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Novel Strategies for Discovery, Validation and FDA Approval of Biomarkers for Acute and Chronic Brain Injury

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1. Introduction

The proposed chapter outlines novel approaches that provide an infrastructure for discovery and validation of new biomarkers of acute brain injury. Approaches to validation can be also applied to existing biomarkers of brain injury in order to provide more rigorous assessment of their clinical utility. Importantly, there are currently no biomarkers of brain injury approved by Food and Drug Administration (FDA). The chapter reviews approaches critical for securing FDA approval of biomarkers of brain injury and disease, focusing on traumatic brain injury (TBI).

The chapter reviews proteomics techniques applied for the first time to discovery of biomarkers of central nervous system (CNS). These techniques include refined mass spectrometry technology and high throughput immunoblot techniques. Output from these approaches can identify potential candidate biomarkers employing systems biology and data mining methods that will also be described.

Once potential biomarkers have been identified, it is important to provide information on their clinical utility for diagnosis, management and prognosis of patients exposed to brain injuries. This section of the chapter will review both preclinical and clinical methods for biomarker validation. Preclinical models discussed include rodent models of closed head injury such as the controlled cortical impact (CCI) device. Consideration will also be given to the design and results from human clinical trials validating biomarkers of mild, moderate and severe traumatic brain injury (TBI). Human studies will include detailed analyses of the biokinetics of different biomarkers in order to understand their utility in acute, subacute and chronic phases of TBI. Consideration will also be given to relationships between levels of biomarkers and magnitude of acute injury, CT imaging profiles, occurrence of secondary insults and long term outcome.

To achieve practical clinical utility, it is important to develop highly sensitive and specific assays for individual biomarkers. Enzyme-linked immunosorbent assays (ELISAs) are the current choice for clinical use, since this assay technology provides reliable, quantitative and accurate data. ELISA technologies relevant to biomarker measurement will be discussed. Once ELISA assays for individual biomarkers have been developed, these assays need to be transferred to devices that are appropriate for the clinical application and medical

environment in which biomarker analyses will be conducted. We will review devices for use in clinical laboratories, emergency rooms (ERs), intensive care units (ICUs) and austere medical environment. In addition, this section will include review developing technologies allowing concurrent assessment of multiple biomarkers (multiplex).

To date, studies of biomarkers for brain injury have been restricted to research applications only. Although there is broad recognition of clinical utility of biomarkers, the FDA has yet to approve any biomarkers of CNS injury of disease. This section of the chapter provides a detailed outline of the regulatory consideration necessary for a biomarker to file for approval by FDA. Importantly, many of these considerations need to be integrated into relatively early stages of biomarker validation, assay development and device selection.

2. Biomarker discovery: Methods and results

2.1 Proteomics/systems biology in the area of neurotrauma

The application of neuroproteomics/neurogenomics has revolutionized the characterization of protein/gene dynamics, leading to a greater understanding of post-injury biochemistry. Neuroproteomics and neurogenomics fields have undertaken major advances in the area of neurotrauma research focusing on biomarker identification. Several candidate markers have been identified and are being evaluated for their efficacy as biological biomarkers utilizing these “omics approaches”. The identification of these differentially expressed candidate markers using these techniques is proving to be only the first step in the biomarker development process. However, to translate these findings into the clinic, data-driven development cycle incorporating data-mining steps for discovery, qualification, verification, and clinical validation is needed. Data mining steps extend beyond the collected data level into an integrated scheme of animal modeling, instrumentation, and functional data analysis.

Proteomics is the identification and quantification of all expressed proteins of a cell type, tissue or organism. The advancement in the field of proteomics has coincided with the completion of the human genome sequencing project (Fenn et al. 1989, Tanaka 1988, Karas & Hillenkamp 1988). In recent years, the term proteomics is often mentioned together with biomarker discovery, as proteomic studies have the capability of identifying sensitive and unique signature protein biomarkers from tissues or biofluids derived from animal models or human clinical samples inflicted with various diseases. Neuroproteomics and neurogenomics, the application of proteomics and genomics in the field of neuronal injury, have been identified as a potential means for biomarker discovery, with the ability to identify proteome dynamics in response to brain injury (Guingab-Cagmat 2009, Haskins et al. 2005, Davidsson & Sjogren 2005, Shin et al. 2002, Celis et al. 2004).

In the area of brain injury, several studies have demonstrated the role of proteomics (Denslow et al. 2003, Katano et al. 2006) and genomics (Redell et al. 2009, Ding et al. 2006) in providing significant insight into understanding changes, modifications and functions in certain proteins post TBI. In addition, genomics and proteomics are powerful, complementary tools that play an important role in the area of biomarker identification. Over the past few years, advances in the fields of neuroproteomics and neurogenomics have led to the discovery of many candidate biomarkers and are becoming the primary methods for initial candidate marker selection (Kobeissy et al. 2008, Wang et al. 2006, Ottens et al. 2007, Nogoy 2007, Ottens et al. 2006). The identification of differentially expressed candidate markers using these techniques is proving to be only the first step in the biomarker

development process. However, to translate these into the clinic, these novel assays require a data-driven development cycle that incorporates data-mining steps for discovery, qualification, verification, and clinical validation (Rifai et al. 2006).

TBI neuroproteomics studies have utilized biofluids such as blood/serum in addition to injured tissue to identify clinical markers that may correlate with injury severity. One of the studies by Burgess et al, evaluated altered differential proteins in normal human post mortem cerebrospinal fluid (CSF) (Burgess et al. 2006). The rationale of using post-mortem CSF is that it resembles a model of massive brain injury and cell death which post-mortem, thus comparing protein profile of post-mortem CSF with brain injury CSF would be ideal for identifying protein markers of injury. Of the 229 proteins identified, a total of 172 were novel and not previously described. The findings showed that the use of post mortem CSF (non-TBI samples) to evaluate altered protein levels mimicked the changes occurring in the brain following a traumatic insult. Furthermore, the identification of differential proteins of intracellular origin in the CSF corroborates the suggestion that there is protein leakage into the CSF following brain injury (Dumont et al. 2004, Hammack et al. 2004). This is a key step in identifying protein markers since, neuronal specific proteins leak from injured brain directly to the CSF.

