5–1.0 mM in the lumen of endoplasmic reticulum (110–112). A long-lasting abnormal increase in Ca^{2+} concentration in cytosol, mitochondria or endoplasmic reticulum has detrimental consequences for the cell (109–113). Mitochondria play a central role in calcium dependent neuronal death (113). In neurons, during glutamate induced excitotoxicity, NMDA receptor over-activity leads to mitochondrial calcium overload, which can cause apoptotic or necrotic cell death (113). Shortly after SCI, Ca^{2+} enters mitochondria through the mitochondrial calcium uniporter (MCU) (114). While the amount of mitochondrial calcium is limited during the resting state of a neuron, they can store a high amount of Ca^{2+} following stimulation (113). Calcium overload also activates a host of protein kinases and phospholipases that results in calpain mediated protein degradation and oxidative damage due to mitochondrial failure (93). In the injured white matter, astrocytes, oligodendrocytes and myelin are also damaged by the increased release of glutamate and Ca^{2+}-dependent excitotoxicity (115). Within the first few hours after injury, oligodendrocytes show signs

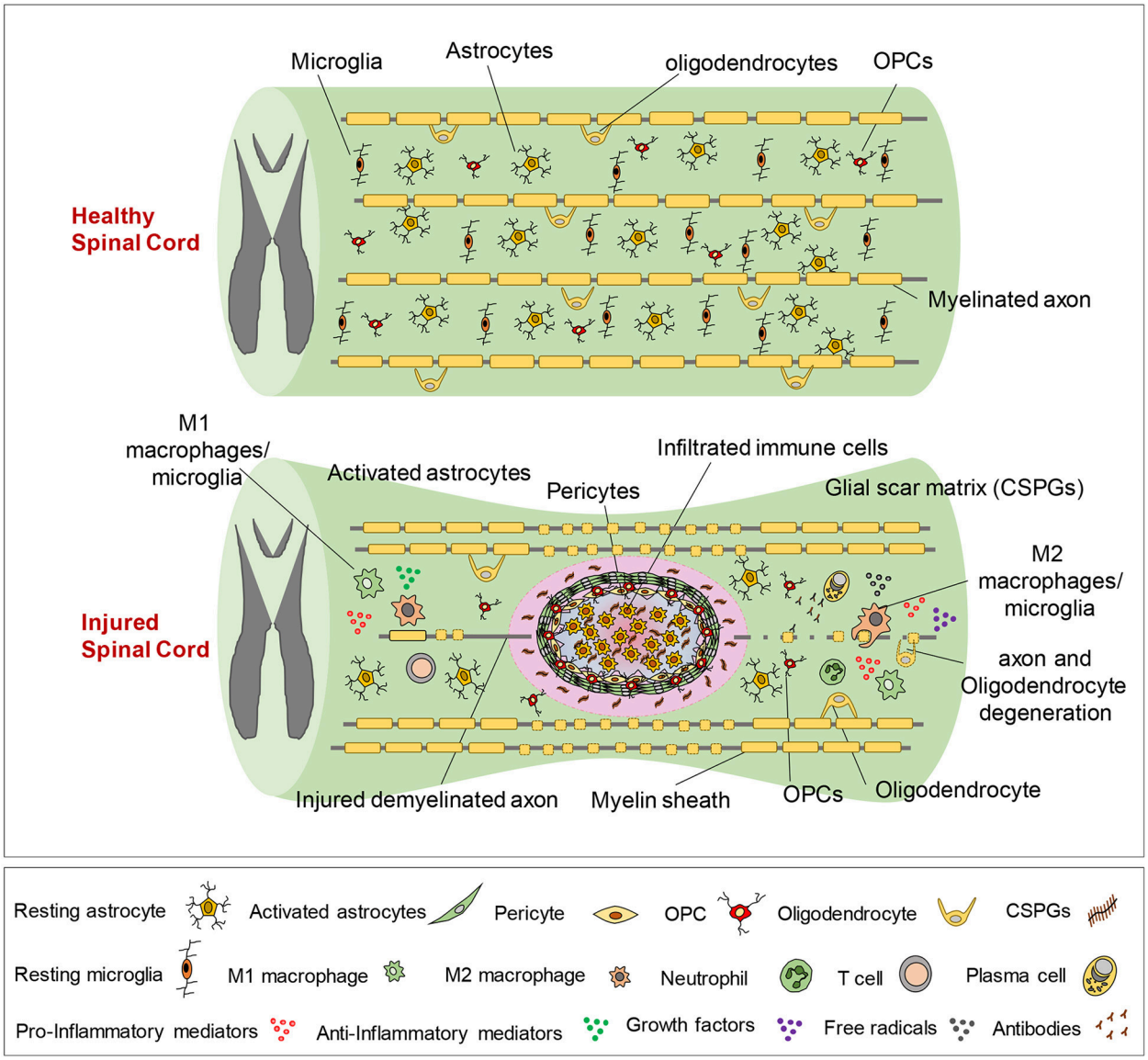

FIGURE 3 | Pathophysiology of traumatic spinal cord injury. This schematic diagram illustrates the composition of normal and injured spinal cord. Of note, while these events are shown in one figure, some of the pathophysiological events may not temporally overlap and can occur at various phases of SCI, which are described here. Immediately after primary injury, activation of resident astrocytes and microglia and subsequent infiltration of blood-borne immune cells results in a robust neuroinflammatory response. This acute neuroinflammatory response plays a key role in orchestrating the secondary injury mechanisms in the sub-acute and chronic phases that lead to cell death and tissue degeneration, as well as formation of the glial scar, axonal degeneration and demyelination. During the acute phase, monocyte-derived macrophages occupy the epicenter of the injury to scavenge tissue debris. T and B lymphocytes also infiltrate the spinal cord during sub-acute phase and produce pro-inflammatory cytokines, chemokines, autoantibodies reactive oxygen and nitrogen species that contribute to tissue degeneration. On the other hand, M2-like macrophages and regulatory T and B cells produce growth factors and pro-regenerative cytokines such as IL-10 that foster tissue repair and wound healing. Loss of oligodendrocytes in acute and sub-acute stages of SCI leads to axonal demyelination followed by spontaneous remyelination in sub-acute and chronic phases. During the acute and sub-acute phases of SCI; astrocytes, OPCs and pericytes, which normally reside in the spinal cord parenchyma, proliferate and migrate to the site of injury and contribute to the formation of the glial scar. The glial scar and its associated matrix surround the injury epicenter and create a cellular and biochemical zone with both beneficial and detrimental roles in the repair process. Acutely, the astrocytic glial scar limits the spread of neuroinflammation from the lesion site to the healthy tissue. However, establishment of a mature longstanding glial scar and upregulation of matrix chondroitin sulfate proteoglycans (CSPGs) are shown to inhibit axonal regeneration/sprouting and cell differentiation in subacute and chronic phases.

of caspase-3 activation and other apoptotic features, and their density declines (116). Interestingly, while glutamate excitotoxicity is triggered by ionic imbalance in the white matter, in the gray matter, it is largely associated with the activity of neuronal NMDA receptors (117, 118). Altogether, activation of NMDA receptors and consequent Ca^{2+} overload

appears to induce intrinsic apoptotic pathways in neurons and oligodendrocytes and causes cell death in the first week of SCI in the rat (119, 120). Administration of NMDA receptor antagonist (MK-801) shortly following SCI has been associated with improved functional recovery and reduced edema (121).

Mitochondrial calcium overload also impedes mitochondrial respiration and results in ATP depletion disabling Na^+/K^+ ATPase and increasing intracellular Na^+ (119, 122–124). This reverses the function of the Na^+ dependent glutamate transporter that normally utilizes Na^+ gradient to transfer glutamate into the cells (119, 125, 126). Moreover, the excess intracellular Na^+ reverses the activity of Na^+/Ca^{2+} exchanger allowing more Ca^+ influx (127). Cellular depolarization activates voltage gated Na^+ channels that results in entry of Cl^- and water into the cells along with Na^+ causing swelling and edema (128). Increased Na^+ concentration over-activates Na^+/H^+ exchanger causing a rise in intracellular H^+ (101, 129). Resultant intracellular acidosis increases membrane permeability to Ca^{2+} that exacerbates the injury-induced ionic imbalance (101, 129). Axons are more susceptible to the damage caused by ionic imbalance due to their high concentration of voltage gated Na^+ channels in the nodes of Ranvier (7). Accumulating evidence shows that administration of Na^+ channel blockers such as Riluzole attenuates tissue damage and improves functional recovery in SCI underlining sodium as a key player in secondary injury mechanisms (130–133).

SCI results in production of free radicals and nitric oxide (NO) (114). Mitochondrial Ca^{2+} overload activates NADPH oxidase (NOX) and induces generation of superoxide by electron transport chain (ETC) (114). Reactive oxygen and nitrogen species (ROS and RNS) produced by the activity of NOX and ETC activates cytosolic poly (ADP ribose) polymerase (PARP). PARP consumes and depletes NAD^+ causing failure of glycolysis, ATP depletion and cell death (114). Moreover, PAR polymers produced by PARP activity, induce the release of apoptosis inducing factor (AIF) from mitochondria and induce cell death (114). On the other hand, acidosis caused by SCI results in the release of intracellular iron from ferritin and transferrin (93). Spontaneous oxidation of Fe^{2+} to Fe^{3+} gives rise to more superoxide radicals (93). Subsequently, the Fenton reaction between Fe^{3+} and hydrogen peroxide produces highly reactive hydroxyl radicals (134). The resultant ROS and RNS react with numerous targets including lipids in the cell membrane with the most deleterious effects (93, 135). Because free radicals are short-lived and difficult to assess, measurements of their activity and final products, such as Malondialdehyde (MDA), are more reliable following SCI. Current evidence indicates that MDA levels are elevated as early as 1 h and up to 1 week after SCI (136, 137).

Oxidation of lipids and proteins is one of the key mechanisms of secondary injury following SCI (93). Lipid peroxidation starts when ROSs interact with polyunsaturated fatty acids in the cell membrane and generate reactive lipids that will then form lipid peroxyl radicals upon interacting with free superoxide radicals (138, 139). Each lipid peroxyl radical can react with a neighboring fatty acid, turn it into an active lipid and start a chain reaction that continues until no more unsaturated lipids are available or terminates when the reactive lipid quenches with another radical (93). The final products of this "termination" step of the lipid peroxidation is 4-hydroxynonenal (HNE) and 2-propenal, which are highly toxic to the cells (138–140). Lipid peroxidation is also an underlying cause of ionic imbalance through destabilizing cellular membranes such as cytoplasmic membrane and endoplasmic reticulum (93). Moreover, lipid peroxidation leads to Na^+/K^+ ATPase dysfunction that exacerbates the intracellular Na^+ overload (141). In addition to ROS associated lipid peroxidation, amino acids are subject to significant RNS associated oxidative damage following SCI (93). RNSs (containing $ONOO^-$) can nitrate the tyrosine residues of amino acids to form 3-nitrotyrosine (3-NT), a marker for peroxynitrite ($ONOO^-$) mediated protein damage (139). Lipid and protein oxidation following SCI has a number of detrimental consequences at cellular level including mitochondrial respiratory and metabolic failure as well as DNA alteration that ultimately lead to cell death (141).

Cell Death in Spinal Cord Injury

Cell death is a major event in the secondary injury mechanisms that affects neurons and glia after SCI (142–145). Cell death can happen through various mechanisms in response to various injury-induced mediators. Necrosis and apoptosis were originally identified as two major cell death mechanisms following SCI (146–148). However, recent research has uncovered additional forms of cell death. In 2012, the "Nomenclature Committee on Cell Death" (NCCD) NCCD defined 12 different forms of cell death such as necroptosis, pyroptosis, and netosis (149). Among the identified modes of cell death, to date, necrosis, necroptosis, apoptosis, and autophagy have been studied more extensively in the context of SCI and will be discussed in this review.

Following SCI, neurons and glial cells die through necrosis as the result of mechanical damage at the time of primary injury that also continues to the acute and subacute stages of injury (7, 150). Necrosis occurs due to a multitude of factors including accumulation of toxic blood components (151), glutamate excitotoxicity and ionic imbalance (152), ATP depletion (153), pro-inflammatory cytokine release by neutrophils and lymphocytes (154, 155), and free radical formation (142, 156–158). It was originally thought that necrosis is caused by a severe impact on a cell that results in rapid cell swelling and lysis. However, follow up evidence showed that in the case of seizure, ischemia and hypoglycemia, necrotic neurons show signs of shrunken, pyknotic, and condensed nuclei, with swollen, irreversibly damaged mitochondria and plasma membrane that are surrounded by astrocytic processes (159). Moreover, necrosis was conventionally viewed as instantaneous energy-independent non-programmed cell death (142, 156). However, recent research has identified another form of necrosis, termed as necroptosis, that is executed by regulated mechanisms.

Programmed necrosis or "necroptosis" has been described more recently as a highly regulated, caspase-independent cell death with similar morphological characteristics as necrosis (160). Necroptosis is a receptor-mediated process. It is induced downstream of the TNF receptor 1 (TNFR1) and is dependent on the activity of the receptor interacting protein kinase 1 (RIPK1)

and RIPK3. Recent studies has uncovered a key role for RIPK1 as the mediator of necroptosis and a regulator of the innate immune response involved in both inflammation and cell death (161). Evidence from SCI studies show that lysosomal damage can potentiate necroptosis by promoting RIPK1 and RIPK3 accumulation (161). Interestingly, inhibition of necroptosis by necrostatin-1, a RIPK1 inhibitor, improves functional outcomes after SCI (150). These initial findings suggest that modulation of necroptosis pathways seems to be a promising target for neuroprotective strategies after SCI.

Apoptosis is the most studied mechanism of cell death after SCI. Apoptosis represents a programmed, energy dependent mode of cell death that begins within hours of primary injury (7). This process takes place in cells that survive the primary injury but endure enough insult to activate their apoptotic pathways (142). In apoptosis, the cell shrinks and is eventually phagocytosed without induction of an inflammatory response (156). Apoptosis typically occurs in a delayed manner in areas more distant to the injury site and most abundantly affects oligodendrocytes. In rat SCI, apoptosis happens as early as 4 h after the injury and reaches a peak at 7 day (156). At the site of injury majority of oligodendrocytes are lost within 7 days after SCI (162). However, apoptosis can be observed at a diminished rate for weeks after SCI (162, 163). Microglia and astrocytes also undergo apoptosis (156, 164). Interestingly, apoptotic cell death occurs in the chronically injured spinal cord in rat, monkey and human models of SCI, which is thought to be due to loss of trophic support from degenerating axons (146, 165).

Apoptosis is induced through extrinsic and intrinsic pathways based on the triggering mechanism (166). The extrinsic pathway is triggered by activation of death receptors such as FAS and TNFR1, which eventually activates caspase 8 (167). The intrinsic pathway, however, is regulated through a balance between intracellular pro- and anti-apoptotic proteins and is triggered by the release of cytochrome C from mitochondria and activating caspase 9 (167). In SCI lesion, apoptosis primarily happens due to injury induced Ca^{2+} influx, which activates caspases and calpain; enzymes involved in breakdown of cellular proteins (7). Moreover, it is believed that the death of neurons and oligodendrocytes in remote areas from the lesion epicenter can be mediated through cytokines such as TNF-α, free radical damage and excitotoxicity since calcium from damaged cells within the lesion barely reaches these remote areas (8, 168). Fas mediated cell death has been suggested as a key mechanism of apoptosis following SCI (144, 169–172). Post-mortem studies on acute and chronic human SCI and animal models revealed that Fas mediated apoptosis plays a role in oligodendrocyte apoptosis and inflammatory response at acute and subacute stages of SCI (173). Fas deficient mice exhibit a significant reduction in apoptosis and inflammatory response evidenced by reduced macrophage infiltration and inflammatory cytokine expression following SCI (173). Interestingly, Fas deficient mice show a significantly improved functional recovery after SCI (173) suggesting the promise of anti-apoptotic strategies for SCI.

SCI also results in a dysregulated autophagy (174). Normally, autophagy plays an important role in maintaining the homeostasis of cells by aiding in the turnover of proteins and organelles. In autophagy, cells degrade harmful, defective or unnecessary cytoplasmic proteins and organelles through a lysosomal dependent mechanism (175, 176). The process of autophagy starts with the formation of an autophagosome around the proteins and organelles that are tagged for autophagy (176). Next, fusion of the phagosome with a lysosome form an autolysosome that begins a recycling process (176). In response to cell injury and endoplasmic reticulum (ER) stress, autophagy is activated and limits cellular loss (177, 178). Current evidence suggests a neuroprotective role for autophagy after SCI (175, 179). Dysregulation of autophagy contributes to neuronal loss (174, 180). Accumulation of autophagosomes in ventral horn motor neurons have been detected acutely following SCI (181). Neurons with dysregulated autophagy exhibit higher expression of caspase 12 and become more prone to apoptosis (174). Moreover, blocking autophagy has been associated with neurodegenerative diseases such as Parkinson's and Alzheimer's disease (182–184). Autophagy promotes cell survival through elimination of toxic proteins and damaged mitochondria (185, 186). Interestingly, autophagy is crucial in cytoskeletal remodeling and stabilizes neuronal microtubules by degrading SCG10, a protein involved in microtubule disassembly (179). Pharmacological induction of autophagy in a hemi-section model of SCI in mice has been associated with improved neurite outgrowth and axon regeneration, following SCI (179). Altogether, although further studies are needed, autophagy is currently viewed as a beneficial mechanism in SCI.

Adaptive and Innate Immune Response in Spinal Cord Injury

Neuroinflammation is a key component of the secondary injury mechanisms with local and systemic consequences. Inflammation was originally thought to be detrimental for the outcome of SCI (187). However, now it is well-recognized that inflammation can be both beneficial and detrimental following SCI, depending on the time point and activation state of immune cells (188). There are multiple cell types involved in the inflammatory response following injury including neutrophils, resident microglia, and astrocytes, dendritic cells (DCs), blood-born macrophages, B- and T-lymphocytes (189) (**Figure 4**). The first phase of inflammation (0–2 days post injury) involves the recruitment of resident microglia and astrocytes and blood-born neutrophils to the injury site (190). The second phase of inflammation begins approximately 3 days post injury and involves the recruitment of blood-born macrophages, B- and T-lymphocytes to the injury site (189, 191–193). T lymphocytes become activated in response to antigen presentation by macrophages, microglia and other antigen presenting cells (APCs) (194). $CD4^+$ helper T cells produce cytokines that stimulate B cell antibody production and activate phagocytes (195) (**Figure 4**). In SCI, B cells produce autoantibodies against injured spinal cord tissue, which exacerbate neuroinflammation and cause tissue destruction (196). While inflammation is more pronounced in the acute phase of injury, it continues in subacute and chronic phase and may persist for the remainder of a patients' life (193). Interestingly, composition and phenotype of

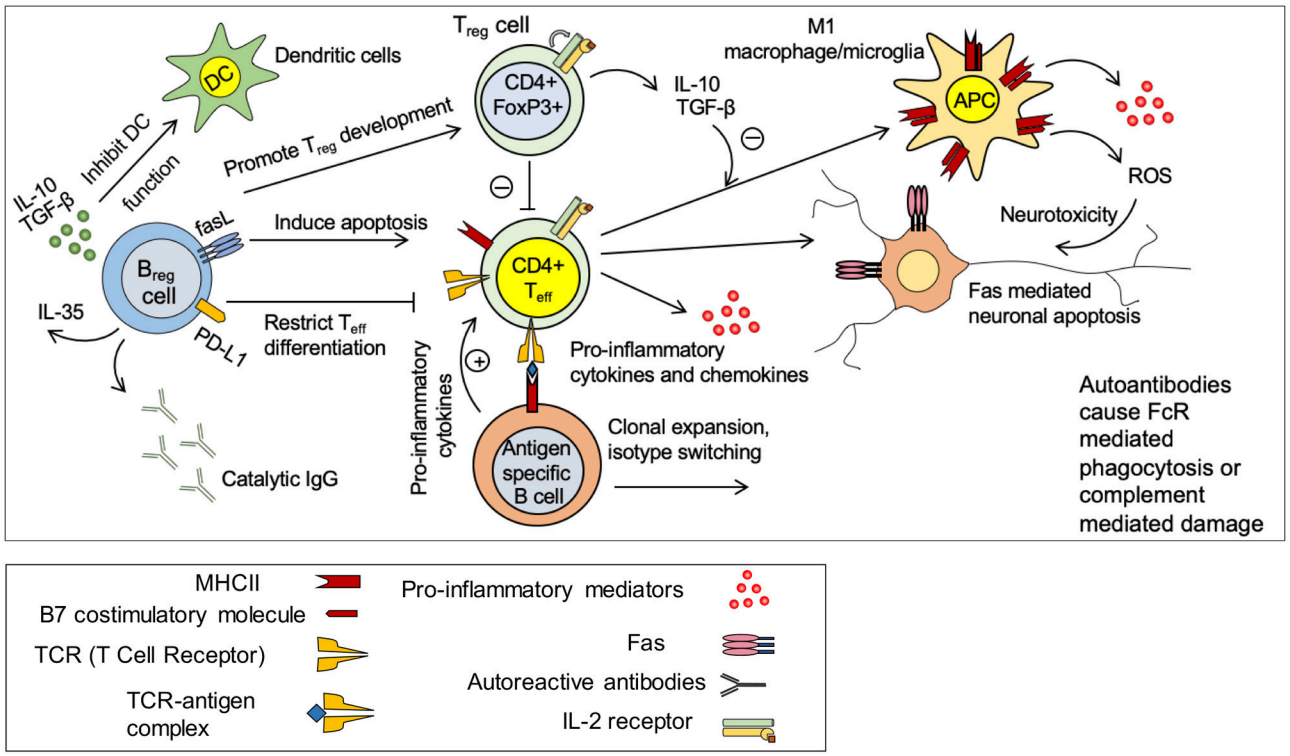

FIGURE 4 | Immune response in spinal cord injury. Under normal circumstances, there is a balance between pro-inflammatory effects of $CD4^+$ effector T cells (T_{eff}) and anti-inflammatory effects of regulatory T and B cells (T_{reg} and B_{reg}). T_{reg} and B_{reg} suppress the activation of antigen specific $CD4^+$ T_{eff} cells through production of IL-10 and TGF-β. Injury disrupts this balance and promote a pro-inflammatory environment. Activated microglia/macrophages release pro-inflammatory cytokines and chemokines and present antigens to $CD4^+$ T cells causing activation of antigen specific effector T cells. T_{eff} cells stimulate antigen specific B cells to undergo clonal expansion and produce autoantibodies against spinal cord tissue antigens. These autoantibodies cause neurodegeneration through FcR mediated phagocytosis or complement mediated cytotoxicity. M1 macrophages/microglia release pro-inflammatory cytokines and reactive oxygen species (ROS) that are detrimental to neurons and oligodendrocytes. B_{reg} cells possess the ability to promote T_{reg} development and restrict T_{eff} cell differentiation. B_{reg} cells could also induce apoptosis in T_{eff} cells through Fas mediate mechanisms.

inflammatory cells change based on the injury phase and the signals present in the injury microenvironment. It is established that microglia/macrophages, T cells, B cells are capable of adopting a pro-inflammatory or an anti-inflammatory pro-regenerative phenotype in the injured spinal cord (191, 197–199). The role of each immune cell population in the pathophysiology of SCI will be discussed in detail in upcoming sections.

Astrocytes

Astrocytes are not considered an immune cell *per se*; however, they play pivotal roles in the neuroinflammatory processes in CNS injury and disease. Their histo-anatomical localization in the CNS has placed them in a strategic position for participating in physiological and pathophysiological processes in the CNS (200). In normal CNS, astrocytes play major roles in maintaining CNS homeostasis. They contribute to the structure and function of blood-brain-barrier (BBB), provide nutrients and growth factors to neurons (200), and remove excess fluid, ions, and neurotransmitters such as glutamate from synaptic spaces and extracellular microenvironment (200). Astrocytes also play key roles in the pathologic CNS by regulating BBB permeability and reconstruction as well as immune cell activity and trafficking (201). Astrocytes contribute to both innate and adaptive immune responses following SCI by differential activation of their intracellular signaling pathways in response to environmental signals (201).

Astrocytes react acutely to CNS injury by increasing cytokine and chemokine production (202). They mediate chemokine production and recruitment of neutrophils through an IL-1R1-Myd88 pathway (202). Activation of the nuclear factor kappa b (NF-κB) pathway, one of the key downstream targets of interleukin (IL)1R-Myd88 axis, increases expression of intracellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), which are necessary for adhesion and extravasation of leukocytes in inflammatory conditions such as SCI (201, 202). Within minutes of injury, production of IL-1β is significantly elevated in astrocytes and microglia (203). Moreover, chemokines such as monocyte chemoattractant protein (MCP)-1, chemokine C-C motif ligand 2 (CCL2), C-X-C motif ligand 1 (CXCL1), and CXCL2 are produced by astrocytes, and enhance the recruitment of neutrophils and pro-inflammatory macrophages following injury (201, 202).

Astrocytes also promote pro-inflammatory M1-like phenotype in microglia/macrophages in the injured spinal cord through their production of TNF-α, IL-12, and IFN-γ (204–206). Interestingly, astrocytes also produce anti-inflammatory cytokines, such as TGF-β and IL-10, which can promote a pro-regenerative M2-like phenotype in microglia/macrophages (201, 207, 208).

Immunomodulatory role of astrocytes is defined by activity of various signaling pathways through a wide variety of surface receptors (200). For example, gp130, a member of IL-6 cytokine family, activates SHP2/Ras/Erk signaling cascade in astrocytes and limits neuroinflammation in autoimmune rodent models (209). TGF-β signaling in astrocytes has been implicated in modulation of neuroinflammation through inhibition of NF-κB activity and nuclear translocation (201, 210). STAT3 is another key signaling pathway in astrocytes with beneficial properties in neuroinflammation. Increase in STAT3 phosphorylation enhances astrocytic scar formation and restricts the expansion of inflammatory cells in mouse SCI, which is associated with improved functional recovery (211). Detrimental signaling pathways in astrocytes are known to be activated by cytokines, sphingolipids and neurotrophins (200). As an example, IL-17 is a key pro-inflammatory cytokine produced by effector T cells that can bind to IL-17R on the astrocyte surface (200). Activation of IL-17R results in the activation of NF-κB, which enhances expression of pro-inflammatory mediators, activation of oxidative pathways and exacerbation of neuroinflammation (200, 212). This evidence shows the significance of astrocytes in the inflammatory processes following SCI and other neuroinflammatory diseases of the CNS.

Neutrophils

Neutrophils infiltrate the spinal cord from the bloodstream within the first few hours after injury (213). Their population increases acutely in the injured spinal cord tissue and reaches a peak within 24 h post-injury (214). The presence of neutrophils is mostly limited to the acute phase of SCI as they are rarely found sub-acutely in the injured spinal cord (214). The role of neutrophils in SCI pathophysiology is controversial. Evidence shows that neutrophils contribute to phagocytosis and clearance of tissue debris (48). They release inflammatory cytokines, proteases and free radicals that degrade ECM, activate astrocytes and microglia and initiate neuroinflammation (48). Although neutrophils have been conventionally associated with tissue damage (48, 215), their elimination compromises the healing process and impedes functional recovery (216).

To elucidate the role of neutrophils in SCI, Stirling and colleagues used a specific antibody to reduce circulating LyG6/Gr1$^+$ neutrophils in a mouse model of thoracic contusive SCI (216). This approach significantly reduced neutrophil infiltration in the injured spinal cord by 90% at 24 and 48 h after SCI (216). Surprisingly, neutrophil depletion aggravated the neurological and structural outcomes in the injured animals suggesting a beneficial role for neutrophils in the acute phase of injury (216). It is shown that simulated neutrophils release IL-1 receptor antagonist that can exert neuroprotective effects following SCI (217). Moreover, ablation of neutrophils results in altered expression of cytokines and chemokines and downregulation of growth factors such as fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) and bone morphogenetic proteins (BMPs) in the injured spinal cord that seemingly disrupt the normal healing process (216). Altogether, neutrophils play important roles in regulating neuroinflammation at the early stage of SCI that shapes the immune response and repair processes at later stages. While neutrophils were originally viewed as being detrimental in SCI, emerging evidence shows their critical role in the repair process. Further investigations are required to elucidate the role of neutrophils in SCI pathophysiology.

Microglia and Macrophages

Following neutrophil invasion, microglia/macrophages populate the injured spinal cord within 2–3 days post-SCI. Macrophage population is derived from invading blood-borne monocytes or originate from the CNS resident macrophages that reside in the perivascular regions within meninges and subarachnoid space (218, 219). The population of microglia/macrophages reaches its peak at 7–10 days post-injury in mouse SCI, followed by a decline in the subacute and chronic phases (20, 220). While macrophages and microglia share many functions and immunological markers, they have different origins. Microglia are resident immune cells of the CNS that originate from yolk sac during the embryonic period (221). Macrophages are derived from blood monocytes, which originate from myeloid progeny in the bone marrow (222, 223). Upon injury, acute disruption of brain-spinal cord barrier (BSB) enables monocytes, to infiltrate the spinal cord tissue and transform into macrophages (222). Macrophages populate the injury epicenter, while resident microglia are mainly located in the perilesional area (222). Once activated, macrophages, and microglia are morphologically and immunohistologically indistinguishable (224). Macrophages and microglia play a beneficial role in CNS regeneration. They promote the repair process by expression of growth promoting factors such as nerve growth factor (NGF), neurotrophin-3 (NT-3) and thrombospondin (225, 226). Macrophages and microglia are important for wound healing process following SCI due to their ability for phagocytosis and scavenging damaged cells and myelin debris following SCI (222, 227).

Based on microenvironmental signals, macrophages/microglia can be polarized to either pro-inflammatory (M1-like) or anti-inflammatory pro-regenerative (M2-like) phenotype, and accordingly contribute to injury or repair processes following SCI (191, 224, 228–230). Whether both microglia and macrophages possess the ability to polarize or it is mainly the property of monocyte derived macrophages is still a matter of debate and needs further elucidation (231–233). Some evidence show that Proinflammatory M1-like microglia/macrophages can be induced by exposure to T_h1 specific cytokine, interferon (IFN)-γ (224, 230). Moreover, the SCI microenvironment appears to drive M1 polarization of activated macrophages (231). SCI studies have revealed that increased level of the proinflammatory cytokine, TNF-α, and intracellular accumulation of iron drives an M1-like proinflammatory phenotype in macrophages after injury (231). Importantly, following SCI, activated M1-like

microglia/macrophages highly express MHCII and present antigens to T cells and contribute to the activation and regulation of innate and adaptive immune response (**Figure 4**) (224, 228). Studies on acute and subacute SCI and experimental autoimmune encephalomyelitis (EAE) models have shown that M1-like macrophages are associated with higher expression of chondroitin sulfate proteoglycans (CSPGs) and increased EAE severity and tissue damage (234–237). *In vitro,* addition of activated M1-like macrophages to dorsal root ganglion (DRG) neuron cultures leads to axonal retraction and failure of regeneration as the expression of CSPGs is much higher in M1-like compared to M2-like macrophages (237, 238). M1-like macrophages also produce other repulsive factors such as repulsive guidance molecule A (RGMA) that is shown to induce axonal retraction following SCI (239, 240). Interestingly, recent evidence shows that IFN-γ and TNFα polarized M1 microglia show reduced capacity for phagocytosis (241), a process that is critical for tissue repair after SCI.

Pro-regenerative M2-like microglia/macrophages, are polarized by T_h2 cytokines, IL-4 and IL-13 and exhibit a high level of IL-10, TGF-β, and arginase-1 with reduced NF-κB pathway activity (224). IL-10 is a potent immunoregulatory cytokine with positive roles in repair and regeneration following CNS injury (242–244). IL-10 knock-out mice show higher production of pro-inflammatory and oxidative stress mediators after SCI (245). Lack of IL-10 is also correlated with upregulated levels of pro-apoptotic factors such as Bax and reduced expression of anti-apoptotic factors such as Bcl-2 (245). SCI mice that lacked IL-10 exhibited poorer recovery of function compared to wild-type mice (245). Our recent studies show that IL-10 polarized M2 microglia show enhanced capacity for phagocytosis (241). We have also found that M2 polarized microglia enhance the ability of neural precursor cells for oligodendrocyte differentiation through IL-10 mediated mechanisms (241). In addition to immune modulation, M2-like microglia/macrophages promote axonal regeneration (224). However, similar to the detrimental effects of prolonged M1 macrophage response, excessive M2-like activity promotes fibrotic scar formation through the release of factors such as TGF-β, PDGF, VEGF, IGF-1, and Galectin-3 (224, 246–248). Hence, a balance between proinflammatory M1 and pro-regenerative M2 macrophage/microglia response is beneficial for the repair of SCI (249).

T and B Lymphocytes

T and B lymphocytes play pivotal role in the adaptive immune response after SCI (194). Lymphocytes infiltrate the injured spinal cord acutely during the first week of injury and remain chronically in mouse and rat SCI (47, 193, 194, 196). In contrast to the innate immune response that can be activated directly by foreign antigens, the adaptive immune response requires a complex signaling process in T cells elicited by antigen presenting cells (250). Similar to other immune cells, T and B lymphocytes adopt different phenotypes and contribute to both injury and repair processes in response to microenvironmental signals (194, 251). SCI elicits a CNS-specific autoimmune response in T and B cells, which remains active chronically (196). Autoreactive T cells can exert direct toxic effects on neurons and glial cells (194, 252). Moreover, T cells can indirectly affect neural cell function and survival through pro-inflammatory cytokine and chemokine production (e.g. IL-1β, TNF-α, IL-12, CCL2, CCL5, and CXCL10) (194, 252). Genetic elimination of T cells (in athymic nude rats) or pharmacological inhibition of T cells (using cyclosporine A and tacrolimus) leads to improved tissue preservation and functional recovery after SCI (194, 253) signifying the impact of T cells in SCI pathophysiology and repair.

Under normal circumstances, systemic autoreactive effector $CD4^+$ helper T cells (T_{eff}) are suppressed by $CD4^+FoxP3^+$ regulatory T cells (T_{reg}) (**Figure 4**) (194, 254). This inhibition is regulated through various mechanisms such as release of anti-inflammatory cytokines IL-10 and TGF-β by the T_{reg} cells (**Figure 4**) (194). Moreover, it is known that T_{reg} mediated inhibition of antigen presentation by dendritic cells (DCs) prevent T_{eff} cell activation (194). Following SCI, this T_{reg} -T_{eff} regulation is disrupted. Increased activity of autoreactive T_{eff} cells contributes to tissue damage through production of pro-inflammatory cytokines and chemokines, promoting M1-like macrophage phenotype and induction of Fas mediated neuronal and oligodendroglial apoptosis (**Figure 4**) (173). Moreover, autoreactive T_{eff} cells promote activation and differentiation of antigen specific B cells to autoantibody producing plasma cells that contribute to tissue damage after SCI (255). In SCI and MS patients, myelin specific proteins such as myelin basic protein (MBP) significantly increase the population of circulating T cells (256, 257). Moreover, serological assessment of SCI patients has shown high levels of CNS reactive IgM and IgG isotypes confirming SCI-induced autoimmune activity of T and B cells (**Figure 4**) (196, 258, 259). In animal models of SCI, serum IgM level increases acutely followed by an elevation in the levels of IgG1 and IgG2a at later time-points (196). In addition to autoantibody production, autoreactive B cells contribute to CNS injury through pro-inflammatory cytokines that stimulate and maintain the activation states of T_{eff} cells (194, 260). B cell knockout mice (BCKO) that have no mature B cell but with normal T cells, show a reduction in lesion volume, lower antibody levels in the cerebrospinal fluid and improved recovery of function following SCI compared to wild-type counterparts (255). Of note, antibody mediated injury is regulated through complement activation as well as macrophages/microglia that express immunoglobulin receptors (193, 255).

The effect of SCI on systemic B cell response is controversial. Evidence shows that SCI can suppress B cell activation and antibody production (261). Studies in murine SCI have shown that B cell function seems to be influenced by the level of injury (262). While injury to upper thoracic spinal cord (T3) suppresses the antibody production, a mid-thoracic (T9) injury has no effect on B cell antibody production (262). An increase in the level of corticosterone in serum together with elevation of splenic norepinephrine found to be responsible for the suppression of B cell function acutely following SCI (261). Elevated corticosterone and norepinephrine leads to upregulation of lymphocyte beta-2 adrenergic receptors eliciting lymphocyte apoptosis (194). This suggests a critical role for sympathetic innervation of

peripheral lymphoid tissues in regulating B cell response following CNS injury (261). Despite their negative roles, B cells also contribute to spinal cord repair following injury through their immunomodulatory B_{reg} phenotype (**Figure 4**) (263). B_{reg} cells control antigen-specific T cell autoimmune response through IL-10 production (264).

Detrimental effects of SCI-induced autoimmunity are not limited to the spinal cord. Autoreactive immune cells contribute to the exacerbation of post-SCI sequelae such as cardiovascular, renal and reproductive dysfunctions (194). For example, presence of an autoantibody against platelet prostacyclin receptor has been associated with a higher incidence of coronary artery disease in SCI patients (265). Collectively, evidence shows the critical role of adaptive immune system in SCI pathophysiology and repair. Thus, treatments that harness the pro-regenerative properties of the adaptive immune system can be utilized to reduce immune mediated tissue damage, improve neural tissue preservation and facilitate repair following SCI.

Glial Scar and Extracellular Matrix

Traumatic SCI triggers the formation of a glial scar tissue around the injury epicenter (266, 267). The glial scar is a multifactorial phenomenon that is contributed f several populations in the injured spinal cord including activated astrocytes, $NG2^+$ oligodendrocyte precursor cells (OPCs), microglia, fibroblasts, and pericytes (268–271). The heterogeneous scar forming cells and associated ECM provides a cellular and biochemical zone within and around the lesion (**Figure 3**) (272). Resident and infiltrating inflammatory cells contribute to the process of glial activation and scar formation by producing cytokines (e.g., IL-1β and IL-6) chemokines and enzymes that activate glial cells or disrupt BSB (267). Activated microglia/macrophages produce proteolytic enzymes such as matrix metalloproteinases (MMPs) that increase vascular permeability and further disruption of the BSB (273). Inhibition of MMPs improves neural preservation and functional recovery in animal models of SCI (273–275). In addition to glial and immune cells, fibroblasts, pericytes and ependymal cells also contribute to the structure of the glial scar (267). In penetrating injuries where meninges are compromised, meningeal fibroblasts infiltrate the lesion epicenter (276). Fibroblasts contribute to the production of fibronectin, collagen, and laminin in the ECM of the inured spinal cord (267) and are a source of axon-repulsing molecules such as semaphorins that influence axonal regeneration following SCI (277). Fibroblasts have also been found in contusive injuries where meninges are intact (268, 270). Studies using genetic fate mapping in these injuries have unraveled that perivascular pericytes and fibroblasts migrate to the injury site and form a fibrotic core in the scar which matures within 2 weeks post-injury (268, 270). SCI also triggers proliferation and migration of the stem/progenitor cell pool of the spinal cord parenchyma and ependyma. These cells can give rise to new scar forming astrocytes and OPCs (278–280). In a mature glial scar, activated microglia/macrophages occupy the innermost portion closer to the injury epicenter surrounded by $NG2^+$ OPCs (**Figure 3**) (267), while reactive astrocytes reside in the injury penumbra and form a cellular barrier (267). Of note, in human SCI, the glial scar begins to form within the first hours after the SCI and remains chronically in the spinal cord tissue (281). The glial scar has been found within the injured human spinal cord up to 42 years after the injury (267).

Activated astrocytes play a leading role in the formation of the glial scar (267). Following injury, astrocytes increase their expression of intermediate filaments, GFAP, nestin and vimentin, and become hypertrophied (282, 283). Reactive astrocytes proliferate and mobilize to the site of injury and form a mesh like structure of intermingled filamentous processes around the injury epicenter (284, 285). The astrocytic glial scar has been shown to serve as a protective barrier that prevents the spread of infiltrating immune cells into the adjacent segments (267, 284, 286). Attenuating astrocyte reactivity and scar formation by blockade of STAT3 activation results in poorer outcomes in SCI (211, 286). Reactive astrogliosis is also essential for reconstruction of the BBB, and blocking this process leads to exacerbated leukocyte infiltration, cell death, myelin damage, and reduced functional recovery (211, 285, 286). Despite the protective role of the astrocytic glial scar in acute SCI, its evolution and persistence in the sub-acute and chronic stages of injury has been considered as a potent inhibitor for spinal cord repair and regeneration (267, 287). A number of inhibitory molecules have been associated with activated astrocytes and their secreted products such as proteoglycans and Tenascin-C (288). Thus, manipulation of the astrocytic scar has been pursued as a promising treatment strategy for SCI (267, 289).

Chondroitin sulfate proteoglycans (CSPGs) are well-known for their contribution to the inhibitory role of the glial scar in axonal regeneration (290–295), sprouting (296–299), conduction (300–302), and remyelination (241, 303–307). In normal condition, basal levels of CSPGs are expressed in the CNS that play critical roles in neuronal guidance and synapse stabilization (90, 308). Following injury, CSPGs (neurocan, versican, brevican, and phosphacan) are robustly upregulated and reach their peak of expression at 2 weeks post-SCI and remain upregulated chronically (309, 310). Mechanistically, disruption of BSB and hemorrhage following traumatic SCI triggers upregulation of CSPGs in the glial scar by exposing the scar forming cells to factors in plasma such as fibrinogen (311). Studies in cortical injury have shown that fibrinogen induces CSPG expression in astrocytes through TGFβ/Smad2 signaling pathway (311). The authors show that intracellular Smad2 translocation is essential for Smad2 signal transduction process and its inhibition reduces scar formation (312). In contrast, another study has identified that TGFβ induces CSPGs production in astrocytes through a SMAD independent pathway (313). This study showed a significant upregulation of CSPGs in SMAD2 and SMAD4 knockdown astrocytes. Interestingly, CSPG upregulation was found to be mediated by the activation of the phosphoinositide 3-kinase (PI3K)/Akt and mTOR axis (313). Further studies are required to confirm these findings.

Extensive research in the past few decades has demonstrated the inhibitory effect of CSPGs on axon regeneration (314, 315). The first successful attempt on improving axon outgrowth and/or sprouting by enzymatic degradation of CSPGs using chondroitinase ABC (ChABC) in a rat SCI model was published in 2002 by Bradbury and colleagues (291). This

study showed significant improvement in recovery of locomotor and proprioceptive functions following intrathecal delivery of ChABC in a rat model of dorsal column injury (291). This observation was followed by several other studies demonstrating the promise of CSPGs degradation in improvement of axon regeneration and sprouting of the serotonergic (295, 297, 299, 303), sensory (293, 298, 316), corticospinal (291, 297, 303, 317), and rubrospinal fibers (318) in animal models of CNS injury. Additionally, ChABC treatment is shown to be neuroprotective by preventing CSPG induced axonal dieback and degeneration (303, 319, 320). Studies by our group also showed that degradation of CSPGs using ChABC attenuates axonal dieback in corticospinal fibers in chronic SCI model in the rat (303). ChABC also blocks macrophage-mediated axonal degeneration in neural cultures and after SCI (238).

The inhibitory effects of astrocytic glial scar on axonal regeneration has been recently challenged after SCI (321). Using various transgenic mouse models, a study by Sofroniew's and colleagues has shown that spontaneous axon regrowth failed to happen following the ablation or prevention of astrocytic scar in acute and chronic SCI. They demonstrated that when the intrinsic ability of dorsal root ganglion (DRG) neurons for growth was enhanced by pre-conditioning injury as well as local delivery of a combination of axon growth promoting factors into the SCI lesion, the axons grew to the wall of the glial scar and CSPGs within the lesion. However, when astrocyte scarring was attenuated, the pre-conditioned/growth factor stimulated DRG neurons showed a reduced ability for axon growth (321). From these observations, the authors suggested a positive role for the astrocytic scar in axonal regeneration following SCI (321). Overall, this study points to the importance of reactive and scar forming astrocytes and their pivotal role in the repair process following SCI (322). This is indeed in agreement with previous studies by the same group that showed a beneficial role for activated astrocytes in functional recovery after SCI by limiting the speared of infiltrated inflammatory cells and tissue damage in SCI (285). It is also noteworthy that the glial scar is contributed by various cell populations and not exclusively by astrocytes (269, 271). Therefore, the outcomes of this study need to be interpreted in the context of astrocytes and astrocytic scar. Moreover, the reduced capacity of the injured spinal cord for regeneration is not solely driven by the glial scar as other factors including inflammation and damaged myelin play important inhibitory role in axon regeneration (323, 324). Taken together, further investigation is needed to delineate the mechanisms of the glial scar including the contribution of astrocyte-derived factors on axon regeneration in SCI.

Role of CSPGs on Endogenous Cell Response and Neuroinflammation

While CSPGs were originally identified as an inhibitor of axon growth and plasticity within the glial scar, emerging evidence has also identified them as an important regulator of endogenous cell response. Emerging evidence has identified CSPGs as an inhibitor of oligodendrocytes (241, 272, 306). Replacement of oligodendrocytes is an important repair process in SCI and other demyelinating conditions such as MS (90). SCI and MS triggers activation of endogenous OPCs and their mobilization to the site of injury (143, 162, 306, 325). *In vitro* and *in vivo* evidence shows that CSPGs limit the recruitment of NPCs and OPCs to the lesion and inhibit oligodendrocyte survival, differentiation and maturation (145, 272, 305, 306, 326). Our group and others have shown that targeting CSPGs by ChABC administration or xyloside, or through inhibition of their signaling receptors enhances the capacity of NPCs and OPCs for proliferation, oligodendrocyte differentiation and remyelination following SCI and MS-like lesions (145, 303, 304, 306).

Mechanistically, the inhibitory effects of CSPGs on axon growth and endogenous cell differentiation is mainly governed by signaling through receptor protein tyrosine phosphatase sigma (RPTPσ) and leukocyte common antigen-related phosphatase receptor (LAR) (327). RPTPσ is the main receptor mediating the inhibition of axon growth by CSPGs (327, 328). Improved neuronal regeneration has been demonstrated in RPTPσ−/− mice model of SCI and peripheral nerve injury (328, 329). Blockade of RPTPσ and LAR by intracellular sigma peptide (ISP) and intracellular LAR peptide (ILP), facilitates axon regeneration following SCI (327, 330). Inhibition of RPTPσ results in significant improvement in locomotion and bladder function associated with serotonergic re-innervation below the level of injury in rat SCI (327). Our group has also shown that CSPGs induce caspase-3 mediated apoptosis in NPCs and OPCs *in vitro* and in oligodendrocytes in the injured spinal cord that is mediated by both RPTPσ and LAR (241). Inhibition of LAR and RPTPσ sufficiently attenuates CSPG-mediated inhibition of oligodendrocyte maturation and myelination *in vitro* and attenuated oligodendrocyte cell death after SCI (241).

CSPGs have been implicated in regulating immune response in CNS injury and disease. Interestingly, our recent studies indicated that CSPGs signaling appears to restrict endogenous repair by promoting a pro-inflammatory immune response in SCI (241, 331). Inhibition of LAR and RPTPσ enhanced an anti-inflammatory environment after SCI by promoting the populations of pro-regenerative M2-like microglia/macrophages and regulatory T cells (241) that are known to promote repair process (224). These findings are also in agreement with recent studies in animal models of MS that unraveled a pro-inflammatory role for CSPGs in autoimmune demyelinating conditions (332). In MS and EAE, studies by Stephenson and colleagues have shown that CSPGs are abundant within "the leucocyte-containing perivascular cuff," the entry point of inflammatory cells to the CNS tissue (332). Presence of CSPGs in these perivascular cuffs promotes "trafficking" of immune cells to induce a pro-inflammatory response in MS condition. In contrast to these new findings, early studies in SCI described that preventing CSPG formation with xyloside treatment at the time of injury results in poor functional outcome, while manipulation of CSPGs at 2 days after SCI was beneficial for functional recovery (333). These differential outcomes were associated with the modulatory role of CSPGs in regulating the response of macrophages/microglia. Disruption in CSPG formation immediately after injury promoted an M1 pro-inflammatory

phenotype in macrophages/microglia, whereas delayed manipulation of CSPGs resulted in a pro-regenerative M2 phenotype (333). In EAE, by products of CSPG degradation also improve the outcomes by attenuating T cell infiltration and their expression of pro-inflammatory cytokines IFN-γ and TNFα (334).

These emerging findings suggest an important immunomodulatory role for CSPGs in CNS injury and disease; further investigations are needed to elucidate CSPG mechanisms in regulating neuroinflammation. Altogether, current evidence has identified a multifaceted inhibitory role for CSPGs in regulating endogenous repair mechanisms after SCI, suggesting that targeting CSPGs may present a promising treatment strategy for SCI.

CONCLUDING REMARKS

Traumatic SCI represents a heterogeneous and complex pathophysiology. While pre-clinical research on SCI has been an ongoing endeavor for over a century, our understanding of SCI mechanisms has been increased remarkably over the past few decades. This is mainly due to the development of new transgenic and preclinical animal models that has facilitated rapid discoveries in SCI mechanisms. Although SCI research has made an impressive advancement, much work is still needed to translate the gained knowledge from animal studies to clinical applications in humans.

AUTHOR CONTRIBUTIONS

AA, SD, and SK-A have all contributed to literature review and writing this manuscript. AA and SK-A contributed to the production of figures. All authors have approved the final version of the manuscript.

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The Young Male Syndrome—An Analysis of Sex, Age, Risk Taking and Mortality in Patients With Severe Traumatic Brain Injuries

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Higher risk taking is particularly characteristic for males between 15 and 35 years, the age when intrasexual competition is the strongest. This fitness-maximizing strategy, however, also has negative consequences; previous data revealed that males have a significantly higher tendency to die in accidents. This retrospective study aimed to assess whether age-related risk taking, often associated with the reproductive competition between males, and referred to as the Young Male Syndrome (YMS), may play a role in the high incidence of severe traumatic brain injury (sTBI) in young males. Derived from the available evidence and the main assumptions of the YMS, we expected that men, especially when they are in the age when their reproductive potential peaks, are more likely to suffer sTBI from highly risky behaviors that also lead to higher mortality. It was also expected that alcohol intoxication makes the demographic pattern of sTBI even more similar to what previous research on the YMS implies. We analyzed demographic data of patients with sTBI ($N = 365$) registered in a clinical database. To this end, we built Generalized Linear Mixed Models (GLMM) to reveal which of the demographic characteristics are the best predictors for risky behaviors leading to sTBI and death as a consequence of the injury. The data suggest that younger people acquired sTBI from riskier behaviors compared to members of older age groups, irrespective of their sex. Moreover, being male and being alcohol intoxicated also contributed significantly to risk-taking behavior. Mortality rate after the injury, however, increased with the age of the patient and did not depend on the riskiness of the behavior. The results indicate that the demographic distribution of the specific patient population in our focus cannot be simply explained by the YMS. However, higher incidence rates of males among the patients are in line with the core assumptions of the YMS. These data indicate that epidemiological studies should also take into consideration evolutionary theories and highlight the importance of age and sex specific prevention strategies.

Keywords: severe traumatic brain injury, young male syndrome, risk taking behavior, age groups, day-of-injury alcohol intoxication

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INTRODUCTION

Demographic Pattern of Risk Taking and Extrinsic Mortality

Males, compared to females, are characterized by more aggressive and competitive behavior in practically all cultures and ages (1–3). They tend to focus on temporary benefits and gains from their actions rather than long-term consequences and costs (4). Higher risk taking is particularly characteristic for the male population between the ages of 15–35 years, the time when intrasexual competition is the strongest. This pattern of risk taking is often referred to as Young Male Syndrome (YMS) (5). The higher willingness of males to engage in risky behavior also manifests in criminal acts. The analysis of law-breaking behavior in Detroit revealed that in robberies and assaults as well as in murders, both victims and offenders were primarily non-related, unemployed, young (18–40 years), single males (5). In a more recent study, Farsang and Kocsor (6) analyzed Hungarian and Australian homicide data to investigate whether the sex and age distributions of both parties correspond to the former findings. They found that both victims and criminal offenders were predominantly males but only the offenders belonged to the young age group (18–34 years) (6). Furthermore, a bulk of studies analyzed the association between dangerous or risky driving habits, and sex and age demonstrating that that young drivers (17–25 years), especially males, were involved in road traffic accidents more often than members of other age groups (7, 8).

Males also have a significantly higher tendency to pass away due to accidents than females, primarily due to the higher probability of being involved in dangerous situations (9–11). This sex difference in external mortality rates among young individuals might be the direct consequence of their fitness maximizing behavioral strategy, which has been suggested to be the ultimate cause of YMS (10).

Epidemiology of Severe Traumatic Brain Injury

This assumption is in concordance with the relatively larger propensity of young males among those who have suffered severe traumatic brain injury (sTBI) (8, 12). TBI represents a major epidemiological problem: the WHO estimates that at the end of this decade it will belong to the three most frequent causes of death (13). In the United Kingdom, one million people will be hospitalized yearly with injuries caused by head trauma (14). In the USA incidence of TBI is as high as 500,000 new cases per year which exceeds the cumulative incidence of stroke and epilepsy (15).

The mortality rate of young European people due to accidents was 17/100,000 cases in 2005. This proportion and the relative number of accidents (e.g., road traffic accidents, motor-vehicle crashes, falls/fallings) caused by alcohol intoxication is much higher among young adults (15–24 years) than in other age groups (8, 12, 16). In various statistical databases, the male/female ratio of TBI cases ranges from 3:1 to 5:1, with a peak age of 35–50 years (8). The burden of these injuries is also reflected in economic consequences as it is primarily affecting the young, active–predominantly male–population. The proportion of physical forces as well as the causes evoking and being responsible for TBI display a broad variation between countries worldwide, reflecting a clear tendency of increased occurrence of TBI in the elderly primarily related to falls. Nevertheless, epidemiological studies have demonstrated that road traffic accidents and interpersonal violence still should be considered as major causes of TBI particularly in the young (17, 18).

