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Blood-Based Biomarkers for Traumatic Brain Injury: Evaluation of Research Approaches, Available Methods and Potential Utility from the Clinician and Clinical Laboratory Perspectives

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Review

Blood-based biomarkers for traumatic brain injury: Evaluation of research approaches, available methods and potential utility from the clinician and clinical laboratory perspectives

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ABSTRACT

Blood-based biomarkers for traumatic brain injury (TBI) have been investigated and proposed for decades, yet the current clinical assessment of TBI is largely based on clinical symptoms that can vary widely amongst patients, and have significant overlap with unrelated disease states. A careful review of current treatment guidelines for TBI further highlights the potential utility of a blood-based TBI biomarker panel in augmenting clinical decision making. Numerous expert reviews on blood-based TBI biomarkers have been published but a close look at the methods used and the astonishing paucity of validation and quality control data has not been undertaken from the vantage point of the clinical laboratory. Further, the field of blood-based TBI biomarker research has failed to adequately examine sex and gender differences between men and women with respect to the clinical care settings, as well as differences in physiological outcomes of TBI biomarker studies. Discussions of tried-and-true laboratory techniques in addition to a few new ones already operating in the clinical laboratory are summarized with a consideration of their utility in TBI biomarker assessment. In the context of TBI biomarkers, the central concerns discussed in this review are the readiness of the clinical laboratory, the willingness of the research environment and the inherent ability of each to radically affect patient outcomes in TBI.

Contents

Abbreviations: TBI, traumatic brain injury; mTBI, mild traumatic brain injury; GCS, Glasgow Coma Score; NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; UCH-L1, ubiquitin c-terminal hydrolase-L1; CSF, cerebral spinal fluid; ELISA, Enzyme-Linked Immunosorbent Assay; IA, immunoassay; LDT, laboratory developed test.

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Introduction

Blood-based biomarkers for traumatic brain injury (TBI) have been investigated and proposed for decades. In recent years, there has been increased focus on mild TBI and concussion with substantial media coverage surrounding concussion in sports [1] and the recognition of TBI as the defining injury for veterans of the Iraq and Afghanistan wars [2]. The current clinical assessment of TBI is largely based on clinical symptoms that can vary widely amongst patients and have significant overlap with unrelated disease states [1,3,4].

Findings generated in the basic research arena have been rapidly integrated into clinically-based studies very often using methods that would not be allowed to operate in the clinical laboratory. Further, many of the published studies provide limited information on assay validation or the use of fundamental laboratory techniques such as statistical quality control. Instead of adding to the accumulating number of expert reviews on TBI biomarkers, we focused on identifying methods used in the study of five of the most prominent biomarkers in the literature. We report on the limited amount of validation and quality control data provided. In fairness, it is possible that the authors of the reviewed publications completed a full, CLIA-approved validation for a high complexity test with the use of established quality control ranges to validate all published data. More likely, the publications selected for review illustrate a pervasive lack of appreciation for the rigorous quality standards required from clinical testing in an attempt to fulfill the promise of translational research expectations.

The clinical laboratory continues to grow in its ability to transform novel, highly complex research assays into routine clinical tests, while at the same time more advanced technology continues to find its way from research into the clinical laboratory. In addition, if sex and gender influences are not fully understood TBI research may fail to address important issues pertaining to the assessment and treatment of TBI. Due to a general ambiguity towards TBI in terms of definition, assessment, and treatment [3] and the fact that 77% of individuals with TBI are male [5], TBI research appears especially prone to gender bias. The subsequent focus of this review is on the clinical approach to TBI, methods used in TBI research, methods available in the modern clinical laboratory and sex and gender differences in TBI biomarker studies.

Current clinical approach to TBI

TBI is a heterogeneous disease with numerous methods to classify patients, most often into mild, moderate or severe TBI, based on clinical severity, injury type and pathophysiology. The most commonly used tool for the assessment of TBI is the Glasgow Coma Score (GCS) [6] in addition to the inclusion of age, medical comorbidity and imaging studies [7,8]. Ideal in its simplicity, criticism of the GCS for TBI classification is based upon a number of confounding factors that may contribute to a skewed score [9].

Mild traumatic brain injury

Mild traumatic brain injury (mTBI) is considerably more common than moderate and severe TBI; however, a risk of serious and long term complications can arise if adequate treatment is not provided. mTBI most often occurs as a result of contact and/or the physical and mechanical forces of acceleration and deceleration.

Concussion in athletics

A concussion is defined as a traumatically induced, transient disturbance of brain function. Concussion is correctly classified as an mTBI, with an important distinction in that not all mTBIs are concussions [1]. In 2012, a consensus statement provided by the 4th International Conference on Concussion in Sport held in Zurich identified common features useful in defining a concussion. These features include cause (direct blow to the head or elsewhere that is transmitted to the head), acute symptoms (short-lived impairment of neurologic function), neuropathological changes (due to functional and not structural changes often missed by imaging studies), a grouping of clinical symptoms which may or may not involve the loss of consciousness, and a step-wise resolution

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of those symptoms [10]. The hallmark symptoms of concussion include confusion and amnesia, with headache and dizziness the most commonly reported symptoms appearing minutes to hours post-injury. Symptoms of severe concussion are non-specific and include nausea, vomiting, headache, sleep disturbances and mood alterations [1].

Current estimates put the number of sports related concussions in the US between 1.6 and 3.8 million per year [11]. Concussions are more common in competition compared to practice, females compared to males [12], and have a higher rate in collegiate compared to high school athletes [13]. In general, concussions are more common and happen at a higher rate in high impact sports such as football, wrestling, soccer, and ice hockey [12], and are seen in higher risk positions on the field such as a catcher in baseball, a linebacker or running back in football, or a goalie in soccer [13]. Other risk factors for concussion include a prior concussion and younger age, while ADHD, mood disorders, learning disorders, and migraine headaches can complicate concussion symptoms and management [1]. The primary cause for the noticeable increase in concussive events is not definitively known but is partly attributed to increased awareness of prevalence and symptoms [14].

