Published in final edited form as:

Expert Rev Mol Diagn. 2011 January ; 11(1): 65–78. doi:10.1586/erm.10.104.

Blood-based diagnostics of traumatic brain injuries

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Abstract

Traumatic brain injury is a major health and socioeconomic problem that affects all societies. However, traditional approaches to the classification of clinical severity are the subject of debate and are being supplemented with structural and functional neuroimaging, as the need for biomarkers that reflect elements of the pathogenetic process is widely recognized. Basic science research and developments in the field of proteomics have greatly advanced our knowledge of the mechanisms involved in damage and have led to the discovery and rapid detection of new biomarkers that were not available previously. However, translating this research for patients' benefits remains a challenge. In this article, we summarize new developments, current knowledge and controversies, focusing on the potential role of these biomarkers as diagnostic, prognostic and monitoring tools of brain-injured patients.

Keywords

biomarkers; clinical management; diagnostics; ELISA; prognostics; proteomics; traumatic brain injury

Background & epidemiology of traumatic brain injury

Traumatic brain injury (TBI) constitutes a major health and socioeconomic problem throughout the world [1,2]. Globally, TBI is a leading cause of death and disability in children and young adults. More than 1.9 million people are estimated to sustain TBI each year in the USA (Figure 1), of whom 50,000 will die as a result of their injuries. Although most TBI is classed as mild, more than 2% of the US population are thought to have a disability caused by a TBI. However, TBI is often described as a 'silent epidemic' [3], since awareness among the public and even clinicians remains low, and no new treatment for TBI has been approved in the past 30 years.

The challenges of accurate diagnosis and monitoring of TBI have created a need for biomarkers that reflect core elements of the disease process.

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

Biomarkers are generally defined as measurable internal indicators of changes in organisms at the molecular or cellular level (markers of stress and malfunction, as well as injury) that can also provide information regarding injury mechanisms. Biochemical markers are consistently being used as diagnostic tools for injuries to specific organs, such as troponine for myocardial infarction, creatinine for renal failure and pancreas amylase and lipase for acute pancreatitis [4]. These blood tests are also used to monitor patients in intensive care settings.

Growing recognition of the importance of biomarkers led to the foundation of the Biomarkers Consortium, which was launched in October 2006 as a public-private partnership, including the NIH, the US FDA (as a part of the FDA's critical path initiative), the Centers for Medicare and Medicaid Services, as well as industry representatives and nonprofit organizations and advocacy groups. In 2007, Von Eschenbach, Commissioner of the FDA, highlighted the role of biomarkers as one technology that the FDA feels is "most likely to modernize and transform the development and use of medicines". However, in spite of this broad-based support, there are currently no FDA-approved biomarkers for TBI or other acute brain injuries. Recently, investigators at Banyan Biomarkers, Inc. (FL, USA) pioneered the application of neuroproteomics to identify and characterize biochemical markers of TBI. At the same time, novel and powerful technologies have made possible the detection of these new biomarkers with unprecedented accuracy and sensitivity. As outlined later, these biomarkers have the potential to revolutionize medical practice and biomedical research of TBI.

Limitations of current approaches to diagnosis of TBI highlight the need for biomarkers

Traditionally, TBI has been acutely diagnosed and classified by neurological examinations (Glasgow Coma Scale [GCS]) and neuroimaging (computed tomography [CT] and MRI).

However, the use of the GCS as a diagnostic tool is subject to a number of important limitations. Stocchetti and colleagues have highlighted the potential inaccurate early assessment of neurological severity of severe TBI patients, noting that in some patient subsets, injury severity can be overestimated [5]. Marion and Carlier reported that most clinicians prefer to use the prehospital GCS score obtained before medications were administered [6]. In view of the high rate of intubation and difficulties in the proper assessment of eye opening, the authors concluded that the GCS motor score was more important than either the eye or the verbal responses in predicting the severity of neurologic injury and outcome. Other recent research has provided evidence that the use of sedative drugs precluded accurate GCS assessment during the first 24 h. Clinicians felt unable to assess the GCS in approximately half of the patients, since they were sedated [7]. Further challenges to diagnosis are presented by the evolving nature of some brain lesions, which can lead to further neurological impairment. In addition, neurological responses after TBI can vary over time for reasons unrelated to the injury. For example, trauma is frequently associated with intoxication by alcohol and drugs. In a US urban trauma center, more than 74% of trauma patients tested positive for illicit or prescription drugs in their blood [8]. A review of studies evaluating the coexistence of alcohol intoxication and TBI found the rate of intoxication at the time of injury to be in the order of 36–51% [9].