In Hungary, falls and auto-vehicular accidents are the leading causes of sTBI (19). In concordance with international epidemiological surveys (20), the most important risk factors associated with sTBI are the following: intoxication with alcohol or drugs, lack of protective devices, violation of traffic rules–particularly speed limits. These factors not only influence the occurrence of the injury but also affect morbidity and mortality, because they enhance the risk of cerebral hypoperfusion and hypoxia, which frequently leads to secondary brain damage. Alcohol intoxication, as one of the main factors associated with human risk taking–both as a cause and a result–, has been suggested to increase the occurrence of unhampered behaviors and risky driving maneuvers (21), the predisposition to violent activities (22), sexual aggression (23), and risky gambling (24).

Young Male Syndrome as an Evolutionary Framework to Explain Prevalence of sTBIs

The demonstration of the ability to overcome dangerous situations, and the prestige which derives from such victories, is attractive for females (25, 26). Males with a tendency to engage in such situations had an advantage in mating opportunities during human evolution and they still have one in modern societies (4, 5, 10). The evolutionary success of the risky fitness-maximizing male strategy, nevertheless, did not mean that it does not have negative consequences as well. In our retrospective study we sought to test whether the age when the reproductive competition between males peaks corresponds to the age when the incidence of severe injuries is the highest. By using a clinical database, we also assessed the riskiness of the underlying causes in the different age groups.

On the basis of the above-detailed etiology of sTBI, and taking into account that risk-taking propensity of males is the highest in adolescence and young adulthood (4), we predicted that males between the ages of 15–35 years acquire sTBI from riskier behaviors (*Hypothesis 1*), which also leads to higher mortality rates, compared to male members of other age groups and females at any age (*Hypothesis 2*).

We also expected that after acute alcohol intoxication, the aforementioned patterns of risk-taking behavior and mortality rates would be even more pronounced. Our prediction was that younger males (15–35 years) who consume alcohol on the day of injury could suffer sTBI from riskier behaviors, whereas intoxicated older males and females at any age suffer severe brain trauma from less risky activities (*Hypothesis 3*). We also predicted that the mortality rate will increase as a result of the riskier behavior in the group of young males (*Hypothesis 4*). To test whether the evolutionary explanation of the demographic

distribution of brain injuries was accurate, we wanted to compare different statistical models to determine which factors (age, sex, alcohol intoxication) contribute the most to risk-taking behavior and mortality.

METHODS

Subjects

The study group consisted of consecutive patients with sTBI ($N = 374$) registered with the Pecs Severe Traumatic Brain Injury Database, with the inclusion criteria of post-resuscitation GCS-score (Glasgow Coma Scale) <9. Data and information of patients were retrieved from the database in 2013. The data on age, sex, injury circumstances, and alcohol consumption were registered between 2002 and 2012. All experimental procedures were carried out with the permission and under the control of the Institutional Review Board of the University of Pecs (IRB number: IRB00003108).

Determination of Age Groups

The group of 374 patients (mean age at the time of injury: 54.0 years, $SD = 20.27$, between 1 and 92 years) with sTBI consisted of 90 females (mean age: 62.4, $SD = 21.81$) and 284 males (mean age = 51.3, $SD = 19.04$). The definition of being "young" varies across publications on YMS (e.g., 0–35, 18–40, etc.), however, we defined 4 age groups to approximate the classification of both evolutionary and clinical studies (see **Table 1**): *group 1* under 15 years, *group 2* between 15 and 35 years (target population according to our hypothesis), *group 3* between 36 and 65 years and *group 4* above 65 years. As the low number of patients under 15 precluded any valid statistical assessment, detailed analysis was performed over this age limit only, so the cohort in the analyses consisted of 365 patients.

Classification of Risk Level

We aimed to assess the degree of risk-taking behavior which led to severe brain injuries. University students ($N = 57$, 47 females, 10 males; mean age = 22.1, $SD = 4.81$) were recruited to judge the riskiness of injury-circumstances on a 5-point Likert-scale (1 = non-risky, 2 = slightly risky, 3 = moderately risky, 4 = considerably risky, 5 = highly risky). All students took part voluntarily. To promote the understanding of the task, we provided and discussed the definition of risk-taking propensity and a couple of examples of different risky activities leading to sTBI. Thus, the students could estimate the riskiness of the behavior behind the injuries, while avoiding mixing it up with the riskiness of the injury in a medical sense.

We defined risk-taking behavior as "a person is consciously seeking situations which are accompanied by *severe consequences*" (27, 28). Examples of the riskiest behaviors/situations are the following: driving a motor-vehicle in an alcohol- or drug intoxicated state; pursuing extreme sports or other dangerous sports (e.g., climbing); driving a motor vehicle at high speed and/or without using seat-belt/coveralls (e.g., helmet); being involved in violent activities (e.g., fights, assaults) in an alcohol- or drug intoxicated state; motorcycling on the roads.

Examples of the least riskiest behaviors/situations have been defined as involvement in an accident unintentionally/unaware of external factors (e.g., an object falls upon the head accidentally; being hit by a motor-vehicle as a pedestrian); unintentional/accidental falls, falling/crashes; falls on the ground/pathway/floor in an alcohol intoxicated state but without being involved in any other risk situation; falls caused by diseases (e.g., epilepsy).

The injury-circumstances varied widely. Accordingly, these examples only gave some direction to help the students make decisions about the degree of riskiness of injury-circumstances. Furthermore, we did not mention examples about some risk-taking behaviors such as gambling or unsafe sex because these were not relevant for our examination.

Since the sex ratio of the university students was not equal, we performed an independent samples *t*-test and a Pearson correlation using SPSS 20.0 to test whether this had any effect on the evaluation of riskiness considering the injury-circumstances. According to the results ($r = -0.021$; $p > 0.05$; $t = 0.155$; $p > 0.05$), the sex distribution did not affect the rating of riskiness of the injury-circumstances. The Cronbach's alpha of estimations given by the raters was 0.977. This is critical, because the very essence of the YMS is that young men have a higher threshold for evaluating an event as risky (29, 30), and, in general, they have higher impetus for sensation seeking (31). Because of that, the skewed distribution of men and women might have potentially distorted the content of the categories. Among the participants who rated the descriptions of events, there was no sign of the aforementioned pattern; both men and women had fairly the same subjective feelings about the riskiness of behaviors that were followed by sTBI.

Following the evaluations, three groups of riskiness were established with K-means Clustering (with SPSS 20.0): *Cluster 1* consisted of low risk injury-circumstances, *Cluster 2* included moderate risk injury-circumstances and *Cluster 3* contained high risk injury-circumstances (examples see in **Appendix 1**). The age distribution of sTBI patients in relation with the level of riskiness is detailed in **Table 2**.

TABLE 1 | Age distribution of patients enrolled.

	Males		Females		Sum	
	Frequency (number)	Mean age, Std. (year)	Frequency (number)	Mean age, Std. (year)	Frequency (number)	Mean age, Std. (year)
Under 15 years	5	$M = 7.4$ Std. = 4.92	4	$M = 12.2$ Std. = 2.87	9	$M = 9.5$ Std. = 4.66
15–35 years	56	$M = 24.9$ Std. = 5.77	7	$M = 23.5$ Std. = 6.47	63	$M = 24.7$ Std. = 5.81
36–65 years	149	$M = 51.4$ Std. = 8.64	29	$M = 50.7$ Std. = 8.77	178	$M = 51.3$ Std. = 8.64
Above 65 years	74	$M = 74.2$ Std. = 5.60	50	$M = 78.6$ Std. = 6.77	124	$M = 75.9$ Std. = 6.45

RESULTS

Determination of the Best Fitting Models

We prepared a series of Generalized Linear Mixed Models (GLMM, SPSS 24.0) (32) to assess which of the potential factors–sex, age, alcohol intoxication–, and the interactions among them, contribute the most to risk-taking behavior, and mortality (**Table 3**). First, our intention with the first two models was to decide whether we should include any random factors in the model. For Model 1 (**Table 4**), we used riskiness as the target variable with a multinomial probability distribution and generalized logit link function, age group, sex and alcohol intoxication as predictors, and year of injury as a random variable. For Model 2 (**Table 5**), we used the same target and predictor variables without any random variable. Both models were significant, but the higher Akaike Information Criterion (AIC) of Model 1 showed that this was not as good as Model 2. Hence, as the latter did not include any random variable, we did not incorporate random factors in the subsequent models.

Our strategy was to create models with all possible variables and interactions, then to omit those factors from the models which were the least significant, one after the other, until a significant model with significant predictors was determined. We started the iterations from four different models: Model 3 with riskiness as the target variable, and age groups and sex as predictors; Model 7 with mortality as the target variable, and age groups, sex and riskiness as predictors; Model 12 with riskiness as the target variable, and age groups, sex and alcohol intoxication as predictors; and Model 14 with mortality as the target variable, and age groups, sex, alcohol intoxication and riskiness as predictors. Thus, we ended up with 31 different models (see **Table 6**), from which seven significant models were appropriate for the evaluation of our hypotheses.

TABLE 2 | Incidence of sTBI according to sex, age and level of riskiness.

		Low risk	Moderate risk	High risk	SUM
Males	Under 15 years	3	1	1	5
	15–35 years	19	21	16	56
	36–65 years	66	54	29	149
	Above 65 years	46	22	6	74
Females	Under 15 years	1	3	0	4
	15–35 years	7	0	0	7
	36–65 years	21	6	2	29
	Above 65 years	46	4	0	50

TABLE 3 | Frequency of day-of-injury alcohol intoxication.

		Occurrence of alcohol intoxication among males and females according to age groups and riskiness			
		Low risk	Moderate risk	High risk	SUM
Males	Under 15 years	0	0	0	0
	15–35 years	0	3	9	12
	36–65 years	1	37	16	54
	Above 65 years	0	15	2	17
Females	Under 15 years	0	0	0	0
	15–35 years	0	0	0	0
	36–65 years	1	3	1	5
	Above 65 years	0	2	0	2

Risky Behavior as the Target Variable

Model 5 (**Table 7**) suggested that the interaction between sex and age group significantly predicted whether the brain injury was caused by highly risky, moderately risky, or non-risky behavior. However, the exponential coefficients were not significant, so it is not possible to draw precise inferences from this relation. In contrast, Model 6 (**Table 8**) showed that age group by itself was a significant predictor for the riskiness of behavior at the time of injury. More precisely, the significant exponential coefficients showed that if a patient's age is between 15 and 35, the chance is about ten times that s/he had suffered brain injury from a highly risky behavior rather than from a low risk behavior, and about three times that the behavior was moderately risky, compared to members of the 36–65 years age group. The relation was similar between the age group of 36–65 and the eldest group with about a five times higher chance for highly risky rather than non-risky behavior. There was no significant difference between highly risky and moderately risky behaviors between these groups.

Considering alcohol intoxication, the predictor variables like age group, sex, alcohol intoxication, and interactions between these resulted in a significant model (Model 13, **Table 9**). However, the value of the AIC is somewhat lower, therefore the model is better, if we eliminate the interactions from the model (Model 2). In this case, the exponential coefficients suggested that people in the youngest age group, in contrast to members of the 36–65 group, were ten times more likely to engage in high risk compared to low risk situations that led to sTBI. A similar, significant relation was found for males compared to females, and alcohol intoxicated compared to not intoxicated ones. A non-significant tendency was also present in the comparison of the 36–65 and the eldest age group, suggesting that members in the younger group had about a five times higher propensity for high risk vs. low risk behavior.

Mortality as the Target Variable

A significant model to predict the likelihood of death after sTBI can be built by including age group, riskiness, the interaction between age and riskiness, and the interaction between sex and riskiness in a GLMM (Model 10, **Table 10**). This model showed that patients in the 15–35 age group were about nine times more likely to survive than those in the 36–65 group, and the latter had about three times higher survival rates compared to the eldest patients. The exponential coefficients for the interaction between age group and riskiness showed that patients between 15 and 35 years had a three times higher chance for survival if the accident happened from a moderately or highly risky behavior compared to a low risk behavior. Neither coefficients for the age group-riskiness interaction, nor the fixed effects of sex and riskiness interaction, nor for fixed coefficients of riskiness were significant. The model had a better fit if we omitted sex-riskiness interaction (Model 11, **Table 11**). In this case, age group was the

TABLE 4 | Model 1 with *year of injury* as random variable and *riskiness* as target variable.

			F	df1	df2	*p*	Exp. Coefficient
Model fit	Akaike Corrected IC	3354.372					
	Accuracy	72.1%					
Fixed effects	Corrected model		8.566	8	355	**<0.001**	
	Age groups		4.219	4	355	**0.002**	
	Sex		6.560	2	355	**0.002**	
	Alcohol intoxication		21.581	2	355	**<0.001**	
Fixed coefficients (high-low/high-moderate)	Intercept					**<0.001/0.014**	189.520/8.881
	15–35					**<0.001/0.050**	0.097/0.325
	36–65					**0.022**/0.126	0.291/0.460
	65+						
	Male					**0.005**/0.302	0.104/0.437
	Female						
	Alcohol intoxicated					**<0.001**/0.902	0.008/1.045
	Not intoxicated						

Coefficients in blank rows are set to zero because these are redundant.
P-values in bold are significant on a $p < 0.05$ significance level.

TABLE 5 | Model 2 without random variables and *riskiness* as target variable.

			F	df1	df2	*p*	Exp. Coefficient
Model fit	Akaike Corrected IC	85.684					
	Accuracy	72.1%					
Fixed effects	Corrected model		5.760	8	355	**<0.001**	
	Age groups		2.854	4	355	**0.024**	
	Sex		4.417	2	355	**0.013**	
	Alcohol intoxication		14.494	2	355	**<0.001**	
Fixed coefficients (high-low/high-moderate)	Intercept					**<0.001/0.044**	189.682/8.885
	15–35					**0.001**/0.108	0.097/0.325
	36–65					0.059/0.210	0.291/0.460
	65+						
	Male					**0.021**/0.398	0.103/0.437
	Female						
	Alcohol intoxicated					**<0.001**/0.920	0.008/1.045
	Not intoxicated						

Coefficients in blank rows are set to zero because these are redundant.
P-values in bold are significant on a $p < 0.05$ significance level.

only significant fixed effect and fixed coefficient, showing that people in the youngest group had a nine times higher survival chance than those in the 36–65 group.

By including alcohol intoxication as a predictor for mortality, the best fitting GLMM was the one with the interaction between age group, sex, and alcohol intoxication (Model 26, **Table 12**). In the 15–35 age group, the survival chance of males after sTBI was about three times higher than that of females, if not intoxicated. Moreover, males in the 36–65 age group were five times more likely to survive if they were alcohol intoxicated on the day of the injury, compared to those who were not. The best model to predict mortality, however, was the one with only age group as a predictor variable (Model 31, **Table 13**), with exponential coefficients suggesting that the increase in age reduced the likelihood of survival.

DISCUSSION

Predictions of the YMS

First, it needs to be emphasized that the analyses we used in this paper did not test whether the demographic distribution of those who suffer *any* kind of accidents is in line with the general predictions of the YMS. A superficial look at the incidents rates (**Tables 2**, **3**) reveals that case numbers of females is less than that of males, suggesting that males are more likely to be involved in accidents (in this case accidents leading to sTBI). We did not test this statistically, on purpose, as the sample is not appropriate for

TABLE 6 | *P*-values of Variables and interactions of the best fitting GLM Models.

		Model 1[a]	Model 2	Model 5	Model 6	Model 10	Model 11	Model 13	Model 26	Model 31
Target variable		**Riskiness**	**Riskiness**	**Riskiness**	**Riskiness**	**Mortality**	**Mortality**	**Riskiness**	**Mortality**	**Mortality**
Model fit	Akaike Corrected IC	3354.372	85.684	60.803	39.824	71.200	52.024	88.066	56.976	21.212
Fixed effects	Corrected model	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	**0.003**	**<0.001**
	Age groups	0.002	**<0.001**		**<0.001**	**<0.001**	0.001	**<0.001**		**<0.001**
	Sex	0.002	0.024					**<0.001**		
	Alcohol intoxication	**<0.001**	0.013					**<0.001**		
	Riskiness					**0.014**	0.505			
	Age group × Riskiness					**0.030**	0.731			
	Sex × Riskiness					0.218				
	Age groups × Sex			**<0.001**				**<0.001**		
	Sex × Alcohol intoxication							**<0.001**		
	Age groups × Alcohol intoxication							**<0.001**		
	Age groups × Sex × Alcohol intoxication								**0.003**	

[a]*Model 1 includes year of injury as a random variable. Values in bold indicate significant effects ($p < 0.05$).*

TABLE 7 | Model 5 without random variables and *riskiness* as target variable.

			F	df1	df2	*p*	Exp. Coefficient
Model fit	Akaike Corrected IC	60.803					
	Accuracy	56.7%					
Fixed effects	Corrected model		3.434	10	353	**<0.001**	
	Age groups × Sex		3.434	10	353	**<0.001**	
Fixed coefficients (high-low/high-moderate)	Intercept					0.998/0.998	3993812637.025/347288055.393
	15–35 × Male					0.998/0.998	0.000/0.000
	15–35 × Female					1.000/1.000	1.249/0.000
	36–65 × Male					0.998/0.998	0.000/0.000
	36–65 × Female					0.998/0.998	0.000/0.000
	65+ × Male					0.998/0.998	0.000/0.000
	65+ × Female						

Coefficients in blank rows are set to zero because these are redundant.
P-values in bold are significant on a $p < 0.05$ significance level.

TABLE 8 | Model 6 without random variables and *riskiness* as target variable.

			F	df1	df2	*P*	Exp. Coefficient
Model fit	Akaike Corrected IC	39.824					
	Accuracy	56.2%					
Fixed effects	Corrected model		6.748	4	359	**<0.001**	
	Age groups		6.748	4	359	**<0.001**	
Fixed coefficients (high-low/high-moderate)	Intercept					**<0.001/0.001**	15.333/4.333
	15–35					**<0.001/0.034**	0.106/0.303
	36–65					**<0.001**/0.111	0.183/0.447
	65+						

Coefficients in blank rows are set to zero because these are redundant.
P-values in bold are significant on a $p < 0.05$ significance level.

TABLE 9 | Model 13 without random variables and *riskiness* as target variable.

			F	df1	df2	*p*	Exp. Coefficient
Model fit	Akaike Corrected IC	88.066					
	Accuracy	73.7%					
Fixed effects	Corrected model		161564623.983	18	345	**<0.001**	
	Age groups		135.814	4	345	**<0.001**	
	Sex		165.920	2	345	**<0.001**	
	Alcohol intoxication		2299285.297	2	345	**<0.001**	
	Age groups × Sex		4906046.901	4	345	**<0.001**	
	Age groups × Alcohol intoxication		115986 170.829	4	345	**<0.001**	
	Sex × Alcohol intoxication		314148 75.741	2	345	**<0.001**	
Fixed coefficients (high-low/high-moderate)	Intercept					**<0.001**/**<0.001**	3397396092.479/147712873.586
	15–35					0.967/**<0.001**	0.876/0.000
	36–65					**<0.001**/**<0.001**	0.000/0.000
	65+						
	Male					**<0.001**/**<0.001**	0.000/0.000
	Female						
	Alcohol intoxicated					**<0.001**/**<0.001**	0.000/2.424
	Not intoxicated						
	15–35 × Male					0.683/**<0.001**	0.270/107509406.062
	15–35 × Female						
	36–65 × Male					**<0.001**/**<0.001**	73856436.793/36792950.197
	36–65 × Female[a]						
	15–35 × Alcohol intoxicated					0.753/**<0.001**	0.116/0.030
	15–35 × Not intoxicated						
	36–65 × Alcohol intoxicated					**<0.001**/**<0.001**	74671103.801/0.413
	36–65 × Not intoxicated[a]						
	Male × Alcohol intoxicated					**<0.001/<0.001**	0.250/1.768
	Male × Not intoxicated[a]						

[a]*Rows with redundant coefficients were removed.*
Coefficients in blank rows are set to zero because these are redundant.
P-values in bold are significant on a $p < 0.05$ significance level.

a general test of the YMS. Instead, our focus was on more specific predictions about risk taking.

With the first hypothesis, we assumed that the younger males in our sample suffer sTBIs from riskier behaviors than older males and females of any age. However, the best fitting model which predicted riskiness included age group as the only significant independent variable (Model 6). Sex was not a significant factor, which contradicts previous observations in which males were found to be more risk taking than females (2, 7). This means that the distribution of patients in the three risk categories can be better explained by age, rather than by biological sex of the patients. As already noted, though the level of risk taking was not affected by sex, this should not conceal the fact that the incidence of females in the sample appears to be much lower than that of males (**Table 2**), prompting us to refrain from criticizing the basic insights of the YMS too sharply.

Mortality pattern, on the other hand, did not correspond at all to the expectations of YMS and contradicted our second hypothesis. That is, younger patients were more likely to survive after the accidents than older ones (Models 10, 11). It does, however, correspond to clinical experience and epidemiological data claiming that decline in regeneration ability and overall health with age makes death after a severe injury more likely (33, 34). More surprisingly, the interaction between age group and riskiness revealed that for younger people risky behavior even *decreases* the likelihood of death. We will discuss this in section Overcoming contradictions: Preparedness to danger in detail.

Nevertheless, we have to exercise caution with the interpretation of our results regarding the negative effect of aging on outcome. Specifically, this study was neither aimed to compare therapeutic decisions-, nor intended to assess the intensity of the treatment at various age groups. Likewise, the intent-to-treat issue was not analyzed either. Data on the effect of aging on outcome are controversial; some authors claim (35, 36) that–though cost-efficiency is relatively low–with higher therapy intensity similar outcome results can be achieved in the elderly, too. Similarly, a bulk of papers (34, 37, 38) point to the existence of a fatalistic approach in the treatment of elderly TBI that could actually work as a "self-fulfilling prophecy."

Effect of Alcohol Intoxication

By including alcohol intoxication into the model we aimed to address the question whether it increases the probability of suffering sTBI from a highly risky behavior. However,

TABLE 10 | Model 10 without random variables and *mortality* as target variable.

			F	df1	df2	*p*	Exp. Coefficient
Model fit	Akaike Corrected IC	71.200					
	Accuracy	64.7%					
Fixed effects	Corrected model		13.081	11	353	**<0.001**	
	Age groups		36.558	2	353	**<0.001**	
	Riskiness		4.347	2	353	**0.014**	
	Age groups × Riskiness		2.704	4	353	**0.030**	
	Sex × Riskiness		1.486	3	353	0.218	
Fixed coefficients (no-yes)	Intercept					0.999	0.000
	15–35					**<0.001**	0.115
	36–65					**0.005**	0.306
	65+						
	Low Risk					0.999	711218554.421
	Moderate Risk					0.999	473510614.812
	High Risk						
	15–35 × Low Risk					**0.036**	2.992
	15–35 × Moderate Risk					0.453	1.525
	15–35 × High Risk						
	36–65 × Low Risk					0.418	1.431
	36-65 × Moderate Risk					0.584	0.771
	36-65 × High Risk[a]						
	Male × Low Risk					0.341	1.140
	Female × Low Risk						
	Male × Moderate Risk					0.060	1.915
	Female × Moderate Risk						
	Male × High Risk					0.999	1021204875.082
	Female × High Risk						

[a] *Rows with redundant coefficients were removed.*
Coefficients in blank rows are set to zero because these are redundant.
P-values in bold are significant on a $p < 0.05$ significance level.

the categorization of the events in which the patients were injured was not fully independent of the information about alcohol intoxication itself. The description of the events included reference to the intoxicated state, therefore the independent raters who participated in the categorization task might have been biased to evaluate intoxicated patients' behavior as highly risky (see section Classification of risk level and **Appendix 1**). Hence, the best approach to consider the models prepared with the inclusion of alcohol intoxication might be that these models test the effect of alcohol on the subjective evaluation of riskiness, rather than on the increase in willingness to take risk. Keeping in mind that alcohol consumption itself is a risky behavior, and that other studies showed a direct effect of alcohol consumption on risk taking (21–24), the lack of full independency of this variable within the models does not affect the interpretation of our results crucially.

Referring back to the hypotheses, we expected that the demographic pattern of risk taking will correspond better with YMS if alcohol consumption is involved in the accident. In fact, it proved to be the only condition when the predictions of the YMS and the hypothesis (Hyp. 3, Models 13, 2) were fulfilled. Alcohol not only made the expected effect more pronounced, in fact it was a crucial variable which contributed to a model consisting of all the expected predictors (i.e., age group and sex) that explain riskiness of the behavior.

The fourth hypothesis, suggesting that mortality rates would be the highest for intoxicated men, had to be rejected. The significant interaction revealed by Model 26 suggests a quite different relation than proposed by the YMS. Men between 36 and 65 years old had higher chances to survive when they were intoxicated. Similar findings were also published by others (39). Experiments on animal models and clinical tests also suggest that low and moderate serum alcohol concentration prior to TBI could have a neuroprotective role (40). Some clinical data, including that of the current study, however, might be the result of a bias in classification of alcohol intoxicated patients; since their level of consciousness is much lower at the time of hospitalization, they might be falsely classified as having sTBI (41). Most of the studies related to alcohol misuse and its effect on post-traumatic life expectancy found a negative effect of intoxication (42, 43), especially when alcohol consumption was chronic (40). It is of note that due to local regulations and protocols serum alcohol levels have only been tested in a fraction of patients enrolled to this study.

Probably because of the confounding impact of alcohol on the recovery of sTBI patients, we obtained the most significant

TABLE 11 | Model 11 without random variables and *mortality* as target variable.

			F	df1	df2	*p*	Exp. Coefficient
Model fit	Akaike Corrected IC	52.024					
	Accuracy	64.7%					
Fixed effects	Corrected model		3.513	8	356	**0.001**	
	Age groups		7.047	2	356	**0.001**	
	Riskiness		0.685	2	356	0.505	
	Age groups × Riskiness		0.506	4	356	0.731	
Fixed coefficients (no-yes)	Intercept					0.424	2.000
	15–35					**0.046**	0.115
	36–65					0.172	0.275
	65+						
	Low risk					0.740	0.743
	Moderate risk					0.815	0.800
	High risk						
	15–35 × Low risk					0.337	3.087
	15–35 × Moderate risk					0.676	1.693
	15–35 × High Risk						
	36–65 × Low risk					0.615	1.647
	36–65 × Moderate risk					0.920 0.899	
	36–65 × High risk[a]						

[a] *Rows with redundant coefficients were removed.*
Coefficients in blank rows are set to zero because these are redundant.
P-values in bold are significant on a $p < 0.05$ significance level.

model for mortality if we eliminated all interactions and variables except age group (Model 31). In sum, the introduction of alcohol intoxication does not help to obtain a good fitting model for mortality after sTBI that could fit the YMS, though it leads to an expected model with respect to risk-taking behavior.

Overcoming Contradictions: Preparedness to Danger

The best fitting models to predict the riskiness of the behavior and mortality after the injury were models 6 and 31, respectively, both listing only age group as a significant predictor. While sex was also expected to predict risk-taking behavior, age negatively affected survival, quite the opposite of what the YMS suggests. At first sight, the fact that the best fitting models are not in line with the YMS could be detrimental to the theory. However, introducing the concept of *preparedness* could help resolve contradictions and fit the current data with previous findings.

Our explanation based on *preparedness* suggest that beside their riskier behavior men are also more prepared for the negative consequences of that behavior. Men's awareness of the riskiness of their own behavior might prevent at least some of the injuries, while women's relative inexperience in dangerous situations could result in a higher number of serious injuries when they are involved. Hence, the proportion of females who take high risks might be lower in the whole population, but they are over-represented in the patient population.

It is often argued that men, in general, have a higher tendency to take risks than women because of the difference in sensation seeking (31) and risk perception (29, 30). It was also pointed out that studies addressing sex differences often fail to overcome the methodological issue that men and women not only perceive the same risks somewhat differently, but also perceive different risks (29). This means that the inclination for risk taking is not always a result of the lack of awareness of the riskiness. In contrast, men sometimes engage in a situation *because* they know that it entails danger. A meta-analysis including 150 studies on risk taking revealed that the tendency for higher risk taking for men indeed exists, and this is not caused by men's underestimation of the riskiness of the situation. Males take more risks even when the possible negative consequences are obvious. Females, in contrast, restrict themselves from even fairly innocuous situations despite avoiding the positive outcomes (28).

The explanation above might also be applied to the results showing that patients between 15 and 35 years who suffer sTBI are more likely to die if the accident happened during a low risk situation. Those who are involved in a dangerous situation and recognize the risks might be able to mitigate the harmful consequences even if the accident happens. Thus, preparedness to danger, rather than risk avoidance, might prevent–or help someone recover from–sTBI. A substantial issue that should also be raised is how external help may increase preparedness. To this end,–and in light of the above observations–it is not sufficient to define overall, general preventive strategies. We should rather stratify our preventive actions tailoring them to the target audience, focusing on various age groups while also considering gender-related features.

The main cause of sTBI in our database (see **Table 14**) is a fall (48.8%), while the second major cause of sTBI in our cohort is represented by auto-vehicular accidents (18.4%) which happened more often among younger individuals (in 63.5% of all patients

TABLE 12 | Model 26 without random variables and *mortality* as target variable.

			F	df1	df2	*p*	Exp. Coefficient
Model fit	Akaike corrected IC	56.976					
	Accuracy	64.7%					
Fixed effects	Corrected model		2.687	10	354	**0.003**	
	Age groups × Sex × Alcohol intoxication		0.320	10	354	**0.003**	
Fixed coefficients (no-yes)	Intercept					0.153	1.526
	15–35 × Male × Alcohol intoxicated					0.999	0.000
	15–35 × Male × Not intoxicated					**0.013**	0.339
	15–35 × Female × Not intoxicated					0.132	0.262
	36–65 × Male × Alcohol intoxicated					**<0.001**	0.208
	36–65 × Male × Not intoxicated					0.070	0.519
	36–65 × Female × Alcohol intoxicated					0.119	0.164
	36–65 × Female × Not intoxicated					**0.015**	0.270
	65+ × Male × Alcohol intoxicated					0.908	0.936
	65+ × Male × Not intoxicated					0.918	1.042
	65+ × Female × Alcohol intoxicated					0.770	0.655
	65+ × Female × Not intoxicated[a]						

[a] *Rows with redundant coefficients were removed.*
Coefficients in blank rows are set to zero because these are redundant.
P-values in bold are significant on a $p < 0.05$ significance level.

TABLE 13 | Model 31 without random variables and *mortality* as target variable.

			F	df1	df2	*p*	Exp. coefficient
Model fit	Akaike Corrected IC	21.212					
	Accuracy	64.7%					
Fixed effects	Corrected model		12.656	2	362	**<0.001**	
	Age groups		12.656	2	362	**<0.001**	
Fixed coefficients (no-yes)	Intercept					**0.021**	1.531
	15-35					**<0.001**	0.241
	36-65					**<0.001**	0.358
	65+						

[a] *Rows with redundant coefficients were removed.*
Coefficients in blank rows are set to zero because these are redundant.
P-values in bold are significant on a $p < 0.05$ significance level.

between 15 and 35 years old). This is followed by crashes and falls from a height (15.6%). These data make necessary that as a note of caution we need to admit that currently we have no plausible ideas about how awareness of the riskiness actually might prevent injury in these situations and what might happen on the behavioral level during the accident. Theories on risk taking would benefit from future studies that aim at clarifying to what extent awareness of riskiness and preparedness to danger prevent or promote involvement in particular situations and how it affects injury severity.

LIMITATIONS

For interpretation of our results we should exercise some caution. Major limitations related to this study are associated with its retrospective nature, meaning that an independent cohort of healthy volunteers were required to assess the risk-taking behavior of the patients. Future studies should utilize prospective design and use tests which evaluate risk-taking attitudes and take pre-injury factors into account. Furthermore, we only focused on a subarea of risk-taking behavior, therefore risk perception and sensation seeking of the patient in our cohort were not addressed in this study. Therefore, we were unable to analyze the general attitudes and propensity behind the involvement in accidents, nevertheless we attempted to identify the riskiness of injury-circumstances. In the future this survey needs to be repeated with a new methodology, wherein acute, incoming patients with sTBI would be asked about injury-circumstances and administered relevant tests to assess risk taking and sensation seeking (e.g., Sensation Seeking Scale created by Zuckerman; Barratt Impulsivity Scale; Big Five–extraversion factor). Besides, as we focused on a specific population and on specific assumptions of

TABLE 14 | Clustering of injury circumstances according to age and gender.

			Clustering of injury circumstances						Total
			Motor-vehicle/road traffic accidents	Falls (on the ground)	Suicide (self-inflicted injuries)	Crashes, falls (from a height)	Violent activities (fights, physical abuses, assaults)	Other reasons (e.g., an object falls upon one's head)	
Male	Age groups	Under 15 years	2	0	0	0	1	2	5
		15–35 years	36	6	0	2	4	8	56
		36–65 years	14	77	8	26	10	14	149
		Above 65 years	6	48	4	14	0	2	74
	Total		58	131	12	42	15	26	284
Female	Age groups	Under 15 years	1	0	0	**2**	0	1	4
		15–35 years	4	0	0	1	0	2	7
		36–65 years	6	12	1	1	1	8	29
		65 years	1	35	0	13	0	1	50
	Total		12	47	1	17	1	12	90
Total	Age groups	Under 15 years	3	0	0	2	1	3	9
		15–35 years	40	6	0	3	4	10	63
		36–65 years	20	89	9	27	11	22	178
		Above 65 years	7	83	4	27	0	3	124
	Total		70	178	13	59	16	38	374

the YMS, we neither tested nor discussed the obvious assumption that males, in general, are represented in patient population in higher numbers than females.

CONCLUSION

The willingness of young males to engage in dangerous situations might be adaptive in terms of fitness maximization. Nonetheless, for some individuals this intense sexual competition can be detrimental to health. The correspondence between the age distribution of the reproductively most active population and those suffering sTBI only partially supports the evolutionary hypothesis about risk-taking behavior. The prevalence of higher external mortality rates of young males, on the other hand, was not present in our data at all, nor did we find any support for the assumption that sTBI acquired from riskier behavior would lead to higher risk of death. In contrast, in our dataset on risky behavior and even alcohol intoxication, the results seem to coincide with lower mortality rates after the injury.

The term YMS refers to a specific demographic pattern of risk-taking behavior. However, this phrasing has not been justified by our data, though our sample is not representative for the whole population, rather it consists of those who suffered serious brain injuries due to accidents. Our results contrast with other studies suggesting that YMS may explain the risk taking behavior and mortality pattern among patients with sTBI (17, 44, 45). However, we propose that men might be more prepared to prevent at least some of the injuries. This might distort the proportions of males and females in the patient population from the patterns expected from evolutionary insights. Still, we wish to highlight that it would be important to convey novel data to re-analyze the correspondence between sex differences in risk-taking behavior and mortality, and the YMS. The adherence to the conclusions in the original work of Wilson and Daly (5) may result in a publication bias, wherein conflicting findings are less likely to be reported. Future research on the relation between risk awareness, risk experience, and preparedness to danger may not only help to form new explanations about the demographic patterns in risk taking and its negative outcomes, but might also open up novel strategies in injury prevention and help reduce the incidence of TBI.

ETHICS STATEMENT

All sensitive personal data of the subjects (e.g., name; address) were anonymized before the retrospective data collection and analysis–consequently all the evaluators of the Likert scale and those who involved in the statistical analysis were blinded to them. All experimental procedures were carried out with the permission and under the control of the Institutional Review Board of University of Pecs (IRB number: IRB00003108). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1,964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

AUTHOR CONTRIBUTIONS

VT: statistical analyses, contributions to the design, and interpretation of data for the work. FK: statistical analyses, revising the content, and interpretation of data. PG: contributions to the conception, revising the content, and interpretation of data. NK and EC: contributions to the conception, and revising the content. AB: provide approval for publication, contributions to the conception, and revising the content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00366/full#supplementary-material

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REVIEW
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Blood Biomarkers for Traumatic Brain Injury: A Quantitative Assessment of Diagnostic and Prognostic Accuracy

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Blood biomarkers have been explored for their potential to provide objective measures in the assessment of traumatic brain injury (TBI). However, it is not clear which biomarkers are best for diagnosis and prognosis in different severities of TBI. Here, we compare existing studies on the discriminative abilities of serum biomarkers for four commonly studied clinical situations: detecting concussion, predicting intracranial damage after mild TBI (mTBI), predicting delayed recovery after mTBI, and predicting adverse outcome after severe TBI (sTBI). We conducted a literature search of publications on biomarkers in TBI published up until July 2018. Operating characteristics were pooled for each biomarker for comparison. For detecting concussion, 4 biomarker panels and creatine kinase B type had excellent discriminative ability. For detecting intracranial injury and the need for a head CT scan after mTBI, 2 biomarker panels, and hyperphosphorylated tau had excellent operating characteristics. For predicting delayed recovery after mTBI, top candidates included calpain-derived αII-spectrin N-terminal fragment, tau A, neurofilament light, and ghrelin. For predicting adverse outcome following sTBI, no biomarker had excellent performance, but several had good performance, including markers of coagulation and inflammation, structural proteins in the brain, and proteins involved in homeostasis. The highest-performing biomarkers in each of these categories may provide insight into the pathophysiologies underlying mild and severe TBI. With further study, these biomarkers have the potential to be used alongside clinical and radiological data to improve TBI diagnostics, prognostics, and evidence-based medical management.

Keywords: traumatic brain injury, TBI, concussion, diagnosis, prognosis, biomarker, biomarkers

INTRODUCTION

Traumatic brain injury (TBI) is a common cause of disability and mortality in the US (1) and worldwide (2). Pathological responses to TBI in the CNS include structural and metabolic changes, as well as excitotoxicity, neuroinflammation, and cell death (3, 4). Fluid biomarkers that may track these injury and inflammatory processes have been explored for their potential to provide objective measures in TBI assessment. However, at present there are limited clinical guidelines available regarding the use of biomarkers in both the diagnosis of TBI and outcome prediction following TBI. To inform future guideline formulation, it is critical to distinguish between different clinical situations for biomarker use in TBI, such as detection of concussion, prediction of positive and negative head computed tomography (CT) findings, and prediction of outcome for different TBI severities. This allows for comparisons to determine which biomarkers may be used most appropriately to characterize different aspects of TBI.

The identification of TBI severity has become a contentious issue. Currently, inclusion in TBI clinical trials is primarily based on the Glasgow Coma Scale (GCS), which stratifies patients into categories of mild, moderate, and severe TBI. The GCS assesses consciousness and provides prognostic information, but it does not inform the underlying pathologies that may be targeted for therapy (5, 6). Furthermore, brain damage and persistent neurological symptoms can occur across the spectrum of TBI severity, limiting the use of GCS-determined injury severity to inform clinical management. Biomarkers in TBI have the potential to provide objective and quantitative information regarding the pathophysiologic mechanisms underlying observed neurological deficits. Such information may be more appropriate for guiding management than initial assessments of severity alone. Since the existing literature primarily focuses on applications of biomarkers in either suspected concussion, mild TBI (mTBI), or severe TBI (sTBI), we will discuss biomarker usage in these contexts.

Concussion is a clinical syndrome involving alteration in mental function induced by head rotational acceleration. This may be due to direct impact or unrestrained rapid head movements, such as in automotive crashes. Although there are over 30 official definitions of concussion, none include the underlying pathology. Missing from the literature have been objective measures to not only identify the underlying pathology associated with the given clinical symptoms, but also to indicate prognosis in long-term survival. Indeed, current practices in forming an opinion of concussion involve symptom reports, neurocognitive testing, and balance testing, all of which have elements of subjectivity and questionable reliability (7). While such information generally reflects functional status, it does not identify any underlying processes that may have prognostic or therapeutic consequences. Furthermore, because patients with concussion typically present with negative head CT findings, there is a potential role for blood-based biomarkers to provide objective information regarding the presence of concussion, based on an underlying pathology. This information could inform management decisions regarding resumption of activities for both athletes and non-athletes alike.

Blood-based biomarkers have utility far beyond a simple detection of concussion by elucidating specific aspects of the injury that could drive individual patient management. For example, biomarkers may aid in determining whether a mTBI patient presenting to the emergency department requires a CT scan to identify intracranial pathology. The clinical outcome for a missed epidural hematoma in which the patient is either discharged or admitted for routine observation is catastrophic; 25% are left severely impaired or dead (8). The Canadian CT Head Rule (9) and related clinical decision instruments achieve high sensitivities in predicting the need for CT scans in mild TBI cases. However, they do this at specificities of only 30–50% (10). Adding a blood biomarker to clinical evaluation may be useful to improve specificity without sacrificing sensitivity, as recently suggested (11). In addition, given concern about radiation exposure from head CT scans in concussion cases, particularly in pediatric populations, identification of patients who would be best assessed with neuroimaging is crucial. Thus, the use of both sensitive and specific biomarkers may serve as cost-effective tools to aid in acute assessment, especially in the absence of risk factors for intracranial injury (12). S-100B, an astroglial protein, has been the most extensively studied biomarker for TBI thus far and has been incorporated into some clinical guidelines for CT scans (13, 14). However, S-100B is not CNS-specific (15, 16) and has shown inconsistent predictive capacity in the outcome of mild TBI (17, 18). Given that several other promising biomarkers have also been investigated in this context, it is important to evaluate and compare the discriminative abilities of S-100B with other candidate blood-based biomarkers for future use.

Blood biomarkers also have the potential to help predict unfavorable outcomes across the spectrum of TBI severity. Outcome predication is difficult; in mTBI, existing prognostic models performed poorly in an external validation study (19). Identifying biomarkers that best predict delayed recovery or persistent neurological symptoms following mTBI would help with the direction of resources toward patients who may benefit most from additional rehabilitation or prolonged observation. In sTBI, poorer outcome has often been associated with a low GCS score (20). However, factors such as intoxication or endotracheal intubation may make it difficult to assess GCS reliably in the acute setting (21, 22). The addition of laboratory parameters to head CT and admission characteristics have improved prognostic models (23). Thus, prognostic biomarkers in sTBI could help determine whether patients are likely to benefit from intensive treatment. Several candidate biomarkers that correlate with various pathologies of mild and severe TBI have been studied (24), but their relative prognostic abilities remain unclear.

Existing reviews on biomarkers in TBI have provided valuable insight into the pathologic correlates of biomarkers, as well as how biomarkers may be used for diagnosis and prognosis (25–31). However, there has been no previous quantitative comparison of the literature regarding biomarkers' discriminative abilities in specific clinical situations. Here, we compare existing studies on the discriminative abilities of serum biomarkers for four commonly studied clinical situations:

detecting concussion, predicting intracranial damage after mTBI, predicting delayed recovery after mTBI, and predicting adverse outcome after sTBI.

MATERIALS AND METHODS

Categories

There has been substantial confusion about the role of blood-based biomarkers in TBI. Therefore, we chose four scenarios in which blood biomarkers might be considered most helpful:

1. To document whether a concussion has occurred, especially when the history is unclear. This might be most useful for professional athletes and military service members, for whom decisions to return to play or to combat could have serious consequences. This assessment relied on individual authors to define concussion, as no single gold standard definition exists.
2. To predict intracranial damage after mTBI (GCS 13–15). This could help decide whether or not a CT scan is indicated to identify occult intracranial lesions with potentially catastrophic consequences.
3. To predict delayed recovery after mTBI (GCS 13–15). This might help direct early rehabilitation therapy to patients at risk of a poor outcome. It could also serve to select these patients as clinical study subjects to evaluate treatment efficacy. This assessment allowed individual authors to define recovery given the variety of clinically relevant endpoints.
4. To predict outcome after sTBI (GCS $\leq$8). This might help alert the healthcare team in cases in which intensive treatment is either helpful or futile, as well as providing prognostic information to the patient's family.

Although several other potential uses of biomarkers have been suggested, we thought these four categories were the most useful clinically and had been covered most thoroughly in the literature. We omitted analysis of publications in which the outcome categories did not conform to the four categories or were unclear. We also elected to limit our analysis to biomarkers measured in peripheral blood and exclude reports of measurements done on CSF, brain tissue, urine, etc. Studies which reported results obtained too long after injury to be of predictive value were not included in the analysis. Cutoff points were 48 h for Category 1, 24 h for Category 2, 72 h for Category 3 and 7 days for Category 4.

Literature Search

We conducted a search of Medline, Embase and the Cochrane library for reports of biomarkers of TBI published in English up to July 2018. The search strategy was limited to articles which included the medical subject headings of both "head injury" and "brain injury," along with either "biomarker(s)" or "marker(s)" in the text. Additional articles were obtained from the bibliographies of selected reports and from the "Similar articles" feature of PubMed. Abstracts limited to animal studies or to samples other than blood were excluded. All other articles were downloaded and reviewed by at least two authors (SS, ZG, KG, DM, LG).

Data Management and Analysis

Data abstracted from each report included TBI category, biomarker(s) measured, time(s) after injury, number of observations, cut point (point dividing positive from negative tests), sensitivity, specificity, area under the receiver operating curve (AUC) (32), any additional features reported (injury mechanism, age of subjects, outcome measured, etc.). If the TBI cohort of a given study was of mixed severity, and at least 70% of the patients met the severity criteria for a certain category, then the study was assigned to that category. Series which included adverse GOS scores were included in the severe injury category, even if fewer than 70% of reported cases had sTBI. For each set of observations, we calculated the AUC if not already provided. We also calculated the Youden J-statistic (33), another measure of diagnostic accuracy. A detailed discussion of diagnostic accuracy is given in the **Supplementary Appendix**.

If multiple reports dealt with the use of the same biomarkers to predict the same outcome, we pooled the data to obtain a single measure. For the AUC, we used a random-effects, inverse variance-weighted meta-analytic model to pool values (34). Since only the maximum J-statistic is used to report on a series of sensitivity/specificity values, we chose only the highest J-statistic measurement for each biomarker. We compared reported biomarkers with how well they predicted outcomes in a given category. We used a previously proposed semi-quantitative scale (35) to rate the accuracy of tests from their AUC's. An AUC above 0.9 is considered excellent, with decreasing intervals of 0.1 through "good," "fair" and "poor." An AUC below 0.6 is graded a "fail."

A number of studies reporting mean biomarker levels were excluded if it was not possible to calculate operating characteristics from the published data. Other reports were excluded for reporting biomarker levels only as combinations of multiple markers or trajectories of a single marker over time.

RESULTS

Literature Search

Our search yielded 2,015 publications, of which 1,034 abstracts were omitted as being unlikely to provide useful data. The remaining 981 articles were downloaded and reviewed. We excluded 233 reviews, editorials, letters to the editor, duplicate reports and other publications containing no original data. Also excluded were 162 case series limited to tissue other than blood (CSF, brain tissue, etc.), 40 reports containing fewer than 10 observations and 346 reports not relevant to the four outlined scenarios or from which operating characteristics could not be calculated. Included for analysis were 200 publications, encompassing a total of 61,722 observations.

The flow chart of the study selection process is shown in **Figure 1**. Included studies are listed by category in **Table S1** (**Supplementary Appendix**), along with the biomarker tested and the number of observations. It should be noted that several reports are listed more than once, owing to their reporting on multiple biomarkers or multiple scenarios. An alphabetical list of abbreviations for biomarkers reported in the tables and the remainder of the manuscript is shown in **Table 1**.

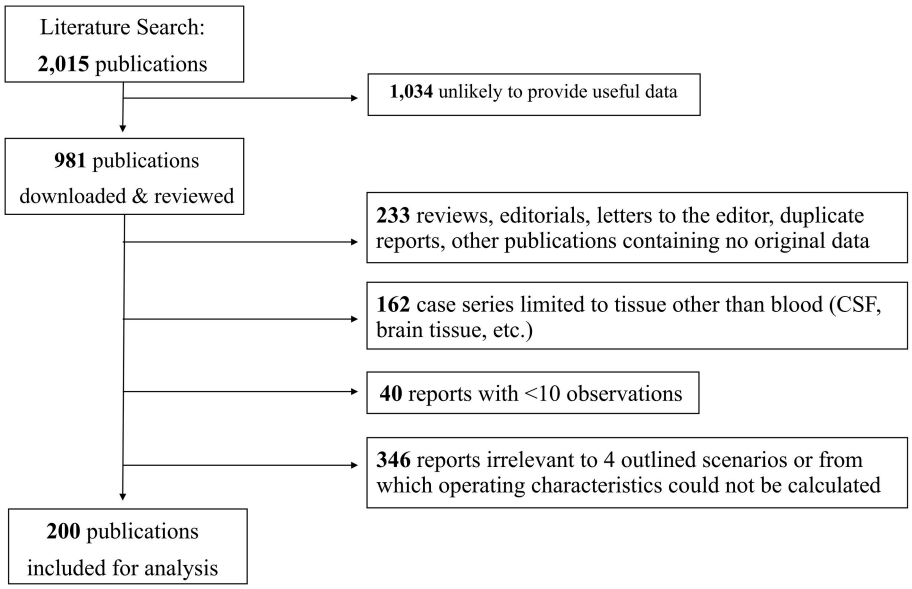

FIGURE 1 | Flow chart of the study selection process.

Analyses

Category 1 (Document Concussion)

There were 9 unique publications, documenting 15 biomarkers and containing a total of 946 observations. Several but not all authors defined concussion based on the 2012 Concussion in Sports Group guidelines (36) or the 2011 Team Physician Consensus Statement (37). **Table 2** shows the pooled values for AUC and the maximum J-statistic obtained for each. Four biomarker panels (copeptin, galectin-3, and MMP-9; GFAP and UCH-L1; 10 metabolites; and 17 metabolites) are in the "excellent" range (AUC≥0.9). The AUC for copeptin, CKBB, and a 10-metabolite panel are also "excellent," and 3 other biomarkers, galectin 3, MMP-9, and occludin rate as "good" (AUC = 0.80➔0.89). However, the observations are few, and no study has been independently verified.

Category 2 (Need for CT Scan After mTBI)

There are 56 publications and 23,316 observations of 24 biomarkers in this category. As shown in **Table 3**, a single report shows excellent operating characteristics for two panels of biomarkers (MMP-2, CRP, and CKBB; UCH-L1 and GFAP), as well as for phospho tau (P-tau) and its ratio with total tau (P-tau/T-tau ratio). The UCH-L1/GFAP panel and P-tau also have excellent J-statistics. The AUC values for GFAP/GFAP-BDP and D-Dimer are in the "good" range; the excellent J-statistic for GFAP/GFAP-BDP is aided by a high specificity. S-100B protein, the most studied biomarker in this category, performs only in the fair category (AUC=0.70➔0.79).

Category 3 (Delayed Recovery After mTBI)

There are 44 publications reporting results of 29 biomarkers in 13,291 observations. Most but not all authors defined delayed recovery as post-concussive syndrome (PCS) at various time points after injury (notably, there is current debate regarding the term "PCS"). As shown in **Table 4**, small studies suggest that ghrelin, glucose, NFL, SNTF, and A-tau have AUC values in the "good" range and show promise for predicting mTBI patients who can be expected to suffer prolonged neurobehavioral or post-concussive symptoms. More commonly-studied biomarkers, such as GFAP, S-100B, NSE, and UCH-L1, have fair to poor discriminating ability.

Category 4 (Poor Outcome After sTBI)

In this category, 85 publications reported 23,442 observations of 59 different biomarkers. As shown in **Table 5**, several biomarkers had "good" ability to predict death, severe disability or other adverse outcomes after sTBI. They include ceruloplasmin, copeptin, D-Dimer, ficolin-3, galectin-3, gelsolin, H-FABP, HMGB1, icORP, IL-1beta, −6 and −8, leptin, MBL, MBP, MIF, NFM, periostin, RDW, S100A12, SCUBE1, SuPAR, TAC, tenascin-C, thrombospondin-1, and T-tau. However, numbers of observations are small, and independent verification is lacking.

DISCUSSION

We have identified leading candidate biomarkers potentially useful for four clinical purposes in TBI, as determined by the highest pooled AUC and J-statistic from the existing data. **Figure 2** provides a visual overview of the candidate biomarkers' anatomical locations. These biomarkers have the potential to be used not as stand-alone diagnostic or prognostic tests, but rather alongside clinical and radiological data in the collective process of forming a clinical decision. In particular, in the absence of acute or chronic behavioral changes, excessive

TABLE 1 | Abbreviations used for biomarkers.

Abbreviation	Full name
A-beta-42	Amyloid beta peptide
A-Tau	Tau-protein A
BDNF	Brain-derived neurotrophic factor
BMX	Tyrosine kinase
CKBB	Creatine kinase B type
CRP	C-reactive protein
C-Tau	Tau-protein C
FDP	Fibrin degradation products
GFAP/GFAP-BDP	Glial fibrillary acidic protein (breakdown products)
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSH	Glutathione
H-FABP	Heart-fatty acidic binding protein
HMGB1	High-mobility group box 1 gene
Hsp70	Heat shock protein
ICAM-1,-5	Intercellular adhesion molecule-1 and−5
icORP	Capacity for induced oxidative stress
IL-1beta, -6, -10	Interleukins
INR	International normalized ratio
LGALS3	Galectin 3
MBL	Mannose-binding lectin
MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein
MDA-LDL	Malondialdehyde modified low density lipoprotein
MIF	Macrophage migration inhibitory factor
MMP-2 -,9	Matrix metalloproteinase-2 and−9
MT3	Metallothionein 3
NCAM	Neuron cell adhesion molecule
NF-H	Hyperphosphorylated neurofilament
NFL	Neurofilament light
NFM	Neurofilament medium
NRGN	Neurogranin
NSE	Neuron-specific enolase
OCLN	Occludin
pNF-H	Phosphorylated neurofilament heavy protein
PRDX-6	Peroxiredoxin
PTT	Partial thromboplastin time
P-Tau	Hyperphosphorylated tau
RDW	Red cell distribution width
S-100A1B, -A12, -B	S-100 calcium-binding proteins
SCUBE1	Signal peptide-cub-egf domain-containing protein-1
SNTF	Calpain-derived αII-spectrin N-terminal fragment
SuPAR	Soluble urokinase plasminogen activator receptor
sVCAM-1	Soluble vascular cell adhesion molecule-1
TAC	Total antioxidant capacity
TIMP-1	Tissue inhibitor of matrix metalloproteinase 1
T-Tau	Total tau
UCH-L1	Ubiquitin C-terminal hydrolase
VWF	Von Willebrand factor

TABLE 2 | Presence of Concussion.

