CONTENTS

COMMENTARY: Lost in translation: understanding the failure of the progesterone/traumatic brain injury Phase III trials
*Future Neurology* Vol. 11 Issue 1

REVIEW: Update on fluid biomarkers for concussion
*Concussion*

EDITORIAL: Stem cell therapies for traumatic brain injury
*Regenerative Medicine* Vol. 10 Issue 8

www.Neurology-Central.com
Lost in translation: understanding the failure of the progesterone-traumatic brain injury Phase III trials

Traumatic brain injury (TBI) remains a major public health problem. The Centers for Disease Control (CDC) reports that there are over 1.7 million cases of TBI annually in USA, accounting for 30% of injury-related deaths. The dozens of Phase III clinical trials evaluating neuroprotective therapeutics have all failed to provide evidence of benefit. This 100% failure rate represents a huge human and economic cost for society and for patients, for whom no effective neuroprotection is available [1]. One of the most promising agents, the neurosteroid progesterone, was recently tested in two independent, Phase III clinical trials. Progesterone seemed to have all the right things going for it in preclinical studies. Hundreds of animal studies reported that the natural form of the hormone could reduce the metabolic cascade of injury that accompanies TBI, ischemic injury, nerve crush and neurodegenerative disorders. Yet despite a promising start in several small Phase II trials, in Phase III testing in almost 2000 patients with moderate to severe TBI, SyNAPSe and ProTECT III, progesterone showed no benefit.

The results of the two trials were published together in the New England Journal of Medicine [2,3] and accompanied by an editorial [4] which argued that the trials “had much in common” with all the other failed trials. Schwamm’s editorial argued that much of the clinical science behind the trials was flawed and biased. However, the trial papers also cited a number of potential confounds within the trials: for ProTECT III, heterogeneity of the injury, pre-existing conditions, resilience of the patients [2]; for SyNAPSe, complexity and variability of the injury, multiple mechanisms of injury varying across patients, insensitivity of outcome measures, absence of reliable biomarkers, the lack of early mechanistic end points [3].

A similar argument has been made by two well-regarded neurologists in discussing the dismal outcomes of trials seeking a safe and effective treatment for ischemic TBI: “One cannot assume that just because clinical trials are expensive and time consuming they are also without serious or even fatal flaws.”

Donald G Stein*
First draft submitted: 10 December 2015; Accepted for publication: 18 December 2015; Published online: 11 January 2016

Keywords
- clinical trial design
- progesterone
- traumatic brain injury
There are multiple reasons to think that the trial results may have been compromised and that both the scientific and clinical communities have to do better if we are ever to succeed in moving forward.”

stroke [5]. These researchers, too, proposed that, as currently performed, animal studies do not effectively model what takes place in the clinical setting, and that the failures to replicate across animal studies lead to the inevitable conclusion that most, if not all, such studies are useless. Some in the scientific community agree that most animal studies do not replicate, the samples are too small and the neural injuries inflicted in the laboratory are too consistent and uniform and not representative of the heterogeneity of the injury and the variance in patients likely to be enrolled in a clinical trial [6,7]. This author thinks the conclusion – that these problems mean that preclinical research is untranslatable – is oversimplified [8], but undeniably, the problems exist and need to be addressed. However, in the case of the progesterone trials the author would argue that the clinical issues are more complex, repeating the same mistakes in trial design that can doom a trial to failure before it starts. It is also a risk to put an exclusive focus on the limitations of the preclinical studies to the exclusion of a close scrutiny of possible problems in the trials themselves. In the face of 100% failure rates, a new approach may be the best strategy in going forward.

In the case of progesterone, it has a very strong biological signal with respect to neuroprotection. A quick search of PubMed using the keywords ‘progesterone, brain injury’ brings up 213 preclinical studies in 23 models of injury, so even if a percentage of the studies were flawed, there is still substantial evidence of neuroprotective benefit following treatment with progesterone [9]. What can be learned from the trials and what can be done better in the future? One cannot assume that just because clinical trials are expensive and time consuming they are also without serious or even fatal flaws. If high cost and complex logistics were an index to success, why would there be such an inordinately high failure rate?

First, in hindsight, analysis of the treatment protocols in both trials, which were almost identical, revealed that no dose-optimization studies were conducted prior to either the Phase I–II or the Phase III trials. The metabolism and pharmacokinetics of drugs in laboratory rats and mice are very different from those in humans. The metabolism of the rodents is four to sixfold higher than humans’. Our recent post hoc analysis [10] found that allometric scaling procedures according to US FDA recommendations were not done – so, in effect, the patients in the progesterone groups received doses approximately sixfold higher than should have been used. Progesterone doses significantly higher than those found to be most effective in rodent studies can lead to receptor saturation and a loss of beneficial effects. In fact, neither very low nor very high doses work – an effect that has also been reported in other drug studies [11,12]. This bell-shaped dose-response curve is well known in pharmacokinetic studies [13–15]. Duration of treatment should also have been optimized via preliminary allometric studies in these trials. It is very likely that the patients should have received the lower dose over a longer period of time to account for the relative metabolism and life-span characteristics of rodents and people [10,16].