Management of concussion can be difficult as the approach to concussion must be individualized for every person due to inter-individual variation in rates of recovery. At a minimum, athletes should obtain a preparticipation physical where a healthcare provider can evaluate the athlete for any current or prior health issues including concussion. If concussion has been an issue for an athlete in the past, additional information should be obtained including, but not limited to, the number of prior concussions, prior recovery, and when the last concussion was sustained. More commonly, athletes are obtaining baseline testing through the use of a sideline assessment tool, balance testing, or neuropsychological testing at the beginning of the season in order to have comparative data after an in-season concussion. Neuropsychological testing can be either computerized or done by pencil and paper, and aims to evaluate the athlete on areas like orientation, memory, reaction time, and concentration. Given the fact that these measures have not been shown to affect short-term or long-term outcomes from concussion, some controversy exists about their utility. Where they do seem to be more useful is in athletes where symptoms have resolved, but concern exists about full recovery. A follow-up administration of a neuropsychological test could show persistent deficits when compared to baseline testing, leading the provider to withhold the athlete until testing results in a return to baseline scores [1].

Once a concussion is determined to be a possibility, on field management includes taking the player out of the game for further evaluation. Generally, the athlete is taken through a sideline assessment tool, designed to assess symptoms, orientation, balance, memory, and concentration. If it is determined that the athlete has had a concussion, he or she is removed from play, and should not be allowed to return to competition on the same day. Acute management may include symptom management with medication for pain, avoidance of bright lights or loud sounds, and physical and cognitive rest. Of note, it is no longer advised to frequently wake a concussed individual or instruct the individual to avoid sleep, as it largely considered a valuable component to the healing process [1].

In general, advanced imaging for concussion is rarely needed, as the results are typically normal. A clinician may proceed to advanced imaging given the presence of more concerning signs such as seizures, evidence of a skull fracture, or focal neurological deficits. CT is the modality of choice in the acute situation, but for prolonged symptoms an MRI may be obtained [10,15]. More recently, imaging modalities like functional MRI, diffusion tensor imaging, and MR spectroscopy are being evaluated for their utility in concussion management with encouraging results, but definitive use has yet to be determined [1].

Development of equipment to protect athletes from injury is an evolving process. Helmets were originally created to reduce the incidence of skull fractures, intracranial bleeding and oral and eye injuries. There is no evidence that headgear reduces the incidence of concussion [1]. Over 40% of concussion symptoms have resolved in 3 days or less, and over 50% of athletes return to play between within 1 to 3 weeks. Unfortunately, approximately 20% of athletes will have symptoms longer than 3 weeks [12,16]. It is difficult to predict who will have profound or prolonged symptoms, but certain signs at presentation have been associated with a worse prognosis. These include greater than 3 symptoms at presentation, a feeling of fogginess, a headache lasting longer than 60 h, and retrograde amnesia. Interestingly, loss of consciousness had no bearing on prognosis [17,18]. It should be noted that a licensed healthcare professional with concussion training should be the one to clear the athlete for play, and more states are passing legislation making this a requirement [1]. Return to activity should be performed in a stepwise manner as outlined in Fig. 1. As the baseline step of the Return to Play Progression, the athlete needs to have completed physical and cognitive rest, and not be experiencing concussion symptoms for a minimum of 24 h. The overall goal of this stepwise approach is the return to competition once the athlete is asymptomatic. Keep in mind, the younger the athlete the more conservative the treatment.

Complications in mTBI

The more serious outcomes from concussion can include post-concussion syndrome (PCS), second impact syndrome (SIS), and chronic traumatic encephalopathy (CTE). PCS is most often described in the setting of mTBI, but its presentation varies widely, as does the criteria for diagnosis. Typically, PCS refers to symptoms of concussion lasting from weeks to months. To date, no studies have shown a significant correlation between severity of injury and PCS; however, past history of concussion may be a risk factor for the development of PCS [19]. SIS

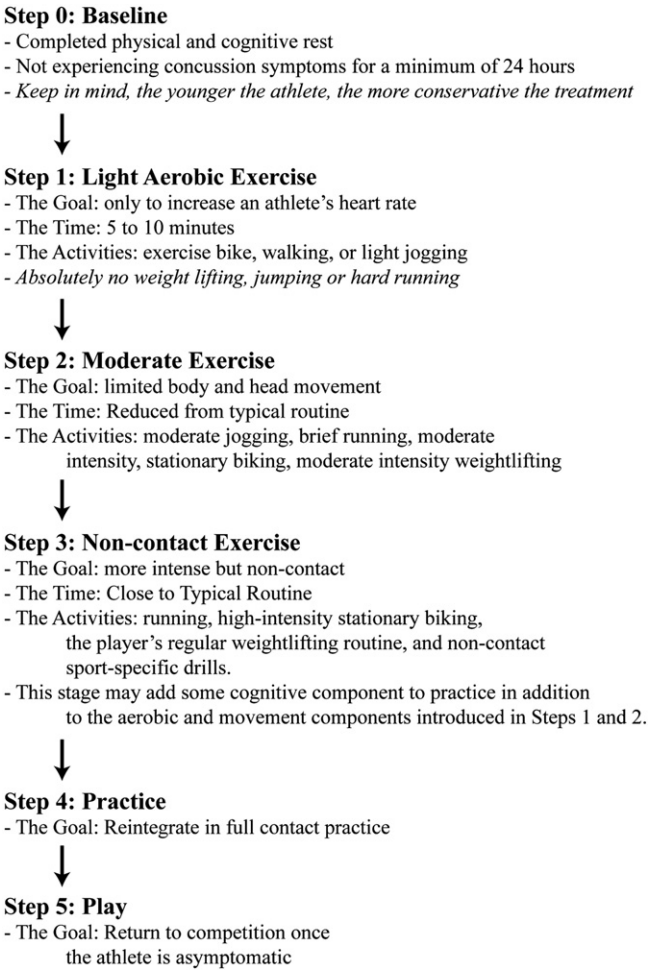

Fig. 1. Steps in the assessment for return to play decisions.

occurs when an athlete returns to competition too soon and sustains another concussion leading to significant sequela, including death [15]. SIS is exceedingly rare and has only been found to occur in athletes younger than 20 years of age. Finally, CTE is an increasingly recognizable phenomena, where the accumulative effects of head trauma lead to earlier symptoms of encephalopathy similar to what is seen in Alzheimer's Disease [20].