Biomarker	#Reports	#Observations	Pooled AUC	Maximum J-statistic
CKBB	1	18	**0.902**	0.602
copeptin	1	55	**0.922**	0.766
GFAP	2	238	0.533	0.030
LGALS3	1	55	**0.849**	0.508
MMP9	1	55	**0.846**	0.655
OCLN	1	55	**0.836**	0.562
panel (10 metabolites)	1	10	**0.976**	0.778
panel (17 metabolites)	1	29	**0.910**	0.76
panel(copeptin, LGALS3, MMP-9)	1	55	**0.968**	0.79
panel(GFAP, UCH-L1)	1	206	**0.940**	
panel(UCH-L1, S-100B)	1	32	0.750	
S-100B	2	108	0.680	0.441
SNTF	1	28	0.760	0.550
T-Tau	1	28	0.740	0.303
A-Tau	1	28	0.750	0.500
C-Tau	1	28	0.711	0.422
Ubiquitin	1	206	0.670	
UCH-L1	1	32	0.740	0.500

*Best results are indicated in boldface. Blank cells = either no data reported in original publication, or pooled AUC was considered too low (below "good" range, <0.80) to calculate J-statistic. See **Table 1** for abbreviations.*

focus on biomarker values may lead to unnecessary testing with negative psychological and economic consequences. Blood biomarkers offer potentially valuable objective information that may augment rather than replace existing tools for clinical assessment and contribute to a holistic approach to management.

Category 1 (Document Concussion)

Two single biomarkers had excellent operating characteristics (AUC>0.9) for documenting concussion. Copeptin, the C-terminal part of the arginine vasopressin (AVP) prohormone, is thought to reflect the hypothalamic pituitary adrenal axis activity as part of the stress response, and serum levels increase in proportion to TBI severity (38, 39). CKBB is an intracellular enzyme that catalyzes the phosphorylation of creatine to phosphocreatine as part of cellular energy homeostasis and is primarily found in oligodendrocytes, which may be due to large energy requirements in these cells (40). The good performance of these biomarkers suggests that both stress axis activation and cellular damage within specific brain areas are involved in the pathophysiology of concussion.

Three other biomarkers, galectin 3, MMP-9, and occludin, had good operating characteristics (AUC = 0.80–0.89) for detecting concussion based on a single study (ref. S17 in **Supplementary Appendix**) while the combination of all 3 yielded an excellent operating characteristic. Galectin-3, a beta–galactoside-binding lectin, was previously found to be expressed in activated microglia after diffuse axonal injury (DAI) (41). MMP-9, a matrix metalloprotease that is expressed in humans early after TBI (42), modifies the brain extracellular matrix and

TABLE 3 | Mild TBI—need for CT scan.

Biomarker	#Reports	#Observations	Pooled AUC	Maximum J-statistic
A-beta-42	1	46	0.689	
BDNF	1	159	0.670	0.839
CKBB	1	92	0.714	
CRP	1	92	0.698	
d-Dimer	2	93	**0.890**	0.669
GFAP/GFAP-BDP	16	2040	**0.831**	0.936
GM-CSF	1	92	0.432	
H-FABP	2	264	0.641	0.293
IL-10	1	133	0.646	0.318
MDA-LDL	1	92	0.497	
MMP-2	1	92	0.616	
MT3	1	306	0.590	
NF-H	1	68	0.717	0.575
NFM	1	52	0.605	0.211
NRGN	1	494	0.510	
NSE	5	844	0.798	0.690
panel(MMP-2, CRP, CKBB)	1	110	**0.964**	0.7190
panel(UCH-L1, GFAP)	1	1947	**0.986**	0.9710
S-100B	30	8464	0.723	0.580
P-Tau	2	350	**0.921**	0.944
T-Tau	6	176	0.666	0.440
P-Tau/T-Tau ratio	2	350	**0.923**	0.816
Ubiquitin	2	302	0.710	0.210
UCH-L1	5	3108	0.700	0.470

*Best results are indicated in boldface. Blank cells = either no data reported in original publication, or pooled AUC was considered too low (below "good" range, <0.80) to calculate J-statistic. See **Table 1** for abbreviations.*

TABLE 4 | Mild TBI—delayed recovery.

Biomarker	#Reports	#Observations	Pooled AUC	Maximum J-statistic
BDNF	1	299	0.585	
BMX	1	63	0.760	0.400
CRP	1	846	0.615	0.330
GFAP	17	1959	0.716	0.850
Ghrelin	1	118	**0.829**	0.659
GSH	1	88	0.773	0.514
ICAM-1	1	118	0.485	
IL-6	1	118	0.535	
IL-8	1	118	0.615	
NCAM	1	118	0.614	
Neuroglobin	1	34	0.682	
NFL	1	35	**0.82**	0.79
Nogo-A	1	34	0.754	
NSE	6	543	0.685	0.691
pNF-H	1	118	0.614	
S-100B	24	2800	0.691	0.810
E-selectin	1	118	0.600	
SNTF	2	73	**0.863**	0.750
regulatory T cells	1	40	0.592	
Testosterone	1	181	0.684	0.786
VCAM-1	2	186	0.654	0.481
A-Tau	1	56	**0.87**	0.77
C-Tau	1	56	0.59	0.28
P-Tau	1	134	0.663	0.350
T-Tau	5	335	0.640	0.863
P-Tau/T-Tau ratio	1	134	0.658	0.300
UCH-L1	7	3158	0.787	0.740

*Best results are indicated in boldface. Blank cells = either no data reported in original publication, or pooled AUC was considered too low (below "good" range, <0.80) to calculate J-statistic. See **Table 1** for abbreviations.*

leads to cerebral edema and disruption of blood-brain barrier (BBB) integrity following TBI (43, 44). OCLN is a regulatory protein at the tight junctions of the BBB that correlates with increased resistance and decreased permeability of the BBB (45). While these findings identify osmotic dysregulation, BBB disruption, cerebral edema, and DAI as potential pathologic correlates of concussion, conclusions regarding clinical utility are limited by the relatively small sample size and lack of independent verification. Furthermore, concern has been raised about the authors' limited characterization of the control group and its subsequent impact on their conclusions (46).

The superior performance of other biomarker panels in this category reflects the multifaceted pathophysiology associated with concussion. These panels appear to successfully gather data about different mechanisms of injury to maximize sensitivity and specificity. The combination of GFAP and UCH-L1, two biomarkers thought to reflect focal mass lesions and diffuse injuries, respectively, also performed at the "excellent" level (ref. S11). However, the combination of UCH-L1 and S-100B only had fair performance, reflective of the poor individual performance of the nonspecific marker S-100B. Incorporation of higher-performing individual biomarkers, such as copeptin and CKBB, into panels may be useful to study in the future. Metabolite panels demonstrated also excellent operating characteristics; some metabolites are thought to reflect altered brain energy metabolism and mitochondrial dysfunction in TBI (47). However, the use of a metabolite panel is limited by variability of specific metabolites used across studies (refs. S10, S12). Given the limitations of these single, small studies, further verification is warranted to identify the best candidate serum biomarkers for a panel to objectively detect concussion.

Category 2 (Need for CT Scan After mTBI)

It should be noted that several clinical decision rules are available to predict the need for CT scan in mild TBI. These rules have near 100% sensitivity. However, their specificities are low (10), resulting in roughly 50% negative CT scans in those patients predicted to need them. A recent report (48) demonstrated that GFAP and UCH-L1 levels were no higher in patients with mild TBI and negative CT scans than in patients with orthopedic but not head injuries. This suggests that low GFAP and/or UCH-L1 levels may be useful in reducing unnecessary CT scans for mild TBI.

TABLE 5 | Severe TBI—adverse outcomes.

Biomarker	#Reports	#Observations	Pooled AUC	Maximum J-statistic
Adiponectin	1	86	0.785	0.604
Base deficit	1	216	0.479	
BDNF	'	170	0.482	
Caspase-Cleaved Cytokeratin-18	1	100	0.685	0.370
ceruloplasmin	1	20	**0.800**	0.600
Cholinesterase	1	188	0.381	0.519
Copeptin	4	422	**0.825**	0.635
Copper	1	20	0.795	0.590
D-Dimer	2	226	**0.819**	0.895
DNA	2	106	0.694	0.430
FDP	1	1266	0.755	0.426
Ferritin	1	69	0.585	0.170
Fibinogen	1	1266	0.712	0.382
Ficolin-3	1	384	**0.823**	0.619
Galectin-3	1	300	**0.808**	0.553
Gelsolin	2	322	**0.805**	0.679
GFAP	10	2448	0.749	0.800
H-FABP	1	49	**0.840**	0.680
HMGB1	1	106	**0.882**	0.657
Hsp70	1	20	0.750	0.500
ICAM-1	1	13	0.498	0.222
ICAM-5	1	170	0.544	
icORP	1	104	**0.870**	0.333
IL-1beta	1	28	**0.871**	0.800
IL-6	3	337	**0.840**	0.840
IL-8	1	20	**0.835**	0.67
IL-10	1	426	0.550	0.265
INR	1	1266	0.738	0.394
Leptin	1	284	**0.875**	0.649
MBL	1	244	**0.832**	0.562
MBP	2	127	**0.831**	0.875
MCP-1	1	170	0.677	
MDA	1	100	0.760	0.370
MIF	1	216	**0.817**	0.547
MMP-9	1	88	0.585	0.340
Nesfatin	1	300	0.786	0.487
NF-H	2	200	0.760	0.552
NFL	1	70	0.700	0.390
NFM	1	12	**0.857**	0.714
NSE	9	911	0.715	0.905
Periostin	1	130	**0.815**	0.506
Platelet count	1	1266	0.618	0.201
PRDX-6	1	170	0.524	
PTT	1	1266	0.748	0.410
RDW	1	122	0.693	0.611
S-100A1B	1	59	0.677	0.3
S100A12	1	306	**0.855**	0.630
S-100B	25	3712	0.762	0.880
SCUBE1	1	113	**0.831**	
Substance P	1	100	0.700	0.360
SuPAR	1	78	**0.801**	0.363
TAC	1	100	**0.830**	0.410
Tenascin-C	1	216	**0.827**	0.590
Thioredoxin	1	216	0.798	0.549
thrombospondin-1	1	402	**0.827**	0.619
TIMP-1	1	100	0.645	0.290
T-Tau	6	344	**0.818**	0.833
UCH-L1	5	195	0.696	0.54
VWF	1	44	0.660	0.32

*Best results are indicated in boldface. Blank cells = no data reported in original publication; pooled AUC was considered too low (below "good" range, <0.80) to calculate J-statistic. See **Table 1** for abbreviations.*

While the current study shows that operating characteristics were good for GFAP and its breakdown products and poor for UCH-L1, the use of both biomarkers in combination had excellent discriminative ability for identifying CT-positive mTBI. Another biomarker panel including MMP-2, CRP, and CKBB also had excellent performance, reflecting mechanisms of generalized inflammation (CRP), local brain inflammation (MMP-2), and cell membrane damage (CKBB, previously discussed in Category 1) (ref. S65). Our analysis shows superior performance of these two biomarker panels to S-100B, the only biomarker with a low-level recommendation for determining the need for a CT scan following mTBI (13). Panels may thus provide a more holistic approach to detecting intracranial injury warranting a CT scan.

Two individual biomarkers performed slightly lower than the panels but still rated as "excellent" based on single studies. The superior discriminative ability of P-tau compared to the more commonly studied T-tau highlights the significance of tau hyperphosphorylation in brain tauopathy. D-dimer also had good performance in this category in a pediatric population (ref. S23), although there are additional clinical scenarios that may cause an elevated D-dimer unrelated to TBI, such as trauma and infection (49). In addition, the applicability of this finding to adults is unknown, and the sample size is small. Further research is warranted to confirm the results of these single studies.

Category 3 (Delayed Recovery After mTBI)

A few less-studied biomarkers performed best for predicting delayed recovery following mTBI. Single studies demonstrated that SNTF, tau A, ghrelin, and NFL all had operating characteristics in the "good" range, outperforming more commonly-studied biomarkers such as GFAP, S100B, NSE and UCH-L1.

Three axon-associated proteins, calpain-derived αII-spectrin N-terminal fragment (SNTF), tau-A, and NFL, may be indicative of DAI, which is thought to be one of the most common pathological mechanisms accounting for long-term dysfunction in all severities of TBI (50–53). SNTF accumulates in damaged axons (54–56) following intra-axonal calcium overload

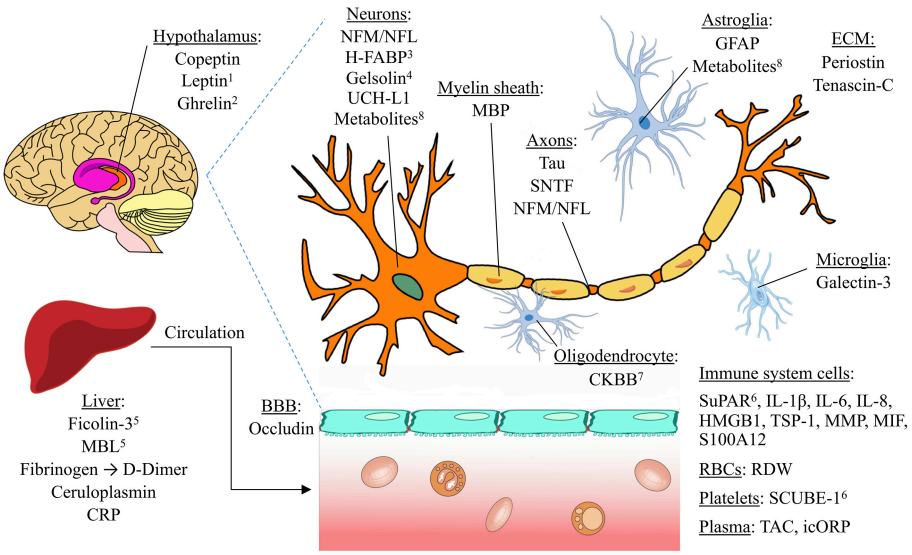

FIGURE 2 | Anatomical locations of potential TBI biomarkers. The biomarkers included in this schematic all rated as "good" (AUC=0.80➔0.89) or better for any of the four clinical situations studied (detecting concussion, predicting intracranial damage after concussion, predicting delayed recovery after concussion, and predicting adverse outcome after severe TBI). Biomarkers with a pooled AUC <0.8 are not shown. [1]Also found in adipose tissue; [2]synthesized in cells of stomach and pancreas; may regulate HPA axis; [3]found mostly in pons; [4]also found extracellularly; [5]lectin pathway of the complement system; [6]also found in endothelial cells. BBB, blood brain barrier. ECM, Extracellular matrix. Image licensed under Creative Commons Attribution-ShareAlike 4.0 International license. https://creativecommons.org/licenses/by-sa/4.0/deed.en. See **Supplementary Material** for image credits and licensing.

and calpain-mediated proteolysis in stretch injury (57, 58). Furthermore, SNTF has been found in degenerating axons after TBI that were undetected by the gold standard marker of transport interruption, amyloid precursor protein (APP) (59). Tau protein may mediate DAI by regulating axon microtubule assembly; (60, 61) tau-A fragments in particular are easily detectable and quantifiable by standard ELISA, perhaps due to their small size, and subsequent ability to cross the BBB (62). NFL is predominantly expressed in subcortical axons and correlates with magnetic resonance diffusion tensor imaging parameters of DAI (63). The included studies found that elevated serum SNTF predicted failure to improve cognitive function at 3 months in CT-negative concussion patients (ref. S83), while tau A and NFL predicted late resolution of post-concussive symptoms in concussed professional ice hockey players (refs. S16, S82). Thus, these proteins may be mechanism-specific biomarkers for identifying patients at risk for persistent cognitive deficits following mTBI.

Ghrelin is an orexigenic peptide hormone that may be linked to stress-induced hypothalamic-pituitary axis (HPA) activation (64, 65) and cognitive dysfunction in neurodegenerative disease (66). In the included study, low values of ghrelin within the first few days following concussion were independently associated with three-month neurocognitive impairment (ref. S89). Thus, ghrelin may be a nonspecific prognostic indicator in mTBI to be used in conjunction with other brain-specific biomarkers such as SNTF.

Reliable biomarkers in this category have the potential to be used in conjunction with radiologic data as well as current predictors of worse outcome after mTBI, such as older age, lower level of education, and pre-existing psychiatric conditions (19). This could help identify patients at risk of persistent disability and the development of additional neurocognitive sequelae. However, as the results for tau-A, SNTF, NFL, and ghrelin were based on a handful of studies with relatively small sample sizes, these candidate biomarkers warrant further investigation regarding their prognostic abilities and rehabilitative implications in mTBI.

Category 4 (Poor Outcome After sTBI)

For predicting mortality and poor outcome in sTBI, there were no biomarkers with operating characteristics in the "excellent" range. However, several biomarkers performed in the "good" range based on single studies, including markers of coagulation and inflammation, structural proteins in the brain, and regulatory proteins in normal homeostasis. The prognostic value of these downstream biological processes suggests that there may be potential for considering some TBIs as systemic rather than primarily localized disorders. Such a holistic approach could have significant implications for both acute and chronic treatments.

Serum biomarkers of coagulation with good ability to predict poor outcome in sTBI include D-Dimer, thrombospondin-1, and SCUBE1. D-dimer is thought to indicate TBI-induced coagulopathy (67–69) that largely occurs secondary to DIC and leads to further cerebral injury (70). Thrombospondin-1 is a thrombin-sensitive, anti-angiogenic factor (71, 72) whose expression is increased after intracerebral hemorrhage (73). SCUBE1 is released from endothelial cells and platelet alpha granules during platelet activation (74, 75). As coagulopathy in isolated TBI is associated with increased mortality (76, 77),

D-Dimer, thrombospondin-1, and SCUBE1 could be important prognostic indicators in sTBI.

Several inflammatory markers with good operating characteristics were identified, including IL-1beta, IL-6, IL-8, HMGB1, ceruloplasmin, ficolin-3, macrophage inhibitory factor (MIF), MBL, galectin-3, S100A12, and SuPAR. HMGB1 had the highest pooled AUC in this category, based on a single study (ref. S165). HMGB1's high expression in the brain (78, 79) suggests that it may be useful for recognizing patients with critical inflammatory responses to brain injury that are associated with severe disability and death. While these markers of inflammation are not specific for brain-localized insults, they may contribute prognostic information by helping to characterize strong inflammatory responses to TBI that contribute to secondary brain injury (80) and ultimately poor outcome. The good performance of periostin and tenascin-C, two extracellular matrix proteins involved in various cell cycle processes including proliferation, migration, differentiation, and apoptosis, suggests that measures of cell turnover in response to injury may have prognostic value as well (refs. S105, S179).

Measurements of the capacity to endure oxidative stress also fared well. The brain is particularly susceptible to oxidative stress due to high oxygen consumption, limited neuron regeneration, and high levels of unsaturated fatty acids in membranes (81). In TBI, the release of reactive oxygen species (ROS) induces inflammation, compromise of the BBB, and cell death (82–85). Quantitation of antioxidants in the brain and the oxidative-reduction potential have subsequently been used to detect oxidative stress (81, 86, 87). The icORP measures the ability of a biological sample to endure an oxidative insult by using an oxidative current to deplete antioxidants in the sample (ref. S92), whereas TAC measures the capacity of antioxidants in a sample to prevent oxidation of a peroxidase substrate (ref. S126). These measures may indicate not only the extent of ongoing ROS-induced damage and inflammation, but also the limited ability of the body to deal with oxidative insults that translate into poor prognosis.

Structural proteins in the brain may also predict outcome as a result of brain-specific injury. High performers identified in this category were MBP, an abundant structural protein of the myelin sheath (88); tau protein, discussed earlier in Category 3 for its ability to predict delayed recovery after mTBI; and NFM, a type IV intermediate filament that contributes to neuron structure, as well as axonal structure and transport (89). Interestingly, the astroglial protein S100B, the most extensively studied biomarker in TBI, had a similar J-statistic but a lower pooled AUC when compared to biomarkers discussed here. Further prognostic studies on these biomarkers in multiple severe TBI populations, particularly on the less-studied MBP and NFM, may allow for better comparison with S-100B.

A handful of proteins involved in homeostatic functions also demonstrated good operating characteristics for predicting poor outcome. Copeptin, which was identified above as a promising marker for detecting concussion, also performed well in this category. This indicates that the degree of stress axis activation has prognostic implications in sTBI, although the prognostic value of copeptin is not limited to TBI (90). Gelsolin mediates cell shape changes & motility (91) and is decreased in acute tissue injury after trauma (92). Leptin, the "satiety hormone," fared well in a pediatric population (ref. S121). It is secreted by adipose tissue (93, 94), is also expressed in the hypothalamus (95), and may play an important role in neuronal and glial maturation (96). H-FABP, which is involved in the intracellular traffic of fatty acids and other hydrophobic ligands, primarily reflects cardiac injury (97) but is also found in smaller concentrations in the brain (98, 99) and other tissues (100). Changes in these markers of osmoregulation, cell motility, energy homeostasis, and fatty acid trafficking may reflect systemic disturbances in sTBI that lead to poor prognosis.

Top-performing biomarkers in this category have the potential to inform which pathologic mechanisms may be most indicative of poor outcome after sTBI. While TBI pathophysiology is undoubtedly complex, making management decisions in this context challenging, the information provided by biomarkers may add value to existing prognostic models (101). The IMPACT (International Mission on Prognosis and Analysis of Clinical Trials) (102) and CRASH (Corticosteroid Randomization After Significant Head Injury) (103) models predict mortality and unfavorable outcome at 6 months after sTBI. Both models take into account age, GCS motor score, pupillary reactivity, and CT classification, and both have been externally validated with comparably reasonable discriminative ability (104). However, lower discriminative performance of these models in a different validation set (105) and at the individual level (106) perhaps indicates the need to update prognostic models to improve generalizability. Validation of promising markers identified in this analysis could potentially lead to the improvement of such models.

LIMITATIONS

This study has a number of consequential limitations. Within each outcome category, the data exhibited considerable heterogeneity. Different patient populations, ages, definitions of outcome, and delays between injury and sampling all detract from the reliability of our findings. In particular, the numerous definitions of concussion and recovery in our included studies limit the strength of our conclusions in these categories. While this variability reflects the heterogeneous nature of these terms in clinical usage, we attempted to identify studies that fit into general categories of clinical interest. Furthermore, the small number of observations, often only a single small study, make statistical comparisons, and stratification (by age, time after injury, etc.) unreliable.

We omitted several otherwise-excellent studies in which levels of a particular biomarker were shown to be significantly associated with the presence of TBI sequelae of interest to us. However, in the absence of operating characteristics or individual subject measurements, we could not calculate how well the biomarker would predict the outcomes of interest. Other studies failed to separate head injuries of different severities or chose outcomes other than those of interest in this study. Biomarkers from tissues other than blood, combinations of biomarkers, and

changes in their levels over time are potentially quite useful but beyond the scope of this study.

The developing field of anti-neuronal autoantibodies could be especially promising for predicting delayed recovery and chronic complications after TBI, across the spectrum of severity levels. An exponential increase in neuroimmunology research over the past decade has contributed to a significant shift in our understanding of anti-neuronal autoantibodies and led to the development of novel blood-based diagnostics for several neurological disorders (107–118). Following the landmark 2007 study that introduced anti-*N*-methyl-D-aspartate receptor (NMDAr) encephalitis (119), IgG autoantibodies against neuronal membrane targets have been implicated in the pathogenesis of various neurological disorders (119–128). Human studies investigating anti-neuronal autoantibodies present in the blood post-TBI have largely investigated the role of TBI-induced (adaptive) IgG autoantibodies, which appear ∼4–6 days following TBI (129–132). However, it has recently shown that serum IgG autoantibodies are present in both human and animal serum, regardless of age, sex or disease state (133–136). The recent discovery that all human blood contains thousands of autoantibodies (133, 136) and that individual autoantibody profiles are influenced by the presence of disease (111, 113, 118, 123, 137) leads to the promising hypothesis that quantification of disease-specific changes in serum anti-neuronal autoantibody titer concentrations can serve as highly sensitive and specific biomarkers of persistent post-TBI neurodegeneration. Indeed, the discovery of non-invasive serum biomarkers such as autoantibody profiling which objectively demonstrate chronic post-TBI neurodegeneration would provide objective information to inform clinical trials for both mechanism discovery and therapeutic intervention.

Genetic variants have been increasingly studied to explain the variability in outcome following TBI. Many single nucleotide polymorphisms (SNPs), single nucleotide substitutions within a gene's coding or regulatory regions, have been identified for this purpose (138–140). In particular, SNPs in genes of proteins involved in dopamine availability and transmission have been targeted, as dopamine dysregulation after TBI is thought to contribute to chronic deficits in memory, attention, and executive function (141). SNPs in both catechol-O-methyltransferase (COMT) and ankyrin repeat and kinase domain-containing 1 (ANKK1) have been associated with a variety of cognitive impairments after predominantly mTBI (142–145), but this association is less clear in sTBI (146–149). A better understanding of which genes are implicated in the neurocognitive response to TBI may shed light on mechanisms of such injury and have both prognostic and therapeutic implications. Future studies will need to clarify the effects of age, gender, ethnicity, environment, and gene-gene interactions on the relationship between gene expression and brain function (150).

Finally, there are questions about the reliability of any blood biomarker as an indicator of brain injury severity. The integrity of the blood-brain barrier, as well as proteolytic degradation of some biomarkers in serum, could affect measured levels (26). Plog et al. hypothesize that the transport mechanisms, which they term the "glymphatic" system, may have a greater influence on biomarker levels than TBI severity itself (151). Thus, clinically relevant manipulations of this system, such as cisternotomy and sleep deprivation, could prevent accurate interpretation of serum biomarker levels. Peripheral surgical trauma also disrupts the BBB and leads to neuroinflammation (152). While comparing the discriminative abilities of CSF biomarkers may bypass these challenges, there exists much more data on blood biomarkers due in part to the ease and convenience with which they may be collected in a variety of settings. Due to these limitations, it must be emphasized that blood biomarkers have value not as isolated diagnostic tests, but rather as adjuncts to clinical, radiological, and other diagnostic information.

CONCLUSION

We have reviewed the literature and identified blood biomarkers with the highest discriminative abilities as determined by operating characteristics in four commonly encountered clinical situations: diagnosing concussion, predicting the need for a CT scan after mTBI, predicting delayed recovery after mTBI, and predicting poor outcome after sTBI. The top performers in each category may provide insight into pathogenic mechanisms of TBI that most influence the measured endpoint. Nonetheless, many challenges remain before these biomarkers can be incorporated into clinical practice. In particular, it remains unclear whether a large panel of biomarkers in addition to clinical assessment will be sufficient to first stratify patients into categories of TBI before more specific biomarker assessments are applied. Alternatively, in the age of precision medicine, biomarker assessment may be tailored to individual patients. Ideally, pre-clinical development will help refine approaches for clinical application.

AUTHOR CONTRIBUTIONS

ZG, SG, LG, DM, and SS contributed to the acquisition, analysis, and interpretation of the data. SS, RS, and DS contributed to study conception and design. ZG, RS, and SS drafted the work, and all other authors (SG, LG, DM, DS) revised it critically for important intellectual content. All authors (ZG, SS, RS, SG, LG, DM, DS) provide approval for publication of the content and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00446/full#supplementary-material

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CASE REPORT
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[18F]FDG, [11C]PiB, and [18F]AV-1451 PET Imaging of Neurodegeneration in Two Subjects With a History of Repetitive Trauma and Cognitive Decline

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Background: Trauma-related neurodegeneration can be difficult to differentiate from multifactorial neurodegenerative syndromes, both clinically and radiographically. We have initiated a protocol for *in vivo* imaging of patients with suspected TBI-related neurodegeneration utilizing volumetric MRI and PET studies, including [18F]FDG indexing cerebral glucose metabolism, [11C]PiB for Aβ deposition, and [18F]AV-1451 for tau deposition.

Objective: To present results from a neuroimaging protocol for *in vivo* evaluation of TBI-related neurodegeneration in patients with early-onset cognitive decline and a history of TBI.

Methods: Patients were enrolled in parallel TBI studies and underwent a comprehensive neuropsychological test battery as well as an imaging protocol of volumetric MRI and PET studies. Findings from two patients were compared with two age-matched control subjects without a history of TBI.

Results: Both chronic TBI patients demonstrated cognitive deficits consistent with early-onset dementia on neuropsychological testing, and one patient self-reported a diagnosis of probable early-onset AD. Imaging studies demonstrated significant [18F]AV-1451 uptake in the bilateral occipital lobes, substantial [11C]PiB uptake throughout the cortex in both TBI patients, and abnormally decreased [18F]FDG uptake in the posterior temporoparietal areas of the brain. One TBI patient also had subcortical volume loss. Control subjects demonstrated no appreciable [18F]AV-1451 or [11C]PiB uptake, had normal cortical volumes, and had normal cognition profiles on neuropsychological testing.

Conclusions: In the two patients presented, the [^{11}C]PiB and [^{18}F]FDG PET scans demonstrate uptake patterns characteristic of AD. [^{11}C]PiB PET scans showed widespread neocortical uptake with less abnormal uptake in the occipital lobes, whereas there was significant [^{18}F]AV-1451 uptake in both occipital lobes.

Keywords: TBI, CTE, neurodegeneration, PET, PiB, amyloid, AV-1451, tau

INTRODUCTION

In vivo imaging characteristics of TBI-related neurodegeneration are currently being studied. Tau-specific positron emission tomography (PET) radiotracers being developed for Alzheimer's disease (AD) have shown promise in detecting tau pathology in patients who exhibit clinical signs of early cognitive impairment, including those with a history of TBI (1–3). [^{18}F]FDG is a well-established PET radiotracer that measures neuronal cell metabolism and has a long history of use in the evaluation of brain metabolic reduction after TBI (4). In a recent study of a group of patients with a history of TBI, [^{18}F]FDG PET demonstrated decreased uptake in clusters of brain voxels compared to normal controls (5). In addition, Pittsburgh compound B ([^{11}C]PiB), an established amyloid-beta (Aβ) PET radioligand, was reported to have increased retention on PET imaging after TBI, the finding supported by postmortem [H-3]PiB autoradiography and Aβ immunohistochemistry in a separate cohort of TBI autopsy cases (6).

In pathology studies, the presence of diffuse Aβ plaques has been observed in resected brain tissue specimens from some severe TBI patients in acute injury phases, including patients of a relatively young age (7). Likewise, in autopsies of fatal-TBI victims, Chen et al. found amyloid plaques in subjects who died within hours of their injuries (8). However, they also found a complete absence of plaques in longer term survivors (27 days—3 years) despite evidence of neuronal Aβ production. Thus, the effect of TBI on AD risk is not clear.

Overlapping clinical and neuroimaging features of TBI- and AD- related cognitive deficits have been suggested to be attributable to synergy between the progression of chronic traumatic encephalopathy (CTE) and the development of early AD (9). Neuropathologic findings from two patients who developed early-onset dementia after moderate-severe Traumatic Brain Injury (TBI) support the hypothesis of post-TBI dementia as a polypathology with features that overlap with several dementia subtypes (10).

MRI evidence of gray matter volume loss is a structural feature common to many forms of dementia. Marketed software for the reproducible detection and analysis of regional brain volumes has shown utility in AD, TBI, and other neurodegenerative disorders (11–14).

We initiated a protocol for *in vivo* imaging of patients with TBI-related neurodegeneration utilizing volumetric MRI and PET imaging indices of cerebral glucose metabolism ([^{18}F]FDG), Aβ burden ([^{11}C]PiB), and tau burden ([^{18}F]AV-1451). The objective of this case report is to present differences in these MRI and PET biomarkers in two patients with a history of chronic, repetitive TBI, one with predominantly blunt-force head trauma, and the other with a history of predominantly repetitive blast trauma.

METHODS

Patient Selection

Both patients were identified after enrollment in research cohorts for studies approved by the Institutional Review Board at the University of Pittsburgh. Patients provided written informed consent for research participation and publication of this case report. Patient #1 was identified as a part of a cohort of patients being evaluated with advanced 3T MRI modalities for chronic TBI. Patient #2 was enrolled in the Targeted Evaluation Action and Monitoring of Traumatic Brain Injury (TEAM-TBI) trial, wherein patients undergo comprehensive clinical evaluations to adjudicate specific post-TBI sequelae. Two age- and gender-matched healthy controls were recruited for comparison.

Patient histories were obtained by trained interviewers and testing was completed by technicians supervised by the senior neuropsychologist (SRB). In both cases, chronic and acute symptoms and diagnoses following TBI were derived from patient-reported outcome measures (PROs) and a neuropsychological test battery. No formal medical record review was performed. Neuropsychological testing included measures of memory (California Verbal Learning Test [CVLT] Short and Long Delay Free Recall), executive function (Halstead Reitan Trail Making Test B [HRTMT-B), Wechsler Adult Intelligence Scale-IV [WAIS-IV] Working Memory Index [WMI], Controlled Oral Word Association Test [COWAT]), and information processing speed (WAIS-IV Processing Speed Index [PSI], HRTMT-A). Patient demographics and Neuropsychological test scores are shown in **Table 1**.

All subjects underwent neuropsychological testing or an evaluation with the Mini-Mental Status Examination upon study entry (16). All neuroimaging studies were conducted over a 2-year period subsequent to neuropsychological testing.

Imaging Protocol

Both patients and one control received structural T1-weighted MRI scans including a Magnetization Prepared Rapid Gradient Echo (MPRAGE) sequence on a 3T Siemens TIM Trio (Siemens Healthcare GmbH, Erlangen, Germany) scanner with a voxel size of $1 \times 1 \times 1$ mm^3. The second control received a structural T1-weighted MPRAGE sequence scan on a 3T Siemens TIM Trio with a modified voxel size of $1 \times 1 \times 1.2$ mm^3, both within recommended parameters for the volumetric software used.

TABLE 1 | Demographic comparisons.

	Age	Gender	Ethnicity	Years education	IQ estimate*	Test validity
Patient #1	60	M	Caucasian	17	101	Yes
Patient #2	54	M	Caucasian	12	092	Yes

**IQ estimated with the North American Adult Reading Test (15).*

TABLE 2 | Neuropsychological test scores*.

	Memory		Executive function			Information processing speed	
	CVLT SDFR	CVLT LDFR	WAIS-IV WMI	COWAT	Trails B	Trails A	WAIS-IV PSI
Patient #1	<0.05	<0.05	0.04	UTC	UTC	0.01	<1
Patient #2	7	<1	18	<1	0.01	0.01	1

**Scores represented as age corrected percentiles. CVLT, California Verbal Learning Test; SDFR, Short Delay Free Recall; LDFR, Long Delay Free Recall; WAIS-IV, Wechsler Adult Intelligence Scale-IV; WMI, Working Memory Index; COWA, Controlled Oral Word Association Test; PSI, Processing Speed Index (21); UTC, Unable to complete.*

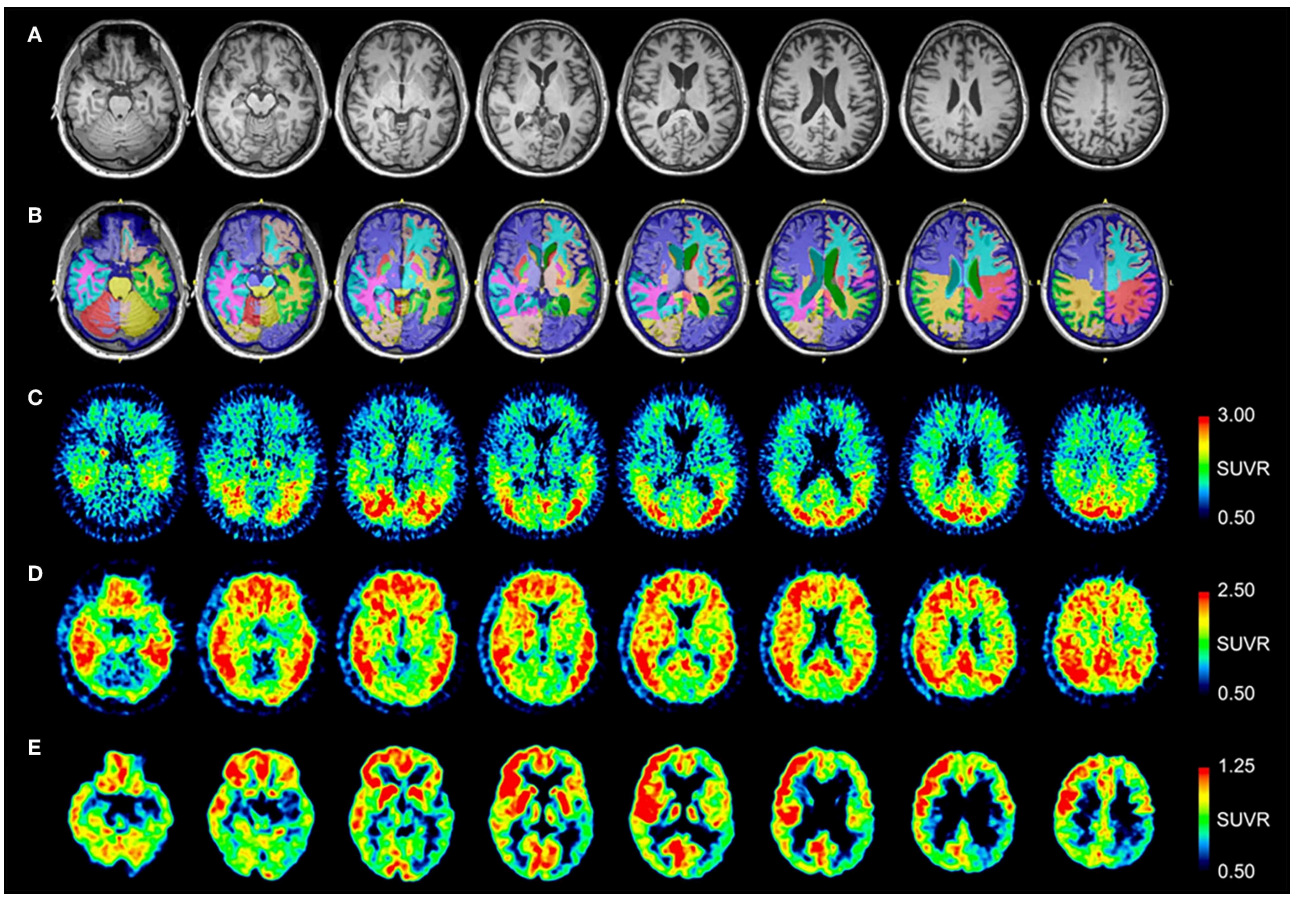

FIGURE 1 | Serial transverse brain sections from **(A)** structural MRI, **(B)** NeuroReader segmentation, **(C)** [^{18}F]AV-1451 PET, **(D)** [^{11}C]PiB PET, and **(E)** [^{18}F]FDG PET for Patient #1. The color bar shows uptake in standardized uptake value ratios (SUVR) with the cerebellar cortex as reference. **(A,B)** The MR image demonstrates evidence of decreased hippocampal volume. Occipital lobe volumes were normal. **(C)** The [^{18}F]AV-1451 PET scan shows broad areas of abnormal intensely increased uptake in the cuneus (FreeSurfer mean [FS] SUVR: 2.39) and lateral occipital regions (FS SUVR: 2.28). There is also abnormally increased uptake in the superior parietal (FS SUVR: 2.19), inferior lateral temporal (FS SUVR: 1.79), and entorhinal brain regions (FS SUVR: 1.72). **(D)** The [^{11}C]PiB PET scan demonstrates abnormally increased uptake in the temporal, parietal, and frontal lobes, as well as the precuneus, in a pattern characteristic of AD. Note that, by comparison, the occipital lobes have relatively less abnormal uptake. **(E)** The [^{18}F]FDG scan shows abnormally decreased uptake in the posterior brain regions. The degree of asymmetry is much lower in the left posterior temporoparietal brain region, also a pattern characteristic of AD.

[^{11}C]PiB PET and [^{18}F]FDG scans for each patient were performed on the same day using a Siemens ECAT Exact HR+ PET scanner (Siemens Healthcare GmbH, Erlangen, Germany). [^{11}C]PiB scanning commenced 40 min after an average injection of 14.8 mCi. Data were acquired in 3D mode over 40–70 min post injection and binned into six 5-min frames. PET data were corrected for attenuation, scatter, and radioactive decay, converted to a 2D data set using Fourier rebinning, and reconstructed using the Direct Fourier (DIFT) method, similar to filtered back projection (FBP), into a 128 × 128 × 63 matrix with voxel sizes of 2.06 × 2.06 × 2.43 mm^3. Images were filtered with a 3 mm Hann window.

[^{18}F]FDG scanning commenced 35 min after an average injection of 7.6 mCi and were acquired over the interval 35–60 min post injection into five 5-min time frames. To minimize contamination of [^{18}F]FDG data by residual ^{11}C radioactivity, procedures were timed such that a minimum of 100 min (five ^{11}C halflives) elapsed between the initial [^{11}C]PiB injection and the start of the [^{18}F]FDG PET scan. Reconstruction and data correction methods were similar to those used for [^{11}C]PiB image processing.

On a different day, [^{18}F]AV-1451 PET imaging was performed on a Siemens mCT-Flow Biograph PET/CT. Scanning commenced 75 min after an average injection of 9.1 mCi [^{18}F]AV-1451. Data were binned into six 5-min frames over a 30 min scan. A low-dose, non-diagnostic CT without contrast was performed immediately before PET acquisition for use in attenuation and scatter correction. PET data were reconstructed via Fourier rebinning (FORE), followed by FBP using manufacturer's software into a 256 × 256 × 109 matrix with voxel sizes of 1.03 × 1.03 × 2.03 mm^3. Reconstruction included corrections for scatter, attenuation, decay, random coincidences, and scanner deadtime. Images were filtered using a 3D 2 mm Hann window.

One control received only a [^{18}F]AV-1451 PET scan, and the second control received only a [^{11}C]PiB PET scan. All control and patient PET scans were conducted with identical protocols.

Image Analysis

PET images were inspected and, if necessary, corrected for interframe motion using the interframe registration tool in the PFUS module of PMOD software (PMOD Technologies LLC, 2015, Zurich, Switzerland). Dynamic PET data were averaged over 40–60 min for [^{18}F]FDG, 50–70 min for [^{11}C]PiB, and 80–100 min for [^{18}F]AV-1451, and each single-frame average PET image was independently registered to the patient's or control's corresponding structural MRI.

For qualitative visual reads of PET, MR images were normalized to Montreal Neurological Institute (MNI) template space using the Unified method within Statistical Parametric Mapping, version 12 (SPM12) software (17). The resulting transformation was applied to corresponding registered and averaged PET images. Cerebellar gray matter (GM) activity was sampled from each normalized PET using the Centiloid reference region (18), and standardized uptake value ratio (SUVR) images were generated by dividing each image by its cerebellar GM activity.

For regional quantification of PET, native-space MR images were processed through FreeSurfer v5.3 software (19). Standard FreeSurfer regions of interest were applied to corresponding PETs to sample radioactivity concentrations, and regional SUVR values were calculated using FreeSurfer's cerebellar cortex as reference.

Volumetric analysis was then performed on the MPRAGE images using NeuroReader (Brainreader, Horsens, Denmark) software (20). For each subject the analysis consisted of registering, segmenting, and measuring regional brain volumes. The regional volumes were corrected for age, gender, and total intracranial volume and compared to a normative control population, resulting in a percentile score.

RESULTS

Patient #1

This 60-year-old Caucasian male (see **Table 1**) had a history of 45-year involvement in scholastic and collegiate athletics,

TABLE 3 | NeuroReader regional brain MRI volumetric results.

	Patient #1 ml (percentile)	Patient #2 ml (percentile)	Control #1 ml (percentile)	Control #2 ml (percentile)
Left hippocampus	2.74 (11.07)*	4.12 (50.87)	3.59 (35.45)	4.13 (52.69)
Right hippocampus	2.86 (12.77)*	4.25 (51.33)	3.49 (30.75)	4.41 (60.51)
Left amygdala	0.78 (14.72)*	1.39 (46.46)	1.63 (58.65)	1.98 (84.06)
Right amygdala	0.83 (12.04)*	1.37 (42.34)	1.78 (63.84)	2.13 (90.56)
Left frontal lobe	209.93 (47.48)	221.24 (59.77)	218.99 (59.38)	230.36 (78.97)
Right frontal lobe	217.64 (56.30)	211.33 (53.37)	217.87 (59.68)	224.87 (73.51)
Left parietal lobe	96.39 (32.18)	106.63 (51.07)	110.73 (57.25)	99.61 (37.82)
Right parietal lobe	101.09 (40.03)	94.42 (36.45)	109.44 (57.27)	102.17 (44.41)
Left occipital lobe	59.17 (69.47)	52.43 (54.49)	60.65 (73.27)	50.83 (48.82)
Right occipital lobe	50.35 (50.83)	50.31 (54.47)	54.21 (63.77)	55.38 (71.36)
Left temporal lobe	99.10 (19.37)*	123.60 (59.33)	115.36 (48.60)	116.94 (53.59)
Right temporal lobe	114.26 (43.30)	116.56 (50.42)	117.89 (52.11)	112.87 (43.64)

**Regional volume with a percentile lower than 25% relative to a normative population after correcting for age, gender, and total intracranial volume.*

including 30 years active participation, followed by 15 years of coaching. During his career, and starting in high school, he self-reported multiple head injuries, both sub-concussive and concussive. He specifically recalls an injury at 21 years of age where he sustained a significant blow to the head, with a post-concussive syndrome that caused him to be removed from practicing for a short period of time. He was never hospitalized or evaluated acutely after any of these injuries.

Shortly before he retired from coaching at age 54, he began to experience a precipitous decline in memory, mood swings, and cognitive difficulties. These symptoms were initially noted by his wife, and they included hypersomnia, irritability, difficulty concentrating, sensitivity to light and memory problems. He was evaluated by a neurologist and diagnosed with early-onset AD. There was no reported family history of early-onset AD. His neuropsychological testing (**Table 2**) demonstrated significant cognitive impairment across multiple test domains, including WAIS-IV Processing Speed Index (<1st percentile), WAIS-IV Working Memory Index (<1st percentile), Trail Making Test A (<1st percentile), and Trail Making Test B (unable to complete) (21).

MRI and PET imaging results are shown in **Figure 1**. Quantitative analysis of the MRI scan demonstrated hippocampal and amygdala volumes to be low (left hippocampus−2.74 ml, 11th percentile; right hippocampus−2.86 ml, 12th percentile; left amygdala−0.78 ml, 14th percentile; right amygdala−0.83 ml, 12th percentile). Left temporal lobe volume was also found to be low (left temporal lobe−99.10, 19th percentile; right temporal lobe−114.26 ml, 43rd percentile). All other cerebral cortex volumes, including the occipital lobe, were normal (left occipital 59.17 ml, 69th percentile; right occipital 50.35 ml, 50th percentile). Selected NeuroReader-derived regional volumes and percentiles are presented in **Table 3**. The [^{18}F]AV-1451 PET scan showed

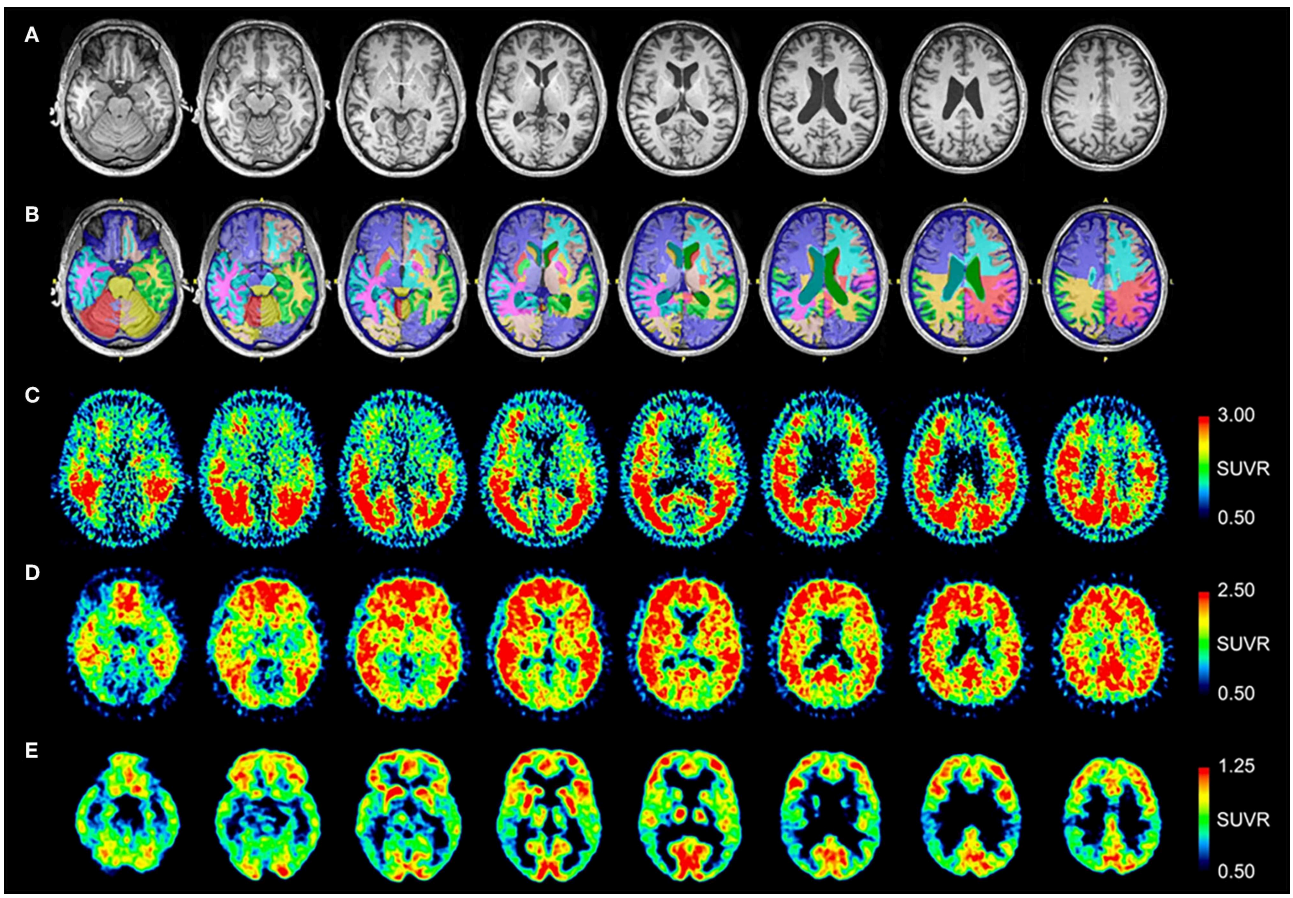

FIGURE 2 | Serial transverse brain sections from **(A)** structural MRI, **(B)** NeuroReader segmentation, **(C)** [^{18}F]AV-1451 PET, **(D)** [^{11}C]PiB PET, and **(E)** [^{18}F]FDG PET for Patient #2. **(A,B)** Quantitative analysis of the MRI scan demonstrated normal volumes. **(C)** The [^{18}F]AV-1451 PET scan is most significant for demonstrating abnormal intensely increased symmetric uptake in the precuneus (FreeSurfer mean [FS] SUVR: 2.97), superior parietal (FS SUVR: 2.86), and lateral occipital regions (FS SUVR: 2.76). There is also abnormally increased uptake in the lateral inferior temporal (FS SUVR: 2.47), entorhinal (FS SUVR: 2.13), lateral orbitofrontal (FS SUVR: 1.78), and superior frontal (FS SUVR: 1.67) regions. **(D)** The [^{11}C]PiB PET scan demonstrates significant abnormally increased uptake in parietal, temporal and frontal lobes, as in Patient #1, demonstrating a pattern characteristic of AD. Note there is negligible accumulation in the occipital lobes (as compared to the [^{18}F]AV-1451 scan). **(E)** The [^{18}F]FDG PET scan shows abnormally and relatively symmetric decreased uptake in the posterior temporoparietal regions of the brain, with sparing of the frontal lobes.

broad areas of abnormal intensely increased [^{18}F]AV-1451 uptake, primarily in the medial and lateral occipital regions, but also in the parietal regions. There was also abnormally increased uptake in the mesial temporal structures. The [^{11}C]PiB PET scan demonstrated abnormally increased uptake in the temporal, parietal, and frontal lobes, as well as the precuneus, in a pattern characteristic of AD, while the occipital lobes had relatively less abnormal uptake. The [^{18}F]FDG scan showed abnormally decreased uptake in the posterior brain regions. In summary, while the [^{11}C]PiB and [^{18}F]FDG PET scans had typical AD patterns, the [^{18}F]AV-1451 appeared more typical for posterior cortical atrophy (PCA) syndrome, a form of dementia that is usually considered an atypical variant of AD (22–24).