A second problematic factor is that the lipid vehicle carriers used in both Phase III trials may not have been neutral in their effects and could have confounded the interpretation of the data. Intralipid, the carrier used in ProTECT, has been used as a detoxification agent in chemical poisoning studies [17] and can increase blood clot formation under certain conditions [18], posing an additional risk for patients with serious brain injury. Intralipid administration has also been shown to influence scores on the Glasgow Coma Scale, which the progesterone trials used to select patients and assign them to their initial categories [19]. Although the literature on this subject is controversial, there is some modest clinical observation that lipid emulsions may reduce mortality in critically ill patients [20]. Overall, the literature indicates that lipid formulations, and more specifically Intralipid, can have both positive and negative effects in patients. Without serum biomarkers to compare the effectiveness of the progesterone to a ‘neutral’ vehicle carrier/solution prior to Phase III testing, the current results are difficult to interpret.

Other issues may also have contributed to the negative outcomes. Patient stratification procedures may have been too broad and encompassing to reveal a 10% difference between the treatment and control groups. In the ProTECT study, patients between 18 and 95 years old were enrolled based primarily on Glasgow Coma Scale score, computed tomography and injury severity regardless of locus of injury. This very wide range of age at time of injury could have led to too much variability to find a difference between the groups.
Both ProTECT and SyNAPSe used the Glasgow Outcome Scale Extended (GOS-E) as their primary measure of treatment efficacy. The GOS-E is a very short, yes/no, quality of life questionnaire with two categories of recovery: good or bad. This is a very blunt instrument for measuring the complexity of brain injury and recovery over time – especially when the testing and follow-ups are done by telephone, as they sometimes are. This issue is controversial and some clinicians have argued that the test cannot adequately reflect the quantitative extent of a patient’s ‘recovery’, especially in individuals with frontal brain injuries that often disrupt executive and emotional functionality [21,22].

The ProTECT trial was conducted in over 50 centers, SyNAPSe in well over 100 hospitals in 21 countries. There can be very large between center differences in outcomes after severe TBI: one report found a threefold increase in outcome variability [25]. Even with the best intentions, discipline and written protocols, it is certainly possible that with such a wide range of hospitals and levels of experience in dealing with TBI, and with different standards of care for TBI, there were protocol exceptions and data entry errors.

It also seems likely that differences in standard of care for moderate-to-severe head injuries would have been a variable, especially in an international trial, but even in the national ProTECT trial it was reported that, compared with national standards, severe TBI patients in the control group had 20% less mortality than expected. This finding could have been due to the well-known tendency for patients enrolled in any clinical trial to get better care, or to the protective effects of the lipid therapy noted above. In any case the 20% differential from the norm could attenuate any differences between the treatment and placebo group.

We have to face the real possibility that, despite all the positive preclinical evidence, and the fact that it is a naturally occurring hormone in human males and females, progesterone may simply not be neuroprotective in humans. However, before we can draw this conclusion, the pharmacologic confounds alone may make it very difficult to interpret the outcomes of the progesterone trials. There are multiple reasons to think that the trial results may have been compromised and that both the scientific and clinical communities have to do better if we are ever to succeed in moving forward. Toward this end the author proposes some changes to the design and conduct of clinical trials:

- Large, double-blind, randomized control trials should not be the gold standard for clinical trial testing. This ‘standard’ should be replaced by more precise adaptive trial design based on computer modeling and Bayesian statistics that support more pragmatic approaches to patient selection and stratification [26]. Adaptive design allows for changes in group assignments based on models and on analysis of data as the trial progresses and meets given criteria, rather than keeping on with ‘standard’ group assignments – a frozen protocol – even when there is strong evidence that the drug is working (or not) [27];

- We now have the technologies to support more selective enrollment of patients based on genomics and imaging to determine which would most benefit from a given treatment. This is a form of ‘precision medicine’ currently being developed and deployed [28]. Smaller but better-designed ‘precision’ trials will be more expensive, but they will have a better chance of yielding meaningful results;

- All clinical studies based on preclinical drug evaluation should be required to perform preliminary optimization studies of dose and duration of treatment in Phase II testing. The optimization should be based on allometric scaling techniques that are now available to clinicians and researchers [10,16,29];

- Combination therapies with drugs that can have synergistic actions when administered together may be more effective than the monotherapies typical of current drug development approaches. Co-morbidities than can affect TBI outcomes need to evaluated and
treated [9,30]. The NIH has called for more attention to combination therapies, but such studies have not yet been much seen in clinical trial designs [31];

- Even though it may take longer and be more costly, oversimplified, nonquantitative outcome measures should be dropped in favor of systematic neuropsychological testing at least as fine grained as the outcome measures used in most animal studies of brain injury/stroke [11,12]. As noted, for humans, such tests are easily available on the NIH website and standardized for just this kind of use. Long-term evaluations of patient recovery need to be incorporated into trial designs. There is no law of neurology stating that if a patient does not show recovery in the first few weeks following treatment, they will never show recovery [32]. Short-term outcomes may not be sufficient to identify long-term benefits of a test drug.

These are just some of the steps that would enable us to design better clinical trials. These designs, along with additional measures, can be adapted to preclinical research methods. The preclinical and clinical studies will require more time, patience (perhaps more patients) and up-front costs, but in the long run may be the best way to finally move forward and ultimately help patients to survive and have a better quality of life than present paradigms can offer. We will not know unless we try.