Neuroimaging techniques, such as CT scanning and MRI, are used to provide to objective information on injury magnitude and location and are not influenced by the aforementioned confounders. However, CT scanning has low sensitivity to diffuse brain damage and the availability and utility of MRI acutely is limited [10,11]. MRI is also very impractical to perform if patients are physiologically unstable, and can lead to confounding diagnoses in

military injuries in which metal fragments are common. Importantly, concerns regarding the consequences of radiation from CT scans [12–14], especially repeated scans for recurrent injuries for the re-evaluation of existing injuries [15], have led to calls for alternative methods of assessing injury.

Furthermore, mild and moderate TBI represent more than 90% of TBI injuries [16]; this injury range represents the greatest challenges to accurate acute diagnosis and outcome prediction. A variety of neurological criteria have been invoked to infer injury severity and predict outcome, including duration of loss of consciousness and post-traumatic amnesia. Unlike severe TBI, there is no universally recognized neurologic assessment scale such as the GCS, nor are there management guidelines. As a result, the GCS has been inappropriately applied in an attempt to classify patients as 'mild TBI' (GCS: 13–15) or 'moderate TBI' (GCS: 9–12). However, even individuals with a GCS score of 13–15 are acutely at risk for intracranial bleeding and diffuse axonal injury and a significant subset of patients will suffer impairment of physical, cognitive and psychosocial functioning [16–20]. This risk has led to the common practice of conducting CT scans on all patients presenting at many emergency departments, thus resulting in a large number of unnecessary CT evaluations.

Repeated mild TBI occurring within a short period (i.e., hours, days or weeks) can be catastrophic or fatal, a phenomenon termed 'second-impact syndrome' [21]. Acute CT or MRI abnormalities are not usually found after these injuries, but levels of some neurotransmitters remain elevated and a hypermetabolic state may persist in the brain for several days after the initial injury [21]. During this time, the brain appears to be particularly vulnerable to additional mild TBI, which may result in severe sequelae, including greatly increased cerebral edema and death.

The widespread recognition of inadequate approaches to diagnose TBI led to a recent NIH conference to assess the current technologies available for the classification of TBI [22]. Participants acknowledged the utility of neurological examinations and imaging technologies, but recognized the need for significant improvement in the diagnosis and classification of TBI, such as the use of biomarkers to supplement functional and imaging-based assessments.

These markers can be altered gene expression, protein or lipid metabolites, or a combination of these changes after traumatic brain injury, reflecting the initial insult (the primary injury) and the evolution of a cascade of secondary damage (the secondary injury). As a consequence, a variety of strategies have been used for biomarker discovery, including transcriptional profiling, proteomic and metabolomic approaches. Recently, studies have assessed lipid metabolism, and the focus has been predominantly on biomarkers of lipid peroxidation, such as F2-isoprostane. Others have highlighted the importance of inflammatory factors on the progression of TBI-associated pathologies. In this article, we will focus on the neuroproteomic approach, presenting data to assess the clinical validation of protein biomarkers in serum in the setting of acute brain injury.

Identification & development of brain-specific biomarkers through neuroproteomics

In the quest to identify potential TBI-specific biomarkers, genomics and proteomics have proven to be powerful, complementary tools that are helpful in revealing specific genes or proteins that are up- or down-regulated as a consequence of TBI in human patients or animal models. With the goal to discover new protein biomarkers that are elevated in the injured brain, proteomic studies have the advantage, since not all transcriptional changes have

concomitant effects on protein levels. Furthermore, in order to qualify as a *bona fide* biomarker candidate that is capable of detecting or monitoring TBI, additional requirements have to be met, including specificity for, and enrichment in brain of, a measurable elevation in biofluids and, ideally, a homologue in an animal model that allows translation of research results to the human situation (Figure 2). Finally, we propose that it would be advantageous to have a panel of complementary biomarkers that have different temporal profiles and cover distinct pathophysiological conditions following TBI (Figure 2) [23].

Several studies have demonstrated the role of proteomics [24–28] in providing significant insight into understanding functional or biochemical changes or modifications in certain proteins following a TBI. Using a targeted approach, specific α II-spectrin (α -fodrin) breakdown products were identified in the brain as a result of calpain and/or caspase proteolysis during the acute necrotic or subacute apoptotic phases, respectively, following a brain injury [29–31]. These protein modifications were not only found to be elevated in injured brain tissues, but also in the cerebrospinal fluid (CSF) and/or serum of affected rats [28,32–34] or humans [35–37].