In one of the TBI studies conducted in our laboratory, 1D-differential gel electrophoresis (DIGE) protein separation in series with mass spectrometry analysis was used to discover putative TBI biomarkers in brain tissues from a rat model (Haskins et al. 2005). These included 57 downregulated and 74 upregulated proteins; however data were not so informative due to limited separation capability. In an advanced study, we utilized a multidimensional separation platform called (CAX-PAGE/RPLC-MSMS) which consisted of different levels of separation including ion chromatography, 1-D gel electrophoresis and mass spectrometry as a novel approach for identifying biomarkers and protein breakdown products (degradomes); for detailed reviews, refer to (Svetlov et al. 2006, Kobeissy et al. 2006). As an application, the CAX/neuroproteomic analysis was employed on cortical samples of rat subjected to controlled cortical impact (CCI) model of experimental TBI (48 hrs post injury). Of interest, our neuroproteomic analysis identified 59 differential protein components of which 21 decreased and 38 increased in levels after TBI. One main advantage of this technique is its ability to elucidate degradomic substrates of different protease systems; thus our data identified the elevated levels of the breakdown products of several proteins (Kobeissy et al. 2006). Several of these are now being investigated as potential biomarkers specific for TBI to assess severity and recovery by evaluating their levels at different time points post TBI.

2.1.1 Data mining coupled neurosystems biology analysis in brain Injury

Coupled to data-mining steps, systems biology (SB) represents a mathematical model capable of predicting the altered processes or functions of a complex system under normal and perturbed conditions. It combines experimental, basic science data sets, proteomic and genetic data sets, literature and text mining, integration with computational modeling, bioinformatics and pathway/interaction mapping methods. When constructed properly, SB databases can provided a context or framework for understanding biological responses within physiological networks at the organism level, rather than in isolation (Chen et al. 2007).

In this regard, “omics” output constitutes one key component of neurosystems biology. It discusses the global changes involved in neurological perturbations integrating the final outcomes into a global functional network map which incorporates potential biomarkers

identified (Grant 2003, Grant & Blackstock 2001). In the area of brain injury, neurosystems biology platform harnesses data sets that, by themselves, would be overwhelming, into an organized, interlinked database that can be queried to identify non-redundant brain injury pathways or convert hot spots. These can be exploited to determine their utilities as diagnostic biomarkers and/or therapeutic targets. The ultimate goals of system biology are: first by exploring the systems component (gene, protein, small molecule, metabolite etc.), help biologist, pharmaceutical companies and doctors to better understand the mechanisms underlying the disease components. Thus, it allows for suitable targets for treatment. Secondly, the systems biology approach enables one to be able to predict the functions and behavior of various components of the system upon varying any on the interconnected component since the whole system will be viewed globally rather than on micro, individual component level (Beltrao et al. 2007).

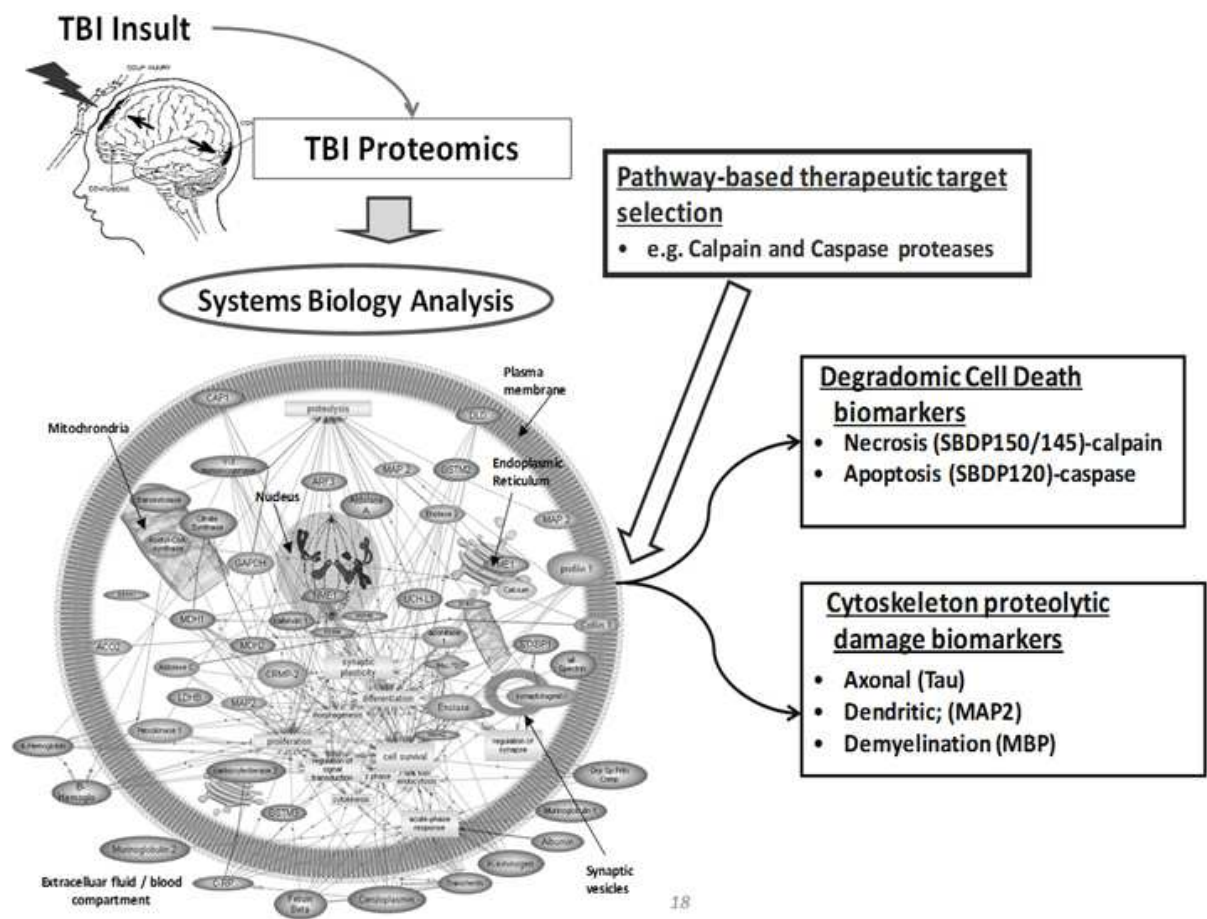


Fig. 1. Systems biology-based therapeutic target identification and target-specific biomarker selection.