Patient #2

This 54-year-old Caucasian male trained in explosive ordinance disposal and served for 26 years on active military duty, including multiple deployments to Iraq and Afghanistan. He was employed a further 7 years as a contractor to a private defense company that focused on training bomb defusing teams. He reported at least 5 episodes of concussive injury to the head over his career, a TBI episode resulting in loss of consciousness in a parachuting accident in 2000, as well as exposure to hundreds of subconcussive blasts while acting as a team leader in Afghanistan defusing improvised explosive devices (IED). In 2007, he was involved in an IED attack where he was exposed to the blast while riding in a vehicle. He self-reported a slow deterioration in his cognition and memory beginning the year after the IED attack in

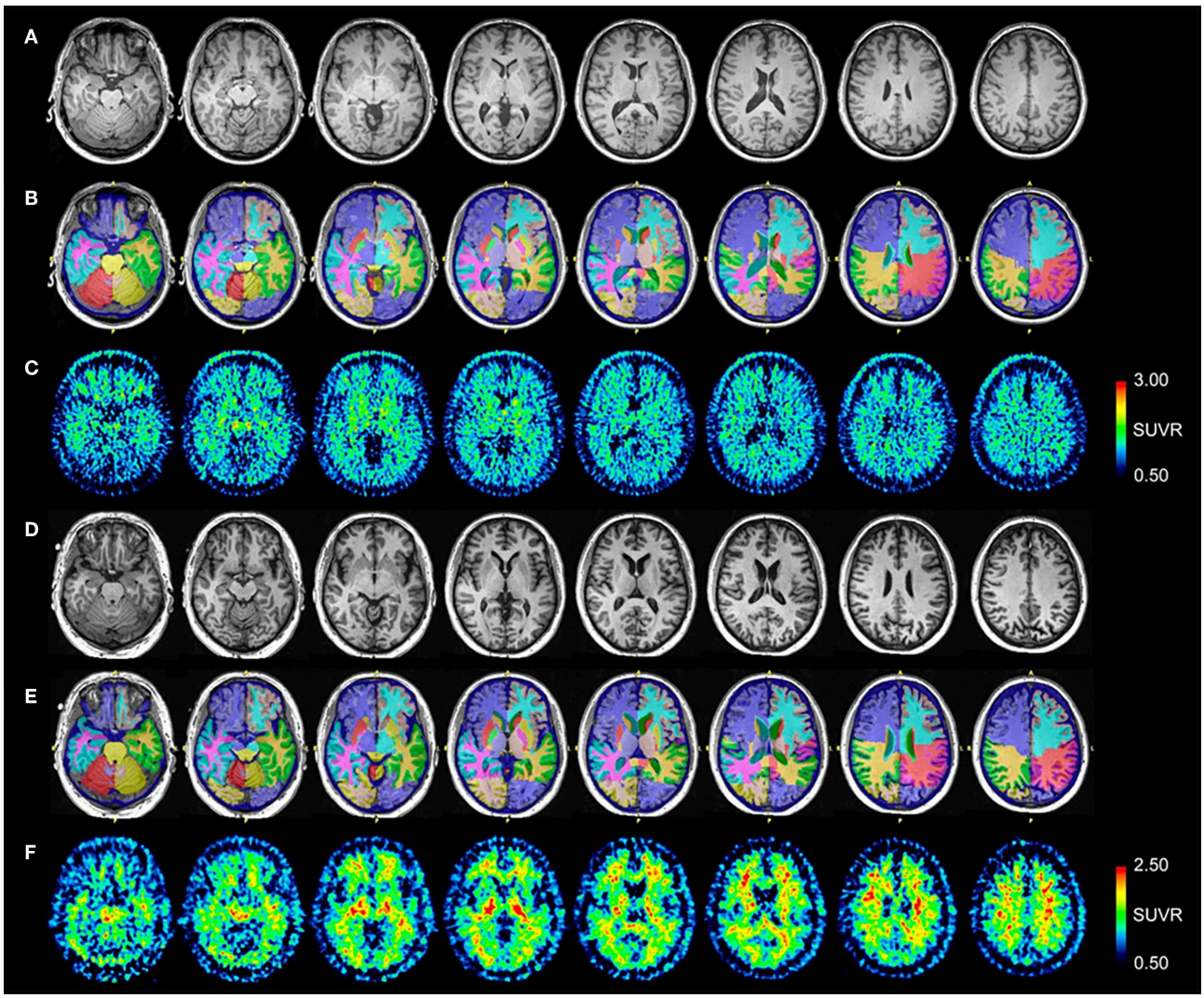

FIGURE 3 | Serial transverse brain sections from **(A)** structural MRI, **(B)** NeuroReader segmentation, and **(C)** [^{18}F]AV-1451 PET scans for Control #1; and **(D)** structural MRI, **(E)** NeuroReader segmentation, and **(F)** [^{11}C]PiB PET scans for Control #2. SUVR color bar scales for each PET radiotracer are identical across all patients and controls. The [^{18}F]AV-1451 image for Control #1 shows mild basal ganglia, substantia nigra, and choroid plexus uptake that reflects a typical pattern of off-target binding. By comparison with both patients, there is negligible occipital, frontal, temporal, and parietal lobe uptake. The [^{11}C]PiB image for Control #2 shows non-specific uptake in white matter regions, but negligible uptake throughout neocortical and subcortical gray matter.

2007. Specifically, he reported significant memory difficulties and manifested a tremor in his hands. Self-reported symptoms were confirmed with his spouse due to his difficulty with memory.

As a part of his enrollment in a research trial, he underwent a neuropsychological test battery (**Table 2**) demonstrating significant cognitive impairment, including WAIS-IV Processing Speed Index (1st percentile), Trail Making Test A (<1st percentile), and Trail Making Test B (<1st Percentile).

MRI and PET results are shown in **Figure 2**. Quantitative analysis of the MRI scan demonstrates normal hippocampal, amygdala, and cerebral cortex volumes (**Table 3**). The [^{18}F]AV-1451 PET scan demonstrates abnormal, intensely increased symmetric uptake primarily in the parietal and lateral occipital regions. There is also abnormally increased uptake in the orbitofrontal, posterior frontal, lateral temporal, and mesial temporal brain regions. The [^{11}C]PiB PET scan demonstrates abnormally increased uptake in parietal, temporal and frontal lobes, as in Patient #1, a pattern characteristic of AD. Note there is negligible accumulation in the occipital lobes (as compared to the [^{18}F]AV-1451 scan). The [^{18}F]FDG PET scan shows abnormally and relatively symmetric decreased uptake in the posterior temporoparietal regions of the brain, with sparing of the frontal lobes. In summary, the [^{18}F]AV-1451, [^{11}C]PiB, and [^{18}F]FDG PET scans demonstrate typical AD patterns of uptake.

Controls

Control #1 is a 58-year-old male and Control #2 is a 64-year-old male. Neither control had a significant history of TBI or neurodegenerative symptoms. Control #1 scored within normal limits on all testing for the neuropsychological battery administered to patients. Control #2 scored 30/30 on the MMSE. Volumetric analysis demonstrated no abnormalities in any brain region. Normalized MRI, NeuroReader segmentation, and [^{18}F]AV-1451 SUVR images for Control #1 are presented in **Figures 3A–C**, respectively. Normalized MRI, NeuroReader segmentation, and [^{11}C]PiB SUVR images for Control #2 are presented in **Figures 3D–F**, respectively.

DISCUSSION

In this case series, we report neuroimaging and clinical findings in two patients with neurodegenerative disease and a history of multiple concussive and subconcussive brain injuries. Neither case reported a family history of early-onset dementia. Both patients had severe neuropsychological impairment as demonstrated on dedicated testing. We utilized an imaging protocol that included volumetric MRI and PET studies including: [^{18}F]FDG metabolism; [^{11}C]PiB, an Aβ specific PET tracer; and [^{18}F]AV-1451, a tau PET tracer. Both patients demonstrated decreased [^{18}F]FDG metabolism in posterior brain regions and global increased uptake of [^{11}C]PiB similar to that demonstrated in AD (25). The pattern of [^{18}F] AV-1451 uptake is most significant in occipital, parietal, and temporal lobes in both patients. This pattern is similar to the uptake pattern of other tau-specific radiotracers that have been studied in patients with suspected CTE (1, 2). While earlier CTE neuropathology stages spare the occipital lobes, global, non-selective tau deposition can be seen in stage IV disease (26). It has previously been reported that predominant PCA with tau deposition can be seen in individuals thought to have a specific phenotype of AD selectively involving the posterior cortical regions, highlighting the potential difficulties of diagnosing trauma-related neurodegeneration from variants of AD using PET radiotracers specific for amyloid and tau (22–24). Neither of our patients, however, demonstrated appreciable occipital lobe atrophy on volumetric analysis, despite the substantial tauopathy and FDG hypometabolism on PET imaging.

It is currently unknown if *in vivo* PET and structural MR imaging demonstrating significant PCA with tau deposition is found commonly in patients with AD and a co-existent history of TBI. However, it has been observed that onset of PCA generally occurs earlier than in typical AD, with most patients experiencing first symptoms in their 50s or early 60s (27). It should be noted that [^{18}F]AV-1451 and other putative tau ligands still require postmortem validation to determine specific pathological substrates for their binding *in vivo*, due to the complexity of tau isoforms, post-translational modifications, and neuropathological aggregates in AD and non-AD tauopathies including CTE (28).

The binding of various tau tracers to different tau isoforms could explain observations of tracer-dependent cortical distributions (29–31). It is possible the posterior cerebral degeneration pattern seen here may reflect the binding of [^{18}F]AV-1451 to one or several tau isoforms or conformations arising from the combined effects of trauma and more intrinsic predilection for development of AD. Nevertheless, the pattern of uptake demonstrated in the patients presented here is not unique to our experience, and similar patterns have previously been reported in suspected CTE and typical and atypical variants of AD (1, 2, 22–24).

This study is limited by its small subject sample and retrospective nature, making it difficult to report significant associations or the predictive ability of these imaging techniques when it comes to *in-vivo* diagnosis of either trauma-related neurodegeneration or multifactorial neurodegenerative syndromes. In addition, there are no diagnostic standards associated with [^{18}F]AV-1451. Thus, the imaging studies presented here should not be viewed as a clinical evaluation and were not performed with diagnostic intent. Instead, our purpose is to use these data to guide evaluation of a larger cohort of patients with suspected traumatic encephalopathy with the goal of validating an *in-vivo* diagnostic panel for trauma-related neurodegeneration.

CONCLUSIONS

Structural T1-weighted MRI and [^{18}F]FDG, [^{11}C]PiB, and [^{18}F]AV-1451 PET hold promise as neuroimaging biomarkers for detecting trauma-related neurodegeneration in living patients with a history of traumatic brain injuries; however, there may be overlap with other neurodegenerative syndromes potentially unrelated to trauma, and distinction between these conditions will require further study.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Review Board of the University of Pittsburgh. The protocol was approved by the Institutional Review Board.

INFORMED CONSENT

All subjects gave written informed consent for research participation and publication of this case report in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

DO, DM, SRB, KE, JS, CL, SB, AP, SP, MI, JMe, WS, CM, and JMo contributed to the conception and design of the study. DM, CL, SP, JMe, and JMo performed image and statistical analyses. RP wrote the first draft of the manuscript. DO, DM, SRB, CL, BL, MI, JMe, and JMo wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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BDNF Val66Met Genetic Polymorphism Results in Poor Recovery Following Repeated Mild Traumatic Brain Injury in a Mouse Model and Treatment With AAV-BDNF Improves Outcomes

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Clinicians have long noticed that some Traumatic Brain Injury (TBI) patients have worse symptoms and take a longer time to recover than others, for reasons unexplained by known factors. Identifying what makes some individuals more susceptible is critical to understanding the underlying mechanisms through which TBI causes deleterious effects. We have sought to determine the effect of a single nucleotide polymorphism (SNP) in Brain-derived neurotrophic factor (BDNF) at amino acid 66 (rs6265) on recovery after TBI. There is controversy from human studies as to whether the BDNF Val66Val or Val66Met allele is the risk factor for worse outcomes after brain trauma. We therefore investigated cellular and behavioral outcomes in genetically engineered mice following repeated mild TBI (rmTBI) using a lateral fluid percussion (LFP) injury model. We found that relative to injured Val66Val carriers, injured Val66Met carriers had a larger inflammation volume and increased levels of neurodegeneration, apoptosis, p-tau, activated microglia, and gliosis in the cortex and/or hippocampus at 1 and/or 21 days post-injury (DPI). We therefore concluded that the Val66Met genetic polymorphism is a risk factor for poor outcomes after rmTBI. In order to determine the mechanism for these differences, we investigated levels of the apoptotic-inducing pro BDNF and survival-inducing mature BDNF isoforms and found that Met carriers had less total BDNF in the cortex and a higher pro/mature ratio of BDNF in the hippocampus. We then developed a personalized approach to treating genetically susceptible individuals by overexpressing wildtype BDNF in injured Val66Met mice using an AAV-BDNF virus. This intervention improved cellular, motor, and cognitive behavior outcomes at 21 DPI and increased levels of mature BDNF and phosphorylation of mature BDNF's receptor trkB. This study lays the groundwork for further investigation into the genetics that play a role in the extent of injury after rmTBI and highlights how personalized therapeutics may be targeted for recovery in susceptible individuals.

Keywords: BDNF, lateral fluid percussion, Val66Met, rmTBI, AAV-BDNF

INTRODUCTION

Traumatic Brain Injury (TBI) is a serious and potentially life-threatening clinical problem. It occurs when there is a force to the head, which results in a disruption of brain function. In 2013, there were 2.5 million TBIs in the United States, 50,000 of which led to death and 70,000 of which led to permanent neurological damage (1). Based on the neurological symptoms that occur after a TBI, the injury is classified either as mild, moderate, or severe. Mild TBI is the most prevalent form of TBI that occurs in the United States. In particular, athletes and military personnel tend to suffer from mild and repeated traumatic brain injuries (2, 3). While the majority of patients who suffer from mild TBI tend to recover over time, it is estimated that around 15% of patients have symptoms that last longer than 3 months and develop into chronic disabilities (4). This problem is only exacerbated when a person is subjected to repeated mild TBI (rmTBI). It is becoming more evident that some athletes and military personnel who have suffered from mild repeated brain injuries can end up developing neurodegenerative diseases (5). TBI should be thought of as an ongoing disease process, rather than a discrete event. The primary injury occurs as the result of mechanical force to the brain. After the initial insult, a secondary injury process occurs as a result of inflammation and secondary mediators. While some patients who suffer from TBI recover quickly and have no obvious long-term symptoms, other patients undergo a prolonged secondary injury phase and have a much harder time recovering (6, 7). One of the reasons that not all individuals respond similarly to rmTBI may be due to the genetic differences that exist in the population. However, specific genes that may affect outcomes after rmTBI have not been identified.

A critical neuronal gene that contains a single nucleotide polymorphism (SNP) is brain–derived neurotrophic factor (BDNF), a neurotrophin that plays a role in neuronal survival and synaptic plasticity (8–10). BDNF has a SNP site, rs6265, at the 66 amino acid position of the BDNF protein, and results in the wildtype Val being replaced with a Met. The Val66Met genotype has been reported to be associated with poor outcomes in a number of disease states such as major depressive disorder (11), anxiety (12), stroke (13), and Alzheimer's disease (14). However, clinical research in TBI patients is not consistent as to whether the Met or the Val polymorphism confers worse outcomes (15–21). The Val66Met polymorphism results in diminished BDNF in dendrites and reduced amounts of BDNF protein released into the synapse upon stimulation (22). (23). In healthy populations, individuals with the Val66Met polymorphism have been found to have impaired cognitive function (24). It seems to follow logically that the Val66Met polymorphism has been shown to result in worse neurocognitive performance after TBI (21), as well as being a risk factor for TBI in combat forces (18–20). However, some recent studies in humans have shown that counterintuitively, after injury, the Val66Val carriers actually exhibit worse recovery compared to the Val66Met carriers (15–17, 25). Specifically, in long-term studies of combat veterans, carriers of the Val66Met polymorphism recovered their executive function back to baseline levels while the Val66Val carriers did not (15, 17). On the other hand, different studies have found no effect of the polymorphism influencing outcomes after injury (26–28). Thus, the clinical research as to which BDNF allele is the risk factor for outcomes after TBI is not all in agreement, and could benefit from a bottom-up, controlled experimental research study as has been done to examine other disease models where genetic polymorphisms may play a role (29). This study will play an important role in helping to ameliorate some of the controversy that exists about the role of the Val66Met allele after TBI.

The situation is complicated by the fact that the BDNF protein has two important and varied forms, the pro form and the mature form. Mature BDNF binds to its receptor trkB and stimulates neurogenesis and cell survival. ProBDNF protein binds to the p75 receptor and activates the apoptotic cascade (30). We have previously shown that the pro form of BDNF and its signaling pathways are preferentially upregulated after TBI (31). While other groups have shown that the Val66Met genetic polymorphism affects levels of total BDNF in dendrites without altering the relative levels of pro and mature BDNF, the effect that this polymorphism has on levels of pro and mature BDNF after injury and the effect this has on injury outcomes have not been elucidated (32, 33). BDNF also has a third form, the truncated form, whose role is less clear but is thought to be generally beneficial (34).

In this investigation, we studied the effect of the BDNF rs6265 genetic polymorphism on cellular, biochemical, and behavioral changes after repeated mild lateral fluid percussion (LFP) brain injury in mice. The LFP model of TBI is a longstanding method used due its ability to mimic injuries seen in humans, by involving both focal and diffuse components (31, 35–37). Moreover, we focused on repeated mild TBI since it is a common yet understudied form of TBI. Cellular changes were investigated at 1 and 21 DPI, in order to ascertain a more complete picture of the various biological processes that are activated at different time points after injury.

We have shown that after rmTBI, Val66Met mice have increased area of inflammation, cell death, neurodegeneration, p-tau, astrogliosis, and activated microglia at 1 and/or 21 DPI in the cortex and hippocampus compared to Val66Val injured mice. When investigating the relative levels of pro and mature BDNF after injury in these mice, we found that injured Met carriers have less total BDNF in the cortex at 21 DPI, and more pro-BDNF relative to mature BDNF in the hippocampus at 1 DPI compared to injured Val carriers. Finally, we show that when Val66Met carriers are treated with an AAV virus vector to overexpress BDNF, we can rescue the high levels of astrogliosis and activated microglia down to the levels observed in Val66Val injured mice. Treatment with AAV-BDNF also improves learning and memory in Val66Met injured mice in the Morris Water Maze paradigm to the level observed in Val66Val injured mice. To our knowledge, this is the first report showing that there is genotypical susceptibility to poor outcomes after TBI that can be rescued by altering neurotrophic signaling.

MATERIALS AND METHODS

Animals

Adult male and female mice aged 10–12 weeks were used in all studies. BDNF mice were generously provided by Dr. Francis S. Lee of Weil Cornell Medical College (38). The mice were created utilizing a targeting vector with or without the point mutation (G196A) which is regulated by the endogenous mouse BDNF promoter. The colony was maintained by crossing BDNF$^{Val/Met}$ mice which yield offspring at Mendelian rates. Mice were housed in a 12 h light/dark cycle with food and water available *ad libitum*. All procedures described were performed in accordance to the NIH guidelines and were approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC). A power analysis was used to determine the appropriate sample size for experiments to reach 80% power; for histology the group size $n = 5$–8, for biochemistry the group size $n = 4$–6, and for behavioral tasks $n = 8$–10 were used to reliably detect changes of the magnitude we are examining ($\alpha = 0.05$) based on the difference seen between experimental groups in our previous publication (31).

Lateral Fluid Percussion Injury

Lateral fluid percussion injury uses a rapid fluid pulse to cause injury to the brain by the displacement of neural tissue. This process has previously been described in detail (35) but has been modified to create repeated mild injury. Briefly, mice were anesthetized using 4–5% isoflurane in 100% O_2 and maintained on 2% isoflurane throughout the procedure. They were placed in a stereotaxic frame, and a trephine-guide 3 mm plastic disc was attached with Loctite glue (444 Tak Pak, Henkel Corporation, Rocky Hill, CT) on the skull, halfway between lambda and bregma, laterally on the right hemisphere. A trephine (3 mm outer diameter) was used to perform a craniectomy. A rigid Luer-loc needle hub (3 mm inside diameter) was secured onto the skull over the opening that was made using cyanoacrylate adhesive and dental acrylic (Henry Schein, Dublin, OH). After a 60 min recovery period, the animals were re-anesthetized and connected to the fluid percussion injury device (Custom Design and Fabrication, Virginia Commonwealth University) through the Luer-loc hub. Once the animals regained normal breathing, before sensitivity to stimulation, a ∼0.8 ATM pulse (15 ms) was generated through the LFP device to strike the intact dura of the brain. Upon return of righting reflex (<4 min for mild injury) the hub was filled with saline and capped. Forty eight hours from the initial injury, a second injury was given. This occurred again at 96 h from the initial injury. This experimental timeline was chosen based on previous studies which have sought to mimic human repeated mild TBIs in a mouse model which controls for the rodent life span (39–42). After the 3rd injury, the hub and dental acrylic were removed and the scalp incision was closed with 3M Vetbond (Fisher Scientific, Waltham, MA). The animals were individually housed after the injury and returned to normal housing conditions. In order to determine humane endpoints, the mice were monitored twice daily. If signs of pain were detected, the vivarium veterinary staff were contacted, and appropriate analgesics were used immediately. Signs of pain and distress included animals that were no longer able to move to get food or water, or showed signs of pain (ex. hunched posture, inappetence, lethargy, decreased body condition). In order to prevent harm and suffering, we gave a surgical pre-emptive analgesia, in the form of an injection of buprenorphrine (0.1 mg/kg SC). During the surgery, the mice were anesthetized with isoflurane while they were in the stereotaxic apparatus. Adequate anesthetic depth was checked for by a no response to toe pinch before surgery commenced. During the surgery procedure, the anesthetic bupivicaine (0.025%) was applied topically to the skull. In addition, the respiratory rate was monitored throughout the entire surgical procedure and the eyes were protected with lubricant. If necessary, post-op pain medication of Carprofen would be given at 5 mg/kg, SC, once a day and continued if signs of pain were observed (not found to be necessary for any mice in this study). With this repeated, mild level of injury, about 5–10% of animals died after the 3rd injury in the chronic post-traumatic period. In this study, we had a mortality rate of 8.8% with 26/295 deaths. The expectation of this mortality was approved by our institutional IACUC. This is a normal and anticipated feature of the LFP TBI model because it mimics human TBI. Mice that underwent the surgical procedure but not the injury were used as sham controls. Assignment of the mice to the LFP or sham group was randomized.

MRI Imaging

Magnetic resonance imaging (MRI) was done on a cohort of mice in order to assess the volume of inflammation as determined by increased relative intensity (ROI). The scans were done utilizing a fast spin echo sequence with a mouse brain coil. Scans were done in the axial position at 1, 7, and 21 days after the final injury. Inflammation was determined through an auto-thresholding to analyze higher intensity areas relative to regular brain tissue. All brains were reviewed with same intensity search and normalized using the Image Scale Factor in the VivoQuant Analysis Software. The region of interest was determined by analyzing areas of increased intensity within specific coordinates in the damaged location of the brain and analyzed blinded to condition. Scans were done at the Rutgers University Molecular Imaging Center with the center's M2 Compact High-Performance MRI (1T).

Immunohistochemistry

To collect tissue for immunohistochemistry, a second cohort of mice were perfused with 0.9% saline, followed by 4% paraformaldehyde at 1 and 21 days after the final injury. After perfusion, the brains were cryoprotected with 30% sucrose for at least 3 days. Sectioning was done in 20 μm thick slices, in a 1:10 series throughout the length of the hippocampus, incorporating the area around the site of injury in the cortex. To measure apoptotic cell death, sections were pretreated with 0.01 M Citrate buffer at 90°C. Anti-cleaved caspase-3 (1:1,000, 9,661, Cell Signaling, Danvers, ME) was then applied overnight, followed by Alexa Fluor 594 goat anti-rabbit (1:1,000, Invitrogen, Waltham, MA). To measure astrogliosis, Glial Fibrillary Acidic Protein (GFAP) antibody was applied overnight (1:500, MAB3402, Millipore, Billerica, MA), followed by Alexa Fluor goat anti-mouse 488 (1:500, Invitrogen, Waltham, MA). To measure

neuronal degeneration, sections were first treated with 1% NaOH and 0.06% $KMnO_4$, then 0.0005% Fluoro-Jade C (AG325, Millipore, Burlington MA)/0.0001% DAPI (D9564, Sigma, St. Louis, MO) was applied for 20 min. To measure microglial activation, IBA1 antibody was applied overnight (1:10,000, 019-19741, Wako Labs, Richmond, VA), followed by Alexa Flour goat anti-rabbit 488 (1:1,000, Invitrogen, Waltham, MA). To measure levels of phosphorylated tau, AT8 antibody was applied overnight (1:500, MN1020, Pierce Antibodies, Waltham, MA), followed by Alexa Fluor goat anti-mouse 488 (1:1,000, Invitrogen, Waltham, MA). All slides were incubated in 4',6-diamidino-2-phenylindole (DAPI) (1:1,000 DAPI in PBS, Sigma, St. Louis, MO). Slides were mounted in Fluoromount-G (Southern Biotech, Birmingham, AL), except for the Flouro-Jade C slides which were mounted in DPX Mountant (44581, Sigma, St. Louis, MO). Visualization of the fluorescent stains was done using a Leica microscope (Model DMIRB, Leica Microsystems, Buffalo Grove, IL). Five to eight animals per time point and treatment were analyzed. Sectioning of tissue was done using a Cryostat (Leica) and collected coronally in 1:10 series throughout the length of the hippocampus. For each biological replicate, the collected sections of brain were counted and the average number of cells per section was calculated. Positive cells were counted in the hemisphere ipsilateral to the injury. In the cortex, for each section, six fields of 40X view (starting at the dorsal midline and moving laterally for three fields of vision, and then the three fields of vision just ventral to the first three) were counted. In the hippocampus, the dentate gyrus as well as the CA1-CA3 were used for quantification of cells. Analysis was performed blind to experimental group and genotype.

Vestibular Rotarod Test

In order to study the vestibular motor abilities of the mice after LFP, the rotarod test was conducted as part of a behavioral battery on a third cohort of mice (**Supplemental Figure 3**). The rotarod test utilized a 36-mm outer diameter, rotating rod whose velocity increased from 4 to 40 rpm over a maximum 180 s interval. Balance and motor function were measured using the latency to fall. Each trial ended when the animal fell off the rotarod. Eight to ten mice per genotype and condition were used. Acclimation and baseline analysis were done 1 day prior to the first injury, using three trials separated by a 1-h inter-trial rest phase. At 1, 7, and 21 days after the last injury, each mouse underwent three trials separated by a 1-h inter-trial rest phase. The same mice were used for each time point and analyzed blinded to condition. The average latency to fall was compared between injured and sham groups.

Balance Beam Test

In order to study fine motor function, the balance beam test was conducted. The beam apparatus consists of a one meter long flat beam with a width of 20 mm, raised 30 cm above the table surface. A black box was placed at one end of the beam as the finish point. The mice were pretested on the beam apparatus for 4 days before the test day for training and baseline measurements. On test day, the mice were observed crossing the beam while the number of paw faults, falls, and relative time to cross were recorded manually. Mice were tested at 7 and 21 DPI, and the same mice were used for each time point. Eight to ten mice per genotype and condition were used. Values were imputed into a predetermined scale to evaluate outcomes with weighted values for the different traits analyzed in order to account for the severity of injury indicated by each. A score of 1 was standard for all mice, the number of falls was added after being multiplied by 2, the number of foot faults were added, and if the mouse crossed the beam in under 5 s, a score of 1 was removed from the final score. Analysis was done blinded to condition.

Morris Water Maze Test

In order to study spatial memory, the Morris water maze test was done. Mice were acclimated to the paradigm and tested for baseline response using a visible platform test 1 day prior to the start of the injury paradigm. The animals were placed in a circular pool (1 m diameter) filled with opaque water containing non-toxic white paint and a clear escape platform marked by a visible rod. To assess learning, the mice were tested using a hidden platform fixed in the northwest quadrant starting 1 day after the last injury. Testing was conducted with four trials a day for 6 days in a row. On the seventh day, a probe test was completed to test memory, where the hidden platform was removed and the time spent exploring the northwest quadrant was recorded. Black and white distal extra-maze cues were positioned on the walls of the room and geometric shaped proximal extra-maze cues were positioned above the walls of the maze. The mice were placed in pseudo-randomly varied quadrants throughout testing, and the time to locate the platform was recorded. Trials were run until the mouse found the platform or was placed there after the maximum trial time of 60 s. At the conclusion of the trial, the mouse was allowed to remain on the hidden platform for 15 s to consolidate learning, followed by removal from the pool and placement onto a heating pad for 10 min. Eight to ten mice per group and condition were used. Data was analyzed blinded to condition. Data was recorded using a video-tracking system (EthoVision XT; Noldus Information Technology, Leesburg, VA).

Western Blot Analysis

The cortex and hippocampus on the ipsilateral side to the injury site were collected from mice at 1 and 21 dpi and flash frozen. Four mice per group and condition were analyzed at each timepoint. Tissue lysates were prepared using T-PER with protease inhibitors and EDTA (Pierce, Rockford, IL). Samples were homogenized for 30 s and then centrifuged for 10 min. The protein content of the supernatant was determined using the bicinchoninic acid (BCA) Protein Assay Reagent Kit (Pierce, Rockford, IL). Equal amounts of protein were loaded onto Bis Tris Gels (Invitrogen, Grand Island, NY). The proteins were transferred onto polyvinylidene difluoride (PVDF)-filter Immobilon-P transfer membranes (Millipore, Billerica, MA). Following blocking in 5% BSA + 5% normal donkey serum overnight at 4°C, the primary antibody was applied overnight at 4°C. Forty microgram of protein was run on a 12% Bis Tris gel and probed for pro and mature BDNF (1:500 BDNF Icosagen, San Francisco, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1,000, Biodesign, Saco, ME) was used

as a loading control. Forty microgram of protein was run on a 4–12% Bis Tris gel and probed for p-trkB (1:1,000, EMD Millipore, Burlington, Ma). trkB was used as a loading control (1:1,000, Sigma-Aldrich, St. Louis, MO). Secondary anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated IgG antibodies were used (1:5,000, GE Healthcare, South Plainfield, NJ). GAPDH protein was visualized by chemiluminescence using the Enhanced Chemiluminescence (ECL) detection kit (Perkin Elmer, Waltham, MA) and all others were visualized using the SuperSignal West Femto Maximum Sensitivity Substance (ThermoFisher Scientific, Waltham MA). Levels of the immunopositive bands were quantified densitometrically using Quantity One version 4.2.1 software on a GelDoc 2000 (Bio-Rad, Hercules, CA). All data is normalized to the sample's own GAPDH and expressed as a fold change relative to the average of the genotype matched sham controls.

BDNF Viral Infusion

An AAV9-CMV-GFP-2A-mouseBDNF construct expressing the wildtype 66Val form of BDNF at a titer of 4.5×10^{13} viral genomes/μL was purchased from Vector BioLabs (Malvern, PA) and dose selection was done in conjunction with the company. Mice were anesthetized using 4–5% isoflurane in 100% O_2 and maintained on 2% isoflurane throughout the procedure. They were placed in a stereotaxic frame, and a 32 G Hamilton Neuro syringe was used to deliver a volume of 0.75 μL at a speed of 0.25 μL/min into both the ipsilateral cortex (AP −1.9 mm, ML, −1.5 mm, DV −1.5 mm) and hippocampus (AP −1.9 mm, ML, −1.5 mm, DV −2.5 mm) of animals 5 min after the final LFP injury. The needle was left in place after injection for 5 min to allow for completion infusion of the drug. The control group received the same injection protocol with a control AAV-CMV-GFP construct 4.5×10^{13} viral genomes/μL purchased from Vector BioLabs. Analysis was done as is standard in the field, assuming dose dependent GFP expression (43, 44).

Statistical Analysis

StatPlus software was used for all data analysis. Groups were compared using Student's two-tailed *t*-test or one-way ANOVA followed by Fisher's PLSD *post-hoc* analysis. $p < 0.05$ is considered statistically significant. Statistical results are presented in the figures and legends and the *p*-values are provided in **Supplemental Figure 4**.

RESULTS

Val66Met Injured Mice Have a Larger Volume of Inflammation Compared to Val66Val Injured Mice at 21 DPI Following rmTBI

Throughout this report, we have compared differences in assay outcomes between the injured and sham groups, as well as between the two injured genotypes Val66Met and Val66Val. To investigate the role that genotype plays on the volume of inflammation after our repeated mild LFP model, we used a 1T MRI to scan the brains of the mice at 1, 7, and 21 DPI. Utilizing T2 fast spin echo sequence imaging scans, we found that by 21 DPI, edema at the site of the craniectomy returned to pre-injury levels in sham condition mice when the same mice were imaged over multiple time points, while in the injured mice there was still edema and swelling present. Some of this edema extended through the hole in the skull left by the craniectomy resulting in extra-axial hyperintensity as part of the injury area (**Supplemental Figure 1**). Due to the persistence of edema, we selected the 21 DPI time point to assess the effect of the BDNF SNP on volume of inflammation. At 21 DPI, there was

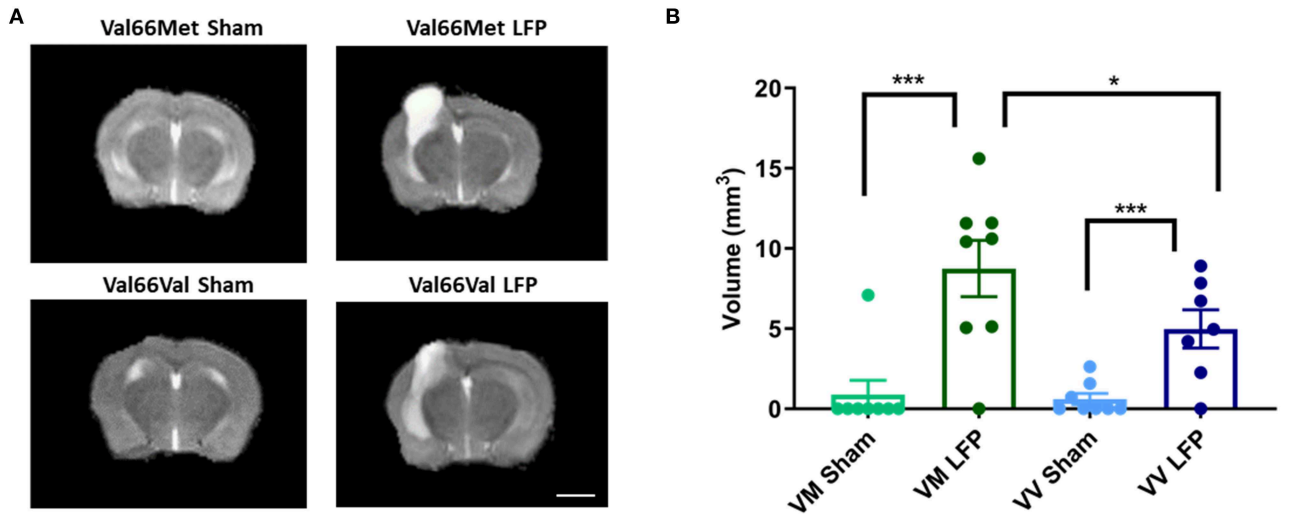

FIGURE 1 | Val66Met injured mice have greater volume of inflammation than Val66Val injured mice. **(A)** Representative MRI images of mice subjected to LFP and sham at 21 days after the final injury. **(B)** Quantification of volume of inflammation in different genotypes, 21 DPI as determined by assessment of hyperintensity ROI. *$p < 0.05$, ***$p < 0.001$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, n =7–8. Scale bar = 3 mm.

a significant difference between the volume of inflammation in sham and injured mice as determined from measuring the hyperintensity volume of T2 MRI scans. Particularly of interest, we saw that Val66Met injured mice had a significantly larger volume of inflammation compared to Val66Val injured mice (**Figure 1**). These data suggest that there are differences in the level of injury occurring between these two genotypes at 21 DPI as seen through the increased edema. However, it is not known which processes are affecting this difference.

Activated Iba1+ Cells Are Increased in Val66Met Injured Mice Compared to Val66Val Injured Mice, While Non-activated Microglia Are Consistent Across All Groups

To study the underlying cellular changes that contribute to the genetic difference in volume of inflammation, we conducted immunohistochemical staining at both 1 and 21 DPI to assess the immediate and longer lasting effects of rmTBI on various cellular processes. As our LFP injury paradigm includes both focal and distal components, we analyzed the ipsilateral cortex to gain an appreciation of the focal components of injury as well as its possible effects on sensorimotor function. We also analyzed the ipsilateral hippocampus to investigate the slightly distal effects of injury and to gain insights into the possible effect on cognitive function. The repeated mild LFP mouse model was chosen because it is able to accurately mimic the injuries that are seen after human repeated mild TBI; there is an acute injury at the point of contact, as well as diffuse injury in other brain areas (45). As expected by this model, we see mild signs of injury on the contralateral side of the brain demonstrated by microglial staining (**Supplemental Figure 5**). Therefore, instead of using the contralateral cortex and hippocampus as controls, we chose to use mice that have undergone craniectomy surgery but no injury (sham) and analyzed the ipsilateral hemisphere of those mice. First, to examine the effect that repeated mild TBI has on the neuroimmune system, we analyzed activated microglia. Activated microglia are an important part of the secondary injury process, have been shown to persist for years after the initial injury, and contribute to long-term neurological dysfunction (46). We used IBA1 as a marker for microglia and we utilized morphology to distinguish activated microglia from non-activated microglia. Non-activated microglia were identified by their ramified appearance, while activated microglia were in either the reactive bushy state or the phagocytic ameboid shape (47). We found that at 1 DPI Val66Met injured mice had significantly more activated microglia than their sham controls in both the ipsilateral cortex and the hippocampus. Notably, we found that at 1 DPI Val66Met injured mice had significantly more activated microglia than the Val66Val injured mice in the ipsilateral cortex or hippocampus (**Figures 2B,D**). These data indicate that Val66Met injured mice have earlier microglial activation than the Val66Val injured mice. By 21 DPI, the Val66Met and Val66Val injured mice both had significantly more

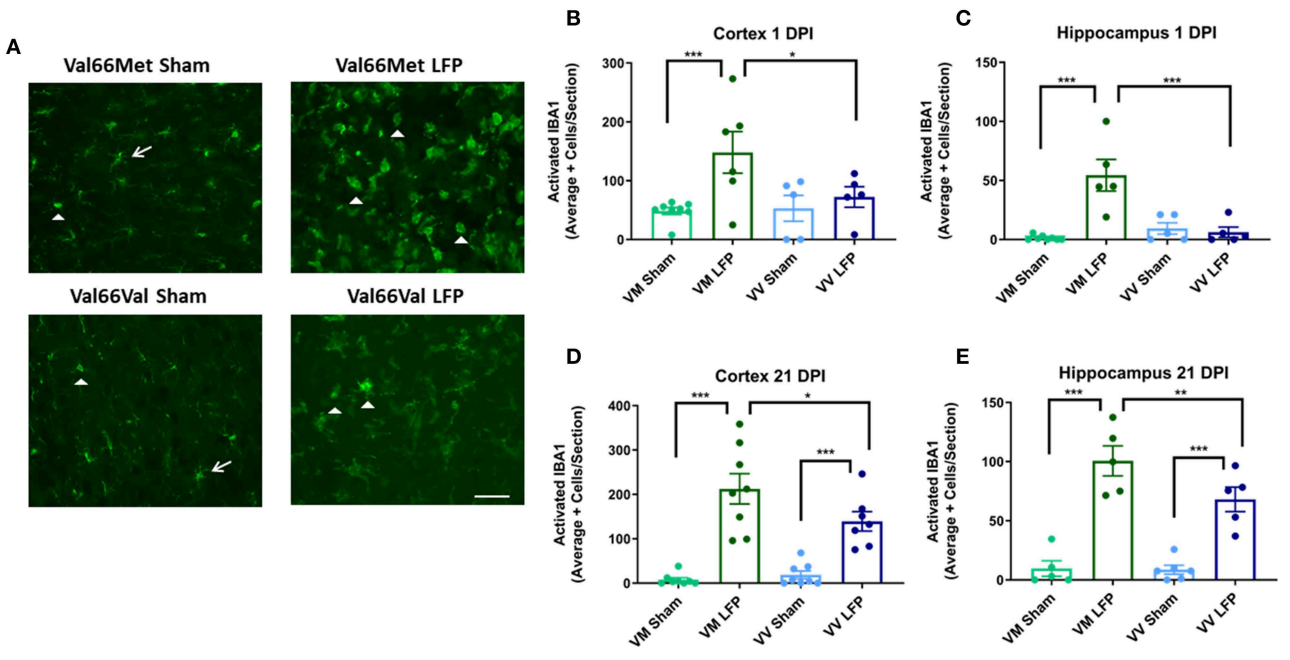

FIGURE 2 | Repeated mild LFP injury causes an increase in ionized calcium binding adaptor molecule 1 (IBA1) positive cells in the brains of injured Val66Met mice compared to injured Val66Val mice at 1 DPI. **(A)** Representative images of cortical sections at 1 DPI stained with IBA1. White arrows indicate resting microglia, white arrowheads indicate activated microglia. Scale bars = 100 μm. **(B–E)** Quantification of the average number of IBA1+ positive cells, broken down into activated and resting categories by morphology, per cortex and hippocampus ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, n =5–8.

activated microglia than their sham controls. Again, we observe a significant difference in the levels of activated microglia in the Val66Met injured mice compared to the Val66Val injured mice at 21 DPI in both the ipsilateral cortex and hippocampus (**Figures 2C,E**). These data suggest that Val66Met injured mice respond to repeated mild injury by activating microglia earlier than Val66Val injured mice, and this increased activation is sustained through 21 DPI.

Levels of Activated Caspase-3+ Cells Are Higher in Val66Met Injured Mice Compared to Val66Val Injured Mice at 1 DPI, but Not at 21 DPI

Since increased levels of neuronal cell death are common following injury (31, 48), we used activated caspase-3 to assess levels of apoptosis. We found that at 1 DPI, both Val66Met and Val66Val injured mice have a significant increase in the number of activated caspase-3 positive cells relative to their sham controls indicating that apoptosis was at higher levels at 1 DPI. Importantly, Val66Met injured mice had a significantly higher number of activated caspase-3 positive cells relative to the Val66Val injured mice, in both the ipsilateral cortex and hippocampus (**Figures 3A–C**) suggesting that Val66Met mice have more cell death after rmTBI than Val66Val mice. However, by 21 DPI levels of cell death had decreased so that there was no detectable difference between the injured mice and their sham controls (**Figures 3D,E**). This is similar to what we have previously seen after single moderate injury by 21 DPI (31). These data suggest that while there are initial genotypic differences in apoptotic cell death after injury with Val66Met exhibiting worse outcomes than Val66Val that these differences are resolved by 21 DPI.

Levels of FluorojadeC+ Cells Are Increased in Val66Met Injured Mice Compared to Val66Val Injured Mice at 1 DPI and 21 DPI

Increased levels of neurodegeneration are common sequelae following injury to the brain (31, 42). We used Fluorojade C (FLJC), a marker for neurodegeneration (49), in order to ascertain the level of neurodegeneration in the ipsilateral cortex and hippocampus. We found that at 1 DPI, Val66Met injured mice had significantly more FLJC positive cells in both the ipsilateral cortex and hippocampus relative to their sham controls (**Figures 4B,C**). At this timepoint, Val66Val injured mice did not significantly differ from their sham controls in numbers of FLJC positive cells in the ipsilateral cortex, although Val66Val mice did differ from their sham controls in the hippocampus. Importantly, the Val66Met injured mice had more FLJC positive cells than the Val66Val injured mice in the ipsilateral cortex at 1 DPI, indicating that the Val66Met injured mice have more neurodegeneration at this early timepoint. By 21 DPI, both the Val66Met injured mice and the Val66Val injured mice had significantly more FLJC positive cells than their sham controls in

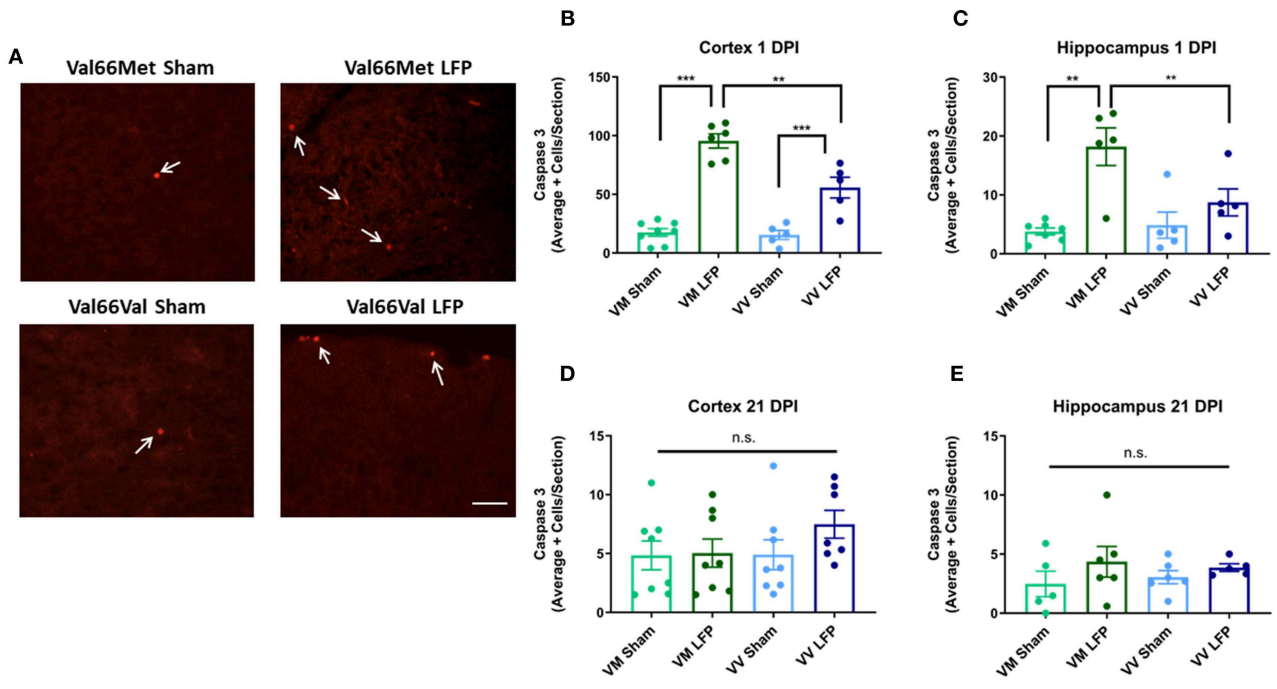

FIGURE 3 | Repeated mild LFP injury causes an increase activated caspase-3 positive cells in brains of injured Val66Met mice compared to injured Val66Val mice at 1 DPI. **(A)** Representative images of cortical sections at 1 DPI stained with activated caspase-3 (indicated by arrows). Scale bars = 100 μm. **(B–E)** Quantification of the average number of activated caspase-3 positive cells per cortical or hippocampal section ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, $n = 5–8$.

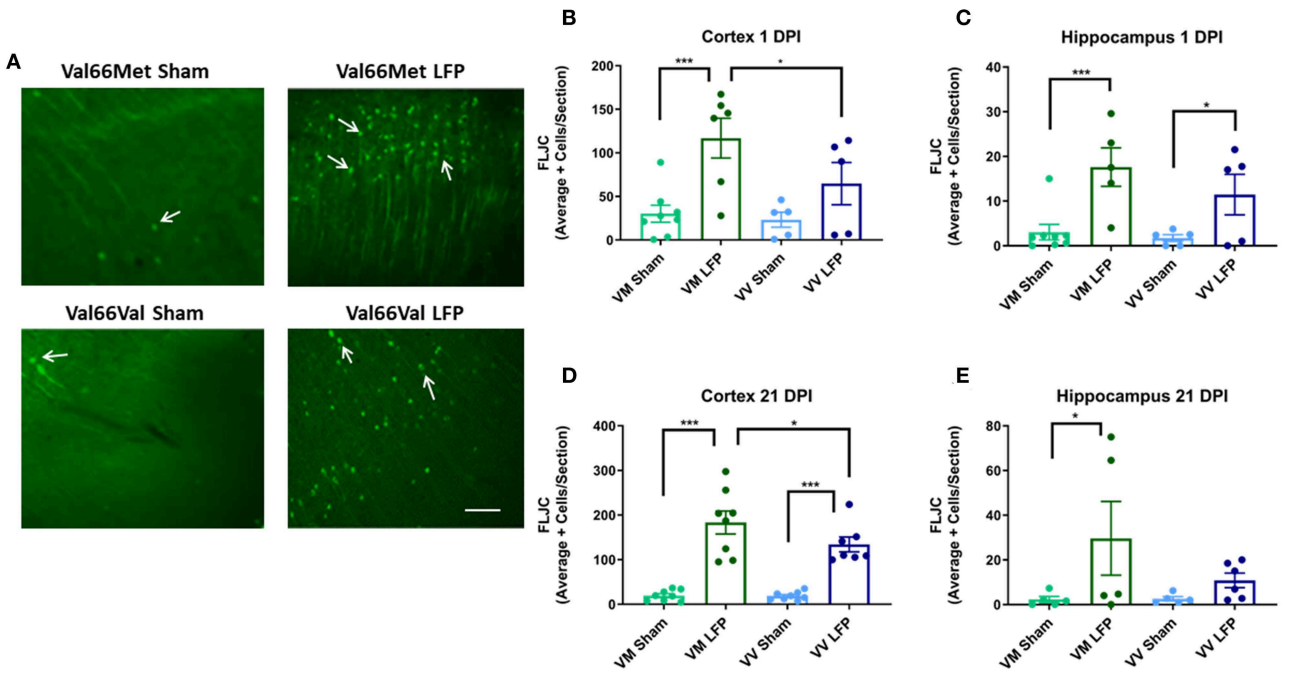

FIGURE 4 | Repeated mild LFP injury causes an increase in Fluoro-Jade C (FLJC) positive cells in the brains of injured Val66Met mice compared to injured Val66Val mice at 1 and 21 DPI. **(A)** Representative images of cortical sections at 1 DPI stained with FLJC (indicated by arrows). Scale bars = 100 μm. **(B–E)** Quantification of the average number of FLJC positive cells per cortex or hippocampus ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, $n = 5$–8.

the ipsilateral cortex, and notably, the Val66Met injured mice had significantly more FLJC positive cells than the Val66Val injured mice at this time point as well (**Figure 4D**). In the hippocampus at 21 DPI there was still a significant difference between the injured Val66Met mice and their sham controls, but no detectable difference between the Val66Val mice and their sham controls (**Figure 4E**). There was also still no detectable difference between the Val66Met and Val66Val injured mice at 21 DPI in the hippocampus. These data suggest that the neurodegeneration process begins as early as 1 DPI and that increased levels of neurodegeneration in response to injury may be sustained until at least 21 DPI in the Val66Met injured mice.

Number of Phosphorylated tau+ Cells Are Increased in Val66Met Injured Mice Compared to Val66Val Injured Mice at 1 and 21 DPI

It has previously been shown that phosphorylated tau can contribute to long-term pathologies in the brain (50). After injury, there are frequently higher levels of phosphorylated tau at the site of injury as well as in distally affected brain areas (51). We investigated the levels of phosphorylated tau at 1 and 21 DPI in both the ipsilateral cortex and hippocampus. We found that there was a significant increase in levels of phosphorylated tau in both Val66Met and Val66Val injured mice compared to their sham controls in the ipsilateral cortex at both 1 and 21 DPI (**Figures 5B,C**). Of note, the Val66Met injured mice had significantly more phosphorylated tau compared to Val66Val injured mice, seen at 1 DPI and sustained through to 21 DPI. This suggests that Val66Met injured mice may have an exacerbated phosphorylated tau reaction after injury that begins at 1 DPI and is sustained until 21 DPI. In the hippocampus, we found that the Val66Met injured mice had significantly more phosphorylated tau than their sham controls at both 1 and 21 DPI, while the Val66Val injured mice did not differ from their sham controls (**Figures 5D,E**). These data suggest that after repeated mild injury, Val66Met mice are uniquely susceptible to mechanisms that result in increased levels of phosphorylated tau in the hippocampus.

The Number of GFAP+ Cells Are Increased in the Ipsilateral Cortex in Val66Met Injured Mice Compared to Val66Val Injured Mice by 21 DPI, but Not at 1 DPI

It is well documented that after injury there is proliferation of glia (31, 42) that can contribute to the formation of glial scarring, inhibition the ability of neurons to regenerate, and prevention of the injured brain from recovering normal morphology and function (52). Using glial fibrillary acidic protein (GFAP), a marker for activated astrocytes, we quantified the level of gliosis after injury. We found that at 1 DPI, both Val66Met and Val66Val injured mice had more astrogliosis than their sham controls in the cortex and hippocampus (**Figures 6B,C**). Interestingly, we found that the Val66Met injured mice had more astrogliosis than

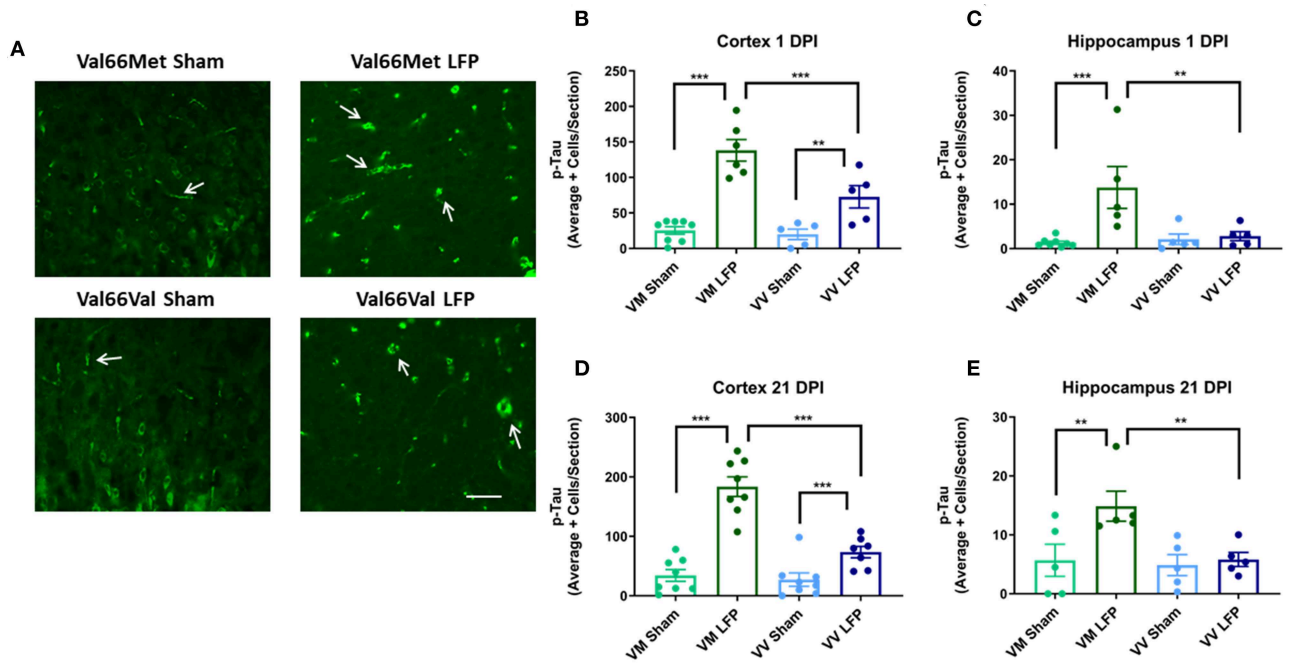

FIGURE 5 | Repeated mild LFP injury causes an increase in phosphorylated tau (p-tau) in the brains of injured Val66Met mice compared to injured Val66Val mice at 1 and 21 DPI. **(A)** Representative images of cortical sections at 1 DPI stained with activated AT8 (indicated by arrows). Scale bars = 100 μm. **(B–E)** Quantification of the average number of p-tau positive cells per cortex or hippocampus ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, $n = 5–8$.