Moderate/severe traumatic brain injury

The establishment of standardized approaches has led to major advances in the treatment of moderate and severe TBI [4,21,22]. A significant complication in treatment protocols for TBI is the high likelihood of accompanying injuries to other organ systems as a result of overall trauma. The initial goal for acute treatment is to prevent hypotension and hypoxia to reduce the impact of secondary injury [23]. Assessment with the GCS may be helpful in guiding early intervention with subsequent treatment involving general emergency management, neurological examination, neuroimaging and possible surgical intervention [4,7,23]. Consistent monitoring of intracranial pressure, cerebral perfusion, fever, glucose levels and prevention of thromboembolism are all important aspects of the treatment for TBI [2,24].

Gender differences in treatment

Farace and Alves describe distinct forms of gender bias in published research on TBI outcomes. In meta-analyzing gender differences in traumatic brain injury outcomes (e.g., death, persistent vegetative state, and severe disability), the researchers reported that of the 9822 published studies available, only 40 described separate outcomes for each sex [25]. Thus, the authors concluded carefully that women fared worse in 85% of the measured variables [25]. Approximately a decade later, researchers at the German Sports University Cologne confirmed the tendency to ignore issues of sex and gender within sport and health related research. A content analysis of journal publications in sports medicine outlets identified only 7.2% of all studies that examined gender/sex as a variable of interest [26,27]. Despite this trend, recent studies indicate that there are significant differences in regard to the epidemiology, diagnosis, and outcomes of TBI between women and men. After examining discharge destinations of 3480 patients, Brown et al. concluded that following TBI, women were significantly more likely than men to be sent to long-term care facilities rather than the in-home setting [28]. Furthermore, differences in long-term disability or life satisfaction following TBI have been reported between women and men, with higher rates of post-concussion (women 50%, men 30%) and disability (women 52%, mean 37%) for women [29]. Moreover, Colantonio et al. [36] found evidence for very different support structures and resources available following release from an acute care setting for female TBI survivors. With a special focus on TBI in the setting of sports, Berz et al. reported that concussed female athletes at the age between 9 and 17 years scored higher on the post-concussion symptom scale compared to male athletes, suggesting that it may take longer for young female athletes to recover following sports-related concussion [30]. Likewise, high school and collegiate female athletes are more likely to suffer concussions compared to male athletes in analogous sports such as soccer, basketball, baseball, softball and ice hockey [13,31–33].

Sex differences in medical outcomes following TBI

In addition to gender differences in acute clinical care settings and with regard to psychosocial outcomes, several researchers have reported sex differences in medical outcomes following TBI including death, vegetative state, disability, and recovery. Many studies have linked sex differences in medical TBI outcomes to sexual dimorphism or differences in brain structure and anatomy [34]. Studies focusing on stroke risk and outcome revealed a protective effect of female sex hormones, such as estrogen and progesterone, in the outcome between women and men following TBI. However, sex differences in TBI have been shown to persist after menopause and in the pediatric population. This suggests that the impact of reproductive steroids may not exclusively explain sexual dimorphism in brain injury [35]. Additionally, TBI has been shown to affect the female cycle and its hormonal cascades by depression of female sex hormones. Colantonio et al. found disturbances (e.g., amenorrhea) within the female cycle up to 60 months after TBI [36]. Furthermore, in determining the causes of clinical depression following TBI, significant differences between women and men have been found with respect to cognitive and emotional adjustment. Women seem to experience higher levels of depression [5], more pain, and report sleep disturbances when compared to men [37–39]. These symptoms emerge with neuroendocrine dysfunction along with pituitary-target hormone disruption following TBI [40,41]. Given the critical correlation of psychosocial well-being and neuroendocrine responses [42], early detection of abnormalities in TBI-specific neuroendocrine biomarkers is important. Neuroscience research over the last decade has focused on cellular and molecular based approaches to identify the mechanisms of TBI and to improve diagnosis and prognosis of TBI. The difficulty lies in the identification of biological markers, which relate to clinical symptoms of TBI and the need to address the lack of understanding of the contribution of gender and sex-specific aspects to TBI biomarker discovery.

Limited laboratory support for TBI

A key component absent from the diagnosis and management of TBI is the use of clinical pathology laboratory results. Proposed blood-based biomarkers for TBI assessment continue to be studied and reported with little translation into routine clinical testing thus far. A recent review highlights the numerous shortcomings of currently proposed TBI biomarkers and is in agreement with the current review that greater adherence to clinical standards in research is required to properly present data to treating physicians [24]. In addition, an often overlooked aspect of blood-based biomarker research is the need for context specific reference intervals and medical decision points to enhance the sensitivity and specificity of putative biomarkers to a level required for their assimilation into the clinical management of TBI patients. Lastly, and perhaps most significantly, biomarker discovery in TBI has predominately focused on the acute stages of injury with little attention given to the utility of blood-based biomarkers at later time points.

Methods available for clinical TBI assessment

Conventional clinical immunoassays

Principles

Antibody-based methods of detection are utilized across nearly all divisions of laboratory medicine and a wide variety of platforms, detection methods and assay principles are available.

Automated platforms commonly utilize turbidimetry or nephelometry for spectrophotometric-based methods, with either rate or equilibrium measurements taken within a few minutes or up to an hour, respectively, after the initiation of the reaction. Both methods rely on the formed immune complex to scatter light and as a result can suffer reduced signal-to-noise levels in specimens such as serum with nonspecific light scattering due to high protein or lipid concentrations.

Antibodies conjugated to various types of labels can be found in automated FDA, approved immunoassays as well as highly manual, laboratory developed assays. Common labels used for detection include enzymes, fluorophores and chemiluminescent compounds. Alkaline phosphatase, β-D-galactosidase, glucose-6-phosphate dehydrogenase and horseradish peroxidase can produce products measured most

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often by chemiluminescence and fluorescence with an inherent amplification of signal by the enzyme label.

Enzyme-Linked Immunosorbent Assay (ELISA) is an antibody-based technique that has been widely used in clinical analyses for several decades. Sandwich ELISAs make use of a capture antibody and a detection antibody to form an antibody–antigen–antibody "sandwich". Sandwich ELISAs are best suited for large antigens such as proteins due to the requirement of two, unique epitopes for capture and detection. Competitive ELISAs are useful for the measurement of small targets such as drugs and drug metabolites where multiple epitopes are not practical. An enzyme-labeled target competes for binding to the capture antibody with any endogenous, non-labeled target present in the sample.