In the field of neurotrauma, identifying and analyzing brain injury-related networks plays important and practical clues relating to biological pathways relevant to disease processes. However, the more important underlying goal in this analysis is to provide important clues that may suggest radically new approaches to therapeutics. Systems modeling and simulation is now considered fundamental to the future development of effective therapies. In the brain injury for example, it has been shown that calpain and caspase proteases are major components in cell death pathways taking part in two destructive proteolytic

pathways that not only contribute to key forms of cell death (necrosis and apoptosis), but also in the destruction of important structural components of the axons (alphaII-spectrin breakdown products (SBDPs) and tau), dendrites (MAP2) and myelin (MBP) (Figure. 1). Interestingly, two different forms of SBDPs reflect either neuronal necrosis (SBDP150 and SBDP145 cleaved by calpain) or neuronal apoptosis (SBDP120 cleaved by caspase-3) (Wang et al. 2005). These SBDPs and other similar neural protein breakdown products can serve as target pathway specific biomarkers as illustrated in Figure 1.

Calpain and caspase proteases are used here as examples of therapeutic targets with proteolytic brain biomarkers representing non-redundant pathways relevant to the pathobiology of these therapeutic targets and the disease itself. TBI (traumatic brain injury); MAP2 (microtubule-associated protein 2); MBP (myelin basic protein); SBDP (spectrin breakdown product).

3. Biomarker validation: Methods and results

Traumatic injury to the brain results in a cellular activation and disintegration, leading to release of cell-type-specific proteins. Measurable amounts of these damage markers are present in cerebrospinal fluid (CSF) and blood. These markers not only indicate the pathoanatomic injury type and the severity of injury but also might provide specific

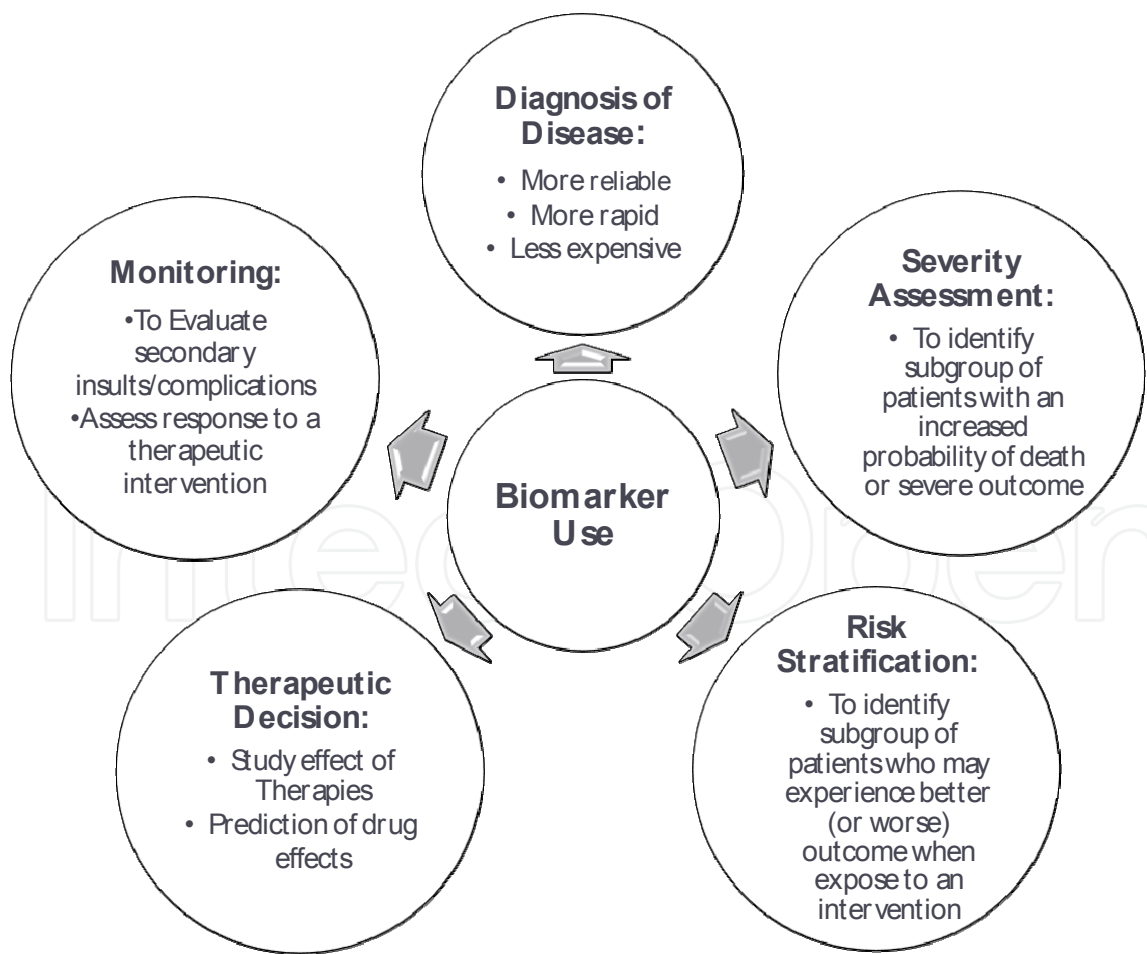


Fig. 2. Potential use of biomarkers in TBI

information about the pathophysiologic mechanisms which can be targeted by therapeutic interventions. Therefore, during the last decade, neurobiochemical markers for TBI have attracted increased attention, they can be used to screen for, diagnose or monitor the patients and to guide targeted therapy or assess therapeutic response (Etzioni et al. 2003, Vitzthum et al. 2005). (Figure. 2) Furthermore, biomarkers might be valuable tool in drug development (Blennow 2010).