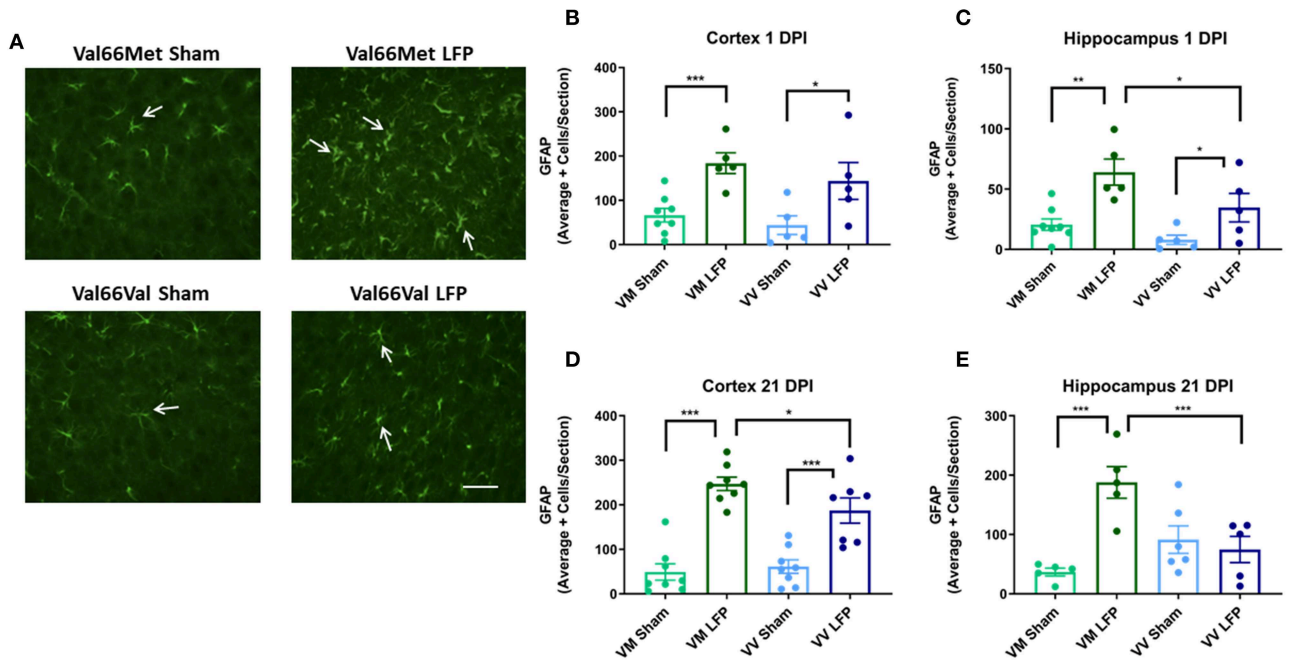

FIGURE 6 | Repeated mild LFP injury causes an increase in glial fibrillary acidic protein (GFAP) positive cells in the brains of injured Val66Met mice compared to injured Val66Val mice at 1 DPI. **(A)** Representative images of cortical sections at 1 DPI stained with glial fibrillary acidic protein (indicated by arrows). Scale bars = 100 μm. **(B–E)** Quantification of the average number of GFAP positive cells per cortex or hippocampus ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, $n = 5–8$.

Val66Val injured mice in the hippocampus at this 1 DPI, but not in the cortex, suggesting that the differential genotypic effects on astrocytes are more evident earlier in the time course in the more distal hippocampal region. By 21 DPI, both the Val66Met and Val66Val injured mice had significantly more astrogliosis than their sham controls in the cortex, although by this timepoint only the Val66Met injured mice remained detectably different from their sham controls in the hippocampus (**Figures 6D,E**). In analyzing genotypic differences, we found that the injured Val66Met mice also had increased astrogliosis relative to Val66Val injured mice in the ipsilateral cortex and hippocampus at 21 DPI. These data suggest that activation of astrocytes after injury is variable based on genotype, and that Val66Met mice have more injury-related cellular changes compared to Val66Val mice.

Injured Met Carriers Have Less Total BDNF in the Cortex at 21 DPI, and More pro-BDNF/mature BDNF in the Hippocampus at 1 DPI Compared to Injured Val Carriers

To examine a possible mechanism underlying the differences in genotypic response to rmTBI, we performed biochemical studies looking at levels of pro and mature BDNF. Previous studies have shown that in naïve mice, Val66Met and Met66Met mice have comparable levels of BDNF compared to their Val66Val counterparts, although they have less BDNF protein released from the dendrites (33). However, to our knowledge no one has assessed the levels of pro and mature BDNF after repeated mild TBI. In order to determine the effect of the Met allele on BDNF levels after injury and whether the Met allele functions in a dose dependent manner, we used Western Blot analysis in Val66Val, Val66Met, and Met66Met mice. The Met66Met group was added to this assay in order to get a more thorough understanding of the effect that the Met allele has on BDNF protein levels, although the comparison of the Met66Met group was not the focus of our study in other assays. We found that injured Met carriers did indeed have less total BDNF in the cortex at 21 DPI compared to injured Val carriers (**Figures 7A–C**). To quantify the amounts of pro and mature BDNF, we analyzed these isoforms separately but combined the mature bands at 14 and 16 kD to account for the differences in molecular weight caused by the Met-His tag on the BDNF transgenic gene. We found that at 1 and 21 DPI, injured Met carriers had more pro-BDNF/mature BDNF than Val carriers (**Figures 7D–F**). These data suggest that there is differential genotypic response to injury by demonstrating a biochemical alteration in the two genotypes and highlight a potential pathway to target for therapies.

Administration of AAV-BDNF to Val66Met Injured Mice Reduces the Level of Astrogliosis and Activated Microglia at 21 DPI to the Levels Seen in Val66Val Injured Mice

Analysis of our data suggests that Val66Met mice have more injury-related cellular responses after repeated mild LFP injury than their sham controls and Val66Val injured mice. Our Western Blot data show that there may be alterations in BDNF levels that are affecting outcomes after injury. Therefore, we injected an AAV-BDNF expressing vector, containing the wildtype 66Val form, immediately after the third injury to increase levels of BDNF in Val66Met injured mice and examined cellular and behavioral outcomes, using injections of AAV-GFP as the control. We previously found differences in both cellular and behavioral outcomes between injured and sham mice using our rmTBI model, but no differences between the Val66Met and Val66Val sham groups (**Figures 2–6**). Therefore, in our rescue study, we decided to focus on the differences we saw between the injured groups and the effect of the treatment on those groups.

We chose to analyze astrogliosis and activated microglia at the 21 DPI timepoint since we previously found significant differences between the two injured genotypes in these markers and this longer time point allows for expression of the AAV-BDNF throughout the cortex and hippocampus on the ipsilateral side with minimal expression on the contralateral side (**Supplemental Figure 2**). Val66Met injured mice treated with the control AAV-GFP had significantly higher levels of GFAP+ cells in the cortex and hippocampus and activated IBA1+ cells in the cortex relative to the Val66Met injured mice treated with the AAV-BDNF and the Val66Val injured mice treated with either AAV-GFP or AAV-BDNF at 21 DPI. The AAV-BDNF treated Val66Met mice had levels of astrogliosis and activated microglia that were similar to levels seen in the Val66Val injured mice, both those treated with the control AAV-GFP and AAV-BDNF (**Figures 8A–F**). These data show that treatment of injured Val66Met mice with AAV-BDNF can decrease astrogliosis and activated microglia relative to injured Val66Met mice treated with control AAV-GFP, suggesting that the AAV-BDNF treatment is able to reduce inflammation after injury.

Treatment of Val66Met Injured Mice With AAV-BDNF Improves Learning at 16 and 17 DPI, but Not Memory at 21 DPI, Back to the Levels Seen in Val66Val Injured Mice

We next examined the effect that our injury paradigm has on cognitive function using the Morris Water Maze to study spatial learning and memory. Previous studies have shown that in naïve mice, there is no difference in learning and memory between Val66Val and Val66Met mice (53). Accordingly, we found no difference between groups in the pre-test done before the injury protocol (**Figure 9A**). However, it has been established that brain injury can have detrimental effects on cognition, especially in animals that have defects in the hippocampus (31, 54). Here, we found that Val66Met injured mice treated with AAV-GFP had a longer latency to find the hidden platform than Val66Val injured mice treated with AAV-GFP at 16 and 17 DPI. Importantly, when the Val66Met injured mice were treated with AAV-BDNF they found the hidden platform significantly faster than the Val66Met injured mice treated with the control AAV-GFP at 16 and 17 DPI, indicating that overexpression of BDNF was able to improve spatial learning at these timepoints (**Figure 9B**).

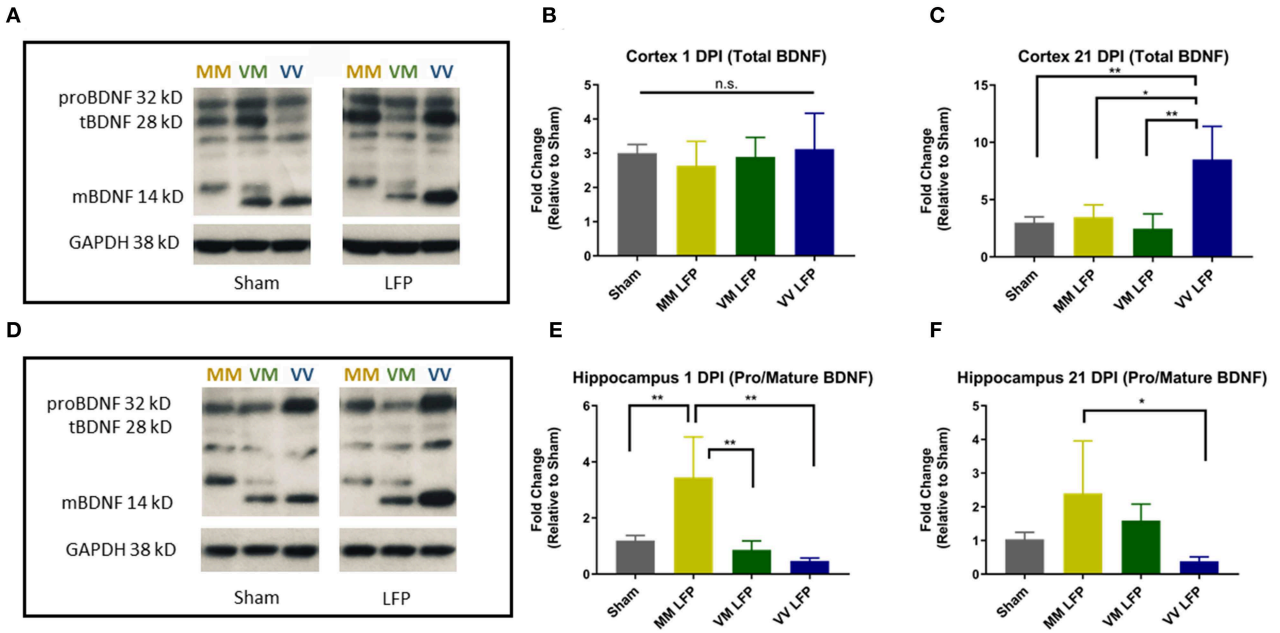

FIGURE 7 | Repeated mild LFP injury causes an increase in proBDNF/mature BDNF expression in Met carriers compared to Val carriers in the hippocampus at 1 and 21 DPI. **(A,D)** Representative Western Blot showing pro and mature BDNF expression in the hippocampus after injury. Each lane represents one animal. **(B,C)** Quantification of protein levels in the cortex at 1 DPI and 21 DPI and **(E–F)** hippocampus at 1 DPI and 21 DPI. All data is first normalized to GAPDH to control for protein loading and then expressed as a fold change relative to the average ± SEM of the time matched sham controls which are represented as a single bar in the graph. $^{*}p \leq 0.05$, $^{**}p < 0.01$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, $n = 4$.

In the Probe Test at 21 DPI, we found that the control AAV-GFP Val66Met injured mice spent significantly less time in the target NE quadrant than control AAV-GFP Val66Val injured mice, indicating impaired spatial memory. However, the AAV-BDNF Val66Met injured mice did not spend significantly more time in the target NE quadrant than the AAV-GFP Val66Met injured mice, indicating that treatment with overexpression of BDNF did not improve spatial memory in these mice (**Figure 9C**). These data suggest that in addition to improving cellular differences seen after injury, that treatment with AAV-BDNF is able to rescue functional cognitive deficits as well.

Treatment of Val66Met Injured Mice With AAV-BDNF Does Not Improve Motor Function at 14 and 21 DPI Back to the Levels Seen in Val66Val Injured Mice

It has previously been shown that following brain injury, there can be deficits in motor ability (54, 55). This is particularly true with our model of LFP, due to the damage that is done at the site of injury to the sensorimotor cortex (56). In order to measure motor ability, we used the rotarod test for vestibular motor function and proprioception and the balance beam test for more subtle differences in motor skills and balance. For the rotarod test, we conducted a pre-test to train the mice on the test, since performance will increase with practice, and to determine if there were any underlying genotypic differences in the mice. We found no differences in the pre-test (**Figure 10A**) and found that at 15 DPI AAV-GFP treated Val66Met injured mice had a shorter latency to fall compared to AAV-GFP Val66Val injured mice, indicating impaired vestibular motor ability in the Val66Met mice after injury. However, treatment with AAV-BDNF did not shorten the latency to fall in the injured Val66Met mice (**Figure 10B**). At 21 DPI, we did not find any differences across groups in latency to fall in the rotarod test, indicating that the vulnerable Val66Met injured mice had endogenously improved vestibular motor ability back to levels comparable with Val66Val injured mice (**Figure 10C**).

For the balance beam test at 21 DPI, we did not observe any difference between the injured Val66Met control AAV-GFP mice and either their Val66Met AAV-BDNF treated counterparts or the Val66Val control AAV-GFP injured mice (**Figure 10D**). These data suggest that while there may be differences in motor ability at earlier timepoints after injury, by 21 DPI Val66Met control AAV-GFP mice that are acutely vulnerable have recovered back to levels comparable with the less vulnerable Val66Val control AAV-GFP mice. Based on these results, as well as an analysis of swim speed in the MWM, we have concluded that there is no locomotor deficit evident at 21 DPI. Therefore, unlike our single moderate LFP paradigm that creates a significant long-term motor deficit (31), our repeated mild LFP appears to generate more subtle short-term motor deficits.

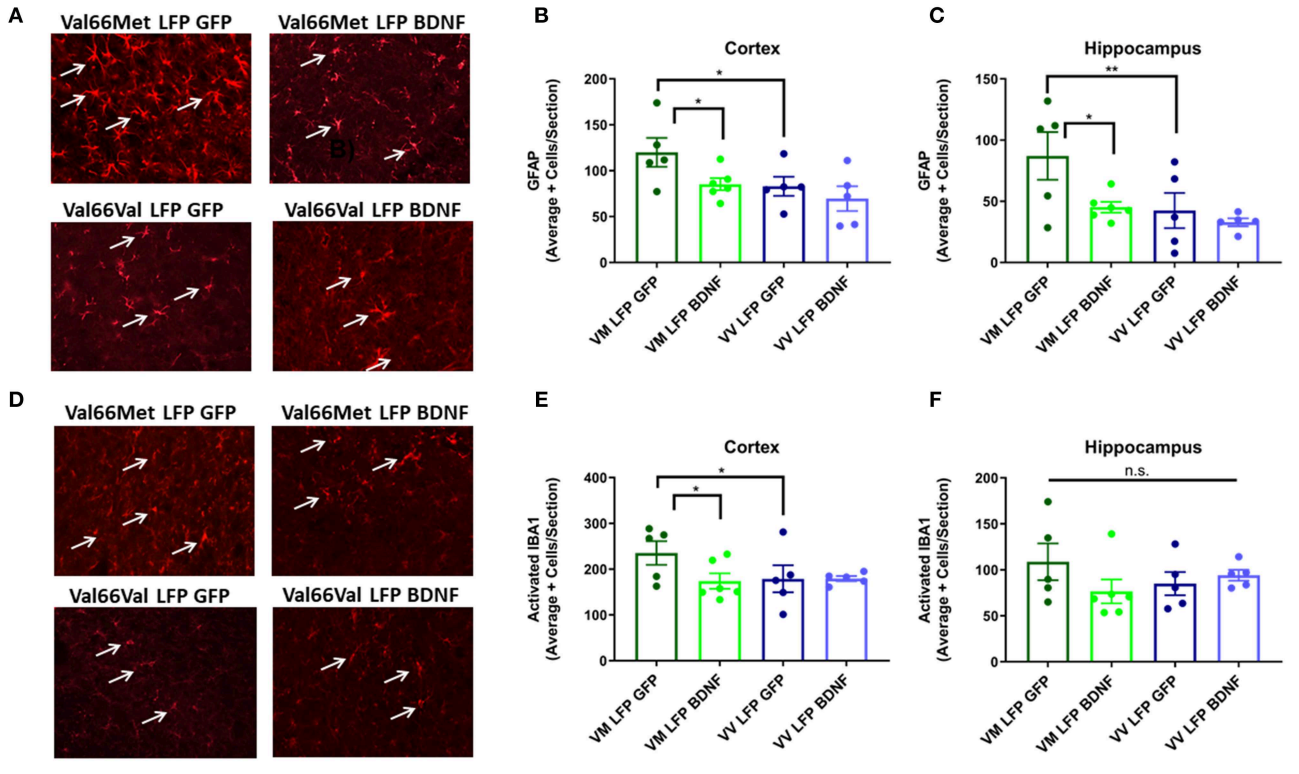

FIGURE 8 | Treatment of injured Val66Met with AAV-BDNF after repeated mild LFP injury causes a decrease in astrogliosis back to injured Val66Val levels in the cortex and hippocampus and activated microglia in the cortex at 21 DPI. **(A)** Representative images of cortical sections at 21 DPI stained with GFAP (indicated by arrows). Scale bars = 100 μm. **(B)** Quantification of the average number of GFAP positive cells per cortex and **(C)** hippocampus ± SEM. **(D)** Representative images of cortical sections at 21 DPI stained with IBA1 (indicated by arrows). Scale bars = 100 μm. **(E)** Quantification of the average number of activated IBA1 positive cells per cortex and **(F)** hippocampus ± SEM. $^{*}p \leq 0.05$, $^{**}p < 0.01$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, $n = 5$–6.

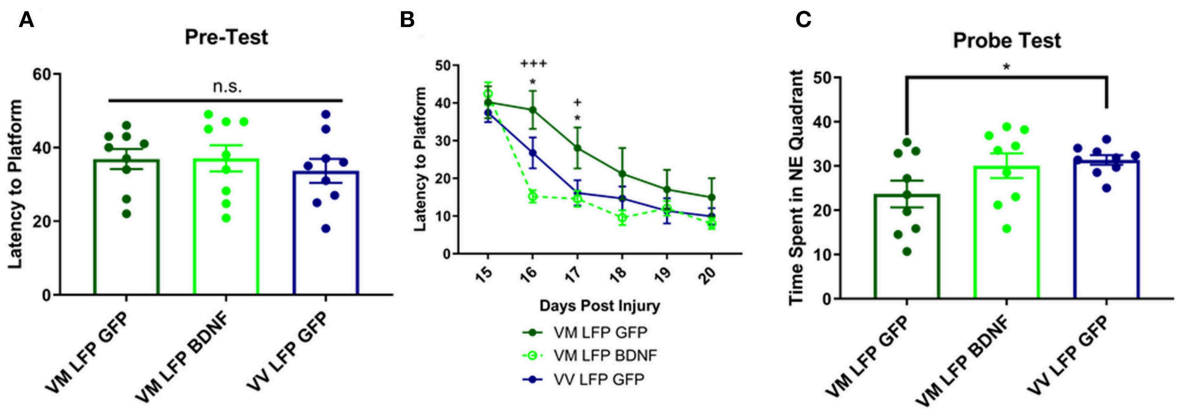

FIGURE 9 | Repeated mild LFP injury causes worse learning and memory in Val66Met injured mice relative to Val66Val injured mice. Treatment with AAV-BDNF in injured Val66Met mice improves learning, but not memory. **(A)** Average latency to platform in pretest phase ± SEM. **(B)** Average latency to platform ± SEM 15–20 DPI. **(C)** Average time spent in the target quadrant in the probe test ± SEM at 21 DPI. $^{*}p < 0.05$ in Val66Met LFP Control relative to Val66Val LFP Control, $^{+}p < 0.05$, $^{+++}p < 0.001$ in Val66Met LFP Control relative to Val66Met LFP BDNF. ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, $n = 9$.

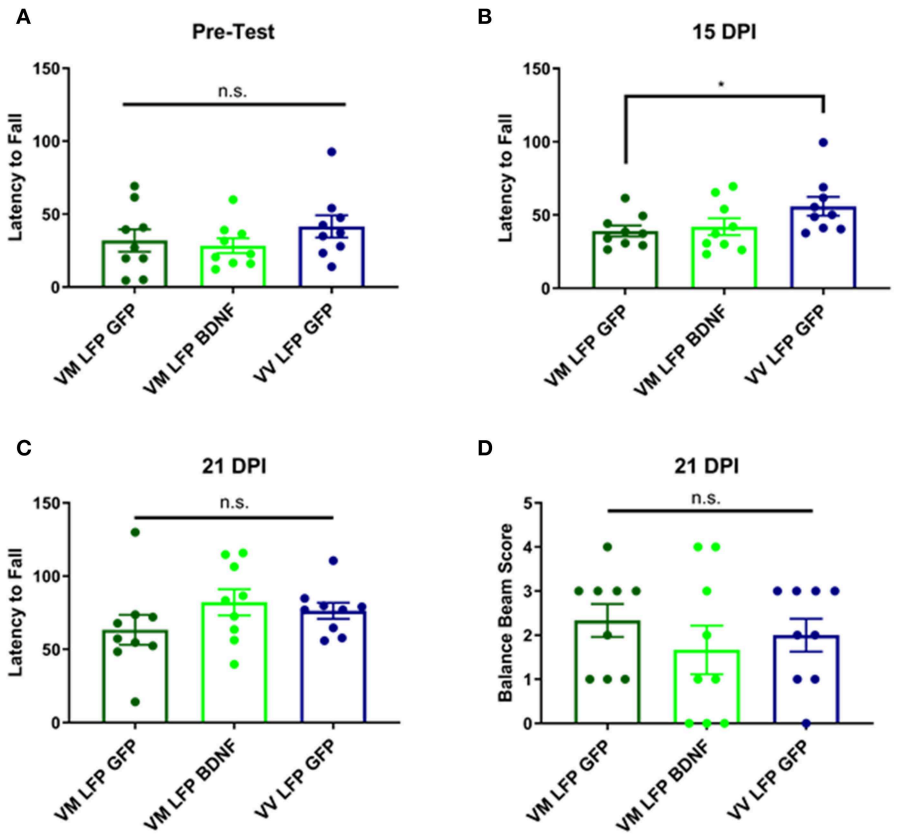

FIGURE 10 | Repeated mild LFP injury causes a deficit in vestibular motor function at 14 days in injured Val66Met mice relative to injured Val66Val. Treatment with AAV-BDNF does not significantly improve this deficit. **(A)** Quantification of latency to fall in the rotarod pretest assay ± SEM **(B)** Quantification of the latency to fall in the rotarod assay ± SEM at 14 DPI and **(C)** 21 DPI. **(D)** Quantification of balance beam score ± SEM at 21 DPI. *$p < 0.05$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups, $n = 9$.

Treatment of Val66Met Injured Mice With AAV-BDNF Increases Levels of Total and Mature BDNF as Well as p-trkB in the Cortex and the Hippocampus Relative to Val66Met Injured Mice Treated With Control AAV-GFP

In order to determine the mechanism by which the AAV-BDNF treatment was working to improve function in these mice, we conducted Western Blot analysis on brain tissue from the cortex and hippocampus after 21 DPI and examined levels of BDNF isoforms as well as trkB activation. We observed that the AAV-BDNF virus does indeed elevate levels of BDNF, particularly mature BDNF, in both the cortex and the hippocampus (**Figures 11A–C**). In addition, we found elevated levels of p-trkB/trkB in AAV-BDNF treated Val66Met injured mice relative to control treated Val66Met injured mice in both the cortex and the hippocampus (**Figures 11D,E**), signifying increased activation of the mature BDNF trkB signaling pathway. Together, these results suggest that disruptions in levels of BDNF play a role in the detrimental effects seen in the Val66Met injured mice and that targeted treatment to elevate BDNF can rescue the effects of repeated mild injury in this vulnerable genotype.

DISCUSSION

In this study, we show that BDNF Val66Met genetic polymorphism results in worse outcomes in terms of imaging, biological correlates, and behavior relative to Val66Val genotype following repeated mild LFP injury in mice. BDNF has been shown to play an important role after injury. Early studies showed that after injury, levels of BDNF protein and mRNA are upregulated but the isoforms were not examined at that time (57–61). Our group demonstrated that after TBI protein levels of proBDNF, proNGF, p75 receptor, and sortilin co-receptor are preferentially upregulated relative to mature BDNF and its trkB receptor which may explain why there is apoptosis and neurodegeneration after injury rather than cell survival and neural regeneration (31). These results are consistent with what has been shown in the literature, that after injury *in vivo* levels of proBDNF protein and mRNA are preferentially upregulated relative to mature BDNF (31, 61, 62). We have also demonstrated

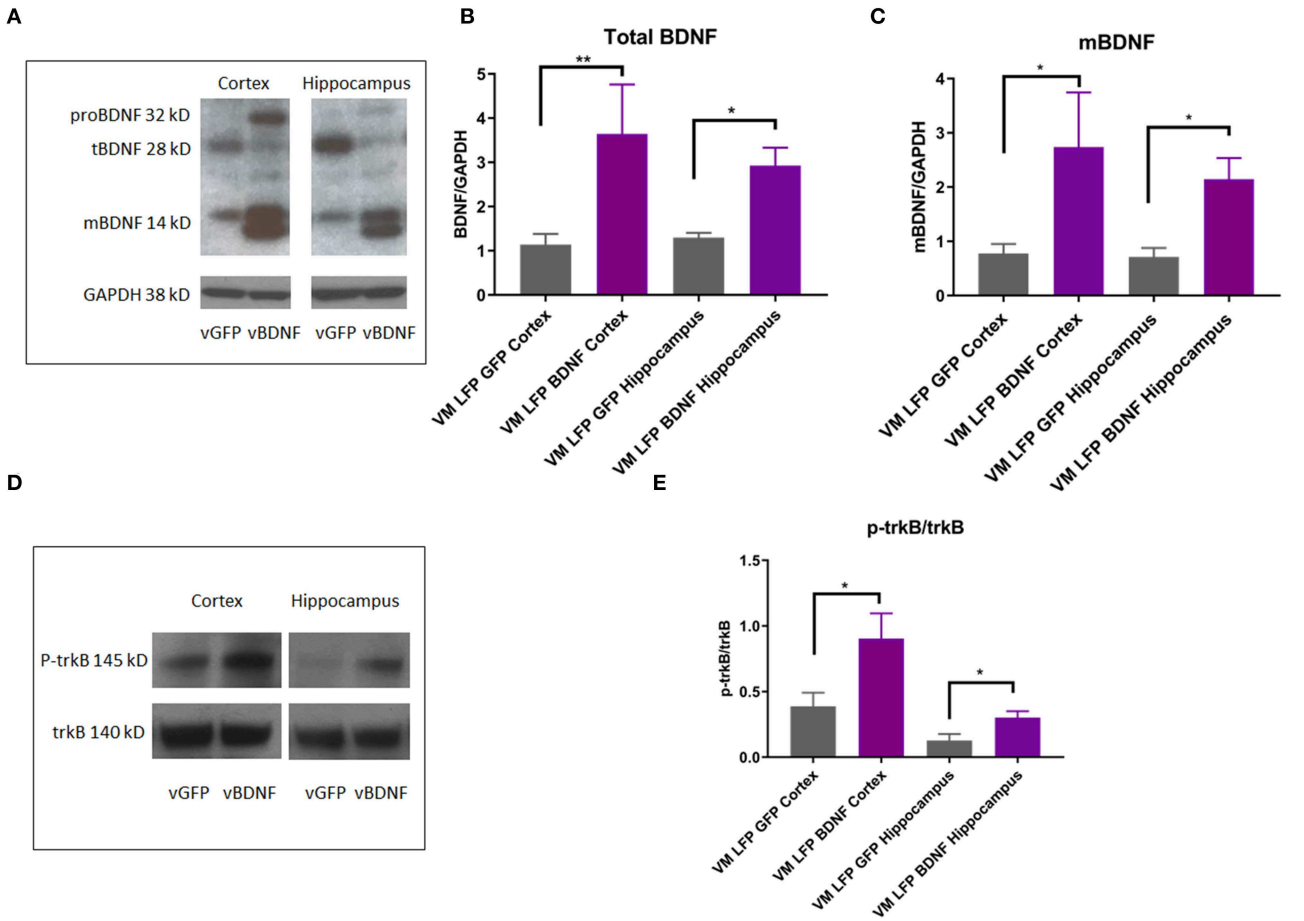

FIGURE 11 | AAV-BDNF injection causes an increase in total BDNF, mature BDNF, and p-trkB expression relative to control AAV-GFP injection in the cortex and hippocampus at 21 DPI in injured Val66Met mice. **(A)** Representative Western Blot showing BDNF expression in the cortex and hippocampus after injury. Each lane represents one animal. **(B)** Quantification of total BDNF protein levels ± SEM at 21 DPI in the cortex and hippocampus. **(C)** Quantification of mature BDNF protein levels ± SEM at 21 DPI in the cortex and hippocampus. All data is first normalized to GAPDH to control for protein loading ± SEM, $n = 6$. **(D)** Representative Western Blot showing p-trkB expression in the cortex and hippocampus after injury. Each lane represents one animal. **(E)** Quantification of p-trkB protein levels at 21 DPI in the cortex and hippocampus. All data is first normalized to trkB to control for protein loading ± SEM, $n = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$, ANOVA Fisher's PLSD *post-hoc* test relative to indicated groups.

that inhibiting p75 signaling or activating trkB signaling using genetic and pharmacological approaches improves cellular and behavioral response to injury (31). Other groups have shown that activating the mature BDNF trkB signaling pathways using molecules (63), neural stem cells (64), physical exercise, and acupuncture (65) can improve outcomes after TBI.

We show that Val66Met genetic polymorphism results in worse outcomes following repeated mild LFP injury. The Val66Met mice had a larger volume of inflammation by 21 DPI relative to Val66Val mice as assessed by MRI. At 1 DPI, there was also more cell death, neurodegeneration, phosphorylated tau, and activated microglia in the Val66Met injured mice compared to the Val66Val mice in the cortex and the hippocampus. By 21 DPI, the amount of cell death was reduced to sham levels while other markers sustained their elevated levels. In addition, astrogliosis became significantly elevated in the Val66Met injured mice relative to the Val66Val injured mice. Since the cortex was the primary site of injury, we found that effects were more dramatic, demonstrating the focal components of injury. We were also able to identify diffuse effects of the injury in the hippocampus in the ipsilateral hemisphere.

Taken together, these results indicate that the Val66Met injured mice respond differentially to a repeated mild injury than Val66Val injured mice starting as early as 1 day after the final injury. This is an important and novel finding since previous research on the effect of the Val66Met genetic polymorphism has been done primarily in clinical studies, with some results concluding Val66Met is the risk factor, while others concluding that Val66Val is more vulnerable. Specifically, while some clinical studies have shown that the Val66Met polymorphism results in impaired neurocognitive performance after TBI (21) and is a risk factor for TBI in combat forces (18–20), others have found that in long-term studies of combat veterans, carriers of the Val66Met polymorphism had recovery of executive function back

to baseline levels while the Val66Val carriers did not (15, 17). Moreover, other studies have shown no effect at all of the polymorphism influencing outcomes after injury (26–28). Our study is the first to investigate the role of the Val66Met genetic polymorphisms on outcomes after rmTBI in a mouse model, and to find that it is the Val66Met genetic polymorphism that is the risk allele in this type of trauma. Moreover, our data is supported by previous work investigating the role of the BDNF Val66Met genetic polymorphism in stroke and spinal cord injury. For example, after stroke there is evidence that humans with the Val66Met polymorphism do worse than those with the Val66Val polymorphism (13, 13, 66, 67).

We show here that in the acute phase, Val66Met mice have more cells undergoing apoptotic cell death than their Val66Val counterparts, and apoptosis has been shown in other studies to contribute to worse outcomes (68). However, after the acute phase of injury, levels of apoptosis often return back to baseline (69, 70), while other processes have been set in motion that continue to have a pathophysiological effect. The degeneration of neurons can be seen in the acute post injury phases as well, but it can have longer lasting effects that persist up until at least 21 days after the final injury (71). In this case, the injured Val66Met mice show higher levels of neurodegeneration which may contribute to the progressive loss of function in neurons in these mice. One specific way in which neurodegeneration can occur is the hyper phosphorylation of tau. When tau is hyper phosphorylated, it will bind to other tau proteins and create aggregates. This is a disruption of normal functioning and can lead to long-term neurodegeneration. One way in which tau phosphorylation can lead to neurodegeneration is by interfering with axonal transport. When this occurs, both retrograde trophic signaling as well as the autophagy process of defective tau proteins is disrupted, leading to long-term problems in function (39, 72, 73). Our findings are in agreement with other studies that have shown that acutely after repeated mild TBI, hyper phosphorylation of tau can emerge, while later stages of tauopathies such as accumulations of hyper phosphorylated tau like neurofibrillary tangles (NFTs) are not yet seen (74). We demonstrate that injured Val66Met mice have higher levels of p-tau than their Val66Val counterparts, which highlights the potential for p-tau to contribute to the differential development of long-term outcomes after TBI.

In addition to cell death and neurodegeneration after injury, it is also common to see inflammation and activation of the neuroimmune response. Astrocytes generally work to maintain homeostasis in the brain, and are known to play an important role in the response to TBI (75). After TBI, astrocytes are activated and repair the damage from the injury. While the actions of astrocytes at baseline tend to be beneficial, after injury the prolonged activation of astrocytes can lead to inflammation and secondary injury processes. In particular, the formation of a glial scar can impair neuronal regeneration and lead to worse long term outcomes (76). We see that 1 day after the final injury, both Val66Met and Val66Val injured mice have an elevation of activated astrocytes, but by 21 days after the final injury the Val66Met injured mice have significantly more activated astrocytes than Val66Val injured mice, suggesting they may have impaired neuronal regeneration due to the formation of a glial scar. Microglia are the resident neuroimmune cells in the brain and are activated as early as 1 day after the final injury and persist until at least 21 days after the final injury in our study. Along with astrocytes, microglia are responsible for initiating the inflammatory response after injury at which point, they become activated. Similar to the role of activated astrocytes after injury, while the initial response may be protective, if the activated microglia persist over time they will contribute to secondary injury processes and worse long term outcomes (77). We have shown that as early as 1 day after the final injury, the Val66Met injured mice have elevated activated microglia relative to Val66Val injured mice, and that this difference persists until at least 21 days after the final injury, perhaps contributing to differential long-term outcomes.

Our findings that the Val66Met polymorphism is a risk allele after rmTBI is consistent with recent reports in the literature. Recent human studies have shown that the Met allele is a risk factor after a single mild TBI in the areas of attention, executive function, memory, and overall cognition (21). In a study that examined emotional symptoms after a single mild TBI, Met carriers were found to have more emotional symptoms than Val carriers (78). The Met allele has also been found to be a risk factor after a single mild-moderate TBI in cognitive language processing speed (27). Interestingly, previous studies have shown that the Met allele was actually protective in long-term executive function after focal frontal TBI in combat veterans (15, 17), perhaps indicating the importance of the SNP interaction with type of injury sustained; however, the retrospective analysis was unable to eliminate confounding factors that may have played a role. In addition to the effects of the Val66Met polymorphism on outcomes following TBI, there have also been reports of its effect after spinal cord injury. Val66Met has also been shown to be a risk factor for a worse clinical presentation in cervical spondylotic myelopathy (79), impaired spinal cord plasticity (80), and low exercise induced serum BDNF levels after spinal cord injury (81) in humans. It is thought that after stroke Val66Met might be a risk factor for poor outcomes (13, 13, 66, 67). However, that the time course of stroke recovery may be more complicated than originally thought, with the Val66Met allele shown to be the risk allele in motor ability acutely (82), and surprisingly, Val66Val allele shown to be the risk factor chronically (83). While yet another study has posited that perhaps Val66Met carriers do not have worse overall recovery but recover using different underlying brain pathways (84). Some research has suggested that the Val66Met allele may cause an altered cytokine response (85, 86). There have been no reports of the effect that the Met66Met genotype has on outcomes after TBI at the time of writing, but studies have shown that the Met66Met allele may confer more risk than the Val66Met allele in terms of anxiety, OCD, and depression (38, 87, 88). Further investigations on the effect of the Met66Met genotype on recovery after TBI in addition to the Val66Met and Val66Val groups would be beneficial to the field.

To our knowledge, this is the first report that shows the effect of the Val66Met SNP on outcomes after repeated mild TBI. Our results provide evidence that the Val66Met genetic polymorphism confers risk after repeated mild TBI.

Furthermore, we have shown that the Val66Met genetic polymorphism alters the upregulation of BDNF that occurs after injury. Previously, our group as well as others, have shown that after TBI *in vivo*, levels of proBDNF protein and its p75 receptor are preferentially upregulated relative to mature BDNF and its trkB receptor (31, 61, 62). Other groups have shown that the Val66Met genetic polymorphism does not affect the relative levels of pro and mature BDNF in naïve mice, despite the fact that the SNP is located in the prodomain of the protein (32, 33). However, importantly, it does decrease intracellular trafficking of BDNF and lowers the activity-dependent secretion of BDNF which can result in functional deficits (24). Here, we have shown that after repeated mild TBI, Met carriers had less total BDNF in the cortex by 21 DPI compared to Val carriers. While we expected to see a dose dependent effect of the Met allele, we found that simply having a single Met allele was significant enough to decrease total BDNF levels. However, it is uncertain at this time whether the Met-allele associated effects are exerted at the level of BDNF secretion or the BDNF promotor. We have also demonstrated that in the hippocampus at 1 and 21 DPI, Met carriers had significantly more pro/mature BDNF compared to Val carriers. Our data therefore suggests that these alterations in BDNF levels after injury in Met carriers may contribute to the worse outcomes seen.

Given that the Val66Met genetic polymorphism appears to be a risk allele following repeated mild TBI and that altered BDNF levels may be a contributing factor, we decided to attempt to rescue the Val66Met injured mice by injecting an AAV that overexpresses wildtype BDNF in the cortex and the hippocampus after injury. Previous studies have employed delivery of BDNF through AAV in animal models as a potential treatment for various brain pathologies, such as depression (89), stroke (43, 90), Alzheimer's disease (91), and spinal cord injury (92, 93). We chose to deliver the AAV treatment immediately after the last injury, and investigated the effect of our treatment on outcomes at 21 DPI because we observed worse outcomes in cellular markers in Met carriers as well as significant differences in BDNF levels in the cortex and hippocampus at this later time point. In addition, focusing on this later time point allows time for the AAV to express sufficiently and allows us to determine the treatment's efficacy in changing longer-term outcomes, which has more translational potential.

Here, we have shown that BDNF overexpression was able to decrease levels of astrogliosis, a well-known marker for poor outcomes after injury, in Val66Met injured mice back to the levels seen in Val66Val injured mice. BDNF overexpression was also able to reduce levels of activated microglia in injured Val66Met mice to levels equivalent to injured Val66Val mice, signaling a reduced pro-inflammatory post injury phase.

In addition to the differences in cellular markers that we observed, we also investigated the effect that AAV-BDNF has on motor and cognitive behavior outcomes. We found that AAV-BDNF is able to increase spatial learning in injured Val66Met mice at 16 and 17 DPI in the Morris Water Maze Test. However, we did not find that treatment with AAV-BDNF had any effect on motor outcomes at 15 DPI or 21 DPI in the rotarod test and balance beam assay, signifying that there are potentially other factors driving the difference between injured Val66Met and injured Val66Val mice in terms of gross vestibular motor ability. These data suggest that the AAV-BDNF treatment had a more potent effect on rescuing hippocampal neurons than on sensorimotor neurons. This may be due to the fact that hippocampal neurons have a high density of trkB receptors and are therefore more responsive to our AAV-BDNF treatment (94).

Given that previous studies have shown the importance of BDNF for hippocampal-dependent processes (95), it is logical that increasing levels of BDNF in these mice improves their recovery in learning assays after injury and may be an important specialized treatment for Val-Met carriers who have lower levels of BDNF compared to their Val-Val counterparts after injury. We found that in these AAV-BDNF treated injured Val66Met mice that injection of the virus increases levels of total and mature BDNF in the cortex and hippocampus, as well as levels of p-trkB at 21 DPI, signifying that the mature BDNF pathway is more activated in AAV-BDNF treated mice relative to control treated mice. These results offer promising evidence that by manipulating the BDNF pathway, we may be able to develop targeted therapies for Met carriers who are more susceptible to poor outcomes after injury.

Since the Val66Met SNP is at the 66 amino acid position which is in the pro domain of the BDNF protein, when the prodomain is cleaved from the mature, the resultant mature BDNF protein will have no altered sequence. However, studies have shown that the genetic polymorphism in the proBDNF gene leads to altered intracellular packaging, which affects the axonal transport of BDNF and results in decreased activity dependent section of BDNF at the synapse (24, 33). Naturally, this can become an issue in disease states such as after TBI, where there is a need for an increased mature BDNF signaling in order to stimulate repair and recovery. In addition, recent work has highlighted the importance of the prodomain itself that has been cleaved off from the mature BDNF protein. Previously thought to be inert, new research has shown that it is in fact an active ligand. Recent work has shown that the pro domain itself has been shown to promote hippocampal long-term depression (LTD) both directly (96), as well as indirectly, by binding to mature BDNF with high affinity and weakening mature BDNF's ability to inhibit hippocampal LTD (97). Importantly, the 66Met substitution changes the structure of the protein, which results in changes of its function, including inhibiting hippocampal LTD *in vivo* (96) and causing acute growth cone retraction *in vitro* (98). In the hippocampus, the pro domain with the Met allele decreases Rac activity, a mediator of synaptic plasticity (98). Newer studies have shown that the 66Met prodomain is also able to disassemble dendritic spines and eliminate synapses in hippocampal neurons, leading to impaired hippocampal dependent fear extinction behavior (99). While the role of the prodomain and the effect that the 66Met substitution are still being investigated, we hypothesize that the prodomain may also play a role in differences in outcomes between Val66Val and Val66Met carriers. Given this knowledge, treatment with AAV-BDNF which supplies the 66Val form of BDNF, may be a useful treatment for other conditions that Val66Met carriers suffer from in addition to its ability to facilitate recovery after rmTBI.

In order to translate these finding into a clinical population, there must be an appreciation for the patient population. In the real world, many people take chronic anti-inflammatory drugs for other indications. Studies have shown that evidence that anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs (NSAIDS) may either impede (100) or improve (101) outcomes after TBI. These issues need to be investigated further, especially in a model of rmTBI (102).

Our study lays important experimental groundwork in the investigation of the genetic underpinning of the differential response to injury seen in the TBI patient population, and highlights the rs6265 SNP and the BDNF signaling pathway as a potential mediator of these differences. However, our study was limited by several factors that future studies should attempt to incorporate. While the benefit of our study is that we could clarify the role of a single SNP, there may be interactions of the rs6265 SNP with SNPs in other genes that could play a role in determining genetic susceptibility. We also maintained strict control over the environment that the mice were in and investigated outcomes at the set timepoints of 1 and 21 DPI. Future studies should investigate the interaction of genetic susceptibility with other factors such environmental influences, longer time points post injury, and age of the subject. Given that the LFP model has diffuse effects in the brain, we should also look at the effects on white matter tracks and other areas of the brain, including the contralateral side. In addition, we used a very controlled and replicable repeated mild LFP injury method. Given that the human TBI data seems to suggest that genetic risk factors may vary based on the different types of injury sustained, future studies should investigate the role of the rs6265 in other forms of injury as well. Finally, we found that treatment with AAV-BDNF with the 66Val form can facilitate recovery after TBI, but we did not investigate the role that 66Met prodomain might be playing after injury. This would be an interesting pathway to investigate more thoroughly, potentially by analyzing signaling through the SorCS2 pathway, or analyzing structural differences between the two forms of the prodomain. There may also be value in exploring other pharmacological treatment approaches; for example, to increase BDNF signaling pathway activation by the use of trkB agonists such as 7,8 DHF. In addition, further research into the daily use of anti-inflammatory drugs by vulnerable genotypes may provide necessary insight into the effect that these drugs have on outcomes after TBI.

Taken together, this study has investigated the role of the Val66Met genetic polymorphism on cellular markers and demonstrated the role that it plays on BDNF levels and signaling after repeated mild TBI. We have explored using overexpression of BDNF as a personalized therapy for the susceptible Met carriers, and highlighted the potential usefulness of targeting BDNF signaling pathways for treatment.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Rutgers University Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

AG, JA, and ST-V conceived and planned the experiments and supervised data collection. AG, ST, SR, and CZ carried out the experiments. DA conducted the imaging analysis. AG, ST, and CZ analyzed data. AG interpreted the results and wrote the manuscript. All authors provided critical feedback and helped to shape the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.01175/full#supplementary-material

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The Increasing Age of TBI Patients at a Single Level 1 Trauma Center and the Discordance Between GCS and CT Rotterdam Scores in the Elderly

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Introduction: Traumatic brain injury (TBI) is frequently encountered in geriatric patients, but there is a paucity of data describing TBI in the elderly. Here, we show the age of patients with TBI is increasing at our medical center and discuss the relationship between age and injury severity with patient outcomes.

Methods: This is a retrospective analysis of 3,179 adult patients with TBI treated at the University of California, Davis Level 1 Trauma Center between 2009 and 2016. Age, Glasgow Coma Scale (GCS), and CT Rotterdam Scores were recorded. Age was analyzed as both a continuous and categorical variable (18–34, 35–50, 51–65, >65 years-old). Extended Glasgow Outcome Scale was obtained at 3 and 6 months and dichotomized into favorable and unfavorable outcomes. Multivariable general linear regression models, chi-square, logistic regression analyses and ANOVA were used for statistical analyses; a $p < 0.05$ was considered significant.

Results: The mean age of patients was 52.2 ± 21.9 years with a male predominance (69%). There was a significant trend ($p = 0.002$) toward an increase in mean age each year, increasing by 4.4 years ($p = 0.008$) over the course of the analysis. Older patients had a higher mean GCS compared to younger patients with the same CT Rotterdam Score ($p = 0.027$), this becoming more pronounced with worse CT Rotterdam Scores. The >65 group had a 4-fold increased risk for unfavorable outcome when compared to the 18–34 group, this effect being most pronounced after mild TBI.

Conclusions: The mean age of TBI patients is increasing at our trauma center. The largest disparity in outcomes across age was seen in patients with a mild GCS and low CT Rotterdam Scores, suggesting that these markers of injury severity may underestimate the severity of injury in the elderly population. This information highlights the need for clinical trials and validation of outcome markers in geriatric TBI.

Keywords: traumatic brain injury, TBI, geriatrics, Glasgow Coma Scale, CT Rotterdam Score, geriatric TBI

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of morbidity and mortality worldwide. In the United States, TBI contributes to ∼30% of deaths related to injury (1), and leads to 2.53 million ED visits, hospitalizations and deaths annually (2). Historically, motor vehicle crash (MVC) has been the leading cause of TBI in the United States, but analysis of recent demographic data highlights a growing proportion of fall-related injuries, surpassing MVCs as the leading cause of TBI in developed countries (1, 3). Similar trends have also been seen in Europe (4).

The major contributing factor to the changing incidence of TBI mechanisms is related to the growing elderly population. Falls are the most common mechanism of injury in the elderly secondary to the effects of age on physical function (5, 6). It is estimated that between 45 and 78% of geriatric patients are frail at the time of injury and this frailty has been associated with a 50% increase in mortality (7–10). One can reason, then, that the incidence of geriatric-related injuries will continue to increase as the elderly population continues to grow. In the United States, the population older than 65 years of age has increased from 12.4% in 2,000 to 14.9% in 2015 and this is expected to increase to 19.6% by 2030 (11, 12). In addition, the number of individuals over the age of 80 is predicted to increase by 10.2 million between 2000 and 2030 (12). Accordingly, the field of geriatric TBI will need to mature along with the aging population to better understand outcome prediction.

Differences in outcomes after TBI are associated with age, with outcomes worsening as early as age 45 (6, 13). While the incidence of TBI in the younger population has remained relatively unchanged, the incidence of TBI is increasing in the elderly population (10, 14, 15). In addition, while patients 65 years and older only account for 15% of the population, they make up 50% of TBI-related deaths (10, 12) and patients older than 75-years old experience twice the rate of TBI (10). This leads to increased resource utilization due to longer hospital stays and more frequent follow-up (6, 10, 13). In addition, acute measurements of injury severity, such as the Glasgow Coma Scale (GCS), may not be reliable predictors of morbidity and mortality in patients over the age of 45 (10). In one study by Livingstone et al. (13), even though older patients had less severe GCS scores, they had worse functional recovery when compared to younger populations.

Through a retrospective analysis of a prospectively maintained TBI registry at a Level 1 Adult Trauma Center, we show that the age of our patient population is increasing and discuss the relationship between age, severity of injury (both radiographic and clinical), and functional outcomes, and highlight the need for better predictive models in geriatric TBI.

METHODS

Patients

This is a retrospective analysis of patient data collected from all adult patients with TBI that were treated at the University of California, Davis Level 1 Trauma Center between 2009 and 2016 as part of an Institutional TBI Registry. As a Level 1 Trauma Center in the United States, all patients, regardless of age, insurance status or ability to pay are stabilized, treated and if necessary, admitted per the Emergency Medical Treatment and Labor Act (EMTALA). Patients included in this registry were all patients seen by the neurological surgery service who met at least one of the following two criteria that prompted consultation: (1) suspected TBI due to clinical history, clinical symptoms, or signs of neurological deficits on physical examination, or (2) abnormal head computed tomography (CT) scan findings after trauma. Neurological surgery consultation is mandated at our institution for all TBI severities, as measured by the GCS. All patients 18-years or older were included in this study and baseline characteristics at the time of injury were obtained, including age, sex, mechanism of injury, and severity of injury. Age was grouped into four categories: 18–34, 35–50, 51–65, and older than 65 years.

Mechanism and Severity of Injury

The mechanism of injury was recorded at the time of neurological surgery consultation and was categorized as assault, automobile vs. pedestrian (AvP), fall, motor vehicle crash (MVC), or "other." Motor vehicle crashes included patients injured in an automobile or a motorcycle. Mechanisms of injury categorized as "other" included penetrating injuries, bicycle accidents, patients found down with no clear mechanism, fall from horse, fall from moving vehicle, sport related accident, or another mechanism not well-characterized. These other mechanisms each had too few incidences to allow for individual categorization. Clinical injury severity was recorded as the post-resuscitation GCS at the time of neurological surgery consultation and categorized as mild (GCS 13–15), moderate (GCS 9–12), or severe (GCS 3–8). The first CT head obtained after the TBI was used to calculate a CT Rotterdam Score, a commonly used measure of radiographic trauma severity that ranges from 1 (mild) to 6 (most severe) (16). Given our low sample sizes with a CT Rotterdam Score of 6, these categories were combined into the category 5 score for analysis.

Outcomes

Trained research assistants performed phone interviews with either the patient or a surrogate to assess outcome, as measured by the Extended Glasgow Outcome Scale (GOSE). The GOSE is the most widely used measure of global functional outcome following TBI and has been recommended as the standard outcome measure for TBI studies (17, 18). Our primary outcome was a dichotomized GOSE (favorable vs. unfavorable) at three and 6 months. Patients with a favorable GOSE included lower moderate disability, upper moderate disability, lower good recovery, and upper good recovery, while unfavorable GOSE included upper severe disability, lower severe disability, vegetative, and dead.

Statistical Analysis

Multivariable general linear regression models were used to examine the effects of age, CT Rotterdam Score, and GCS on the Glasgow Outcome Score. Chi-square analyses were used to examine relationships between categorical variables. Multivariable logistic regression was used to examine effects of

age, CT Rotterdam Score, and GCS on categorical neurological outcomes (favorable or unfavorable) at 3 and 6 months. For logistic regression models, the age group "18–34" was used as the reference. ANOVA was used to examine relationships between categorical and continuous variables. A p of < 0.05 was considered significant. All statistical analyses were performed using SAS software v.9.4 (SAS Institute Inc., Cary, NC). Prior to analysis, approval was obtained from the UC Davis Institutional Review Board.