While these targeted approaches have provided several unique biomarker candidates, a rigorous exploration of all potential biomarker candidates requires an unbiased global proteomic approach. Kochanek *et al.* [26], Haskins *et al.* [38] and Ottens [39] have shown that 2DE, cation/anion exchange chromatography-coupled to 1D gel electrophoresis (CAX-PAGE) and MS/MS technology, respectively, go a long way toward accomplishing such a task [26,38]. Using a combination of these technologies and an experimental TBI model, we were able to identify 21 and 41 proteins to be up- or down-regulated in rat brains 24 h following a controlled cortical impact [40]. The workflow of this neuroproteomic approach is illustrated in Figure 3. Using the controlled cortical impact model, we complemented the mass spectrometry approach with an antibody panel-based high-throughput immunoblotting process, a method comprising the use of 1000 monoclonal antibodies to probe replicates of both control and TBI tissues (48-h postinjury). Although not exhaustive, this process allowed us to identify many additional protein biomarker candidates, as well as brain ischemia and a new penetrating brain-injury model [41,42]. These candidate biomarkers can be readily confirmed by testing additional tissue or CSF samples with the available monoclonal antibodies [41]. It is worth noting that both the CAX-PAGE-reverse-phase liquid chromatography–MS/MS and high-throughput immunoblotting methods are capable of identifying proteolytic truncations of brain proteins – a major TBI-induced post-translational modification event [35].

Identification of several hundred putative biomarkers raised the need to reduce this portfolio into a more manageable size. Employing systems biology to place all biomarker candidates into distinct converging and nonredundant pathophysiological pathways (Figure 4), we were able to identify a number of emerging pathways, including those of necrotic and apoptotic cell death, cytoskeleton damage (e.g., axonal, dendritic and myelin), synaptic dysfunction, neuronal cell body injury, glial injury, neuroinflammation (including microgliosis) and possible neuroregeneration. The next task involved strategic selection of one to two promising biomarkers representative of each of these distinct pathways or events. This had led us to identify proteolytic markers spectrin breakdown product (SBDP)150 and SBDP145 as reporters of calpain-mediated necrotic injury, SBDP120 as a marker for caspase-mediated apoptosis, ubiquitin C-terminal hydrolase-L1 (UCH-L1) as a novel candidate neural cell body injury marker, MAP2 as a marker for dendritic injury, EMAPII as a reporter for microgliosis, glial fibrillary acidic protein (GFAP) as gliosis marker and synaptotagmin-BDP and CRMP2-BDP as putative biomarkers for synaptic damage and neuroregeneration, respectively (Figure 4) [40].

Identification of this panel of potential TBI biomarkers was followed with the development of sensitive sandwich ELISAs for determining their concentrations in biofluids and allowing their validation in animal studies or in human clinical studies. To date, elevation of UCH-L1 as a consequence of TBI has been demonstrated in CSF and blood of rats [32], in human CSF [43] and in human serum [Wang KK *et al.*, Unpublished Data]. In addition, SBDP145 and SBDP120 have been validated as tandem TBI markers in rat [32] and in human CSF [44,45], and the utility of GFAP as a marker for severe TBI was recently confirmed [46,47]. The development of ELISA for other markers is currently in progress.

Assay development

The most common methodology for measuring serum levels of protein biomarkers is by two-sided sandwich immunoassays, whereby the capture antibody is typically attached to a solid surface and detection of the captured target analyte is achieved through a directly or indirectly labeled detection antibody (Figure 5). In ELISA, signal generation is mediated through an enzyme that is directly or indirectly attached to the detection antibody. The variety of surface structures, labels and detection methods now available to the assay developer has resulted in a plethora of diagnostic assay systems and devices, and a multiplicity of different assay formats. The system of choice depends on the specific application and, hence, is driven by specific analytical and commercial performance requirements. Key drivers typically include sensitivity, specificity (**Box 1**), speed, cost, system complexity and the amount of sample required, as well as the need for sample preparation.

