Rapid Analytical Methods for On-Site Triage for Traumatic Brain Injury*

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Abstract

Traumatic brain injury (TBI) results from an event that causes rapid acceleration and deceleration of the brain or penetration of the skull with an object. Responses to stimuli and questions, loss of consciousness, and altered behavior are symptoms currently used to justify brain imaging for diagnosis and therapeutic guidance. Tests based on such symptoms are susceptible to false-positive and false-negative results due to stress, fatigue, and medications. Biochemical markers of neuronal damage and the physiological response to that damage are being identified. Biosensors capable of rapid measurement of such markers in the circulation offer a solution for on-site triage, as long as three criteria are met: (*a*) Recognition reagents can be identified that are sufficiently sensitive and specific, (*b*) the biosensor can provide quantitative assessment of multiple markers rapidly and simultaneously, and (*c*) both the sensor and reagents are designed for use outside the laboratory.

1. INCIDENCE, SEVERITY, AND COSTS OF TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is defined as injury to the brain caused by external physical forces. The injury can be classified as penetrating (from firearms, shrapnel, or other pointed objects) or nonpenetrating. Penetrating TBI tends to be focused on a specific region of the brain and is usually characterized by hemorrhages, hematomas, and lesions. Nonpenetrating TBI is harder to determine because its effects are spread throughout part or all of the brain, causing disruption of neurological functions and diffuse axonal damage. In most cases, this type of injury is caused by the rapid acceleration and deceleration of the brain in a linear plane, in a rotational direction, or in a combination of the two. Macroscopic lesions may not be present upon imaging after injury.

Although the overall rate of death related to TBI has decreased, it is still a major cause of death and disability worldwide and takes a toll physically, emotionally, and economically (1). An estimated 1.7 million people in the United States who sustain TBI require medical treatment annually (1–4). Whereas most brain injuries result in concussion or mild TBI, 16% of all TBI cases require hospitalization and 3% of patients succumb to the injury. It is estimated that a large percentage (up to 25%) of people with TBI never seek medical care. The leading causes for such civilian brain injuries are automobile accidents, sports, and accidental falls. Although most cases of TBI are associated with accidental injury, there are numerous cases in which the injury occurs as a result of violence or firearms (nonmilitary) (5). Violence-related TBIs can be the result of an intentional assault to the head caused by direct contact with fists or hard objects or a consequent fall. An indirect violence-related injury can be caused by intense shaking, as is seen in shaken baby syndrome.

TBI has also received significant attention in the military community. Although improvements in battlefield medical response and widespread use of body armor have decreased mortality rates well below those of previous conflicts, an ever-larger number of military personnel are returning home with significant wounds (6). Estimates of deployed personnel affected with some form of TBI range upward of 20% (7–10), and over 212,000 cases of combat-associated TBI were clinically diagnosed between January 2000 and February 2011 (11). TBI has thus been considered the signature injury of the current conflicts in Iraq and Afghanistan. The U.S. military health care system spent $192 million on direct and purchased care for TBI patients in fiscal year 2010 alone (12).

TBI can have a long-range impact on the life of a person—medically, emotionally, and economically. Even mild TBI can last weeks to months before the symptoms subside. Changes in brain function due to the injury can lead to alterations in mood, reactions, memory, perceptions, and motor coordination. Severe TBI can result in the patient requiring medical and rehabilitation treatment for the remainder of his or her life. It is estimated that the economic cost of TBI in the United States is over $50 billion dollars annually (1).

Perhaps more worrisome are the long-term sequelae associated with TBI. Causal relationships between penetrating TBI and both unprovoked seizures and premature death have been established. Additional positive correlations exist among moderate to severe TBI and unemployment, aggressive behavior, depression, Alzheimer's disease–type dementia, and long-term social function in both children and adults (13). Furthermore, tau tangles and β-amyloid plaques, hallmarks of neurodegenerative diseases such as Alzheimer's, are observed shortly after injury and may still be present even years after a single moderate-to-severe TBI or repetitive head injuries (tau tangles only) (14–16).

2. CURRENT DIAGNOSTIC METHODS

The primary method for identifying nonpenetrating TBI or concussion is based on self-reporting or reporting by a witness that an insult to the head (blunt object or blast injury) has occurred and that the injured person has had symptoms such as loss of consciousness, dizziness, headache, nausea and vomiting, and confusion. Initial assessment by emergency personnel or primary physicians is the Glasgow Coma Scale (GCS), which classifies the TBI as mild, moderate, or severe (**Table 1**) (17; **http://www.traumaticbraininjury.com/content/symptoms/glasgowcomascale.html**). The GCS is a rapid assessment based on level of consciousness and coherence as determined by eye responses in addition to verbal and motor capabilities. This evidence-based assessment is routinely carried out by emergency medical technicians and emergency room personnel to rapidly triage the patient. However, other injuries, age, and breathing apparatus may interfere with verbal and motor responses. In addition, medications that are known to cause drowsiness, such as allergy medications, can affect the validity of this test.

Another evidence-based assessment used by physicians is the Acute Concussion Evaluation (ACE) form (1). It is part of a toolkit developed by the Centers for Disease Control and Prevention. The symptom checklist includes changes in physical, cognitive, emotional, and sleep characteristics after the incident, as well as risk factors due to previous concussions, migraines, attention-deficit/hyperactivity disorders, and psychiatric history. An aspect of this evaluation considers changes in physical and cognitive activities upon exertion, given that some symptoms may not be observable without exertion. A similar assessment, known as the MACE (for Military Acute Concussion Evaluation), is used by the U.S. Department of Defense for initial screening of military personnel (18, 19). The possibility that some personnel memorize the answers so that they can pass the test and return to duty has raised concerns about the effectiveness of the test and implications for the safety of the military unit.

More recently, two other neurocognitive tests, the ANAM (Automated Neuropsychological Assessment Metrics) and the ImPACT (Immediate Post-Concussion Assessment and Cognitive Testing), have been employed by the military and sports organizations, respectively, for postconcussive evaluation of cognitive functions (18, 20–22). They provide useful tools for evaluating an injured person's ability to return to duty or to the playing field. However, a baseline test prior to any possible head injury needs to have been performed for determining changes in attention, memory, or thinking abilities. Concerns have been expressed about the results from such tests when used on site because the results can be affected by fatigue, stress, and medications (23). Both tests yield between 25% and 34% false-positive results when tested in combat or sports environments. The tests may be more useful for monitoring recovery following a concussion than for initial diagnosis of mild to moderate TBI.