RESULTS

Population Characteristics

A total of 3,179 patients were included in this analysis (**Table 1**). The mean age of the entire cohort was 52.2 ± 21.9 years and 69% were male. From 2009 to 2016, there was a significant trend ($p = 0.002$) toward an increase in average age each year, such that mean age increased by 4.4 years ($p = 0.008$) over the course of the analysis (**Figure 1**). Patients > 65 years of age was the most frequently encountered age group, representing 30% of all patients in the cohort, followed by the group aged 18–35 years (28%). Fall was the most frequent mechanism of injury (39.0%), an incidence more than double that of any of other mechanism (**Table 1**). The relationship between mechanism of injury and categorical age was significant ($p = 0.008$), such that patients who suffered a fall were nearly three times as likely to be in the oldest category when compared to other mechanisms of injury.

Severity of Injury

Seventy percent of patients presented with a mild GCS, while 14 and 16% presented with a moderate or severe GCS, respectively. Chi-square analysis showed that GCS was affected by age ($p = 0.003$), such that the older patients were more likely to present with a mild GCS (**Table 2**). The CT Rotterdam Score was known for 1,969 (62%) patients. Linear regression showed that the relationship between GCS and the CT Rotterdam Score varied with age, such that older patients (>65 group) had a higher average GCS score ($p = 0.027$) compared to younger patients with the same CT Rotterdam Score. This separation in GCS from the oldest age group to the younger groups was more pronounced as the CT Rotterdam Score worsened (**Figure 2**).

Outcome at 3 Months

GOSE at 3-months was known for 2,713 patients (85% of the entire cohort) and was significantly affected by categorical age ($p < 0.001$), such that the 51–65 age group was 1.5 (OR = 1.5, 95% CI 1.2–2.0) times more likely to have an unfavorable outcome when compared to the 18–34 age group (**Table 2**). The oldest age group (> 65 years old) had the largest risk of unfavorable outcome, being 3.7 (OR = 3.7, 95% CI 3.0–4.6) times more likely to have an unfavorable outcome when compared to the 18–34 age group (**Table 2**). In a logistic regression model with age as the continuous and sole explanatory variable, every year older increased the odds of an unfavorable outcome by 2.6% at 3 months (OR = 1.026, $p < 0.001$). When controlling for GCS and its interaction with age, there continued to be a significant effect ($p < 0.001$) on GOSE, with the relationship being more pronounced following mild and moderate injuries, while outcomes following severe injuries were similar across age groups (**Table 2**). The interaction between injury severity as measured by the CT Rotterdam Score and age was statistically significant ($p < 0.0001$) showed similar trends (**Table 3** shows representative ORs at three different CT Rotterdam Scores).

Outcome at 6 Months

GOSE at 6-months was known for 2,464 patients (78% of the entire cohort). The relationship between age and outcome was similar to what was seen at 3-months, such that the 51–65 age group and > 65 age group had 1.8 (OR = 1.8, 95% CI 1.4–2.3) and 4.2 (OR = 4.2, 95% CI 3.32–5.2) times the likelihood of having an unfavorable outcome when compared to the 18–34 age group (**Table 2**), respectively. In a logistic regression model with age as the continuous and sole explanatory variable, every year older increased the relative odds of an unfavorable outcome by 2.9% at 6 months (OR = 1.029, $p < 0.001$). When controlling for GCS, age continued to have a significant effect ($p = 0.03$) on outcome, similar to the relationships seen at 3-months. When controlling for injury severity as measured by the CT Rotterdam Score, age continued to have a significant effect ($p < 0.0001$) on the GOSE at 6 months (**Table 3**). The change in outcome from three to 6 months was significantly affected by age ($p < 0.001$), such that the younger age groups were more likely to have improved overtime than the two older age groups, although the effect size was small (**Table 2**).

DISCUSSION

As the population of the United States continues to age, our understanding of the presentation and outcome following geriatric TBI will need to evolve. The data presented here confirms that in our medical center, the average age of patients suffering a TBI increased by 4.4 years from 2009 to 2016 and that patients older than 65 years were more frequently encountered than younger age groups. This institutional trend mirrors what is happening at the national level, where the incidence of TBI continues to increase in the elderly population, particularly in patients >75 years of age, while incidence in the younger population has remained relatively unchanged (10, 14, 15). In fact, TBI-related hospital visits among the oldest segment of the U.S. population has actually exceeded population growth in recent years (14). In line with the increasing age of the TBI population, fall was the most commonly encountered mechanism of injury in our cohort (39% of the population), followed by MVC (20%). An injury of the elderly, nearly 80% of patients who suffered a fall in our cohort were older than the age of 50 and is consistent with other published reports (14, 15, 19). This is consistent with the elderly populations increased risk for frailty and multiple co-morbidities (5, 7, 10).

Most of the patients in our cohort suffered a mild TBI, while much of the literature focuses on patients with moderate-to-severe injuries. In this study, the interaction between age group and GCS on admission was statistically significant, such that patients older than 65 years were more likely to present with a mild GCS. To our knowledge, this is only

TABLE 1 | Demographics of 3,179 traumatic brain injury patients seen at the University of California Davis Medical Center between 2009 and 2016.

	Age group (years)				
	All	**18–34**	**35–50**	**51–65**	**>65**
Number of patients (% total)	3,179 (100)	885 (28)	549 (17)	785 (25)	960 (30)
Age, years ± SD	52.2 ± 21.9	24.9 ± 4.7	43.4 ± 4.8	57.7 ± 4.2	79.0 ± 8.3
Gender (% male)	2207 (69)	717 (81)	470 (85)	505 (64)	515 (54)
GCS, *n* (%)*					
Mild	2221 (70)	534 (60)	356 (65)	557 (71)	774 (81)
Moderate	451 (14)	157 (18)	87 (16)	108 (14)	99 (10)
Severe	507 (16)	194 (22)	106 (19)	120 (15)	87 (9)
Mechanism of Injury, *n* (%)					
Assault	443 (14)	189 (21)	140 (23)	94 (13)	20 (2)
AvP	291 (9)	94 (11)	77 (13)	81 (11)	39 (4)
Fall	1241 (39)	109 (12)	143 (23)	267 (37)	722 (75)
MVC	629 (20)	300 (34)	113 (18)	130 (18)	86 (9)
Other	575 (18)	1933 (22)	142 (23)	147 (20)	93 (10)
CT Rotterdam Score *n* (%)	*n* = 1,969	*n* = 569	*n* = 349	*n* = 406	*n* = 645
1	76 (4)	36 (6)	11 (3)	15 (4)	14 (2)
2	841 (43)	236 (41)	157 (45)	164 (40)	284 (44)
3	744 (38)	176 (31)	112 (32)	171 (42)	285 (45)
4	183 (9)	67 (12)	44 (13)	31 (8)	41 (6)
5	97 (5)	34 (6)	19 (5)	23 (6)	21 (3)
6	28 (1)	20 (4)	6 (2)	2 (0)	0

*AvP, auto vs. pedestrian; CT, computed tomography; GCS, Glasgow Coma Scale; MVC, motor vehicle crash; n, sample size; SD, standard deviation; *Differences in GCS noted across age groups (chi-square, p = 0.003).*

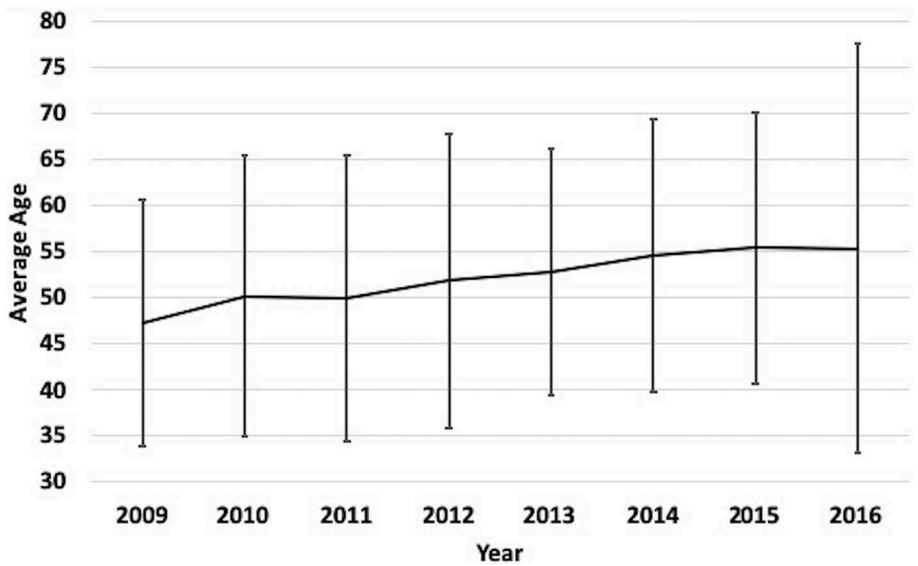

FIGURE 1 | Yearly mean age of TBI patients at our medical Center from 2009 to 2016. Label: Bars indicate standard deviation.

the second study that has compared the distribution of TBI severity across age categories. The first was an analysis of the National Trauma Data Bank National Sample Program TBI cases and did not find a difference in TBI severity across several age categories, with most patients presenting after mild injury (14). Differences in this study and ours may be related to sample size (522,882 vs. 3,179 patients) and age categories, with the previous study having smaller age ranges (10 year increments starting after the age of 45). Regardless of these study differences, it's clear that most elderly patients are presenting with a mild GCS, and, as we and others have shown, suffer worse outcomes.

TABLE 2 | The effects of patient age and GCS on outcomes at 3 and 6 months following traumatic brain injury.

Age group (years)		Unfavorable outcome at 3 months *n* (%)	Unfavorable outcome at 6 months *n* (%)	Odds ratio unfavorable outcome 3 months OR (95% CI)	Odds ratio unfavorable outcome 6 months OR (95% CI)
All patients	18–34	228 (31)	181 (27)	**Reference**	**Reference**
	35–50	139 (32)	135 (31)	1.2 (0.9–1.6)	1.3 (1.0–1.6)
	51–65	258 (40)	213 (41)	1.5 (1.2–2.0)	1.8 (1.4–2.3)
	>65	555 (62)	508 (61)	3.7 (3.0–4.6)	4.2 (3.3–5.2)
Mild GCS	18–34	29 (7)	26 (6)	**Reference**	**Reference**
	35–50	37 (4)	34 (13)	2.9 (1.7–5.0)	2.3 (1.3–4.0)
	51–65	118 (27)	91 (16)	5.6 (3.4–9.2)	4.9 (3.0–8.1)
	>65	400 (56)	363 (55)	20.0 (12.6–31.6)	18.3 (11.5–28.9)
Moderate GCS	18–34	48 (36)	33 (27)	**Reference**	**Reference**
	35–50	25 (36)	27 (36)	1.3 (0.7–2.4)	1.6 (0.9–3.0)
	51–65	50 (55)	41 (60)	2.8 (1.5–5.3)	4.0 (2.1–7.6)
	>65	79 (86)	73 (81)	10.9 (5.4–22.3)	11.0 (5.7–21.4)
Severe GCS	18–34	151 (81)	122 (72)	**Reference**	**Reference**
	35–50	139 (78)	74 (74)	1.0 (0.5–1.8)	1.2 (0.7–2.0)
	51–65	258 (80)	81 (83)	0.9 (0.5–1.8)	2.0 (1.0–3.7)
	>65	555 (90)	72 (89)	2.0 (0.9–4.5)	3.2 (1.5–6.9)

Unfavorable outcome was defined as upper severe disability, lower severe disability, vegetative, and dead as graded by the Extended Glasgow Outcome Score.

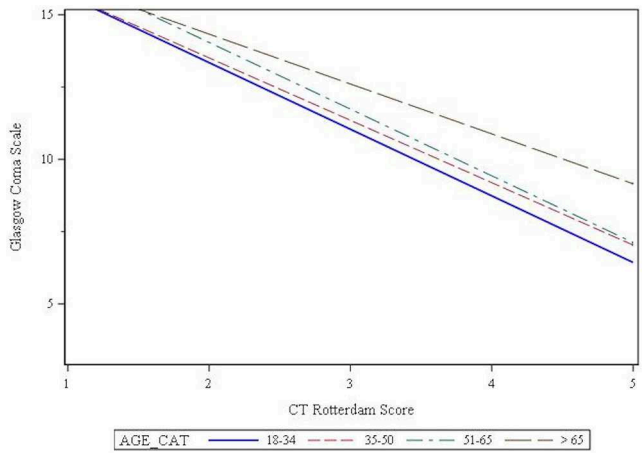

FIGURE 2 | Interaction effects of age and CT Rotterdam Score on Glasgow Coma Scale.

TABLE 3 | The effects of patient age and representative CT Rotterdam Scores on outcomes at 3 and 6 months following traumatic brain injury.

CT Rotterdam Score	Age group (years)	Odds ratio unfavorable outcome 3 months OR (95% CI)	Odds ratio unfavorable outcome 6 months OR (95% CI)
1	18–34	**Reference**	**Reference**
	35–50	1.7 (0.9–3.4)	2.9 (1.7–5.0)
	51–65	3.0 (1.6–5.7)	5.6 (3.4–9.2)
	>65	16.2 (9.1–28.7)	20.0 (12.6–31.6)
3	18–34	**Reference**	**Reference**
	35–50	1.4 (1.0–1.9)	1.3 (0.7–2.4)
	51–65	2.2 (1.7–3.0)	2.8 (1.5–5.3)
	>65	4.4 (3.7–5.8)	10.9 (5.4–22.3)
5	18–34	**Reference**	**Reference**
	35–50	1.1 (0.6–2.3)	0.9 (0.5–1.8)
	51–65	1.7 (0.8–3.4)	0.9 (0.5–1.8)
	>65	1.2 (0.6–2.3)	2.0 (0.9–4.5)

Unfavorable outcome was defined as upper severe disability, lower severe disability, vegetative, and dead as graded by the Extended Glasgow Outcome Score.

The results of this analysis show that the relationship between age and unfavorable outcome at three- and 6-months post-injury is continuous. This has been previously been demonstrated after moderate and severe TBI, with some studies suggesting an inflection point toward worse outcomes beginning between ages 30 and 60 years (20). In our study, this relationship remains continuous when mild injuries are incorporated, with no inflection point being found. In particular, we found that the relative odds of an unfavorable outcome at 3- and 6-months increased by 2.6 and 2.9%, respectively, for every year older. This relationship was preserved when age was used as a categorical variable, with the two oldest age groups having significantly higher odds ratios for unfavorable outcome at both three- and 6-months post-injury when compared to the youngest age group.

The effect of age on outcome was most pronounced in patients who presented with a mild GCS; the effect was still statistically significant in patients with a moderate injury, but to a lesser extent. Surprisingly, many patients suffered an unfavorable outcome following a mild TBI, occurring in 6–16% of the youngest three age groups. This cause of such a high rate is not known, but could likely be related to uncontrolled confounders, such as the presence of polytrauma. In the oldest age group, 55% suffered an unfavorable outcome following a mild brain injury. The relationship between GCS and age is complex, with multiple studies showing that the elderly routinely present with a worse Abbreviated Injury Score of the Head (AIS) when compared

to younger patients with the same GCS (14, 19, 21). While we were not able to retrospectively calculate AIS, we did find a statistically significant interaction effect between age and CT Rotterdam Scores on GCS, such that older patients presented with a better GCS than their younger counterparts with similar CT Rotterdam Scores. In addition, this effect of age was most prominent in patients with the worst CT Rotterdam Scores. Several theories have been postulated to explain this discrepancy between GCS and other measures of injury severity in the elderly. Salotolla et al. (21) postulate that the elderly patients have a blunted or delayed physiological response to trauma when compared to younger patients, which could include a lesser neuroinflammatory response or differences in vasoreactivity and cerebral edema formation. In addition, brain atrophy is common in the elderly, which may allow for expansion of mass lesions without significant neurological symptoms. In this scenario, the AIS or CT Rotterdam Score would overestimate injury severity secondary to a large mass lesion with little or no change in the GCS. However, analysis of our cohort suggests that CT Rotterdam Scores may underestimate injury severity, particularly in patients with lower CT Rotterdam Scores, as age had a much more profound effect on outcome when CT Rotterdam Scores were low. It is clear, then, that we do not have a definitive tool to understand and characterize TBI severity in the geriatric population. Given the elderly have worse outcomes even with better GCS scores at presentation, the admission GCS may not be an appropriate measure of injury severity. This has direct ramifications regarding patient care, as the geriatric patient may not receive the necessary acute or longitudinal interventions they need in light of a relatively high GCS. Further studies, including use of other severity scores like the AIS and CT Rotterdam Score, are necessary to develop predictive models of outcome in the vulnerable elderly population.

In patients who suffered a severe TBI, no statistically significant differences in outcome were seen among the four age groups at 3-months. However, by 6-months, outcomes were once again statistically worse in the two oldest age categories when compared to the youngest. It is well-established that elderly patients have slower rates of functional and cognitive recovery (15, 22) and this was seen in our population as whole, in which more patients from the youngest age group showed neurological improvement from 3–6-months than the other age groups. It is not entirely clear why elderly patients show slower recovery after TBI. Some have postulated a bias in clinical care resulting in slower acute interventions, higher likelihood to withdraw care, and increased likelihood to be discharged to a nursing facility instead of acute rehabilitation in light of poor rehabilitation tolerance (10, 14, 15, 22). It is also possible that our measurements of functional outcome do not accurately capture the functional status of the elderly population following TBI (15). While the GOSE is the most widely used measure of global functional outcome following TBI and has been recommended as the standard outcome measure for TBI studies, it has not been validated in the elderly population. One group showed continued improvement in functional outcome in the elderly up to 1-year following severe TBI using the Health Related Quality of Life Measure, an improvement that was not appreciated using the GOSE (23). In addition, the GOSE does not distinguish disability related to neurological impairment from that of systemic injury or illness; one could then postulate that in the setting of increased co-morbidities in the elderly, GOSE scores remain low in the elderly secondary to non-neurological conditions. Future work is necessary to validate functional outcome markers in the elderly.

Strength and Limitations

The main strength of this study is the large sample size from a prospectively collected database from a busy Level 1 Trauma Center that manages all patients with TBI regardless of age, insurance status, or ability to pay. Accordingly, it is likely that our patient population better reflects the diversity of the TBI population as a whole than do clinical trials with strict inclusion and exclusion criteria.

A major limitation of this study includes the registry not containing all pertinent data, including data regarding patient comorbidities, presence and severity of systemic injuries, coagulopathy, surgical interventions, hospital complications, length of stay and post-discharge disposition, rehabilitation, and care. These uncontrolled confounding factors are known to contribute to patient outcome and further research is necessary to control for these factors across age groups. It is also possible that the mechanism of injury was not always accurately known by the treating team. We were not able to determine which patients transferred from UC Davis after presenting to a different hospital first, and therefore were not able to determine the effects of transfer time or measure any differential treatment strategies based on the patients' severity of injury or age. Patients who were lost to follow-up also introduce bias that we were not able to control for.

CONCLUSION

The proportion of patients with TBI that are elderly is likely to continue to increase as the age of the population increases. Age is a major independent risk factor for unfavorable functional outcome when controlling for injury severity, both by radiographic and clinical severity scores. The largest disparity in outcomes across age was seen in patients who present with a mild GCS and CT Rotterdam Scores, suggesting that these markers of injury severity may underestimate the severity of injury in the elderly population. This information has clinically meaningful ramifications and highlights the need for clinical trials and validation of outcome markers in the elderly TBI population.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available because dissemination of data is not included in the study's IRB approval. Requests to access the datasets should be directed to Ryan Martin, MD at rymartin@ucdavis.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UC Davis Interval Review Board. Written

informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

NG, AT, and RM participated in the literature search and participated in data collection. NG, AT, MW, KS, and RM participated in the study design, participated in data interpretation, and participated in critical revision. NG, MW, and RM participated in data analysis and participated in writing.

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Serum SNTF, a Surrogate Marker of Axonal Injury, Is Prognostic for Lasting Brain Dysfunction in Mild TBI Treated in the Emergency Department

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Mild traumatic brain injury (mTBI) causes persisting post-concussion syndrome for many patients without abnormalities on conventional neuroimaging. Currently, there is no method for identifying at-risk cases at an early stage for directing concussion management and treatment. SNTF is a calpain-derived N-terminal proteolytic fragment of spectrin (α_{II}-spectrin1-1176) generated in damaged axons following mTBI. Preliminary human studies suggest that elevated blood SNTF on the day of mTBI correlates with white matter disruption and lasting brain dysfunction. Here, we further evaluated serum SNTF as a prognostic marker for persistent brain dysfunction in uncomplicated mTBI patients treated in a Level I trauma center emergency department. Compared with healthy controls ($n = 40$), serum SNTF increased by 92% within 24 h of mTBI ($n = 95$; $p < 0.0001$), and as a diagnostic marker exhibited 100% specificity and 37% sensitivity (AUC = 0.87). To determine whether the subset of mTBI cases positive for SNTF preferentially developed lasting brain dysfunction, serum levels on the day of mTBI were compared with multiple measures of brain performance at 90 days post-injury. Elevated serum SNTF correlated significantly with persistent impairments in cognition and sensory-motor integration, and predicted worse performance in each test on a case by case basis (AUC = 0.68 and 0.76, respectively). SNTF also predicted poorer recovery of cognitive stress function from 30 to 90 days (AUC = 0.79–0.90). These results suggest that serum SNTF, a surrogate marker for axonal injury after mTBI, may have potential for the rapid prognosis of lasting post-concussion syndrome and impaired functional recovery following CT-negative mTBI. They provide further evidence linking axonal injury to persisting brain dysfunction after uncomplicated mTBI. A SNTF blood test, either alone or combined with other markers of axonal injury, may have important utilities for research, prognosis, management and treatment of concussion.

Keywords: mild traumatic brain injury, prognostic biomarker, diffuse axonal injury, blood test, post-concussion syndrome, calpain, SNTF

INTRODUCTION

Mild traumatic brain injury (mTBI), often referred to as concussion, is the most common neurological injury, occurring at an estimated annual incidence in the United States of 1.4–3.8 million (1). For TBI sufferers with mild initial symptoms, conventional neuroimaging such as non-contrast head CT scan is usually negative, and post-concussion symptoms commonly resolve within a few hours or days. Nevertheless, in a significant subset of these uncomplicated mTBI cases, brain dysfunction is persistent and its effects debilitating, sometimes for years (2–7). Unfortunately, there is currently no therapeutic or rehabilitation intervention clinically proven to promote long-term brain functional outcomes after CT-negative mTBI. Moreover, there is no established method for identifying at an early stage those individuals at risk of experiencing lasting brain dysfunction. For mTBI management, challenges remain to make neurobiologically informed decisions on suitability for return to work, school, play, or military service, and assess the vulnerability to repetitive injuries. Progress has been made toward understanding the pathophysiology of mTBI that leads to chronic post-concussion syndrome. In particular, diffuse axonal injury (DAI) has emerged as a likely primary structural correlate for chronic brain functional impairment after mTBI. Axonal pathology is reportedly extensive in human mTBI cases who then died shortly thereafter from other causes (8). In a large animal model mimicking the human biomechanics of mTBI, DAI accompanied by localized blood-brain barrier disruption are the only observable histopathologies (9–12). From advanced neuroradiology techniques such as diffusion tensor imaging, white matter microstructural disruption is strongly expressed in some mTBI patients (13–16). A blood biomarker whose levels relate to mTBI-induced chronic brain dysfunction and the DAI underlying it could have major applications related to mTBI.

SNTF is an N-terminal proteolytic fragment of spectrin (α_{II}-spectrin1-1176) that is generated by the calpain family of calcium-activated proteases under neurodegenerative conditions (17–19). SNTF is normally undetectable in neurons and their axons, but is formed within injured neurons via calcium overload and calpain-mediated spectrin degradation, after which it is released (20). SNTF preferentially accumulates within damaged axons in both small and large animal models of mTBI (21–24) as well as in human TBI cases examined post-mortem (24). In a preliminary study of mTBI patients treated in the emergency department, blood SNTF levels in the acute post-injury period are associated with white matter abnormalities detectable with diffusion tensor imaging, as well as a measure of cognitive dysfunction persisting for at least 3 months (25). Serum SNTF also increases rapidly in a subset of concussed professional ice hockey players, and distinguishes those that experience lasting symptoms requiring prolonged delay in their return to play (26). Taken together, these studies support the hypothesis that SNTF is a biologically plausible surrogate marker for DAI, and its blood elevation following mTBI provides preliminary biomarker evidence linking DAI with long-term brain functional impairment. This hypothesis is further supported by studies of additional candidate markers for mTBI. In sports-related concussion, the axon-enriched cytoskeletal proteins tau and neurofilament L (NFL) also increase in the blood acutely after injury, where their levels above threshold relate to the persistence of symptoms (27, 28) and, in the case of tau, correlate with the elevation in SNTF (26). However, studies of tau and NFL as prognostic markers for mTBI treated in the emergency department included complicated TBI cases with intracranial lesions discernable on non-contrast head CT scan, which may be prognostic for worse long-term outcomes (29). Thus far, neither marker has shown utility in this setting for the prognosis of uncomplicated mTBI (30–32). Biomarkers tied to brain elements other than the axon also have been investigated as candidates for mTBI. Peripheral blood levels of a tandem of the astrocyte-enriched glial fibrillary acidic protein (GFAP) and the neuron-enriched ubiquitin C-terminal hydrolase-L1 (UCH-L1) identify cases of complicated TBI with intracranial lesions and distinguish them with high fidelity from cases of mTBI (33–37). However, these and other well studied proteins such as S100β and neuron-specific enolase have yet to demonstrate prognostic utility in the much larger uncomplicated mTBI patient population (27, 31, 37).

Given the urgent and currently unmet need for a rapid and technically simple prognostic test for mTBI, it is important to further assess surrogate markers for DAI measurable in the blood. Here, we conducted a study evaluating serum SNTF as a rapid prognostic marker for persistent brain dysfunction in mTBI patients treated in the emergency department of a Level I trauma center. The study excluded complicated TBI, and focused on 95 mTBI participants who either were confirmed as head CT negative or did not meet criteria to receive a head CT scan. We compared the serum levels of SNTF on the day of mTBI with its levels in uninjured controls, and evaluated prognostic relationships between the day of injury serum SNTF concentrations and the presence of persistent brain dysfunction, using a battery of tests of cognitive and sensory-motor-integration performance, conducted at two time points in the chronic post-injury period.

METHODS

Study Participants

The prospective study of mild traumatic brain injury (mTBI) treated in the emergency department was reviewed and approved by the Institutional Review Boards of Regions Hospital, St. Paul, and the University of Pennsylvania (#810115). All participants in this study provided written informed consent, or assent if written consent was given by a minor's parent. They were recruited and assessed with approval from and according to the ethical guidelines of the Institutional Review Board of Regions Hospital. All procedures were conducted in accord with the ethical standards of the Helsinki Declaration of 1975, as revised in 2000.

This study on SNTF (α_{II}-spectrin1-1176) as a potential diagnostic and prognostic marker examined sera of 40 healthy control participants (20 males and 20 females) and 95 mild TBI patients (55 males and 40 females), with the latter samples collected within 24 h of injury. The time between mTBI and blood sampling ranged from 2 to 22 h, with 87% collected from 2 to

7 h post-injury. The mTBI cases are typical of those evaluated in the emergency department setting and then discharged without hospital admission. They presented with initial GCS scores of 13–15 and were recruited from a convenience sample of patients treated in the emergency department of the level I trauma center at Regions Hospital, St. Paul. The mTBI patients were > 9 years of age, had an injury to the head from blunt trauma or acceleration-deceleration forces, experienced observed or self-reported confusion, disorientation, or impaired consciousness (<5 min), and one or more of the following concussion symptoms: dizziness, headache, fatigue, irritability, vomiting. Non-contrast head CT scans were performed on 80 mTBI study participants, with the remaining 15 cases not meeting criteria to receive a head CT scan. Decisions to perform CT scan were made based on clinician experience and were frequently guided by the use of clinical decision rules (38). All of the mTBI study participants were discharged without hospital admission.

Exclusion criteria for enrollment were: age under 10 years, non-English speaking, abnormal acute intracranial CT findings, blood alcohol level >200 mg/dl, previous head injury within the past 30 days, pre-existing neurological disorder, or pre-existing psychiatric disorder. In the sections that follow herein, we refer to cases having an initial GCS of 13–15 either not meeting criteria for head CT scanning or with negative findings on a non-contrast head CT scan as mTBI. Healthy controls were free of known diseases, and no history of TBI.

Brain Functional Assessments

After enrollment, the mTBI participants were administered the Standardized Assessment of Concussion (SAC) and the Rivermead Post-Concussion Symptom Questionnaire (RPCSQ). The RPCSQ tested 16 different self-reported symptoms and was performed by study participants on a weekly basis for up to 90 days post-injury ($n = 58$ at 90 days).

At 30 and 90 days post-injury, mTBI study participants were administered a battery of five neurocognitive performance tests: the Stroop Color and Word, Digit Span, Trail Making, Buschke Selective Reminding, and Controlled Oral Word Association test. From 65 to 67 participants completed these cognitive tests at 90 days, depending on the test. One mTBI patient, an 86 years old female, was excluded from the comparative analyses of serum SNTF levels and neurobehavioral test performance, owing to the established deleterious effects of this advanced age on performance in the battery of cognitive and sensory-motor integration tests (39–46). To assess recovery of cognitive function, 30 days scores were subtracted from 90 days scores for each concussion case in the Stroop Color and Word Interference Test ($n = 65$), measuring cognitive function under stress.

Sensory-motor integration was evaluated using the Grooved Pegboard test ($n = 65$ at 90 days). The speed to complete the test and the number of errors made were determined for both the dominant and non-dominant hands. For comparative assessment of serum SNTF levels in relation to persisting sensory-motor integration dysfunction, participants were dichotomized to either good or bad functional groups, with the cutoff criteria for the latter defined as either taking 80 s or more to complete the dominant hand test (placing them at least 20% above the mean of all mTBI patients), or committing two or more dominant hand errors. Eleven mTBI cases (17%) met these criteria at 90 days post-injury for dysfunctional sensory-motor integration.

SNTF Second Generation Immunoassay

Development of a second-generation electrochemiluminescence-based SNTF sandwich immunoassay improved our previously published method, increasing the detection sensitivity for serum SNTF by more than an order of magnitude (25, 26), and facilitating quantification of the low SNTF levels in sera from healthy control subjects. One major change involved the derivation of an ultra-high affinity rabbit monoclonal cleavage site-specific antibody for SNTF, selected based on its superior performance as the detector in the sandwich immunoassay format. The rabbit monoclonal was derived under contract with Abcam. Rabbits were immunized with the synthetic peptide CAQQEVY, a mimic of the neoantigenic site at the carboxy-terminal region of the amino-terminal half of human α_{II}-spectrin generated by calpain cleavage between residues tyrosine 1,176 and glycine 1,177 (19, 47). The peptide was conjugated via its cysteine side chain to carrier proteins keyhole limpet hemocyanin and bovine serum albumin (BSA) using maleimide chemistry, with the former conjugate used for the initial immunization and all but the final boost, and the latter for the boost prior to the hybridoma fusion. Hybridomas were screened by a two-step procedure, initially by testing conditioned media against ELISA plates coated with the peptide-BSA conjugate, and subsequently evaluating all of the initial positives as detector antibodies in electrochemiluminescence sandwich immunoassay with SNTF standard. Following subcloning and a second round of the two-step screening, the rabbit monoclonal antibody 43–8 was selected for scale-up production, and purified to >95% homogeneity using protein A affinity chromatography. Specificity of the rabbit monoclonal for SNTF was established by Western blot analysis (>1,500-fold selectivity for SNTF over intact α_{II}-spectrin), and by the more than order of magnitude difference in signal in sandwich immunoassay between SNTF-enriched and SNTF-poor brain extracts.

SNTF was quantified in human sera by the following modifications of our original immunoassay method (25, 26). Mouse monoclonal anti-α_{II}-spectrin capture antibody (clone D8/B7; BioLegend) was biotinylated, and bound to 96 well electrochemiluminescence plates spot coated with streptavidin (MesoScale Discovery), then the wells blocked with BSA. The mouse monoclonal reacts with the SH3 domain spanning residues 967–1,026 and recognizes SNTF as well as the intact α_{II} subunit, but not carboxy-terminal α_{II}-spectrin derivatives of calpain and caspase proteolysis referred to in the literature as SBDPs 150, 145, or 120 (48), all of which lack this domain. Sera were diluted to 40%, incubated at 25 µl/well for 2 h, the wells washed, and incubated for 1 h in SNTF-specific rabbit monoclonal 43–8 at 0.1 µg/ml, and finally for 1 h in goat-anti-rabbit IgG-Sulfotag (species cross-adsorbed; MesoScale Discovery). Electrochemiluminescent signals were quantified using the QuickPlex SQ120 imaging system (MesoScale Discovery), and standardized against serial dilutions of either partially purified brain-derived SNTF (25) or

recombinant human SNTF (rhSNTF). The latter was generated by digesting purified recombinant human full-length α_{II}-spectrin (Origene) with purified human calpain I (Millipore-Sigma; 100:1 molar ratio). The second generation SNTF immunoassay has an experimentally determined lower limit of detection (LLOD) of 0.31 Units SNTF (1 Unit corresponds to the amount in nanoliters of SNTF-containing standard per ml), corresponding to 32 femtograms rhSNTF/well. The lower limit of quantification (LLOQ) of the second generation immunoassay is 0.55 U, or 58 femtograms rhSNTF/well. The coefficient of variation, determined using 16 human serum samples analyzed a minimum of three times, is 13.6%. Spiking experiments adding known amounts of SNTF standard to pooled healthy human control serum demonstrated equivalent and high recovery across the full range of SNTF concentrations. Systematic testing of repetitive freeze-thaw demonstrated recovery of SNTF from human serum was >95% per cycle over two cycles.

SNTF was evaluated in the human sera in a blinded experimental design without knowledge of any of the study participant data. To ensure the specificity of the method for SNTF and rule out non-specific signals derived from heterophilic substances sometimes present in human sera (49), parallel wells were reacted in the same procedure as above, except that the SNTF-specific rabbit monoclonal detector was replaced with purified normal rabbit IgG at the same concentration. This procedure abolished signal, as did replacing the α_{II}-spectrin-specific capture antibody with a biotinylated mouse monoclonal of the same isotype but targeting a different protein. Human serum samples were evaluated using duplicate wells per experiment in a minimum of two independent experiments per sample. As a control for assessing cross-plate variability, a pooled serum sample from healthy controls without neurological injury was evaluated on every plate.

Statistical Analyses

Group comparisons of mean serum SNTF levels between healthy controls and mTBI cases or dichotomized serum SNTF concentrations in relation to brain functional endpoints were evaluated by the non-parametric Mann-Whitney *U*-test. Receiver Operator Characteristics (ROC) curve analyses comparing mTBI and control participants or brain functional measures for mTBI cases on the basis of their serum SNTF levels also employed the Mann-Whitney *U*-test. The comparative analyses of serum SNTF between controls and mTBI cases as well as with measures of chronic brain functional status were conducted on the mTBI group as a whole ($n = 95$), as well as the subgroup of 80 mTBI cases confirmed to have negative non-contrast head CT scans. All of the analyses were conducted with GraphPad Prism 8.0 software.

RESULTS

An Improved Second Generation SNTF Immunoassay Quantifies Serum Levels in Uninjured Controls

Prior studies in experimental animal models and relatively small numbers of cases of human TBI have provided evidence that SNTF may be a biologically plausible blood biomarker for the subset of concussions that lead to diffuse axonal injury and persisting brain dysfunction (25, 26). SNTF is the predominant amino-terminal fragment of the α-subunit of the actin-binding cytoskeletal protein non-erythroid spectrin derived from cleavage by the calcium-activated calpain protease family (17–19). It is a distinct post-translationally modified protein (α_{II}-spectrin1-1176) from the carboxy-terminal spectrin breakdown products, sometimes referred to in the literature as SBDPs (17–19, 48, 50, 51). In order to compare the serum SNTF level between healthy controls and uncomplicated mTBI cases on the day of injury and assess its prognostic relationship with long-term brain functional impairment, we developed an improved second-generation electrochemiluminescence-based SNTF immunoassay with high analytical validity (see *Methods* for details). The new immunoassay reliably quantified SNTF in sera from uninjured controls, with levels of the marker in 38 of 40 control sera along with all 95 mTBI patients taken within 24 h of injury being above the lower limit of quantification of 0.55 U (58 femtograms rhSNTF), and for all 135 study participants above the lower limit of detection of 0.31 U (32 femtograms rhSNTF).

Study Cohorts and Effects of Age and Sex on Serum SNTF

With an objective to investigate SNTF as a prognostic blood biomarker for identifying mTBI patients at high risk of developing persisting brain dysfunction and disability, we first compared serum SNTF levels in mTBI cases on the day of injury with healthy control participants. We then analyzed SNTF levels in mTBI cases in relation to cognitive and sensory-motor test performance for up to 90 days post-injury. **Table 1** shows the breakdown of the study cohorts by mean age and sex, along with the mTBI cohorts evaluated longitudinally for concussion symptoms on the day of injury, and at 30 and 90 days post-injury. Among healthy controls, serum SNTF levels were consistently low, ranging from 0.40 to 1.53 U (**Figure 1A**). Marker levels did not vary appreciably as a function of age (panel A, $r^2 = 0.01$), did not differ significantly between males and females (not shown), and did not change in either males or females as a function of age (panel C; males $r^2 = 0.0$; females $r^2 = 0.01$). Similar observations were made in the mTBI patients, in whom serum SNTF did not differ as a function of sex (panel B; $p > 0.5$) or change appreciably with aging (panel D; males $r^2 = 0.01$; females $r^2 = 0.00$).

Diagnostic Accuracy of Serum SNTF on the Day of mTBI

Compared to its level in the serum of neurologically normal uninjured controls, serum SNTF increased substantially on the day of mTBI. As shown in **Figure 2**, mean serum SNTF levels within 24 h of injury were 92% higher (panel A; $p < 0.0001$) and median levels were 63% higher (panel B; $p < 0.0001$). Strikingly, whereas many of the mTBI cases had serum SNTF levels indistinguishable from healthy controls, a subset had levels above those observed in all 40 controls, and up to 9-fold higher than the mean control value. Receiver Operator Characteristics-Area Under The Curve (ROC-AUC) analysis demonstrated that the level of serum SNTF on the day of injury strongly distinguished mTBI cases from uninjured controls (panel C; AUC

TABLE 1 | Study demographics.

		Means		
	N	**Age yrs (Range)**	**Males (*N*)**	**Females (N)**
Healthy controls	40	39.3 (22–63)	40.9 ± 2.2 (20)	37.7 ± 2.3 (20)
Mild TBI				
@ <24 h	95	35.6 (10–86)	31.9 ± 2.3 (55)	40.5 ± 3.0 (40)
@ 1 month	75	36.1 (11–70)	32.5 ± 2.5 (45)	41.6 ± 3.7 (30)
@ 3 months	65	36.4 (11–70)	35.4 ± 2.9 (39)	38.0 ± 3.7 (26)
Mild TBI/CT-				
@ 3 months	53	37.4 (11–70)	35.0 ± 3.0 (33)	41.4 ± 4.4 (20)

The N per mild TBI group represents the number of participants that performed all of the cognitive and sensory-motor performance tests at the given time point post-injury.

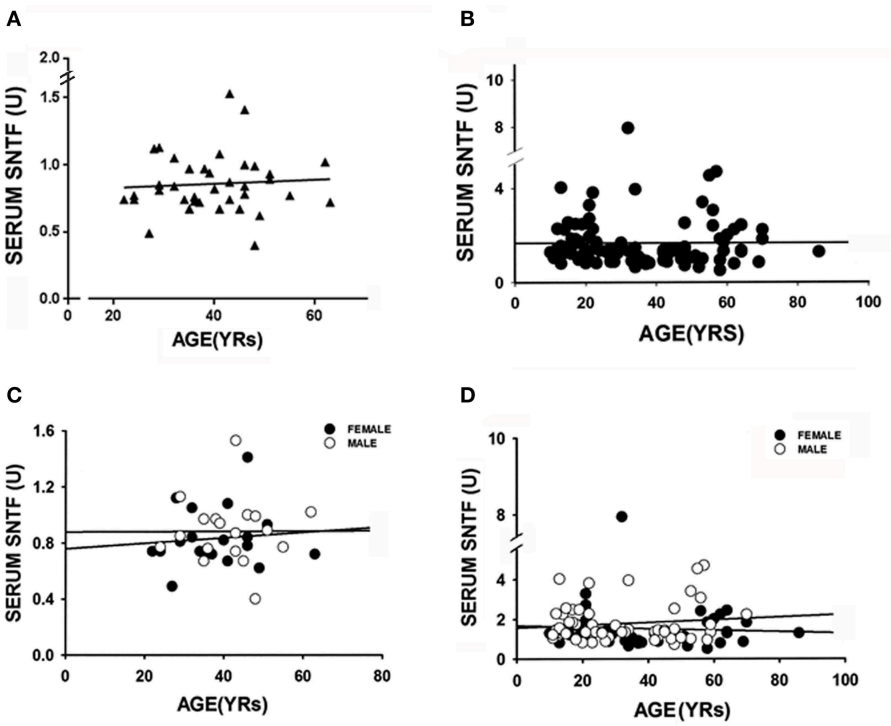

FIGURE 1 | Neither age nor sex affects serum SNTF levels in either healthy controls or acutely following mild TBI. Each study participant was evaluated for serum SNTF a minimum of 4 times. Relationships between serum SNTF, age and sex were evaluated by regression analysis and Mann-Whitney *U*-test. **(A)** Serum SNTF concentration is depicted vs. the age of 40 healthy adult control subjects. Linear regression analysis shows there is no relation between serum SNTF and adult age in non-neurologic injury controls. **(B)** Serum SNTF concentration on the day of injury is depicted vs. the age of 96 mTBI study participants. Linear regression analysis shows there is no relation between serum SNTF on the day of mTBI and the age of the patient. **(C)** Serum SNTF is depicted as a function of age of healthy adult control males and females. Once again, there is no appreciable change in SNTF of either males (open circles; $n = 20$) or females (filled circles; $n = 20$) with adult aging. **(D)** Serum SNTF on the day of injury is depicted as a function of age of male (open circles; $n = 55$) and female (filled circles; $n = 40$) in the mTBI study participants. There is no appreciable change in SNTF as a function of the age of mTBI patients in either males or females.

$= 0.87$, $p < 0.0001$). At a cutoff value of 1.54 U, serum SNTF was 100% specific and 37% sensitive for the diagnosis of mTBI (i.e., 40 of 40 healthy controls were SNTF negative, whereas 35 of 95 mTBI cases were SNTF positive). At a lower cutoff of 1.45 U, serum SNTF retained 97.5% specificity and exhibited 40% sensitivity. The latter cutoff value was used for all of the group analyses that follow, whereas comparative evaluations between long-term brain functional performance tests and serum SNTF on a per case basis were made by ROC-AUC analyses without a pre-determined biomarker cutoff.

In subgroup analysis, serum SNTF increased on the day of mTBI in both the confirmed head CT-negative (1.72 U ± 0.12, $n = 80$) and non-scanned (1.50 U ± 0.23, $n = 15$) cases compared to healthy controls (0.86 U ± 0.03, $n = 40$; $p < 0.0001$ for both comparisons). In contrast, there was no significant difference in SNTF levels between the mTBI cases not referred for head CT

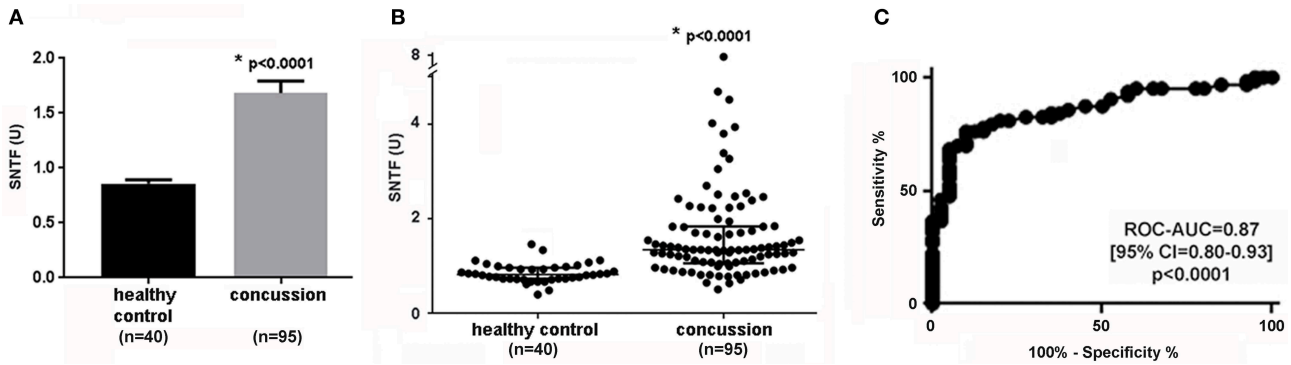

FIGURE 2 | Serum SNTF is significantly elevated on the day of mTBI and is diagnostic for a subset of the injuries. Between group comparisons and ROC-AUC analyses were evaluated by Mann-Whitney *U* test. Each study participant was evaluated for serum SNTF a minimum of 4 times. **(A)** In group analysis, mean serum SNTF levels on the day of mTBI are 92% higher than their mean levels in healthy controls. The bars represent the S.E.M. Between group statistical comparison by two-tailed *t*-test. **(B)** In group analysis, median serum SNTF levels on the day of mTBI are 63% higher than their median levels in healthy controls. The filled circles represent each case, and the bars represent quartiles. Note that whereas some of the mTBI cases have serum SNTF levels indistinguishable from the range of the controls, a subset have SNTF levels markedly elevated over the entire control group. Between group statistical comparison by Mann-Whitney *U*-test. **(C)** In ROC-AUC curve analysis, serum SNTF levels on the day of mTBI distinguish a subset of injured patients from healthy controls. At a cutoff value of 1.54 U, serum SNTF is 100% specific and 37% sensitive for mTBI. The AUC is 0.87, the 95% confidence interval is 0.80–0.93, and is significantly different from chance ($p < 0.0001$; Mann-Whitney *U* test).

and confirmed as head CT negative ($p > 0.5$). In ROC-AUC analysis comparing the head CT-negative mTBI subgroup with controls, serum SNTF on the day of injury once again strongly distinguished the two groups (AUC = 0.87; $p < 0.0001$) in a manner indistinguishable from the overall mTBI group. In this study, blood was obtained from 2 to 7 h post-injury in 88% of the mTBI cases, and there was no discernable relationship over this time frame between serum SNTF levels and the delay between injury and sampling ($r^2 = 0.02$; $p = 0.3$).

Evidence That Serum SNTF on the Day of mTBI Prognoses Lasting Cognitive Dysfunction and Poor Cognitive Functional Recovery

Given that in a subset of uncomplicated mTBI cases serum SNTF concentration is elevated on the day of injury above the range of neurologically normal controls, and only some mTBI cases experience discernable long-lasting brain functional problems, the question arises whether the rapid injury-induced SNTF increase relates to impaired brain performance in the chronic post-injury period. To address this issue, we compared SNTF levels on the day of mTBI with the scores from a battery of tests evaluating cognitive and sensory-motor function at 30 and 90 days post-injury. Additionally, the Standardized Assessment of Concussion served as a measure of the initial severity of symptoms on the day of injury, and the Rivermead Post-Concussion Symptom Questionnaire (RPCSQ) evaluated self-reported cognitive, somatic, and emotional symptoms throughout the 90 days observation period. Cognitive function in the chronic post-injury period was studied by serial assessments using the Stroop Color and Word, Digit Span, Trail Making, Buschke Selective Reminding, and Controlled Oral Word Association tests.

As shown by group analyses (**Tables 2**, **3**), the serum SNTF concentration sampled within 24 h of mTBI correlated significantly with a subset of cognitive functional measures at 90 days after the injury. In the Stroop Color and Word test (**Table 2**), the SNTF-positive mTBI group scored poorer on the cognitive interference, color score, and color score *T* test components, with the biomarker positive mTBI group exhibiting significantly impaired performance compared with the SNTF-negative mTBI group on each of these measures. In ROC-AUC analysis, serum SNTF positivity on the day of injury significantly distinguished cases with the worst 20% of cognitive interference scores at 90 days (<49 words) from those with better test performance (>49 words; AUC = 0.68; $p = 0.028$; data not shown). In subgroup analysis of the mTBI cases with confirmed negative head CT findings, participants positive for serum SNTF on the day of injury also performed significantly worse on the Stroop cognitive interference and color score *T* test components than their SNTF-negative mTBI counterparts (**Table 2**).

Serum SNTF on the day of injury predicted not only impaired cognitive performance at 90 days post-injury, but also poor recovery of cognitive function under stress. Taking the difference in the Stroop interference test scores between 30 and 90 days as a measure of cognitive recovery, 41 of 65 mTBI participants improved performance over the 60 days recovery assessment period, whereas 24 showed either no improvement or worse scores. The decile showing the worst recovery of cognitive stress function had a mean SNTF level (2.57 ± 0.44) on the day of injury significantly higher than the quartile exhibiting the best cognitive recovery (1.48 ± 0.13 U; $p = 0.004$). Additional differences were observed in the median SNTF level between the worst recovering decile and the best recovering quartile (2.43 vs. 1.31 U; $p = 0.03$).

TABLE 2 | Serum SNTF on the day of mild TBI relates to cognitive and sensory-motor performance scores at 3 months post-injury.

Performance test	SNTF- (*N*)	SNTF± (*N*)	*P*-value
Stroop color and word			
SNTF > 1.45 U:			
Interference Score-T (words)	56.9 ± 1.5 (36)	51.5 ± 1.3 (30)	**0.015**
Color Score	75.7 ± 2.6	68.3 ± 2.1	**0.031**
Color Score-T	49.0 ± 2.2	43.3 ± 1.8	**0.050**
CT-/Interference Score-T	57.5 ± 1.9 (27)	51.8 ± 1.5 (26)	**0.040**
CT-/Color Score	74.8 ± 2.8	68.3 ± 2.3	0.068
CT-/Color Score-T	48.6 ± 2.4	42.5 ± 2.0	**0.046**
Dominant hand speed (sec)	61.4 ± 1.6 (35)	71.0 ± 4.1 (30)	0.12
Dom hand speed (>16 yrs)	60.1 ± 1.6 (30)	72.8 ± 4.8 (25)	**0.033**
Dominant hand errors	0.26 ± 0.07	0.80 ± 0.22	**0.040**
Non-Dominant hand speed	67.6 ± 2.1	71.9 ± 2.7	0.21
Non-Dominant hand errors	0.54 ± 0.15	0.70 ± 0.17	0.44
	Mean SNTF (U)		
	<80 s (*N*)	**≥80 s (*N*)**	***P*-value**
Dominant hand speed	1.71 ± 0.14 (57)	2.58 ± 0.46 (8)	**0.019**

The group analyses of cognitive and sensory-motor integration performance at 90 days post-injury are compared between all SNTF-negative and SNTF-positive mTBI cases, and again for cases confirmed as head CT-negative, using a cutoff value for serum SNTF concentration on the day of injury (1.445 U) that is 97.5% specific for mTBI. Cognitive performance scores were worse for both the overall mTBI SNTF-positive subgroup and for the SNTF-positive subgroup confirmed as CT negative compared to their SNTF-negative counterparts. For the sensory-motor pegboard test, mTBI cases SNTF-positive on the day of injury made significantly more dominant hand errors, and the adult mTBI subgroup also had significantly worse dominant hand speed. Serum SNTF levels were quantified a minimum of four times for each study participant. Statistically significant differences by Mann-Whitney U-test are shown in bold.

TABLE 3 | Serum SNTF on the day of mild TBI in relation to other brain performance scores at 3 months post-injury.

Performance test	SNTF- (sem; *n*)	SNTF ± (sem; *n*)	*P*-value
Rivermead post-concussion symptom questionnaire			
SNTF > 1.45 U COG-3	1.8 ± 0.6 (32)	2.6 ± 0.6 (26)	0.38
RPQ-13	7.1 ± 1.9 (32)	8.7 ± 2.0 (26)	>0.5
Standardized assessment of concussion			
SNTF > 1.45 U	24.7 ± 0.4 (61)	24.9 ± 0.5 (35)	>0.5
Digit span			
SNTF > 1.45 U	18.0 ± 0.8 (36)	16.4 ± 0.8 (29)	0.15
Controlled oral word association			
SNTF > 1.45 U	39.3 ± 2.1 (37)	38.9 ± 2.4 (30)	>0.5
Buschke selective reminding			
SNTF > 1.45 U Reminding	54.6 ± 1.3 (37)	53.6 ± 1.4 (30)	>0.5
SNTF > 1.45 U Delayed	8.73 +/ 0.41 (37)	9.31 ± 0.37 (30)	0.26
Trail making			
SNTF > 1.45 U; Trail A speed (s)	22.5 ± 2.2 (35)	21.9 ± 1.5 (29)	>0.5
SNTF > 1.45 U; Trail B speed	52.3 ± 3.7 (35)	62.6 ± 6.7 (29)	0.16
SNTF > 1.45 U; Trail A errors	0.53 ± 0.13 (36)	0.55 ± 0.15 (29)	>0.5
SNTF > 1.45 U; Trail B errors	0.94 ± 0.45 (36)	1.28 ± 0.44 (29)	0.47

*In contrast to the significant performance impairments of SNTF-positive participants compared to the SNTF-negative cases in the test results depicted in **Table 2**, for other tests of cognitive function and self-reported symptomatic scores there was no significant difference as a function of serum SNTF status. The Rivermead, Digit Span, Controlled Oral Word Association, Bushke Selective Reminding, and Trail Making test data shown are at 90 days post-injury. The Standardized Assessment of Concussion was conducted on the day of injury. Serum SNTF was quantified a minimum of 4 times per mTBI study participant.*

As shown in **Figure 3**, ROC-AUC analysis demonstrated that serum SNTF on the day of injury predicted with significance mTBI cases falling into the worst recovering decile compared with both the best recovering decile (**Figure 3A**; AUC = 0.90, $p = 0.014$) and the best recovering quartile (**Figure 3B**; AUC = 0.79; $p = 0.029$).