Disclaimer
The opinions expressed in this editorial are strictly those of the author.

Financial & competing interests disclosure
DG Stein has been conducting preclinical research on progesterone as a treatment for brain injury for more than 25 years. DG Stein holds patents on the use of progesterone for traumatic brain injury and other forms of brain injury, although none of this technology is currently licensed. No fees or royalties are currently obtained from the research although the author does receive occasional honoraria for colloquia and scientific workshops presented on this topic. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References


Severe traumatic brain injury (sTBI) is easily diagnosed by clinical examination and standard neuroimaging techniques. However, mild TBI (mTBI) or concussion (the two terms are used interchangeably in the literature), defined as a head trauma resulting in brief loss of consciousness and/or alteration of mental state [1], is much harder to objectively detect. Concussion causes no gross pathology, such as hemorrhage, and no abnormalities on a conventional computed tomography scan of the brain [1], but rather rapid-onset neuronal dysfunction that usually resolves over a few days to weeks. Presumably, exposure to head injury has been a feature of human existence for millennia, and the human brain has evolved normal adaptive and recovery processes.

A chronic syndrome, likely related to repetitive concussions, has not received much attention until recently. However, in 1928, Martland published a case series describing symptoms such as slowed movement, tremor, confusion and speech problems in a group of former boxers, which he called ‘the punch drunk syndrome’ [2]. A few years later, Millspaugh called this syndrome dementia pugilistica [3]. In 1973, Corsellis and colleagues published a case series in which the brains of 15 retired boxers were studied and the neuropathology of what we now know as chronic traumatic encephalopathy (CTE) was first described [4]. The molecular pathology of this condition resembles aspects of Alzheimer’s disease, but with some distinctions discussed below. Boxing was and still is the particular sport in which athletes voluntarily expose themselves to repetitive concussion. However, a number of other sports, for example, American football, ice hockey, rugby and martial arts other than boxing, have now developed in such a way that professional participation involves a considerably increased risk of repetitive concussions. More recently, case reports have shown pathologically confirmed CTE in contact sports athletes other than boxers and in former combat military personnel [5–7]. This has resulted in renewed interest in the potential for long-term neurodegenerative changes to...
occur after concussive and subconcussive repetitive head trauma [8]. In addition to case reports showing pathology in ex-sportsmen, there is epidemiologic evidence suggesting increased dementia risk after documented head injury [9], but apart from these types of descriptive studies there is a lack of systematic research on the problem.

Neuronal dysfunction in concussion is at least partly caused by direct damage to axons and other structures in the CNS. Approximately 15% of concussion patients suffer persisting cognitive dysfunction [10,11] and diffuse axonal injury (DAI) appears to be the most important underlying pathology in such cases [12]. The dysfunction may also relate to tau pathology that spreads around the brain in CTE [13,14]. However, what initiates this destructive and neurotoxic spreading cascade only in some individuals and not others is unknown.

It is important to remember that head injury and concussion are not synonyms (one head injury may not impact the brain at all, whereas another, depending on how the mechanical forces of the head blow are transferred to the brain, may impact the brain profoundly), which may be a problem in epidemiological studies. It is also clear that different sports and other risk activities have different types of contact and, therefore, probably different types of lesion. What are the damaging types of lesion and what types of damage are reversible and what are not reversible? How long does it take to recover from any particular type of damage? What are the outcomes of playing long-term professional sports today, when everything is faster, the players are heavier and the protective gear for the head appears less effective than protective gear for other parts of the body?

We now need systematic research on injury mechanisms in concussion and how they relate to CTE. Fluid biomarkers in TBI may relate directly to brain injury, released principally from neurons, glia or blood vessels or to response mechanisms such as microglial and astrocytic migration and activation, which may be protective or deleterious. Most individuals with concussion do not develop CTE. However, in some individuals there may be a transition from an injury/recovery pattern to a persistent, progressive process representing the initiation of a neurodegenerative process and a transition to the earliest phases of CTE. We hypothesize that this may relate to the extent, frequency and cumulative effect of brain injury together with variation in individual vulnerability to injury. It is possible that intrinsic (genetically determined) risk factors for Alzheimer’s, Parkinson’s or tauopathies such as progressive supranuclear palsy will also be risk factors for the development of CTE. Identification of individuals in the earliest phases of CTE is of crucial importance in terms of instituting appropriate advice on subsequent exposure, and in selecting individuals for future trials of disease-modifying therapies. Fluid biomarkers have the potential therefore to help to define: the severity of TBI; adaptive and recovery processes following TBI; and the transition between a normal injury/recovery pattern and a currently irreversible and inexorable CTE process. In this review, we start by discussing the different fluids that may be used as samples in which concussion biomarkers may be measured. We thereafter discuss technical aspects of the biomarker analysis. We then review the literature published so far on candidate biomarkers for concussion. Finally, we discuss the special case of CTE and emphasize the need for biomarker development for this disease entity.