Major limitations in antibody-based assays have been well documented [43,44]. On the one hand, broad cross-reactivity of the antibodies used can enhance sensitivity by detecting multiple, structurally similar antigens [45,46]. On the other hand, this same cross-reactivity can result in poor specificity if non-related antigens are detected. In the presence of high antigen concentrations, false negatives can occur and is referred to as a "hook effect" while anti-reagent antibodies present in the sample can result in both false negative and false positive results. Reduction of these anti-reagent interactions requires the presence of non-immune serum or non-specific IgG from the same species where the assay antibodies were originally raised. Lastly, matrix components can significantly impact the performance of a developed ELISA necessitating the independent validation of performance characteristics across all desired specimen types and most often require the use of matrix-matched calibrators and controls.

Utility in biomarker studies

The majority of immunoassay-based methods currently available in the clinical laboratory provide independent analysis of each target of interest. Required sample volumes for nephelometric, turbidimetric and ELISA assays vary with limited capacity for multiplexed measurements. As a result, investigating the five putative markers listed above would require five separate aliquots of sample in addition to any excess sample needed in the event that dilution or repeat testing is required.

Multiplexed immunoassays

A growing interest in the simultaneous measurement of multiple analytes for diagnostic and prognostic purposes has immense implications for the clinical laboratory. Unfortunately, relatively few platforms capable of multiplexed, antibody-based measurements of proteins or small molecules have a significant presence in laboratory diagnostics in comparison to traditional single target assays. The multiplexed assays that are available can be divided into planar and suspension microsphere designs.

Principles

Planar immunoassays are similar in principle to the classic, single target immunoassays routinely used in most clinical diagnostics. The major difference with a multiplexed format is the application of numerous, unique microspots arranged in a reproducible, two-dimensional layout with each microspot containing specific capture antibodies. Each microspot will form an antibody–antigen–antibody sandwich after addition of a detection antibody. Although each microspot has the same method of detection, e.g., chemiluminescence, specificity of the signal is imparted by the *x*,*y* location. Intensity of the signal is used to determine concentration in quantitative assays through the use of a standard curve. Not surprisingly, reproducibility in *x*,*y* position and the amount of capture antibody deposited in the microspot are of outstanding concern and can introduce unwanted variability to the measurement system.

Suspension microsphere immunoassays are by far the more common design currently used for multiplexed antibody-based measurements [47] and are based upon the principles of flow cytometry. Capture antibodies are immobilized on unique microspheres that are distinguishable by size or fluorescence. An antibody–antigen–antibody sandwich will form through the use of a labeled detection antibody. Each formed sandwich will use the same type of label (e.g., fluorescence of phycoerythrin) that is used to determine the amount of antigen in the sample. The unique fluorescence signature of each bead present in the multiplexed assay provides the means for identification. Quantitation is possible by comparison of the resulting signal to that of a standard curve. Uniformity of microsphere size, fluorescence signal, and the amount of immobilized capture antibody are all possible sources of variability.

Utility in biomarker studies

Multiplexed immunoassays are routinely used in biomarker studies and have proven their utility in clinical diagnostic settings where measurements of multiple analytes are needed [48,49]. In addition, a single, simultaneous measurement is advantageous in situations where sample volume is a limiting factor. The use of a multiplexed approach is not without limitations, specifically in an environment as heavily regulated as the clinical laboratory. High complexity testing requires a comprehensive method validation including characterization of accuracy, intra and interassay imprecision, linearity, limits of detection, limits of quantitation and recovery. Further, quality control procedures increase in complexity in proportion to the degree of multiplexing. Matrix matched, quality control materials at multiple levels for each analyte are required in addition to established acceptability ranges for each. Multiplexed assays present further complications in regard to quality control acceptance criteria if one or more QC results fail to meet expected criteria for some but not all analytes.

Mass spectrometry

Mass spectrometry has been a pioneering technology for the field of laboratory medicine as nearly all disciplines from biochemical genetics to microbiology have benefited from its introduction [50]. Sample preparation methods range from a simple dilution of a sample to highly complex solid phase and liquid–liquid extractions. Gas chromatography and liquid chromatography remain the most prominent separation methods prior to analysis by single quadrupole, triple quadrupole and time-of-flight mass spectrometry [43].

Principles

In its simplest form, linear quadrupoles use a combination of radio-frequency and direct currents to stabilize the flight path of gas phase ions from the ionization source through a vacuum to the detector. Continuously sweeping the voltage settings during the analysis allows for all ions within an established mass to charge range (m over z or m/z) to eventually reach the detector. Alternatively, discrete combinations of voltages can be used to allow only specific ions of a predetermined m/z to reach the detector. Single quadrupole analysis coupled with gas chromatography remains a commonly used technique for qualitative toxicology screens [51].

Triple quadrupole mass spectrometry is frequently referred to as tandem mass spectrometry and is the technique of choice for clinical laboratories conducting quantitative measurements with mass spectrometry [43]. Two mass filtering quadrupoles (Q1 and Q3) are situated before and after a middle quadrupole collision cell (q2). At any given time, voltages within Q1 are set to stabilize transmission of ions of a single m/z, q2 is filled with an inert gas causing collision induced dissociation (fragmentation), while Q3 voltages are set to stabilize a single fragment of a specific m/z. Specificity of tandem mass spectrometry arises from the requirement of a given analyte to have the correct m/z precursor ion and the correct m/z product ion. The combination of a precursor and product ion is termed a transition and the majority of clinical tandem mass spectrometry assays monitor one transition for

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quantitation and monitor separate transitions for qualification to further enhance specificity.

Time-of-flight mass spectrometry is arguably the easiest form of mass spectrometry to conceptualize but is less prominently used in the majority of clinical mass spectrometry laboratories. Gas phase ions within an entire m/z range are introduced into a high vacuum flight tube by a uniform voltage. The resultant velocity of the ions is proportional to their m/z ratio, with low m/z ions traveling faster than high m/z ions towards the detector. Comparison to a reference solution containing known compounds at specific m/z ratios provides the positive identification of the analyte of interest. Coupled to liquid or gas chromatography, time-of-flight mass spectrometry provides a high-resolution, high accuracy method for detecting large numbers of analytes in a single sample. Time-of-flight mass spectrometry has begun to be applied successfully for routine drug analysis in various biological matrices [52–56].