3.1 Clinical evaluation

However, several studies have measured a variety of neurochemical substances in the CSF or blood, and a number of proteins synthesized in astroglial cells or neurons have been proposed as markers of cell damage in the CNS and after TBI, to date, none has been approved for clinical use. Critical criteria of the diagnostic performance of a clinically valid biomarker for TBI include its diagnostic accuracy and predictability.

The diagnostic accuracy of a test is the proportion of correctly classified patients (the sum of true positive and true negative tests) and is determined by calculating the test's sensitivity, specificity, likelihood ratio and receiver operating characteristic (ROC) curve (Bossuyt et al. 2003). Sensitivity is the ability to detect a disease in subjects in whom the disease is truly present (i.e., a true positive), and specificity is the ability to rule out the disease in subjects in whom the disease is truly absent (i.e., a true negative). Likelihood ratio (LHR), used to assess diagnostic value of a test, is the likelihood that a given test result would be expected in a patient with the specific disease compared to the likelihood that that same result would be expected in a patient without the target disorder. Two dimensions of accuracy might be considered, the LHR for a positive test (positive LHR) and the LHR for a negative test (negative LHR) (Albert 1982, Altman 1991). ROC curve is the graphical way of presenting the sensitivity (true positive rate) versus false positive rate ($1 - \text{specificity}$). A ROC curve enables the determination of appropriate cut-points, depending on the intended clinical utility of the test (Zweig & Campbell 1993). The area under the curve (AUC) is a measure of predictive discrimination: 50% is equivalent to random guessing and 100% is perfect prediction.

Although sensitivity and specificity are the most commonly provided variables in diagnostic studies, they do not directly apply to many clinical situations because the physician would rather know the probability that the disease is truly present or absent if the diagnostic test is positive or negative rather than probability of a positive test given the presence of the disease (sensitivity). This information is provided by the diagnostic predictability. Diagnostic predictability establishes the ability of the test to predict the presence or absence of disease for a given test result and is determined by calculating the positive and negative predictive values. The positive predictive value denotes the proportion of patients with positive test results who have the disease, and the negative predictive value defines the proportion of patients with a negative test who do not have the disease. The predictive values of a test vary with the prevalence of the disease in the population examined. Bayes' theorem allows calculation of the predictive values for any prevalence of disease using the prevalence and the sensitivity and specificity measures derived from previous studies (Altman 1991).

To demonstrate its clinical utility, a novel biomarker needs to be evaluated in a series of human studies (phase 1–4 trials) in order to establish the performance characteristics. The phase 1 examines whether the biomarker is significantly different for diseased patients as compared to those known not to have the disease. If a satisfactory discrimination between patients and controls is proven, the following step is to determine diagnostic accuracy

(phase 2). Phase 3 evaluates the performance of the test in the target population (Sackett & Haynes 2002). Phase 3 assesses whether the measurement of the biomarkers modifies outcome and influence therapeutic interventions and the subsequent effect on health outcome (intervention studies) (Sackett & Haynes 2002). Phase 4 trial is also known as post-marketing surveillance trial because this phase can be done after the marker has been made commercially available.

3.2 Novel candidate biomarkers

Many publications can be found for candidate biomarkers, although the initially promising results have often not been confirmed. Here, we review novel biomarkers that have shown high sensitivity and specificity in at least two independent studies. Biomarkers reflect damage and release from each of the major cell types/structures in brain parenchyma, therefore they can be divided into neuronal/synaptic and glial biomarkers (Table 1). We also discuss selected candidate biomarkers related to cellular and subcellular origins.

Biomarker	Location	Function/ Pathogenic process	Comment
Neuronal and Axonal Markers			
NSE	Prominently in the cytoplasm of neurons	Upregulated NSE is released from damaged axons to maintain cellular homeostasis.	Also present in erythrocytes and platelets
UCH-L1	Cell Body (perikarya)	Protein de-ubiquitization	Implicated in familial Parkinsonism
SBDPs	Axons	Cortical cytoskeleton matrix support	Pathology--dependent generation of α II-spectrin breakdown products (SBDPs): Calpain generates SBDP145 as a signature event in acute neuronal necrosis Caspase-3 generates SBDP120 as a signature event in delayed neuronal apoptosis.
Phosphorylated neurofilament	Predominantly in axons	Main component of the axonal cytoskeleton	Increased serum concentrations of this protein are expected to provide a specific measure of axonal injury
Glial Markers			
S100b			
GFAP	Major protein constituent of glial filaments in astrocytes	Cytoskeleton support	Induced during “reactive astrogliosis” after TBI

Table 1. Biomarkers for TBI

3.2.2 Neuronal and axonal markers

Neuronal and axonal proteins could prove to be valuable biomarkers as these molecules might correlate with cognitive function and long term outcome.

3.2.2.1 Neuron-specific enolase

Neuron-specific enolase (NSE) is a glycolytic pathway enzyme, localized predominantly in neuronal cytoplasm. Several studies demonstrated that NSE is a sensitive and specific indicator of neuronal cell death (Selakovic et al. 2005, Oertel et al. 2006). In addition, studies have been conducted examining CSF and serum NSE levels from adults with severe TBI, and their relationship with severity of injury and clinical outcome. Increased CSF and serum levels of NSE have been reported after TBI. NSE concentrations were also associated with severity of injury, CT scan findings and outcome (Selakovic et al. 2005, Herrmann et al. 2000, Ross et al. 1996). Although, NSE was originally identified in neurons, further studies have shown the protein in erythrocytes and platelets. False positive values have been reported in the setting of combined CNS injury plus shock and in the setting of hemolysis (Piazza et al. 2005).