**Marker matrices**

**Cerebrospinal fluid**

Cerebrospinal fluid (CSF) is a clear fluid that surrounds the brain, provides mechanical support and helps clear metabolites from the brain parenchyma together with direct transport across the blood–brain barrier, clearance via the glymphatic system and clearance via the recently discovered meningeal lymphatic vessels [15,16]. The total CSF volume is approximately 150 ml and the production and clearance rates are approximately 20 ml/h. Twenty to 30% of the CSF volume is derived directly from the brain; 70–80% is a choroid plexus-derived filtrate of plasma. CSF is sampled through a lumbar puncture, which is a harmless procedure with postlumbar puncture headache as the sole potential complication [17]. Standard operating procedures for CSF sampling and handling have been established and the procedure can be done in outpatients [18]. The main advantage of CSF as a matrix in which to measure markers of CNS injury is that it communicates freely with the brain interstitial fluid that bathes the neurons. Biochemical changes in the brain are thus reflected in the CSF, which may be regarded as an accessible, although by no means perfect, sample of the brain interstitial fluid. Further, CSF has low protease activity and most molecules do not change upon sampling provided the sample is not contaminated by blood. The main disadvantage is that lumbar puncture may be regarded as impractical to perform in emergency settings and in clinical studies. Another limitation is that it is presently unclear how clearance of brain metabolites into the CSF relates to clearance via the glymphatic and/or lymphatic system of the brain; potentially, biomarkers we thought would be well reflected in the CSF may escape detection if their main clearance pathway is through these newly described systems directly into the blood.
Blood

The other major biofluid for the measurement of concussion markers is blood (serum or plasma). Blood is more accessible than CSF but most CNS-enriched markers are present in blood at very low concentrations that necessitate the employment of ultrasensitive techniques that can measure in the femtomolar range (most standard immunochemical techniques cannot reach this analytical sensitivity). The blood–brain barrier also poses a challenge in the analysis of CNS injury markers in blood. Normally, the blood–brain barrier restricts the release of CNS-enriched proteins and peptides into the bloodstream and, for some molecules, specific transport mechanisms exist. In concussion, there may be a transient opening of the blood–brain barrier, which could increase the blood concentrations of CNS-enriched molecules, also in the absence of direct injury to the structures they are thought to represent. It may thus be difficult to tell to what extent a peak in the blood concentration of such a biomarker reflects CNS injury or blood–brain barrier damage/dysfunction. Reliable blood biomarkers for blood–brain biomarker integrity relevant to CNS injury markers would be a major contribution to the field (the best established biomarker so far for blood–brain barrier function is the CSF/serum albumin ratio, which obviously necessitates access to both body fluids [19]). Further, most intracellular proteins released into the bloodstream undergo degradation and/or modification by proteases and other enzymes and for most of the biomarker candidates discussed below, the normal half-life is unknown. The dilution of CNS proteins into 4 l blood instead of 150 ml CSF may also contribute to the low concentrations of CNS-derived molecules in the blood.

Saliva, urine & tears

It is possible that some CNS-derived proteins are eventually excreted into body fluids other than CSF and blood. The presence of the axonal protein tau in saliva has been demonstrated using mass spectrometry [20]. The same research group has also detected Parkinson-related α-synuclein and DJ-1 in this body fluid [21]. However, the relationship between salivary concentrations of these proteins and processes within the CNS is far from clear and no conclusive data on disease association have been reported so far. Similar lines of reasoning are relevant to tears and urine, although renal clearance of potential concussion biomarkers may be a more physiological pathway than salivary or lacrimal clearance.

Measurement techniques

Most fluid markers of CNS injury are proteins or protein fragments that can be measured using immunochemical or MS-based techniques (or combinations thereof).

ELISA has become established as a standard method for the measurement of proteins in biofluids. The general principle is that a capture antibody directed against one epitope on the target analyte is immobilized on a surface, whereafter sample and labeled detector antibody (directed against another epitope on the same analyte) are added sequentially between blocking and washing steps to remove unspecific signal. Capture and detector antibodies are in molar excess so that most of the target analyte is captured in a sandwich between the antibody pair. Many ELISAs and ELISA-like techniques can reach lower limits of quantification of 10–100 pg/ml, but measuring even lower concentrations, as is needed for most brain-specific proteins in the blood, is a challenge. Auto-antibodies against the target analyte or heterophilic antibodies (e.g., endogenous anti-mouse IgG antibodies) that may react with the antibodies in the assay may block epitopes or bridge the capture and detector antibodies (replacing the analyte), giving falsely low or high signals. Interference from heterophilic antibodies may be blocked using polyclonal mouse IgG or commercially available blockers.

To allow for ultrasensitive measurement, two new techniques have entered the market: Erenna and Simoa [22]. The magnetic bead-based Erenna system can detect molecules at femtogram/ml concentrations using Single Molecule Counting (SMC) technology in which labeled detector antibodies are released from the captured immunocomplexes and counted one by one. Simoa is based on the isolation of individual immunocomplexes on magnetic beads using standard ELISA reagents. The main difference between Simoa and conventional immunoassays lies in the ability to trap single beads in femtoliter volume wells. This compartmentalization of the detection reaction allows for a digital readout of each individual bead to determine if it has bound the target analyte or not. In theory, this type of assay thus measures at the single molecule level, which may also be true when using the SMC approach of Erenna. Another upcoming technique for ultrasensitive biomarker quantification is proximity ligation assay, which builds on the principle that recognition of target proteins by two, three or more antibodies can bring in proximity DNA strands conjugated to the antibodies. The DNA strands can then participate in ligation reactions, giving rise to molecules that can undergo rolling circle amplification for highly sensitive detection [23]. The same potential interferences as for ELISA, applies to these types of measurement techniques as well, as they are all antibody based.