A recent addition to the battery of on-site tests for neurological impairment is the King-Devick test for concussion (24). This test does not assess TBI but has been suggested as a useful

Table 1 Traumatic brain injury categories

Severity	Glasgow Coma Scale	Alteration in consciousness[a]	Loss of consciousness	Posttraumatic amnesia	Imaging (CT/MRI)
Mild	13–15	≤24 h	0–30 min	≤1 h	Negative
Moderate	9–12	>24 h	30 min–24 h	1–7 days	Positive or negative
Severe	–3–8	>24 h	≥24 h	≥7 days	Positive

[a]Dazed, confused, even momentary loss of consciousness. Abbreviations: CT, computed tomography; MRI, magnetic resonance imaging.

and simple method for evaluating athletes for concussion as a result of sports injury. The test evaluates impairment of eye movements, attention, language, and other correlates of suboptimal brain function. It is not quantitative, but it may be a good "first start" for on-site evaluation because it can be performed by non–technically trained operators.

If symptoms and the evidence-based assessments indicate possible concussion, the next step is further evaluation with imaging of the head and brain. Computerized axial tomography (CAT or CT) scans are used to detect skull fractures, large hemorrhages, and hematomas (25). Although CAT scans may be useful in identifying injury to the brain region in severe and moderate TBI, they are often not useful in mild TBI in which there is no obvious damage to the brain (26). Magnetic resonance imaging (MRI) can be employed to obtain finer resolution and more information on the brain tissue but is often not performed because there are many drawbacks, including the length of time needed to perform the evaluation, complications due to metal embedded in the body, and lack of patient tolerance for the closeness and noise of the device. Recently, newer MRI techniques have been used that can discern microscopic damage to the brain. Susceptibility-weighted imaging (SWI), diffuse tensor imaging (DTI), magnetic resonance spectroscopy imaging (MRSI), and other imaging techniques have demonstrated applicability for analysis of particular aspects of heterogeneous TBI pathologies (27). SWI is often used for hemorrhage detection, whereas DTI is used for edema quantification and axonal injury identification. MRSI is used to measure brain metabolites and various biochemical processes in vivo at the cellular level. Although these imaging techniques are sensitive, their use is limited by factors such as availability, size, cost, and the need for trained operators. At this stage, these technologies are impractical for military applications in theater and more routine triage by emergency personnel.

Although imaging is currently considered the gold standard for TBI diagnosis, there is a need for rapid, sensitive, and specific diagnostic tests that can be used for on-site triage, as well as for follow-up patient management and treatment purposes. An easy-to-use, analytical diagnostic method to provide information about neurobiological processes in the affected individual has the potential to determine TBI status. In addition, this method would probably decrease the incidence of over- and undertriage of potential patients, as well as provide important data for the development of more effective therapeutic interventions. Although there are presently no objective, blood-based diagnostic tests for TBI, there is a significant drive toward development of analytical tests for assessing biochemical markers that could be used to determine the severity and type of TBI and the pathological sequelae from brain injuries.

3. PATHOPHYSIOLOGY OF TRAUMATIC BRAIN INJURY

To understand how rapid detection of biomarkers could provide information on the type and the severity of TBI, we present a brief background of the pathophysiology of TBI. Damage from TBI progresses in stages. The first stage, primary injury, is characterized as direct tissue damage resulting from mechanical force on the brain and includes contusions, lacerations, and damage due to shearing. Secondary injury, however, develops as a sequela of primary injury via a series of biochemical, cellular, and physiological processes that contribute to tissue damage and cell death. The timeline involved in these secondary processes extends from several minutes to weeks or even years after the initial insult. The myriad pathways and processes involved in secondary injury are interrelated and produce a complicated mixture of physiological responses and symptoms that overlap considerably with those associated with other neuropathologic syndromes, such as amyotrophic lateral sclerosis and Parkinson's, Alzheimer's, and Huntington's diseases.

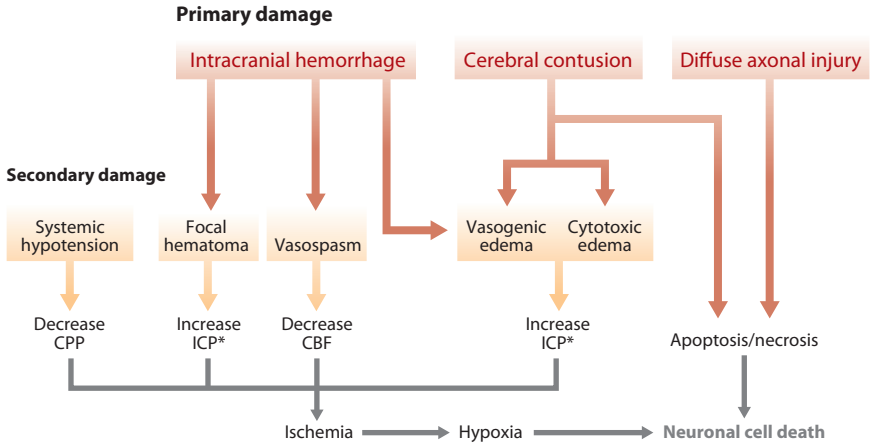

Figure 1

Pathophysiology of brain injury. Abbreviations: CBF, cerebral blood flow; CPP, cerebral perfusion pressure; ICP, intracranial pressure. The asterisks indicate the presence of ICP biomarkers C-tau, GFAP, and S100β.

3.1. Physiological Processes

On a gross anatomical level, initial damage occurs when the brain experiences rapid acceleration and deceleration, an overpressure wave associated with a blast, or penetration of foreign material. Depending on the type of injury, tissue is mechanically damaged upon impact with the skull or in diffuse regions through shear, lacerations, and tearing of cellular membranes and tissue structures (**Figure 1**). The blood-brain barrier and vasculature may likewise be disrupted, resulting in direct access of blood constituents to the brain tissues, where they could influence injury processes. In extreme cases, diffuse axonal injury (DAI) may occur where shear forces are sufficient to cause widespread disruption of axon structures and myelin sheaths, resulting in impaired axonal transport. DAI is the underlying injury in shaken baby syndrome.