3.2.2.2 Ubiquitin C-terminal hydrolase

Ubiquitin C-terminal hydrolase (UCH-L1), a neuron-specific cytoplasmic enzyme, is highly enriched in neurons representing between 1 and 5% of total soluble brain protein (Lincoln et al. 1999). This protein is involved in either the addition or removal of ubiquitin from proteins that are destined for metabolism (via the ATP-dependent proteasome pathway) (Laser et al. 2003), thus playing an important role in the removal of excessive, oxidized or misfolded proteins during both normal and neuropathological conditions, such as neurodegenerative disorders (Kobeissy et al. 2006). Because of the important function and its high brain specificity, UCH-L1 has been proposed as a novel biomarker for TBI. CSF levels of UCH-L1 in CSF have been found significantly increased in severe TBI patients compared with uninjured patients, with significant associations observed between levels of UCH-L1 in CSF, injury severity and clinical outcome (Papa et al. 2010). A study has been conducted investigating the exposure and kinetic metrics of UCH-L1 in adults with severe TBI, and their relationship with severity of injury and clinical outcome (Brophy et al. 2010). A strong correlation between CSF and serum median concentrations and biokinetics, especially during the acute period, and relationships with clinical outcome were observed (Brophy et al. 2011). Furthermore, a recent study reported that serum concentrations of UCHL1 were associated with abnormal blood brain barrier (BBB) permeability, suggesting that UCH-L1 might be used to monitor BBB disruption in patients with TBI (Blyth et al. 2011).

3.2.2.3 Spectrin Breakdown Products (SBDPs)

Alpha II-spectrin is primarily found in neurons and is abundant in axons and presynaptic terminals (Riederer et al. 1986). The protein is processed to breakdown products (SBDPs) of molecular weights 150 kDa (SBDP150) and 145 kDa (SBDP145) by calpain and is also cleaved to a 120-kDa product (SBDP120) by caspase-3. Calpain and caspase-3 are major executioners of necrotic and apoptotic cell death, respectively, during ischemia or TBI (Ringger et al. 2004, Pineda et al. 2007, Mondello et al. 2010). Thus, a unique feature of this technique is the ability to concurrently detect calpain and caspase-3 proteolysis of α -II-spectrin, providing crucial information on the underlying cell death mechanisms. In addition, distinct temporal release patterns of CSF SBDP145 and SBDP120 were reported to reflect different temporal characteristics of protease activation (Mondello et al. 2010). Pineda

et al., employing Western blot analyses, reported elevated levels of SBDPs in CSF from adults with severe TBI and their significant relationships with severity of injury and outcome (Pineda et al. 2007). Increased CSF SBDP levels were found to be significantly associated with mortality in patients with severe TBI. In addition, the temporal profile of SBDPs in nonsurvivors also differed from survivors (Mondello et al. 2010). These data suggest that SBDPs may provide crucial information not only on severity of brain injury, but also on underlying pathophysiological mechanisms associated with necrotic and apoptotic cell death.

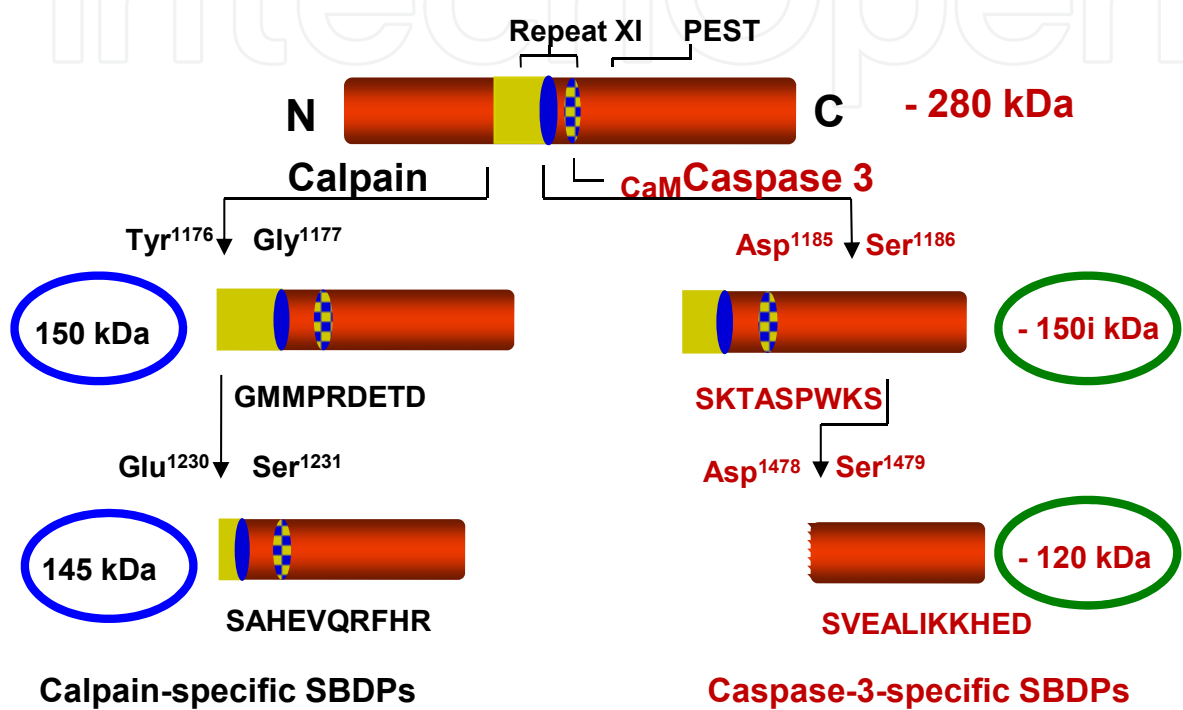


Fig. 3. Brain Injury-Dependent Generation of SBDPs

3.2.2.4 Phosphorylated neurofilament

A recent addition to the growing number of markers of brain injury is the heavily phosphorylated form of the major neurofilament subunit NF-H (pNF-H). Because pNF-H is specific for axons, increased serum concentrations of this protein are expected to provide a specific measure of ongoing axonal damage or degeneration. Increased pNF-H concentrations in serum and CSF have been observed following a variety of CNS damage and disease states, both in animal models and in human patients (Anderson et al. 2008, Boylan et al. 2009, Ganesalingam et al. 2011, Douglas-Escobar et al. 2010).