MS-based explorative proteomics has been applied to discover novel biomarkers in complex samples such as CSF and plasma for many years. More recently, how-
ever, antibody-independent selected or parallel reaction monitoring (SRM or PRM)-based MS techniques have been developed for the quantitative measurement of proteins and protein fragments in a manner that is stable enough to allow for use on large sample series and in clinical laboratory practice [24]. SRM-based MS is a method that can be expected to grow into a complementary or alternative technique to immunochromatographic assays in the analysis of protein markers in the near future [25].

**Fluid markers of acute mild traumatic brain injury**

Candidate fluid markers of acute mild TBI are summarized in Figure 1.

**CSF markers**

Axonal injury in concussion can be identified and monitored using CSF levels of the intra-axonal proteins neurofilament light (NF-L) and tau [26,27], measured by ELISA. NF-L is a structural protein that is highly expressed in large-caliber myelinated axons that extend subcortically into deeper brain layers. These are the primary targets when a blow to the head applies rotational forces to the brain inducing DAI and NF-L leakage from injured axons into the CSF. After a boxing bout, NF-L concentrations in CSF correlate with the number of received head blows [26,27]. Similar results have been obtained using an assay for phosphorylated neurofilament heavy protein [28]. A recent case report on a knocked-out amateur boxer showed that it took 8 months before his CSF NF-L concentration normalized [29]. This result resonates with neuropathological analyses showing that axonopathy can continue for years after TBI [30].

Tau is primarily expressed in thin unmyelinated axons and may respond more to cortical contusions than rotational brain injuries. However, CSF concentrations of tau change after both concussive and subconcussive head blows in a manner similar to NF-L but with a lower amplitude in the changes [27].

---

**Figure 1. A neuron, an astroglial cell, a blood vessel and diffuse Aβ deposits close to a synapse.** Candidate fluid biomarkers for concussion are indicated. NSE is a protein highly expressed in the neuronal soma, but also in red blood cells. UCHL1 is a de-ubiquitinating enzyme highly expressed in neurons, but also in gonads and lung tissue. α-SNTF is an axonal injury marker generated by the calpain family of calcium-activated proteases. SNTFs accumulate in the neuronal somatodendritic part of injured axons following traumatic brain injury. NF-L and NF-H are intra-axonal structural proteins highly expressed in large-caliber axons. Tau is an intra-axonal structural protein highly expressed in thin, unmyelinated axons. S100B and GFAP are astroglial proteins. S100B is CNS-enriched but not specific, while GFAP appears to be highly CNS-specific. Aβ and other APP fragments may represent increased amyloidogenic APP-processing and diffuse plaque formation in response to axonal injury in traumatic brain injury.

Studies in TBI models and on human brain tissue samples, as well as in brain interstitial fluid have demonstrated that APP accumulates in neurons and axons after brain trauma with axonal damage and that there is release of amyloid β (especially aggregation-prone Aβ42) into brain interstitial fluid with plaque formation around damaged axons [31]. In spite of this, there are no clear changes in CSF levels of secreted APP or Aβ fragments in concussion [26,27].

**Blood markers**

In regard to blood biomarkers for concussion, recent data show promising results for tau, when measured on the ultrasensitive Simoa platform discussed above. A tau assay has been established on this platform and the first pilot studies have shown: a strong correlation between serum tau concentrations and neurological outcome in resuscitated cardiac arrest patients [32], increased tau concentrations in plasma from Olympic boxers [33], increased tau concentrations in plasma from patients with Alzheimer’s disease [34], increased plasma tau concentrations in concussed ice hockey players 1 h after the injury; the degree of tau elevation correlated with the number of days it took until the players were free from symptoms [35]; and increased tau concentrations in plasma from military personnel who had been deployed within the previous 18 months and reported or had medical records on having had a TBI [36]. The dynamics of tau changes in blood and CSF following acute brain injury appears to be distinct; tau levels in CSF and plasma do not correlate [34] and tau elevations following acute brain injury stay much longer in CSF (weeks) than in blood (days) [32,37]. Possibly, tau is degraded when entering the bloodstream. A recent study by Al Nimer et al. used conventional ELISA for detection of NF-L in serum of TBI patients, showing increased serum concentrations in patients with the most severe neurotrauma [38]. The analytical sensitivity of the assay would, however, not allow for measuring serum NF-L in patients with mTBI or concussion and the correlation with CSF NF-L concentrations was very low, in contrast to a recently published ultrasensitive assay based on the Simoa platform [39], which is now being examined in relation to TBI.