Edema frequently occurs after TBI and may be attributed to fluid buildup in tissues following disruption of the blood-brain barrier and damage to cell walls in the cerebral vascular endothelia (vasogenic edema), osmotic imbalances between blood and tissue (osmotic edema), and cellular responses to biochemical cascades (cytotoxic edema). Cerebrovascular autoregulation—the ability of the cerebral vasculature to constrict or dilate in response to cerebral perfusion pressure—is often impaired following TBI; vasospasm may also be present in severe cases and is highly correlated with a negative prognosis. Increased intracranial pressure caused by cerebral edema and/or hematoma formation may lead to hypoperfusion, accumulation of lactic acid, and ischemia; posttraumatic ischemia has been associated with poor neurologic outcome.

3.2. Cellular and Biochemical Processes

The physiological changes that occur with TBI are correlated to processes that occur on a cellular and biochemical scale (**Figure 2**). These cellular and biochemical processes are not yet fully understood, but they constitute a complex series of interrelated and often overlapping processes that contribute to secondary injury. At least three major mechanisms are responsible for secondary cellular damage following TBI: excitotoxicity, necrosis or apoptosis, and oxidative stress. The mobilization and migration of immune cells also play a key role in mounting an inflammatory response to TBI. Each of these biochemical cascades, discussed briefly below, represents a potential

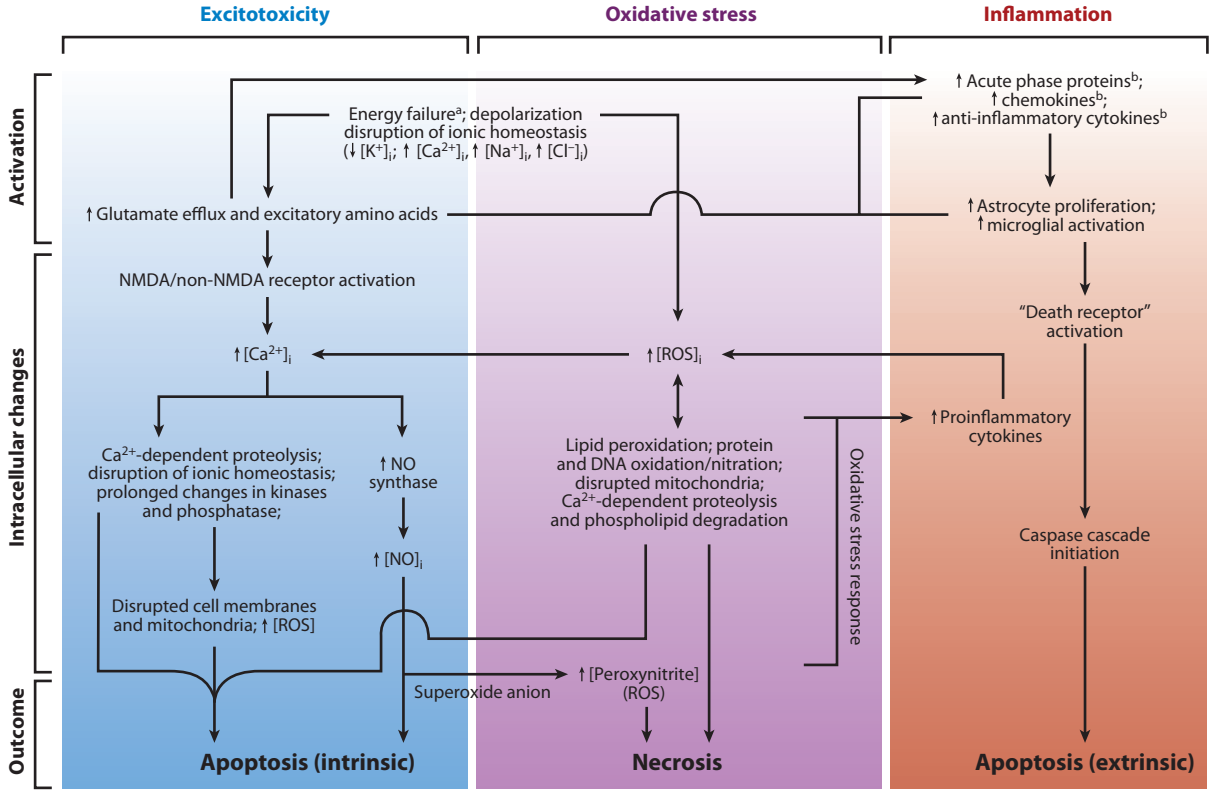

Figure 2

Biochemical cascades following brain injury. The up arrow indicates an increase or upregulation; the down arrow indicates a decrease or downregulation. The brackets with subscript i enclose an intracellular element. Superscript a refers to energy failure (↑ glycolysis, ↓ ATP, ↓ pH); superscript b refers to acute-phase proteins [e.g., C-reactive protein, amyloid A, interleukin (IL)-1, IL-6, tumor necrosis factor α], anti-inflammatory cytokines (e.g., IL-10, transforming growth factor β), and chemokines [e.g., intercellular adhesion molecule 1, macrophage inflammatory protein (MIP)-1, MIP-2]. Abbreviations: NMDA, *N*-methyl-D-aspartic acid; NO, nitric oxide; ROS, reactive oxygen species.

source of biomarkers that may be used to determine the location and severity of injury. Note that many components of these pathways are not associated exclusively with TBI but may be present in other neuropathological conditions. Several excellent, in-depth reviews of the biochemistry and cell biology associated with TBI pathology have recently been published (32–35).

3.2.1. Excitotoxicity. Excitotoxicity is defined as continuous and excessive stimulation of postsynaptic receptors by a neurotransmitter, resulting in cell injury and death. As the sentinel event for injury by excitotoxicity following TBI (36), glutamate levels rise within synapses and extracellular spaces as a result of its release from neuronal cell synapses, through disruption of the blood-brain barrier, and extravasation at the site of mechanical impact.

At the same time, numerous ligand-gated ion channels are activated, leading to efflux of K^+, massive influx of Na^+ and Ca^{2+} into postsynaptic neurons, and prolonged membrane depolarization. The resultant disruption of ionic homeostasis in turn stimulates energy-dependent pumps (presumably to restore ionic balance), increasing metabolic demand and activating glycolysis. As a result of increased glycolysis, cellular ATP stores become depleted, leading to the accumulation

of lactic acid in brain tissues. Simultaneously, glutamate transport into astrocytes and microglia, which is required to maintain correct extracellular glutamate levels under physiological conditions, is inhibited by quinolinic acid, another excitotoxic compound released with glutamate.