Utility in biomarker studies

The majority of putative TBI biomarkers are large molecular weight proteins at the same time that clinical protein analysis by mass spectrometry is still currently out of reach for the average clinical mass spectrometry laboratory [57,58]. The multiplex capability of mass spectrometry has already been realized for small molecule analysis and targeted approaches to clinical proteomics are being actively pursued [59] and beginning their introduction into the clinical laboratory [60]. Despite a clear advantage and successful application in research and discovery, multiplexed analysis of putative biomarkers in the clinical laboratory by mass spectrometry has yet to be fully realized.

Point of care devices

The utility of point-of-care (POC) testing in laboratory medicine is a continuously debated topic often focusing on ease of use, turn-around-times, staff training requirements, central laboratory oversight, associated costs and increasing interest in accurately documenting clinical outcomes associated with their use [61]. POC testing has become commonplace in hospital emergency locations for cardiac marker assessment, coagulation and drug abuse testing in addition to glycemic control both in the clinical setting and for personal use at home [62]. Although the controversies that surround POC testing are not likely to be resolved easily or completely, the small, portable nature of a POC instrument together with the reduced requirement for sample volume and faster TAT [63] make these devices attractive for in-the-field assessments of TBI.

Principles

POC instruments vary considerably in complexity, analytical principle and overall size. The POC designs that are most relevant to TBI assessment include lateral flow and cartridge or cassette devices and are predominantly based upon similar principles to larger-scale sandwich or competitive immunoassays discussed in the Principles section. One key difference that can enhance the quality of the POC device is the incorporation of a built-in control to indicate successful operation of the assay due to the predominantly single-use nature of POC testing. Detection methods include visual observation by the operator, charge-coupled device cameras, absorbance, surface plasmon resonance, evanescent wave, fixed-polarized ellipsometry and diffraction [64–68]. The application of microfluidic technologies to POC testing provides a potential mechanism for small volume, cost-effective measurement across a wide range of applications [64].

Utility in biomarker studies

The availability of a portable, POC device for TBI assessment has major applications into the exiting diagnostic workflow; however, given even a near perfect POC device from an analytical standpoint the success of such a device relies heavily on the utility of the incorporated biomarkers. Numerous studies have been published on the application of POC devices for TBI assessment and the need to move from a single biomarker to a biomarker panel is widely appreciated [69]. Based upon the recognized difficulties with the transition of promising research findings into a clinical TBI biomarker panel, it is unlikely that a single panel will provide the required sensitivity and specificity to adequately serve all categories of TBI across the wide array of affected populations.

Putative biomarkers for the assessment of TBI

The list of putative biomarkers for traumatic brain injury continues to grow as does the conflicting results of their utility in various injury paradigms. The most thoroughly investigated biomarkers to date include S100B, Neuron Specific Enolase (NSE), Glial Fibrillary Acidic Protein (GFAP), Myelin Basic Protein (MBP), and Ubiquitin C-terminal Hydrolase-L1 (UCH-L1). Each of these markers is discussed briefly in the following sections with emphasis placed upon the reported performance characteristics of the various methods used.

S100B

Outcomes and findings

S100B belongs to the calcium binding EF-hand protein group with the origins of the family name based upon their solubility in a 100% saturated solution of ammonium sulfate at neutral pH [70]. The diverse roles of S100 proteins are still being identified in normal and abnormal settings and extend beyond simple calcium sensing [71]. S100B has compared favorably to CSF-serum albumin quotient, the gold standard for assessing blood–brain barrier permeability [72]; however, other studies have reported contradictory results [73,74]. The majority of reviewed studies indicated that S100B measurement, either acutely or at several time points, can distinguish injured from non-injured patients [75,76] with an uncertain degree of utility in predicting mortality. Urine measurements have been reported as an early predictor of short-term mortality but perform no better than previously determined serum measurements [77]. At present, S100B has largely become an acceptable biomarker of TBI; however, studies have begun to highlight the need to incorporate clinical symptoms instead of S100B concentration in isolation on the basis of inconsistent results across published studies [78]. APOE genotype was observed to correlate with maximal S100B concentration [79], and the recent report of the presence of auto-antibodies in the serum of football players with repeated blood–brain barrier disruption [80], based upon S100B measurements, illustrates the growing complexity in the laboratory assessment of TBI.

Performance characteristics of identified methods

The reviewed studies listed in Table 1 reporting on the utility of S100B predominantly used commercially developed immunoassays using ELISA-based or variations of chemiluminescent-based detection. Numerous studies made use of commercially available assays offered on automated chemistry immunoassay analyzers, a clear indicator of the prevalence and interest regarding S100B assessment. In addition, two relatively recent publications are available comparing several, commercially available immunoassays [81,82]. None of the reviewed assays explicitly indicated complete verification of assay performance or the use of quality controls and acceptability ranges. One assay cited validation data taken from the manufacturer and a separate study on the acceptability of the kit for use in urine measurements [77]. Of interest, one cited publication concluded that results from two, independent commercially available immunoassays were not interchangeable in serum and provided no correlation whatsoever in urine [83]. One assay established age specific reference intervals in 236 healthy children using an automated immunoassay [84].

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Table 1
S100B publications reviewed.