Another promising blood marker of concussion is the 1176 residue N-terminal fragment of α-spectrin, termed SNTF, which is a protein that accumulates preferentially in damaged axons [40]. SNTF is normally undetectable in axons, but is generated following stretch injury by intra-axonal calcium overload and spectrin proteolysis mediated by the calpain family of calcium-activated proteases [41,42]. SNTF increases measurably in the blood after TBI, including CT-negative mTBI [43,44], and was recently found to be a predictive marker in sports-related concussion [45].

Other candidate blood biomarkers for brain injury in concussion include NSE, UCHL1, as well as the astroglia-enriched S100B and GFAP [46]. Clinically relevant changes in serum concentrations for S100B [47] and GFAP [48] have been reported to detect radiographically apparent intracranial injury and the former protein has been included as a biomarker that could reduce the number of unnecessary CT scans of the brain in new clinical guidelines for the management of head injury [47]. In contrast to tau and SNTF, none of these markers has a prognostic relationship with patient outcomes in concussion with negative brain computed tomography scan findings [46]. NSE, UCHL1 and S100B are also expressed in extracerebral tissues, which restricts their interpretability in multitrauma [46].

**CTE**

CTE is a neurodegenerative disease associated with repetitive head trauma [49]. Although initially believed to affect only boxers, the at-risk population has expanded to encompass a much wider demographic, including American football players, ice hockey players, wrestlers and military veterans. This expansion has garnered considerable media attention and public concern for the potential neurodegenerative effects of head trauma. There are no established autopsy-verified clinical criteria for CTE and no neuroimaging or fluid markers of the disorder, although the gross morphological changes seen in advanced CTE may be visualized using standard neuroimaging techniques [50]. The molecular pathology of CTE is characterized by tau-positive neurofibrillary tangles and neuropil threads that may be present in all regions of the brain with or without Aβ pathology [49]. Neurofibrillary tangles in CTE form preferentially in the superficial cortical layers, rather than in deeper layers as is more common in AD, with focal accumulations at the depths of the sulci [51]. Another distinctive feature is perivascular tau deposition [51].

In regards to in vivo markers of the molecular pathology in CTE, preliminary findings from positron emission tomography (PET) scanning using PET ligands for brain tau in retired national football league players have recently been reported; five retired players were compared with five matched controls without a concussion history and displayed higher overall signals of tau deposition in their brains [47]. Amyloid PET imaging in traumatic brain injury has yielded ambiguous data, possibly due to the diffuse nature of trauma-related Aβ deposits that are less prone to bind amyloid ligands than neuritic plaques [53].
No data on fluid markers of tau and Aβ pathology have been presented so far but clinical biomarker studies with longitudinal follow-up of patients with chronic or progressive symptoms after TBI, for example, the Diagnosing and Evaluating Traumatic Encephalopathy using Clinical Tests (DETECT) study [54], are in progress and will tell us more about the pathogenesis, risk factors and clinical course of CTE, and how CTE can be diagnosed and monitored with the help of biomarkers.

Could markers of mild TBI/concussion help prevent CTE?
The primary goal of biomarker research in TBI is not to develop techniques to identify moderate to severe brain injury, as such already exist, but rather to identify molecular changes in mTBI/concussion to indicate if the brain was injured by a head injury and to monitor the recovery process. Most current research suggests that the risk of long-term symptoms following concussion is highest in individuals who have received repetitive concussions before the brain has recovered properly [49]. Identifying incomplete recovery following concussion using an objective test and prolong the rehabilitation phase in such cases could potentially help to reduce the incidence of CTE.

Conclusion & future perspective
Several promising CSF and blood markers of concussion exist but the field is not yet mature enough to rank them according to diagnostic accuracy. Their potential predictive value in regard to time to complete recovery and risk of incomplete recovery/CTE needs to be established. Further, we need more knowledge on release and clearance mechanisms of the candidate biomarkers and how their blood concentrations relate to blood–brain barrier dysfunction. Another outstanding research question is if additional information could be gained by combining different markers with each other, for example, by quantifying the extent of both the axonal injury and the inflammatory response using different biomarker combinations. All this should be relevant not only for sports-related concussions but also for the many concussions that are not sports related. Information gained using biomarkers for TBI could potentially be used to identify at-risk cases most appropriate for enrollment in clinical research studies and therapeutic trials.

Financial & competing interests disclosure
Work in the authors’ laboratories is supported by the Swedish Research Council (H Zetterberg, K Blennow), Swedish State Support for Clinical Research (H Zetterberg, K Blennow), the Torsten Söderberg Foundation (K Blennow), the Knut and Alice Wallenberg Foundation (H Zetterberg), the Wolfson Foundation (H Zetterberg, J Hardy), Fimuresstiftelsen (H Zetterberg), Parkinson’s UK (HR Morris), the Medical Research Council UK (HR Morris), the Welsh Assembly Government (HR Morris), Teva (HR Morris), the Ipsen Fund (HR Morris), MND Association (HR Morris), the PSP Association (HR Morris), CBD Solutions (HR Morris) and the Drake Foundation (H Zetterberg, HR Morris, J Hardy). H Zetterberg and K Blennow are listed as co-inventors on a US patent application for plasma tau as a brain injury marker. K Blennow has served on advisory boards for Eli Lilly, Kyowa Kirin Pharma, Pfizer and Roche. H Zetterberg and K Blennow are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. HR Morris reports personal fees from Teva, Abbvie, UCB, Boehringer-Ingelheim and GSK. HR Morris is a co-applicant on a patent application related to C9ORF72 – method for diagnosing a neurodegenerative disease (PCT/GB2012/052140) pending. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Open access
This work is licensed under the Creative Commons Attribution 4.0 License. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

Executive summary

• Concussion is difficult to diagnose clinically.
• Objective biomarker tests for brain injury in suspected concussion would be an important diagnostic tool.
• Such biomarker tests could also help to tell when the injurious process has resolved.
• Several cerebrospinal fluid tests for brain injury in concussion are at hand.
• Ultrasensitive assays have made it possible to measure CNS-specific proteins in the blood.