Excitotoxicity is further exacerbated by an intricate cross talk between the inflammatory cascade and glutamate receptors, a process described as immunoexcitotoxicity (38). Immunoexcitotoxicity is postulated to play an important role in chronic traumatic encephalopathy and the so-called two-hit syndrome. When primed by prior insults, microglia may become hyperreactive, releasing high levels of cytokines and other immune mediators, as well as glutamate, aspartate, and quinolinic acid. Furthermore, tumor necrosis factor α (TNF-α) can upregulate glutaminase, resulting in enhanced conversion of glutamine to glutamate within the astrocytes and thereby increasing astrocyte intracellular glutamate stores with potential for release. Finally, as parts of a vicious circle, proinflammatory cytokines further activate release of excitotoxins from microglia and astrocytes, while excitotoxins likewise activate release of proinflammatory cytokines from the same cells; moreover, oxidative stress (induced by inflammatory cytokines and excitotoxins) further interferes with glutamate clearance and intensifies immunoexcitotoxicity over a prolonged period of time (37). This effect is further enhanced through TNF receptor–mediated increases of ionotropic glutamate receptor trafficking, rendering microglial membranes more sensitive to stimulation by glutamate.

3.2.2. Apoptotic and necrotic pathways. Excitotoxic depolarization and activation of ion channels lead to tremendous increases in ionic flux. One result is a significant accumulation of free intracellular Ca^{2+} both due to massive influx of Ca^{2+} and through its release from intracellular stores, such as mitochondria. These increased levels of intracellular Ca^{2+} activate a number of Ca^{2+}-dependent enzymes (e.g., proteases such as calpains and caspases, phospholipases, and endonucleases), thereby activating necrotic and/or apoptotic pathways and eventually leading to cell death.

Necrotic pathways proceed primarily through calpain-mediated degradation of cytoskeletal elements such as spectrin, neurofilaments, and microtubule-associated protein 2, leading to loss of mitochondrial and overall structural integrity of the brain cells (30). Structural damage, such as dendrite beading, microtubule disruption, and reduction in organellar structure, occurs through the action of quinolinic acid, another excitotoxic compound. Recent intriguing results indicate that mechanical stress or strain on integrins may result in similar cytostructural damage due to integrin-mediated activation of downstream effectors of cytoskeletal rearrangement, neurofilament polymerization, and microtubule instability (39); integrin-mediated pathways may also play a role in TBI-induced vasospasm (40).

Caspases mediate neuronal apoptosis via both intrinsic and extrinsic pathways. The intrinsic apoptotic pathway involves the recruitment and activation of caspases by cytochrome *c* released from disrupted mitochondria. Extrinsic apoptosis involves the binding of TNF family proteins to their corresponding receptors, formation of death-inducing signaling complexes, and subsequent recruitment of caspases. Programmed cell death may also be induced following TBI by a caspase-independent pathway (41, 42). Whether caspase dependent or caspase independent, apoptotic processes may involve an interplay between prodeath and prosurvival signaling pathways and may extend for hours or months beyond the initial brain injury. Furthermore, because some dying cells exhibit characteristics of both necrotic and apoptotic pathways, it has been proposed that these pathways are not mutually exclusive but rather that there exists a continuum between the two.

3.2.3. Oxidative stress. Secondary injury by oxidative stress is related to the destructive actions of high levels of intracellular reactive oxygen species (ROS) and free radicals following TBI. Both

the formation of ROS and free radicals and their injurious effects are closely linked to apoptosis, excitotoxicity, and inflammation, although the full extent of these interactions is still not fully understood.

Whereas ROS and free radicals are always present at low levels within neuronal cells, intracellular levels increase drastically following TBI. This increase may represent an increase in ROS and free radical production, a decrease in clearance or destruction, or both; it has been attributed to numerous factors acting alone or in concert. High intracellular Ca^{2+} concentrations (resulting from glutamate excitotoxicity) upregulate the expression of several enzymes catalyzing production of free radicals (e.g., nitric oxide synthase, xanthine oxidase); nitric oxide production is also induced through the production and release of inflammatory cytokines within the brain. Additionally, this increase in intracellular Ca^{2+} alters mitochondrial membranes, disrupting the electron-transport chain and allowing leakage of electron-reduced oxygen intermediates. Additional ROS production is favored by local acidosis and interaction between nitric oxide and superoxide radicals, yielding peroxynitrite, which is postulated to be among the most damaging free radical species (43). Apoptotic signaling molecules may further increase ROS generation within mitochrondria and participate in permeabilization of the outer mitochondrial membrane.

Oxidative stress within injured cells can become self-propagating. When modified through tyrosine nitration, superoxide dismutase, which regulates ROS and free radical levels in healthy mitochondria, becomes inactive, leading to further oxidative damage. Additionally, numerous inflammatory mechanisms are activated under oxidative stress; proinflammatory cytokines in turn activate cyclooxygenase and stimulate the eicosanoid cascade. The net result of these additional processes is increased production of ROS, leading to a cycle of increasing potential for oxidative damage.

When levels of these free radicals are sufficient to overwhelm the cellular mechanisms designed to protect against oxidative damage (e.g., superoxide dismutase, catalase, glutathione peroxidase), the resultant stress causes peroxidation of cellular membranes, protein and DNA oxidation/nitration, and inhibition of the mitochondrial electron-transport chain. In turn, mitochondrial dysfunction—due to damage from free radicals, disruption of Ca^{2+} homeostasis, and proteolytic breakdown of spectrin—may also lead to the loss of structural and functional integrity of the cells, culminating in neuronal cell death.

3.2.4. Inflammatory pathways. Inflammation is often encountered as an early pathological response in TBI. Proinflammatory cytokines [e.g., interleukin (IL)-1β, IL-6, TNF] are released by astrocytes and microglia (28). Activation of prostaglandins, additional cytokines, and the complement system leads to induction of chemokines and cellular adhesion molecules. Release of chemokines stimulates recruitment and infiltration of polymorphonuclear leukocytes, T cells, monocytes, and macrophages to sites of primary and secondary injury through the blood-brain barrier, as well as activation of resident central nervous system (CNS) microglia. Recruitment and infiltration typically peak at two to three days after insult, with a return to control levels over the following days to weeks. The interplay between various components of the immune system, inflammatory cascade, and growth factors, combined with the dual roles of cytokines (neurodestructive versus neuroprotective), although deleterious in the acute phase, may set the stage for regenerative and reparative processes in later, chronic stages (29–31).