One trivial possibility that could account for differences in long-term cognitive performance between the SNTF-positive and –negative mTBI groups is potential imbalance between the two in the initial severity of the mTBI. However, as shown in **Table 3**, scores on the Standardized Assessment of Concussion conducted on the day of injury demonstrated that the differences in long-term cognitive performance and functional recovery in relation to acute serum SNTF levels were not due to any appreciable difference in the initial severity of the concussion symptoms between the serum SNTF-positive and -negative groups.

The relationships in mTBI cases between serum SNTF concentrations and cognitive functional test scores in the chronic post-injury period and were subtle. Unlike for multiple readouts for the Stroop cognitive test, four other assessments of cognition at 90 days after mTBI did not reveal significant performance differences in association with the day of injury serum SNTF levels. Scores on the Digit Span, Trail Making, Buschke Selective Reminding, and Controlled Oral Word Association tests did not differ with statistical significance between the SNTF-negative and –positive mTBI groups, although the SNTF-positive mTBI participants trended toward worse performance in the Digit Span, Trail Making B, and Buschke Delayed Reminding tests (**Table 3**). In addition, the Rivermead Post-Concussion Symptom Questionnaire, a self-reporting tool for assessing cognitive problems via the COG-3 sub-score, or emotional and somatic symptoms via the RPQ-13 sub-score, did not reveal self-reported symptomatic differences that associated with the day of injury serum SNTF concentration.

Evidence that Serum SNTF on the Day of mTBI Is Prognostic for Persistent Impairment in Sensory-Motor Integration

In addition to the evidence associating serum SNTF positivity on the day of injury with long-lasting impairment in cognition

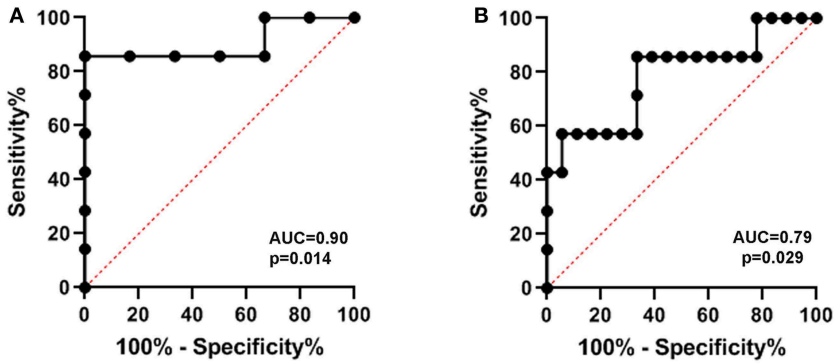

FIGURE 3 | Serum SNTF on the day of mTBI predicts impaired long-term recovery of cognitive function under stress. Serum levels of the biomarker were evaluated a minimum of 4 times, and analyzed in relation to brain functional measures by Mann-Whitney *U* test. Recovery of cognitive stress function was determined as the difference in the Stroop Color and Word IS-T scores between 90 and 30 days post-injury. In ROC-AUC analyses, serum SNTF on the day of injury was significantly higher in mTBI with the decile exhibiting the worst functional recovery compared to either the best recovering decile **(A)** or best recovering quartile **(B)**.

measured using the Stroop Color and Word test, the SNTF-positive mTBI patients also exhibited worse sensory-motor integration function at 90 days post-injury, as assessed using the Grooved Pegboard test. As shown in **Table 2**, the SNTF-positive mTBI group completed the task with a slower dominant hand speed that trended toward significance, and made significantly more dominant hand errors ($p = 0.04$). The association between serum SNTF on the day of mTBI and 90 day sensory-motor integration performance was stronger for the adult subgroup of mTBI cases. For mTBI participants over the age of 16, the SNTF-positive cases exhibited significantly slower dominant hand speed (72.8 s ± 4.8; $n = 25$; $p = 0.033$) compared to the SNTF-negative subgroup (60.1 s ± 1.6; $n = 30$). The SNTF-positive mTBI group also exhibited slower speed and made more errors with the non-dominant hand, although these trends were not statistically significant.

To evaluate the prognostic strength with which serum SNTF on the day of injury predicted persisting dysfunction in sensory-motor integration, ROC-AUC analysis compared performance groups based on criteria for poor functional cases that reached threshold for either slow dominant hand speed (>80 s) or dominant hand errors (2 or more; **Figure 4**). Eleven of the 65 cases of mTBI cases (17%) met these criteria for persisting impairment in sensory-motor integration. The SNTF cutoff of 1.45 U predicted sensory-motor dysfunction at 90 days post-injury with an AUC = 0.76 ($p = 0.006$), a sensitivity of 91% and a specificity of 64%.

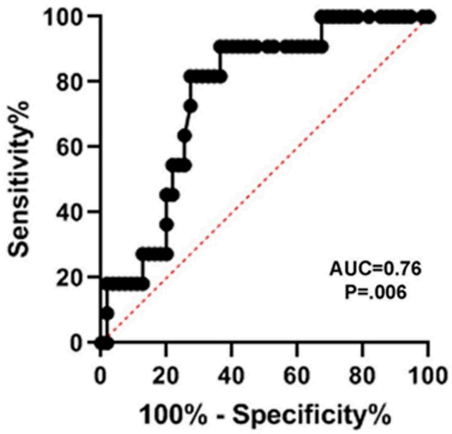

FIGURE 4 | Serum SNTF on the day of mTBI predicts persisting dysfunction in sensory-motor integration at 90 days post-injury. Serum SNTF levels were analyzed for each participant a minimum of 4 times, and evaluated in relation to sensory-motor performance scores by Mann-Whitney *U* test. Sensory-motor integration function was assessed at 90 days post-injury by the Grooved Pegboard test ($n = 65$). Criteria for poor test performance were either a slow dominant hand speed to complete the test of >80 s or commission of two or more dominant hand errors. Seventeen percent of the mTBI cases met these criteria for dysfunctional sensory-motor integration at 90 days. In ROC-AUC analyses, SNTF distinguished cases with impaired sensory-motor integration at 90 days from those not meeting criteria for long-lasting dysfunction (AUC = 0.76, $p = 0.006$).

DISCUSSION

SNTF has been reported to increase in the blood in a subset of uncomplicated mTBI patients treated in the emergency department and after sports-related concussion treated in the athletic training room, but until now has been evaluated in only relatively small numbers of cases. In the present study of 95 mTBI patients treated in a Level I trauma center emergency department setting for whom non-contrast head CT scans were either negative ($n = 80$) or not indicated ($n = 15$), serum levels of SNTF within 24 h of injury are significantly higher by 92% compared with a group of 40 neurologically normal controls matched for age and gender. A serum SNTF cutoff yielding 97.5% specificity (i.e., 39 of 40 controls were SNTF-negative) identifies 40% of mTBI cases as SNTF-positive (**Figure 2**; AUC = 0.87; $p < 0.001$). By comparing the functional status of SNTF-positive and -negative mTBI patients, the subset with elevated SNTF on the day of injury exhibits evidence of brain dysfunction on multiple performance tests that persist for at least 90 days

post-injury. In group analyses, dichotomized SNTF discriminates lasting dysfunction in multiple measures of cognition (**Table 2**). It also predicts poor recovery of cognitive stress function from 30 to 90 days post-injury, separating the worst recovering decile from the best recovering quartile and decile, with AUCs of 0.79 ($p = 0.029$) and 0.90 ($p = 0.014$), respectively (**Figure 3**). Serum SNTF on the day of injury separates mTBI patients not only on the basis of their chronic cognitive function in the Stroop Color and Word test, but also relates strongly to persistent dysfunction in sensory-motor integration. At 90 days post-injury, the SNTF-positive mTBI group is slower to perform the Grooved Pegboard test with their dominant hand and commits significantly more errors ($p = 0.04$) than the SNTF-negative mTBI group. Significant associations with dominant hand speed and errors are even more pronounced for the adult subgroup of SNTF-positive mTBI cases over age 16. On a case-by-case basis, serum SNTF levels on the day of injury predict sensory-motor integration performance deficits at 90 days post-injury in the mTBI group overall with an AUC of 0.76 ($p = 0.006$), 91% sensitivity and 64% specificity (**Figure 4**). In contrast, serum SNTF does not vary appreciably as a function of age or sex in either healthy controls or on the day of injury in mTBI patients (**Figure 1**). These results coupled with the evidence from cognitive test performance suggest that a serum SNTF blood test on the day of injury might have important applications for rapid prognosis of mTBI.

The findings reported here from the study of 135 participants confirm and extend previous preliminary studies on small numbers of cases of SNTF as a blood biomarker for mTBI treated in the emergency department and for sports-related concussion treated in the athletic trainer's room (25, 26). Here, the development and analytical validation of a next generation SNTF electrochemiluminescence immunoassay with markedly improved detection sensitivity enables quantification of serum SNTF in healthy control as well as mTBI subjects, with all 135 participants above the lower limit of detection. There is abundant evidence that the SNTF elevation found in the blood in a subset of mTBI patients on the day of their injury derives in large part from its formation in damaged axons in the brain, leakage into the interstitial fluid, and efflux into the blood. It is a calpain-derived fragment of the α_{II}-subunit of spectrin (17, 19), a large tetrameric cytoskeletal protein that binds to rings of actin filaments and links them to the inner face of the axolemma, spacing them at regular intervals (52). SNTF is not present in detectible amounts in the brain under normal conditions, but forms specifically in response to intra-axonal calcium overload, accumulates within damaged axons (18, 19), and serves as a marker for axonal cytoskeletal disruption in numerous studies of small and large animals models of mTBI, as well as in the human brain (21–24). Supporting this hypothesis, the rise in blood SNTF concentration on the day of mTBI correlates with white matter tract microstructural changes discernable by diffusion tensor imaging at 2 weeks post-injury (25). Furthermore, after concussion in professional ice hockey players, serum SNTF levels correlate with those of tau, another axon-enriched cytoskeletal protein (26). A host of histopathological and diffusion tensor imaging studies provide strong evidence that diffuse axonal injury is a structural correlate for chronic brain dysfunction following mTBI (8, 13–16). Consequently, we propose that SNTF is a biologically plausible surrogate blood biomarker for the axonal damage contributing to persisting brain functional impairments and post-concussion syndrome after mTBI.

At present there are no blood biomarkers or advanced neuroimaging methods proven and clinically validated for the rapid prognosis of mTBI. The biomarker tandem of glial fibrillry acidic protein and ubiquitin C-terminal hydrolase-L1 has consistently demonstrated utility as a blood test for identifying cases of complicated TBI presenting with mild initial symptoms (GCS 13–15) that exhibit intracranial lesions on non-contrast head CT scan (33–36). Unfortunately, in cases of mTBI with negative head CT findings, which is a far more common neurological injury, these markers have not shown prognostic utility (31, 37). In studies of sports-related mTBI evaluating concussed professional ice hockey players and boxers after a bout, tau and neurofilament L are elevated in the blood, and in the former group and in a manner similar to SNTF, predict the persistence of post-concussion symptoms based on the length of the delay in return to play. On the other hand, studies published to date of tau and NFL as candidate markers for TBI treated in the emergency department have included cases with discernable intracranial lesions on head CT scan, thus precluding evaluation of these markers for prognosis of mTBI (30–32). Strikingly, SNTF, tau, and neurofilament L are all important structural proteins of the axon whose blood levels rise after sports-related concussion (26–28), supporting the hypothesis that they all represent surrogate blood biomarkers for the diffuse axonal injury underlying functionally deleterious mTBI. This hypothesis will require further evaluation, as will the prospect for improved mTBI prognosis using a multivariable panel of biologically plausible markers.

There is confusion in the literature regarding the identity of SNTF and its characterization as a biomarker for mTBI and other acute brain injuries, owing to inconsistencies with nomenclature and the frequent lack of distinction from other calpain-derived proteolytic fragments of the α_{II}-subunit of spectrin. The discovery that non-erythroid spectrin is a preferred high affinity calpain substrate came with the identification of major breakdown products of the α_{II}-subunit, referred to initially as spectrin BDPs (17). Sequencing of the BDPs and development of cleavage site-specific antibodies identified the preferred calpain cleavage sites in the subunit (19, 49, 50), thereby defining the amino acid sequences of each fragment. Subsequent publications referred to the two major carboxy-terminal α_{II}-spectrin fragments as SBDP150 and 145, and did not distinguish SNTF from these SBDPs (53–56). Whereas the SBDP carboxy-terminal fragments have also been studied as candidate cerebrospinal fluid and blood biomarkers for acute brain injuries, neither has shown promise as a blood diagnostic or prognostic for mTBI. In contrast, SNTF is the predominant amino-terminal calpain derivative and has been widely studied as a histological and biochemical marker for axonal degeneration and the necrotic mode of neuronal death (19, 21, 57–60). Since SNTF continues to be confused with the SBDPs in the literature, we propose for purposes of clarity adoption of the following nomenclature

for calpain-derived α_{II}-spectrin breakdown products: SNTF for representing spectrin α_{II} 1-1176, SBDP150 for representing α_{II} 1177-2472, and SBDP145 for representing α_{II} 1231-2472.

The present study of SNTF as a diagnostic and prognostic blood marker for lasting functional impairment after mTBI has several limitations. Advanced neuroimaging is not available from this study for examining directly the interrelationships between rapid elevations in serum SNTF, persisting brain functional problems, and white matter microstructural disruption. The association between serum SNTF elevations and the lasting effects of mTBI on cognitive performance is complex, with many tests failing to uncover any significant prognostic relationship. This issue is complicated by the well-established influences of subject intelligence level and degree of education on performance of a number of cognitive tests (39–46, 61–67). When applying tests in which performance varies widely across cases independently of mTBI, it can be challenging to discern the long-lasting effects of the injury itself. In this regard, multiple components of the Stroop Color and Word test that associate with a rapid serum SNTF increase, the Symbol Digit Modalities Test linked to blood SNTF levels previously (25), accounting for age as a covariate in cognitive testing, and the serial evaluation of these tests over a prolonged recovery time may offer advantages for future clinical assessments of long-term cognitive dysfunction and impaired functional recovery after mTBI. In addition to cognitive assessment, lasting impairments in sensory-motor integration after mTBI may be discernable using the Grooved Pegboard test. Finally, at present this study lacks comparative information on tau, neurofilament L, and other potential biomarkers for diffuse axonal injury that may have strengths when combined with SNTF in multivariable biomarker analyses. Future work will be required to address these outstanding issues and conduct a large multi-site clinical validation study of an SNTF-inclusive panel of axonal injury biomarkers.

A clinically validated prognostic blood test for mTBI tied to the pathophysiology that underlies chronic brain functional impairments would have a number of vitally important uses in precision medicine and clinical research. These include the ability to (i) give patient prognosis, facilitating neurobiologically informed decisions on return to work, school, or participation in sports or military activities; (ii) initiate rehabilitation protocols more quickly after injury; (iii) stratify mTBI to enrich for at-risk cases for participation in well-controlled clinical research studies of the underlying mechanisms and the therapeutic benefit of drug interventions and rehabilitation protocols; (iv) apply a surrogate marker for the rapid quantitative evaluation of target mechanism engagement and therapeutic benefit; and (v) encourage mTBI evaluation and treatment in the emergency department, to reduce the problem of concussion under-reporting. In summary, the results of this study of 135 participants show that, in an emergency department setting, the blood level of SNTF on the day of a CT-negative mTBI strongly discriminates a subset of injury cases and is prognostic for persistent impairments in cognitive and sensory-motor function. They provide further evidence for the important contribution of axonal injury to persisting brain dysfunction after mTBI, and suggest a day of injury SNTF blood test, either alone or combined with other markers of axonal injury, may have utility in the rapid prognosis of chronic brain dysfunction and impaired functional recovery.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board, Regions Hospital, St. Paul, MN and Institutional Review Board, University of Pennsylvania, Philadelphia, PA. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

This project was conceived and developed by RS, SW, and MZ. SW and LH directed participant enrollment and were responsible for serial neurobehavioral assessments and compilation of the brain functional data sets. HC and RS performed quantitative and biostatistical analyses of serum SNTF levels comparing healthy control and mTBI participants and relating their levels to neurobehavioral outcomes in the long-term post-injury period. RS and MZ wrote the manuscript. DS supported the development of the second-generation biomarker immunoassay.

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Conflict of Interest: RS was the inventor on issued patents and pending patent applications related to an SNTF blood test for the diagnosis and prognosis of concussion. The patents are assigned to the Trustees of the University of Pennsylvania. DS was a consultant for Abbott Laboratories.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Is Salivary S100B a Biomarker of Traumatic Brain Injury? A Pilot Study

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Traumatic brain injury (TBI) results in short and long-term disability neurodegeneration. Mild traumatic brain injury (mTBI) represents up to 85% of head injuries; diagnosis and early management is based on computed tomography (CT) or in-hospital observation, which are time- and cost- intensive. CT involves exposure to potentially harmful ionizing radiation and >90% of the scans are negative. Blood-brain barrier (BBB) damage is suspected pathological event post-TBI contributing to long-term sequelae and a reliable and rapid point-of-care test to screen those who can safely forego acute head CT would be of great help in evaluating patients with an acute mTBI. In this pilot study, 15 adult patients with suspected TBI (mean age = 47 years, range 18–79) and 15 control subjects (mean age = 33 years, range 23–53) were enrolled. We found that the average salivary S100B level was 3.9 fold higher than blood S100B, regardless of the presence of pathology. $[S100B]_{saliva}$ positively correlated with $[S100B]_{serum}$ (Pearson' coefficient = 0.79; $p < 0.01$). Salivary S100B levels were as effective in differentiating TBI patients from control subjects as serum levels (Control vs. TBI: $p < 0.01$; Serum $ROC_{AUC} = 0.94$ and Saliva $ROC_{AUC} = 0.75$). I These initial results suggest that measuring salivary S100B could represent an alternative to serum S100B in the diagnosis of TBI. Larger and confirmatory trials are needed to define salivary biomarker kinetics in relation to TBI severity and the possible roles of gender, ethnicity and age in influencing salivary S100B levels.

Keywords: peripheral biomarkers, blood-brain barrier, saliva, mild traumatic brain injury, S100B

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INTRODUCTION

Traumatic brain injury (TBI) is common and it accounts for > 3 million emergency department visits per year in the United States alone (1). It has been widely recognized that there is need for improved clinical guidelines to diagnose TBI and to predict risk of downstream consequences. In particular, a consistent and rapid diagnosis of mild TBI (mTBI) based on blood analysis has been widely studied (2, 3). Serum S100B has been incorporated into Scandinavian guidelines for the management of acute mTBI, and serum GFAP and UCHL-1 have recently been cleared by the US Food and Drug Administration as an aid in the

diagnosis of mTBI (2, 4). Although serum markers such as GFAP, UCH-L1 or S100B can assess for the presence of intracranial bleeding from TBI with an excellent negative predictive value using sensitive immunoassays, there is currently no translation of this technology to a saliva-based point-of-injury or point-of-care (POI/POC) device. Existing salivary tests for TBI are based on detection of pathologic forms of nucleic acids (5), but no POI/POC salivary test exists for the most common blood protein markers used in the evaluation of neurologic disorders.

Concussions, or mTBI, result in axonal disruption, neuroinflammation, and glial activation (6) which are major pathologic mechanisms underlying of cognitive and sensory impairments. In addition, mTBI can be associated with a rapid disruption of blood-brain barrier (BBB) integrity followed by eventual development of brain damage [e.g., (7–9)]. BBB disruption after TBI is also a risk factor for secondary brain hemorrhage (10–12). It is therefore important to detect early BBB disruption to predict the development of persistent post-concussion disability.

The peripheral BBB reporter S100B (13, 14) has been shown to correlate with several indices of BBB disruption including albumin coefficient (15), gadolinium enhancement (16–18), or CT scans (19). Most importantly, S100B increases also after a minor head injury (mTBI) and is useful to identify the absence of intracranial bleeding. In comparison to CT-based diagnosis of mTBI, S100B serum levels accurately identified negative intracranial bleeding, with a negative predictive value of 99% (20–23).

While the development of POI/POC devices is in the works, almost all POC prototypes use blood samples to measure biomarkers. Saliva is an attractive biological fluid, which has some advantages over blood testing in pre-hospital settings and direct to consumer home-based applications. Blood components, such as platelets or red blood cells, require a removal process before an analysis can be completed. In addition, blood samples cannot be drawn when paramedics or trained personnel are not present. In addition, risk of infection from blood products and the time and equipment necessary to separate blood components could make POC blood diagnostic challenging.

In an attempt to overcome this, we developed a prototype test for salivary S100B, which is being implemented in a laboratory medicine and in a POI/POC format. We report here the preliminary findings obtained by analyzing S100B in saliva of control subjects and mTBI patients. We enrolled patients with Glasgow Coma Scale (GCS) of 14–15 on admission to capture the mild forms of TBI which constitute the target population for our salivary S100B test.

METHODS

All patients (≥18 years old) presented to the emergency department at Penn Presbyterian Medical Center < 6 h after an injury involving head trauma warranting a head CT for clinical evaluation, with a GCS of 14 or 15. Patients were excluded from the study if pregnant, in custody, and/or diagnosed with skull fractures. Written informed consent was obtained from the participants of the study for the publication of the identifiable data.

The Galveston Orientation and Amnesia Test (GOAT) was used to assess for a diminished capacity to consent. For this preliminary investigation, fifteen TBI subjects and fifteen control subjects were enrolled. Control subjects were defined as individuals who have no history of TBI in the past 6 months and do not display any neurological symptoms.

Demographic history, comorbidities, and clinical course of the injury, including GCS score, trauma mechanism, and loss of consciousness, were obtained at initial assessment by a treating physician, then confirmed by study staff when blood and salivary samples were collected.

Patients being treated in the hospital had an intravenous (IV) catheter placed for standard hospital monitoring. Blood was collected < 6 h from mTBI by draining off the IV whenever possible, otherwise collection was done by venipuncture. All control samples were collected by venipuncture. Whole blood was processed for serum, within 30 min of collection. After centrifugation, serum was transferred into cryovials labeled with a unique subject ID number and stored at −80C in 0.5 ml aliquots.

Saliva was collected with a commercially available collection system (Salivette^{TM}) at time of blood draws. The subjects were asked to chew a plain cotton roll for 1 min to stimulate salivation. The rolls with the absorbed saliva were then centrifuged to removed food remnants, insoluble material, and cell debris. The resulting supernatant was stored in 0.5 ml aliquots at −80C.

S100B was measured, in both saliva and serum, by chemiluminescence and automated sandwich ELISA (LIAISON; Diasorin, Stillwater, MI) (24). The Limit of Detection is 0.02 ng/mL, and the lower-upper range is 0.02–30 ng/mL. The inter- and intra-coefficient of variation are 9.4 and 7.2%, respectively.

To assess the significance of differences between groups we used Minitab 19 and linear regression/ Mann-Whitney tests. Because clinical data are not usually normally distributed, they are also reported as median and interquartile ranges (**Table 2**). Outliers were removed in Origin 2019 Pro by Grubbs test. ROC were obtained with JMP 14 (SAS).

RESULTS

The patients' demographic and clinical characteristics are shown in **Table 1**. Comparison of all data points (serum vs. saliva, **Figure 1A**) revealed that levels of the biomarker S100B are higher in saliva than blood. **Figure 1B** shows the correlation between serum and salivary S100B levels. Line slope is 3.9, the Adj. R-Square 0.38, Pearson's coefficient 0.7 and the p value $p < 0.01$.

Next, we tested the hypothesis that S100B in saliva is as effective as blood S100B in detecting TBI vs. control. The results are shown in **Figure 1C**. We used a non-parametric test (Mann-Whitney) to determine the statistical significance of the observed increases in salivary and blood S100B after a diagnosis of mTBI. Significantly elevated levels of both salivary and blood S100B were measured in mTBI patients compared to controls ($p <$

TABLE 1 | Demographic and clinical characteristics of the patients and controls.

ID		Gender	Ethn.	Age	GCS at admission	Time to collection of samples	CT findings (0,1)	CT descriptive	Symptoms amnesia, vomiting, et.
SB-1001	CONTROL	Male	Cauc.	29	NA	NA	NA	NA	NA
SB-1002	CONTROL	Male	Cauc.	25	NA	NA	NA	NA	NA
SB-1007	CONTROL	Male	Cauc.	29	NA	NA	NA	NA	NA
SB-1009	CONTROL	Male		53	NA	NA	NA	NA	NA
SB-1012	CONTROL	Male	Cauc.	31	NA	NA	NA	NA	NA
SB-1020	CONTROL	Male	AA	23	NA	NA	NA	NA	NA
SB-1021	CONTROL	Female	Cauc.	32	NA	NA	NA	NA	NA
SB-1024	CONTROL	Male	Cauc.	37	NA	NA	NA	NA	NA
SB-1025	CONTROL	Female	Cauc.	30	NA	NA	NA	NA	NA
SB-1026	CONTROL	Male	Cauc.	35	NA	NA	NA	NA	NA
SB-1027	CONTROL	Male	Cauc.	28	NA	NA	NA	NA	NA
SB-1028	CONTROL	Female	Cauc.	27	NA	NA	NA	NA	NA
SB-1029	CONTROL	Male	Cauc.	41	NA	NA	NA	NA	NA
SB-1030	CONTROL	Female	Cauc.	26	NA	NA	NA	NA	NA
SB-1014	CONTROL	Male	AA	53	NA	NA	NA	NA	NA
SB-1003	TBI	Male	Cauc.	77	14	4:20	0		LOC
SB-1004	TBI	Male	Cauc.	76	15	4:19	1	Acute left frontal subarachnoid hemorrhage	AOC, LOC, PTA
SB-1005	TBI	Female	Cauc.	61	15	4:51	0		PTA, AOC
SB-1006	TBI	Female	AA	21	15	3:58	0		mild PTA, LOC
SB-1008	TBI	Male	AA	29	15	5:02	0		PTA, Dizziness, Headache
SB-1010	TBI	Female	AA	55	14	2:43	0		LOC, PTA
SB-1011	TBI	Male	AA	18	15	4:58	0		LOC, PTA
SB-1013	TBI	Female	AA	33	15	3:40	0		LOC, Headache
*SB-1015	TBI	Male	Cauc.	25	15	5:00	0		LOC, Headache
*SB-1016	TBI	Male	AA	41	15	2:38	0		LOC
*SB-1017	TBI	Female	AA	68	15	5:02	1	Acute right extra axial hemorrhage hematoma	LOC, nausea, vomiting
SB-1018	TBI	Male	AA	58	15	1:46	0		LOC
SB-1019	TBI	Male	AA	39	15	4:55	0		Headache
*SB-1022	TBI	Female	AA	28	15	2:39	0		LOC, headache
SB-1023	TBI	Male	AA	79	15	3:34	0		Headache

*The * points to outliers detected by Grubbs test. Cauc, Caucasian; AA, African-American; AOC, LOC, Alteration, loss of consciousness; PTA, post-traumatic amnesia.*

0.01) (**Table 2**). ROC curves for saliva and serum are shown in **Figure 1D**. The two curves were not statistically different ($p > 0.05$). Finally, we performed an analysis of variance to detect if potential confounders (ethnicity, age, and gender) other than TBI had influenced blood or serum levels of S100B. We conducted a multivariate linear regression that included saliva and blood S100B (continuous variable) as outcomes, group as fixed effect/predictor, and age/race/sex as covariates. A limitation of our study is the unequal repartition of AA and Caucasian subjects among control and TBI cohorts. The model revealed that these covariates did not have significant influence on the outcomes. we report the results without covariates and also with race (we prioritized race, given the small sample size) as a covariate. Both models showed significant group differences in blood (without covariate $\beta = -0.301$, $SE = 0.074$, $p = 0.001$; with race $\beta = -0.230$, $SE = 0.110$, $p = 0.050$) and saliva (without covariate $\beta = -2.768$, $SE = 0.986$, $p = 0.011$; with race $\beta = -3.321$, $SE = 1.492$, $p = 0.038$).

DISCUSSION

To our knowledge, this is the first study demonstrating a correlation between salivary and blood S100B levels in adults with mTBI. The results have shown that: (1) salivary levels of the astrocytic protein S100B are higher than those measured in serum; (2) the diagnostic properties of S100B in blood are similar

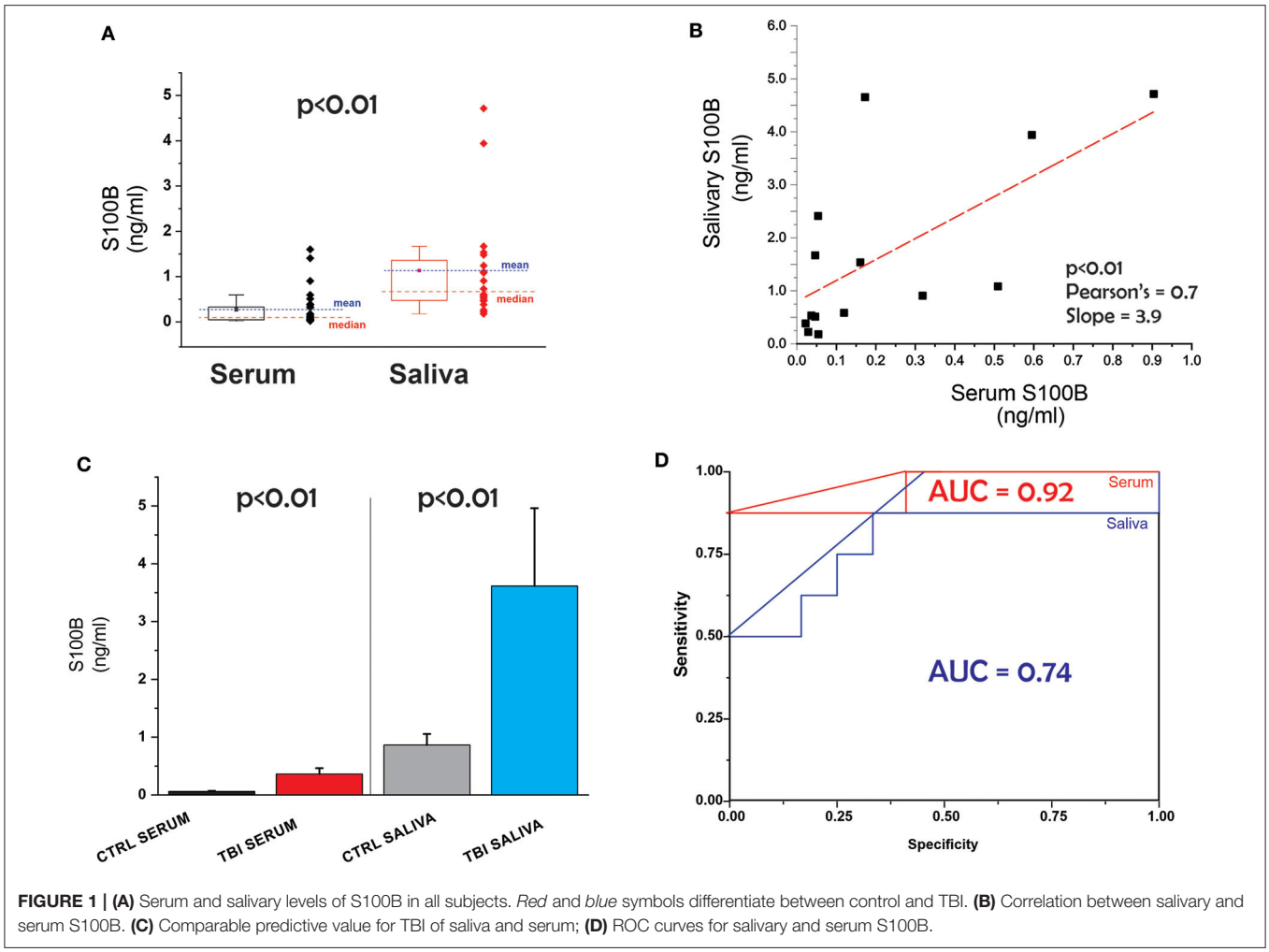

FIGURE 1 | (A) Serum and salivary levels of S100B in all subjects. *Red* and *blue* symbols differentiate between control and TBI. **(B)** Correlation between salivary and serum S100B. **(C)** Comparable predictive value for TBI of saliva and serum; **(D)** ROC curves for salivary and serum S100B.

TABLE 2 | Data reported as median and interquartile values.

		N	Mean	Standard Error	Standard Deviation	Coefficient of Variation	Minimum	Quantile	Median	Maximum
Blood	**CONTROL**	15	0.058	0.008	0.032	54.620	0.022	0.037	0.047	0.122
	TBI	14	0.502	0.129	0.483	96.270	0.037	0.170	0.353	1.600
Saliva	**CONTROL**	15	0.849	0.159	0.615	72.430	0.223	0.466	0.584	2.411
	TBI	8	3.620	1.340	3.800	105.150	0.180	0.950	2.740	11.920

to those in saliva; (3) a correlation exists between salivary and blood levels of S100B in control and post-TBI conditions.

A crucial aspect of TBI diagnostics is the fact that although serum markers such as GFAP, UCH-L1 or S100B already rule out clinically important concussion sequelae with an excellent negative predictive value and a low limit of detection in laboratory-based approaches, there is currently no translation of this technology to a saliva-based POI or POC device. The POI/POC platforms currently in development use blood where platelets, leukocytes and red blood cells require a cumbersome removal process prior to protein analysis. The equipment used to process serum/plasma and measure biomarkers in blood is not always available. Finally, a saliva-based POI/POC device would solve several of the problems associated with blood diagnostics in TBI or other neurological diseases. The development of new diagnostic methods to rapidly screen for TBI related brain damage is of utmost significance for head-contact sports, military and emergency head trauma settings. The use of saliva in place of serum or plasma samples could further streamline the diagnostic routine, simplifying the execution of testing.

Saliva is an important biofluid for evaluation of health and disease in human subjects. The use of saliva for diagnostics has advantages, but an obstacle in the progression of this field has been lack of a detailed understanding of how dynamic passage

of biomarkers from blood into saliva occurs. To substantiate the use of salivary diagnostic approach, several research groups have reported their pilot data. For example, Di Pietro et al. (25) recruited small sample size ($n = 6$ concussion, $n = 6$ controls) and screened 800 human microRNA expression levels in saliva samples. Five microRNAs were significantly elevated in the concussion group compared to the control group, yet these genes were non-brain specific. Subsequently, (26) identified 5 panels of microRNA, which are related to neuronal functional integrity, to be significantly elevated in saliva in pediatric concussion patients (avg. age of 14 years). The salivary microRNA measure was particularly useful in detecting prolonged concussion recovery, with 85% accuracy in detecting patients with prolonged concussion symptoms from their acute symptomatic counterparts. It is worth also noting that whether measured in blood or saliva, S100B is an indicator of blood-brain barrier disruption (23, 27). Finally, we have recently published a paper describing the pharmacokinetic aspects of biomarkers' origin, fate and distribution (28). This was limited to traditional body fludis (blood, urine). We now expanded this to encompass salivary biomarkers (13).

To highlight the prospective advantage of various biomarkers in saliva-based diagnostics, we recently developed a computer algorithm to reproduce the passage of small molecular weight protein from blood into saliva (13). This program was originally designed as a "physiologically-based pharmacokinetic model to describe the distribution of brain-derived biomarkers in blood" (28). Its main structure was expanded to include a new compartment, namely an idealized salivary gland receiving its vascular supply by the external carotid. The venous output was approximated by jugular vein branches. To approximate the combined contribution of protein extravasation along transcellular and paracellular pathways crossing capillary endothelial cells and salivary gland epithelia, we used a simple mass transfer equation to quantify the process. While this approach gave an insight into the kinetics of passage of protein from blood to saliva (13), it did not predict significantly higher S100B levels in saliva vs. blood. A question that will be answered by future studies thus relates to the several-fold change in S100B levels in saliva vs. serum (**Figure 1A**).

The main limitation of this report is the small numbers of TBI cases. Larger studies in children and adults are being performed in the US and Europe. Regardless of its preliminary nature, this initial results provides evidence supporting the continuous studying of salivary S100B in TBI diagnostics. Also, we believe that the implementation of salivary S100B into clinical guidelines could be cost and time saving. An additional limitation is the use of normal subjects as controls: a population of non-trauma victims (e.g., orthopedic emergency room patients) may have been more appropriate. It is important to underscore that in a small pediatric study (29), salivary S100B was not increased in polytrauma no-TBI patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB University of Pennsylvania. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DJ directed the study. RD-A and ES performed all the clinical studies. KK and NM assisted in data analysis and manuscript preparation. RD-A and DJ led the overall effort.

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Conflict of Interest: DJ is the founder of FloTBI, Inc. a company developing the salivary test presented herein.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Risk for Misdiagnosing Chronic Traumatic Encephalopathy in Men With Anger Control Problems

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Background: There are no validated or agreed upon criteria for diagnosing chronic traumatic encephalopathy (CTE) in a living person. In recent years, it has been proposed that anger dyscontrol represents a behavioral clinical phenotype of CTE. This is the first study to examine the specificity of the diagnostic research criteria for traumatic encephalopathy syndrome (TES, the clinical condition proposed to be CTE) in men from the US general population who have anger dyscontrol problems. It was hypothesized that a substantial percentage of these men would meet the research criteria for TES.

Methods: Data from 4,139 men who participated in the National Comorbidity Survey Replication, an in-person survey that examined the prevalence and correlates of mental disorders in the United States, were included in this study. Men who were diagnosed with intermittent explosive disorder in the past year were the clinical sample of interest (n = 206; 5.0% of all men in the database), and the remaining men were used as a comparison sample. They were classified as meeting the research criteria for TES if they presented with the purported supportive clinical features of CTE (e.g., impulsivity/substance abuse, anxiety, apathy, suicidality, headache).

Results: In this sample of men from the general population with intermittent explosive disorder, 27.3% met a conservative definition of the proposed research criteria for CTE (i.e., traumatic encephalopathy syndrome). If one assumes the delayed-onset criterion is present, meaning that the men in the sample are compared to former athletes or military veterans presenting with mental health problems years after retirement, then 65.0% of this sample would meet the research criteria for TES.

Conclusions: These results have important implications. Using conservative criteria, at least one in four men from the general population, who have serious anger control problems, will meet the symptom criteria for TES. If one considers former athletes and

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military veterans with anger control problems who present many years after retirement and who experienced a documented decline in their mental health, nearly two-thirds will meet these research criteria. More research is needed to examine risks for misdiagnosing TES and to determine whether anger dyscontrol is a clinical phenotype of CTE.

Keywords: athletes, anger, depression, suicidal ideation, suicide, brain concussion, brain injuries, chronic traumatic encephalopathy

INTRODUCTION

There is tremendous interest in chronic traumatic encephalopathy (CTE). In the twentieth century, CTE was considered to be a neurological disorder affecting a subgroup of long-career boxers (1, 2), and the clinical features were usually described as reflecting rather obvious chronic brain damage and cognitive impairment (3, 4). Varying degrees of neurological hard signs, such as abnormal reflexes and hemiparesis, and extrapyramidal signs, such as slurred or dysarthric speech, gait abnormalities, and tremor, were described (2, 4–11). The extent to which CTE is static or progressive or whether its course reflects two or more different clinical conditions has never been clear (1, 2, 7, 10, 12–17), and many authors conceptualized CTE as a progressive parkinsonian-like neurological disorder, and others have not.

In its modern form, CTE is considered to be a postmortem neuropathological diagnosis (18, 19), with the defining pathological feature being the accumulation of hyperphosphorylated tau (p-tau), in a patchy distribution at the depths of the cortical sulci around small vessels (18, 19). This specific neuropathology has been identified after death in the brains of young athletes (20, 21), active NFL players (20, 21), former collegiate athletes from multiple sports (20), retired boxers (20), retired professional hockey players (20), retired NFL players (18, 20, 21), and military veterans (22). P-tau accumulates in the brain in normal aging and in numerous neurodegenerative diseases (23–28), but researchers have asserted that it does not accumulate in a *patchy distribution* in the depths of sulci in association with aging or other diseases (18, 19). However, this assertion remains in doubt because CTE neuropathology has been identified in some people from the general population with no known exposure to repetitive neurotrauma and in association with substance abuse, temporal lobe epilepsy, multiple system atrophy, amyotrophic lateral sclerosis, and other neurodegenerative diseases (29–36).

The extent to which the neuropathology of CTE causes specific clinical symptoms and problems is unclear (19, 37); there is no agreed-upon way to diagnose CTE in a living person, and there is major interest in developing and validating clinical diagnostic criteria. At present, validated diagnostic criteria do not exist, although several sets have been proposed (13, 38–40). Preliminary proposed research criteria for "traumatic encephalopathy syndrome (TES)" (39) include three core features of CTE: (i) "cognitive," (ii) "behavioral" (i.e., anger dyscontrol), and (iii) "mood" (i.e., depression or hopelessness). These core features are used to define diagnostic "subtypes" or "variants" according to the research criteria. In addition to a subtype, two supportive features must be present [i.e., impulsivity, anxiety, apathy, paranoia, suicidality, headache, motor signs, a progressive clinical course, or a delayed onset of symptoms (e.g., after retirement from sport)].

A major gap in the literature is that there are very few published studies relating to the specificity of the proposed research criteria for TES (41, 42). This is the first study to examine the research criteria for TES (39) in men from the US general population who have intermittent explosive disorder (IED). We chose to study these men from the general population because the "behavioral" subtype of TES is defined as follows: "Being described as emotionally explosive (e.g., having a "short fuse" or being "out of control"), physically violent, and/or verbally violent, as reported by self or informant, by history of treatment, or by clinician's report. A formal diagnosis of IED would meet this criterion but is not necessary (39). We hypothesized that a substantial percentage of these men from the general population would meet the proposed research criteria for TES.

METHODS

Participants

The National Comorbidity Survey Replication (NCS-R), conducted between February 2001 and April 2003 (43, 44), examined the prevalence and correlates of mental disorders in the United States (45–49). The interview for this survey was conducted in the homes of a nationally representative sample of adult respondents (N = 9,282, 4,139 men and 5,143 women) (44). The clinical sample of interest was obtained by applying a filter to the publicly available NCS-R database selecting all "male" participants meeting the criteria for a *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (*DSM-IV*) IED in the past year (i.e., variable = "D_IED12"). This filter resulted in the inclusion of 206 men; an incidence rate of 5.0% of all men in the database. The mean age of this sample was 32.9 years (median = 30.5, SD = 12.1, interquartile range = 23–41, range = 18–83). Their race was reported as follows: white = 65.5%, African Americans = 12.6%, Hispanic = 11.2%, Asian = 2.4%, and all other races = 8.3%. Their level of education was as follows: 0–11 years = 22.3%, 12 years = 33.0%, 13–15 years = 30.6%, and 16 or more years = 14.1%. Their employment status was as follows: employed = 73.8%, unemployed = 1.9%, and not in the labor force = 23.8%. Their relationship status was described as 56.3% married, 32.0% as never married, and 11.7% as divorced, separated, or widowed. The remaining 3,933 men were considered to be a sample representing the general population. The mean age of this sample was 44.4 years (median

= 43.0, SD = 17.0, interquartile range = 31–56, range = 18–93). Their race was reported as follows: white = 74.8%, African Americans = 10.8%, Hispanic = 9.4%, Asian = 2.0%, and all other races = 3.0%. Their level of education was as follows: 0–11 years = 15.4%, 12 years = 28.9%, 13–15 years = 27.9%, and 16 or more years = 27.8%. Their employment status was as follows: employed = 72.1%, unemployed = 6.8%, not in the labor force = 20.5%, and missing = 0.5%. Their relationship status was described as 62.4% married, 22.2% never married, and 15.4% divorced, separated, or widowed.

The NCS-R Protocol

Researchers from the Survey Research Center of the Institute for Social Research at the University of Michigan conducted the survey using laptop computer-assisted personal interviews. The core diagnostic assessment, conducted with 9,282 respondents, included the following modules: household listing, screening, depression, mania, irritable depression, panic disorder, specific phobia, social phobia, agoraphobia, generalized anxiety disorder, IED, suicidality, services, and pharmacoepidemiology. The diagnoses were derived from the World Mental Health Survey Initiative Version of the World Health Organization Composite International Diagnostic Interview, a fully structured lay-administered diagnostic interview that generates both *International Classification of Diseases, 10th Revision* (50) and *DSM-IV* (51) diagnoses. The NCS-R database is publicly available, and we accessed it at http://www.icpsr.umich.edu/icpsrweb/ICPSR/studies/20240.

Research Criteria for Traumatic Encephalopathy Syndrome

The research criteria for "traumatic encephalopathy syndrome" (39) include three proposed core features of CTE: (i) "cognitive," (ii) "behavioral" (i.e., anger dyscontrol), and (iii) "mood" (i.e., depression or hopelessness). We selected a sample of men who would definitively meet the core criterion for "behavioral" in that they were diagnosed with *DSM-IV* IED within the past 12 months. The "supportive features" for a diagnosis of the syndrome include impulsivity, anxiety, apathy, paranoia, suicidality, headache, motor signs, a documented decline in functioning or a progression of symptoms, or a delayed onset—such as having problems at least 2 years after the end of a career in contact sports. Two or more supportive features must be present. For the present study, we selected five of the nine supportive features, available in the NCS-R database, for the primary analyses (i.e., impulsivity, anxiety, apathy, suicidality, and headache). Other supportive features, such as paranoia, motor signs, decline in functioning, and delayed onset of symptoms were deemed to be less reliable or missing variables in the NCS-R database. The neurotrauma exposure criterion for TES is broad and diverse and includes any one of the following: (i) four of more concussions; (ii) two or more moderate or severe TBIs; (iii) involvement in "high exposure" contact sports (e.g., American football, ice hockey, lacrosse, rugby, wrestling, and soccer) for a minimum of 6 years, including at least 2 years at the college level (or higher); (iv) military service (including, but not limited to, combat exposure to blast and other explosions as well as non-combat exposure to explosives, or to combatant training or breaching training); or (v) history of any other significant exposure to repetitive hits to the head (including, but not limited to, domestic abuse, head banging, and vocational activities such as door breaching by police). This criterion could not be applied because the information was not available in the NCS-R database.

The rates of screening positively for TES are presented in two ways. First, the rate at which the men meet two of the five supportive criteria is presented. Second, the rate at which the men meet one of the five supportive criteria is presented because this simulates the normal clinical situation in which the delayed-onset criterion and/or the decline in functioning criterion would be met. The "delayed onset" criterion requires "delayed onset of clinical features after significant head impact exposure, usually at least 2 years and in many cases several years after the period of maximal exposure," and the "documented decline" criterion requires a "progressive decline in function and/or a progression in symptoms" (39). Any former athlete or military veteran who met the neurotrauma exposure criteria and retired in their 20s or 30s, for example, who had mental health or neurological problems consistent with "TES" anytime between the ages of 40 years and the time of their death would meet the delayed-onset criterion.

RESULTS

The prevalence of IED in men in the National Comorbidity Survey Replication was 9.7% for lifetime, 5.0% for past year, and 2.1% for past 30 days. Lifetime diagnoses of major mental disorders, and mental disorders experienced over the past year and past 30 days, stratified by group, are presented in **Table 1**. Lifetime experiences with headaches, suicidality, anger, and chronic pain, stratified by group, are presented in **Table 2**. Compared to men in the general population, those with IED have a greater lifetime history of many disorders, including but not limited to attention-deficit/hyperactivity disorder [20.9 vs. 3.6%; $\chi^2 = 135.5$, $p < 0.001$, relative risk (RR) = 5.7, 95% confidence interval (CI) = 4.1–7.9], conduct disorder and oppositional defiant disorder (both 22.3 vs. 4.5%; $\chi^2 = 122.1$, $p < 0.001$, RR = 5.0, 95% CI = 3.6–6.7), alcohol abuse (45.6 vs. 14.5%; $\chi^2 = 141.3$, $p < 0.001$, RR = 3.2, 95% CI = 2.6–3.7), drug abuse (30.1 vs. 8.8%; $\chi^2 = 100.4$, $p < 0.001$, RR = 3.4, 95% CI = 2.7–4.3), major depressive episode (35.9 vs. 13.8%; $\chi^2 = 75.5$, $p < 0.001$, RR = 2.6, 95% CI = 2.1–3.2), mania (16.0 vs. 2.5%; $\chi^2 = 115.6$, $p < 0.001$, RR = 6.4, 95% CI = 4.3–9.3), generalized anxiety disorder (18.0 vs. 4.7%; $\chi^2 = 72.2$, $p < 0.001$, RR = 4.1, 95% CI = 2.9–5.7), panic disorder (12.6 vs. 2.8%; $\chi^2 = 68.3$, $p < 0.001$, RR = 3.8, 95% CI = 2.7–5.3), adult separation anxiety disorder (17.5 vs. 4.3%), social phobia (i.e., social anxiety disorder, 21.8 vs. 10.3%; $\chi^2 = 26.9$, $p < 0.001$, RR = 2.1, 95% CI = 1.6–2.8), and a specific phobia (21.4 vs. 8.3%; $\chi^2 = 40.6$, $p < 0.001$, RR = 2.6, 95% CI = 1.9–3.4). Over the past 12 months, those with IED were 6.9 times more likely to meet criteria for *DSM–IV* alcohol abuse disorder (17.5 vs. 2.5%; $\chi^2 = 137.4$, $p < 0.001$, RR = 6.9, 95% CI = 4.7–9.9), and 4.4 times more likely to meet criteria for *DSM–IV* major depressive episode (23.8 vs. 5.4%; $\chi^2 = 111.4$,

TABLE 1 | DSM-IV disorders.

	Men with intermittent explosive disorder ($n = 206$)			Men from the general population ($n = 3,933$)		
	Lifetime *n* (%)	Past 12 months *n* (%)	Past 30 days *n* (%)	Lifetime *n* (%)	Past 12 months *n* (%)	Past 30 days *n* (%)
Substance Use Disorders						
Alcohol abuse	94 (45.6%)	36 (17.5%)	11 (5.3%)	569 (14.5%)	100 (2.5%)	31 (0.8%)
Alcohol dependence	48 (23.3%)	17 (8.3%)	7 (3.4%)	227 (5.8%)	44 (1.1%)	18 (0.5%)
Drug abuse	62 (30.1%)	16 (7.8%)	6 (2.9%)	345 (8.8%)	55 (1.4%)	20 (0.5%)
Drug dependence	28 (13.6%)	6 (2.9%)	2 (1.0%)	109 (2.8%)	15 (0.4%)	7 (0.2%)
Nicotine dependence	41 (19.9%)	26 (12.6%)	18 (8.7%)	259 (6.6%)	122 (3.1%)	79 (2.0%)
Intermittent explosive disorder	206 (100%)	206 (100%)	88 (42.7%)	197 (5.0%)	0 (0.0%)	0 (0.0%)
Mood Disorders						
Dysthymia	17 (8.3%)	12 (5.8%)	8 (3.9%)	106 (2.7%)	57 (1.4%)	26 (0.7%)
Major depressive episode	74 (35.9%)	49 (23.8%)	24 (11.7%)	543 (13.8%)	213 (5.4%)	77 (2.0%)
Bipolar I	13 (6.3%)	11 (5.3%)	7 (3.4%)	25 (0.6%)	16 (0.4%)	9 (0.2%)
Bipolar II	5 (2.4%)	5 (2.4%)	3 (1.5%)	31 (0.8%)	23 (0.6%)	13 (0.3%)
Bipolar subthreshold	20 (9.7%)	14 (6.8%)	7 (3.4%)	82 (2.1%)	42 (1.1%)	14 (0.4%)
Hypomania	5 (2.4%)	2 (1.0%)	1 (0.5%)	39 (1.0%)	16 (0.4%)	6 (0.2%)
Mania	33 (16.0%)	27 (13.1%)	12 (5.8%)	99 (2.5%)	52 (1.3%)	21 (0.5%)
Anxiety Disorders						
Generalized anxiety disorder	37 (18.0%)	26 (12.6%)	17 (8.3%)	184 (4.7%)	90 (2.3%)	39 (1.0%)
Panic attack	103 (50.0%)	43 (20.9%)	17 (8.3%)	867 (22.0%)	260 (6.6%)	82 (2.1%)
Panic disorder	26 (12.6%)	17 (8.3%)	8 (3.9%)	109 (2.8%)	50 (1.3%)	24 (0.6%)
Agoraphobia without panic disorder	11 (5.3%)	10 (4.9%)	4 (1.9%)	63 (1.6%)	37 (0.9%)	20 (0.5%)
Agoraphobia with panic disorder	7 (3.4%)	6 (2.9%)	0 (0.0%)	36 (0.9%)	22 (0.6%)	12 (0.3%)
Posttraumatic stress disorder	17 (8.3%)	10 (4.9%)	2 (1.0%)	119 (3.0%)	58 (1.5%)	35 (0.9%)
Adult separation anxiety disorder	36 (17.5%)	11 (5.3%)	8 (3.9%)	169 (4.3%)	47 (1.2%)	18 (0.5%)
Social phobia	45 (21.8%)	31 (15.0%)	14 (6.8%)	405 (10.3%)	208 (5.3%)	89 (2.3%)
Specific phobia	44 (21.4%)	31 (15.0%)	22 (10.7%)	328 (8.3%)	197 (5.0%)	137 (3.5%)
Adolescent/Developmental Disorders						
Attention deficit disorder	43 (20.9%)	26 (12.6%)	NA	143 (3.6%)	61 (1.6%)	NA
Conduct disorder	46 (22.3%)	8 (3.9%)	NA	177 (4.5%)	14 (0.4%)	NA
Oppositional defiant disorder	46 (22.3%)	5 (2.4%)	NA	177 (4.5%)	17 (0.4%)	NA

$p < 0.001$, RR = 4.4, 95% CI = 3.3–5.8) than men from the general population.