Assay sensitivity is generally the key requirement for biomarkers that are to be measured in blood, and this is particularly true for brain proteins, which are prevented from entering the blood in healthy individuals owing to the blood–brain barrier. Assay sensitivity is most often defined as the lower limit of detection (i.e., the smallest amount of analyte that can be detected above the mean background with reasonable statistical significance, a value typically set as the mean background plus two to three standard deviations) [48]. Note that other definitions and more sophisticated calculations have been used [48,49]. For clinical assays that correlate biomarker concentration with disease severity or outcome, the more important number is the lower limit of quantitation, as well as a variety of other parameters (e.g., accuracy, precision, specificity and linearity) that characterize assay performance. Determining these parameters is an essential part of the final stages of assay development [42,50] and is critical before submission of an application to the FDA for an investigational device exemption can be considered.

However, there are many steps in assay development before this stage is reached, and the most critical by far is the development of antibodies with the appropriate affinity and specificity. While one or more antibodies with the appropriate specificity may have been used during the early phases of biomarker development and validation, they may not be suitable for the development of sensitive assays. The reason is that the validation of a biomarker does not necessarily require a quantitative assay, or even a very sensitive assay. All that is required for this purpose is an assay that is sufficiently sensitive to detect the target biomarker in the sample of most patients, so that its presence can be statistically correlated with disease status. Much more stringent criteria, however, will be placed on an assay that has to detect and quantify the biomarker in a single sample for the purpose of making a diagnosis. This concept is referred to as 'fit-for-purpose' assay development [50].

For two-sided sandwich ELISAs, one typically chooses the most specific antibody as the capture antibody (typically a monoclonal antibody), while the detection antibody may be less specific, since most of the sample materials can be washed away before the secondary

antibody is added. Of course, there are other considerations that are of equal if not higher importance, such as the affinity (dissociation constant $[K_D]$), which is the ratio of the dissociation rate constant and the association rate constant (i.e., the off-rate divided by the on-rate) [48]. Thus, for assays where total assay time is less important than sensitivity, antibodies with a poor on-rate are acceptable if the overall affinity is good. However, for short assay times, antibodies with fast on-rates are obviously needed. It follows that antibodies selected to work in research applications (often at times with overnight capture of target) may prove unsuitable when the assay is to be ported to a point-of-care (POC) system that requires an assay time of 30 min or less. The kinetic characteristics of the antibodies involved are therefore critical and can place a high value on a specific monoclonal antibody or even a polyclonal serum. However, there are natural limits to the affinity of antibodies [41], and assay optimization involves all system components.

The second most important contributor to assay sensitivity is the detection system. An ideal assay would be homogeneous and not require a detection antibody [48,51–55], whereby target capture is detected by the resulting change in the physicochemical properties of the binding surface [56–66], or by displacement of a labeled hapten (competitive assay) [67,68]. Although such systems have evolved in recent years to approach the sensitivity of ELISA assays [34,46,53–55], the most powerful detection systems typically involve a sophisticated label and detection strategy or even a signal amplification step, such as immuno-PCR or biobarcode technology. The increased use of such amplification techniques often results in a decrease in assay precision, and an increase in assay and instrument complexity (for a review, see [69]). With the goal of keeping assay development and instrument costs at a minimum, the two-sided sandwich ELISA with detection based on absorbance, fluorescence or chemiluminescence still appears to be the most convenient and robust system, and has been the method of choice for research laboratories in the TBI field.

Platform development

Translation of successful biomarker identification, assay development and validation in clinical studies into a clinically useful and commercially successful diagnostic product requires the transfer of such assays to an appropriate instrument platform that is suited to its specific application (Figure 6). Measurement of biomarkers starts at the accident site or in the emergency room – measurement is best performed on small, portable POC systems that require little or no sample preparation and provide a result in 30 min or less. Biomarkers that can be used for diagnosis days or even weeks after injury are suitable for reference laboratories, which demand high throughput and high accuracy. The Military has even more stringent requirements, since assay systems have to be small, light weight, simple, rugged and capable of performing in extreme environments to be far forward deployable in a theatre of operations.