4. TRAUMATIC BRAIN INJURY BIOMARKER DEVELOPMENT

Early intervention and improved outcome after TBI require an objective assay to determine severity and prognosis. Initial data indicate that pathological changes that occur in the brain immediately

after injury can be detected in the blood or cerebrospinal fluid (CSF) through biochemical markers. This subset of biomarkers can be measured and evaluated as indicators of neurological damage. Studies measuring protein expression associated with brain injury have suggested that the magnitude of change and temporal patterns of identifiable proteins in blood or CSF can be associated with the severity and outcome of the injury, which has potential for diagnosis, prognosis, and therapeutic monitoring (44–47). The identification of biomarkers for brain injury is faced with several challenges, the most daunting of which is the complex, nonhomogeneous structure and functionality of the brain, wherein each tissue or cell type may be nonuniformly susceptible to different types or severity of injury.

The search for an ideal biomarker or set of biomarkers is ongoing. Ideal TBI biomarkers meet the criteria of high specificity for brain tissue, high sensitivity for brain injury, rapid appearance in interstitial fluids specifically after injury, release in a time-locked sequence with the injury, and correlation with neurological scores and/or neuroimaging data (48). An objective assay of biomarkers that meet these attributes would allow for early diagnosis of brain injury, when treatment is potentially curative; the assessment of the severity and specificity of the injury, for more targeted intervention; and monitoring of the progression of the condition and response to treatment.

4.1. Identifying Biochemical Markers of Traumatic Brain Injury

Typically, CSF is used as a reference sample for most proteomic studies of brain injury because it provides the most comprehensive proteomic information relevant to and reflective of the status of the brain, allowing for the detection of biomarkers unable to cross an intact blood-brain barrier (49–52). Injured or diseased neurons and support cells of the CNS shed cytoplasmic proteins (e.g., S100, NSE, GFAP, and MBP) and proteolyzed or modified structural proteins from neuronal processes (e.g., pNF-H, c-tau, and SBDP) into interstitial fluids (**Table 2**) (44, 46, 53–55).

However, the relative ease of access to human blood, as well as the established clinical and scientific infrastructure for its analysis, has made blood a preferred material for diagnostics and discovery. Despite the advantages of using blood, it poses analytical challenges due to its complex proteome. For example, protein concentrations exhibit wide dynamic ranges in blood. The concentrations of hundreds of proteins in the blood proteome are in constant flux; materials are exchanged between healthy and apoptotic or necrotic cells. Although conventional thought suggests that most protein and peptide biomarkers that leak into the blood are found in relatively low concentrations, there may be a difference of 3 to 12 orders of magnitude in biomarker concentrations (56, 57). Thus, the blood proteome cannot be fully resolved with a single proteomic technique. Although mass spectrometry (MS) has evolved to detect trace quantities of proteins and peptides, the dynamic range of detection by MS limits the comprehensive profiling of complex samples (58).

Significant advances in proteomic technologies coupled with high-powered bioinformatic systems are helping to overcome problems associated with the complexity and heterogeneity of the brain proteome (59–62). Such approaches are being applied to large-scale, quantitative analysis and identification of biomarkers for brain injury (56, 59, 63–66). Biomarker discovery via high-throughput proteomics is characterized by two main approaches: global proteomic profiling (58, 67) and targeted proteomic profiling (68, 69, 70, 71). The former identifies potential biomarkers by comparing samples from normal and injured individuals to find changes in concentrations of proteins that could be associated with TBI. The latter quantifies specific proteins identified as candidate markers by use of selective reagents. Methods include the enzyme-linked immunosorbent assay (ELISA) and microarrays that identify and quantitate RNA or antigen.

Table 2 Candidates for biochemical markers for traumatic brain injury (TBI)

Candidate TBI biomarker	Expression	Physiological characteristics	Timeline	Sample source	Normal serum levels (ng ml^{-1})	Diagnostic value	Comments
S100β (21 kDa)	Astrocytes, Schwann cells, adipocytes, chondrocytes, melanocytes	Gliosis, BBB compromise	Detection 24 h postinjury, peak levels after 48–72 h, serum half-life of 60–120 min	CSF serum	<0.05	>1.13 ng ml^{-1}; correlates with ↑ mortality, ↑ ICP, poor GCS and poor GOS, loss of BBB integrity	Marker for malignant melanoma, renal and/or intestinal ischemia, also observed increase in S100β in trauma patients with no head injury
NSE (78 kDa)	Neurons, neuroendocrine cells, oligodendrocytes, thrombocytes, erythrocytes	Neuronal injury	Detection 6 h postinjury, peak levels after 72 h, serum half-life of 24 h	Serum	<12.5	>21.7 ng ml^{-1}; correlates with ↑ mortality, poor GCS, poor GOS	Marker for small cell lung cancer, ischemic stroke, neuroblastomas, neuroendocrine bladder tumor
GFAP (50 kDa)	Astrocytes	Gliosis	Detection 24 h postinjury	Serum	<0.03	>1.50 ng ml^{-1}; correlates with ↑ mortality, ↑ ICP, poor GCS, and poor GOS	Exclusively found in brain, may not be sensitive enough for mild TBI
MBP (18.5 kDa)	Myelin	Demyelination	Detection 48–72 h postinjury and persistence for 2 weeks, serum half-life of 12 h	Serum	<0.3	↑ MBP correlates with poor GOS	Marker for white matter injury
UCH-L1 (24 kDa)[a]	Neurons, neuroendocrine cells	Neuronal cell body injury	Detection 2 h postinjury, peak levels after 24 h (in rodent model), detection 6 h postinjury, peak levels after 6–24 h (in human CSF)	CSF serum	ND	↑ UCH-L1 level correlates with ↑ mortality, ↑ postinjury complication, poor outcome tested 6 months postinjury	Brain-enriched marker

pNF-H (200 kDa)	Neurons	Axonal injury	Detection 6 h postinjury, peak levels after 24–48 h (in rodent model)	CSF serum	ND	↑ pNF-H level correlates with the volume of the injured cortex	Sensitivity of this marker not yet investigated in human brain injury
C-tau (30–50 kDa)	Neurons	Axonal injury, BBB compromise	Detection 6 h postinjury, no significant increase after 24 h due to reestablishment of BBB integrity (in rodent model)	CSF	ND	>0 ng ml^{-1} (serum) or >1,600 ng ml^{-1} (CSF); correlates with ↑ ICP, poor GCS; conflicting reports regarding correlation to outcome; loss of BBB integrity	Proteolytic fragment of microtubule-associated protein tau, exclusively found in brain
SBDPs (120, 145, and 150 kDa)[a]	Neurons	Neural necrosis/ apoptosis, axonal injury	Peak levels 2 days postinjury	CSF serum	ND	↑ SBDP level correlates with poor GCS, poor GOS, and ↑ lesion size; ↓ SBDP level decrease between 6 and 96 h postinjury correlates with better outcome	Proteolytic fragments of neuronal cytoskeletal protein, αII-spectrin; ↑ SBDP150/120 during apoptosis via caspase-3 activation; SBDP150/145 during necrosis via calpain activation