3.2.3 Glial markers

Several candidate glial markers have been proposed. In the section below, we focus on s100 and GFAP, which are the most extensively examined glial markers in TBI.

3.2.3.1 S100B

S-100b is a low-affinity calcium-binding protein that is primarily expressed and secreted astrocytes (Xiong et al., 2000). The biological function of S100B is still somewhat unclear.

Intracellularly, this protein is involved in the regulation of a large variety of cell activities and in the regulation of cell morphology (Goncalves et al., 2008, Kleindienst & Bullock 2006). S100B is also released into the extracellular space where seems to have both toxic/degenerative and trophic/reparative roles depending on the concentration (Goncalves et al., 2008). S100B found in low levels in healthy individuals, rises rapidly after head injury. The serum half-life of s100 is about 30 to 90 minutes and can be detected soon after injury (Jonsson et al., 2000). Several studies have demonstrated effectiveness of S100B as a diagnostic marker in the setting of TBI (Romner et al. 2000, Raabe et al. 1999). Increased levels of this protein marker have been shown to correlate with injury severity, mortality, and poor neurologic outcome following severe TBI (Vos et al. 2004). In addition, since 1995 several studies have investigated the clinical utility of this biomarker to predict normal CT findings after minor head injury in adults (Ingebrigtsen et al. 1995, Biberthaler et al. 2006). However, several observations concerning S100B have tempered the original enthusiasm regarding the usefulness of this protein as brain damage biomarker. One of the limitations to the use of S100B as a potential screening agent for brain injury is the lack of specificity. Indeed, S-100b, originally considered specific to astroglia in the central nervous system, is present in other extracerebral tissues such as adipocytes, chondrocytes and bone marrow cells (Donato 2001). High serum S-100b levels have been observed after extracranial trauma and burns (Anderson et al. 2001, Romner & Ingebritsen 2001). In addition, increased S100B may reflect either glial damage or astrocytic reactions to neural injury, referred to as reactive astrogliosis, which might have beneficial or detrimental physiological purposes (Herrmann et al. 2003, Kleindienst et al. 2007). This has raised concerns as to whether the serum levels of this protein actually correlate with the degree of brain damage or are more reflective of other processes. Finally, because of the high normative values in the pediatric population, S100B is not a useful marker in children (Piazza et al. 2007, Berger et al. 2006).

3.2.3.2 Glial fibrillary acidic protein

Glial fibrillary acidic protein (GFAP), a monomeric intermediate filament protein found in the astroglial skeleton, is not found outside the central nervous system and is thus considered a marker worth focusing upon in the search for brain specificity (Vos et al. 2004, Missler et al. 1999). In addition GFAP can be measured in peripheral blood (van Geel et al. 2002, Missler et al. 1999).

Recent evidence indicates elevates serum GFAP levels in various types of brain damage, ranging from neurodegenerative disorders (Baydas et al. 2003) and stroke (Herrmann et al. 2000) to traumatic brain injury (TBI) (Vos et al. 2004, Nylen et al. 2006, Pelinka et al. 2004). Clinical studies have proposed that the serum GFAP level is a reliable marker of primary brain damage after TBI with the further advantage that it is not released in situations of multiple traumas without brain injury (Vos et al. 2004, Pelinka et al. 2004). GFAP has also been demonstrated to be a potential useful biomarker to predict clinical outcome. Recently, Vos and colleagues showed a high specificity of GFAP in predicting death or unfavorable outcome at 6 months (0.93– 0.95), with a falsepositive rate for unfavorable outcome below 5% (Vos et al. 2010).

4. Assay development and hand-held devices

A variety of different analytical assay formats have been developed to measure biomarkers in biofluids such as cerebrospinal fluid (CSF) and plasma and serum (Mondello et al. 2011).

The most commonly used format is a two-sided enzyme-linked sandwich immunoassay (ELISA). This format is based on the coating of a solid surface in a plate to capture the biomarker/analyte of interest, followed by incubation with a directly or indirectly-labeled detection antibody. In the ELISA, this label is of enzyme-based, but there are a variety of different surface structures, labels and detection methods available. These include fluorescence, chemiluminescence, optical (absorbance) and electro-chemiluminescence.

Key elements in the consideration of the assay format are the analytical performance of the assay in terms of sensitivity and specificity, which assessed as specific assay parameters. The importance of these assay parameters and their assessment can be found in a recent paper by Mondello et al. (Mondello et al. 2011). Over the past few years, a practical iterative approach “fit-for-purpose” approach to biomarker assay development and validation has evolved, which centers around the “intended use” of the assay and the regulatory requirements associated with it (Lee et al. 2006). Assays “evolve” through different stages from the original assay development phase through an analytical qualification and validation phase. The underlying concept here is to fully understand the analytical performance of these assays for their intended use, before testing on clinical samples. Laboratories performing testing on human specimen for diagnosis, prevention or treatment of any disease are certified under the Clinical Laboratory Improvement Amendments (CLIA) of 1988 or have similar accreditation outside the US. These standard practices frequently required for CLIA certification were published by the Clinical and Laboratory Standard Institute (CLSI). In May 2001, the Food and Drug Administration (FDA) issued guidance for industry for bioanalytical method validation of assay to support pharmacokinetic (PK) assessment of small molecule drugs (Guidance for Industry on Bioanalytical Method Validation: availability. Fed. Regist. 66, 28526-28527, 2001).