A large number and variety of fluidic platforms and detection systems have been developed during the past three decades and combined into diagnostic devices ranging from the closed, high-throughput immunoassay analyzers that are standard in clinical laboratories of major hospitals, to small, simple, low-cost devices that can be used by any untrained individual. The difference, of course, is the performance in terms of accuracy and precision. For tests that do not require quantization but simply identify the presence of a biomarker beyond a certain threshold, a simple lateralflow strip assay is typically sufficient [41], as demonstrated by the commercial success of over-the-counter devices to screen for food allergens, or as pregnancy or drug tests. It is therefore tempting to speculate that such devices may be suitable for the screening of TBI, at least to eliminate the possibility of brain trauma in vehicle accidents or in sports injuries in the acute situation. Whether this is feasible depends on the level of biomarkers in the blood of patients with mild concussive events as opposed

to the variance of that level in normal, unaffected individuals. The requirements for sensitivity and precision may make this a difficult, if not impossible task, although our preliminary data provide some hope in this regard. In addition to the ELISAs, future developments will focus on the development of a miniaturized POC, which can be transported out in the 'field', near or at the site of patient care, and can be miniaturized to the size of a handheld device. These POC devices use different technologies, such as flow strips, and reduce the assay time to 15–20 min, which is required in these emergency settings. In the near future, it is more likely that a POC device with more sophisticated performance features will provide a laboratory assessment of mild, moderate and severe TBI, and allow the differentiation between affected and unaffected brain tissue akin to a CT scan, but without the drawbacks of high cost and radiation exposure. In close collaboration with our colleagues at the Department of Defense (DoD; WA, USA) and the Walter Reed Army Institute for Research (MD, USA) [41], we aim to close this gap in TBI diagnostics (Figure 6).

TBI biomarkers, clinical studies & results/status of biomarker research

Several potential biomarkers have been identified and assessed in the last decade; our group has a long-standing interest in biomarker research.

In broad terms, useful biomarkers may be specific to brain injury or may be markers of inflammation and/or other biochemical and physiological processes, such as degeneration, repair and apoptosis.

However, several studies have measured a variety of neurochemical substances in the CSF or blood, and a number of proteins synthesized in astroglial cells or neurons have been proposed as markers of cell damage in the CNS and after TBI to date [70–80], but none have proved sufficiently useful to justify routine clinical use.

Important hierarchical criteria for a clinically valid biomarker for TBI includes:

- Demonstration that the biomarker is significantly modified in diseased patients compared with controls (uninjured subjects, as well as subjects with multiple injuries but no head injury);
- Assessment of the diagnostic properties of the biomarkers in the acute setting, as well as in the subchronic phase after TBI;
- Comparison of the diagnostic properties of the biomarker to existing tests (GCS and neuroimaging);
- Demonstration that the measured level of the biomarker aids in medical decision making.

This article will summarize some of our work while also integrating a number of important studies in the field.

S-100 β has received considerable attention as a marker of CNS injury. As the principal low-affinity calcium-binding protein in astrocytes [81] it is considered a marker of astrocyte injury or death. Studies have reported that S-100 β serum levels consistently correlate with both GCS scores and neuroradiologic findings at hospital admission [82–85]. Two groups have reported that S-100 β serum measurements after TBI significantly differentiated patient groups with mild and severe injuries [86,87]. Moreover, several studies consistently reported a correlation between S-100 β serum levels and outcome [82,84–90]. Nevertheless, S-100 β , initially considered unique to the nervous system, is present in other tissues, including adipocytes and chondrocytes, and high serum levels have been observed in trauma patients

without head injuries [91–93]. Moreover, the lack of specificity in settings of hemorrhagic shock or circulatory arrest, such as in cardiopulmonary bypass, makes the use of S-100 β as a potential screening agent for brain injury in neurointensive care impractical [94,95]. Finally, S-100 β is not a useful marker in children less than 2 years of age, owing to high normative values in that age group [96,97]. These factors also create methodological difficulties when comparing different immunoassays for S100B with regard to specificity and sensitivity. The recognition of S100B by certain commercial kits is affected by calcium, and certain antibodies used in commercial kits cross-react with S100A1 (reviewed in [98,99]). Therefore, although S-100 β remains promising as an adjunctive marker, its utility as a biochemical diagnostic remains controversial.

Neuron-specific enolase is located in the cytoplasm of neurons and is probably involved in increasing neuronal chloride levels during the onset of neural activity [100]. This marker is thought to assess damage to the functional cells of the brain (i.e., the neurons), and a rapid appearance in serum after head injury has been reported [87,88,101]. Despite these promising characteristics, several studies show conflicting results. The slow elimination makes it difficult to assess the amount of primary damage and impossible to distinguish between primary and secondary injuries [102,103]. Neuron-specific enolase is also released in the blood by hemolysis, which may be a serious source of error [104,105].

Myelin basic protein, specific to the myelin sheath of CNS myelin, can be released into serum by brain damage or demyelinating diseases and appears to be a promising marker of TBI [106–108]. Although CSF and serum levels in TBI patients have demonstrated excellent specificity, sensitivity has been limited [109].