[a]TBI biomarkers identified by proteomic approaches. Up arrow indicates an increase or upregulation; down arrow indicates a decrease or downregulation. Abbreviations: BBB, blood-brain barrier; CSF, cerebrospinal fluid; GCS, Glascow Coma Scale; GFAP, glial fibrillary acidic protein; GOS, Glascow Outcome Scale; ICP, intercranial pressure; MBP, myelin basic protein; ND, not detected; NSE, neuron-specific enolase; pNF-H, phosphorylated neurofilament H; SBDP, spectrin breakdown product; UCH-L1, ubiquitin C-terminal hydrolase. Data from References 44, 46, 49, 54, 55, 73, 74, and 108–110.

The number of studies to date that have employed neuroproteomic analysis for the identification of previously unidentified protein biomarkers for TBI is still low. However, from these studies a handful of candidates have emerged, such as UCH-L1 and SBDP (**Table 2**) (61, 72–74). These and other biochemical markers show promise for TBI diagnostics and underscore the significant potential of a proteomics-based approach for biomarker discovery.

4.2. Biochemical Marker Validation

Notwithstanding the inherent challenges associated with biomarker discovery and development in general, which have been extensively reviewed (58, 67, 75), the most significant obstacle to the advancement of TBI biomarker research has not been the ability to identify candidate biomarkers. The literature is replete with long lists of proteins and peptides discovered through global proteomic profiling. Although many putative TBI biomarkers have shown potential for monitoring the effectiveness of therapeutic interventions, to date none has been proven to have diagnostic and prognostic value. Thus, rigorous validation—ascertaining that a marker or a set of markers is specific and predictive of TBI—remains the principal obstacle to developing an effective diagnostic assay.

The quantitative assessment of putative biomarkers for TBI across large cohorts presents a major challenge to this field. Even the most extensively studied markers, S100β, NSE, GFAP, MBP, and c-tau (**Table 2**), cannot distinguish TBI patients from non-TBI patients in many cases (53). These markers exhibit high serum concentrations in association with neurological conditions or diseases unrelated to TBI. Furthermore, biomarker assay validation must overcome the heterogeneity of patient population (in terms of age, sex, genetic makeup, physiological and metabolic states, lifestyle choices such as endurance training, and underlying chronic diseases) and the lack of standardized quantification across various laboratories performing TBI studies (76). These challenges have imposed additional complexity that further limits the usefulness of biomarkers currently identified for the clinical evaluation of TBI patients (77, 78).

Reagent development must keep pace with the discovery of putative biomarkers. Biomarker candidates have traditionally been validated with quantitative immunoassays (e.g., sandwich ELISAs) that are unique for each analyte. The extensive neuroproteomic changes observed after TBI make it highly probable that previously unknown biomarkers will be identified by proteomic approaches. Doing so will then require the development of antibodies with high affinity and specificity for each candidate biomarker in need of validation. At present, quantitative assays available for most human proteins (79) and the development of highly sensitive sandwich ELISAs for testing large numbers of candidate biomarkers are both costly and resource prohibitive.

It is unlikely that a single biomarker will be able to provide the sensitivity and specificity needed for most TBI applications. In fact, it has been frequently proposed that a combination of markers, namely a biomarker panel, will be needed to adequately evaluate the progression of TBI pathology over time, much less to discriminate among different pathologies (80, 81). To reach this goal and to provide more predictive outcomes, advances in the aforementioned proteomic technologies will be critical to the identification, assembly, and validation of such a panel of biomarkers. At the same time, the commercial development of rapid diagnostic tests to measure these markers needs to progress.

5. BIOSENSORS WITH POTENTIAL FOR RAPID TRIAGE

A rapid evaluation of the extent of injury can expedite an appropriate response to TBI, whether in a military or civilian situation. At the current time, such decisions are often made on the basis of a

visual inspection of the injured person, evaluating such factors as mental acuity. Ideally, a point-of-care diagnostic system providing a quantitative measure of the different processes involved in cellular or tissue damage would discriminate between mild, moderate, and severe TBI. Fueled by the identification of biochemical markers for cancer, heart attack, infectious disease, genetic disorders, and intoxication, investigators are currently developing analogous biochemical tests for TBI markers for point-of-care applications. Progress toward the development of biosensors for TBI is discussed below from two points of view. First, we review the publicly reported efforts currently geared specifically toward TBI diagnostics. Then, on the basis of the wide variety of portable biosensors that have recently evolved, we provide examples of commercially available biosensors with potential for TBI diagnostics if a panel of validated biomarkers becomes established and appropriate reagents are available.

5.1. Biosensors Under Development for Detecting Biomarkers of Neuronal Damage or Related Inflammation

Although they are not biosensor platforms per se, several lateral flow tests for biochemical markers of TBI have been described. Lateral flow tests are inexpensive and lightweight, but sensitivity can be a serious issue unless the levels of biomarkers are suitably high or some type of signal amplification is integrated. Venkatasubbarao and colleagues (84) have developed a multiplexed, quantitative lateral flow test strip for four biochemical markers: GFAP, S100, NSE, and F2-isoprostane. The system comprises a fourplex lateral flow immunoassay with quantum dot–labeled antibodies and a battery-powered reader to distinguish the binding of four different targets on the basis of differential fluorescence emissions. Evaluation of sera from 20 patients with severe TBI (GCS $\leq$ 8) 10 h after injury revealed increases in the levels of three of the markers (all except F2-isoprostane), which then declined over the next 140 h. Although these results appear promising, the mean values for the 20 patients showed a large coefficient of variation. Because neither the sensitivity nor the precision of the assay was reported, it is not possible to tell if the high variability was due to differences in the individual patients or the assay itself.