Publication	Study description	Reported method	Validation data	QC included	Reported findings
Blyth et al. [138]	Serum measurements compared to CSF albumin quotient Monomeric transthyretin also measured	ELISA (Nanogen, CA)	Limit of detection	Use of controls referenced	Concentrations correlated with BBB permeability
Kleindienst et al. [139]	Prospective study using CSF and serum measurements	Electrochemiluminescence IA (Roche Cobas e411)	Limited of detection Analytical measurement range	None referenced	Concentrations in CSF and serum not correlated with outcome Concentration in serum not a confirmed surrogate of BBB integrity
Pham et al. [74]	Characterization of tissue specificity for S100B serum measurements	ELISA (DiaSorin, MN) ELISA (Fujirebio Diagnostics, Japan) Electrochemiluminescence IA (Roche Diagnostics, IN)	Limit of detection Accuracy (Method comparisons)	None referenced	No relationship between serum concentration and Body Mass Index Characterization of CNS release of the B–B homodimer of S100B
Vos et al. [99]	Prospective study using serum measurements S100B also measured	STAT IntraOperative IA (Future Diagnostics, Netherlands)	None provided	None referenced	Validation of previously established cutoff concentrations Correlation with outcome prediction
Bellander et al. [73]	Prospective study using CSF and serum measurements in severe TBI Complement system activation and NSE also measured	Chemiluminometric IA (DiaSorin, Italy)	None provided	None referenced	Complement activation parallels increased S100B concentrations Concentration in serum not a confirmed surrogate of BBB integrity No difference in acute S100B concentrations between injury groups
Bohmer et al. [76]	Prospective study of severe TBI using CSF NSE and GFAP also measured	ELISA (DiaSorin, Italy)	*Referenced Rainey et al.*[140] Limit of detection	None referenced	S100B concentrations were increased compared to controls Significant association between concentration and clinical outcome
Bouvier et al. [84]	Reference interval establishment in children for S100B	Electrochemiluminescence IA (Roche Diagnostics, IN)	Linearity Intra-assay imprecision Limit of detection *Referenced Alber et al.* [141]	None referenced	Age specific reference intervals established Inverse correlation with head circumference reported
Rodriguez-Rodriguez et al. [77]	Prospective study using serum and urine measurements	Electrochemiluminescence IA (Roche Diagnostics, IN)	*Referenced Hallen et al.* [83] Imprecision (manufacturer provided) *Limit of detection* Analytical measurement range	None referenced	Urine measurements used as a predictor of mortality Urine equivocal in predictive power compared to serum
Wolf et al. [78]	Predictive power assessment of S100B concentration with clinical symptoms NSE also measured	Electrochemiluminescence IA (Roche Diagnostics, IN)	None provided	None referenced	S100 concentration useful in conjunction with clinical symptoms

Neuron specific enolase

Outcomes and findings

Neuron Specific Enolase (NSE) belongs to the superfamily of enolase enzymes predominantly known for their glycolytic role but their importance in a wide array of cellular functions and disease states continues to unfold [85]. Alpha, beta and gamma enolase have all been implicated in diverse pathologies consistent with their expression patterns and the gamma isoenzyme has been studied extensively in TBI due to its increased expression in neurons and cells of neuronal origin [85–87]. NSE in combination with S100B has been reported to aid in detecting intracranial hemorrhage in mild TBI when used in conjunction with clinical symptoms [78]. NSE on its own was reported to correlate with Glasgow Coma Score for moderate and severe TBI with modest sensitivity and specificity in predicting poor neurological outcomes [88]. The incorporation of NSE into a panel of biomarkers measured in CSF identified severe TBI patients for up to three days post-injury [76]. In pediatric patients, measurement of NSE over time was used to develop trajectory models for classification of outcome [89]. In direct comparison to other proposed biomarkers of TBI, NSE proved useful but less so in the acute phase of injury [90]. In the setting of mild TBI, a combination of NSE with S100B was found to add value in early prognosis of patients [91]. Recently, APOE genotype was observed to correlate with maximal NSE concentration; however, the finding was not statistically significant [79].

Performance characteristics of identified methods

The reviewed studies listed in Table 2 reporting on the utility of NSE used commercially developed immunoassays using ELISA-based or variations of chemiluminescent-based detection. The availability of commercial NSE immunoassays is predominantly attributed to its role in small-cell lung cancer [92,93], with one publication available on the performance characteristics of a commercially offered assay in the context of its use as a diagnostic test for small-cell lung cancer [94]. The majority of reviewed publications provided no validation data, with one publication referencing work conducted in an animal model [76] and others citing manufacturer claims. None of the reviewed assays explicitly indicated complete verification of assay performance or the use of quality controls and acceptability ranges. Of interest, one publication used a shotgun proteomic approach to identify candidate targets from CSF of severe TBI patients and identified several, prominent biomarkers including NSE [95].

Glial fibrillary acidic protein

Outcomes and findings

Glial Fibrillary Acidic Protein (GFAP) is an intermediate filament classically considered a marker of astrocyte reactivity with eight different isoforms expressed across numerous subsets of astrocytes. Since its initial days as an astrocytic-specific marker, GFAP has been shown to be

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Table 2
NSE publications reviewed.

Publication	Study description	Reported method	Validation data	QC included	Reported findings
Meric et al. [142]	Comparison of serum NSE between head and non-head trauma	Electrochemiluminescence IA (Roche Diagnostics)	Reference interval	None referenced	Increase in NSE in head trauma Correlation between NSE concentrations and GCS
Berger et al. [89]	Trajectory analysis in children	ELISA (International Point of Care, CA)	None provided	None referenced	Outcome correlated with trajectory of biomarkers
Honda et al. [90]	Comparison of NSE measurements in serum to alternate biomarkers S100B and GFAP also measured	ELISA (Alpha Diagnostics, TX)	None provided	None referenced	Potentially useful in the acute phase of injury
Bohmer et al. [76]	Prospective study of severe TBI using CSF S100B and GFAP also measured	Electrochemiluminescence IA (Roche Diagnostics, IN)	*Referenced Busnello et al.*[143] *(animal model)* Imprecision	None referenced	NSE concentrations were increased compared to controls Acute elevations of NSE accurately predicted mortality
Topolovec-Vranic et al. [91]	Prospective measurement of NSE in serum in mild TBI S100B also measured	Electrochemiluminescence IA (Roche Diagnostics, Quebec)	None provided	None referenced	Added value in early assessment for prognosis
Wolf et al. [78]	Predictive power assessment of NSE concentration with clinical symptoms S100B also measured	Electrochemiluminescence IA (Roche Diagnostics)	None provided	None referenced	NSE concentration useful in conjunction with clinical symptoms

widely expressed in numerous cell types inside and outside the central nervous system [96]. GFAP measurements have provided promising data on injury pathway indication [97], focal versus diffuse injuries [98], and prediction of morbidity and mortality [99–101] while other studies provide a less positive outlook [102] or the need to measure more specific break down products [103,104].