The percentages of men with IED meeting supportive criteria for TES are presented in **Table 3**. "Impulsivity," as reflected by a diagnosis of alcohol abuse or drug abuse in the past year, was present in 20.4%. Problems with anxiety were present in 49.0%, and suicidality was present in 5.3%. Apathy was reported by 10.7%. A significant problem with headaches was reported by 15.0%. Overall, 27.3% met the criteria for two or more supportive features for the syndrome and thus when combined with their diagnosis of IED would meet the clinical criteria for TES. We could not apply the criteria relating to delayed onset (e.g., anger control problems in a middle-aged man who played college football) or progressive worsening of symptoms (i.e., over at least a 1-year duration). Assuming that one of those two criteria was met, then only one additional criterion from **Table 3** would be necessary to meet research criteria for the syndrome. The proportion of the IED sample who met one or more of the criteria was 65.0%.

DISCUSSION

This is the first study to examine a proposed set of research criteria for the diagnosis of TES (39) in a sample of men from the general population with IED. There were three primary important findings. First, anger attacks are common in men in the general population, with one in four men reporting them at some point during their lifetime (**Table 2**). Second, in the general population, the lifetime prevalence of IED is 9.7% in the present study, and men with this disorder have a high lifetime prevalence of other disorders that have been proposed to be clinical features of CTE and TES, such as alcohol abuse (45.6%), drug abuse (30.1%), and major depressive episode (35.9%). In other words, men in the US general population with severe anger control problems are likely to experience other symptoms, problems, and disorders that researchers have proposed to be characteristic of CTE and TES (**Tables 1**, **2**). Finally, the rate of meeting the symptom criteria for TES in men from the US general population who have serious anger control problems,

TABLE 2 | Headaches, chronic pain, anger, and suicidal behavior.

	Men with intermittent explosive disorder (n = 206)				Men from the general population (n = 3,933)			
	Endorsed		Missing		Endorsed		Missing	
Condition	*f*	%	*f*	%	*f*	%	*f*	%
Headaches								
Ever had frequent or severe headaches	54	26.2	14	6.8	396	10.1	1,728	43.9
Still have severe headache or received treatment	31	15.0	152	73.8	206	5.2	3,537	89.9
Chronic pain								
Ever had chronic back/neck problems	66	32.0	14	6.8	707	18.0	1,728	43.9
Still have back/neck problems or receive treatment in past year	44	21.4	140	68.0	474	12.1	3,226	82.0
Ever had any other chronic pain	26	12.6	14	6.8	272	6.9	1,728	43.9
Still have chronic pain or received treatment in past year	19	9.2	180	87.4	176	4.5	3,661	93.1
Ever had arthritis/rheumatism	40	19.4	14	6.8	533	13.6	1,732	44.0
Anger Attacks (Ever in Life….)								
Anger attack leading to breaking item of some value	181	87.9	0	0	1,074	27.3	3	0.1
Anger attack leading to hitting/attempt hitting person	137	66.5	0	0	977	24.8	5	0.1
Anger attack leading to threat of harm to person	43	20.9	137	66.5	599	15.2	979	24.9
Irritability and anger in past month								
Feel irritable/grumpy*	104	50.5	9	4.4	595	15.1	1,235	31.4
Feel mad/angry	95	46.1	9	4.4	398	10.1	1,234	31.4
Feel angry and out of control	33	16.0	27	13.1	64	1.6	2,318	58.9
Feel urge hit/push/hurt someone	26	12.6	27	13.1	58	1.5	2,318	58.9
Feel urge to break/smash something	32	15.5	27	13.1	61	1.6	2,318	58.9
Suicidal Ideation and Behavior								
Ever seriously thought about committing suicide	57	27.7	36	17.5	395	10.0	697	17.7
Seriously thought about committing suicide in past 12 months	11	5.3	148	71.8	58	1.5	3,538	90
Ever made a plan for committing suicide	27	13.1	148	71.8	121	3.1	3,538	90
Made a suicide plan in the past 12 months	4	1.9	179	86.9	14	0.4	3,812	96.9
Ever attempted suicide	20	9.7	148	71.8	97	2.5	3,539	90
Attempted suicide in the past 12 months	3	1.5	186	90.3	11	0.3	3,836	97.5

*%, percentage; f, frequency; n, number. *Those who rated experiencing irritability or anger as some of the time, most of the time, or all of the time were included; GAD, Generalized Anxiety Disorder; IED, intermittent Explosive Disorder. Missing values included values missing from the system, don't know responses, and/or refused to respond responses.*

during the past year, is high (**Table 3**). The average age of this sample was 33, 50% were between the ages of 23 and 41 years, and 25% were older than 41 years. In other words, a large percentage of this sample was of a similar age of men who have retired from contact or collision sports or retired from the military. As such, if we assume the "delayed onset" supportive feature is met, then only one additional supportive feature is necessary to diagnose TES. As such, approximately two of three men from the general population, who have serious anger control problems, meet the proposed research diagnostic criteria for TES.

It is essential to appreciate that anger dyscontrol and aggressive behavior are complex and multifactorial in causation. There are many reasons why former athletes or military veterans might have anger control problems. Temperamental (52–54) and personality (55, 56) factors have been linked to risk of anger dyscontrol and aggression. Adverse events in childhood, such as abuse and neglect, have been associated with increased risk of future anger control problems (57–59). Men who had abusive or aggressive fathers are statistically more likely to also be abusive or aggressive (57). Some boys might choose certain high contact, collision, or combat sports in part due to innate aggressiveness (60, 61). As such, a certain degree of anger dyscontrol and aggressiveness may represent longstanding behavioral and personality characteristics in some former athletes, as has been speculated by authors writing about former boxers (2, 4, 5, 8, 62–64). These longstanding characteristics could be amplified or exacerbated by life stress, depression, anxiety, substance abuse, chronic cumulative brain damage, and a number of neurological and neurodegenerative diseases. Life stress (65), financial problems (66), marital problems (66), and substance abuse (67, 68) are all associated with anger control problems. Military veterans with posttraumatic stress disorder frequently have anger control problems (69–71). Men who develop a depressive disorder are also at risk of having anger attacks (72, 73). Anger attacks in men with depression have been assumed to be related to the depressive disorder, as opposed to reflecting a primary underlying IED. People with TBIs sometimes develop problems with anger dyscontrol and

TABLE 3 | Percentage of men meeting research criteria for supportive features of traumatic encephalopathy syndrome.

	Intermittent explosive disorder (n = 206)	General population (n = 3,933)
"Impulsivity. Impaired impulse control, as demonstrated by new behaviors, such as excessive gambling, increased or unusual sexual activity, substance abuse, excessive shopping or unusual purchases, or similar activities (39)." Definition: DSM-IV diagnosis of alcohol abuse in past year (D_ALA12) or drug abuse in past year (D_DRA12).	20.4%	3.4%
"Anxiety. History of anxious mood, agitation, excessive fears, or obsessive or compulsive behavior (or both), as reported by self or informant, history of treatment, or clinician's report. A formal diagnosis of anxiety disorder would meet this criterion but is not necessary (39)." Definition: DSM-IV diagnosis, in the past year, of generalized anxiety disorder (D_GAD12), agoraphobia without panic disorder (D_AGO12), agoraphobia with panic disorder (D_AGP12), panic disorder (D_PDS12), social phobia (i.e., social anxiety disorder; D_SO12), posttraumatic stress disorder (D_PTS12), or rating any of the following as 1 = often in the past month: nervousness, fidgety, tense (SC9D); worry too much about things (NSD1F); suddenly scared no reason (NSD1B); or feel frightened (NSD1H).	49.0%	14.9%
"Apathy. Loss of interest in usual activities, loss of motivation and emotions, and/or reduction of voluntary, goal-directed behaviors, as reported by self or informant, history of treatment, or clinician's report (39)." Definition: No interest in things over the past month (rated as 1 = often; NSD1G).	10.7%	2.2%
"Suicidality. History of suicidal thoughts or attempts, as reported by self or informant, history of treatment, or clinician's report (39)." Definition: Seriously thought about committing suicide in past 12 months (SD3), made a suicide plan in past 12 months (SD5), or attempted suicide in past 12 months (SD10).	5.3%	1.5%
"Headache. Significant and chronic headache with at least one episode per month for a minimum of 6 months (39)." Definition: Reports a current problem with severe headaches and/or treatment for headaches (CC4C).	15.0%	5.2%
One or more of the above criteria are met.	65.0%	20.1%
Two or more of the above criteria are met.	27.3%	5.7%

Variable names from the publicly available NCS-R database are provided.

aggressiveness, particularly after sustaining a single severe TBI (74, 75). However, the associations between cumulative mild injuries to the brain and anger or aggression are not well-understood. Finally, problems with anger and aggression can occur as a result of a neurological disorder, such as a stroke (76–78), or during the course of a neurodegenerative disease, such as Alzheimer disease (79, 80).

Modern researchers studying CTE have emphasized psychiatric and behavioral problems as being common (39), and those who are younger and who have less neuropathology have been conceptualized as being more likely to have this proposed "mood" or "behavioral" subtype or phenotype of TES and CTE (81). The mechanisms by which small amounts of p-tau in specific brain regions drive complex changes in behavior, such as depression and anger control problems, have not been studied in a meaningful way and are unknown. Nonetheless, modern researchers seem to suggest that virtually any clinical or psychosocial problem present prior to death in someone who has CTE neuropathology in their brain identified on postmortem examination must have that problem as a direct result of the CTE neuropathology. For example, the two leading research groups in the United States have asserted that clinical features of CTE include (i) depression and anxiety (18, 82, 83); (ii) suicidality (18, 81, 83–87); (iii) poor financial decisions, financial problems, and bankruptcy (82); (iv) gambling (39); (v) excessive shopping or unusual purchases (39); (vi) marital problems, separation, and divorce (87); and (vii) substance abuse (39). This approach to defining clinical features represents association by assertion, or *circulus in probando*, as opposed to being based on empirical research or rigorous clinicopathological correlation.

In their review of all known cases of CTE, published in 2009, McKee et al. (20) documented that 17 of the 41 cases (41.5%) published in the twentieth century had a personal history of aggression or violence. We reexamined the 41 case studies, and without question, some former boxers believed to have CTE had documented anger control problems and violent behavior during their lifetime (4–6, 8, 10, 64). In contrast, other former boxers were described as showing euphoria (6, 15), a child-like demeanor (4), or "fatuous cheerfulness" (6). The demographic and clinical characteristics of the 17 cases with aggressive behavior are summarized in **Table 4**. According to the new proposed criteria for TES, a former boxer (or contact sport athlete) could be diagnosed as having the behavioral variant of TES if he had developed clinical problems "at least 2 years after a period of maximal exposure" (39) and had any one of the following problems: excessive gambling, unusual sexual activity, excessive shopping or unusual purchases, anxiety, excessive fears, obsessive-compulsive disorder, any anxiety disorder, or suicidality. To our knowledge, there was never a case that matched any of those characteristics in the twentieth century, based on our review of the case information presented in the tables in McKee et al. and our review of the six published studies that reported the case histories of those 17 people. As seen in **Table 4**, the aggressive behavior and volatility displayed by these former boxers generally co-occurred with other obvious neurological signs of brain damage, such as dysarthric speech,

TABLE 4 | Twentieth century case studies of presumed CTE with a history of violence or aggressiveness ($n = 17$) from McKee et al. (20).

Case #	Year*	Age sport began	Years in sport	Initial symptoms and problems	Age of onset of symptoms	Years between retirement and symptoms	Interval between symptom onset and death	Age at death	Cognitive changes	Memory loss	Dementia	Parkinsonism or obvious neurological abnormality***	CTE pathology (88)
8	1967	16	7	Cognitive decline, hemiparesis	25	1	33	58	Yes	Yes	Yes	Yes	N/A
10	1968	15	20	Headaches	36	0	10	46	No	No	No	Yes	N/A
11	1968	19	12	Headaches	31	0	15	46	Yes	Yes	No	Yes	N/A
12	1968	16	16	Slurred speech, gait change	40	8	5	45	Yes	Yes	No	Yes	N/A
15	1973	11	14	Violent outbursts	25	0	38	63	Yes	Yes	No	Yes	Yes CTE
17	1973	16	14	Confusion, falls	30	0	33	63§	Yes	Yes	No	Yes	Yes CTE
19	1973	18	18	Irritability, memory loss, aggression	36	0	25	61	Yes	Yes	No	Yes	No ARTAG
20	1973	13	25	Gait, speech	37	0	46	83	Yes	Yes	No	Yes	Yes CTE
21	1973	16	20	Dysphoria, violence	54	18	8	62	Yes	Yes	Yes	Yes	Yes CTE
22	1973	17	23	Ataxia, falls, weakness	60	20	11	71	Yes	No	No	Yes	Yes CTE
24	1973	NR	NR	Memory loss	40	NR	27	67	Yes	Yes	Yes	NR	Yes CTE/AD
25	1973	NR	NR	Confusion	48	NR	19	67	Yes	Yes	Yes	Yes	No ARTAG
26	1973	14	16	Speech, delirium	43	4	14	57§	Yes	Yes	Yes	Yes	No AD
28**	1973	NR	NR	Aggression	NR	NR	NR	91	No	No	No	No	No Diagnosis
32	1992	NR	>25	NR	NR	NR	NR	63	Yes	Yes		Yes	N/A
34	1996	NR	15	NR	NR	NR	NR	33	Yes	Yes	Yes	NR	N/A
41	1999	NR	10	Cognitive decline, ALS-like syndrome	64	NR	NR	67	Yes	Yes	Yes	Yes	N/A

*This information was derived from **Table 2** in McKee et al. (20). All cases were reported to have been boxers with the exception of a circus clown who was an achondroplastic dwarf, aged 33 at the time of death, who had 15 years of exposure as a circus clown and participation in dwarf throwing competitions reported by Williams and Tannenberg (89). *1967 = Constantinidis and Tissot (90), 1968 = Payne (64), 1973 = Corsellis et al. (4), 1992 = Hof et al. (91), 1996 = Williams and Tannenberg (89), 1999 = No listed authors (92), 2018 = Goldfinger et al. (88); **Case 28 said to have boxed professionally in his youth. He was described as being "considered active and mentally alert up to the time of his death" (4); AD, Alzheimer's disease; ARTAG, Aging-related tau astrogliopathy; CTE, chronic traumatic encephalopathy; N/A, not applicable; NR, not reported; §Case 17 "Age at Death" reported as 63 years in Corsellis et al. (4) and 62 years in Goldfinger et al. (88); Case 26 "Age at Death" reported as 57 years in Corsellis et al. (4) and 56 years in Goldfinger et al. (88); ***Obvious neurological abnormality includes two or more of the following: movement abnormalities, decreased facial movement, slowed movements, tremor, rigidity, falls, ocular abnormalities, ptosis, reduced upgaze, gait problems: staggered gait, slowed gait, shuffled gait, ataxia, reduced coordination, speech changes: slowed speech, slurred speech, dysarthria, dysphagia, spasticity.*

gait problems, and parkinsonism, and virtually all had cognitive impairment or dementia (2, 4, 5, 10, 64). Moreover, authors sometimes noted that aggressive behavior seemed to be a longstanding problem (4, 5, 10), perhaps contributing to their chosen career of boxing.

Our study has three important limitations. First, we have no information on the subjects' concussion history, and it is likely that some of the men in our case series experienced one or more concussions during the course of their lives because concussions are very common in men in the general population (93, 94). Second, we were unable to study the "exposure history" criterion in the research definition of TES. It is possible that some of the men included in this study would have met the exposure criterion. It is important to appreciate that *all* former professional soccer players, hockey players, boxers, and American or Canadian football players meet the exposure criteria for repetitive neurotrauma. The exposure criterion is very inclusive and simply requires that the person played one or more sports (e.g., boxing, American football, ice hockey, lacrosse, rugby, wrestling, or soccer), for a minimum of 6 years [with 2 at the college level (or equivalent) or higher], which resulted in "multiple impacts to the head" that can be concussions or "subconcussive trauma" (i.e., with no clinical symptoms) (39). Moreover, military service or police training involving exposure to blasts, explosives, combat, or breaching is listed as a source of exposure sufficient to meet criteria. It is very likely that some men who participated in the NCS-R study played contact sports, at least at the high school level. No information relating to lifetime history of sports participation was available in the NCS-R database. Finally, we were not able to align precisely the results of the NCS-R interviews on to the research criteria for TES, which are broader and more inclusive than what we could study. We did not include two categories of supportive features: (i) paranoia and (ii) motor signs. Moreover, within the category of impulsivity, we did not include "excessive gambling," "increased or unusual sexual activity," or "excessive shopping or unusual purchases." If we had more variables that aligned with all the supportive features criteria, the rate of identifying TES in this sample would have been greater.

In conclusion, we examined the proposed research criteria for the behavioral subtype of TES in a large sample of men with serious anger control problems who were selected from a nationally representative sample of men from the US general population who underwent a thorough in-person psychiatric interview yielding *DSM-IV* diagnoses. Using liberal criteria, we discovered that approximately two of three of these men could be identified as having TES. Researchers have not established a clinicopathological correlation between anger control problems and the region-specific accumulation of hyperphosphorylated tau believed to characterize CTE (19), so researchers and clinicians should not assume that anger control problems in a former athlete or military veteran are caused by CTE neuropathology. Anger control problems described in the case histories of boxers in the twentieth century were not considered to be a "behavioral" phenotype or subtype of CTE, nor were they described as a core clinical feature. More research is needed to examine risks for misdiagnosing TES, which appear considerable based on the results of this study. In addition, more research is needed to determine whether anger dyscontrol is a clinical phenotype of CTE.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://www.icpsr.umich.edu/icpsrweb/ICPSR/studies/20240.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Michigan. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GI and AG contributed to the conception and design of the study. GI analyzed the database. All authors drafted and revised the manuscript and approved the submitted version.

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Conflict of Interest: GI serves as a scientific advisor for BioDirection, Inc., Sway Operations, LLC, and Highmark, Inc. He has a clinical and consulting practice in forensic neuropsychology, including expert testimony, involving individuals who have sustained mild TBIs (including athletes). He has received research funding from several test publishing companies, including ImPACT Applications, Inc., CNS Vital Signs, and Psychological Assessment Resources (PAR, Inc.). He has received research funding as a principal investigator from the National Football League, and salary support as a collaborator from the Harvard Integrated Program to Protect and Improve the Health of National Football League Players Association Members. He acknowledges unrestricted philanthropic support from ImPACT Applications, Inc., the Heinz Family Foundation, and the Mooney-Reed Charitable Foundation. AG has a clinical practice in neuropsychology involving individuals who have sustained sport-related concussion (including current and former athletes). He has been a contracted concussion consultant to Rugby Australia since July 2016. He has received travel funding from the Australian Football League (AFL) to present at the Concussion in Football Conference in 2013 and 2017. Previous grant funding includes the NSW Sporting Injuries Committee, the Brain Foundation (Australia), and the Hunter Medical Research Institute (HMRI), supported by Jennie Thomas, and the HMRI, supported by Anne Greaves. He is currently funded through an NHMRC Early Career Fellowship, and Hunter New England Local Health District, Research, Innovation and Partnerships Health Research & Translation Center and Clinical Research Fellowship Scheme, an Australian-American Fulbright Commission Postdoctoral Award, and the University of Newcastle's Priority Research Center for Stroke and Brain Injury.

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Concussion Disrupts Normal Brain White Matter Microstructural Symmetry

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Injuries and illnesses can alter the normal bilateral symmetry of the brain, and determining the extent of this disruption may be useful in characterizing the pathology. One way of quantifying brain symmetry is in terms of bilateral correlation of diffusion tensor metrics between homologous white matter tracts. With this approach, we hypothesized that the brains of patients with a concussion are more asymmetrical than those of healthy individuals without a history of a concussion. We scanned the brains of 35 normal individuals and 15 emergency department patients with a recent concussion. Fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD) were determined for regions of interest (ROI) defined by a standard white-matter atlas that included 21 bilateral ROIs. For each ROI pair, bilateral correlation coefficients were calculated and compared between the two subject groups. A symmetry index, defined as the ratio between the difference and the sum of bilateral measures, was also calculated for each ROI pair and compared between the groups. We found that in normal subjects, the extent of symmetry varied among regions and individuals, and at least subtle forms of structural lateralization were common across regions. In patients, higher asymmetry was found overall as well as in the corticospinal tract specifically. Results indicate that a concussion can manifest in brain asymmetry that deviates from a normal state. The clinical utility of characterizing post-concussion pathology as abnormal brain asymmetry merits further exploration.

Keywords: acute concussion, bilateral homolog, diffusion tensor imaging (DTI), mild traumatic brain injury (mTBI), magnetic resonance imaging (MRI)

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INTRODUCTION

Although the functional and anatomical lateralization of the brain is well recognized, what is more obvious, and thus can easily be taken for granted, is its normal structural symmetry. There may be various ways to quantify bilateral symmetry of the brain, but one approach is to do so for the microstructure of white matter tracts in terms of correlation between the metrics of magnetic resonance (MR) diffusion imaging. As is known, correlations of diffusion metrics between

homologous pairs of tracts are generally high in healthy individuals (1–3). This verification supports an assertion that bilateral asymmetry reflects disruption of a normal state when found in patients with injuries or illnesses and that determining the extent of this disruption is useful in characterizing the pathology (2, 4, 5). Still, it needs to be noted that there are normal asymmetries as well as symmetrical abnormalities. To examine *abnormal* brain asymmetry, at least three considerations are needed: (1) the extent and similarity of the impact of an injury or illness on bilateral regions vary; (2) the degrees of normal symmetry vary among regions (1); and (3) no two injuries or illness progressions are alike (2).

Here, using MR diffusion tensor imaging (DTI) we studied white matter microstructural symmetry of adults with and without a concussion, with the hypothesis that the brains of patients with a concussion are more asymmetrical. We investigated abnormal brain asymmetry by: (1)′ examining whether any white matter tract is potentially more vulnerable to concussion than others; (2)′ benchmarking the normal extent of symmetry between homologous pairs of white matter tracts; and (3)′ examining deviations from these benchmarks at an individual level. We also controlled for potential developmental variables by examining only the data from subjects 18 years or older.

This report constitutes a secondary substudy of a larger study related to concussion with a focus on a normative characterization of eye movement performance in a variety of cohorts (6, 7) and is cross-sectional case-control in design. MR images were collected from predefined groups of subjects. Some imaging results not overlapping with the present report, comparing concussed young athletes and patients from the emergency department (ED), have been published (8). Here we focused on ED patients because a substantial number of adults were included in the sample and their demographic characteristics agreed well with those of the control pool.

METHODS

Subjects

The protocols for subject enrollment and assessment were approved by the Institutional Review Board of Weill Cornell Medical College (Approval number: 1201012120). Patients from the ED with concussion within the past 2 weeks and healthy control subjects with no history of head injury, aged 7 years or older, were recruited for the imaging study. Control subjects were recruited through flyers posted at colleges, office buildings, community centers, and other facilities in the New York City area. A concussion was defined as an event of blunt impact on the head, with loss of consciousness (LOC), post-traumatic amnesia (PTA), or at least one of the following symptoms: dizziness, nausea, headaches, balance problems, blurred or double vision, or feeling dazed/confused. Although for the purpose of this research we did not rely on formal medical diagnosis of concussion, this definition is consistent with the guidance of the American Academy of Neurology (9). Prior to data collection, written informed consent by adult subjects, or legal guardians of minor subjects with the minors' assent, was obtained in accordance with the Declaration of Helsinki. Subjects' symptoms and cognitive performance were assessed with an extensive battery of tests as reported elsewhere (7).

As stated in Introduction, only the data from subjects 18 years and older were analyzed for this substudy. These subjects were required to have a high school diploma or GED, or for 18-year olds, set to graduate high school on time. Exclusion criteria as per the parent study were a prior history of eye disease, neurological/psychiatric conditions, substance abuse, or contraindications for MR imaging. For ED patients with concussion, additional exclusion criteria were acute intoxication at the time of the concussion and LOC or PTA for more than 24 hours. Of the total of 42 patients, 15 were 18 years or older. Recruited patients were MR-scanned as soon as scheduling allowed. The scan took place with a mean (SD) of 10.5 (2.8) days following the injury. A total of 38 control subjects were MR-scanned, of whom 35 were 18 years or older. A comparison of the groups' demographic characteristics is reported in Results.

Magnetic Resonance Imaging

The methods for MR image acquisition, quality inspection, and processing were described previously (8). Briefly, on a 3T Siemens Trio scanner, whole-brain diffusion imaging with 128 × 128 × 60 cubic voxels of 2 mm dimensions was conducted using an echo-planar imaging sequence (TE = 85 ms, TR = 7500 ms) with one $b = 0$ s/mm^2 scan and $b = 1{,}000$ s/mm^2 in 64 diffusion directions. Images were processed with tools within the Functional MRI of the Brain (FMRIB) Software Library (10). They were corrected for eddy currents and subject motion and registered to the $b = 0$ s/mm^2 volume using the FMRIB's Linear Image Registration Tool (11). Image volumes were checked for excessive subject movement between diffusion weighted images and were accepted for mean and median movement being <2 mm. Non-brain voxels were excluded using the Brain Extraction tool (12). Using the diffusion-weighted data, a diffusion tensor model was generated using DTIFIT, from which fractional anisotropy (FA), mean diffusivity (MD), radial diffusivity (RD), and axial diffusivity (AD) were determined at each voxel. These interdependent metrics together characterize local water diffusion properties.

Tract-Based Spatial Statistics (TBSS) were used to perform non-linear registration on the FA volumes to the FMRIB58_FA standard-space, constructed from the average of 58 FA images of healthy adult subjects (13, 14). After brain volumes were registered into the common space, a mean FA skeleton was generated using a threshold of FA ≥ 0.2 to limit the analysis to white matter voxels. TBSS alignment and white-matter skeleton generation were performed separately for each subject group. Masks were applied corresponding to the 48 white matter tracts labeled in the Johns Hopkins University white-matter atlas (15). The regions of interest (ROI) included 21 pairs of bilateral tracts: anterior corona radiata; anterior limb

Abbreviations: ED, emergency department; DTI, diffusion tensor imaging; MR, magnetic resonance; AD, axial diffusivity; RD, radial diffusivity; MD, mean diffusivity; FA, fractional anisotropy; LOC, loss of consciousness; PTA, post-traumatic amnesia; ROI, region of interest; SD, standard deviation.

of the internal capsule; cingulum-cingulate gyrus; cingulum-hippocampus; cerebral peduncle; corticospinal tract; external capsule; fornix/stria terminalis; inferior cerebellar peduncle; medial lemniscus; posterior corona radiata; posterior limb of the internal capsule; posterior thalamic radiation; retrolenticular part of the internal capsule; superior cerebellar peduncle; superior corona radiata; superior fronto-occipital fasciculus; superior longitudinal fasciculus; sagittal stratum; tapetum; and uncinate fasciculus. Note that in this atlas, the middle cerebellar peduncle was not defined bilaterally. For each subject, within-ROI mean values of the four DTI metrics were determined.

Statistics

Group-wise matching in age was tested with an F-test and a two-sample *t*-test. Matching in sex was tested with a chi-square test. The alpha level was set to 0.05.

To identify particular white matter tracts as potentially vulnerable to concussive impacts, we compared DTI metric values of each of the 48 ROIs between the two groups using a two-sample *t*-test without assuming equal variances, where data from all subjects were entered for each ROI. A statistically significant difference between the means was taken as an indication of propensity for concussive injury. The alpha level was corrected for multiple comparison using Holm's method considering the number of ROIs (stepping down from $\alpha/48 = 0.00104$, $\alpha/47 = 0.00106$, and so on, until no further null hypothesis could be rejected) (16). We inspected group differences in variances in DTI metrics using an F-test, where data from all subjects were entered for each ROI. Furthermore, we examined whether there was an overall trend in mean or variance differences across ROIs using a paired *t*-test with pairing by ROI, where the within-group mean or variance of 48 ROIs were entered.

For each of 21 ROI pairs for each DTI metric, Spearman's correlation coefficients ρ between the two sides of the brain were calculated for the two groups. A one-tailed paired *t*-test, paired between groups for each ROI, was used to test the hypothesis that the mean bilateral correlation was reduced in patients compared to controls. To test whether specific ROIs could be identified as having lower correlations in patients, the ρ values were compared using Fisher's method (17). Based on the hypothesis that correlations would be reduced in patients, these tests were also one-tailed. The alpha level was corrected for multiple comparison using Holm's method.

Differences between patients and controls in bilateral symmetry of DTI metrics were also examined using an index defined as the ratio between the difference (right minus left) and the sum of bilateral measures within individual. By definition, the value of this symmetry index ranges from −1 to 1, with 0 indicating perfect symmetry. Distributions of symmetry indices were visualized with box plots. The bottom and top of each box indicate the first (Q1) and third (Q3) quartiles, and the middle line or symbol the median. The whiskers indicate the minimum and maximum of all the data excluding outliers whose values were larger than $Q3 + w \cdot (Q3 - Q1)$ or smaller than $Q1 - w \cdot (Q3 - Q1)$, where $w = 1.5$. We expected that the index values of some patients would fall outside the normal spread. Specifically, we hypothesized that there would be ROIs in which values of the symmetry index were more variable among patients than controls. This hypothesis was tested using a one-tailed F-test. The alpha level was corrected for multiple comparison using Holm's method considering that there were 21 bilateral ROI pairs.

Although normal white matter microstructural asymmetry is already recognized (1), we sought to document it as well using the symmetry index. We applied a two-tailed one-sample *t*-test to the control sample to identify tracts whose symmetry index values deviated from zero. Again, the alpha level was corrected for multiple comparison using Holm's method.

Finally, it was hypothesized that, with abnormal values of symmetry indices expected among patients, the *magnitudes* of the symmetry indices across ROIs would be overall larger in patients than controls. This hypothesis was tested by taking within-individual averages of the absolute values of symmetry indices across ROIs and applying a one-tailed *t*-test between the two groups.

RESULTS

The mean (SD) ages of the ED patient and control groups were 35.5 (12.7) and 40.7 (15.4) years old, respectively. The patient group was 60% female while the control group was 66% female. The two groups did not differ significantly in terms of distributions in age [$F_{(34,14)} = 1.41$, $p = 0.50$; $|t(48)| = 1.13$, $p = 0.26$] or sex [$\chi^2(1) = 0.15$, $p = 0.70$].

Group-wise comparisons of FA values of 48 ROIs yielded statistical differences after correction for multiple comparison in the middle cerebellar peduncle, right corticospinal tract, splenium of the corpus callosum, right posterior limb of the internal capsule, and right superior fronto-occipital fasciculus (**Table 1**). Comparisons of MD values yielded differences in the bilateral superior fronto-occipital fasciculus, genu and body of the corpus callosum, left anterior corona radiata, right anterior limb of the internal capsule, and right posterior limb of the internal capsule. Comparisons of RD values yielded differences in the right superior fronto-occipital fasciculus, right posterior limb of the internal capsule, middle cerebellar peduncle, genu and splenium of the corpus callosum, left anterior corona radiata, and left external capsule. Finally, comparisons of AD values yielded differences in the left superior fronto-occipital fasciculus.

The above listed tracts were identified as possibly vulnerable to concussion. Even so, across ROIs, averaged FA values tended to be larger in the patient group [$|t(47)| = 6.15$, $p < 0.0001$], and MD, RD, and AD values smaller in the patient group [MD: $|t(47)| = 7.56$, $p < 0.0001$; RD: $|t(47)| = 6.87$, $p < 0.0001$; AD: $|t(47)| = 4.73$, $p < 0.0001$], substantiating diffuse effects of concussion. The statistically significant group differences found for the middle cerebellar peduncle in FA and RD values (**Table 1**) opposed these trends, however. In addition to the centrality measure, for each DTI metric, some ROIs indicated group differences in variances at the alpha level of 0.05. Although only the variances of MD values in the left cerebral peduncle showed a statistically significant group difference [$p < 0.0001$, $F_{(34,14)} = 8.88$] when the alpha level was corrected for the number of ROIs, a general difference in the shapes of distributions could be

TABLE 1 | White matter tracts that yielded a statistically significant difference between patients with concussion and control subjects in each DTI metric.

	Patient mean	Control mean	$\|t\|$	df	p
FA					
Middle cerebellar peduncle	0.563	0.598	5.92	35.7	<0.0001
Cortico-spinal tract, R	0.616	0.579	4.30	28.6	0.0002
Splenium of corpus callosum	0.819	0.801	3.87	45.4	0.0003
Posterior limb of internal capsule, R	0.701	0.678	3.72	33.1	0.0007
Superior fronto-occipital fasciculus, R	0.548	0.507	3.79	27.0	0.0008
MD					
Superior fronto-occipital fasciculus, L	6.31×10^{-4}	6.68×10^{-4}	4.57	36.3	<0.0001
Genu of corpus callosum	6.92×10^{-4}	7.25×10^{-4}	4.36	45.5	<0.0001
Anterior corona radiata, L	7.13×10^{-4}	7.46×10^{-4}	4.29	40.9	0.0001
Superior fronto-occipital fasciculus, R	6.30×10^{-4}	6.69×10^{-4}	4.39	32.0	0.0001
Anterior limb of internal capsule, R	6.85×10^{-4}	7.10×10^{-4}	4.15	40.0	0.0002
Posterior limb of internal capsule, R	6.95×10^{-4}	7.18×10^{-4}	4.24	31.9	0.0002
Body of corpus callosum	7.73×10^{-4}	8.03×10^{-4}	3.68	43.1	0.0006
RD					
Superior fronto-occipital fasciculus, R	4.14×10^{-4}	4.60×10^{-4}	6.03	46.5	<0.0001
Posterior limb of internal capsule, R	3.56×10^{-4}	3.86×10^{-4}	4.73	34.7	<0.0001
Middle cerebellar peduncle	4.68×10^{-4}	4.27×10^{-4}	4.65	22.8	0.0001
Genu of corpus callosum	2.67×10^{-4}	3.09×10^{-4}	4.16	40.2	0.0002
Anterior limb of internal capsule, R	4.05×10^{-4}	4.29×10^{-4}	4.08	44.5	0.0002
Splenium of corpus callosum	2.43×10^{-4}	2.72×10^{-4}	4.02	42.3	0.0002
Anterior corona radiata, L	4.93×10^{-4}	5.29×10^{-4}	3.72	36.7	0.0007
External capsule, L	5.28×10^{-4}	5.52×10^{-4}	3.49	43.9	0.0011
AD					
Superior fronto-occipital fasciculus, L	1.03×10^{-3}	1.08×10^{-3}	4.29	27.6	0.0002

Group means and the results of two-sample t-tests are shown. Statistical significance was determined by correction of α-levels for multiple comparison. L, left; R, right; df, degrees of freedom.

noted. Specifically, the variances of FA values were significantly different between the two groups paired for ROIs [$|t(47)| = 3.27$, $p = 0.0020$], lower in the patient group. Tighter clustering of FA values are visualized in **Figure 1** for $2 \times 4 = 8$ ROIs. There was no statistical group difference across ROIs in variances for MD, RD, or AD.

Bilateral correlation for the control group varied widely across ROIs, but ranged similarly among the DTI metrics: $0.30 \leq \rho \leq 0.89$ for FA, $0.33 \leq \rho \leq 0.89$ for MD, $0.19 \leq \rho \leq 0.89$ for RD, and $0.14 \leq \rho \leq 0.84$ for AD. Across the metrics, the anterior corona radiata, posterior corona radiata, and superior longitudinal fasciculus were among the consistently better-correlated ROIs, with ρ-values 0.75 or above (for example, **Figures 1A–C**, open circles). On the other hand, the uncinated fasciculus, fornix/stria terminalis, retrolenticular part of the internal capsule, and superior fronto-occipital fasciculus were among the consistently less-correlated ROIs, with ρ-values 0.70 or below.

Against the normative benchmark that bilateral ROIs have variable but mostly high ρ-values, in all DTI metrics but AD, ρ-values were significantly lower in the patient group than the control group [FA: $t(20) = 2.40$, $p = 0.013$; MD: $t(20) = 3.08$, $p = 0.003$; RD: $t(20) = 2.67$, $p = 0.007$; AD: $t(20) = 0.01$, $p = 0.49$]. Thus, bilateral symmetry was generally disrupted in patients as a group. Comparison of ρ-values associated with each bilateral ROI for the patient and control groups, after correcting the alpha level for multiple comparison, specifically identified the corticospinal tract as having a statistically significant reduction in the patient group (FA: $z = 2.47$, $p = 0.007$, **Figure 1D**; RD: $z = 3.25$, $p < 0.001$). Note that the corticospinal tract was also among the tracts with a statistically significant difference between the two subject groups (**Table 1**, larger FA in patients). Thus, this tract stood out as possibly generally vulnerable as well as variably sensitive to concussion.

That normal variations in bilateral symmetry differed among ROIs was also characterized by the heterogeneity in the inter-individual variability of symmetry index values for the control group (**Figure 2**, open boxplots). The cingulum-hippocampus (ROI 4), tapetum (ROI 20), and uncinate fasciculus (ROI 21) were among those with the largest inter-individual variability across the four DTI metrics, while the anterior limb of the internal capsule (ROI 2), superior longitudinal fasciculus (ROI 18), and superior corona radiata (ROI 16) were among those with the smallest. The patient group also showed differences in the inter-individual variability of symmetry index values. However, between-group comparisons indicated that the variance in symmetry was larger in patients in the corticospinal tract yet again, as indicated in **Figures 2B–D** by longer whiskers for

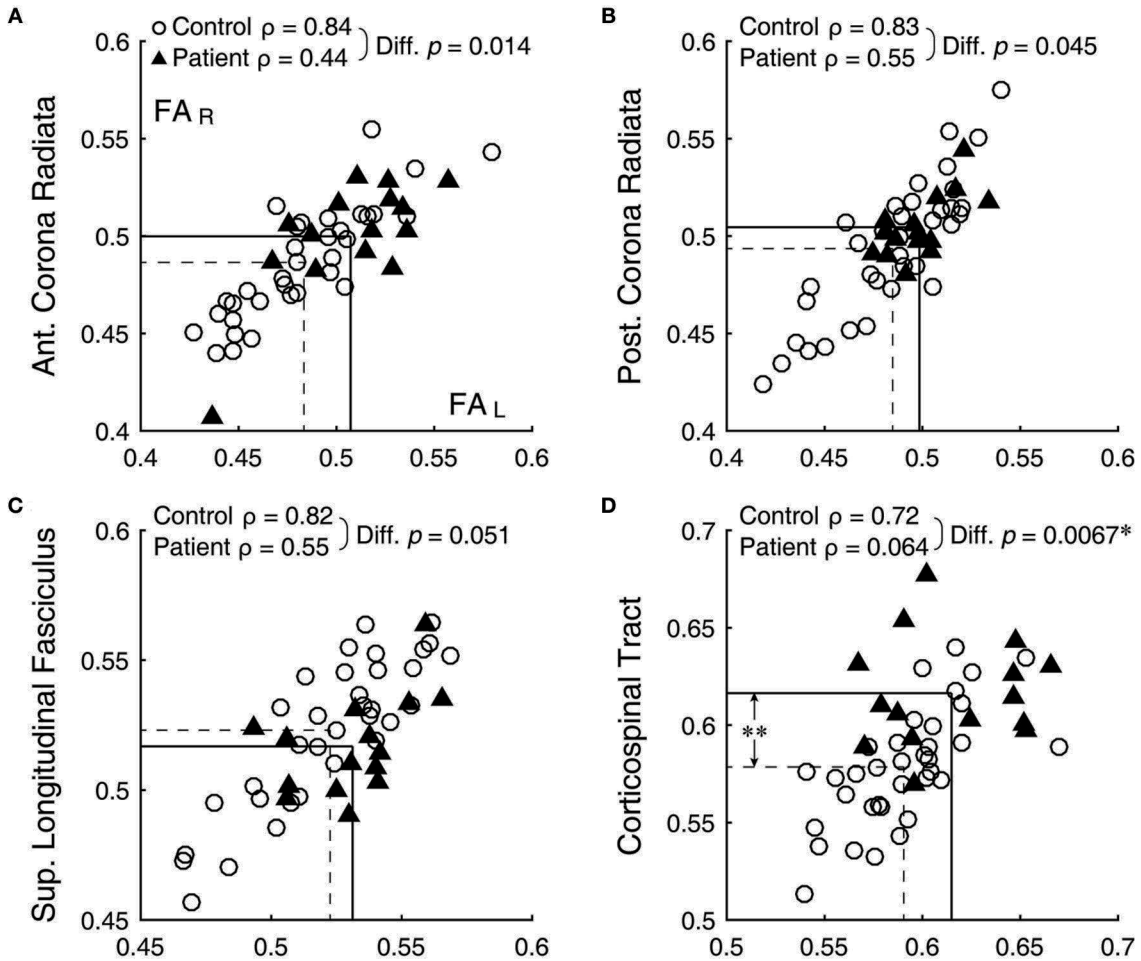

FIGURE 1 | Bilateral correlation of FA values. **(A)** anterior corona radiata, **(B)** posterior corona radiata, **(C)** superior longitudinal fasciculus, and **(D)** corticospinal tract. Open circles and filled triangles indicate control and patient subjects, respectively, with the left and right FA values represented by the abscissa and ordinate, respectively. Dashed lines indicate means of the control group and solid lines those of the patient group. *Significant difference (with correction for multiple comparison) between the ρ-values. **Significant difference (with correction for multiple comparison) between the means (see **Table 1**).

ROI 6, marked by a rectangle [MD: $F_{(34,14)} = 0.17$, $p < 0.0001$; RD: $F_{(34,14)} = 0.14$, $p < 0.0001$; AD: $F_{(34,14)} = 0.29$, $p < 0.0016$].

Figure 2 also suggested that the distributions of symmetry index values often veered from zero. Indeed, in the majority of the bilaterally paired tracts of control subjects, a statistically significant deviation from zero was found for one or more DTI metrics (**Table 2**). Thus, at least subtle forms of structural lateralization were common in normal brains.

Individual averages of symmetry index magnitudes were statistically larger in patients than controls in all DTI metrics but MD [FA: $t(48) = 1.92$, $p = 0.030$; MD: $t(48) = 1.65$, $p = 0.052$; RD: $t(48) = 2.38$, $p = 0.011$; AD: $t(48) = 2.52$, $p = 0.007$]. A separate examination, of within-individual across-ROI variations in symmetry index magnitudes, could not ascribe the larger asymmetry in the patient group to contributions by a select few patients with consistently extreme bilateral asymmetry. Thus, asymmetry in patients was in one way or another exaggerated beyond the asymmetry that was broadly observed in normal brains.

DISCUSSION

Using DTI metrics, we characterized bilateral white matter microstructural symmetry-asymmetry in normal individuals, and compared it to that of ED patients with a recent concussion. In normal subjects, the extent of symmetry varied among regions and individuals, and at least subtle forms of structural lateralization were common across regions. In patients, higher asymmetry was found overall as well as in a specific region, namely the corticospinal tract. Findings from two separate approaches, namely assessments of correlation and symmetry index, were in agreement. Results indicate that a concussion can manifest in brain asymmetry that deviates from a normal state.

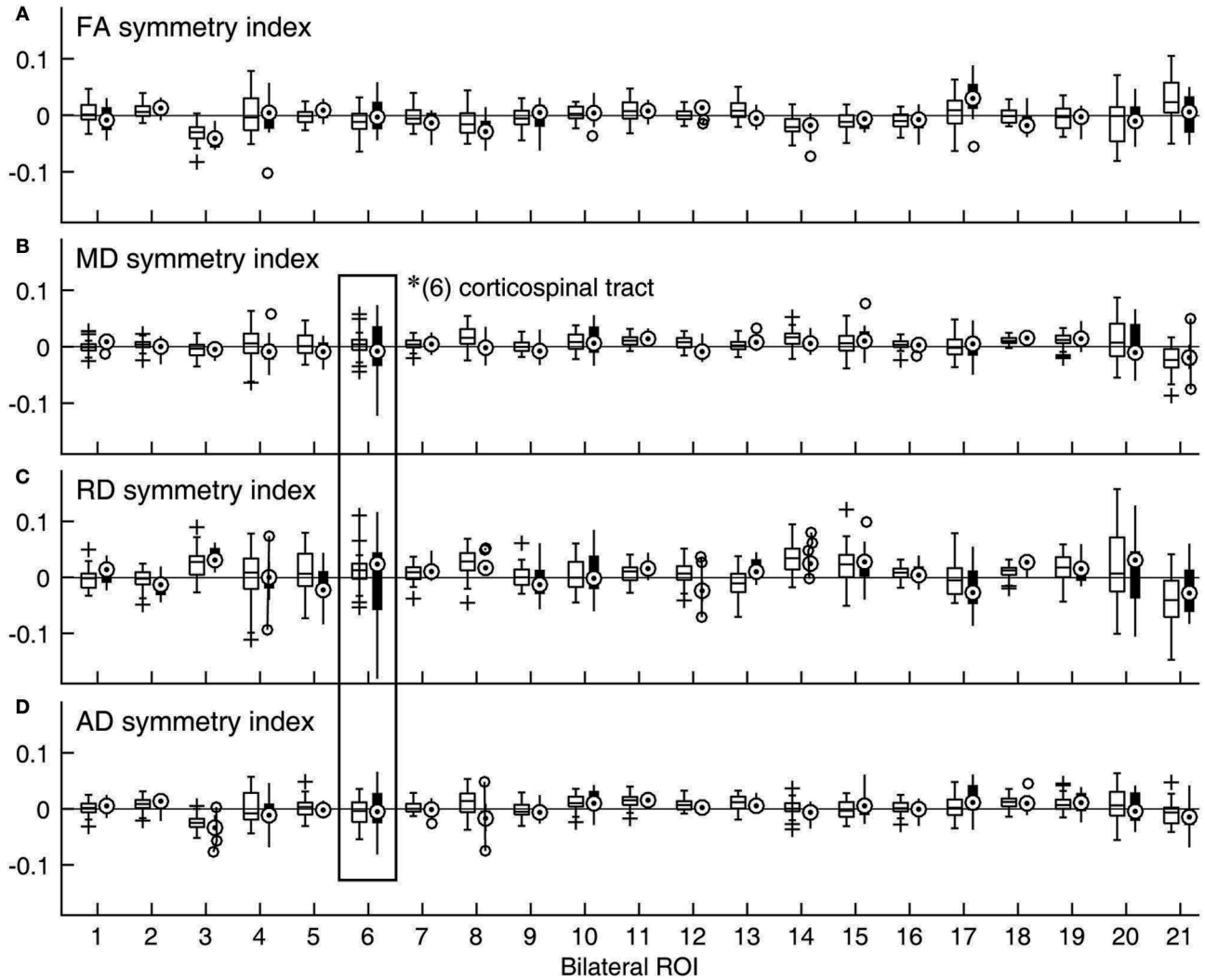

FIGURE 2 | Distributions of symmetry indices across ROIs and DTI metrics expressed as boxplots. **(A)** FA, **(B)** MD, **(C)** RD, and **(D)** AD. A symmetry index value of zero indicates bilaterally equal DTI metric values (horizontal lines). For a given ROI, the data for the control group are shown with an open box on the right side, while those for the patient group are shown with a filled box on the left side. *Significant group difference (with correction for multiple comparison). (1) anterior corona radiata; (2) anterior limb of internal capsule; (3) cingulum-cingulate gyrus; (4) cingulum-hippocampus; (5) cerebral peduncle; (6) corticospinal tract; (7) external capsule; (8) fornix/stria terminalis; (9) inferior cerebellar peduncle; (10) medial lemniscus; (11) posterior corona radiata; (12) posterior limb of internal capsule; (13) posterior thalamic radiation; (14) retrolenticular part of internal capsule; (15) superior cerebellar peduncle; (16) superior corona radiata; (17) superior fronto-occipital fasciculus; (18) superior longitudinal fasciculus; (19) sagittal stratum; (20) tapetum; (21) uncinate fasciculus.

Overall, values of FA tended to be higher, and MD, RD, and AD lower in our patient sample compared to controls, with a notable inconsistency in the middle cerebellar peduncle. FA values also tended to cluster more tightly in patients than controls, possibly indicating a ceiling effect. While the trend in FA may indicate an effect consistent with that reported for late acute injury (5–7 days post-concussion) (18), on account of our 2-week post-concussion recruitment window, our patient subjects likely represented a wide range of phases of response to the injury and changing diffusion characteristics (18, 19). Regardless of the MR scan timing, however, abnormality could be signaled as a deviation from normal benchmarks because the symmetry approach is based on intra-individual comparisons (5). Despite this benefit, the timing issue is still a weakness of this study. That is, although it is unlikely that the brain is affected evenly by the initial physical impact to the head, secondary effects involving commissural or bilateral changes (20) may alter symmetry-asymmetry characteristics over time. This possibility should be explored in a longitudinal study.

In this study, the corticospinal tract, identified at the medulla and the pons level in the atlas we used (15), stood out as a locus of asymmetry. Previously, we addressed whether anatomical findings after a concussive event could be biased in particular populations by examining patients with concussion

TABLE 2 | White matter tracts with statistically significant asymmetry among normal individuals.

	Mean	SD	$\|t(34)\|$	p
FA				
Cingulum-cingulate gyrus	−0.031	0.018	10.20	<0.0001
Retrolenticular part of internal capsule	−0.019	0.017	6.54	<0.0001
Superior cerebellar peduncle	−0.012	0.016	4.38	0.0001
Uncinate fasciculus	0.029	0.039	4.34	0.0001
Superior corona radiata	−0.010	0.013	4.21	0.0002
Anterior limb of internal capsule	0.008	0.013	3.56	0.0011
Posterior thalamic radiation	0.009	0.017	3.29	0.0023
MD				
Superior longitudinal fasciculus	0.011	0.007	9.05	<0.0001
Posterior corona radiata	0.012	0.010	6.87	<0.0001
Retrolenticular part of internal capsule	0.015	0.015	5.69	<0.0001
Fornix/stria terminalis	0.017	0.018	5.64	<0.0001
Uncinate fasciculus	−0.023	0.025	5.33	<0.0001
Sagittal stratum	0.011	0.013	5.01	<0.0001
Posterior limb of internal capsule	0.007	0.011	4.12	0.0002
Superior corona radiata	0.005	0.008	3.24	0.0026
Medial lemniscus	0.009	0.017	3.18	0.0032
RD				
Retrolenticular part of internal capsule	0.034	0.026	7.61	<0.0001
Fornix/stria terminalis	0.027	0.026	6.12	<0.0001
Cingulum-cingulate gyrus	0.025	0.026	5.76	<0.0001
Uncinate fasciculus	−0.040	0.044	5.34	<0.0001
Superior longitudinal fasciculus	0.009	0.012	4.78	<0.0001
Superior cerebellar peduncle	0.021	0.033	3.75	0.0007
Superior corona radiata	0.007	0.012	3.72	0.0007
Sagittal stratum	0.015	0.025	3.41	0.0017
AD				
Cingulum-cingulate gyrus	−0.025	0.012	12.11	<0.0001
Posterior corona radiata	0.015	0.013	6.49	<0.0001
Superior longitudinal fasciculus	0.012	0.011	6.29	<0.0001
Medial lemniscus	0.012	0.013	5.03	<0.0001
Anterior limb of internal capsule	0.008	0.010	4.45	0.0001
Posterior limb of internal capsule	0.007	0.010	4.16	0.0002
Posterior thalamic radiation	0.010	0.014	4.00	0.0003
Sagittal stratum	0.009	0.014	3.67	0.0008

Group means and SDs of the symmetry index and the results of one-sample t-tests are shown. Statistical significance was determined by correction of α-levels for multiple comparison.

from ED and athlete cohorts separately (8). The particularity of the corticospinal tract could be due to its vulnerability as well as variable sensitivity to concussion in general, but the mechanisms of injuries elucidating this particularity could not be identified due to the study design. Alternatively to the mechanistic explanation, it is possible that injury to the corticospinal tract leads to signs such as balance or coordination problems, for which a concussed patient may be directed to an ED. Such a cohort may have been well-represented in our ED patient sample. If so, other patient cohorts, such as athletes and soldiers, may be found with different or additional patterns of asymmetry from those reported here. Of note is a recent report on soldiers with a past concussion (5), which also identified the corticospinal tract as well as inferior longitudinal fasciculus (a tract not studied presently) as loci of increased asymmetry compared to military controls without a history of concussion. It is further conceivable that an individual in a typical head impact situation, unaccompanied by a person trained to recognize signs and symptoms of concussion, may not be seen by a medical professional immediately, until other symptoms or signs become a nuisance. Such type of patients may present still different patterns of brain asymmetry.

We defined the ROIs according to a standard white-matter atlas that is readily available (15). The use of this atlas allowed for objective quantification of white matter characteristics that

could be compared across subjects, and the approach can be easily replicated in other laboratories. However, anatomical details that can be provided by the atlas are limited in terms of the number of the specified white matter structures and sensitivity to small, localized changes; therefore, asymmetry identified with the atlas may not be directly relevant to correlates of possible functional impacts of a concussion.

Lastly, the present findings cannot be generalized across ages because we selected adult subjects to control for potential developmental variables. Aging after maturity may also interact with the state of white matter symmetry following a concussion (5). Characterization of brain symmetry in normal development and aging would be an important avenue of research by itself as well as in defining abnormality.

To conclude, interpretation of brain asymmetry requires prudence. We showed that bilateral asymmetry reflecting disruption of a normal state can be found in patients after a concussion, but the study bore a number of further questions. The clinical utility of characterizing post-concussion pathology as abnormal brain asymmetry merits further exploration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Weill Cornell Medical College. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JM, JG, and PM designed experiments and oversaw data collection. JMM, GS, and EP processed the imaging data. JM conducted the statistical analyses and drafted the manuscript. All authors contributed to the interpretation of data and to revising the work. All authors contributed to the article and approved the submitted version.

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