A second lateral flow immunoassay used concentrations of circulating pyruvate dehydrogenase (85) as a measure of oxidative stress, and correlated brain gliosis and brain injury (**Figure 2**) in a mouse model. The qualitative assay only compared blood samples before and after trauma; it never presented quantitative data on levels in normal or TBI samples. Not only did the test require measurements before trauma, there were no controls to determine whether the decrease was due to a so-called high-dose hook effect—a false-negative effect commonly encountered in lateral flow immunoassays. Much more characterization would be required to determine the clinical utility of an assay based on pyruvate dehydrogenase concentrations for TBI assessment.

A collaboration led by Joseph Wang and Evgeny Katz (82, 83) has resulted in development of a biosensor based on multienzyme cascade processing of five biomarkers characteristic of TBI. Norepinephrine, lactate, and glucose were considered input signals, and norepiquinone and NADH were defined as the output signals to monitor the concerted operation of the enzymes lactate oxidase, horseradish peroxidase, and glucose dehydrogenase. The unique feature of this biosensor is the built-in logic for analysis of complex biochemical inputs. The integration of biocomputational logic gates into the analysis is intended to reduce the complexity of the data by generating output signals with the information encoded to discriminate between different pathophysiological conditions. Both optical and electrochemical readout methods were employed. Although three of the five biochemical markers were evaluated at physiologically relevant levels, the ability of these markers to discriminate TBI from other traumas was not at all clear. Whether this original

approach will be practical for TBI or will be relevant more as a model for exemplifying digital biosensor networks for any application remains to be determined.

At the U.S. Naval Research Laboratory, the authors of this review are developing multiplexed assays for at least five biochemical markers of TBI that yield results in less than 1 h (86). Biomarkers currently included in the assay are GFAP, UCH-L1, NSE, and S100β (**Table 2**), but additional biochemical markers can be added to the array as they are validated in clinical studies and the recognition reagents are developed. The intention with this array-based system is to quantify markers representing different aspects of TBI pathobiology in a single test to assess the severity and progress of TBI in patients (87). To address the issue of sensitivity, which typically poses a problem for rapid tests, an abbreviated tyramide signal amplification (TSA) (88) was added to the assay protocol. Although the TSA protocol provided a 100-fold improvement in signal, this procedure involves one additional step, which may prove too cumbersome for triage in a military battlefield scenario.

Although most of the diagnostic systems under development measure circulating biochemical markers of neural damage, imaging methods that measure physiological markers of TBI should not be ignored. Sonography is being explored to detect intracranial hemorrhage by measuring the diameter of the optic nerve sheath (25, 89, 90). This method has potential and portable sonography equipment is available, but the technique is not yet ready for routine clinical use (91). The miniaturization of optoelectronic devices and the emergence of new acoustic and photonic imaging technologies, such as two-photon systems that can image under the skin, suggest that portable imaging systems may also prove highly valuable in evaluating TBI at the point of care.

5.2. Field-Usable Biosensors That Can Detect Multiplexed Biochemical Markers

There is a relatively long list of biosensors that can screen blood products for multiple targets by using arrays of sandwich immunoassays without significant sample preparation. Because ELISA, the method most frequently used to validate the presence of suspected biomarkers in TBI patients, performs sandwich assays against individual targets, the transition to a biosensor that already performs such immunoassays would appear straightforward. The two main issues affecting the transition from ELISA to biosensor are (*a*) antibody specificity and (*b*) assay sensitivity.

Absolute specificity for the target of interest is critical when integrating multiple single-target immunoassays into a multiplexed test. ELISA assays are generally performed in a one-target-per-well format; thus, the antibody reagents that work well in ELISAs generally are not screened for cross-reactivity against each other. The degree to which the specificity has been established against a panel of similar targets is also highly variable. Furthermore, many antibodies have been developed by use of peptide fragments or denatured antigens. Although these antibodies work against denatured molecules in formats such as Western blots, they often do not recognize circulating native proteins and thus are not useful for screening untreated blood, serum, or plasma. The development of an immunoassay against an array of biomarkers requires screening many antibodies in a combined format for specificity and sensitivity against each target. During this screening process, the issue of nonspecific binding must be carefully addressed. As the number of targets increases, the total concentration of reagents also increases. This increase may cause a rise in the background signal as well as an accompanying increase in variability. The net result is often a decrement in assay sensitivity if the chemistry at the sensor surface is not controlled sufficiently to account for these changes. In addition to specificity, antibodies for biochemical markers must have rapid binding kinetics with sufficiently high affinity to detect the low concentrations of biochemical markers most commonly encountered in the circulation. The sensitivity required to distinguish

changes in biomarker concentrations (ranging from picograms per milliliter to low nanograms per milliliter) is generally lower than that obtained by most currently deployed biosensor tests.

Each user community imparts additional constraints on biosensors depending on the intended use. In general, the biosensor should be portable, fast (<30 min), battery operated or electricity free, rugged, inexpensive, as sensitive as necessary, and fully automated. An excellent compendium of biosensors that use antibodies for detection is included in the *Chemical*, *Biological*, *Radiological Technology Survey*, recently compiled by Emanuel & Caples (92). Information obtained from an open invitation to sensor developers was compared; the rankings included evaluation for field use (easily portable, simple to operate, single use or reusable with minimal cleaning). **Table 3** compares devices with demonstrated capability for detecting proteins in clinical matrices in conditions compatible with field operations. Although Emanuel & Caples emphasized use for battlefield triage, these systems are also appropriate for use by emergency medical personnel responding to a reported injury. In all the systems listed in **Table 3**, the ability to perform multiple simultaneous assays is featured; such multiplex assay capability is crucial for any sensor designed for TBI diagnosis based on biochemical markers. Additional systems are under development by the clinical community for minimally invasive diagnostic procedures for diagnosis of infection or trauma (93–98); many of these diagnostics may also be adaptable for the analysis of biochemical markers of TBI. Clearly, the transition of a multiplexed test for TBI from prototype demonstration to patient evaluation could be expedited if the diagnostic device passes regulatory hurdles, establishing its capability for sensitivity, precision, and reliability.