References


2 Maarland H. Punch drunk. JAMA 91 1103–1107 (1928).


Update on fluid biomarkers for concussion  

Review


The clinical problem

Traumatic brain injury (TBI) is a common problem with unmet therapeutic needs. Although most cases of TBI are concussions that usually resolve over a few weeks, a great number of patients will suffer chronic disability from TBI-associated encephalopathies. Such conditions include focal contusions related to low-impact falls and diffuse axonal injury (DAI) from ultrafast loading of axons due to rotational acceleration in the course of motor vehicle crashes and other scenarios. Focal contusions are impact injuries featured by intraparenchymal hemorrhage with edema and ischemia in the inferior frontal and temporopolar regions leading to neuronal cell death and secondary axonal degeneration. DAI is an impulse injury associated with dynamic loading of axons and represents the commonest neuropathology across TBI causes and degrees of severity. Clinical problems caused by traumatic contusions and DAI have no satisfactory treatments besides symptomatic alleviation with physical/occupational/speech–language therapy and the empirical use of CNS-acting drugs. Clinical trials of small molecules have been unsuccessful [1]. Inspired by some earlier success in models of ischemic brain injury, stem cell transplantation has shown some preclinical efficacy, primarily in models of focal traumatic brain injury.

Stem cells from bench to bedside

Recent discoveries of the ability of exogenous neural stem cells to become successfully incorporated into the neural parenchyma have refuted earlier notions on the mature nervous system as an environment unfavorable to ongoing developmental events [2,3]. Such neural stem cells are derived from fetal neural tissue, embryonic stem cells or somatic cells induced to pluripotency with specific transcription factors. Scaling up production is challenging for fetal cells. Embryonic stem cells and induced pluripotent stem cell sources are theoretically inexhaustible and extremely pliable and have been induced to a large number of neuronal or glial fates including motor [4] and dopaminergic [5] neurons as well as oligodendrocytes [6]. Work with human neural stem cells in animal models was instrumental in the initiation of pioneering clinical trials in motor neuron disease (NCT01348451, NCT01730716) and spinal cord injury (NCT01772810). Although it is too early to know the outcomes, these were landmark developments in regenerative neuroscience and are already followed by early trials in other disorders. The therapeutic effects of neural stem cells in these conditions are presumably due to a combination of synaptic physiological actions and the synaptic release of neuroprotective molecules [7]. In an interesting turn of events, regenerative medicine based on stem cells may look more and more like regenerative medicine based on neurotrophins and trophic cytokines in the 1990s, perhaps with the added benefit that...
stem cells can produce these molecules indefinitely (Figure 1).

Preclinical modeling of TBI
The last 20+ years have seen considerable efforts in modeling TBI by cause or mechanism. These models are usually classified into focal and diffuse. Focal models include the weight drop, controlled cortical impact injury and mid-line fluid percussion injury. Diffuse models are produced via inertial or impact acceleration. Modifications of fluid percussion injury have both focal and diffuse elements. These models have not been adequately tested in nude rats or SCID mice, in other words, subjects appropriate for stem cell transplantation, but this need has begun to be more recently addressed. Controlled cortical impact and impact acceleration models offer complementary opportunities for regenerative medicine: the former is a primary contusional injury with secondary axonal degeneration, whereas the latter is a model of DAI with secondary effects on neurons. Based on paradigms worked out in spinal cord injury, the previous models can be used to optimize neuronal-versus oligodendrocyte-based cell therapies: neuronal precursor transplants can be best optimized in models like controlled cortical impact, whereas oligodendrocyte precursor transplants can be best worked out in models of DAI. This contention does not imply that contusions are best treated with neuronal and DAI with oligodendrocyte transplants. As in the case of spinal cord injury, both neurons and oligodendrocytes may need to be replaced and a mixed transplantation approach would probably work best in clinical TBI scenarios [8].

Stem cell transplantation as experimental therapy for TBI
Because of the complexity of TBI, we need to identify specific targets for repair guided by pathological mechanisms. Such tasks may include replacing dead neurons or protecting injured axons and cell bodies and promoting axonal repair and regeneration (Figure 2). Neuronal degeneration or death is encountered in both focal injury and DAI. Focal TBI (contusion) and DAI present different challenges in this regard: contusions may respond to targeted transplantation but cell death is acute; transplantation in DAI may need to be multifocal, but perikaryal degeneration is slow and may or may not lead to cell death. Although axonal repair/remyelination as a therapeutic target is best established in spinal cord injury, demyelination may also contribute to axonal degeneration and disconnection within brain circuits.