In view of the diverse of biomarker assays for either PK, drug development or diagnostic purposes, neither the FDA bioanalytical validation nor the CLSI guidelines fully meet the needs of drug development and diagnostic applications of biomarker assays, which has resulted in additional recommendations for example by the American Association of Pharmaceutical Scientists (AAPS). A more comprehensive overview can be found in Lee et al, 2006. While it is important to develop “fit-for purpose” biomarker assays and analytically define and understand their performance, this procedure should be mirrored in the standardized collection of biofluids to be analyzed. For example, the Alzheimer Disease Neuroimaging initiative (ADNI) has identified the crucial parameters in the collection and storage primarily of CSF, which has resulted in the formulation of standard operating procedures (SOPs) and the quality-control assessment of collected samples. This has also led to the formation of a global CSF consortium, which based on the availability of biofluids such as CSF collected under standardized conditions allowed the identification of the variability when performing biomarker assessment at multiple sites (Mattson et al. 2011).

The translation of a “fit-for-purpose assay into a clinically useful and commercially viable diagnostic product requires the transfer of such an assay onto an appropriate platform, suitable for its intended use. While the laboratory assays described above are typically performed in a clinical reference laboratory setting over a course of hours, it is desirable to perform the measurement of acute biomarkers of brain injury in point-of-care (POC) type setting, outside the clinical laboratory. This includes the accident site, emergency room, sidelines of a sports field and military field. Rather than performing the biomarker assay in a benchtop multiwell plate format, in these setting a smaller and portable POC system is desirable, which requires little to no sample preparation and can performed as a rapid

diagnostic test in 30 min or less. In the battle field, these requirements are even more stringent in that the assay system should be carried around as handheld device, simple and lightweight and withstand extreme environments.

Over the last couple of years a variety of different POC platforms have been developed (Price & Kricka 2007). These POC assay platform comprise different design concepts and detection technologies based on their intended use. Glucose testing in diabetic patients is one of successful examples of POC test. The first blood glucose measurements were carried out with small paper strips invented and introduced by Ames in 1965. A measurement is performed by adding a drop of blood to a strip wherein the reaction of glucose in the blood results in the development of blue color and is compared to a color chart for analysis.

For tests, which do not require quantification, but simply detect the presence of a biomarker such as pregnancy test, a lateral flow-strip design might be sufficient. These are membrane strips, through which a small volume of sample liquid is transported by capillary action. These lateral flow strips are based on the binding of labels in the presence of biomarker through an immunochemical reaction, typically, small gold particles.

The assessment of biomarkers in the POC or handheld device format might require a more semi-quantitative assessment and here solutions have been developed, which mimic several of the assay steps a central laboratory test in a miniaturized format. One of the earliest examples is the i-STATr system (Abbott Laboratories, Abbott Park, IL) for measuring electrolyte, coagulation, glucose, cardiac markers such as troponin, and other tests. Wet assay reagents are stored in a cartridge and are actively pumped by the device through mechanical displacement to perform the various steps of the assays. Other examples such as AtolyzerR, originally developed by Atonomics, and the Roche cobas h232R exist and many more new technologies are being developed. A modification is the Philips MagnotechR system, which replaces the liquid manipulation steps by magnetically controlled movement. The implementation of molecular-diagnostic-based POCs also shows promise for the future. These assays typically include a polymerase-chain reaction-based amplification step.

All these implementations of the POC tests in a miniaturized cartridge-type format are designed to allow a high level of assay control. The overall assay performance is influenced by minor variation for example in timing of the individual assay steps and can result in large overall assay performance variability. The successful adaptation of any biomarker assay to a POC/handheld device is dependent on the requirement for sensitivity and precision in its intended use. The development of new POC technologies and solutions shows great promise for the adaptation of CNS biomarker assays in unmet medical needs.

5. Strategy for regulatory approval by FDA

Biomarkers, whether proteomic based or based on more traditional technologies, can be used for a variety of purposes. Generally speaking, the term 'biomarker' describes any measurable diagnostic indicator used to assess the presence or risk of disease. For the US Food and Drug Administration (FDA), the term 'diagnostic' includes the diagnosis, screening and prediction of disease. But it also encompasses other uses such as staging or the prognosis of disease, monitoring patients and the effectiveness of a treatment and/or optimizing treatment outcomes by aiding health-care providers in medical and therapeutic decision making.

The FDA has been involved in the regulation of medical devices, including in vitro diagnostic devices (IVDs) since 1976 when the US Congress established laws for the

regulation of medical devices under the Medical Device Amendments act (REF. 8). According to the Medical Device Amendments act, for an in vitro diagnostic (IVD) device to enter the US market it must comply with a set of rules and regulations in order to prove safety and effectiveness for its intended use. In 21 CFR 860.7(d)(1) device safety requires “the probable benefit to health from use of device for its intended use and conditions of use ... outweigh any probable risk.” In 21 CFR 860.7(e)(1) device effectiveness requires “that in a significant portion of the target population, the use of the device for its intended use and conditions for use ... will provide clinically significant results.” In addition, a new assay is required to demonstrate an adequate analytical performance (appropriate accuracy and precision) and clinical performance (sensitivity, specificity and some indication of clinical utility) (21 CFR 807; 21 CFR 814).

In order to be commercialized in a kit, newly discovered biomarkers device must follow specific pathways. Investigational studies of diagnostic devices can be performed with various design configurations, but they must conform to FDA requirements. The researchers are required to apply for an investigational device exemption (IDE) before initiating the study, as described in Title 21 of the Code of Federal Regulations 812 (21 CFR 812)(US Food and Drugs Administration [FDA], 2006). The IDE submission should describe the nature of the proposed study, include details of informed consent and ensure patient protection and that the risks associated with participation in the study will be clearly communicated to individuals.

After the appropriate investigational studies have been completed, the FDA requires premarket submissions before a test can be approved for clinical use in the United States. Depending on the nature of the test and its classification, the product could be reviewed as a 510(k) premarketing clearance with a 90-day timeline or Premarket Approval (PMA) with a 180-day timeline (21 CFR 807; 21 CFR 814). FDA considers three classes of devices (class I, II, or III) (FDA, 2009, 2010).