Ubiquitin C-terminal hydrolase-L1 was previously used as a histological marker for neurons owing to its high abundance and specific expression in neurons. This protein is involved in either the addition or removal of ubiquitin from proteins that are destined for metabolism (via the ATP-dependent proteasome pathway) [110], thus playing an important role in the removal of excessive, oxidized or misfolded proteins during both normal and neuropathological conditions in neurons [111]. A recent study reported that levels of UCH-L1 in CSF were significantly increased in severe TBI patients compared with control subjects, with significant associations observed between levels of UCH-L1 in CSF and injury severity measures, including GCS, evolving lesions on CT and 6-week mortality [112]. Our research team examined initial UCH-L1 serum levels from 27 patients with mild TBI and 101 severe head-injured patients [Hayes RL *et al.*, Unpublished Data]. UCH-L1 was demonstrated to be a sensitive and specific biomarker of TBI. Furthermore, although limited by a small subject population, in mild-TBI, group data suggest that higher levels of UCH-L1 are potentially associated with the presence of intracranial lesions that are detectable on CT. A study has been conducted recently examining serum UCH-L1 levels from adults with severe TBI, and their relationship with severity of injury and clinical outcome [43]. UCH-L1 peaked early after injury and levels in serum were significantly increased in TBI patients compared with uninjured controls. UCH-L1 serum concentrations were associated with severity of injury, CT scan findings and outcome. UCH-L1 was shown as the only independent predictor of in-hospital mortality (adjusted odds ratio: 2.74; 95% CI: 1.537–4.896), and also as a strong predictor of death 6 months postinjury. In addition, a rigorous biokinetic analysis performed on UCH-L1 concentration in serum and CSF of severe TBI patients revealed a strong correlation between CSF and serum exposure and kinetic characteristics, especially during the acute period. In addition, a significant relationship between serum exposure metrics and survival at 3 months postinjury was observed [Brophy *et al.*, Pers. Comm.].

Glial fibrillary acidic protein represents the major part of the cytoskeleton of astrocytes, is found only in glial cells of the CNS [113,114] and may, therefore, be considered to be a specific marker for CNS disease, and is also involved in various neuronal processes, including maintenance of the blood–brain barrier [115]. Increased serum GFAP levels have been reported in patients suffering from severe head trauma [116]. Recently, other reports have confirmed that serum GFAP is a specific marker of brain damage after head trauma [89,101,117,118]. GFAP has also been demonstrated to be a potential useful biomarker to predict clinical outcome. Ongoing studies in our research group have shown that serum GFAP levels are significantly higher in patients who died 6 months postinjury than in those who are alive.

Our laboratory has also focused on α II-spectrin proteolysis as a biochemical marker of CNS injury [34,119]. α II-spectrin is primarily found in neurons and is abundant in axons and presynaptic terminals [120]. The protein is processed to breakdown products (SBDPs) of molecular weights 150 kDa (SBDP150) and 145 kDa (SBDP145) by calpain and is also cleaved to a 120-kDa product (SBDP120) by caspase-3. Calpain and caspase-3 are major executioners of necrotic and apoptotic cell death, respectively, during ischemia or TBI [33–35,121]. Thus, a unique feature of this technique is the ability to concurrently detect calpain and caspase-3 proteolysis of α II-spectrin, providing crucial information on the underlying cell death mechanisms.

Pineda *et al.*, employing western blot analyses, reported elevated levels of SBDPs in CSF from adults with severe TBI and their significant relationships with severity of injury and outcome [35]. Recently, CSF SBDP levels were found to be significantly higher in patients who died compared with those who survived. Increased levels of SBDP145 and SBDP120 were found to be strong predictors of death at 3 months postinjury (odds ratio: 5.9 for SBDP145, and 18.34 for SBDP120). In addition, the temporal profile of SBDPs in nonsurvivors also differed from survivors [36]. Taken together, these data suggest that SBDPs may provide crucial information not only on severity of brain injury, but also on underlying pathophysiological injury mechanisms associated with necrotic and apoptotic cell death. Our laboratories are currently focusing on developing a serum-based assay to detect these SBDPs in blood.

TBI biomarkers & clinical trial design

According to the Cochrane Library, there have been more than 200 unsuccessful clinical trials assessing potential therapies for TBI, and there are currently no FDA-approved therapies. As early as 2002, a NIH workshop recognized the need for development of more refined surrogate measures to improve the design and execution of clinical trials in head injury [122].