6. CHALLENGES FOR BIOMARKER-BASED TRIAGE

As highlighted above, one of the most significant challenges facing the development of rapid diagnostic sensors for TBI is the identification of specific biochemical markers. A multifarious syndrome, TBI involves many interrelated processes such as neuronal cell signaling, inflammation, hemostasis and ischemia, cellular metabolism, and intracellular signaling mechanisms, many of which, unfortunately, are shared by other neurodegenerative syndromes. The most promising solution for TBI triage is to quantify a suite of biochemical markers, determined over a period of time, and to assess time-associated changes in the array of biomarkers as a whole as an indicator of TBI. Such a chronological approach to biomarker analysis should facilitate rapid diagnosis of moderate and severe TBI and help distinguish it from the more slowly developing neuropathologies. However, the magnitude and time course for each process involved in TBI pathology—and, hence, each biochemical marker—depend on the type and severity of injury, which complicates even the best-controlled, highly rigorous chronologic biomarker studies.

A further challenge relates to the models used to determine which biochemical markers are the most appropriate for diagnostic tests. In addition to the normal variations inherent in any studies involving human subjects, biomarker immunoassays may be also affected by the presence of autoantibodies directed against brain-specific proteins in opiate addicts (99, 100) and in patients with neurological disorders (101); these autoantibodies may dampen or even eliminate any measurable change in biomarker levels that might be used for diagnostic purposes. The prevalence and timing of multiple traumatic events, as well as application of any medical interventions prior to biomarker sampling (102), may further cloud the study of biomarkers in blast-injured subjects or individuals involved in automobile accidents.

To circumvent the variability inherent in human subjects, many researchers have turned to rodent models. The small size, relatively low cost, and genetic homogeneity of rodent populations allow statistically significant numbers of replicates to be used with appropriate and rigorous controls. Furthermore, the conditions for traumatic insult are generally reproducible and, in many

Table 3 Commercial biosensors demonstrated to detect proteins in clinical samples[a]

Sensor	Company	Protein detection limit (ng ml^{-1})	Evaluation in clinical fluids	Immunoassay readout technology	Cost of instrument/tests	Speed (min)
Arrayed imaging reflectometry bioassay system	Research International	0.001–1.0	Blood	Highly multiplex refractometry	$30,000/~$20	<30
Advanced Liquid Logic	Advanced Liquid Logic	<1	Blood, nasal, and throat swabs	Digital microfluidics	$10,000–50,000/$12–20	15–30
mBio MQ	mBio Diagnostics	1–100	Blood, serum (5–10 μl)	Evanescent fluorescence	$5,000/$10–20	15–30
Magnetic immunochromato-graphic test system	MagnaBio Sciences	<1	Blood, nasal, and throat swabs; urine	Magnetic colloid labels and magnetic detection	$4,000/$2.5–3	2–15
MSA biosensors 2,200R and 4,000	MSA	<1	Blood	Magnetic bead labels, optical readout	2,200R: $16,000<$504,000: $18,000/<$75 for 8-plex	2–15
MSD cartridge reader	MesoScale Diagnostics	<1	Blood, nasal, and throat swabs; urine	Electrochemi-luminescence	To be determined	15–30
QTL biosensor 2,200R	QTL Systems	<1	Blood	Magnetic bead capture, fluorescence readout	$15,000/<$50	2–15
RAPTOR	Research International	<1	Blood, urine	Evanescent fluorescence	$49.5/$210 for 30 samples	2–15

[a]Information was provided by suppliers and extracted from Reference 92.

cases, are adjustable (103, 104). However, conditions that give rise to multiple traumatic insults such as those encountered in real-life scenarios with human subjects are only infrequently described. Moreover, brain size and anatomy may not scale directly from rodents to humans, and functional assignations may not be directly translatable to human models (102). Although larger animal studies are being developed to bridge this gap (104, 105), the use of anesthesia to minimize the discomfort of subject animals may produce unintended artifacts. Clearly, additional studies using multivariate analysis must be undertaken to eliminate nonrelevant information and to account for these individual variations (106, 107).

The final challenge lies in integrating the biomarker analyses into an appropriate detection platform. Although plate-based ELISAs provide an excellent example of high-throughput screening, performing multiple assays in parallel at multiple time points becomes logistically cumbersome, even when fully automated processing is available. We have described numerous biosensors that are either in development or commercially available that have potential for use in TBI diagnosis. The ability to perform multiplexed assays simultaneously and obtain quantitative or semiquantitative data is a function of the parameters measured, the detector configuration, and the sensor instrumentation itself. Assay sensitivity and the time-to-result, however, depend on the characteristics of the antibodies used. If suitable sensitivity cannot be attained in the platform used, there are many reagents and techniques that have the potential to improve sensitivity. However, integration of these reagents and methodologies may require additional manipulations (which increase the manual hands-on time or the complexity of automated processing), additional or longer incubations (which increase the overall time-to-result), or physical modifications to the instrumentation (which can be both costly and labor intensive).

TBI is a complex and potentially chronic condition that presents with a broad spectrum of symptoms that can result in permanent disabilities. To date, researchers and clinicians have relied primarily on neuroimaging and behavioral measures for limited diagnosis, prognosis, and treatment of brain injuries, as parallel efforts are made to advance the discovery and development of biochemical markers for TBI. However, these methods cannot differentiate TBI from other neurological conditions. Any one approach or any one biomarker is unlikely to provide the sensitivity and specificity needed for most TBI applications. Only through a complementary, multimodal approach that combines neuropsychological evaluations with analytical neuroimaging and biochemical techniques will we obtain the critical ability to determine the severity and formulate a prognosis of TBI and the opportunity for early intervention and improved clinical outcome.

SUMMARY POINTS

1. Currently, responses to stimuli and questions, loss of consciousness, and altered behavior, along with neurocognitive tests, are used for rapid assessment of brain injury and to determine the need for more definitive diagnostic imaging. These rapid methods are susceptible to false-positive and false-negative results for TBI due to stress, fatigue, medications, and microlesions (imaging).
2. Even with mild TBI, neurological damage can result in behavioral and physiological changes with both short-term and long-term consequences.
3. Effective biomarkers are critical for early diagnosis both to assess the severity of the injury for more targeted intervention and to monitor the progression and response to treatment.

4. An ideal biomarker for the identification of TBI (including mild forms) would have high sensitivity and specificity for molecules from the brain, reflect the pathology of the damage, appear in blood shortly after injury with levels proportional to injury, and correlate with neurological scores and imaging.
5. Because no single biomarker that is indicative of TBI alone has been identified, panels of biomarkers (S-100β, GFAP, UCH-L1, NSE, etc.) show promise for differentiating between different types and levels of injuries in a timely manner.
6. Point-of-care devices that can rapidly detect multiple biomarkers in blood would provide the information needed for appropriate treatment, thereby improving long-term outcomes.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Contents