Performance characteristics of identified methods

The reviewed studies listed in Table 3 reporting on the utility of GFAP used laboratory developed ELISA-based tests and one commercially developed immunoassay. For several of the laboratory ELISA tests, substantial validation data was provided in the form of a reference to the original method publication [99,100]. The publication containing the greatest amount of method validation data provided a near complete validation for a clinical assay conducted in a CLIA-certified laboratory [105]. None of the reviewed studies clearly indicated the use of established quality control samples or acceptability ranges; however, a single assay specified the use of a negative control [99]. Of note, several studies reported on the detection of GFAP break down products (GFAP-BDP) with one reviewed publication reporting on the cross reactivity profile for the antibodies used in their specific laboratory developed sandwich ELISA [103]. Of interest, one study in a cell culture injury model measured GFAP by LC–MS/MS targeted quantitation using an isotope dilution approach (IDA) [106].

Myelin basic protein

Outcomes and findings

Myelin Basic Protein (MBP) is a critical constituent of the insulating myelin sheath surrounding axons. Though typically referred to as a singular protein, there are numerous isoforms of MBP ranging from 14 kDa to 21.5 kDa with the predominate isoform varying amongst species [107]. The interest in MBP as a biomarker of TBI has lessened in comparison to S100B, NSE and GFAP in large part due to an observed lack of clinical sensitivity [108].

Performance characteristics of identified methods

The reviewed studies listed in Table 4 reporting on the utility of MBP used commercially available assays from both Canada and the United States. No validation data was provided in any of the studies reviewed nor was there reference made to quality control materials used during sample analysis. Commercially available ELISA kits are typically marketed as research use only (RUO) with the responsibility falling to the clinical laboratory to verify any performance characteristics established and reported by the manufacturer.

Ubiquitin C-terminal Hydrolase-L1

Outcomes and findings

Ubiquitin C-terminal Hydrolase-L1 (UCH-L1) is an E2 ubiquitin-conjugating enzyme expressed in neurons where it functions to add and remove ubiquitin to proteins intended for degradation [109]. UCH-L1 is one of the newest proposed biomarkers for TBI and as a result there is limited data available to demonstrate its utility. The published data suggest that UCH-L1 may be a useful serum biomarker for severe TBI [110–112] and the ratio of GFAP to UCH-L1 in the serum has been proposed as a method to determine focal versus diffuse TBI injuries [97,98].

Performance characteristics of identified methods

The reviewed studies listed in Table 5 reporting on the utility of UCH-L1 used laboratory developed ELISA-based tests with validation data predominantly resting on a single publication with limited imprecision, linearity and detection limit data in an animal model of TBI [113]. Of the five publications reviewed three indicated the use of internal controls [97,98,112]. One publication provided evidence of recovery data by comparing expected versus calculated calibrator concentrations and

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Table 3
GFAP publications.

Publication	Study description	Reported method	Validation data	QC included	Reported findings
Lumpkins et al. [101]	Prospective study of severe TBI using serum	ELISA (Biovendor, NC)	None provided	None referenced	Persistent elevation after two days predictive of increased mortality
Vos et al. [99]	Prospective study using serum measurements S100B also measured	STAT IntraOperative IA (Future Diagnostics, Netherlands)	*From Vissers et al.*[144]	Controls without detectable GFAP	Validation of previously established cutoff concentrations Correlation with outcome prediction
Wiesmann et al. [100]	Prospective study using serum measurements S100B also measured	Dissociation-enhanced lanthanide fluorescence immunoassay (LDT)	Reference interval establishment From Missler et al. [105] Imprecision Analytical measurement range Accuracy (Method comparison to ELISA — Van Geel et al., [145]) Linearity Sample stability Analytical measurement range Recovery Linearity Intra- and interassay imprecision Interferences (hemolysis, lipemia) Hook effect Sample stability	None referenced	Elevated after trauma Rapid decline in concentration
Bohmer et al. [76]	Prospective study of severe TBI using CSF NSE and S100B also measured	ELISA (LDT)	*From Tramontina et al.*[146] Analytical measurement range Linearity Interassay imprecision Recovery Interference (phosphorylated GFAP)	None referenced	Acute GFAP assessment of limited utility No significant association between concentration and clinical outcome
Mondello et al. [97]	Ratio to UCH-L1	ELISA (LDT)	None provided	Internal controls referenced	GFAP/UCH-L1 > 1 observed in focal mass lesions GFAP/UCH-L1 < 1 observed in diffuse injury
Mondello et al. [98]	Prospective study using serum measurements Ratio to UCH-L1	ELISA (BioVendor, Czech Republic)	None provided	None referenced	Biomarkers may be useful to determine injury pathway
Metting et al. [102]	Prospective study of mild TBI using serum	ELISA (Biovendor GmbH, Germany)	Limit of Detection Intra-assay imprecision Analytical measurement range	None referenced	Correlation between concentration and imaging studies Not an independent factor of outcome

Table 4
MBP publications reviewed.

Publication	Study description	Reported method	Validation data	QC included	Reported findings
Yamazaki et al. [147]	Acute head injury NSE also measured	Disequilibrium radioimmunoassay	None provided	None referenced	Internal jugular and peripheral blood concentrations equivocal
Berger et al. [148]	Prospective study in children NSE and S100B also measured	ELISA (SynX Pharma, ON, Canada)	None provided	None referenced	MBP concentrations increased in majority of pediatric TBI
Berger et al. [149]	Prospective study in children NSE and S100B also measured	ELISA (Nanogen Corp, CA)	Established cutoff values per Berger et al. [148]	None referenced	Potential utility of MBP as a screening test for infant TBI
Berger et al. [150]	Prospective study in children NSE and S100B also measured	ELISA (Nanogen Corp, CA)	Established cutoff values per Berger et al. [148]	None referenced	Biomarker peak time varied amongst TBI type
Berger et al. [89]	Trajectory analysis in children	ELISA (International Point of Care, CA)	None provided	None referenced	Outcome correlated with trajectory of biomarkers

imprecision of <10% and <15% for CSF and serum, respectively, using this recovery data [111]. One study noted earlier measured UCH-L1 by LC–MS/MS targeted quantitation using an isotope dilution approach (IDA) in a cell culture injury model [106].

Gender differences in biomarker studies

Considerations of sex and gender in blood-based TBI biomarker performance

The sensitivity and specificity of any quantitative test are dependent on the cut-off value above or below which the test is positive [114]. Accordingly, disregarding sex/gender can lead to gender bias in TBI research leading to test verification bias resulting in a lower observed specificity of clinically appropriate TBI biomarkers [115].