A growing number of studies with systemically administered stem cells may disclose novel mechanisms of neural injury and repair. Cells typically used in this approach are derived from bone marrow and include mesenchymal, multipotent adult and mononuclear stem cells. Bone marrow-derived stem cells are easy to access, require simple or no manipulation and have no attached immune rejection concerns if they are patient derived. Mononuclear cells have already entered several clinical trials: these cells are relatively small and thence not trapped in the lungs (first-pass effect) after intravenous administration, whereas less than 4% of intravenously injected mesenchymal cells reach arterial circulation [10]. Bone marrow-derived cells have shown efficacy in stroke models. Because of questionable penetration into brain, one of the proposed therapeutic mechanisms is the modulation of immune response [10]. Consistent with this view, there is growing evidence for the role of spleen as an organ that modulates neural injury [11]. The presumed trophic effects of systemically delivered bone marrow cells require further clarification. One study that has shown good blood–brain barrier penetration in controlled cortical impact injury also found an increase in levels of neurotrophins and VEGF in brain [12], whereas other studies have shown poor penetration [13]. This discrepancy may be due to first-pass effects or the particulars of experimental models. At any rate, it is unclear how trophic effects can be induced if these cells do not cross the blood–brain barrier [14].

Although parenchymal or intravenous administration of stem cells in models of TBI and stroke is common, intrathecal or intraventricular delivery is also being used.

Figure 1. The progression from trophic factor therapies in the 1990s (left) to stem cell therapies in the 2000s (right) and now to the realization that part of stem cell efficacy may be mediated via release and transduction of trophic signals targeted and amplified via synaptic contacts (bottom).
Adapted with permission from [21].
with some benefits. The potential of such strategies, especially the intraventricular route, for diffuse or multifocal effects may be greater compared with that of parenchymal strategies, especially if stem cells can be enticed to migrate to the lesion sites and differentiate into neural cells [15]. However, the consistency and comparative advantages of such effects are far from established.

The outcomes of preclinical testing of stem cells in TBI models have been recently reviewed by one of us [16]. Positive effects were observed in most studies with a small mean effect size that was more pronounced with modified or ‘enhanced’ cells. Transplantation within the lesion (for focal TBI) had a larger effect size than intravenous or ventricular delivery. Unfortunately, many of these studies have methodological problems. In addition, there is as yet no common standard for the assessment of outcome measures. Furthermore, a synthesis of studies using different stem cell preparations is extremely difficult. Also, the majority of TBI studies using human stem cell do not assess cell survival, thus clouding our understanding of potential mechanisms of action. Although transplanted stem cells such as neuronal precursors are fully capable of forming mature synapses with host structures in the brain and spinal cord [17], the physiological status of these synapses and their specific role in restoring function has not been characterized. Functionality of regenerated synapses is important not only for the purpose of conveying appropriate physiological signals but also for transsynaptic trophic support. The application of optogenetic strategies may prove critical in solving this problem [18].

**Special considerations for focal TBI & DAI**

Most published stem cell experiments in TBI are on focal models: about half of them used some form of controlled cortical impact, and the rest are equally split between weight drop and fluid percussion. For reasons explained in the previous paragraph, the field is behind stroke and spinal cord injury. Although a variety of benefits have been reported, integration with the host has not been demonstrated. Also unresolved are the questions of optimal dosing and dose scaling to man; this is a tricky issue because, at least based on our experience on spinal cord injury, dose escalation alters the dynamics of engraftment, migration and fate [19]. Even less can be said about stem cell therapies for models of DAI, although the field can borrow from spinal cord injury that invariably involves trauma in axonal tracts. In contrast to spinal cord injury, where axons course in restricted areas, DAI involves disparate axon tracts that would be difficult to transplant at the same time. Therefore, in the case of DAI, the choice of transplantation route (systemic, ventricular or parenchymal) and location of transplant (if we select parenchymal delivery) are critical. A recent study has shown that, in sharp contrast to neuronal progenitors, human oligodendrocyte progenitors do not colonize and differentiate locally but rather migrate massively along white matter tracts and remain within the white matter, often ensheathing themselves around host axons [20].

**Conclusion**

After multiple failures in clinical trials of single and combination agents, TBI is in dire need for effective treatments. The nature of some of the key lesions invites the consideration of the toolkit of regenerative medicine, including stem cell transplants. Important advancements in preclinical stem cell therapeutics and the popularity of TBI models create unprecedented opportunities for discoveries that could push this stalled field forward. Although there is no lack of interesting data, a great disparity in models, cell preparations, and reported outcomes detract from an enthusiastic endorsement of stem-cell therapeutics for TBI at this time. Consortia to establish guidelines for TBI modeling and NIH initiatives supporting collaborative and replication platforms are urgently needed: it makes no sense to only fund original research that would make little difference in and of itself and would be difficult to replicate or integrate with other studies. In the course of experimenting with stem cell therapeutics, one of
the greatest promises is the discovery of physiological molecular signals that afford protection or promote recovery in the adult brain.

Financial & competing interests disclosure

This work was supported by a Maryland Technology Development Corporation (TEDCO) grant 2015-MSCRF1-1718 to Koliatsos VE. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References