There are a number of areas in which incorporation of biomarkers could significantly improve clinical trial design and execution. Injury magnitude and risk assessment is an important criterion for determining patient eligibility for TBI clinical trials.

Obviously, it is important to have patients with similar magnitudes of injury in different treatment groups. At present, the GCS is the primary, if not the exclusive, entry criterion assessment tool for injury magnitude. Given the difficulties associated with accurate GCS assessment, as outlined previously, biomarkers could provide an objective, quantitative tool to measure injury magnitude.

Secondary brain insults worsen neurologic outcome after TBI [123–125]. In an effort to prevent occurrences of these insults, physiologic vital signs (e.g., intracranial pressure, mean arterial blood pressure and tissue oxygenation) are routinely assessed in intensive care

environments, although they are usually recorded only intermittently in the medical record. Conventional manual recording of vital signs can underestimate the total number of secondary insults [126]. Undetected occurrence of secondary insults and increased neurologic damage in different treatment groups can significantly enhance variability in clinical trials. Detection of increases in biomarker levels, in conjunction with physiological assessment and management, could provide critical information to reduce the number of undetected secondary events and allow for the stratification of patients by occurrence of these insults.

As mentioned previously, management of severe TBI patients can importantly influence outcome [127], potentially by reducing the number of secondary insults. In spite of vigorous educational programs, the American Association of Neurological Surgeon Guidelines for Management of Severe TBI Patients are not followed uniformly. Moreover, even when efforts are made, failure to rigorously standardize clinical management in different centers could contribute to outcome variability in severe TBI clinical trials. For example, in a recent trial assessing the effects of moderate hypothermia on severe TBI [128], treatment effects for the five largest centers varied between 14% positive and 20% negative. Although there were significant differences in cerebral perfusion pressure management among centers, investigators did not detect a correlation with treatment effect and attributed center differences to other baseline variables. In any case, the systematic assessment of biomarkers on admission and during the course of management could provide critical insights into potential differences between patient cohorts among centers [129].

Expert commentary

Despite progress over the past 10–15 years, advances in basic science have not yet led to new treatments of clinically proven benefit. Current classification systems have been proved to be no longer sufficient and clinical trials in TBI have had methodological problems posed by the heterogeneity of TBI and poor understanding of its pathology and prognosis. Therefore, there is an urgent need for significant improvement in the diagnosis and classification of TBI, including in the use of biomarkers to supplement functional and imaging-based assessments. Furthermore, the current diagnostic criteria for mild and moderate TBI rely on GCS, the clinical identification of loss of consciousness along with detection of one or more abnormal neuroimaging results, including CT and MRI. Biomarkers could obviously be useful in a routine clinical diagnostic setting; however, more detailed guidelines are needed on how biomarkers can be integrated into current diagnostic procedures to screen individuals with mild or moderate TBI who are at risk of deterioration and later cognitive deficits. Studies relating to these issues are only just emerging.

At this time, one of the limitations is the lack of consensus for the biomarkers that should be used. Furthermore each study uses different methods. Studies with larger populations will certainly be helpful.

The standardization of procedures and assays' validation is essential to push the field forward. Progress will also require the development of new high-sensitivity assays. Newer generations of current assays are enabling the measurement of biomarkers at concentrations that are not reliably detected with prior generations, improving overall diagnostic accuracy and sensitivity. The assessment of the clinical significance of low-level elevations of biomarkers and the prognostic relevance in patients with TBI have to be addressed in future studies. Furthermore, the variation in biomarker levels between centers is well known in clinical chemistry. These types of variation are probably the result of variations in clinical procedures as well as batch-to-batch variation in the biomarker assays. These sources of variability are routinely controlled by rigorously implemented quality control protocols and

standard operations procedures. Quality controls include standardization of the clinical procedures, processing and analysis. Standardized protocols should minimize variation caused by differences in pre-analytical and laboratory procedures, and, thus, allow direct comparisons of biomarker levels between laboratories and between publications. To overcome batch-to-batch variation in biomarker assays, biomarker kit vendors will need to implement new standards for quality control. Assays should exhibit low overall variability in calibration curves and strict limits of variability across batches. To achieve these goals, stringent quality control of critical reagents, including antibodies and calibrators, is needed. In the long term, the aim is that the quality control program will serve as the basis for a more general introduction of biomarkers into routine clinical practice and multicenter clinical trials.