Markers of TBI have distinctive features and cellular origins representing a diversity of cellular injuries and injury patterns following different types of primary injuries. The specific impacts of gender/sex on the most common blood-based TBI biomarkers S100B, NSE, MBP, GFAP, and UCH-L1 are not well investigated or documented. Even though S100B is one of the most extensively studied biomarkers [116,117], limited studies have focused on the influence of sex/gender on the utility of this biomarker. In 1992, van Engelen et al. were the first to examine whether age and sex were associated with an increase of S100B levels in cerebrospinal fluid (CSF) [118]. Despite collection of 79 specimens of CSF from children and adults no sex dependency was observed. Subsequently, Wiesmann et al. detected similar increases in S100B plasma levels of healthy subjects between 18 and 25 years of age but they could not confirm an effect of sex on alterations in S100B levels within this age group [119]. In examining a younger population, Gazzolo et al. investigated 1004 healthy children (males, n = 482; females, n = 522) age 1 month to 15 years and found significantly higher peripheral S100B concentrations in female pediatric patients [120]. A relationship between age and sex was shown as well by Sannia et al. as was a dependency of S100B urine concentrations in late preterm infants on the gestational age and infant's sex [121]. Even though sex differences in healthy adults have not been confirmed, sex-specific differences in the increase of serum S100B levels in adult depressive patients have been reported. Examination of 54 adult patients with major depression found significantly higher serum S100B levels in female patients compared to male patients and confirmed the presence of sexual dimorphism in altered neurophysiology [122].

Like the protein S100B, NSE is not secreted into extracellular space under physiological conditions. However, NSE is detectable in extracellular fluids after axon injury due to leakage and up-regulation in an attempt to maintain homeostasis [117]. Given its location and abundance, NSE in body fluids should possess relatively high specificity and sensitivity for axonal injury [117]. So far, clinical studies show no effect of patient's sex on NSE levels in human biological fluids. Initially, Abramson et al. [123] excluded sex-specific effects on NSE levels in aqueous humor. Furthermore, NSE levels in the CSF of patients with and without neurological disorders were observed to vary independent of the patient sex [124-126]. Similarly, a study on CSF concentrations of NSE in children and adults with diagnostic lumbar puncture found no sex dependency but a relative increase of NSE with age was observed, emphasizing the necessity of age-matched reference intervals [118]. Over the course of time, little influence of sex on NSE levels has been reported for serum levels of epileptic and depressive patients [127] or lung cancer patients [93,128]. Interestingly, a study by Nygaard et al. in 1998 demonstrated age- and sex-dependency of NSE concentrations in CSF, increasing with age from 21 to 84 years and comparatively higher concentrations in male than in female patients with various surgical procedures in spinal anesthesia [129]. This sex and age-related dependency of NSE has not been shown elsewhere and the explanation for this finding remains unclear.

In addition to S100B and NSE, UCH-L1 is one of the most widely studied biomarkers for TBI [130]. To date, only a few studies have

Table 5
UCH-L1 publications reviewed.

Publication	Study description	Reported method	Validation data	QC included	Reported findings
Brophy et al. [112]	Comparison of CSF and serum measurements	ELISA (LDT)	From Liu et al. [113] (animal model) Limited imprecision Linearity LOD (calculated)	Internal controls referenced	Correlation between CSF and serum Elevated in TBI compared to controls Serum measurements correlated with 3 month survival
Papa et al. [110]	Prospective study in CSF	ELISA (LDT)	None provided	None indicated	Elevated after severe TBI 24-hour concentration predictive of 6-week mortality
Mondello et al. [97]	Serum measurements; ratio to GFAP	ELISA (LDT)	None provided	Internal controls referenced	GFAP/UCH-L1 > 1 observed in focal mass lesions GFAP/UCH-L1 < 1 observed in diffuse injury
Mondello et al. [98]	Prospective study using serum measurements Ratio to GFAP	ELISA (BioVendor, Czech Republic)	None provided	None referenced	Biomarkers may be useful to determine injury pathway
Mondello et al. [111]	CSF and Serum measurements in severe TBI	ELISA (LDT)	Recovery by calibration verification Limited imprecision using recovery data	Internal controls referenced	GCS score correlated with UCH-L1 in CSF and serum

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described sex-specific aspects in regard to UCH-L1 levels in CSF or peripheral blood. In 2012 Mondello et al. evaluated the ratio between GFAP and UCH-L1 concentrations (GNR) to investigate the relationship of this ratio with neuroimaging profiles in patients with severe brain trauma. They observed that GNR was independent of sex, age, GCS score and the mechanism of injury [98].

Similar to S100B, NSE, and UCH-L1, peripheral levels of MBP appear to be a very specific marker of TBI although no results on MBP in CSF are currently available [131]. In 1992, van Engelen et al. obtained reference values for MBP in children and adults of different ages and both sexes for the first time. They reported an increase of MBP in CSF with age but no significant differences between female and male patients between 7 months and 66 years of age [118].

Comparable to other TBI biomarkers, GFAP concentrations do not seem to be influenced by sex at first glance. In a study to develop an ELISA assay for human autoantibody to glial fibrillary acidic protein in Alzheimer's disease, the titer of GFAP antibody showed no significant correlation with age or with sex in the control serum [132]. Nevertheless, studies using animal models to demonstrate astroglial reactivity showed sexual dimorphism in the distribution of GFAP indicating an influence of sex steroids [133–137].

Conclusion

In summary, a brief overview of common TBI biomarkers – S100B, NSE, GFAP, MBP, and UCH-L1 – provides contradictory results in regard to the overall utility in diagnostic and prognostic roles at the same time highlighting the often overlooked influence of gender on biomarker performance. The growing consensus in society and health sciences regarding the necessity to improve excellence in scientific research by incorporating the categories of sex and gender into the research design is accompanied by a need to fully validate the performance of assays used in clinical research. The clinical laboratory has an unprecedented number of new and long-standing methods available to aid in medical decision making and guide diagnosis. As sex and gender aspects in blood-based TBI biomarker performance need further attention, so too must a higher priority be given to the proper validation of all research methods used in the clinical setting, the use of quality control materials and the explicit incorporation of this data into publications. Based upon the elegant array of methods currently operating in clinical laboratories world-wide, it seems that the clinical laboratory not only is ready for its role in TBI assessment but also is poised to help lead by example in generating high quality, reproducible data.

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