The 510(k) process is used when the new test measures an existing FDA classified analyte (class I or II) where there exists a commercially available predicate test method that has been cleared by the FDA or that was in commercial distribution before May 28, 1976 (21 CFR 860.84). Premarket clearance requires the sponsor to provide information for the new product including its intended use and classification and ‘substantially equivalence’ to the predicate device. This ensures that a high level of safety and effectiveness is maintained. In addition the sponsor must show characterization of analytical capability of the test (e.g., specificity and accuracy, precision and linearity by correlating patient studies against the predicate device) (<http://www.fda.gov/oc/mdufma/cover sheet.html>).

The PMA process is used when the test is classified as class III; that is, either it is associated with high risk (e.g., when the outcome determines cancer treatment or diagnosis) or the clinical utility of the marker or the technology of the measurement are novel and no predicate device can be identified (21 CFR 814). FDA requires the sponsor to submit the same data required for 510(k) as well as clinical outcomes data, where the level of the marker is related to disease status defined by clinical criteria (<http://www.fda.gov/oc/mdufma/cover sheet.html>).

In 1997, since some new biomarkers have no obvious predicate devices and do not have safety concerns, FDA created a new hybrid ‘de novo’ or ‘risk-based’ classification (21 CFR 814). This process allows a new biomarker to be regulated as in a 510(k), but requires the demonstration of clinical effectiveness.

Therefore, in the absence of a predicate device and depending on the intended use and clinical utility, either a PMA or a ‘de novo’ process will be required for a new protein of interest before it achieves commercial availability in a kit or device.

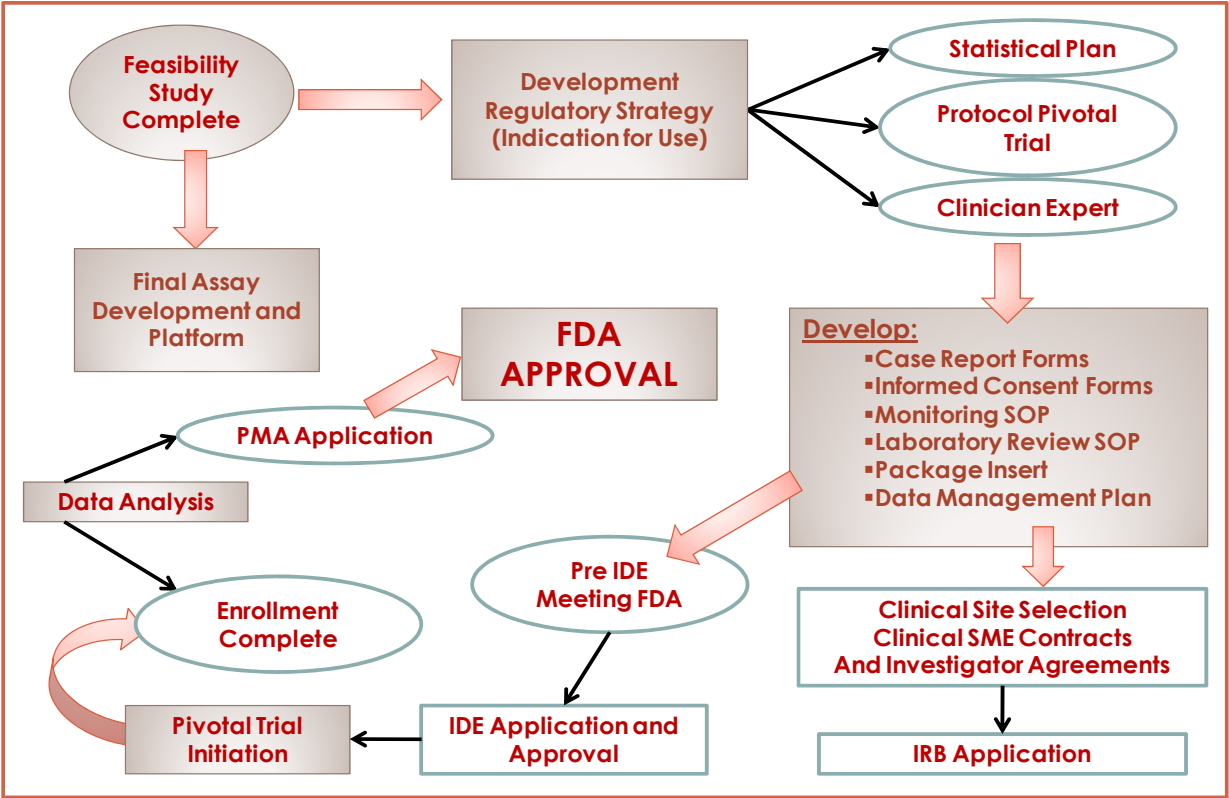


Fig. 4. Regulatory Pathway for PMA Application

6. Conclusion

This chapter provides an introductory review of techniques that have been applied to biomarker identification and clinical validation. Demonstration of clinical utility and compliance with regulatory requirements is critical for the commercialization of novel biochemical markers but also formidable, uncertain and costly.

At present, there are still many unanswered questions in this area of research, however, such as the best statistical methods to analyze the large volume of data generated, the role of potential demographic and clinical confounders, such as age, sex, and on the other hand the broad and complex spectrum of types and severities of brain injuries. In addition, issues such as sample integrity and preservation, normalization, and appropriate control data also must be given careful consideration. A multimarker strategy will probably be needed to provide a greatly expanded approach to the detection of brain injury, elucidating its pathogenesis and making it possible to guide and monitor the therapy in new ways and ultimately to improve outcome.

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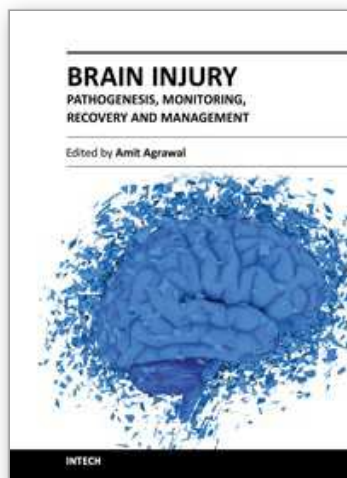
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