When implementing these biomarkers in clinical practice, financial considerations will be of importance. The cost of combined analysis of blood biomarkers will have to be compared with CT scans or structural MRI investigations.

Finally, we should recognize that no single treatment can be uniformly appropriate across the wide range of clinical manifestations of TBI or multiple mechanisms underlying these injuries. Clinical trial methodologies require refinements, including the implementation of approaches to deal with the inherent heterogeneity of TBI populations. Blood biomarkers might serve as tools for categorizing TBI patients, allowing potential therapies to be tested in those who are most likely to benefit, as well as to act as surrogates for drug efficacy. Biomarkers might also serve as valuable tools in drug development, providing important information on pathobiological responses to TBI and potential targets of therapy.

Five-year view

Blood biomarkers have a potential high diagnostic value in the context of TBI. The combination of these biomarkers and structural and/or functional brain imaging (CT or MRI) should provide increased diagnostic accuracy compared with the individual use of these diagnostic modalities.

The groups of biomarkers discussed in this review are usually considered individually. Further studies are needed to further define the added diagnostic value when multiple biomarker modalities are combined. Moreover, a multimarker strategy could be useful in refining risk stratification and for categorizing patients with TBI.

The application of proteomics, the evaluation of proteins using mass spectrometric analysis coupled with high-pressure liquid chromatography, is likely to yield totally new classes of biomarkers. Large platforms that would facilitate the study of hundreds of proteins are likely to become available, elucidating mechanisms of injury and allowing the identification of potentially complex pathophysiological alterations. The ultimate goal of this research is to develop more individualized treatment, targeting the specific needs of a given patient.

The seriousness and complexity of the problems posed by TBI have been underestimated. Current classification systems are no longer sufficient. No clinical method is available for an accurate assessment of severity of injury and outcome in patients with TBI, especially in mild TBI, since mild TBI patients have only subtle disturbances.

Box 1. Criteria for evaluation of traumatic brain-injury biomarkers

- Sensitivity: the capacity of a biomarker to identify patients who have disease (the proportion of subjects with disease that are correctly classified as positive)

- Specificity: the capacity of a biomarker to identify subjects who do not have disease (the proportion of subjects without disease that are correctly classified as negative)
- Positive-predictive value: the likelihood that an individual with a positive test result has the disease (the number of true-positive cases divided by all cases with a positive test)
- Negative-predictive value: the likelihood that an individual with a negative test does not have the disease (the number of true-negative cases divided by all cases with a negative test)

Studies evaluating the diagnostic performance of a biomarker should include determination of the molecule's sensitivity, specificity, positive-predictive value and negative-predictive value.

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• of interest

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Figure 1. 'The silent epidemic'

(A) Average annual numbers of brain injury-related emergency department visits, hospitalizations and deaths by external causes. (B) Percentage of average annual traumatic brain injury-related emergency department visits, hospitalizations and deaths by external causes. Motor vehicle accidents were the leading cause of traumatic brain injury (50%), followed by falls (21%) and assaults (12%).

Figure 2. Brain-injury biomarker genesis, distribution and temporal profile as detected in blood
(A) Paradigm for brain-injury biomarker studies. **(B)** Traumatic brain-injury biomarkers show a distinct temporal profile (trendline analysis).

BBB: Blood–brain barrier; BDP: Breakdown product; CSF: Cerebrospinal fluid; H: Hours.

Figure 3. Proteomics-based biomarker discovery

CCI: Controlled cortical impact; LQC: Log quality control/check; MS: Mass spectrometry; RPLC: Reverse-phase liquid chromatography.

Figure 4. Systems biology-based selection of candidate traumatic brain-injury biomarkers, representing nonredundant and convergent pathways

BDP: Breakdown product; CRMP: Collapsin response mediator proteins; EMAP: Endothelial-monocyte activating polypeptide; GFAP: Glial fibrillary acidic protein; MBP: Myelin-basic protein; NSE: Neuron-specific enolase; SBDP: Spectrin breakdown product; TBI: Traumatic brain injury; UCH-L1: Ubiquitin C-terminal hydrolase-L1.

Figure 5. Basic sandwich antibody-based detection of traumatic brain-injury diagnostic biomarkers and the process of the development and validation of such assays and their clinical diagnostic utility testing

CV: Coefficients of variation; TBI: Traumatic brain injury.

Figure 6. Platform options for different applications

POC: Point of care; VA: Veterans affairs.